



## Mitochondrial functionalism in HIV-infected children receiving antiretroviral therapy

Constanza Morén Núñez

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**UNIVERSITAT DE BARCELONA  
FACULTAT DE MEDICINA**

**MITOCHONDRIAL FUNCTIONALISM IN  
HIV-INFECTED CHILDREN RECEIVING  
ANTIRETROVIRAL THERAPY**

Memòria de Tesi per a optar al grau de Doctora per la Universitat de Barcelona

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El que certifico a Barcelona a 05 de desembre de 2011.

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Doctor.

El que certifico a Barcelona a 05 de desembre de 2011.

**Clàudia FORTUNY GUASCH**



***No es valiente el que no tiene miedo,  
sino el que sabe conquistarlo.***

*Nelson Mandela*





*A la Ita*

A mi madre

A mi padre



## ***Agraiments***



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FORWARD



The present Thesis has been performed in the Mitochondrial Functionalism Laboratory, Muscle Research Unit, Internal Medicine Department of the Hospital Clínic of Barcelona, located in the *Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)*, in the Faculty of Medicine of the University of Barcelona (UB)- Hospital Clínic of Barcelona. This Thesis was initiated in 2007 through a grant from the *Fundación para la Investigación y Prevención del SIDA en España (FIPSE)* and it has been co-tutorized by Dr. Òscar Miró i Andreu, from the Emergency Department of the Hospital Clínic of Barcelona and Dra. Clàudia Fortuny Guasch, associated professor of UB, from the Infectious Diseases Unit, Pediatrics Department of the Hospital Sant Joan de Déu of Barcelona. The obtention of one more grant from FIPSE, within the *Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER)*, whose Principal Investigator is Dra. Glòria Garrabou Tornos, has permitted the continuation of the studies in the last period of this Thesis.

This Thesis entitled: “Mitochondrial functionalism in HIV-infected children receiving antiretroviral therapy” has explored the mitochondrial status in the context of HIV- infection and antiretroviral therapy in pediatric patients, a major issue with special relevance in this population group.



## LIST OF ABBREVIATIONS



A	Adenosine
Å	Angstrom
$\Delta\psi_m$	Mitochondrial membrane potential
ABC	Abacavir
ACoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
ANT	Adenine nucleotide translocase
APAF-1	Apoptotic protease activator factor 1
ARV	Antiretroviral
ATP	Adenosine triphosphate
ATox	Ascorbate oxidation
AU	Absorbance unit
AZT	Zidovudine
Bp	Base pair
C	Cytidine
CI	Complex I or NADH-CoQ reductase
CII	Complex II or succinate-CoQ reductase
CIII	Complex III or CoQH <sub>2</sub> - cytochrome c reductase
CIV	Complex IV or cytochrome c oxidase
CV	Complex V or ATP synthase
CAD	Caspase activated DNase protein
CCCP	Carbonyl cyanide m-chlorophenyl-hydrazone
Cellox	Endogenous respiration of the cell
CO	Carbon monoxide
CO <sub>2</sub>	Carbon dioxide
CoQ	Coenzyme Q or ubiquinone (in its oxidized form)
CoQH <sub>2</sub>	Coenzyme Q or ubiquinone (in its reduced form)
COX	Cytochrome c oxidase
CRABP-1	Cytoplasmic retinoic acid binding protein type 1
CytC	Cytochrome c
D loop	Displacement loop of mitochondrial genome
d4T	Stavudine



DCPIP	2,6-dichlorophenolyndophenol
ddC	Zalcitabine
ddI	Didanosine
ddNTP	Dideoxynucleosides triphosphate or dideoxynucleotides
DLV	Delavirdine
DMFA	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DNTB	5,5'-dithio-bis 2-nitrobenzoic acid
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
EI	Entrance inhibitor
Env	Envelope gene
ES	Elite suppressors
ETC	Electron transport chain
F <sub>0</sub>	Hydrophobic subunit of complex V
F <sub>1</sub>	Subunit of complex V facing the matrix
FADH <sub>2</sub>	Flavin adenine dinucleotide
FDA	Food and drug administration
Fe-S	Iron-sulfur cluster
FI	Fusion inhibitors
FMN	Flavin mononucleotide
Fp	Flavoprotein subunit
FTC	Emtricitabine
g	Units of gravity, relative centrifugal force
G	Guanosine
G3Pox	Glycerol-3-phosphate oxidation
Gag	Group specific antigen
GMox	Glutamate-malate oxidation
Gp	Glycoprotein
H <sup>+</sup>	Hydrogen proton
H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
H strand	Heavy strand of mitochondrial DNA

HAART	Highly active antiretroviral therapy
HAE	Hydroxyalkenals
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSP <sub>1</sub>	Heavy strand promoter type 1
HSP <sub>2</sub>	Heavy strand promoter type 2
HSV	Herpes simplex virus
HTLV	Human retrovirus T- cell leukemia family
IDV	Indinavir
II	Integrase inhibitor
Ip	Iron protein subunit
KCN	Potassium cyanide
KDa	Kilo Dalton
L strand	Light strand of mitochondrial DNA
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LHON	Leber's hereditary optic neuropathy
LIP	Lymphoid interstitial pneumonia
LPN	Lopinavir
LRP	Low density lipoprotein-receptor related protein
LSP	Light strand promoter
LTNP	Long term non progressors
LTR	Long terminal repeat
M-Tropic	Viral monocytotropic strains
MDA	Malondialdehyde
MELAS	Mitochondrial encephalomyopathy lactic acidosis stroke-like episodes
MERRF	Myoclonic epilepsy with ragged red fibers
MI	Maturation inhibitor
MIP	Macrophage inflammatory protein
MNGIE	Mitochondrial neurogastrointestinal encephalopathy
MRC	Mitochondrial respiratory chain
mRNA	Messenger RNA

MPTP	1- methyl- 4 phenyl 1,2,3,6 tetrahydropyridine
MPP <sup>+</sup>	1-methyl-4phenyl pyridinium
MSA	Methanesulfonic acid
MtDNA	Mitochondrial DNA
mTERF	Mitochondrial transcription terminator
MTG	Mitotracker Green
MtRNA	Mitochondrial RNA
MtSSB	Mitochondrial single-strand binding protein
MtTFA	Mitochondrial transcription factor A
NADH	Reduced nicotinamide adenine dinucleotide
NARP	Neuropathy ataxia and retinitis pigmentosa
nDNA	Nuclear DNA
Nef	Negative factor gene
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NFV	Nelfinavir
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NO	Nitric oxide
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
O <sub>2</sub> <sup>-</sup>	Superoxide anion
OD	Optic density
O <sub>H</sub>	Replication origin of the heavy strand mitochondrial DNA
OH <sup>-</sup>	Hydroxyl anion
O <sub>L</sub>	Replication origin of the light strand mitochondrial DNA
ONOO <sup>-</sup>	Peroxynitrite
OXPHOS	Oxidative phosphorylation system
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate saline buffer
PCR	Polymerase chain reaction
PEO	Progressive external opthalmoplegy
PEP	Phosphoenolpyruvate
P <sub>i</sub>	Inorganic phosphate
PI	Protease inhibitors

PK	Pyruvate kinase
PKB	Protein kinase B
PMox	Pyruvate-malate oxidation
Pol	Polymerase gene
PPAR- $\gamma$	Peroxisome proliferator activated receptor $\gamma$
PTP	Permeability transition pore
Q	Oxidized form of ubiquinone or coenzyme Q
QH <sub>2</sub>	Reduced form of ubiquinone or coenzyme Q
RANTES	Regulated upon activation normal T expressed and secreted
Rcf	Relative centrifugal force
Rev	Regulator of expression of viral protein gene
RITOLS	Ribonucleotide incorporation throughout the lagging-strand
RNA	Ribonucleic acid
R-OOH	Hydroperoxides
ROS	Reactive oxygen species
rpm	Revolutions per minute
RRF	Ragged red fibers
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RTV	Ritonavir
s	Svedberg units
SDF-1	Stromal cell-derived factor type 1
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulphate
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SMAC	Second mitochondria-derived activator of caspases
SOD	Superoxide dismutase
Sox	Succinate oxidation
SQV	Saquinavir
SREBP-1	Sterol regulatory element-binding protein type 1
RTV	Ritonavir
T	Thymidine

T- Tropic	Viral lymphotropic strains
T20	Enfuvirtide
TAR	Transactivation response element
Tat	Transactivator of transcription gene
TCA	Tricarboxylic acid cycle
TDF	Tenofovir diproxil fumarate
TEMED	Tetramethylethylenediamine
TFAM	Transcription factor A
TFB1M	Mitochondrial transcription factor type B1
TFB2M	Mitochondrial transcription factor type B2
TNB	2-nitro-5-benzoic
TNF $\alpha$	Tumor necrosis factor alpha
tNRTI	Nucleotide analogue reverse transcriptase inhibitor
tRNA	Transfer RNA
UTR	Untranslated region
VDAC	Voltage dependent anion channel
Vif	Virion infectivity factor gene
VLDL	Very low density lipoprotein
Vpr	Viral protein R
Vpu	Viral protein U gene
WCLB	White cell lysis buffer
ZDV	Zidovudine
$\gamma$ -pol	$\gamma$ DNA polymerase
3TC	Lamivudine

# INTRODUCTION

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## **1- THE MITOCHONDRION**

### **1.1. DEFINITION**

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The mitochondrion (*Et.*: from Greek *μίτος*, *mítos*: filament, and *κόνδρος*, *kóndros*: grain) is a cell organelle, of maternal inheritance, located in the cytoplasm of most of the eukaryotic cells. The size and morphology is similar to the bacteria, from 0.5 to 1  $\mu\text{m}$  of diameter and 10  $\mu\text{m}$  in long (Scheffler 2010).

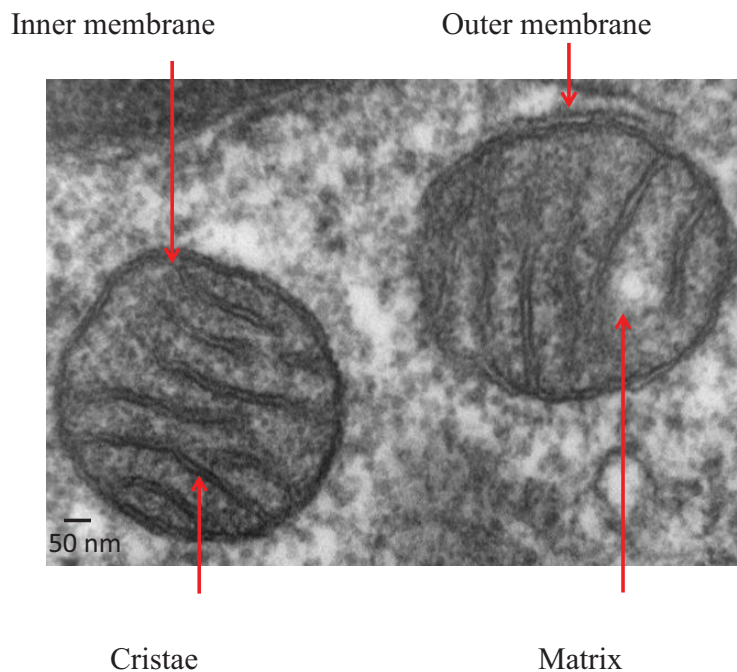
This organelle is present in a variable number within the cells depending on the energetic requirements of the tissue. The most energy demanding will contain a higher number of mitochondria.

The mitochondria are indispensable for the cell viability and they are involved in very important processes, such as calcium homeostasis, heat production, apoptosis, cell respiration, energy production of the cell, or the anabolism and catabolism of the metabolites among others.

These organelles are the energy generators of the cells, as they provide energy through the formation of molecules of adenosine triphosphate (ATP), which is the major energy currency of the cell. The ATP synthesis is coupled to the cell respiration in a process which is called oxidative phosphorylation (OXPHOS).

The mitochondria are constituted by a double membrane (**Figure 1**). The external membrane, which is permeable to many solutes and makes possible the interchange of molecules with the cytosol; and the internal membrane which is impermeable and it is found as a folded structure constituting the mitochondrial cristae, where the enzymatic complexes of the mitochondrial respiratory chain (MRC) are located. Together with the chloroplasts in the plant cell, they are the only organelles containing its own deoxyribonucleic acid (DNA), or mitochondrial DNA (mtDNA) and an autonomous transcriptional and translational capacity, encoding for some proteins of the MRC.





**Figure 1.** Mitochondrial structure. Transmission electron microscope image of mammalian lung tissue. Source: <http://remf.dartmouth.edu/images/mammalianLungTEM/source/8.html>

## 1.2. HISTORY

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The discovery of the mitochondria was a collective fact. A proof of this is the high number of terms that have been referred to the same organelle: *blepharoplasts*, *chondrioplasts*, *chondriosomes*, *chondriospheres*, *fila*, *fuchsinophilic grains*, *Korner*, *mitogel*, *parabasal bodies*, *sarcosomes*, *interstitial bodies*, *plasmosomes*, *plastochondria*, *bioblasts*... It was not until 1918 that Cowdry and Lehninger started systemizing and unifying all these terms.

When, in the XIX century, morphologists with their light microscopes discovered grains (chondria) and filaments (mito), they could not be sure that they were all looking at the same functionality distinct structure in different cells and, undoubtedly, they were not. However, variability in number and shape must have been apparent. A more systematic approach became possible when selective staining methods were used (fuchsin, Altman, 1890; crystal violet, Benda, 1898 and Janus green, Michaelis, 1898). The following dates were significant hallmarks in the final mitochondrial history:

- 1880-1888- Probably the first observations of the mitochondria were made by the Swiss botanic Kolliker who noticed the presence of some grains in the insect cells and gave them the name of *sarcosomes* (Sastry 2004).
- 1882- Flemming W. (German biologist) discovered a series of inclusions at which denominated *fila* (Flemming W 1882).
- 1884- They were also observed by Altmann R, who later on described a series of corpuscles, which he speculated to be independent parasites with their own metabolism and called them *bioblasts*. This fact was later accepted and the structures were described as mitochondria.
- 1889- The term mitochondrion, so known by us, was created by Benda C., who named these organelles that appeared brightening in crystal violet dyes.
- 1912- Kingsbury linked these organelles with the cell respiration (Gupta PK 2006).
- 1913- Warburg OH. discovered the association with the enzymes of the MRC (Gupta PK 2006).
- 1934- The mitochondria were isolated for the first time from liver homogenates (Gupta PK 2006).
- 1948- Lehninger and Kennedy E. demonstrated that both the Krebs cycle, or tricarboxylic acid cycle (TCA) and the beta-oxidation take place in the mitochondrial matrix and that the OXPHOS is located inside the mitochondria. In the same period, Ephrussi B. suggested the existence of some genetic material in the cytosol different to the nuclear genome and speculated about its association to these organelles (Ephrussi B 1949).
- 1961- The biochemist Mitchell P. postulated the Chemiostatic Theory, receiving the Nobel Prize Chemistry in 1978. This theory explained the mechanism of coupling of the electron transfer and the ATP synthesis during the so-called OXPHOS process (Kocherginsky 2009).
- 1962- Luft R. and collaborators (Luft R 1962) associated, for the first time, a disease with a mitochondrial alteration in a patient who presented a non thyroid hyper-metabolism and to which gave the name of Luft disease. Since then, many mitochondrial alterations (mitochondriopathies) have been described.
- 1963- The presence of mtDNA was definitively discovered by Margit MK. Nass and Sylyan Nass (Nass MM 1963).

### 1.3. MITOCHONDRIAL ORIGIN: ENDOSYMBIOTIC THEORY

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The famous scientific Lynn Margulis and her collaborators reformulated an ancient hypothesis about mitochondrial origin around 1980, the endosymbiotic theory (Margulis L 1975).

This theory postulates that, 1,500 million years ago, a prokaryotic cell was phagocytosed by another prokaryotic or primitive eukaryotic cell, without being digested and, subsequently, they fused together in the evolution process. A permanent symbiosis was produced between both cell types. The former provided energy, in ATP form, utilizing the molecular oxygen as an oxidant and the host cell offered a stable media rich in nutrients.

This symbiosis, or mutual benefit, made possible that the prokaryotic cell ended belonging to the principal organism becoming an inherent part of the system, the mitochondrion. During the evolution, an important part of the mitochondrial genes are supposed to have been transferred to the nucleus, in a way that the mitochondrion is not viable outside of the host cell and *vice versa*.

Many factors support the endosymbiotic theory, for instance, the fact that the bacteria and the mitochondria present many similar characteristics, as the size, the structure, the compounds of the membrane, the way of producing energy, the presence of an own genetic material different from the nuclear DNA, or the affection of mitochondria derived from the use of some antibiotics, among others.

### 1.4. STRUCTURE

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The mitochondria are not static structures inside the cells. They are plastic structures, able to be deformed, they can be fused and fissioned. The number of mitochondria within the cells varies depending on the energetic requirements. The total number of the mitochondria in the cell is called cell chondriome.

The mitochondria present two membranes of very different characteristics, between which the inter-membrane space is located.

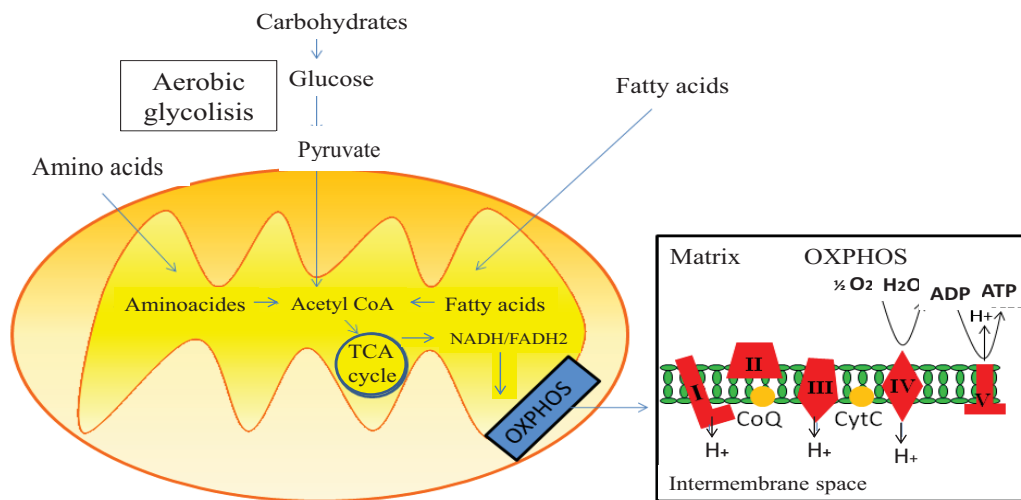
**1.4.1. THE OUTER MEMBRANE:** Is a lipid bilayer permeable to the ions, metabolites and polypeptides. Its permeability is given by some proteins which are called porins or voltage-dependent anion channel (VDAC), which form transmembrane pores or channels. These pores make possible the flux of big molecules of 10 kDa and approximately 20 Å in diameter.

**1.4.2. THE INNER MEMBRANE:** As opposite to the outer membrane, this membrane is impermeable to most of the small molecules and ions, it is rich in cardiolipin, it lacks of pores, it presents more proteins and it is highly selective. Some transmembrane transport proteins and translocases are found on it. These proteins make possible the flux of ions and molecules through it: pyruvic acid, adenosine diphosphate (ADP), ATP, oxygen, water (H<sub>2</sub>O) or fatty acids. From these transmembrane proteins and translocases, the following are highlighted:

- Adenine nucleotide translocase (ANT), which deals with the transportation the cytosolic ADP to the mitochondrial matrix and translocates the synthesized ATP to the cytosol in the OXPHOS process.
- Phosphate translocase, which translocates cytosolic phosphate together with a proton (H<sup>+</sup>) to the matrix. This phosphate is essential for ADP phosphorylation in the OXPHOS process.

This membrane is highly folded and forms invaginations, so-called mitochondrial cristae, which increase the surface area, in order to provide a location of the enzymatic complexes of the OXPHOS system.

**1.4.2.1. THE OXIDATIVE PHOSPHORYLATION (OXPHOS) SYSTEM:**



**Figure 2.** Mitochondrial metabolism. CoQ: Coenzyme Q, CytC: Cytochrome c, OXPHOS: oxidative phosphorylation system, H<sup>+</sup>: protons.

The mitochondria are involved in anabolic and catabolic processes of many molecules. In the catabolic systems ATP is obtained from the degradation of different metabolites (carbohydrates, amino acids, fatty acids). Each metabolite has a different metabolic pathway and all converge in the production of acetyl-coenzyme A (ACoA), which enters in the Krebs cycle being degraded to carbon dioxide (CO<sub>2</sub>) and H<sub>2</sub>O. These catabolic processes are carried out in the mitochondrial matrix and generate reducing power in form of some intermediates: nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH<sub>2</sub>). Both, in their reduced forms, transfer electrons to the MRC in order to synthesize ATP through the oxidative phosphorylation. This OXPHOS is based on the synthesis of ATP coupled to the oxygen consumption or cell respiration (**Figure 2**).

This system is constituted by the electron transport chain (ETC), which is formed by 4 enzymatic complexes (complex I or CI-, CII, CIII and CIV), 2 mobile electron carriers [coenzyme Q (CoQ) and cytochrome c (CytC)] and a fifth complex, called ATP synthase or complex V (CV) (Andreu AL 2003). The electrons flow through the MRC by means of oxide reduction (or redox) reactions ending in the complex IV, where the oxygen is the final acceptor of the electrons and it is reduced into H<sub>2</sub>O. This is an exergonic process (a favorable reaction from a higher energetic status to a lower one) and the released energy is used to export protons (H<sup>+</sup>), from the mitochondrial matrix to the intermembrane space) against gradient, through complexes I, III and IV. Complex V returns these protons to the matrix forward gradient leading to ATP synthesis (**Figure 3**).

It is believed that complexes assemble in supra-molecular structures, known as super-complexes (Schägger H 2000).

- **The complex I**, also known as NADH dehydrogenase or NADH- CoQ reductase (CI), contains flavin mononucleotide (FMN) and it transfers electrons to CoQ or ubiquinone. The overall reaction catalyzed by this complex is described as follows:



CoQ and CoQH<sub>2</sub> refer to the oxidized and reduced form of ubiquinone, respectively. The reaction is accompanied by a transfer of 4 protons from the matrix side to the intermembrane space.

This apparently simple reaction requires a 45 subunits (>900 kDa) complex in mammalian mitochondria (it is the biggest complex of the MRC). Seven of these subunits (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) are encoded by the mitochondrial genome in mammals and other organisms.

Its function is to oxidize the NADH, which has been produced through the Krebs cycle in the mitochondrial matrix and to transfer the electrons to CoQ in order to reduce it. It has got an “L” shape with a horizontal arm integrated to the inner membrane and a vertical arm which is projected to the matrix, where it contacts to NADH. The CI contains iron- sulfur clusters (or centers) (Fe-S) involved in the electron transfer (Scheffler 2010).

Specific inhibitors: Over the years the analysis of this complex and its activity in mitochondria has been aided by the discovery of a variety of highly specific, naturally occurring inhibitors. The best known of these is rotenone, an insecticide of vegetal origin, used as a fish poison. Another inhibitor is the antibiotic piericidin A. Some of these are being developed not only as potential insecticides but also for various pharmacological purposes. The “designer drug” MPTP (1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine) can be converted to the active metabolite 1-methyl-4-phenyl pyridinium (MPP<sup>+</sup>) by monoamine oxidase and transported into dopaminergic neurons. At sufficiently high concentrations (mM) it is a specific inhibitor of CI. This mechanism is thought to be responsible for the death of neurons in the *substantia nigra*, and hence the symptoms of parkinsonianism, in humans and experimental primates (Scheffler 2010).

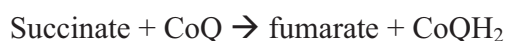
- **The complex II**, succinate dehydrogenase, or succinate- CoQ reductase (CII), which is exclusively encoded by the nuclear genome, transfers electrons to the CoQ. It is the simplest and smallest of all the complexes of the MRC, with only 4 peptides.

The 2 largest peptides constitute the peripheral portion of the complex and function as the enzyme succinate dehydrogenase in the Krebs cycle. They are associated with the membrane through 2 integral membrane proteins, also referred to as the “anchor” proteins.

The electrons from the oxidation of the succinate to fumarate are channeled through this complex to ubiquinone. Thus, complex II links the Krebs cycle directly to the MRC.

Cofactors, cytochrome b<sub>558</sub> and metal ions also constitute CII. A flavin is linked covalently to the largest peptide, yielding the flavoprotein subunit (Fp). This Fp is intimately associated with the iron-protein subunit (Ip), made up of a peptide containing 3 non-heme Fe-S centers: [2Fe-2S], [3Fe-4S], [4Fe-4S]

The overall reaction catalyzed by complex II is:



There is a lack of proton pumping in this complex.

Specific inhibitors: The most useful inhibitor for CII has been the substrate analogue malonate, which binds competitively and specifically at the active site on the Fp subunit of the complex (Scheffler 2010).

- **The coenzyme Q (CoQ)** (as well as cytochrome c) acts as an electron carrier between the complexes. It is a quinone with a lateral polyisoprenoid chain, which acts as a receptor center of the electrons (in hydrogen form), proceeding from CI and CII and as a donor to the CIII.

- **The complex III** or CoQH<sub>2</sub>-cytochrome c reductase transfers electrons from the CoQH<sub>2</sub> to the cytochrome c (Cyt c). Some cytochromes (Cyt b<sub>562</sub>, Cyt b<sub>566</sub>, Cyt c<sub>1</sub>) and a Fe-S cluster were found on it.

The overall reaction catalyzed by this complex is:

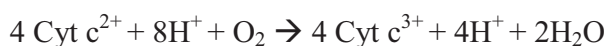


Similar to the reaction taking place in complex I, the oxidation of one of the substrates (CoQH<sub>2</sub>) and the transfer of electrons to the mobile carrier (cytochrome c) is coupled to the transfer of 4 protons across the inner mitochondrial membrane. One peptide of the complex is encoded by the mitochondrial genome and incorporated as cytochrome b. The other peptides (10 in mammals) are encoded by the nuclear genome, synthesized in the cytosol and imported for assembly in the inner mitochondrial membrane (Scheffler 2010).

Specific inhibitors: The antibiotic antimycin A is the best known of the inhibitors for the CIII.

- **The cytochrome c** is a mobile carrier heme-protein located in the external face of the mitochondrial inner membrane. Its function is to transfer the electrons from CIII to CIV.

- **The complex IV** or cytochrome c oxidase (COX) transfers electrons to the oxygen molecule reducing it into 2 molecules of water. The overall reaction catalyzed is the following:



Molecular oxygen is the terminal electron acceptor, the mobile carrier cytochrome c is reoxidized and 2 protons are transferred to the intermembrane space.

The mammalian CIV contains 13 subunits (~160 kDa). The 3 largest subunits (I, II, III) are encoded by the mitochondrial genome, synthesized in the matrix and represent the most important active center of the complex. For the remaining subunits the nomenclature is: IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc and VIII).

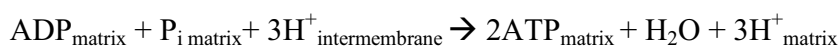
Specific inhibitors: The classical inhibitor for CIV is the potassium cyanide (KCN) (Scheffler 2010).

- **The complex V** or ATP synthase plays a key role in OXPHOS being involved in the synthesis of ATP from ADP and inorganic phosphate ( $P_i$ ). This endergonic reaction is coupled to redox reactions and electron transport. The reaction is driven by a proton gradient. This complex is constituted by two functional subunits:  $F_0$  and  $F_1$ . The former is a hydrophobic structure integrated to the inner membrane which contains the transmembrane channel where the protons pass through, from the intermembrane space to the mitochondrial matrix. It consists of a ring made of 10-14 c-subunits and one a-subunit. The latter one,  $F_1$ , is orientated to the mitochondrial matrix and contains the catalytic synthase activity. It is constituted by five types of polypeptide chains ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$ ), which have a stoichiometric relation of 3:3:1:1:1, respectively.  $\alpha$  and  $\beta$  subunits alternate constituting a hexameric ring. Both bind nucleotides, but only subunits  $\beta$  participate directly in the catalysis, while  $\alpha$  subunits trigger a regulatory function. The connecting part between  $F_0$  and  $F_1$  is the central part of the ATPase and made of  $\epsilon$  and  $\delta$  proteins (Jonckheere AI 2011).

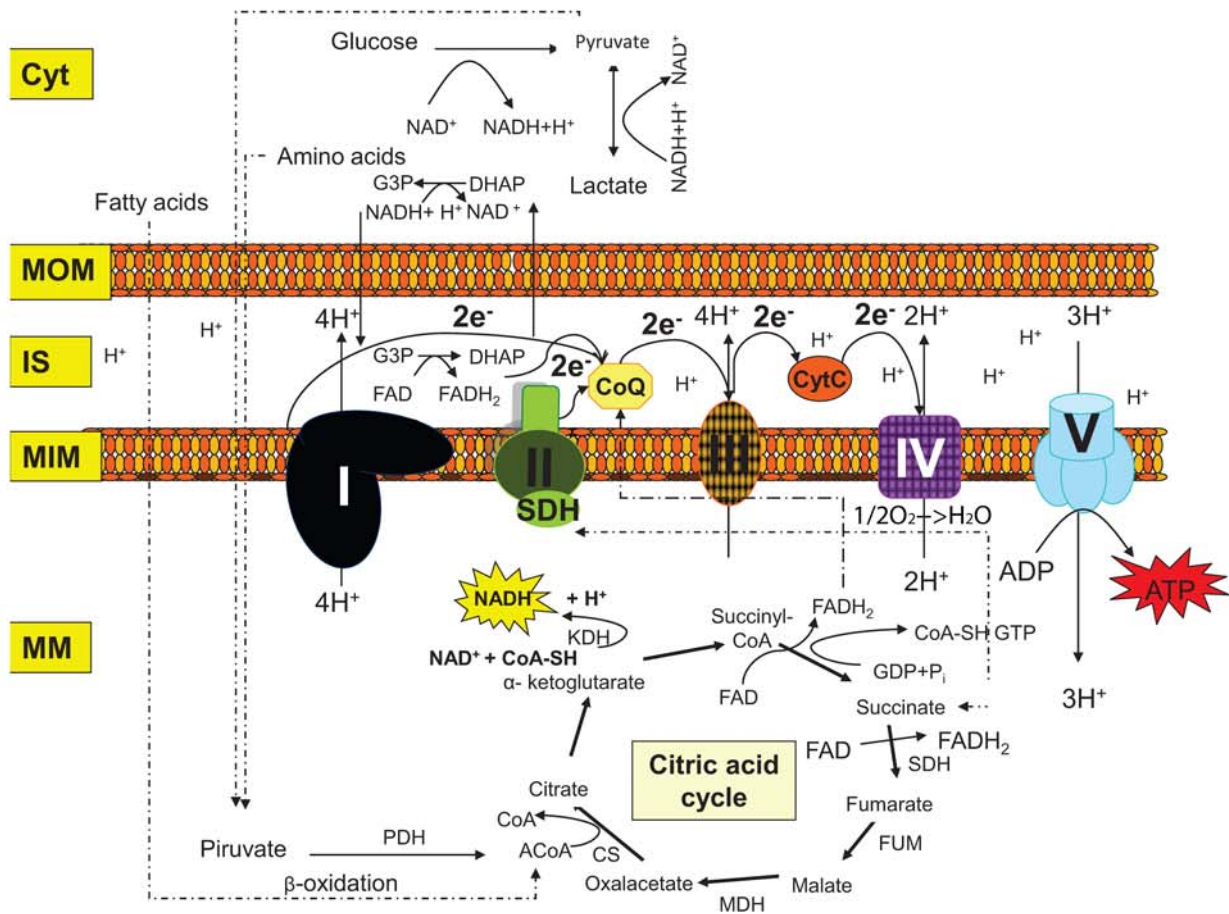
The detailed mechanism of ATP synthesis by CV is based on the rotational catalysis proposed by P. Boyer et al in 1997 (Boyer PD 1997) (Jonckheere AI 2011).  $\alpha$  subunits rotate and there is the inter-conversion of 3 different conformations. Thus, the function of CV may act as a turbine which moves to produce energy. The c ring and the central part, constituted by  $\gamma$  and  $\epsilon$ , is the mobile unit or rotor and the exterior column and the hexameric  $\alpha_3\beta_3$  ring represent a structure that rotates in response to the proton motive force.

Specific inhibitors: The antibiotic oligomycin is the highly specific inhibitor of the subunit  $F_0$  of the CV.

The overall reaction that takes place in this complex is:







**Figure 3.** Mitochondrial respiratory chain. SDH: Succinate dehydrogenase, MM: Mitochondrial matrix, MIM: Mitochondrial inner membrane, IS: Intermembrane space, MOM: Mitochondrial outer membrane, Cyt: cytosol, DHAP: dihydroxyacetone phosphate,  $\text{NAD}^+$ : Nicotinamide adenine dinucleotide, NADH: Reduced nicotinamide adenine dinucleotide, CoA-SH: Reduced coenzyme A, CoQ: Coenzyme Q, CytC: Cytochrome c, CS: Citrate synthase; ACoA: Acetyl coenzyme A, FAD: Flavin adenine dinucleotide,  $\text{FADH}_2$ : Reduced flavin adenine dinucleotide, ADP: Adenosine diphosphate, ATP: Adenosine triphosphate, G3P: Glycerol-3-phosphate, PDH: Pyruvate dehydrogenase, GTP: Guanosine triphosphate, GDP: Guanosine diphosphate.

**1.4.3. THE INTERMEMBRANE SPACE** is the space delimited between the outer and the inner membrane. It has a similar composition to that of the cytosol and a high concentration of protons as a result of the pumping carried out by the enzymatic complexes of the MRC. The carnitine, which is a molecule involved in the transport of fatty acids from the cytosol to the

mitochondrial matrix where they will be oxidized by the beta-oxidation process, is located in this intermembrane space.

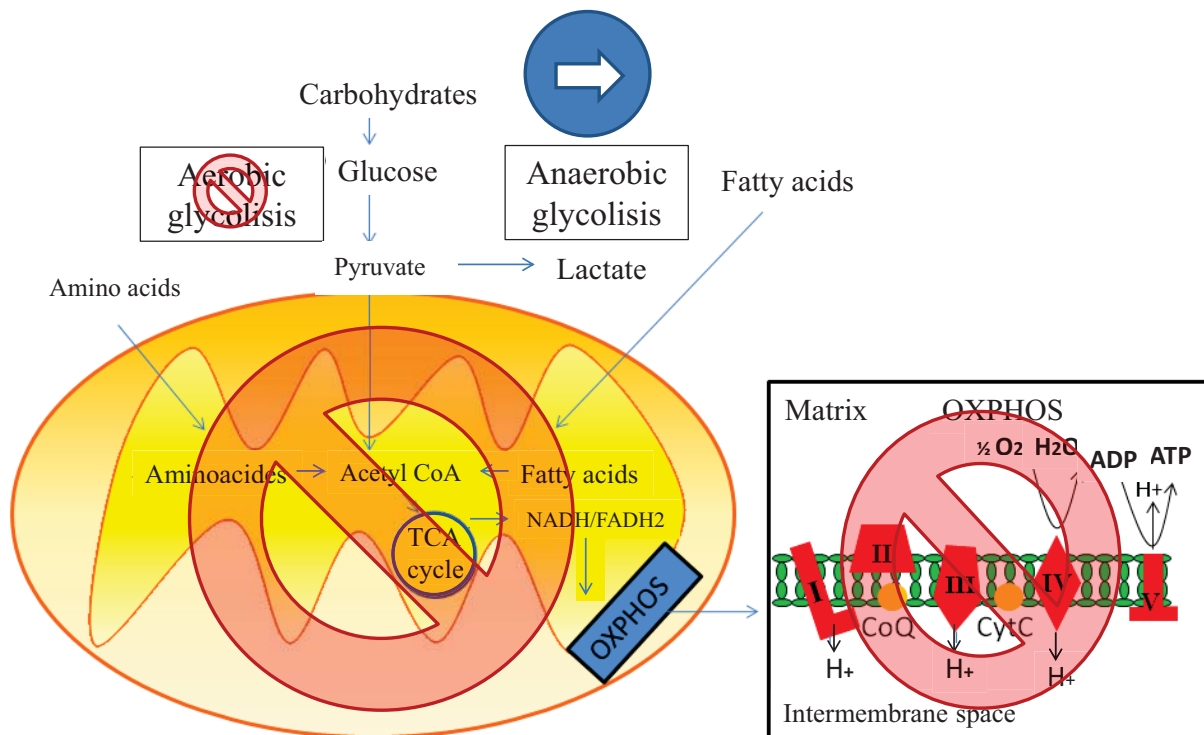
**1.4.4. THE MITOCHONDRIAL MATRIX** (or mitosol) contains ions, metabolites to oxidize and the genetic material of the mitochondrion. Mitochondrial genome is found in the matrix as a circular double stranded DNA, similar to the bacteria genome, together with mitochondrial ribonucleic acid (mtRNA) and 55S type ribosomes that make possible the synthesis of some mitochondrial proteins. Many important metabolic pathways for the cell viability take place in the matrix, as the Krebs cycle, the beta-oxidation of the fatty acids, the oxidation of amino acids and urea and heme-groups synthesis, among others.

## **1.5. MITOCHONDRIAL RESPIRATION**

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The mitochondrion is the organelle responsible for oxygen consumption. The respiration is the process of oxidizing nutrient molecules to CO<sub>2</sub> and H<sub>2</sub>O in order to synthesize ATP in the OXPHOS process. This oxygen consumption takes place in CIV.

In abnormal conditions, such as hypoxia or alterations in mitochondrial functionalism, the mitochondria are unable to consume oxygen and metabolic pathways are readjusted to continue the obtaining of reduction power and to produce energy in an anaerobic process (**Figure 4**). In these conditions, the resulting pyruvate after the catabolism of the carbohydrates is not imported into the mitochondria, albeit it is converted into lactate through the cytosolic enzyme lactate dehydrogenase.



**Figure 4.** Anaerobic metabolism in hypoxia or in case of mitochondrial deficiencies.

In case of mitochondrial abnormalities or hypoxia, the lactate concentration increases in the bloodstream, proceeding from its synthesis in skeletal muscle, liver, nervous and lymphoid system. The rank of lactate concentration in normal conditions oscillates from 0.5 to 2.4 mmols/l. However, in hypoxia conditions or in mitochondrial alterations, lactate synthesis increases in the tissues, increasing as well in the bloodstream, while the pH decreases, since the lactate is an organic acid which, at physiologic pH (7.3) releases protons and acidifies the blood.

## 1.6. REACTIVE OXYGEN SPECIES

The reactive oxygen species (ROS) are intermediate metabolites derived from the oxygen and most of them are produced in the mitochondria in case of OXPHOS dysfunction. These species are free radicals (some of their electrons are uncoupled) and they are considered highly oxidants, unstable and with the capacity to harm most of the cellular structures, such as proteins, lipids, carbohydrates and genetic material. Some examples of ROS are the superoxide anion ( $O_2^-$ ) and

the hydrogen peroxide ( $H_2O_2$ ), which are relatively stable, although hydroxyl anion ( $OH^-$ ) and peroxynitrite ( $ONOO^-$ ) are highly reactive. All of them derive from superoxide anion, which is mainly generated in complexes I and III of MRC through some redox reactions of the CoQ, which in its semi-reduced form is able to auto-oxidize and go back to its oxidized form, transferring an electron and turning the molecular oxygen into  $O_2^-$ .

There are many antioxidants, such as the superoxide dismutase (SOD), able to convert  $O_2^-$  to  $H_2O_2$ , the catalase or peroxidase is able to turn  $H_2O_2$  into  $H_2O$ , and the glutathione peroxidase which catalyzes the  $H_2O_2$ , as well as hydroperoxides (R-OOH) and the lipid peroxides (resulting from the attack of ROS to the membrane cell), into  $H_2O$ . There are many antioxidant non-enzymatic molecules such as vitamins E and C, carotenes, quinones, glutathione and other metallic elements, such as selenium, zinc, iron or copper, among others. All of them are able to reduce ROS levels. In normal conditions, all the antioxidant mechanisms minimize ROS production and thus, they act as protective systems against oxidative stress. However, in the presence of mitochondrial dysfunction, ROS increase beyond the threshold of detoxification. These ROS are able to attack all the cell structures, including mitochondria which are particularly vulnerable. The release of ROS can be associated to the presence of exogenous toxic compounds affecting mitochondria. For instance, it has been described an increase of ROS production linked to human immunodeficiency virus (Wanchu A 2009) and antiretrovirals (Manda KR 2011).

## **1.7. THE MITOCHONDRIAL MEMBRANE POTENTIAL**

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The electrons flow through the MRC produce energy release which is used for proton pumping at complexes I, III and IV levels, from the matrix to the intermembrane space. The accumulation of the protons against gradient in the intermembrane space produces a proton-motor force which impels the ATP synthesis in the complex V, through the entrance of these protons into the matrix forward gradient. As a consequence of the loss of protons and positive ions through the mitochondrial internal membrane, there is a -180 mV difference in the electric potential between both sides of the internal membrane, known as mitochondrial membrane potential ( $\Delta\psi$ ). In case of mitochondrial dysfunction it is possible a depolarization of the membrane, meaning a loss of the mitochondrial membrane potential. Eventually, this depolarization can be accompanied by the release of many apoptogenic factors from the intermembrane space: cytochrome c, second

mitochondria-derived activator of caspases (SMAC) or its homologous in mouse (DIABLO), apoptosis-inducing factor (AIF) or endonuclease G, among others.

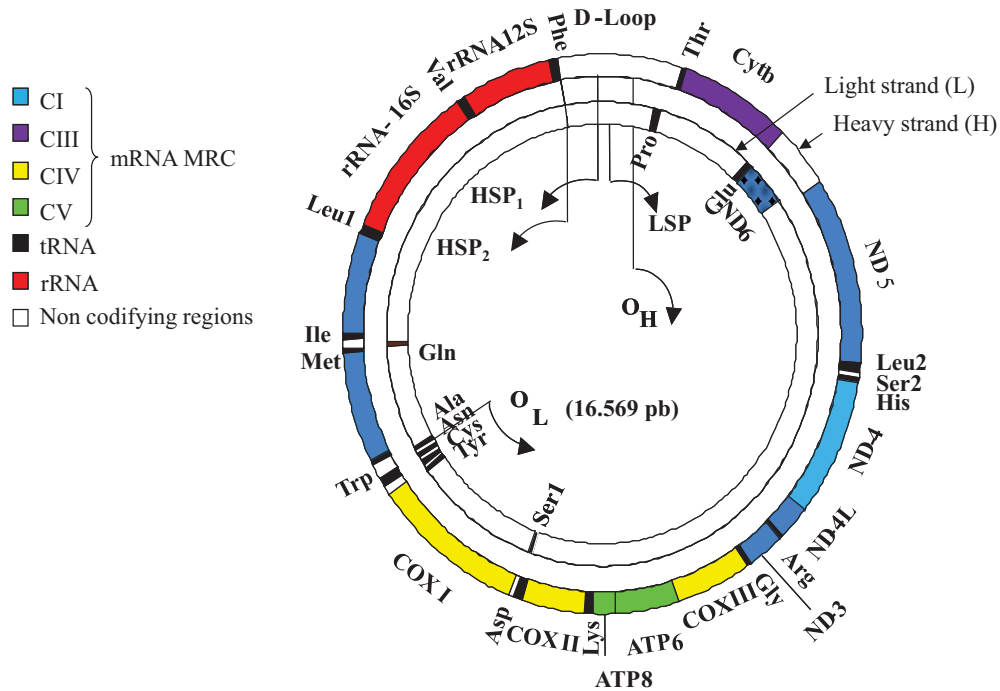
## 1.8. THE MITOCHONDRIAL GENOME

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The genetic material of the mitochondria (very similar to bacteria chromosomes) is found in the matrix, near the inner membrane, in a variable number. Its size and structure change depending on the different species. In humans, it consists of a double stranded circular DNA molecule with 16.569 base pair (bp) and it is associated to some proteins and organized in a supra-molecular structure, the nucleoids. During the past three decades, hundreds of mitochondrial genomes from animals, plants, and fungi have been characterized genetically and even sequenced. The first complete mitochondrial genome to be sequenced from any organism was that of humans (Anderson S 1981) and soon thereafter the bovine sequence was completed (Anderson S 1982).

The study of the variations in the inter-individual sequence of the mtDNA has contributed to the knowledge of the populations and the human migrations over the history of the evolution. These variations of the mitochondrial genome are generated by polymorphisms proceeding from a common ancestral mtDNA and are known as haplogroups. Eighteen out of them have been described. Some of them have been associated to the longevity, diabetes, Parkinson disease development, or sperm motility. A given mutation responsible for a mitochondriopathy may have different phenotype depending on the corresponding haplogroup.

The mtDNA contains 37 genes which transcription results in long primary transcripts that have to be processed to yield to two ribosomal RNA (rRNA) (both involved in mitochondrial translational processes), 22 transfer RNA (tRNA) and 13 messenger RNA (mRNA) which produce 13 polypeptides that constitute part of four enzymatic complexes of the OXPHOS system (CI, CIII, CIV and CV) (**Figure 5**).



**Figure 5.** Scheme of a molecule of mitochondrial genome.

The mitochondrial genes are replicated, transcribed and translated inside the matrix, through catalysis by the enzymes that are codified by nuclear genes and imported to mitochondria (Clayton DA 2000). Note that mtDNA replication needs to be coordinated with nuclear DNA and there must be a connection between both genomes (**Table 1**). Strand elongation of mtDNA is achieved by the mtDNA polymerase ( $\gamma$ ) that proceeds in the usual 5'-3' direction.

**Table 1.** Protein subunits encoded by mitochondrial genome (mtDNA) or by nuclear genome (nDNA):

Subunits	CI	CII	CIII	CIV	CV	Total
MtDNA	7	0	1	3	2	13
nDNA	~ 33	4	10	10	12	> 69
Total	~ 40	4	11	13	14	> 82

Mitochondrial DNA is polyploid. As opposite as nuclear DNA which is diploid (it presents 2 copies of each gene per cell), a mitochondrion may present from 2 to 10 molecules of mtDNA. Since in a cell there may be from 100 to 1000 mitochondria, depending on the tissue, the number of mtDNA copies oscillate from hundreds to thousands.

Mitochondrial genome is a double stranded DNA constituted by a heavy strand (H strand) and a light strand (L strand). Such difference in density is due to a higher content of guanines and thymines in the H chain. Most of the genes are encoded by the H chain, since it carries information for the subunits of CI (ND1, ND2, ND3, ND4 and ND5), CIII (cytochrome c), CIV (COXI, COXII and COXIII) and CV (ATPase 6 and ATPase 8), for the 12S and 16S rRNA and for 14 tRNA (Scheffler IE 2010).

The replication of mtDNA is not synchronized with cell division. There are two origins of replications, one for each chain. This issue is still controversial. The replication is carried out exclusively by the  $\gamma$  DNA polymerase enzyme, which is a protein constituted by two subunits that are nuclear encoded. It also carries out the exonuclease activity in order to repair mistakes in the process.

Mitochondrial DNA does not follow a genetic recombination (Elson JL 2001), even this is a controversial issue (Eyre-Walker A 2001) (Piganeau G 2004) (Scheffler IE 2010).

The organization of the genes is very well-compacted. There are no introns and all the codifying sequences are localized nearby (Anderson S 1981). Most of the structural genes which codify for proteins, present some nucleotides overlapped with the contiguous gene. Most of these structural genes lack a termination code, and this is generated by post- transcriptional polyadenylation (Scheffler IE 2010).

Mitochondrial genome contains a non codifying region from approximately 1000 bp, which is called Displacement Loop (D-loop), localized between genes tRNA<sup>Phe</sup> and tRNA<sup>Pro</sup>. This is a control region consisting of a triple helix generated in the initial replication stages. The replication origin of the heavy strand ( $O_H$ ) is located in the D-loop, as well as the promoters for the transcription of both strands (LSP or light strand promoter and HSP<sub>1</sub> and HSP<sub>2</sub> or heavy strand promoter types 1 and 2). D-loop sequence is highly variable and it is often used for evolution, migration, parental and population genetic studies.

Mitochondrial DNA is exclusively maternally inherited. The oocyte carries almost the totality of the mitochondria in the fecundation process. The spermatozoa concentrate their mitochondria in the tail to motive the head to the oocyte and finally it only brings the nucleus (Scheffler IE 2010). Even in the presence of some mitochondria proceeding from spermatozoa, it is believed that they would be eliminated by ubiquitination processes (Sutovsky P 1999). On the other hand, mtDNA is distributed at random to the filial cells by mitotic segregation during cell division.



Consequently, the cells may receive a different content of wild or mutated mtDNA coexisting in a variable percentage in a given mitochondrion, a cell or a tissue, thus generating heteroplasmy. The phenotype may change depending on the level of heteroplasmy and it is expressed according to a threshold effect, meaning the percentage of mutated mtDNA molecules necessary for a mutation to be present in the phenotype (Scheffler IE 2010). Normally the threshold effect varies around 60-80% of heteroplasmy (Lightowlers RN 1997).

It is remarkable that mitochondrial genetic code is different from the universal code, described for the nuclear genome, in six codons: UGA, AGA, AGG, AUA, AUU and AUG (Scheffler IE 2010); which, in turn, are only recognized by mitochondrial tRNA (**Table 2**).

Both H and L chain are transcribed to 3 polycistronic molecules which, after a proteolytic process, form mature mRNA, tRNA, and rRNA.

**Table 2.** Differences in codon usage in nucleus and mitochondria

<b>Codon</b>	<b>Nuclear DNA</b>	<b>MtDNA</b>
<b>UGA</b>	STOP	Trp
<b>AGG, AGA</b>	Arg	STOP
<b>AUA, AUU</b>	Ile	Met and Initiation
<b>AUG</b>	Met and Initiation	Initiation

There is a high mutation rate in mitochondrial genome, reaching from 10 to 20-fold the one of the nuclear genome. This characteristic can be due to:

- An excess of ROS, which can impair mtDNA (Scheffler IE 2010).
- The lack of histones and linked proteins which protect the structure.
- The lack of efficient repair mechanisms (Shen CC 1995) (Stierum RH 1999).

### **1.8.1. MITOCHONDRIAL DNA REPLICATION**

Replication and transcription of mtDNA are basic processes which are closely related to both the function and the biogenesis of the organelle.

γ- DNA polymerase (γ-pol) is the only enzyme responsible for mitochondrial DNA replication. It is codified by the nuclear gene POLG and it is imported to the mitochondria where it exerts its polymerase activity in the mtDNA replication and exonuclease activity in the repair mechanism process.

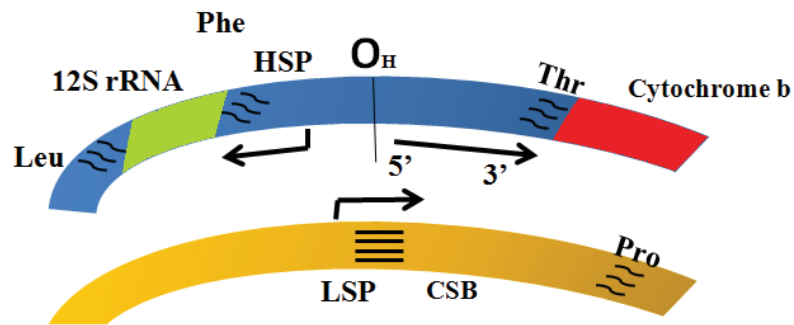


In mtDNA replication, many factors are involved (**Figure 6**), most of them are also involved in the transcriptional process, since in the initiation of the replication it is required the presence of some RNA primers which are the result of a digestion of the transcript. The RNA polymerase synthesizes and the endoribonuclease cut the primer of RNA (required for the mtDNA replication), the mitochondrial transcription factor A (TFAM, mtTFA), which enables the initiation of the transcription, the helicase (TWINKLE) and the topoisomerases I and II, necessary to add or relax a torsion in mtDNA while it forwards in the replication fork, and the mitochondrial proteins binding to the simple stranded mtDNA, named, mitochondrial single-strand binding protein (mtSSB), are all involved.

There are three postulates to explain mtDNA replication:

- a) Strand-displacement or strand-asymmetric (Clayton DA 1982). According to this theory, both strands are synthesized in one direction and continuously in  $5' \rightarrow 3'$  (as most prokaryotes). This theory supports that, as mtDNA contains two origins of replication ( $O_H$  and  $O_L$ ), the replication of mtDNA starts from the heavy chain in the  $O_H$  located at the D-loop and proceeds in only one direction  $5' \rightarrow 3'$ , continuously. For the initiation of the replication of the heavy strand, the primer is a fragment of a transcript from the light strand. Thus, the replication is asynchronous and asymmetric in both strands. Each strand initiates the replication in different times, from different origins and each fork forwards continuously  $5' \rightarrow 3'$ .
- b) Strand-coupled leading- and lagging-strand synthesis (Holt IJ 2000)
- c) Ribonucleotide incorporation throughout the lagging-strand (RITOLS) (Yang MY 2002) (Yasukawa T 2006).

The two latter theories support a bidirectional replication (as in the eukaryotes), with a continuous synthesis  $5' \rightarrow 3'$  in the heavy strand and a discontinuous synthesis  $3' \rightarrow 5'$ , using RNA primers for the formation of Okazaki fragments in the light strand. The strand-coupled leading- and lagging-strand synthesis supports the coexistence of bidirectional synthesis in coexistence with the traditional unidirectional model. However, the RITOLS theory only backs the bidirectional model.



**Figure 6.** The D-loop region of mtDNA and the flanking genes. HSP and LSP are the heavy strand and light strand promoters. A processed transcript from the LSP serves as the primer for DNA replication from the  $O_H$  origin.

### 1.8.2. MITOCHONDRIAL DNA TRANSCRIPTION

The inter-genome communication is essential for mtDNA transcription and organelle function. Many molecules involved in the mitochondrial transcriptional process are encoded by the nuclear genome (including RNA polymerase or transcription factors).

This process requires the mitochondrial RNA polymerase and the mtTFA in combination with either the mitochondrial transcription factor B1 (TFB1M) or the B2 (TFB2M) (Falkenberg M 2002). Finally, the mitochondrial transcription terminator (mTERF) is required at the end of the transcription (Daga A 1993). As opposite as the nuclear genome, the mitochondrial transcription is symmetric and complete, as well as continuous from the promoters of the transcription in the light strand (LSP) or in the heavy strand (HSP<sub>1</sub>, HSP<sub>2</sub>), located at the D-loop. Three polycistronic transcripts are originated, containing the product of the transcription of all the genes codified in the light strain (through the LSP), all the genes codified in the heavy strand (through the HSP<sub>1</sub>) or exclusively the two rRNA primers (through the HSP<sub>2</sub>). Once processed, these polycistronic transcripts will generate the different types of mitochondrial RNA: 13 mRNA, 22 tRNA and 2 rRNA. The location of the genes is alternate to ensure that, in the maturation process, the genes codifying for rRNA and mRNA are separated by tRNA. The tRNA molecules are cut and separated, thus leading to the release of mRNA and rRNA (that afterwards will be modified to become mature transcripts). In the maturation process many enzymatic activities are necessary: endonucleotidic digestion in the 3' and 5' ends of the tRNA, the addition of the CCA sequence in

the 3' end of the tRNA and the polyadenylation in the 3' end of both the rRNA and mRNA (Fernández-Silva P 2003). Most of the structural genes mRNA lack of the termination codon (codified in the mtDNA), albeit it is generated in the polyadenylation post-transcriptional process of the 3' end (Ojala D 1981). They lack of the characteristic capsule of the 5' end (Montoya J 1981) which is aggregated to the mRNA of the nuclear genome of the eukaryotes and, finally, they also lack of the untranslated regions, which are characteristics of 3' and 5' ends of the nuclear mRNA.

### **1.8.3 MITOCHONDRIAL DNA TRANSLATION**

The mitochondrial translational system in the matrix is an independent molecular machinery consisting of several molecules encoded by the nuclear and mitochondrial genomes. As most of the mitochondrial processes, there must be a proper inter-genomic coordination. Around 800-1500 proteins are found in mitochondria at a relative abundance and this amount varies from tissue to tissue. Of those, only 13 are encoded by the mitochondrial genome, but each is essential for oxidative phosphorylation. Thus, the main function of the mitochondrial translation machinery is to synthesize 13 proteins for the assembly of the OXPHOS system. Mitochondrial ribosomes and general translation factors are similar to their prokaryotic counterparts, but a closer look can identify some aspects of translation in mitochondria which differ significantly: changes in tRNA structure and use of altered codons, changes in the fine structure of ribosomes, cis-acting elements of mRNA and initiation factors. The similarities of the mitochondrial ribosomes to the prokaryotes ones make them sensitive to some antibacterial antibiotics. Thus, the antibiotics that inhibit the protein synthesis in bacteria, also inhibit mitochondrial protein synthesis becoming the causative toxic agent of mitochondrial failure. For instance, the aminoglycosides are able to produce deafness (Leroux L 1950), chloramphenicol has been related to aplastic anemia (Rich ML 1950) and Linezolid has been associated to hyperlactatemia (Palenzuela L 2005), all related to mitochondrial protein synthesis inhibition.

Mitochondrial ribosomes, as the cytoplasmic ones, are ribonucleotide complexes consisting of ribosomal proteins associated to rRNA, giving rise to two subunits: the big one presents 16SrRNA and the small subunit presents 12SrRNA. Both 12S and 16S rRNA are codified in the mitochondrial genome. However, most of the proteins (~80) constituting mitochondrial

ribosomes and the translation factors are nuclear encoded and they are translated in cytoplasmic ribosomes and imported to mitochondria (O'Brien 1996).

## 1.9. MITOCHONDRIA AND APOPTOSIS

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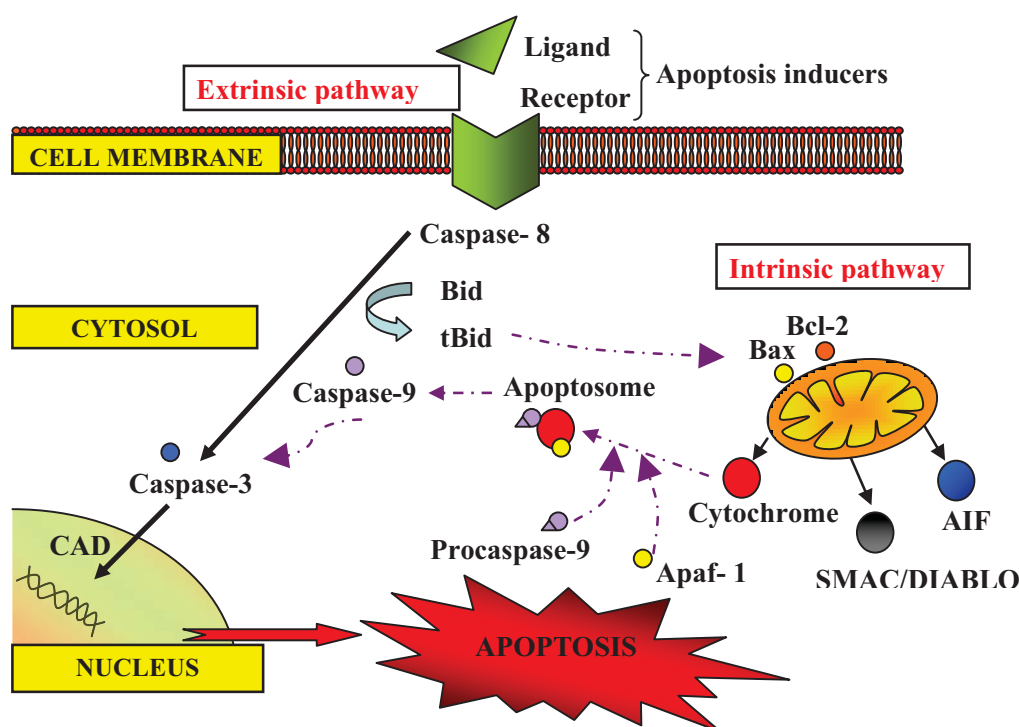
Apoptosis is the programmed cell death orchestrated by genes within the affected cell. It is a process with common genetic pathways evolved over a wide phylogenetic range. Apoptosis is a mechanism able to eliminate unwanted cells under three major circumstances: (a) development and homeostasis, (b) as a defense mechanism against genetically damaged and hence potentially tumor cells, and (c) natural senescence and aging. The phenomenon is primarily found in multicellular, eukaryotic organisms, although it has been argued that cell death may have evolved in single-celled organisms as a defense strategy (Sheffler 2010). It has become useful to subdivide the process of apoptosis into several phases or stages. The final phase encompasses a number of characteristic changes such as cell shrinkage, chromatin degradation and nuclear fragmentation, and loss of plasma membrane integrity. It appears that these changes are essential for rapid recognition of the dying cell by its neighbors, which phagocyte the remnants and efficiently eliminate any debris that would otherwise cause an inflammatory response by the immune system.

A family of cysteine proteases referred to as caspases are involved in cell death. In a global view, apoptosis is accompanied by extensive proteolytic cleavage, and there is, in fact, a cascade of such cleavages, since these caspases exist as inactive precursors (pro-caspases) that must be cleaved after a specific aspartate residue before they can be assembled into heterotetramers and activate proteases.

Mitochondria play a central role in the apoptotic process (Green DR 1998) by participating either in the extrinsic or in the intrinsic pathway (**Figure 7**). In the extrinsic pathway, the stimulus may be an external one, received by a cell surface receptor. The union of the apoptotic inducer ligand to the corresponding receptor leads to the activation of the caspase-8 in the cytosol which, in turn, activates by proteolysis the Bid proapoptotic protein, which belongs to Bcl-2 protein family. Bcl-2 proteins can be classified as either pro-apoptotic or anti-apoptotic. Truncated Bid protein (t-Bid) is translocated to the mitochondria where it interacts with other proapoptotic members of Bcl-2 family, as Bax protein. Subsequently, the apoptotic process continues in mitochondria,

where it converges with the intrinsic pathway. The activated caspase-8 can also directly activate other caspases, such as effector caspase 3, and induce apoptosis without any mitochondrial participation. However, mitochondrial implication in extrinsic apoptosis enhances the activation signals of the cell death, initiated by caspase-8, through the release of mitochondrial proapoptotic molecules. In the intrinsic pathway, regulated by mitochondria, the stimulus is internal, as the result of the action of a drug, toxin, radiation damage, nourishment shortage and, in general, a given stress situation. Of course, drugs, toxins, radiation, etc, are also ultimately external influences, but the difference between these two mechanisms, extrinsic and intrinsic, becomes apparent during the following phase: the signal transduction (Lindsay J 2011).

Active, healthy mitochondria exhibit a transmembrane potential ( $\Delta\psi_m$ ) across the inner mitochondrial membrane. A pH and electrochemical gradient across the inner membrane is set up as the result of electron transport through the ETC, with an excess of positive charge on the outside, that is, in the intermembrane space. In cells undergoing apoptosis, a fall in  $\Delta\psi_m$  is observed as one of the relatively early events in many different cell types, prior to DNA fragmentation. When the induction of apoptotic process occurs, there are changes in the ratio of proapoptotic (e.g. Bax, Bak, t-Bid, Bim, Bad and Bik) and antiapoptotic proteins (e.g. bcl-2). This ratio regulates the permeability of the outer mitochondrial membrane and its imbalance leads to the breakdown of the  $\Delta\psi_m$  which, in turn, involves the so-called mitochondrial “permeability transition”, which is the result of the opening of a permeability transition pore (PTP). A massive opening of such pores can collapse  $\Delta\psi_m$ , stop OXPHOS, arrest import of proteins into mitochondria and induce leakage of cytochrome c and other mitochondrial apoptogenic proteins, such as SMAC-DIABLO, out into the cytosol. On the other hand, damaged mitochondria release an apoptogenic protein, apoptosis inducing factor (AIF), which is a 50 kDa intermembrane protein, with protease activity but no nuclease activity. Cytochrome c binds the cytosolic protein APAF-1 which, in turn, interacts with procaspase-9 to form a multiprotein complex so-called apoptosome. The apoptosome induces the activation of caspase-9 by proteolysis, and caspase-9 activates effector caspase-3. This induces an irreversible activation of a cascade of reactions catalyzed by caspases to activate, finally, a caspase activated DNase protein (CAD) which, at the end, will cleavage nDNA leading to the cell death.



**Figure 7. Apoptosis pathways. Extrinsic pathway.** The ligand inducer of apoptosis binds to its receptor. Activation of **caspase-8** protein and cascade of reactions catalyzed by caspases (cell cysteine proteases). Action of DNase protein activated by caspase (**CAD**) which degrades DNA and induce cell death. Caspase-8 also activates by proteolysis the **Bid** protein in its truncated form (**tBid**) which interacts in mitochondrial with **Bax** protein or other proapoptotic members of the family Bcl-2. Subsequently the process converges with the intrinsic pathway and the mitochondria acts as an enhancer. **Intrinsic pathway.** A signal within the cell produces changes in the proapoptotic/antiapoptotic ratio of the Bcl-2 family proteins. Some proapoptotic compounds are released: Apoptosis inducing factor (**AIF**), which activates apoptotic reactions, not dependent of caspases, the Second mitochondria derived activator (**SMAC**) or its homologous in mouse (**DIABLO**), **cytochrome c**, which binds to the apoptotic proteases activator factor (**APAF-1**) and to the **procaspase-9** to give rise to the **apoptosome**. This activates **caspase-9** and the process continues with the cascade reactions of the caspases, as above mentioned.

## 1.10. MITOCHONDRIAL DISEASES

Mitochondrial diseases are those associated to alterations in the organelle and normally they are reflected in an alteration of the mitochondrial parameters, often leading to mitochondrial dysfunction. They are referred as mitochondriopathies and include a wide spectrum of

pathologies. Most of the systems in our organism can be a target for mitochondrial diseases, as all human cells contain mitochondria, except erythrocytes.

The knowledge about mitochondrial diseases has increased substantially and, currently, some treatments such as vitamins, cofactors or final electron acceptors have been administered in order to minimize the manifestations (Pedrol E 2005) (Artuch R 2006).

Mitochondriopathies are classified in two groups, the genetic mitochondrial diseases (those which are inherited or spontaneous in the absence of a toxic compound); and those which are acquired by the presence of exogenous toxic compounds.

### **1.10.1. GENETIC MITOCHONDRIAL DISEASES**

In 1962 Luft disease, related to an uncoupled OXPHOS system, was described for the first time and this fact represented a first approach to mitochondrial diseases (Luft R 1962).

Nowadays, more than 50 of these mitochondriopathies have been characterized and, in general, the mitochondrial disorders have been well-established and classified (Andreu AL 2003). These mitochondrial diseases result from punctual mutations, deletions, insertions, duplications and different levels of depletion of mtDNA (**Figure 8**): (1) Currently, around 200 punctual mutations in mtDNA have been identified (<http://mitomap.org>). They are associated to many pathological phenotypes and they either affect structural genes that encode polypeptides of the OXPHOS system, or genes that encode mitochondrial tRNA and rRNA (Zeviani M 1997): (a) myo-encephalopathy, lactic acidosis and stroke-like episodes (MELAS), (b) myoclonic epilepsy with ragged red fibers (MERRF), (c) mitochondrial neurogastrointestinal encephalopathy (MNGIE) (González-Vioque E 2011), (d) neuropathy, ataxia and retinitis pigmentosa (NARP) or (e) Leber's hereditary optic neuropathy (LHON) are some of the best known (Andreu AL 2003). Most of them are maternally inherited, however, some cases can be sporadic. (2) More than 100 deletions and approximately (3) 17 duplications or insertions of a given fragment of mtDNA have also been identified. Simple deletions and duplications are considered as sporadic rearrangements which are generated during embryogenesis, although in some cases they can be present at oocytes and, thus, they can be maternally transmitted (Chen X 1995). Normally they are associated to three pathological phenotypes: Kerns Sayre syndrome (Zeviani M 1988), progressive external ophthalmoplegy (PEO) (Moraes CT 1989) and Pearson syndrome

(Casademont J 1994) (Odoardi F 2003). Due to the high compactness of mitochondrial genome, multiple or simple deletions, imply a total or partial loss of mitochondrial genes. Multiple deletions, responsible for many mitochondrial diseases, are also associated to alterations in the inter-genomic communications, interfering in mtDNA integrity. A decrease of mtDNA molecules, known as mitochondrial genome depletion, is also possible. This decrease of the number of mtDNA copies may trigger a dysfunction in the OXPHOS system, leading to a cell disability in obtaining energy. Many patients presenting mtDNA depletion manifest hypotonia and lactic acidosis or hepato-renal dysfunction at the first days of life (Tritschler HJ 1992) (Morris AA 1998). Severe depletions may cause death at the first years of life, oscillating from 70 to 98% in the most severe cases of depletion. Depletion, as some deletions, may be caused by a defect in the inter-genomic communication between nucleus and mitochondria. It is possible that a mutated nuclear gene, involved in mitochondrial biogenesis, interferes in the regulation of the amount of mtDNA (Taanman JW 1997) (Bodnar AG 1993). A given defect in the replication process may also lead to alterations in mtDNA copy number (Hirano M 2000). For instance, 54 mutations affecting the nuclear gene POLG have been described. The nuclear gene POLG codifies the catalytic subunit of  $\gamma$ -pol, which is the only enzyme responsible for the mtDNA replication and repair. The consequent alterations of a defect in this POLG nuclear gene are the presence of either punctual mutations, deletions or depletions of the mtDNA (**Figure 8**) (Andreu AL 2004).

A mutation in mtDNA may lead to different phenotype variants and different molecular affections may lead to the same clinical manifestation. At a histological level, mitochondrial alterations may appear in muscle tissue as huge mitochondria, accumulated at subsarcolemic levels, known as ragged red fibbers (RRF) (Andreu AL 2003). It also is possible to detect lipid inclusions in the affected cells, or to detect function alterations of the mitochondria through immune-histochemical reactions with cytochrome c oxidase, known as COX negative cells, and an increase of succinate dehydrogenase reaction, known as SDH positive cells.

In normal conditions, during oogenesis, the number of mitochondria increases while the number of mtDNA molecules does not, thus, leading to a bottleneck phenomena in the mitotic segregation of the mtDNA molecules (Marchington DR 1998). The consequence is a considerable decrease of the ratio mtDNA/mitochondrion to 1 unit. This fact reduces enormously the mitochondrial genetic variability of the next generations, who will receive low mtDNA



content. Thus, most of the oocytes are homoplasmic, with normal mtDNA as well as the genotype of the individual at birth. However, occasionally there may exist a mutated mtDNA subpopulation in the germinal line. Then the mitotic segregation of mitochondria is carried out at random during cell division. When heteroplasmy is present at embryogenesis, the mitochondrial segregation (at random) generates differences in the percentage of mtDNA mutated molecules among in the different tissues, while, when the heteroplasmy is present at the individual development, there are differences among the cells of a given tissue.

There is an important variability in the clinical manifestations related to mitochondrial defects.

The clinical heterogeneity associated to mitochondrial diseases is due to some of the mtDNA characteristics and specific tissue properties: the heteroplasmy, differences in the energetic requirements of the different tissues, in the mutation distribution of the different tissues and tissue-dependent capacity to eliminate abnormal mitochondria.

In general, the phenotypic variability of genetic mitochondriopathies is due (among others) to the heteroplasmy, the threshold effect (it is necessary to pass a given number of mutated molecules to manifest the phenotype), the mitotic segregation (at random) and the nuclear genome.

Mitochondriopathies can affect many physiologic systems, overall, those of high energy requirements, that is, a high mitochondrial activity for their normal function, such as nervous, muscle, endocrine or audio-visual systems. In post-mitotic tissues, such as skeletal muscle, nervous cells and pancreas, which have evolved to a  $G_0$  phase due to their high specialization characteristics, the capacity of discard abnormal mitochondria is reduced, so that this kind of tissues may be affected by mitochondrial defects as well. In this sense, the most common clinical manifestations derived of mitochondrial impairment are neuromuscular alterations, deafness, short size, blindness, pancreatitis, diabetes, hepatopathies, cardiomyopathies, lactic acidosis, or exercise intolerance (Andreu AL 1999) (DiMauro S 2001), among others.



described in HIV infected patients. The association of AZT drug and the inhibition of  $\gamma$ -pol was discussed for the first time (Dalakas MC 1990) (Herzberg NH 1992).

The similarities between secondary adverse events of some treatments or the clinical manifestations derived from some intoxications and the symptomatology of genetic mitochondrial diseases, lead to the association of a mitochondrial etiopathogenesis in both toxicity and secondary effects of a given therapy.

The mitochondrial lesion induced by an exogenous compound may affect different levels, such as the mtDNA replication, mtRNA transcription, mitochondrial translation processes, enzymatic activities of the MRC, oxygen consumption, ROS production, mitochondrial membrane potential and mitochondrial biogenesis. These alterations may appear as an isolated effect derived from a specific lesion mechanism. However, a continuous exposition to the toxic agent may trigger a global lesion and this fact could represent a threat for the cell viability. Otherwise, it is possible that, when the exogenous toxic exposition is interrupted, the mitochondrial alterations are recovered, particularly in non postmitotic cells.

Additionally, mitochondrial implication in most pathologies has been widely proposed. Some of these diseases are schizophrenia (Ben-Shachar D 2004) (Rosenfeld M 2011) (Verge B 2011), Alzheimer's dementia (Rodriguez-Santiago B 2001) (Zhu X 2004) (Casademont J 2005) (Coskun P 2011) and other neuropsychiatric disorders (Marazziti D 2011), cancer (Herrmann PC 2003) (Mallery SR 2004) (Erol A 2005) (Fogg VC 2011) (Kamp DW 2011), inflammation processes (Grau JM 1994) (Kamp DW 2011), amyotrophic lateral sclerosis (Vijayvergiya C 2005) (Cozzolino M 2011), Parkinson's disease (Cardellach F 1993) (Coskun P 2011), multiple sclerosis (Andrews HE 2005) (Campbell GR 2011), some forms of diabetes mellitus type II (Lowell BB 2005) (Yang H 2011), and ageing (Grau JM 1994) (Barrientos A 1997) (Singh KK 2004) (Green DR 2011), among others.

## **2- HUMAN IMMUNODEFICIENCY VIRUS (HIV)**

### **2.1. HISTORY OF HIV**

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Some relevant dates are remarkable in the history of HIV:

- 1981- The first cases of human immunodeficiency virus type 1 (HIV-1) infection arose in USA, in a series of young homosexual men communities (Gottlieb MS 1981). The clinical symptoms were similar in all cases: Kaposi's sarcoma, opportunistic infections such as pneumonia caused by *Pneumocystis carinii*, *Candida albicans*, and lymphadenopathies (Centers for Disease Control, CDC 1981). A deficit in the immune system, caused by a decrease of CD4+ T-lymphocytes, resulted in the death of the patients. The disease was described as acquired immune deficiency syndrome (AIDS). Soon after the infection spread out to many different social groups and subsequently it was considered as a pandemic by the World Health Organization.
- 1982- Four cases of AIDS arose in lactants, surely infected by mother-to-child transmission (MMRW, 1982).
- 1983- The essence of the risk groups suggested an infectious agent of blood and sexual transmission (Francis DP 1983). The epidemiologic and immune-pathogenic studies, first suggested a retrovirus as the causal agent of the infection. Indeed, in 1983 HIV-1 was identified by the French group, headed by Montagnier, as the retrovirus responsible of the infection (Barre-Sinoussi F 1983). These investigations were based in previous studies about the development of tumors derived from retrovirus in the decade of 1960. For instance, the discovery of the reverse transcriptase enzyme (RT), present in all retrovirus (Temin HM 1970) (Baltimore D 1970), made possible the development of sensitive techniques for virus detection in cultured cells by Gallo R. Initially, it was believed that HIV belonged to HTLV family (human retrovirus causing T-cell leukemia and it was named HTLV-III), since both present a similar transmission pattern and they mainly infect T-cells. However, later on, it was confirmed that this retrovirus differed from HTLV family and belonged to Lentivirus genus.
- 1984- HIV was isolated and its presence was demonstrated in patients developing AIDS and in asymptomatic patients belonging to risk groups (Levy JA 1984). These observations lead to the knowledge of the so-called window period, in which the virus is incubated for months (even years) before the clinical manifestations are plausible.

- 1985- Development of a test to detect HIV antibodies in blood (Weiss SH 1985) (Sarngadharan MG 1984) (Safai B 1984).
- 1986- Isolation of HIV type-2 in patients from West Africa. This HIV-2 variant also causes AIDS, although it is less frequent and virulent and its transmission is more difficult.
- 1987- Introduction of ZDV as the first ARV drug against HIV infection, which inhibits the RT of the virus.
- Currently- Even the efforts and the achieved goals in all the fields regarding of HIV infection, the epidemic is still increasing. An effective, organized worldwide response against AIDS is required, including better prevention programs, ARV drugs development, more and better distributed economic resources, dis-stigmatization of the disease, consciousness about the importance of such an epidemic, also in the governmental classes, and universal access to low cost ARV drugs (Sepkowitz KA 2001). The establishment of latently infected CD4+ T-lymphocytes represents a reservoir of HIV infection that is not appreciably affected by effective ARV chemotherapy. Unless an approach to purging this reservoir is identified, effective management of HIV infection requires lifelong treatment. Although substantial insights about the latent reservoir have been made, our understanding about the details of the latency mechanism and potential targets to eliminate latently infected cells is too primitive to achieve a cure without a great deal of basic research to elucidate some of these areas (Richman DD 2011).

## **2.2. ORIGIN OF HIV**

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Although the accurate origin of HIV has not been completely elucidated, it is believed that HIV proceeds from a virus which causes immunodeficiency in African monkeys, the simian immunodeficiency virus (SIV) (Gao F 1999). Curiously, in the same area, new cases of AIDS were also detected. Due to the similarity of SIV and HIV, it is considered that the latter proceeds from the former. The existence of two types of SIV that co-infected simultaneously a given population of common chimpanzees gave rise to a genetic exchange of both and the origin of HIV. Probably, the transmission from monkeys to humans was possible through the blood contact derived from the hunting of such species.

It is believed that the HIV was first transmitted to humans around 1930 and afterwards the apparition of different HIV subtypes took place (Cohen J 2000) (Hooper E 2000).

### 2.3. CHARACTERISTICS OF HIV

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Human immunodeficiency virus is a member of the genus *Lentivirus*, part of the family *Retroviridae* (International Committee on Taxonomy of Viruses 2002). HIV is present in the bodily fluids as free virus particles and within infected immune cells causing AIDS, since it mainly infects CD4<sup>+</sup> T-cells, macrophages and dendritic cells in order to carry out its vital cycle. HIV kills CD4<sup>+</sup> T-lymphocytes by three mechanisms: (a) by direct viral killing of infected cells, (b) by increasing rates of apoptosis in infected cells and (c) by killing of infected CD4<sup>+</sup> T-cells by CD8 cytotoxic lymphocytes that recognize infected cells. When CD4<sup>+</sup> T-cell numbers decline below a critical level, cell-mediated immunity is lost, and the organism becomes progressively more susceptible to opportunistic infections.

There are two types of HIV: HIV-1 and HIV-2, homologous in 40-60% of proteins. Most cases of HIV infection are caused by HIV-1, since HIV-2 is largely confined in West Africa, is much less frequent and less infective and virulent than the former. In this Thesis, only HIV type 1 is considered.

HIV-1 is, in turn, subclassified in different subtypes with specific geographical distributions (Osmanov S 2002): A (West, East and Center Africa, East Europe), B (North and South America, Europe, East Asia), C (South Africa and South Asia, Etyopia), D (East Africa) and E (Southeast Asia).

Infection of HIV occurs by the transfer of blood semen, vaginal fluid, pre-ejaculate or breast milk.

HIV is transmitted by:

- Parenteral transmission: through direct contact with blood of an infected person. E.g. blood transfusion, organ transplant or contaminated needles, among others.
- Sexual transmission: through direct contact with blood and seminal fluids of an infected person.
- Vertical/perinatal transmission: from the infected mothers to their newborns during pregnancy, delivery or breastfeeding. In the absence of treatment, the transmission rate up to birth is around 25%. However, the cesarean section, the ARV treatment and the avoidance of breast feeding have dramatically reduced this risk to less than 1% (Coovadia H 2004).

## 2.4. STRUCTURE OF HIV

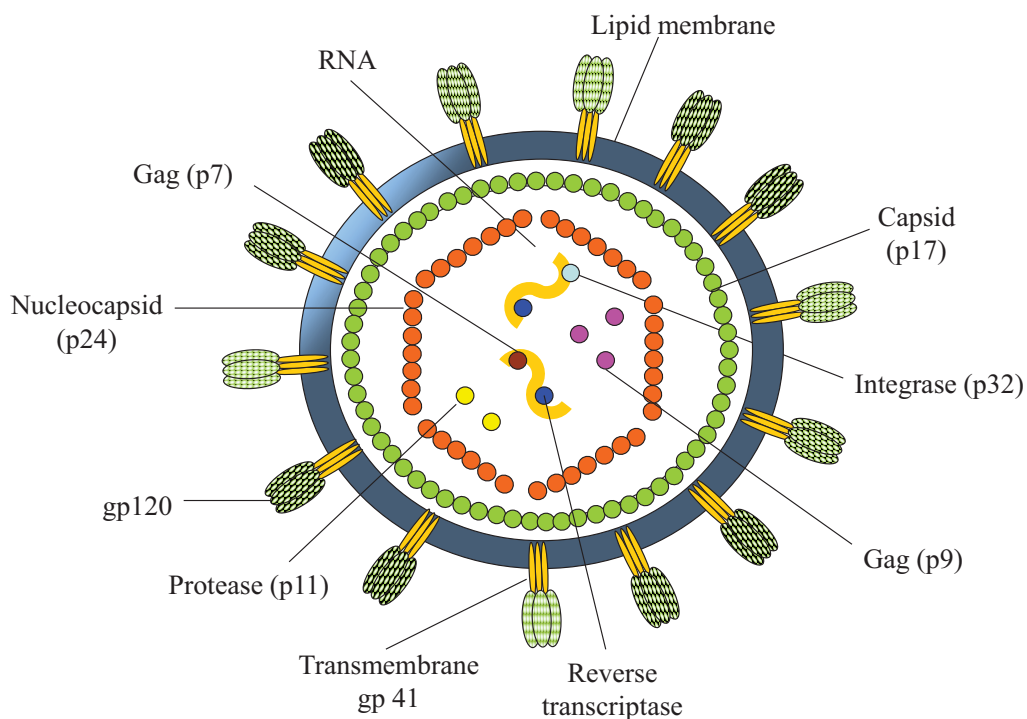
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Human immunodeficiency virus is a spherical particle of 120 nm of diameter. Its structure is constituted by three layers (**Figure 9**):

- **Lipid envelope:** This is the most external enfolding bilayer of phospholipids, which has been taken from the host infected cell. Thus, it contains class I and II histocompatibility antigens and some adhesion molecules which facilitate the contact with target cells. It also contains 72 copies of a viral glycoprotein (gp) complex, which is called Env. Env protrudes through the surface to the exterior. These gp complexes are constituted by a cap of three gp120 and a stem of three gp41, anchored to the viral envelope molecules. It enables the virus to attach and fuse the target cells to initiate the infectious cycle. Both surface proteins have been considered as possible targets of future treatments or vaccines (National Institute of Health 1998).

- **Capsid or matrix:** Spherical intermediate structure constituted by protein p17.

- **Nucleocapsid or core:** Icosahedral internal structure, constituted by protein p24. It contains the viral genome, the nucleoproteins p9 and p7, and the required machinery for the viral replication (reverse transcriptases, integrases and proteases).



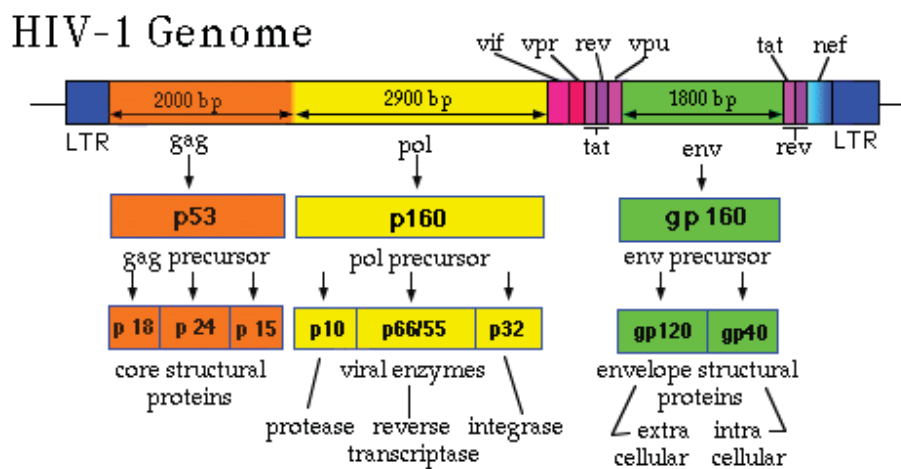
**Figure 9.** HIV structure.

## 2.5. VIRAL GENOME

The HIV genome is constituted by two identical molecules of single stranded RNA (9.5 kb each) located at the core. As a retrovirus, its RT enzyme converts viral RNA into proviral double stranded DNA.

HIV genome is characterized by a high mutation rate, due to the mistakes of RT enzyme during the retro transcription from RNA to DNA and to the recombination capacity of the different viruses that may coexist within a cell. Consequently, HIV presents a high genetic variability, which hampers both the defense response of the immune system and the creation of an effective vaccine against the virus.

HIV RNA is constituted of 9 genes (*gag*, *pol*, *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu*, and sometimes a tenth *tev*, which is a fusion of *tat* *env* and *rev*), encoding 19 proteins. Three of these genes (*gag*, *pol* and *env*) encode for structural proteins (**Figure 10**). The six remaining genes are regulatory genes that control the ability of HIV to infect cells (**Table 3**).



**Figure 10.** LTR-long terminal repeats; repetitive sequence of bases. **Gag**-group specific antigen gene, encodes viral nucleocapsid proteins: p24, a nucleoid shell protein, MW=24000; several internal proteins, p7, p15, p17 and p55. **Pol**-polymerase gene; encodes the viral enzyme, protease (p10), reverse transcriptase (p66/55; alpha and beta subunits) and integrase (p32). **Env**-envelope gene; encodes the viral envelope glycoproteins gp120 (extracellular glycoprotein, MW=120 000) and gp41 (transmembrane glycoprotein, MW=41000). **Tat**: encodes transactivator protein. **Rev**: encodes a regulator of expression of viral protein. **Vif**: associated with viral infectivity. **Vpu**: encodes viral protein U. **Vpr**: encode viral protein R. **Nef**: encodes a 'so-called' negative regulator protein. Source: EEB Web Site/HIV Lab-Site Maintained by Jeff Huckaby, Yale University.



The list of HIV genes and their corresponding function is detailed below:

**Table 3.** HIV genes.

GENES	PRECURSOR	PROT	FUNCTION
<i>Gag</i>	p55	p170	Structural protein of the matrix
		p24	Structural protein of the nucleocapsid
	p15	p9	Structural protein of the nucleocapsid
		p7	Structural protein of the nucleocapsid, associated to viral RNA
<i>Pol</i>	p190	p11	Protease
		p13	RNase
		p51, p66	Reverse transcriptase
		p32	Integrase
<i>Env</i>	gp160	gp120	Extracellular protein, enables the fusion HIV-cell
		gp41	Transmembrane protein, enables the fusion HIV-cell
<i>Tat</i>		p14	Transcriptional activators
<i>Rev</i>		p18	Regulates the expression of structural genes
<i>Nef</i>		p27	Necessary for viral replication and progression of the disease
<i>Vif</i>		p23	Regulates the infectivity of HIV
<i>Vpr</i>		p15	Weak transcriptional activator
<i>Vpu</i>		p16	Regulates maturation and release of viral particles

## 2.6. HIV REPLICATION CYCLE

Like all obligate intracellular pathogens, HIV-1 extensively utilizes the host machinery during replication. Since only a reduced number of viral proteins are encoded by the HIV-1 genome, HIV-1 must take advantage of multiple host cell functions in order to successfully replicate (Friedrich BM 2011).

When HIV enters into the target cell, the viral RNA genome is converted into double-stranded DNA by the virally encoded RT that is transported along with the viral genome in the virus particle. The resulting viral DNA is then imported into the cell nucleus and integrated into the cellular DNA by a virally encoded integrase and host co-factors (Smith JA 2006). Once integrated, the virus may become latent, allowing the virus and its host cell to avoid detection by the immune system. Alternatively, the virus may be transcribed, producing new RNA genomes and viral proteins that are packaged and released from the cell as new viral particles that begin the replication cycle. Thus, the viral cycle is divided in four stages (**Figure 11**):

- **Fusion and entry of HIV:** The contact HIV-host cell is mediated by the binding of both the viral gp120 and CD4 receptor of the host cell (Dalgleish AG 1984). Glycoprotein 120 suffers a conformational change and interacts with a co-receptor causing, in turn, conformational changes in viral gp41, which enters in the cell membrane. This is a key step required for the fusion. Subsequently, HIV fusions with the target cell membrane and release its genetic material (viral RNA) into the cytoplasm of the cell, together with viral proteins.

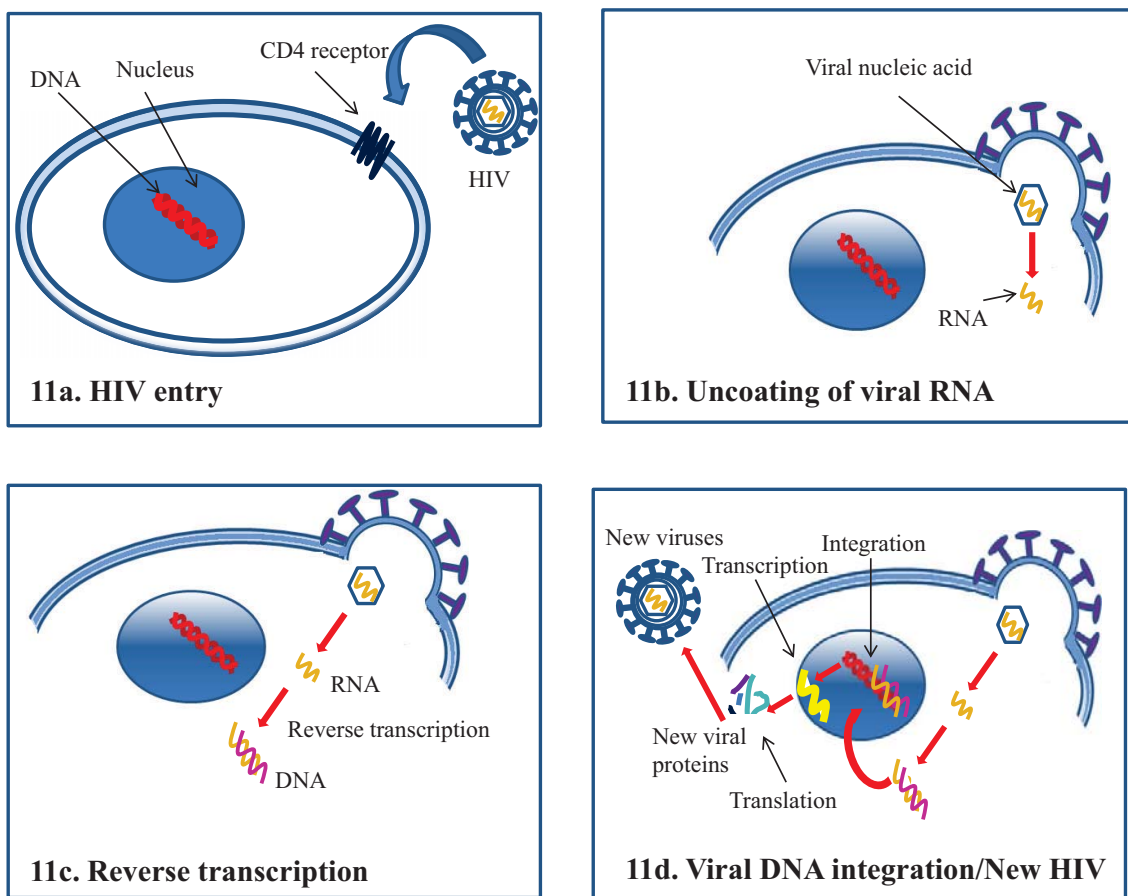
- **Reverse transcription and integration of proviral DNA:** Single stranded RNA is converted into double stranded DNA through the viral RT enzyme activity. This RT enzyme consists of three domains: DNA polymerase-RNA dependent, RNase and DNA polymerase-DNA dependent. Reverse transcription is initiated by the binding of a tRNA primer to the viral RNA. Then the first complementary proviral DNA strand is synthesized, viral RNA is degraded by ribonuclease H of the RT and the second proviral DNA strand is synthesized. Reverse transcriptase does not present any proof reading activity, giving rise to a high mutation rate and an important genetic variability of HIV.

The viral proteins help double stranded proviral DNA to reach the nucleus and it integrates into the cell genome, through integrase enzyme of the virus. In case of entrance of HIV into a quiescent cell, the proviral DNA will accumulate in the cytoplasm without any integration, leading to a latency situation. The latent provirus existing as a reservoir within the quiescent cells enormously hamper the finding of an effective endogenous system or treatment against HIV, since they can avoid the immunological and exogenous control. Moreover, the available treatments are not able to destroy latent provirus.

-**Expression of the viral genome:** Once proviral DNA has integrated into the nuclear genome of the target cell, some viral proteins, together with cellular transcription factors, such as nuclear factor kappa-light-chain enhancer of activated B cells  $\kappa\text{B}$  (NF-  $\kappa\text{B}$ ) induce the replication and transcription of the viral genome. Initially, the transcription leads to the synthesis of the regulatory proteins of the HIV (Tat, Rev and Nef). Tat binds to the transactivation response element (TAR), located in the long terminal repeat (LTR) of the viral genome and induces transcription, resulting in the synthesis of viral mRNA. The mRNA is produced as a unique transcript which is transported to the cytoplasm and will be processed in many different RNA of different size. This conversion from big precursor molecules of mRNA into small active

functional molecules is mediated by post-translational proteolysis mediated by the protease of HIV. This step is necessary for the generation of viral particles with infective capacity.

- **Assembly and new viral particles:** All the functional viral compounds assemble giving rise to new viral particles that are released by a budding process into the blood stream in order to infect other cells. Life span of HIV in plasma is of six hours. To keep a constant viral concentration in the organism,  $10^9$ - $10^{10}$  new viral particles are produced in a day. This fact hampers the finding of an effective treatment against the virus.



**Figure 11.** (11a) HIV entry. HIV binds to an area of the cell membrane. (11b) After merging with the cell membrane, the virus removes the outside protein coat and exposes its nucleic acid. (11c) To get HIV nucleic acid into the form where the human cell machinery can copy it, HIV must convert its RNA into DNA in the reverse transcription process. (11d) The viral DNA is integrated into the human DNA. This is then transcribed into RNA and translated into viral proteins.

## 2.7. TROPISM OF HIV

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The term tropism refers to which cell types the virus infects. HIV mainly affects CD4<sup>+</sup> T cells, although it can also affect to other great number of cells: macrophages, monocytes, CD8<sup>+</sup> T-lymphocytes, B-lymphocytes, natural killers, dendritic cells, endothelial cells, gastrointestinal epithelial cells, fibroblasts or microglia cells among others. The entry to the target cells is mediated through the interaction of the surface proteins gp120 and the CD4 molecule on the target cells and also with the chemokine co-receptors (Chan DC 1997). The principal co-receptors of the host cell that interact with HIV are two: CCR5 and CXCR4 and they define three types of HIV strains. HIV that use only the CCR5 receptor are called R5; those that use only CXCR4 are called X4, and those that use both, X4R5.

- **X4 strains** of HIV are lymphotropic (T-tropic), also known as syncytia-inducing strains, since they induce the formation of multinuclear giant cells resulting from the fusion of an infected cell, through gp120 of the surface, and the CD4 molecules of the non infected cells. They replicate in primary CD4<sup>+</sup> T-cells and use the  $\alpha$ -chemokine receptor, CXCR4, for entry. They appear during the progression, in the advanced stages of the infection (Feng Y 1996) (Connor RI 1997) (Coakley E 2005).

- **R5 strains** of HIV-1 are macrophage, monocytophagic (M-tropic) or non-syncytia-inducing strains. They use the  $\beta$ -chemokine receptor CCR5 for entry and are able to replicate in macrophages, CD4<sup>+</sup> T-cells, monocytes and dendritic cells (Deng H 1996) (Coakley E 2005). This CCR5 co-receptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype. Indeed, macrophages play a key role in several critical aspects of HIV infection. They appear to be the first cells infected by HIV and perhaps the source of HIV production when CD4<sup>+</sup> T-cells become depleted in the patient.

- **Dual-tropic** HIV-1 strains are able to use both CCR5 and CXCR4 as co-receptors for entry.

The natural ligands of CCR5 co-receptor are: (a) the macrophage inflammatory receptor 1 $\alpha$  (MIP-1 $\alpha$ ) chemokine, (b) MIP-1 $\beta$  and (c) regulated upon activation normal T expressed and secreted (RANTES). All of them are peptides produced by CD8<sup>+</sup> T-cells which are able to block the entrance of M-tropic R5 strains of HIV into CD4<sup>+</sup> T-cells (Cocchi F 1995). On the other hand, the natural ligand of the CXCR4 co-receptor is the stromal cell-derived factor type 1 (SDF-1), which inhibits the entrance of T-tropic R4 HIV strains into the T-cells.

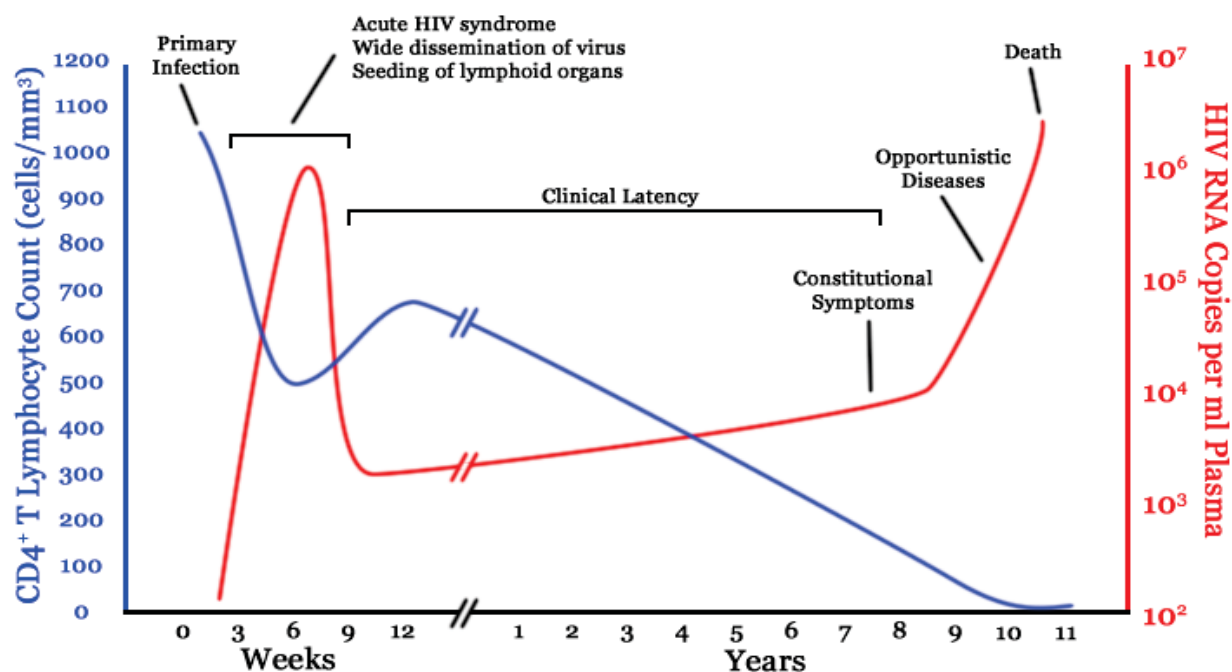
A given polymorphism, mutation or genetic characteristic, may offer a resistance against HIV infection or its progression. For instance, homozygosis in a deletion of 32 bp in the gene that codifies for CCR5 co-receptor is related to a natural resistance against HIV infection (Liu R 1996). Heterozygosis in the same deletion is related to long term non progressors (LTNP) (a group of HIV infected population who control the infection for many years without ARV drugs producing neutralizing antibodies more frequently than other HIV patients) (Braibant M 2006) as it is associated with a slow progression of the infection (Michael NL 1997).

## 2.8. SYMPTOMS AND PROGRESSION OF THE DISEASE \_\_\_\_\_

HIV infection is associated with a progressive decrease of the CD4<sup>+</sup> T-cell count and an increase in viral load. The stage of infection can be determined by measuring the CD4<sup>+</sup> T-cell count and the viral load of the patients.

The stages of HIV infection are: (a) acute infection (also known as primary infection), which lasts for several weeks and may include symptoms such as fever, lymphadenopathy, pharyngitis, myalgia or mouth and esophageal sores. (b) the latency stage involves few or no symptoms and can last from two weeks to twenty years or more and (c) AIDS, defined by low CD4<sup>+</sup> T-cell counts (less than 200/ $\mu$ l), increased viral loads, various opportunistic infections and cancers (**Figure 12**).

A small population of HIV-1 infected patients, classified as HIV controllers or LTNP, is able to retain high levels of CD4<sup>+</sup> T-cells without ARV therapy. However, most have detectable viral load and, without ARV drugs, the infection will probably progress to AIDS, albeit more slowly than normal progressors. Otherwise, there is a small percentage of HIV-1 infected population who maintains CD4<sup>+</sup> T-cells and have undetectable viral load without treatment. They are known as elite controllers or elite suppressors (ES).



**Figure 12.** Normal progression of HIV infection. Source: Image Hiv-timecourse.png, Jurema Oliveira 2005. Category: AIDS [Category: Infectious Diseases].

## 2.9. EPIDEMIOLOGY AND GLOBAL TRENDS IN CHILDREN

The development of an effective therapy for HIV infection has substantially reduced the death rate and has converted HIV infection in a chronic disease in those areas where these drugs are available (Palella F 1998) (Steinbrook R 2001). However, as the life expectancy of people with HIV has increased in countries where ARV are widely used, the continuing spread of the disease has increased the number of people living with HIV (**Table 4**) (**Figure 13**).

- 1999- 2005- More than 25 million people were killed by AIDS.
- 2003- 5 million newly infected people, from which 630,000 were children.
- 2009- 33.3 million people with HIV, 1.8 million AIDS-related deaths and 2.6 million new infections took place.

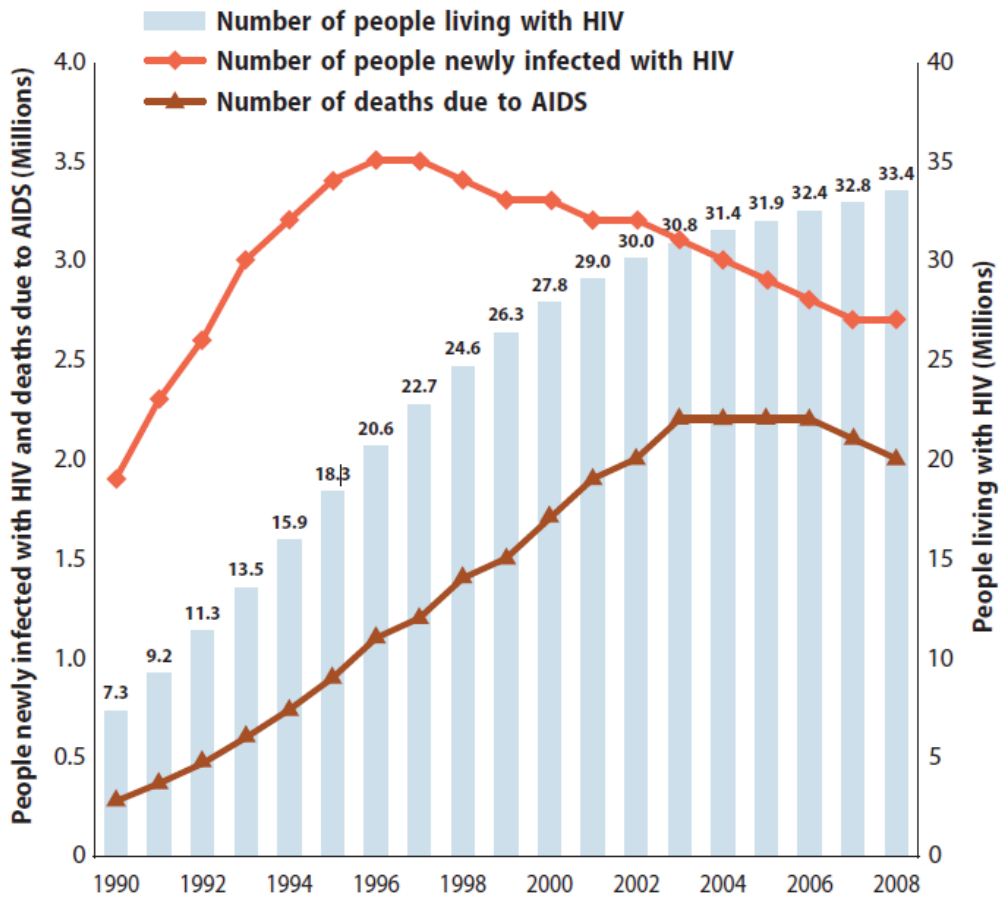
**Table 4.** Epidemiological data of HIV and AIDS.

<b>YEAR</b>	<b>1997</b>	<b>2004</b>	<b>2009</b>
New infections	3.2 x10 <sup>6</sup>	3 x10 <sup>6</sup>	2.6 x10 <sup>6</sup>
People with HIV	22.7 x10 <sup>6</sup>	31.4 x10 <sup>6</sup>	33.3 x10 <sup>6</sup>
AIDS deaths	1.2 x10 <sup>6</sup>	2.1 x10 <sup>6</sup>	1.8 x 10 <sup>6</sup>

Heterosexual contact has become the main transmission pathway of HIV, although most of the pediatric patients are infected by vertical transmission. Women from 15 to 49 years old represent half of the global population infected by HIV. One percent of the worldwide population from 15 to 49 years old is infected by HIV.

Sub-Saharan Africa is, by far, the worst affected region with an estimated 22.5 million people currently living with HIV (67% of the global total), 1.3 million AIDS-related deaths (72% of the global total) and 1.8 million of new infections (69% of the global total). However, between 2001 and 2009 the number of new infections declined a 19% in this area. Asia is the second-worst affected region with 4.9 million people living with HIV (15 % of the global total) (Joint United Nations Program on HIV/AIDS 2010).

**Number of people living with HIV, newly infected people with HIV, AIDS-related deaths**



**Figure 13.** Number of people living with HIV, newly infected people with HIV and AIDS-related deaths in the period 1990-2008. Source: Lois Jensen (2010). The Millennium Development Goals: Report 2010. New York: United Nations Department of Economic and Social Affairs.

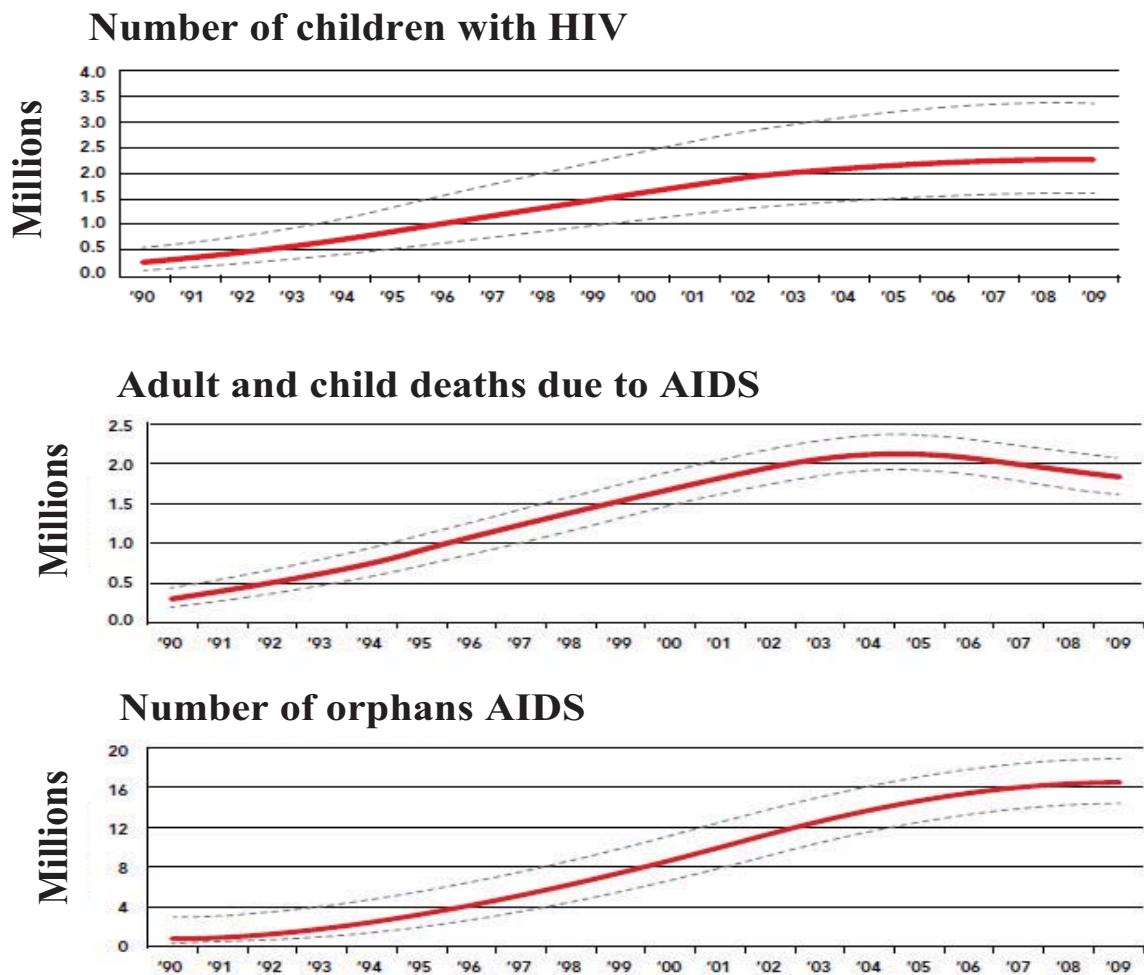
New infections among children are decreasing. As access to services for preventing the vertical transmission of HIV has increased, the total number of children being born with HIV has dropped. An estimated 370,000 [230,000–510,000] children were newly infected with HIV in 2009 (a drop of 24% from five years earlier).



Globally, deaths among children younger than 15 years of age are also declining. The estimated 260,000 [150,000–360,000] children who died from AIDS-related illnesses in 2009 were 19% fewer than the estimated 320,000 [210,000–430,000] who died in 2004 (**Figure 14**). This trend reflects the steady expansion of services to prevent transmission of HIV to infants and an increase (albeit slow) in access to treatment for children.

- 2010- HIV-1 infections in approximately 40 million people worldwide, with almost 5 million new individuals becoming HIV-infected each year (Friedrich BM, 2011).

### GLOBAL HIV TRENDS IN CHILDREN



**Figure 14.** Number of children living with HIV, adult and child AIDS-related deaths and number of orphans due to AIDS in the period (1990-2009). Source: UNAIDS report on the global AIDS epidemic, 2010

## 2.10. HIV IN CHILDREN

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Vertical transmission is the main type of infection in the pediatric population. The infection can occur during pregnancy, at delivery or through the breastfeeding. In developing countries, three out of four children are infected at delivery. Some risk factors for mother-to-child transmission are the vaginal delivery, membrane break during more than 4 hours, use of intravenous drugs during pregnancy, prematurity and low weight at birth, high maternal viral loads, low maternal CD4+ T-cell counts and advanced stage of the disease. In most of the cases, the diagnosis of HIV vertical transmission is performed in the first weeks of life: viral genome is detected by polymerase chain reaction (PCR) techniques in a 93% of infected newborns at 15 days of life. The sensitivity and specificity of these tests increase up to 96-99% at the age of 1 month (Dunn D et al 1995). The precocity at the diagnosis enables a fast initiation of highly active antiretroviral therapy (HAART) in the acute stage of the infection.

In patients who have acquired HIV infection by vertical transmission, the acute infection is not associated with the acute retroviral syndrome that occurs in 60% of adult primo-infections. Normally HIV replicates in CD4+ T-cells and progressively destroys the immune system. In children, as the immune system is not completely developed, immune suppression, as well as AIDS, are faster. Consequently, in the first years of life, viral loads remain high in the plasma of a child.

The first symptoms of vertical HIV infection may be unspecific and with a prolonged evolution. Opportunistic infections appear in patients with severe immune suppression and, in most of the cases, they have a worst evolution than in adults (such as pneumonia caused by *Pneumocystis jirovecii*).

In vertical transmission, there are mainly two evolutive patterns for the progression of HIV infection: rapid progressors (20%) and slow progressors (80%). Clinical manifestations during the first months of life will determine the prognosis. For instance, pondo estatural lateness, HIV-associated encephalopathy and pneumonia caused by *Pneumocystis jirovecii*, are predictors of a rapid progression, while paroditis or lymphoidea interstitial pneumonia are linked with a slow evolution. It has also been described a third group of children (<5%): the very slow progressors, who remain long time with normal CD4+ T-cell counts and low viral loads, without any treatment.

In lactants (< 1 year old) not receiving ARV, the natural clinical and immunological evolution of HIV is very fast: 15% of the patients will develop AIDS or die, 50 % of the patients will present moderate immune suppression levels (15-25% CD4+ T-cell counts) and 20% of the patients will present high immune suppression (CD4+ T-cell counts below 15%) (Gray L 2001) (Dunn D 2003). Even in presence of normal levels of CD4+ T-cell counts, some patients develop opportunistic infections (Dunn D 2003).

Furthermore, in lactants, there is a lack of any reliable biomarker of the progression of the disease. The plasmatic viral load is around 200,000 copies/ml during the first year and there is no defined threshold to identify patients with high risk of rapid progression (Mayaux MJ 1996).

In children (> 1 year old) the progression of infection decreases after 12 months of life (Gray L 2001) and there are useful risk biomarkers of the progression: viral loads over 100,000 RNA-HIV copies/ml (Mofenson LM 1997) (Palumbo PE 1998), decrease of CD4+ T-cell counts below 15% (Immunological Category 3) or clinical development of AIDS (Clinical Category C).

Currently, the most commonly used prognostic markers are viral loads and CD4+ T-cell counts.

### **2.10.1. CLINICAL CATEGORIES OF HIV INFECTION IN PEDIATRICS:**

Currently, HIV-infected children are clinically classified accordingly to four categories (Guidelines for the Use of Antiretroviral Agents in Pediatric Infection, 2011):

#### **a) Category N: Not symptomatic**

Children who have no signs/secondary symptoms considered to be the result of HIV infection or who have only one of the conditions listed in Category A.

#### **b) Category A: Mildly symptomatic**

Children with two or more of the following conditions, but none of conditions listed in Categories B and C.

- Lymphadenopathy ( $\geq 0.5$  cm at more than two sites; bilateral = one site).
- Hepatomegaly.
- Splenomegaly.
- Dermatitis.
- Parotitis.
- Recurrent/persistent upper respiratory infection, sinusitis or otitis media.

**c) Category B: Moderately symptomatic**

Children who have symptomatic conditions, other than those listed for Category A or Category C, that are attributed to HIV infection. Examples of conditions in Clinical Category B include, but are not limited to, the following:

- Anemia (hemoglobin <8 g/dl), neutropenia (<1,000 cells/mm<sup>3</sup>), or thrombocytopenia (<100,000 cells/mm<sup>3</sup>) persisting  $\geq$  30 days.
- Bacterial meningitis, pneumonia or sepsis (single episode).
- Candidiasis oropharyngeal (thrush) persisting  $\geq$  2 months in children age > 6 months.
- Cardiomyopathy.
- Cytomegalovirus infection with onset before age 1 month.
- Diarrhea, recurrent or chronic.
- Hepatitis.
- Recurrent herpes simplex virus (HSV), stomatitis (>2 episodes within one year).
- HSV bronchitis, pneumonitis or esophagitis with onset before age 1 month.
- Recurrent herpes zoster ( $\geq$  2 episodes) involving at least 2 distinct episodes or more than 1 dermatome.
- Leiomyosarcoma.
- Lymphoid interstitial pneumonia (LIP) or pulmonary lymphoid hyperplasia complex.
- Nephropathy.
- Nocardiosis.
- Fever lasting  $\geq$  1 month.
- Toxoplasmosis with onset before age 1 month.
- Varicella, disseminated.

**d) Category C: Severely symptomatic.**

Children who have any condition listed in the 1987 surveillance case definition for AIDS (Centers for Disease Control and Prevention, 1987), with the exception of LIP (which is a Category B condition).

- Serious bacterial infections, multiple or recurrent (any confirmation of at least two culture-confirmed infections within a 2-year period), of the following types: septicemia, pneumonia, meningitis, bone or joint infection, or abscess of an internal organ or body cavity (excluding otitis media, superficial skin or mucosal abscesses and indwelling catheter-related infections).

- Candidiasis, esophageal or pulmonary (bronchi, trachea, lungs).
- Disseminated coccidioidomycosis.
- Cryptococcosis, extrapulmonary.
- Cryptosporidiosis or isosporiasis with diarrhea persisting >1 month.
- Cytomegalovirus disease with onset of symptoms at age > 1 month (at a site other than liver, spleen or lymph nodes).
- Encephalopathy (at least one of the following progressive findings present for at least two months in the absence of a concurrent illness other than HIV infection that could explain the findings): (a) failure to attain or loss of developmental milestones or loss of intellectual ability, verified by a standard developmental scale or neuropsychological tests; (b) impaired brain growth or acquired microcephaly demonstrated by head circumference measurements or brain atrophy demonstrated by computerized tomography or magnetic resonance imaging (serial imaging is required for children < 2 years of age); (c) acquired symmetric motor deficit manifested by two or more of the following: paresis, pathologic reflexes, ataxia, or gait disturbance.
- HSV infection causing a mucocutaneous ulcer that persists for >1 month, or bronchitis, pneumonitis or esophagitis for any duration affecting a child >1 month of age.
- Histoplasmosis, disseminated.
- Kaposi's sarcoma.
- Lymphoma, primary, in brain.
- Lymphoma, small, non cleaved cell (Burkitt's) or immunoblastic or large cell lymphoma of B-cell or unknown immunologic phenotype.
- *Mycobacterium tuberculosis*, disseminated or extrapulmonary infection.
- *Mycobacterium*, other species or unidentified species, disseminated (at a site other than or in addition to lungs, skin, or cervical or hilar lymph nodes).
- *Mycobacterium avium* complex or *Mycobacterium kansasii*, disseminated (at a site other than or in addition to lungs, skin, or cervical or hilar lymph nodes).
- *Pneumocystis jirovecii* pneumonia.
- Progressive multifocal leukoencephalopathy.
- Salmonella (non typhoid) septicemia, recurrent.
- Toxoplasmosis of the brain with onset at >1 month of age.

- Wasting syndrome in the absence of a concurrent illness other than HIV infection that could explain the following findings: persistent weight loss >10% of baseline, or below 5th percentile in children, plus: chronic diarrhea ( $\geq 2$  loose stools per day for > 30 days, or documented fever for  $\geq 30$  days, intermittent or constant).

### 2.10.2. IMMUNOLOGICAL CATEGORIES OF HIV INFECTION IN PEDIATRICS:

Currently, immunological situation in HIV infection at the pediatric age is classified in three categories (Table 5).

**Table 5.** Immunological categories of HIV infection in children.

Immunological category	<12 months		1-5 years		6-12 years	
	CD4 /mm <sup>3</sup>	%	CD4/mm <sup>3</sup>	%	CD4 /mm <sup>3</sup>	%
<b>Category 1: no immune suppression</b>	$\geq 1500$	$\geq 25\%$	$\geq 1000$	$\geq 25\%$	$\geq 500$	$\geq 25\%$
<b>Category 2: Moderate immune suppression</b>	750-1499	15-24%	500-999	15-24%	200-499	15-24%
<b>Category 3: Severe immune suppression</b>	< 750	< 15%	< 500	< 15%	< 200	< 15%

Source: Centers for Disease Control and Prevention, 1994. Guidelines for the Use of Antiretroviral Agents in Pediatric Infection, 2011.

## 2.11- ANTIRETROVIRAL THERAPY

### 2.11.1. HISTORY

The following dates are remarkable in the history of ARV treatment:

- 1985- Anti-HIV activity was demonstrated in ZDV (Mitsuya H 1985). Zidovudine is a nucleoside thymidine analogue which blocks viral replication through the inhibition of the RT of the HIV. During many years, this has been the only ARV able to stop the progression of the infection. However, its administration in monotherapy was insufficient and carried secondary adverse events such as myopathy (Hamilton JD 1992) (Volberding PA 1995).
- 1987- Food and drug administration (FDA) approved the first ARV drug against HIV, ZDV.

- 1991-1994- These years are considered the most lethal period of HIV infection. Introduction of alternative ARV nucleoside analogues drugs against HIV, such as didanosine (ddI), zalcitabine (ddC) and stavudine (d4T) with a similar mechanism of action to that of ZDV. The first clinical assays in monotherapy in pediatric patients demonstrated a significant amelioration of the clinical manifestations (growing and neurological development) and virological and immunological parameters of the patients (McKinney RE 1991) (Butler KM 1991) (Lewis LL 1996) (Kline MW 1998).

- 1995- The effectiveness of the use of two combined nucleoside analogues rather than the monotherapy was demonstrated (Hammer SM 1996). Studies of bitherapy (ZDV and lamivudine or 3TC, ZDV and ddI) in children confirmed the same features (McKinney RE 1998). Development of the protease inhibitors (PI), able to block the protease enzyme of the virus, as a new family of drugs (saquinavir, SQV; ritonavir, RTV and indinavir, IDV).

- 1996- Implementation of the highly active ARV therapy (HAART) through the combination of more than one ARV drug family. Incorporation of the first non nucleoside reverse transcriptase inhibitor (NNRTI), nevirapine (NVP). Demonstration of the effectiveness of the addition of a PI combined with two nucleoside analogues (Cameron DW 1998) (Brodt HR 1997). In children is also demonstrated that the introduction of PI in the ARV schedules ameliorates the progression of the infection, rather than in bitherapy (Luzuriaga K 1997) (Nachman SA 2000) (Yogev R 2002).

- 2003- Development of the fusion inhibitors (FI) (enfuvirtide, T20) as a new ARV class, which block the entrance of the HIV into the cell.

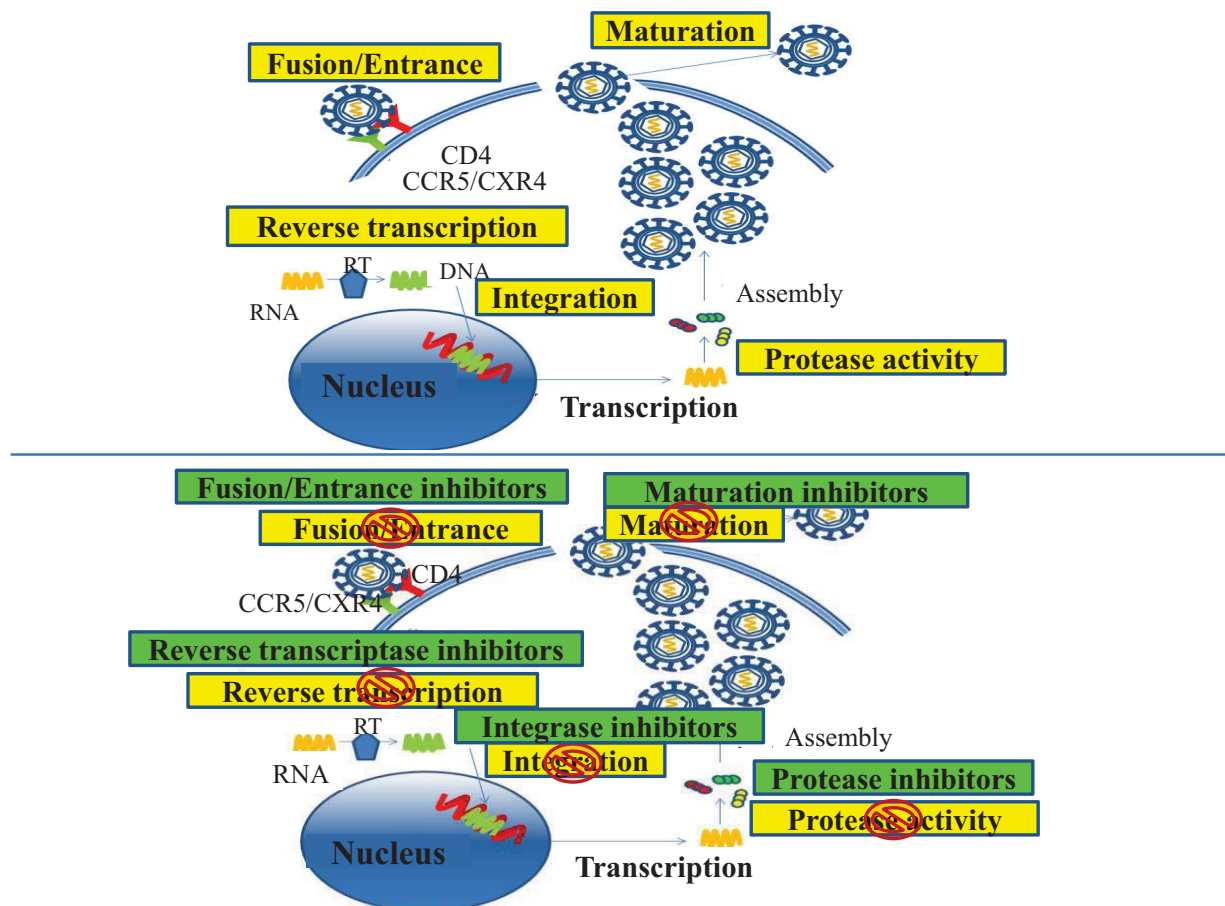
Currently- The implementation of HAART has dramatically improved mortality and morbidity of HIV infection by decreasing viral load to undetectable levels and increasing CD4+ T-cell counts. Furthermore, the decrease of the necessary dose and number of pills has entailed a better adherence to the therapy. In developed countries, HIV infection is considered a chronic disease rather than a lethal infection. On the other hand, with the implementation of ARV drugs, the presence of many secondary adverse events as well as resistant strains, need to be taken into account. In this sense, eventually, some changes in the therapy schedules are required. The efforts of the scientific community are centered in finding a very effective anti-HIV therapy with a minimum effective dose and derived secondary effects. However, ZDV is still one of the most common ARV administered in the therapy.



### 2.11.2. CLASSIFICATION AND MECHANISM OF ACTION OF ANTIRETROVIRALS

Highly active ARV therapy is the combination of more than one type of ARV drugs. Nowadays, ARV drugs are classified in 5 families depending on the stage of the viral cycle in which they interact (**Figure 15**):

- 1) RT inhibitors: 1a) Nucleoside reverse transcriptase inhibitors (NRTI), 1b) Non-nucleoside reverse transcriptase inhibitors (NNRTI), 2) Protease inhibitors (PI), 3) Fusion inhibitors (FI) and Entrance inhibitors (EI), 4) Maturation inhibitors (MI), 5) Integrase inhibitors (II)



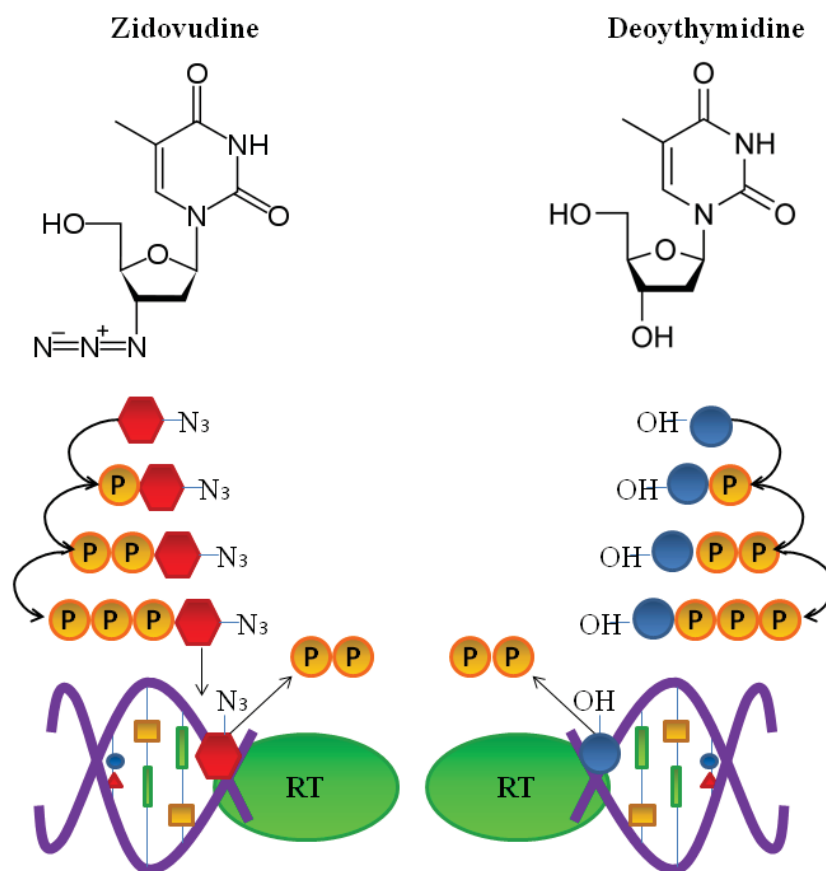
**Figure 15.** HIV replication cycle and the stages in which the different antiretrovirals act. RT: Reverse transcriptase.



### ***2.11.2.1. Nucleoside and nucleotide reverse transcriptase inhibitors***

Transcriptase inhibitors block the viral RT, the DNA polymerase RNA- dependent enzyme, responsible of the conversion from viral single stranded RNA into proviral double stranded DNA, able to be integrated in the nuclear genome. They are sub-classified in two groups: NRTI and NNRTI.

Nucleoside reverse transcriptase inhibitors are dideoxynucleosides with the same structure of deoxynucleosides, although with a substitution of the hydroxyl group in the 3' –OH position by another radical. Their structure forces them to compete with the natural nucleosides (adenosine, A; thymidine, T; cytidine, C; guanosine, G), acting as substrates of the RT. AZT and d4T are thymidine analogues; ddC, 3TC, and emtricitabine (FTC) are cytidine analogues; ddI is an inosine analogue which is transformed in dideoxyadenosine before execute its function; abacavir (ABC) is a guanosine analogue (**Table 6**). To become functional they must be phosphorylated by the endogenous cell kinases and only the resulting triphosphate (dideoxynucleoside triphosphate, ddNTP) is able to inhibit viral RT (**Figure 16**). When NRTI are incorporated by the RT into the synthesizing DNA they stop its continuation, blocking the phosphodiester bond (between the 5' phosphate group of the synthesizing DNA and the substituted 3' OH of the NRTI).



**Figure 16.** Prior activation through the phosphorylation process of NRTI and natural deoxynucleotides. Competition between zidovudine (ZDV) and endogenous natural deoxynucleotide deoxythymidine into the growing viral DNA strand. NRTIs present a substitution of the 3' hydroxyl group (OH) by another chemical group, such as azido group ( $N_3$ ), in case of ZDV. Thus the synthesis of DNA is inhibited since the phosphodiester bond is not viable.

There is a nucleotide analogue (tNRTI), known as tenofovir disoproxil fumarate (TDF), available against HIV. It is an analogue of the adenosine 5'-monophosphate nucleotide and it contains already a phosphate group on its structure, so it only requires the addition of two phosphate groups to be active. It has a powerful anti-HIV activity, a good resistance profile, a great tolerability, a high bioavailability and with a simple dosage (once per day).

In general, NRTI are often administered in only one dose every day and they do not interact excessively with other drugs, being potent inhibitors of HIV replication. These characteristics conferred them the suitable properties to become the backbone of the ARV schedules nowadays.

Clinical secondary effects: lactic acidosis, polyneuropathy, pancreatitis, lipodystrophy (body fat abnormalities).

**Table 6.** List of available NRTI and tNRTI ARV. Highlighted in grey color those available in children.

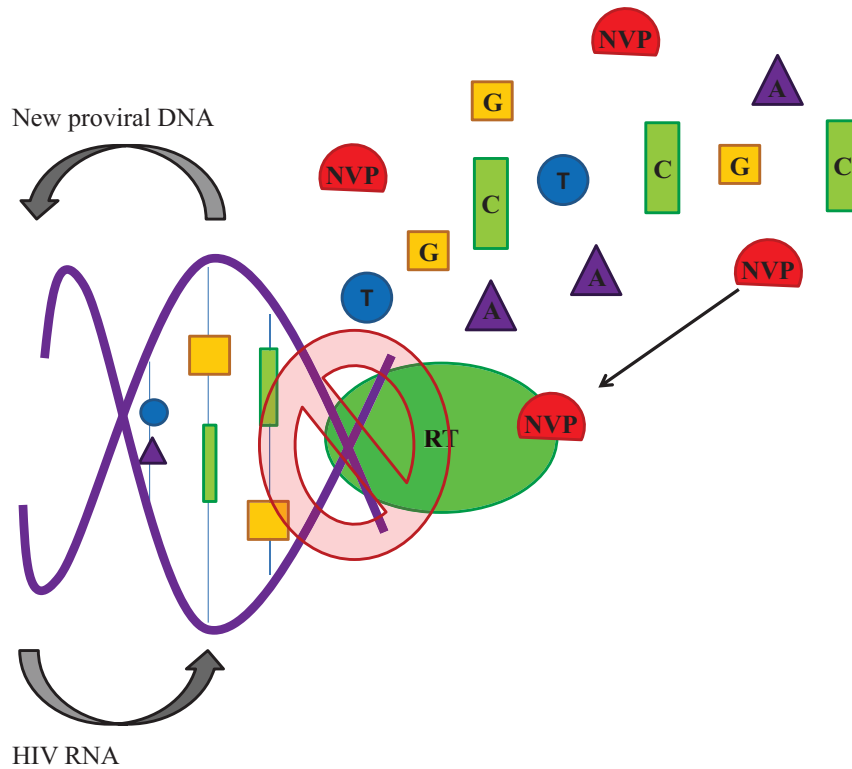
<b>NRTI</b>	<b>Available presentation</b>	<b>Advantages of the use in pediatrics</b>	<b>Disadvantages/secondary events</b>	<b>FDA approval</b>
Zidovudine (ZDV)	Capsules Syrup Endovenous Tablets/Pills	Wide experience in pediatrics, even in the neonatal period Administration with meals	Avoid d4T combination Hematologic toxicity Administration/12h Pharmacological interactions	March 1987
Didanosine (ddI)	Capsules Tablets/Pills Syrup		Administration /12h on empty stomach Interactions with absorption of various drugs, as PI	October 1991
Stavudine (d4T)	Capsules Syrup	Administration with meals	Avoid ZDV combination Administration /12h	June 1994
Zalcitabine (ddC)	Tablets/Pills		Administration on empty stomach	June 1992
Lamivudine (3TC)	Tablets/Pills Syrup	Administration with meals	Administration /12h	November 1995
Abacavir (ABC)	Tablets/Pills Syrup	Administration with meals	Hypersensitivity reaction in 5% of cases Administration /12h	December 1998
Emtricitabine (FTC)	Capsules	Administration /24h with meals		July 2003

<b>tNRTI</b>	<b>Available presentation</b>	<b>Advantages of the use in pediatrics</b>	<b>Disadvantages/secondary events</b>	<b>FDA approval</b>
Tenofovir (TNF)	Tablets/Pills	Administration /24h with meals	Nephrotoxicity	October 2001

### **2.11.2.2. Non-nucleoside reverse transcriptase inhibitors**

Non-nucleoside reverse transcriptase inhibitors are common compounds in the ARV schedules. Their function, as well as NRTI, is to inhibit the viral RT. As opposite as NRTI, they do not need to be activated within the cell and they do not compete with natural nucleosides, but directly interact

with the RT and block its activity by a conformational change and a blockage of the catalytic center of the enzyme (**Figure 17**).



**Figure 17.** Mechanism of action of NNRTI (non-nucleoside reverse transcriptase inhibitors (e. g. Nevirapine, NVP), by binding to RT (reverse transcriptase of the HIV) which is blocked after a conformational change.

Non-nucleoside reverse transcriptase inhibitors are often administered in triple combination, normally with NRTI, exerting a high anti-HIV activity. Some of the most frequently used are efavirenz (EFV) and NVP, due to the high tolerability and simple dosage, as opposite to delavirdine (DLV), which requires a more complex administration (**Table 7**).

Clinical secondary effects: Nevirapine has been associated to hepatotoxicity (Mallolas J 2006).

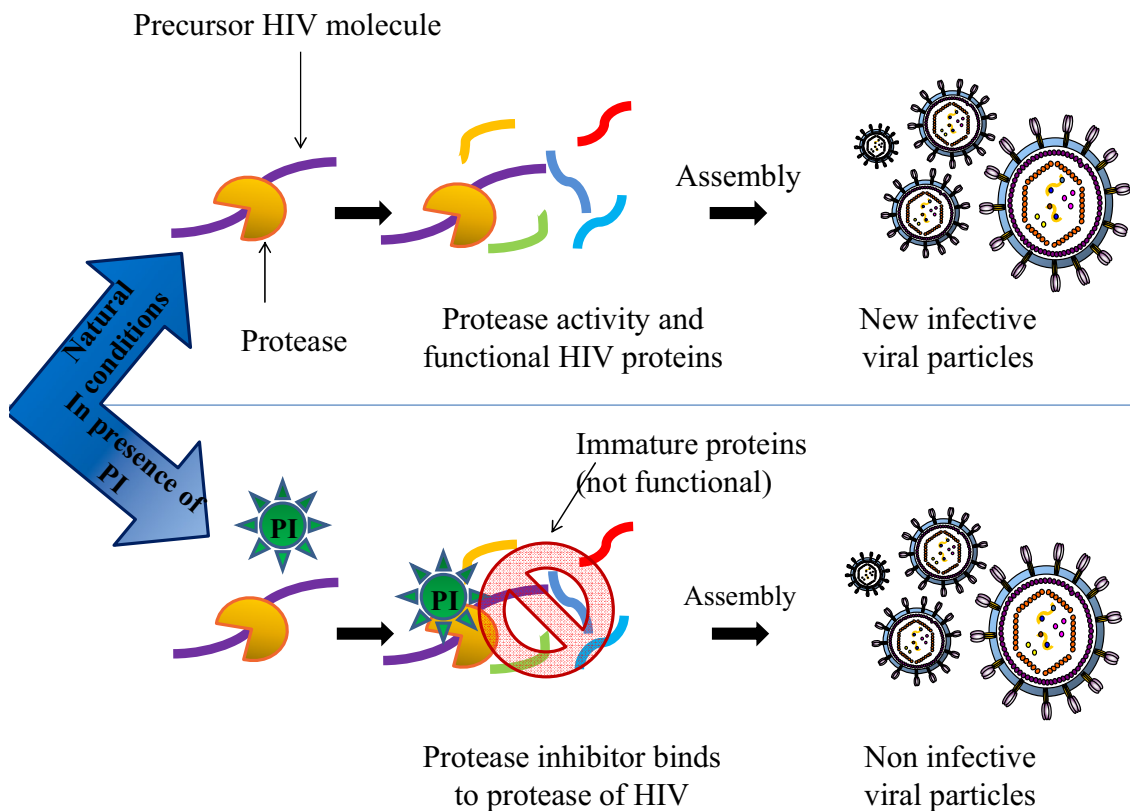
**Table 7.** List of available NNRTI ARV. Highlighted in grey color those available in children.

	Available presentation	Advantages of the use in pediatrics	Inconvenients/ Secondary events	FDA approval
Nevirapine (NVP)	Syrup Tablets/Pills	Administration /12h with meals	Cutaneous Rash Hepathotoxicity	June 1996
Efavirenz (EFV)	Capsules Tablets/Pills	Administration /24h, better at night, with meals	Teratogenic in monkeys Pharmacological interactions. Secondary effects in central nervous system	September 1998
Delavirdine (DLV)	Tablets/Pills	Administration with meals	Interactions with many drugs	April 1997

### 2.11.2.3. Protease inhibitors

Protease inhibitors are often administered in combination with other drugs in HAART.

They inhibit the protease activity of HIV, which is responsible for the post-translational proteolysis of the inactive precursor molecules of HIV making them functional. Thus, PI block the maturation and activation of viral proteins and, consequently, the formation of new infective viral particles (**Figure 18**).



**Figure 18.** Mechanism of action of protease inhibitors (PI). Protease inhibitors bind to viral protease and inhibit the enzyme blocking the assembly of new infective viral particles.

Once HIV has entered into the target cell, the reverse transcribed proviral DNA integrated into the nuclear genome of the host cell is transcribed to mRNA which, in turn, contains the information for the precursor polyprotein Gag-Pol product. This Gag-Pol is an initial protein that has to be processed by proteolysis in order to become functional. These proteolytic processes are induced by the viral aspartyl protease, which cuts this precursor Gag-Pol in structural and functional proteins, which will assemble giving rise to new viral particles in the so-called maturation stage. Protease inhibitors block the protease of HIV leading to an interruption of the maturation process. Thus, the inhibition by PI occurs at an advanced phase of the viral cycle, since these ARV act against the already infected cells which have integrated the viral genome into their nucleus and have begun the translational processes.

Most of PI are metabolized in the liver, through P450 cytochrome, therefore, the potential pharmacokinetic interaction with other drugs is remarkable and it is required to pay attention at the scheduled doses and the derived toxicity resulting after the intake of these drugs. Some PI are administered in combination, often powered by ritonavir (RTV), in order to ameliorate the plasmatic levels (**Table 8**).

Clinical secondary effects: lipodystrophy, metabolic syndrome, insulin resistance and diabetes or cardiovascular risk.

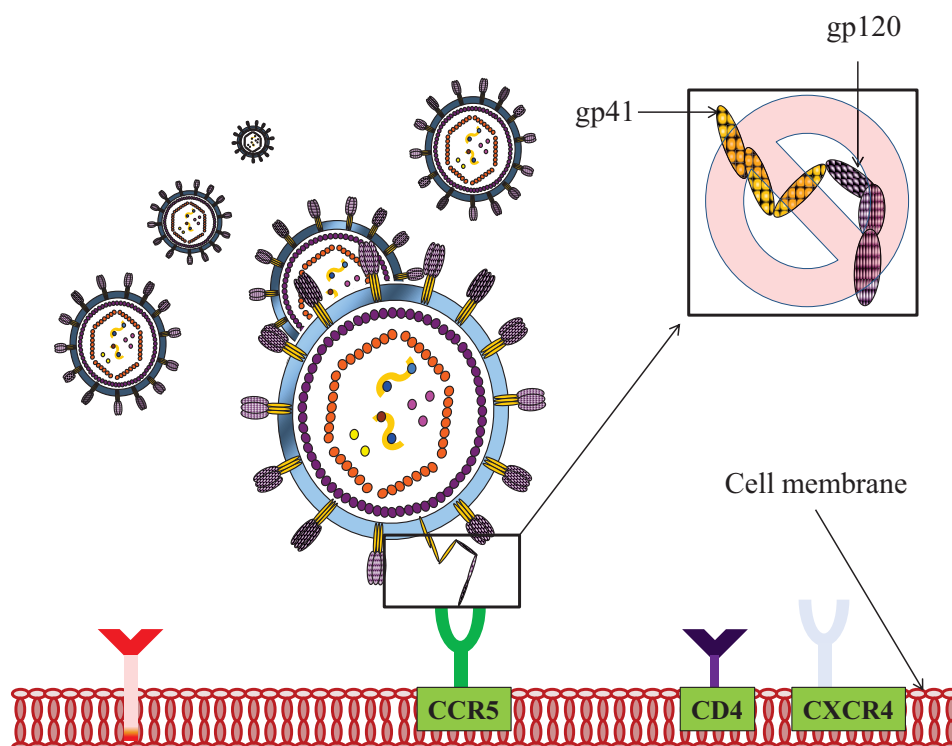
**Table 8.** List of available PI ARV. Highlighted in grey color those available in children.

	Available presentations	Advantages of the use in pediatrics	Inconvenients/ secondary events	FDA approval
Nelfinavir (NFV)	Syrup Tablets/Pills	Administration with meals	Administration /8-12h Diarrhea	March 1997
Indinavir (IDV)	Capsules	Administration with meals	Administration /8h Hyperbilirubinemia Nephrolitiasy Administration on an empty stomach	March 1996
Ritonavir (RTV)	Syrup Capsules	Administration with meals Well-tolerated in children	Administration /12h Lipodystrophy and dislipidemya	March 1996
Lopinavir /ritonavir (LPV/r)	Syrup Capsules		Administration /12h Major dose with NVP or EFV Lipodystrophy and dislipidemya	September 2000
Amprenavir (APV)	Syrup Capsules	Administration with meals	Administration /8-12h Severe cutaneous Rash	April 1999
Saquinavir (SQV)	Capsules	Administration after meals	Administration /8-12h	November 1997
Tipranavir / ritonavir (TPV /r)		(being studied)		
Fosamprenavir / ritonavir (FPV /r)		(being studied)		
Atazanavir / ritonavir (ATV/r)	Capsules	Administration with meals Administration /24h	Crossed resistance with other IPs Hyperbilirubinemya	

#### 2.11.2.4. Second generation drugs

##### *Fusion inhibitors*

Fusion inhibitors act by blocking the fusion between HIV membrane and the target cell (**Figure 19**). They block the entrance by the interaction with the proteins of the HIV envelope. They play an important role in the anti-HIV activity, since they stop the infection of new cells. Enfuvirtide, also known as T20 is the first of this drugs category (**Table 9**). It consists of 36 amino acids polypeptide which needs to be administered subcutaneously. It has been applied in adults and in children over 6 months of life, normally when resistances have become plausible. Its effectiveness is limited, although its tolerability is high. The implementation of a new molecule, BMS 663068, which is now in second phase of clinical trial will be possibly available in 2013-2014.



**Figure 19.** Mechanism of action of Fusion inhibitors (FI). FI block the viral proteins constituting the HIV envelope and inhibit its fusion with the host cell.



Clinical secondary effects: slight reaction in the application zone. Possible nauseas, diarrheas, vomits, headache and insomnia.

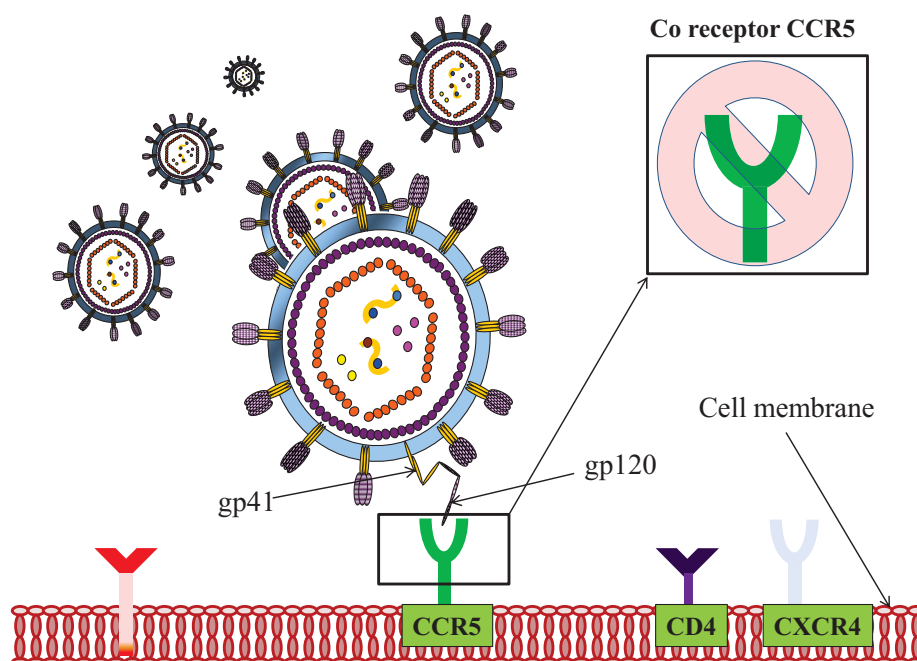
**Table 9.** List of available FI ARV.

	<b>Available presentation</b>	<b>Advantages of the use in pediatrics</b>	<b>Inconvenients/ secondary events</b>	<b>FDA approval</b>
Enfuvirtide (T20)	Subcutaneous punctions	No crossed resistances with other ARV	2 injections/ day Local pain Local hypersensitivity	March 2003

### ***Entrance inhibitors***

Entrance inhibitors are an alternative ARV to PI and RT inhibitors that, as well as FI, trigger the effects without entering to the cells and acting in the most initial stages of the HIV replication (**Figure 20**). Entrance inhibitors block the entrance of the virus into the host cell by inhibiting CD4 receptor or CCR5/CXR4 co-receptors, and promoting a conformational change, where the virus needs to be anchored. Maraviroc is the first FDA-approved ARV drug to target a cellular factor (**Table 10**), HIV coreceptor CCR5 and serves to intercept viral-host protein-protein interactions mediating entry. Note that maraviroc is only able to block R5 HIV strains and it is required to improve the sensitivity of the tests detecting exclusively CCR5-tropic HIV strains in order to ensure the effectiveness of the treatment.

Clinical secondary effects: There is still scarce data on secondary adverse events derived from maraviroc.



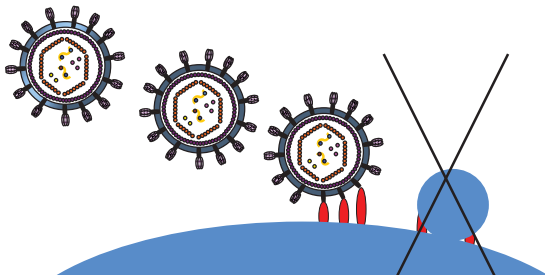
**Figure 20.** Mechanism of action of entrance inhibitors (EI). EI block the receptors of the host cell, so that unable the fusion between HIV and cell.

Recent large-scale small interfering RNA (siRNA) and short hairpin RNA (shRNA) screens have revealed over 1,000 candidate host factors that potentially support HIV-1 replication, and have implicated new pathways in the viral life cycle. These host proteins and cellular pathways may represent important targets for future therapeutic discoveries (Zhou H 2008).

The importance of FI and EI relies in the concept that, since the interaction among HIV and ARV, or among host cell and ARV (respectively) is an extracellular interaction, it entails a lower cytotoxicity (**Figure 21**).

**Table 10.** List of available EI ARV.

	Available presentation	Advantages of the use in pediatrics	Inconvenients/ secondary events	FDA approval
Selzentry® Maraviroc - bloq CCR5	No pediatric formulation	Susceptibility of HIV to a new class of ARVs Can give with food	Ineffective against CXCR4 or mixed/tropic viral strains Limited data on pediatric dosing or safety. Multiple drug interactions	April 2007



**Figure 21.** Both EI and FI inhibit the entrance of the HIV into the host cell blocking the potential new infections.

### *Integrase inhibitors*

Integrase inhibitors are able to block the integrase enzyme of the HIV, thus, inhibiting the integration of the viral genome into the nuclear genome of the cell. They are used in combination with other ARV. HAART schedules including an II lead to a faster recovery of CD4 T-cell counts, although the clinical implications of this fact are still unknown. Currently, Raltegravir is the first and unique available II and it is only administered in case of resistances to other ARV (**Table 11**). Its inclusion is recommended in combination with other ARV drugs and its effectiveness has been reported elsewhere (Cooper DA 2008).

**Table 11.** List of available II. Highlighted in grey color those available in children.

	Available presentation	Avantatges of the use in pediatrics	Inconvenients/ secondary events	FDA approval
Isentress® Raltegravir MK-0518	Oral tablets	Susceptibility of HIV to a new class of ARV Can give with food	Limited data on pediatric dosing or safety	October 2007

### *Maturation inhibitors*

Maturation inhibitors prevent the last step of the viral cycle, the assembly and maturation of the viral particles. They inhibit the protein articulation within the infected cells to form new HIV infective copies. Currently there is still no commercial MI available. Although PA-457, (also known as Bevirimat or MPC4326) has shown promising antiviral effects in phase I and II clinical trials, some polymorphisms conferring resistances to these ARV have been described (Lu W 2011) (Adamson CS 2010).

### **2.11.3. ANTIRETROVIRAL DRUGS AT THE PEDIATRIC AGE:**

As in adults, in the last years, the use of HAART and the development of new drugs and families of ARV have led to a dramatic decrease of the mortality and morbidity associated to HIV infection at the pediatric age (Watson DC 1999) (Sanchez JM 2003) (Gibb DM 2003).

The pathogenesis of HIV infection and the virologic and immunologic characteristics that have to be taken into account for the ARV schedules are common to all infected patients, although some points should be considered as specific for the pediatric age (Public Health Service Task Force, 2004. Guidelines for the Use of Antiretroviral Agents in Pediatric Infection 2011):

- a) Most of the cases are infected by vertical transmission.
- b) Most of the infected children by vertical transmission are exposed to ZDV or other ARV drugs during pregnancy, delivery or neonatal period.
- c) Specific virological tests are needed for HIV diagnosis, to completely elucidate an infection through vertical transmission before 18 months of life.
- d) Clinical and virological manifestations of HIV infection in vertical transmission have distinct characteristics due to the immaturity of the immune system.
- e) CD4+ T-cell counts normality values change depending on the age.
- f) Pharmacokinetic parameters of ARV differ depending on the age and the maturation stage of the organs responsible for their metabolization.
- g) The adherence to ARV drugs differs at the pediatric age (lactants, children and adolescents). It is required to obtain drugs with adequate organoleptic characteristics and well-prepared and

adapted for the pediatric age, with simple administration regimens that minimize the impact in the quality of lives of the patients and relatives.

h) Monitorization of ARV treatment is needed and modifications in ARV schedules are based in the clinical evolution, the viral and immunological status of the patients (**Table 12**).

i) The use of the ARV drugs in the pediatric age is sustained in their effectiveness experienced in adulthood and clinical assays security in I and II phases in children.

j) It is important not to forget that future new options in the treatment may be required, overall, concerning crossed resistance between drugs.

k) A close following in the neuropsychological and somatic evolution of the child is needed, in order to early identify any clinical manifestation derived from the disease or from the therapy.

l) In lactants infected by vertical transmission, the rapid and aggressive progression of HIV entails that most of the experts support a precocious HAART initiation, regardless of the clinical, immunological and virological status of the patient. Receiving ARV therapy, maintained suppression of viral replication and clinical-immunological recovery of the patients have been described (Luzuriaga K 1997) (Luzuriaga K 2000) (Hainaut M 2000) (Faye A 2002).

m) In children (> 1 year old) progression of the disease of HIV infection decreases after 12 months of life (Gray L 2001). This fact enables the use of conservative therapeutic strategies in the management of pediatric patients (>1 year), as in adults, to minimize the toxicity and the impact in the quality of life derived of the treatment.

**Table 12.** Recommendations for the initiation of ARV therapy in HIV-infected pediatric patients over 1 year old.

Clinical category <b>C</b> , or, Immunological category <b>3</b>	To initiate HAART
Clinical category <b>A</b> or <b>B</b> , or, Immunological category <b>2</b> (*), or, Viral load >100-250,000 RNA-HIV/mL copies	To assess HAART initiation
Clinical category <b>A</b> and immunological category <b>1</b>	To postpone HAART

(\*) In patients below 4 years, with high risk of encephalopathy, some experts advice to initiate the treatment with CD4+ T-cell counts below <20%.

In all ages it is recommended to initiate HAART with a minimum of 3 drugs to completely suppress viral replication, achieving undetectable viral loads, as well as to improve immunological function and clinical situation.

The proper combination for initiation to HAART regimen in pediatric patients is:

-According to **American Treatment Guidelines** (Guidelines for the Use of Antiretroviral Agents in Pediatric Infection 2011);

2 NRTI (ZDV+3TC, ZDV+ddI, d4T+3TC) plus 1 PI (Lopinavir/ritonavir or LPN/r, nelvinavir or NFV, RTV) backbone or 2 NRTI plus 1 NNRTI backbone (EFV in children > 3 years old, NVP in children < 3 years old, or children unable to swallow a pill).

-According to **European Treatment Guidelines** (Sharland M 2004);

2 NRTI (AZT+3TC, AZT+ddI, 3TC+ddI, ABC+3TC) plus 1 PI (LPN/r, NLV) and 1 NNRTI (NVP in children < 3 years old, and NVP or EFV in children > 3 years old).

Some studies have described the effectiveness and safety of the combination of 4 drugs (1 or 2 NRTI, EFV or NVP and NLV), which can be useful in selected cases, such as vertical infected lactants with high viral loads or with rapid clinical or immunological progression. (Wiznia A 2000).

#### **2.11.4. WHY CHANGE ANTIRETROVIRAL THERAPY?**

A change in the ARV schedule is due to:

- a) A failure of the initial schedule with an evidence of a progression of the disease (clinical, virological or immunological parameters). In this case, a possible failure in the adherence should be considered and a resistance test of HIV to ARV should be assessed before changing the therapy.
- b) Intolerance or toxicity derived from the administered ARV. In this case, the change to a new ARV schedule should introduce drugs with different secondary effects than those that triggered the toxicity.
- c) Scientific evidences that demonstrate a better response with a given combination of drugs.

The new ARV schedule follows the same recommendations as in initial therapy and, in most of the cases, it will include a minimum of two NRTI combined with one or more NNRTI, PI or, in

special cases, TDF, T20 and other recent drugs (for compassionate use or clinical assays in children).

A number of factors need to be considered in making decisions about initiating and changing ARV therapy in children:

- The severity of HIV disease and risk of disease progression, as determined by age, presence or history of HIV-related or AIDS-defining illnesses, level of CD4 T-cell immune suppression and magnitude of HIV plasma viremia.
- Availability of appropriate drug formulations and pharmacokinetic information on appropriate dosing in the pediatric population.
- Potency, complexity (e.g., dosing frequency, food and fluid requirements), and potential short and long-term adverse effects of the ARV regimen.
- Effect of initial regimen choice on later therapeutic options.
- The child's ARV treatment history.
- The presence of ARV drug-resistant virus.
- The presence of comorbidity, such as tuberculosis, hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, or chronic renal or liver disease.
- Potential drug interactions.
- The ability of the caregiver or the child to adhere to the regimen.

(Source: Guidelines for the Use of Antiretroviral Agents in Pediatric Infection 2011)

### **3- INTERACTIONS BETWEEN HIV/ANTIRETROVIRALS AND MITOCHONDRIA**

#### **3.1. INTERACTION BETWEEN HIV AND MITOCHONDRIA**

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Mitochondrial impairment was associated to HIV itself for the first time in 2002. H. Côté and collaborators (Côté HC 2002) described a mitochondrial DNA depletion in PBMC of HIV infected patients who had never received ARV. Later, these results were confirmed in PBMC (Miura T 2003) (Chiappini F 2004) (Polo R 2003) (Gourlain K 2003) and adipose tissue (Cherry CL 2002).

HIV is associated to mitochondrial impairment by triggering apoptosis. It is well-known the capacity of many viral proteins to induce apoptosis (Ferri KF 2000).

- **The viral protein Vpr** causes proapoptotic effects and enhances Fas-induced apoptosis in human T-cells (Arokium H 2009). It binds to mitochondrial adenine nucleotide translocase or ANT (ATP/ADP translocator, which is a protein located in the inner membrane that interacts with the proapoptotic Bax protein), inducing the permeabilization of the mitochondrial inner membrane and the release of cytochrome c to the cytosol, which activates caspases pathway and, thus, apoptosis (Muthumani K 2002) (Jacotot E 2000).

- **The HIV protease** induces apoptosis through the proteolytic degradation of the antiapoptotic Bcl-2 protein (Strack PR 1996). It also induces apoptosis by the activation of caspase-8 which, in turn, exerts the release of mitochondrial apoptogenic protein to the cytosol. It has been described a positive correlation between viral protease and apoptosis in vivo and in vitro (Nie Z 2002).

- **The viral protein Tat:** (a) reduces the expression of mitochondrial isoenzyme superoxide dismutase 2, which is an endogenous inhibitor of the permeability of the mitochondrial membrane (Westendorp MO 1995) (Williams MD 1998) (Creaven M 1999) and triggers loss of  $\Delta\psi$  (Macho A 1999); (b) increases the expression of Fas ligand in the T-cells, inducing apoptosis (Li-Weber M 2000); (c) induces apoptosis in non infected T-cells, through the secretion of Tat by the infected cells. The secreted Tat is received by the non infected cells and it binds the cytoskeletal microtubules of the host cell, affecting its stability and triggering mitochondrial apoptosis (Chen D 2002) (Li CJ 1995) (Huo L 2011).

- **Env protein** promotes changes in the rate of proapoptotic Bax and antiapoptotic Bcl-2 proteins, by increasing the former and reducing the latter, and activates mitochondrial apoptosis (Torre D



2005). Recently, it has been described that Env glycoprotein-mediated bystander apoptosis depends on the expression of the CCR5 coreceptor and the Env fusogenic activity (Joshi A 2011).

- **Nef protein** activates the production of Fas ligand in infected T-cell and induces apoptosis (Zauli G 1999) (Lenassi M 2010). In the non infected cells, Nef induces apoptosis independently of Fas ligand, through its binding to cell surface receptors (Otake K 1994) (Lenassi M 2010).

- **Gp120** induces mitochondrial apoptosis through the depolarization of the mitochondrial membrane and the release of cytochrome c to the cytosol, once it contacts the co-receptor of the host cell (Roggero R 2001).

Additionally, and partly as a result of increased apoptosis, cells infected by HIV show an imbalance between oxidants and antioxidants.

Nitric oxide (NO) has antiviral effects and it increases within the cell in presence of HIV. On the contrary, NO and peroxynitrite (ONOO<sup>-</sup>) contribute to the oxidative damage of the cells and the direct inhibition of the mitochondrial respiration (Torre D 2002).

HIV infection produces an increase in TNF $\alpha$  levels, which is an apoptotic inductor, a cytokine produced in most of the inflammatory and immunologic reactions. It is produced in T-lymphocytes as a response against HIV, and it also promotes replication of HIV in T-cells through the activation of the transcription NF- $\kappa$ B (Israel N 1989). The increase of TNF $\alpha$  production is associated with obesity and insulin resistance (Recasens M 2004) and with a pathogenic status, muscle wasting and cachexia. Many HIV infected patients developing AIDS, present some symptoms compatible with weakness and muscle waste, known as wasting syndrome (Grunfeld C 1992). TNF $\alpha$  can also induce apoptosis in adipose tissue (Prins JB 1997) and block adipocyte differentiation (Xing H 1997) and induce lipolysis (Green A 1994), inhibit transcription of genes involved in adipogenesis and lipogenesis or in glucose capture.

In general, HIV derived apoptosis affects either infected and non infected CD4<sup>+</sup> T-cells (Luciani F 2004), contributing to the typical leukopenia of infected patients. Some studies have demonstrated that T-cells of HIV infected patients are more susceptible to apoptosis than those of non infected patients (Meyaard L 1992) (Gougeon ML 1993) (Finkel TH 1994) (Muro-Cacho CA 1995) (Patki AH 1997).

The use of HAART minimizes the HIV-related mitochondrial impairment by the decrease of the viral load to undetectable levels. On the contrary, HAART is also linked to secondary effects, as discussed in the following subheading. Thus, mitochondrial toxicity is, at the end, determined by

the viral load and the ARV intake. In the clinical practice, occasionally, it is difficult to distinguish if mitochondrial abnormalities are linked to HIV or HAART.

### **3.2. INTERACTIONS BETWEEN ANTIRETROVIRALS AND MITOCHONDRIA**

The use of HAART has dramatically reduced mortality and morbidity associated to HIV infection and AIDS, by maintaining the viral load undetectable and the CD4+ T-cell counts within the normality values. However, this lifelong treatment has been linked to many secondary effects. Many different and heterogeneous clinical adverse events have been associated to HAART: allergies, hypersensitivity to NRTI, hepatotoxicity, fever, general unwell, gastrointestinal alterations, anemia, leukopenia, hyperpigmentation of the skin, hyperglycemia derived from insulin resistance or diabetes mellitus, renal alterations, (mainly derived from IDV and TDF), decrease of the bone mass, myopathy (associated to ZDV) (Grau JM 1993) (Wiwanitkit 2011), hyperlactatemia and lactic acidosis (associated to d4T), pancreatitis (mainly related to ddI, but also to d4T, 3TC and ddC), peripheral neuropathy (associated to ddI, d4T and ddC) and central nervous system alterations, such as depression, mood changes or insomnia, among others (related to EFV). From the above list, pancreatitis, hyperlactatemia and lactic acidosis are considered the most severe secondary effects, able to cause death, and, undoubtedly, all they are related to mitochondrial abnormalities. Indeed, mitochondrial deficiencies explain the etiopathogeny of most of the HAART-related clinical manifestations.

In general, mitochondrial alterations are greater in those high energy demanding tissues, with a reduced cellular turnover and a high respiratory dependence. The most affected tissues are mainly the skeletal muscle, peripheral nerves, subcutaneous fat, peripheral blood mononuclear cells, kidneys, heart, liver and reproductive system.

Normally, the clinical manifestations are plausible soon after the toxic agent is administered. However, in some cases, it appears after months or even years of the initiation of the therapy. It is important to take into account the pharmacokinetic interactions of the different ARV, since it is possible a synergistic effect among them (Negredo E 2008). On the other hand, once the exposition to the ARV has finished, many adverse effects normally disappear.

**Myopathy:** Zidovudine, was the first and unique available drug administered in monotherapy from the last 80s until about the middle of 90s. Soon it was related to myopathy, as a consequence of mitochondrial alterations (Grau JM 1993) (Grau JM 1994). The clinical related symptoms are weakness, muscle pain and high levels of creatine kinase. In a histological approach, this disease is characterized by the ragged red fibers, which indicate the presence of abnormal mitochondria and which correlate with the severity of the disease and the administered dose of AZT (Dalakas MC 1990) (Grau JM 1989). The mtDNA depletion, caused by the inhibition of the  $\gamma$ - pol, in the skeletal muscle relies at the etiopathogenic basis of the myopathy development. Subsequently, it was found that AZT-related myopathy was reverted once the drug was withdrawn (Masanés F 1998). Currently, the inclusion of new available drugs to combine, entails a reduction of the toxic dose of AZT and it is unlikely to find severe cases of myopathy associated to AZT administration. Otherwise, mtDNA depletion has been demonstrated to be a NRTI class-effect, with a different intensity among each drug, being thymidine derivatives the most harmful from a mitochondrial point of view.

**Pancreatitis:** In general, the use of NRTI has been related to pancreatitis development. Particularly, the use of ddI alone or in combination with d4T has been associated with the development of pancreatitis. Some specific mutations, such as, cystic fibrosis transmembrane conductance regulator and serine protease inhibitor kazal-1 mutations, have been recently reported to increase the risk of pancreatitis in the general population (Tozzi V 2010).

**Lipodystrophy:** The inclusion of PI in 1996 entailed the apparition of other secondary adverse events, such as hyperlactatemia, lactic acidosis, pancreatitis, diabetes, or polyneuropathy (Aboulafia DM 1997) (Ault A 1997). Protease inhibitors were soon associated to lipodystrophy syndrome (Carr A 1998), characterized by body fat abnormalities, such as an increase in the fat (lipohypertrophy) in the abdominal diameter, a decrease in the fat (lipoatrophy) of the face and the extremities (Huff A 1997) (Munk B 1997) (Mishriki YY 1998), an increase in the breast fat and in the cervical area (buffalo hump) (Saint-Marc T 1998) (De Luca A 1998). The redistribution of the body fat is often accompanied by metabolic alterations, such as hyperlipidemia, hypercholesterolemia and hyperglucemia (Sullivan AK 1997).

Lipodystrophy has been associated to both PI and some NRTI (Lo JC 1998). Brinkman et al. suggested for the first time the mitochondrial dysfunction as the etiopathogenic cause of the syndrome, supporting the hypothesis of the mitochondrial toxicity associated to the ARV therapy (Miró Ò 2000). The suspects of a mitochondrial dysfunction as the causative agent, relied on the fact that many HIV-infected patients with lipodystrophy, had a phenotypical similarity with some aspects of Madelung's disease, the symmetric multiple lipomatosis, which is a genetic mitochondriopathy. This rare mitochondrial alteration is characterized by the presence of multiple subcutaneous fat bodies in the dorsal cervical zone, shoulders and upper part of the extremities, accompanied by peripheral neuropathy, hepatopathy and glucose intolerance. This disease is characterized by the presence of mtDNA alterations and a decrease of enzymatic activity of CIV of MRC (Klopstock TP 1994). In this context,  $\beta$ -oxidation is involved in the lipolysis process, by which fatty acids enter in the oxidative metabolism of mitochondria to obtain energy. Therefore, a given mitochondrial alteration would limit the oxidation process of the fatty acids, producing an accumulation of lipid inclusions.

**Hyperlactatemia and lactic acidosis:** These secondary adverse events are characterized by an increase of the levels of lactic acid in the bloodstream, which can either be symptomatic or asymptomatic. When the lactate levels range from 2 to 5 mmols/l, the increase is considered hyperlactatemia, whereas an increase up to more than 5 mmols/l, accompanied by a decreased blood pH, is considered lactic acidosis. Both clinical manifestations are able to cause death (80% of the patients who reach > 10 mmols/l) (Falco V 2003), through acute hepatic failure.

### **3.2.1. NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS-RELATED TOXICITY**

It has been widely described that mitochondrial toxicity is derived from NRTI, due to their affinity with  $\gamma$ -pol, the only enzyme responsible for mtDNA replication. Nucleoside reverse transcriptase inhibitors compete with the natural nucleosides, inhibiting the RT of the HIV, and as a secondary effect, inhibiting the  $\gamma$ -pol enzymatic activity and, consequently, the replication of the mitochondrial genome. Thus, they have been associated to mtDNA depletion which may lead to mitochondrial dysfunction, since mtDNA codifies for 13 polypeptides, 22 tRNA and 2

rRNA, depending on the level of depletion. At the end, mitochondrial impairment could compromise the viability of the entire cell. Mitochondrial toxicity depends on the dose and concentration of the administered NRTI and mtDNA depletion is observed after a prolonged period of exposition to the ARV. The mitochondrial dysfunction derived from mtDNA depletion is not always found, since a threshold of mtDNA depletion is required to trigger mitochondrial failure in a functional level.

It is remarkably that not all the NRTI drugs have the same intensity or capacity to produce mitochondrial toxicity, as they present different affinities with  $\gamma$ -pol enzyme, as described (Lim SE 2001) (Kakuda TN 2000). Indeed, a ranking of toxicities have been in vitro established as follows (from the most to the less harmful): ddC>ddI>d4T>>AZT>3TC>ABC=TDF.

Mitochondrial DNA depletion can occur in connection with three different pathogenic mechanisms: a) through the direct inhibition of the  $\gamma$ -pol without any incorporation of the NRTI to the polymerizing strand, or the terminators of the mtDNA polymerizing strand, b) by inducing errors during replication, or c) by reducing the exonuclease repairing capacity of the  $\gamma$ -pol.

Nucleoside reverse transcriptase inhibitors have also been reported to cause mitochondrial damage independent to  $\gamma$ -pol inhibition, as they can interfere with CIV function (e.g. ZDV), mtRNA transcription or transport of substrates into the organelle (ADP/ATP or succinate) (Galluzi L 2005) (Dalakas MC 1994) (Mallon PW 2005).

### **3.2.2. NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS- RELATED TOXICITY**

Although NNRTI have not been associated to mtDNA alterations, they are related to mitochondrial apoptotic pathways, leading to mitochondrial secondary toxic effects.

Non nucleoside reverse transcriptase inhibitors have been associated to some adverse events, such as a slight increase of apoptosis. This has been specially attributed to EFV, which may alter the proliferation of T-lymphocytes as well (Pilon AA 2002) and the differentiation in vitro of adipocytes, triggered by the sterol regulatory element binding protein (SREBP-1c) transcription factor (Hadri KE 2004). Both EFV and NVP have been described to cause mitochondrial membrane depolarization, which in case of NVP is sufficient to induce the apoptosis cascade (Karamchand Leshern 2008).

### **3.2.3. PROTEASE INHIBITORS- RELATED TOXICITY**

Protease inhibitors are highly associated to lipodystrophy syndrome onset. The secondary effects derived from the administration of these ARV are due to the unspecific capacity of the drug, not only to block the protease from the HIV, but also the other proteins from the host cell, which are involved in lipid and carbohydrates metabolism as well as in the mitochondrial biogenesis (Carr A 1998) (Martinez E 1998). The alterations in glucose and lipid metabolism often lead to an energetic failure that can derive in apoptosis.

Protease inhibitors induce apoptosis in the subcutaneous adipose tissue and hyperlipidemia, through the binding to two proteins that are involved in the regulation of the lipid metabolism: (a) the cytoplasmic retinoic acid binding protein type 1 (CRABP-1) and (b) the low density lipoprotein (LDL) receptor related protein (LRP) (Carr A 1998).

The cytoplasmic retinoic acid binding protein type 1 is a ubiquitous protein that binds intracellular retinoic acid and produces catabolism to cis-9-retinoic through cytochrome P-450 3A. This cis-9-retinoic acid is the ligand of RXR receptor, which forms a heterodimer with the transcription factor PPAR- $\gamma$  (peroxisome proliferator activated receptor  $\gamma$ ) in the nucleus of the adipocytes. In normal conditions, PPAR- $\gamma$ -RXR heterodimer simulates the differentiation and proliferation of the adipocytes and inhibits apoptosis of adipose tissue.

The PI have been related to a decrease in cis-9-retinoic acid, thus, producing a decrease in PPAR- $\gamma$ -RXR function, leading to an increase of the apoptosis in the peripheral adipocytes, a decrease in the adipocyte differentiation and, consequently, a development of the peripheral lipoatrophy (Dowell P 2000). Subsequently, there is a reduction of the lipogenesis and an increase of lipolysis (leading to hyperlipidemia) and of the central adipose stores, accumulation of fat in the breast and insulin resistance. Since mitochondria are involved in apoptosis process, an alteration of these organelles would trigger the cell death in the subcutaneous adipose tissue, through the release of cytochrome c and AIF to the cytosol. Some studies have described the presence of apoptosis in the subcutaneous adipose tissue of lipoatrophic patients receiving PI (Domingo P 1999).

There is controversial data with respect to the apoptotic effects derived from the PI in the literature. While some authors describe the proapoptotic effects associated to these drugs, some others argue an antiapoptotic effect, due to the inhibition of the receptors of the Fas and TNF- $\alpha$ ,

which are apoptotic regulator ligands. It has been reported a possible protective role of the PI on the loss of mt $\Delta\psi$  of the T-cells, which is a characteristic of the initial stages of apoptosis (Matarrese P 2003) (Sloand EM 2000).

The complex LRP-LPL (being LRP an hepatic receptor, also expressed in the endothelia with the lipase lipoprotein), degrades flow triglycerides into fatty acids, that will enter into the adipocytes to be stored. In the presence of PI, the drugs bind LRP, triggering its inhibition and leading to an increase of the lipid levels in blood.

The development of lipoatrophy or lipohypertrophy depends on the different function of the central and the peripheral adipose tissue, and on the tissue specificity of the ARV. The central adipose tissue has a higher lipolytic activity than the peripheral adipose tissue and, therefore, it is more dependent on mitochondria. In case of mitochondrial dysfunction, the lipolysis process would be limited, triggering a lipid accumulation in the central and dorsal-vertical adipocytes similar to the multiple symmetric lipomatosis. On the other hand, the lipoatrophy of the peripheral adipose tissue has been related to the apoptosis of the adipocytes of the region, caused by the mitochondrial dysfunction derived from the ARV intake.

It has been reported that PI inhibit the translocation of the transcription factor SREBP to the nucleus. The sterol regulatory element binding protein is involved in the lipogenesis and adipocyte differentiation. It is associated to the activation of the PPAR- $\gamma$  and it increases the capture and synthesis of fatty acids, inhibits lipolysis and increases the capture of glucose as energetic substrate, through the carrier of glucose GLUT-4, which is insulin-dependent. In the presence of PI, SREBP is inhibited, leading to an increase of fatty acids and glucose in the bloodstream and contributing to lipoatrophy.

It has also been suggested that PI inhibit some proteases involved in the degradation of SREBP, leading to an increase in the triglycerides production in the liver and, consequently, to an increase in the production of lipoproteins VLDL (very low density lipoprotein) and apolipoprotein B, both of which are carriers of the triglycerides to the adipocytes, where they will be stored. Furthermore, PI are related to a decrease of the capture of triglycerides, leading to an increase in the bloodstream.

On the other hand, it has been suggested a direct interaction of PI with GLUT-4, inhibiting the capture of glucose in skeletal muscle and adipose tissue and leading to an insulin resistance.



The increase of the plasmatic concentrations of triglycerides and glucose are the main adverse effects resulting of the described interactions. This is linked to insulin resistance which, together with both alterations in glucose and lipid metabolism is known as metabolic syndrome.

#### **3.2.4. SECOND GENERATION DRUGS-RELATED TOXICITY**

Due to the relatively recent creation of the remaining ARV families (FI, EI, II and MI), there is still scarce data about the potential secondary adverse effects derived from these second generation drugs. However, the potential cytotoxicity expected from most of them is low, as they suppress the viral cycle at very early stages. For instance, EI and FI stop the replication of HIV before it enters into the host cell, and II block the integration of the viral genome into the nuclear DNA, thus, inhibiting both the transcription and translation processes by extracellular interactions.





HYPOTHESIS



It is widely known that HIV and ARV drugs trigger mitochondrial impairment in adults. However, their effects in perinatally-infected children have been poorly explored. For this reason, the main hypothesis of the present Thesis was to demonstrate that mitochondrial abnormalities are present in HIV-infected pediatric patients treated with ARV.

It is expected to find mitochondrial alterations in asymptomatic perinatally HIV-infected children.

This mitochondrial lesion, manifested in a depletion of the mitochondrial genome, would lead to a reduction of the mitochondrial protein synthesis or to a mitochondrial dysfunction and, as a last resort, compromising the cellular viability. However, it is also possible that the presence of homeostatic mechanisms in mitochondria entails a proper function of some complexes, even in the presence of mitochondrial genome depletion. Rather than a localized mitochondrial alteration in a specific enzymatic activity, it is possible that HIV and ARV cause a diffuse damage in the organelle which may be observed in a general assessment of the respiratory chain.

In case of a mitochondrial alteration, either in asymptomatic or symptomatic patients, it would be expected a more evident presentation of mitochondrial toxicity in case of the latter.

If our hypothesis of an evidence of mitochondrial toxicity derived from HIV and ARV in children is confirmed, we believe that, once the detrimental agent is withdrawn, a recover of the mitochondrial affectation is possible.

Mitochondrial impairment may change depending on the type of HAART regimen, leading us to use mitochondrial parameters as a biomarker or a trail to find the best therapeutic options in the choice of different HAART schedules. In this context, the intensity of mitochondrial impairment over time would be higher in children receiving first generation NRTI which, in turn, have been demonstrated to present a higher mitochondrial toxicity in vitro, than those under second generation NRTI.



## OBJECTIVES



In order to study and test our hypothesis, the main objectives of the present Thesis are:

### **General Objective**

To test if HIV and ARV mechanisms of mitochondrial toxicity found in adults are present in perinatally HIV-infected children.

### **Specific Objectives**

**Objective 1:** To elucidate whether ARV treatment or HIV infection were exerting a mitochondrial toxic effect in asymptomatic perinatally HIV-infected pediatric patients receiving HAART.

**Objective 2:** To investigate if hypothetical alterations in the mitochondrial genome of asymptomatic HIV-infected children receiving ARV are downstream reflected at transcriptional, translational and functional levels. In case of mitochondrial dysfunction was present, to test whether MRC alterations are focalized or diffuse.

**Objective 3:** To determine mitochondrial status in lipodystrophic HIV-children and compare them to a group of asymptomatic children and to a group of uninfected controls.

**Objective 4:** To evaluate whether a 12-month interruption of ARV is able to improve or revert these hypothetical mitochondrial alterations at molecular and/or clinical level.

**Objective 5:** To compare mitochondrial toxicity derived from different HAART schedules in a longitudinal 2-year follow-up assessment of immunovirological and mitochondrial status under first or second generation NRTI. To elucidate whether those NRTI demonstrated to present high mitochondrial toxicity in vitro present a major toxicity in vivo as well.





## METHODS



## **1. PATIENTS**

The inclusion of patients in our studies has been conducted through the collaboration of the tertiary-care pediatric center Hospital Sant Joan de Déu and the Hospital Clínic of Barcelona.

All the patients and controls were voluntarily recruited for the studies at the pediatric hospital. Local ethics committee approval and informed consent from parents or legal guardians have been obtained in all cases.

Extensive information about specific patient or control characteristics is detailed in each study. From a general point of view, we have taken into account the following criteria:

- General characteristics of controls and patients: The controls recruited for the studies are uninfected controls, whereas the cases are perinatally HIV-infected patients.

- General classifications of HIV-infected patients: HIV-infected cases have been classified in several groups, depending on whether they receive ARV therapy or not, depending on whether they receive a specific type of therapy, or depending on whether they present lipodystrophy or not.

- General criteria of exclusion: cases who have reached adulthood, cases with familial history of genetic or inherited mitochondrial diseases, cases receiving an antibiotic or other substances potentially affecting mitochondrial function (oxazolidinones, aminoglycosides or statins, among others), cases receiving a concomitant therapy (immune suppressors such as interferon or ribavirin; or immune modulators such as interleukin-2).

The changes of therapy derived from a therapeutic failure or secondary effects are quite usual in HIV-infected patients, leading to heterogeneous characteristics in each case. This fact often hampers the selection and recruitment of a large number of patients with comparable characteristics.

## **2. SAMPLES**

The studies included in the present Thesis have been performed in peripheral blood mononuclear cells (PBMC) obtained by puncture from the forearm of patients and controls. All measurements of mitochondrial parameters enable to part from isolated and cryopreserved material, except flow

cytometry and polarographic experiments, in which the sample must be fresh. All experimental procedures are extensively described in each study.

### **3. ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS**

The isolation of PBMC is performed either through a Ficoll's gradient, a protocol including a series of centrifugations, or by means of the tempered dextran method. Both procedures are detailed below.

#### **3.1. FICOLL'S GRADIENT**

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Normally a volume of 20 ml of peripheral blood extracted by a forearm puncture is used to perform the mitochondrial studies in adults. In case of extractions of pediatric patients, the volume to part from is much more reduced (5-10 ml). In newborns is possible, to obtain, at the most, 2.5 ml of blood (not in the forearm).

The blood sample is recruited in EDTA tubes (Vacutainer <sup>TM</sup> EDTA) which contain the anticoagulant Ethylenediaminetetraacetic acid.

Before starting the protocol, it may be useful to isolate the plasma by centrifuging the blood sample for 15 minutes at 1,500 relative centrifugal force (rcf or g). The plasma will remain in the upper phase after centrifugation. A given volume of the plasma will be removed, aliquoted and cryopreserved at -80°C for further studies. After removing this plasma, it is required to put back the same volume of phosphate saline buffer (PBS). Thereafter, we can start the Ficoll's gradient procedure, as follows (**Figure 22**).

- . Blood is diluted 1:1 proportion with PBS.
- . The mixed volumes of blood and PBS are placed carefully in a volume of 10-15 ml of Ficoll, previously tempered, which is the isolating solution of the PBMC (Histopaque®-1077, Sigma Diagnostics, St. Louis, MO). There will be 2 heterogenic phases: a) the Ficoll at the bottom of the Falcon tube and b) the blood mixed with PBS in the upper part.

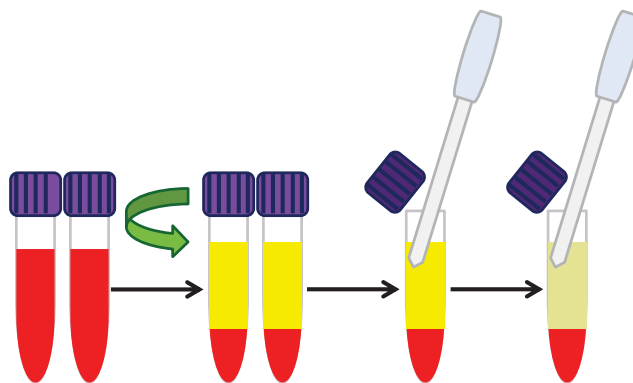
- Centrifuge 30 minutes at 670 rcf in a refrigerated centrifuge at 20°C. This step separates the blood content depending on the different densities. The erythrocytes and the granulocytes aggregate to the poly-sucrose of the Ficoll solution and accumulate to the bottom of the tube.
- We collect PBMC that form an inter-phase in the middle part of the tube, between the plasma (which remains at the upper part with the platelets) and the erythrocytes (which appear at the bottom).
- The collected PBMC will be dissolved in a volume of 15-20 ml of PBS. And we will proceed to the washings.
- Centrifuge for washing 10 minutes at 350 rcf and discard supernatant.
- Dissolve the pellet in 300-600 µl of PBS and centrifuge again in the same way. These washings discard most of the platelets that may remain in PBMC fraction.

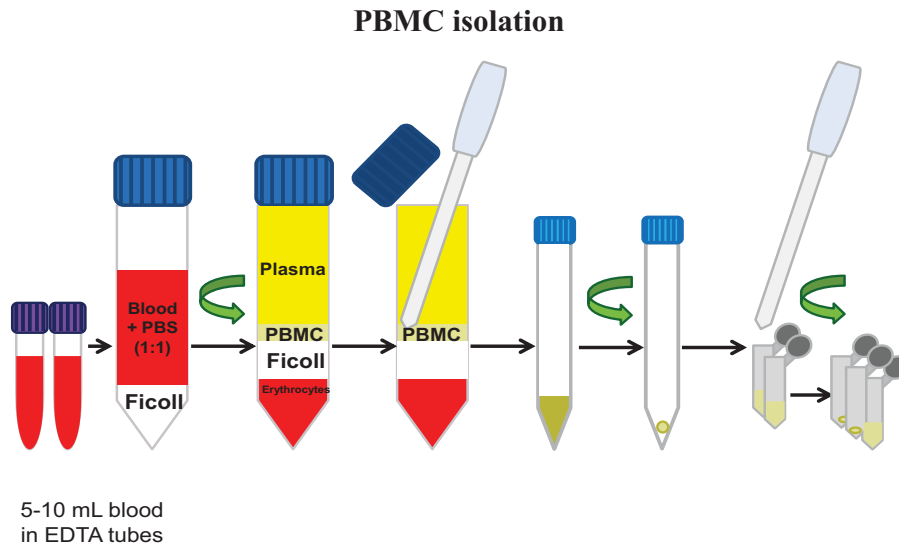
Finally, we will obtain the isolated PBMC (lymphocytes and monocytes) which can remain either dried as a pellet or dissolved in PBS. It is allowed to aliquot and cryopreserve the samples at – 80°C.

Optionally, to minimize the platelet content, at the beginning of the protocol it is possible to carry out a centrifugation of 25 minutes at 100 rcf at room temperature. The first resulting fraction contains plasma and platelets of the blood that will be discarded. The remaining cells will be reconstituted in PBS until obtaining the initial volume of the blood from which we parted from. From this point we will start the protocol, as explained.

In cases of hemolytic samples, with a high presence of erythrocytes, it is possible to use the reactive ACK lysing buffer (Lonza, BioWhittaker ®, Walkersville, MD, USA).

### Plasma isolation





**Figure 22.** Plasma and PBMC isolation by Ficoll's gradient method.

### 3.2. TEMPERED DEXTRAN

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In case of an extremely reduced blood volume at our disposal, for instance, in case of samples proceeding from lactants, another methodology to obtain PBMC might be suitable, the protocol of the tempered dextran. This protocol is detailed below:

- Prepare the dextran at 6%. Weigh 1.5 g of dextran in 25 ml of PBS.
- Place 5 ml of blood in a conic tube (it can be performed with 0.5 ml).
- Add 2 ml of tempered dextran (it can be performed at 0.2 ml).
- Mix gently by inversion.
- Incubate at 37°C during 30-40 minutes.
- After the incubation 2 layers will be observed, well-defined in white and red colour.
- Collect the white layer and transfer it to another conic tube of 15 ml (or 1.5 ml in case of reduced volume).
- Add 10 ml of PBS (or 1 ml if less volume).

- Mix gently. Centrifuge 10 minutes at 1000 ±100 revolutions per minute (rpm).
- Discard the supernatant by decantation.
- Dilute the pellet in 2 ml of distilled water (or 0.3 ml in case of small volumes) and mix with vortex 30 seconds. With less time a non proper lysis of the erythrocytes would occur and in case of overtime leucocytes would be impaired, so that the technique would be wrong.
- Neutralize immediately with PBS until 15 ml (or 1.5 ml in case of small volumes).
- Mix several times. Centrifuge 10 minutes at 1,000 ±100 rpm.
- Decant and dissolve the pellet in 2 ml of PBS (or with 0.2 ml for small volumes).
- Mix with vortex.
- Cryopreserve.

#### **4. QUANTIFICATION OF THE TOTAL PROTEIN CONTENT**

All the mitochondrial enzymatic activities should be relativized by the total protein amount of the sample.

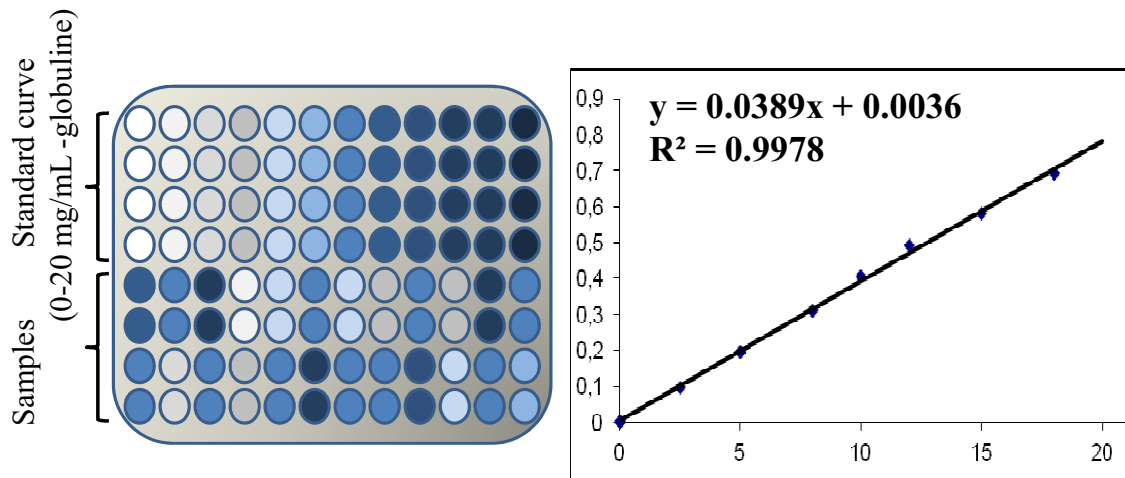
The quantification of the total protein content within the cell is possible through several methods (including Bradford, Lowry or BCA).

In the studies included in the present Thesis the protein quantification has been carried out by Bradford's methodology, described in 1976 (Bradford M 1976).

This method consists in a colorimetric reaction measured at an absorbance of 595 nm. The absorbance is dependent and positively correlates with the protein concentration of each sample, so it shows a darker colour when its protein content is higher. It consists in the maximum displacement of absorption of the acid solution Coomassie Brilliant Blue G-250 reactive (BIO-RAD Laboratories, München, Germany) when it binds to the proteins.

In the experiment it is included an internal calibration curve with known concentrations from 0 to 20 mg/ml of  $\gamma$ -globulin. Eventually it will be required to make dilutions in order to adjust the sample values within the pattern curve. The procedure is carried out in a 96-well plaque which will be read in a spectrophotometer. The absorbance values of the unknown samples are extrapolated to the calibration curve to determine the protein amount (**Figure 23**).





**Figure 23.** 96-well plaque with the standards and the unknown samples (left) and an example of a standard curve (0-20 mg/ml  $\gamma$ - globulin) (right).

## 5. QUANTIFICATION OF THE MITOCHONDRIAL MASS

It is possible to quantify the mitochondrial mass or mitochondrial content through three methodologies described below.

### 5.1. CITRATE SYNTHASE ACTIVITY

The citrate synthase (EC: 4.1.3.7) is an enzyme of the Krebs cycle that remains constant in the mitochondria and has been established as a reliable marker of the number of the mitochondria (Barrientos A 2002) (Palloti F 2001) (Zeviani M 1991). The technique to assess the enzymatic activity of the citrate synthase is based on the spectrophotometric measurement of the enzyme activity at 412 nm and at 37°C.

**Principle:** The citrate synthase catalyzes the formation of citrate parting from oxalacetate and acetyl-CoA. The reduced Coenzyme A (QH<sub>2</sub>) generated in the reaction converts the 5,5'-dithiobis 2 nitrobenzoic acid (DTNB) into 2-nitro-5-benzoic (TNB) which absorbs at a 412 nm wave

length. The activity of the citrate synthase is assessed by the measurement of the increase of the absorbance of TNB at 412 nm (**Figure 24**).

Method

Preparation of reaction mixture:

- 20  $\mu$ l 5  $\mu$ M DNTB.
- 30  $\mu$ l 10 mM Acetyl Coenzyme A.
- 10  $\mu$ l 10% Triton X-100.
- 100  $\mu$ l 1 M Tris HCl pH 8.1.
- 770  $\mu$ l H<sub>2</sub>O.

Assay:

Read at the spectrophotometer at 37°C and 412 nm wavelength. Initial calibration on air.

Incubate the cuvettes at 37°C during 5 minutes.

Read the baseline every 15 seconds during 4 minutes.

Initiate the reaction with 50  $\mu$ l of 10 mM oxalacetate in 100 mM Tris HCl pH 8.1.

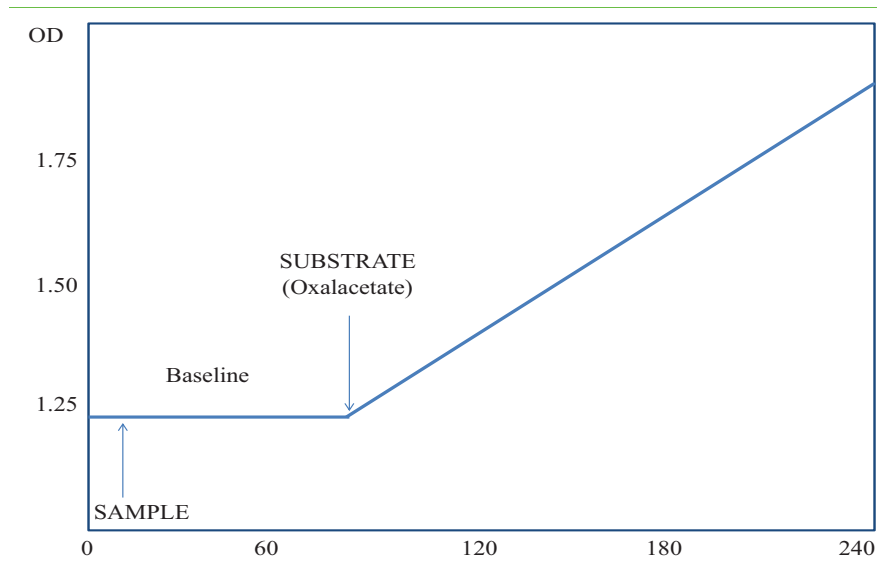
Read the reaction every 15 seconds during 4 minutes.

Calculations

It is necessary to subtract the baseline from the total activity: specific abs/min= total abs/min-  
baseline abs/min.

Extinction coefficient of DNTB is  $\Sigma = 13.6$ .

Units: nmols/min·mg protein.



**Figure 24.** Citrate synthase enzymatic activity measurement by spectrophotometry. OD: Optic density. The reaction is initiated by the addition of oxalacetate.

## 5.2. VOLTAGE DEPENDENT ANION CHANNEL LEVELS

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Voltage dependent anion channel (VDAC) is a structural nuclear-encoded porin voltage dependent anion channel located in the mitochondrial outer membrane with a structural function, which is considered a reliable biomarker of the mitochondrial mass. The quantification of VDAC is carried out through immune detection by western blot.

### Preparation of the samples

We part from 20  $\mu\text{g}$  of protein and load 20  $\mu\text{l}$  of final volume (sample, water and loading buffer) to each well, in a thick gel- 1.5 mm comb and separator.

Note: The volume of 4x loading buffer must be 5  $\mu\text{l}$  (for a total volume of 20  $\mu\text{l}$ ).

Prepare the samples in 0.2 ml eppendorf and to be boiled in the thermocycler at 99.9°C during 5 minutes to denaturalize the proteins. Make a spin in the centrifuge and leave them on ice.

A marker of the molecular weight will be also loaded (5-7  $\mu\text{l}$  without being boiled).

### Preparation of the electrophoresis gel

Prepare 10 ml of separator gel at 13%:

- 4.73 ml of sterilized Tris 1.5 pH 8.8.

- 690 µl of bi-distilled sterilized H<sub>2</sub>O.
- 4.33 ml of acrylamide 30%-bisacrylamide 0.5%.
- 94.8 µl of sodium dodecyl sulphate (SDS) 10%.
- 100 µl of ammonic persulphate 10% → polymerizing agent.
- 8 µl of tetramethylethylenediamine (TEMED) → polymerizing agent.

Add immediately the separator gel to the cassette (approximately 5-6 ml) with a Pasteur pipette, until the last line of the cassette and thereafter add ethanol 70% to align the gel and remove the bubbles.

- Wait 15-20 minutes for polymerization.

Prepare 3 ml of concentrator gel at 7%:

- 1.5 ml sterilized Tris 0.25 pH 6.8.
- 850 µl of bi-distilled sterilized H<sub>2</sub>O.
- 730.33 µl of acrylamide 30%-bisacrylamide 0.5%.
- 30 µl SDS 10%.
- 30 µl of ammonium persulphate 10% → polymerizing agent.
- 3 µl of TEMED → polymerizing agent.
- Clean the ethanol over the separator gel with water. Place a comb twisted to one side of the cassette and add the concentrator gel with a Pasteur avoiding bubbles.
- Wait 15-20 minutes until polymerization.
- Build the electrophoresis cubette, prepare 1x electrophoresis buffer and fill up the cubette.
- Load the samples and connect the cubette to the voltage source at 120 V until migration front is almost gone out of the cassette (approximately 3 hours).

#### Transfer to the membrane

- Prepare 1x transfer buffer (5x buffer, methanol and H<sub>2</sub>O) (1:1:3).
- Full a tray with the buffer and wet Whatman papers, sponges and membrane. Assemble the structure inside the tray.
- Place the structure into the cubette and connect it to the electric supplier at 400 mA approximately 1 hour.

Note: In case of having a machine disposal to do the transfer, the time process is reduced up to 7 minutes.

- Dismantle the structure, keep the membrane wet and proceed to immune detection. An example of an immune blot is provided (**Figure 25**).

#### Blocking

- Prepare blocking solution (0.1% Tween 20; 5% powdered milk in PBS).
- Add 10 ml to the membrane.
- Shake it at room temperature for 1 hour.

#### Incubation with the primary antibody

- Dilution 1/1,000 of the VDAC primary antibody (31 kDa) and 1/30,000  $\beta$ -Actin (47 kDa), as a sample loading control, with 1% powdered milk in PBS and 0.05% Tween 20.
- Incubate and shake overnight at 4°C.

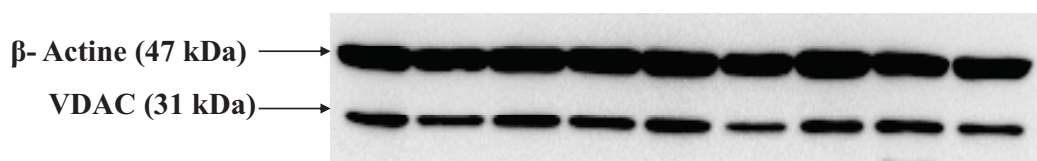
#### Washing

- 3 washing of 10 minute each, at room temperature, keep shaking with the washing solution (PBS and 0.1 % Tween 20).
- Add revealing solution to the membrane for 1 minute and capture the image in LAS 3000/4000 in chemiluminescence mode.

#### Quantification

ImageGauge Program.

The results are expressed as the ratio of the mitochondrial porin and the total protein content within the cell: VDAC/ $\beta$ -Actin.



**Figure 25.** Quantification of the mitochondrial and total protein content by western blot. kDa: kiloDalton, VDAC: structural voltage-dependent anion channel (a nuclear encoded porin of mitochondrial location),  $\beta$ -Actin: nuclear encoded cytoplasmic protein.

### 5.3. MITOTRACKER GREEN FLUORESCENCE

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Mitotracker green (MTG) is a mitochondrial-selective fluorescent label, commonly used in flow cytometry to count mitochondria. The quantification of the number of mitochondria with this MTG dye must be performed in fresh samples.

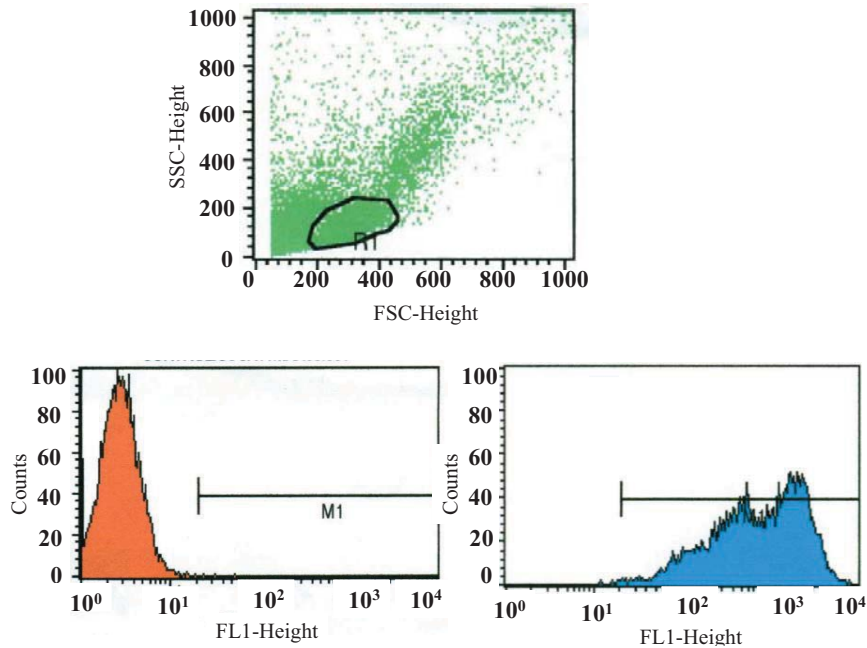
#### Method

Isolate PBMC and proceed to the cell counting in Neubauer chamber.

Start from approximately 750,000 cells to carry out the experiment.

Add this amount of PBMC to two cytometer tubes:

- Blank= negative control which will contain the sample without dye.
- MTG= Dyed sample.
- Centrifuge 7 minutes at 250- 300 rcf.
- Discard supernatant and dissolve in 1 ml of complete media (89% RPMI media 1640, 1% SS Penicillin- Streptomycin- L-Glutamine), 10% Foetal Calf serum (inactive 30 minutes at a 56°C bath).
- Add 10 µl of intermediate solution of MTG (20 µM in dimethyl sulfoxide, DMSO) into the dyed sample in the cytometer tube (200 nM, final concentration) and incubate for 30 minutes at 37 °C in darkness.
- For all the tubes (blank/MTG) add 1 ml PBS and centrifuge 7 minutes at 250-300 rcf.
- Discard supernatant and dissolve in 400 µl of PBS.
- Analyze with FACScan/FACScalibur (**Figure 26**).



**Figure 26.** In green colour, dot plot showing the subpopulation of CD4<sup>+</sup> T cell type. In orange colour, an histogram showing the unspecific fluorescence. In blue colour, an histogram the fluorescence corresponding to the MTG.

## 6. DNA EXTRACTION

Total DNA extraction is performed through phenol-chloroform technique which consists in:

### **-PBMC lysis and protein digestion**

Using sterilized material.

Prepare the mix in 2 ml eppendorf as follows:

- 125 µl proteinase K (2 mg/ml) (-20°C).
- 750 µl lysis buffer (White cell lysis buffer, WCLB) (4°C).
- 50 µl of SDS 10% (room temperature).
- Incubate and shake overnight at 37°C.
- **Separation of total DNA** (in the extractor hood).

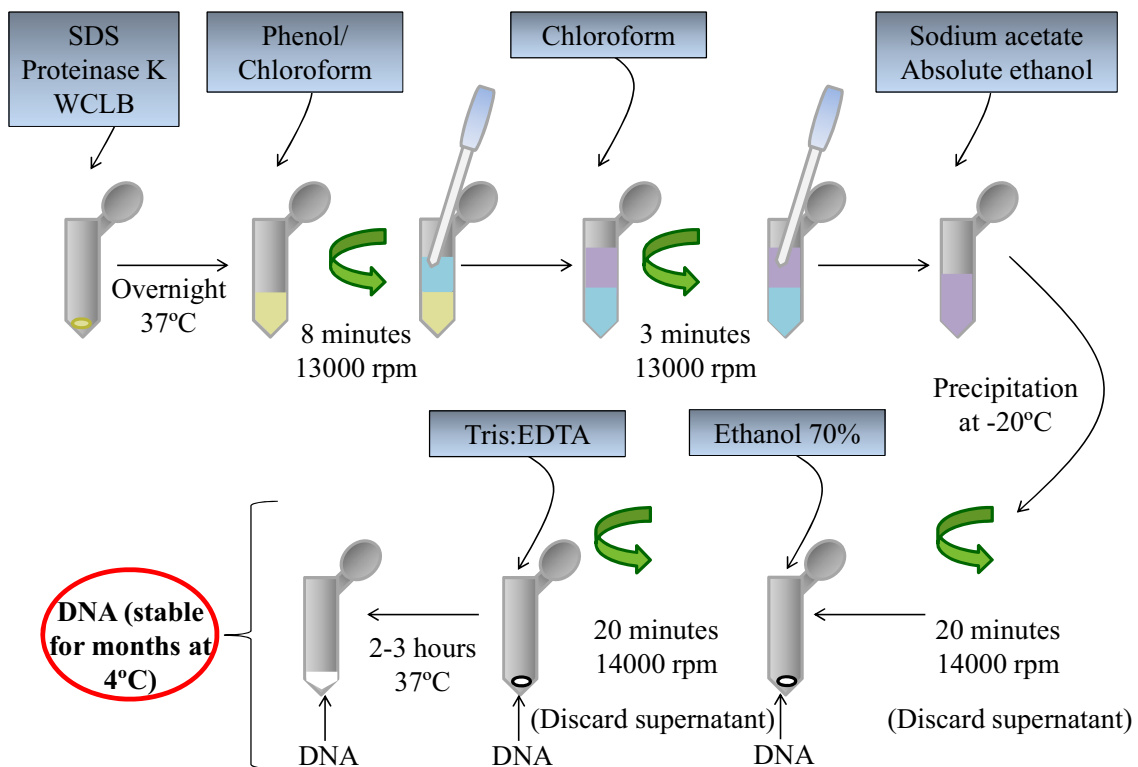
- After overnight incubation add to each sample an equal volume of phenol-chloroform (1/2) (800-900  $\mu$ l) (it contains 2 phases, we will avoid the phase on the surface corresponding to Tris pH 8 buffer).
- Mix until it gets a milky aspect.
- Centrifuge 8 minutes at 13,000 g.
- Keep the supernatant (upper phase) which corresponds to the inorganic fraction containing DNA. Lower phase is the organic one with phenol-chloroform, proteins and lipids that will be discarded, and the inter-phase containing the products that still have not been digested. Lower phase and inter-phase are discarded in a special bin for toxic chlorate products and Tripure-derived products.
- Add an equal volume of chloroform into the retrieved phase (1/2) (800-900  $\mu$ l) and gently mix to discard the remaining proteins.
- Centrifuge 3 minutes at 13,000 rcf.
- Collect again the upper phase.
- Precipitation of total DNA**
- Add 40  $\mu$ l sodium acetate 3M pH 5.2.
- Add 1 ml of absolute and cold ethanol (it is important to respect this reactive order of addition for a proper precipitation).
- Precipitation at -20°C during 2 hours or overnight.
- Centrifuge 15-20 minutes (it is possible to increase the time to optimize the extraction) at 13,000 rcf. A small pellet, which corresponds to the total DNA, will appear.
- Decant supernatant in a receptacle, preserve the pellet and leave the eppendorf opened.
- Add 600  $\mu$ l of ethanol 70% and dissolve the pellet.
- Centrifuge 20 minutes at 13,000 rcf (it is possible to increase the time to optimize).
- Decant the supernatant and keep the pellet.
- Dry the remainder drops of ethanol by capillarity with a sterile tip and leave the eppendorfs opened to evaporate the ethanol.
- Add 50-75 $\mu$ l (depending on the obtained pellet amount) of buffer TE 10:1 (Tris 10 mM-EDTA 1 mM) (autoclaved, pH 7.5, 4°C).
- Vortex, spin in the centrifuge. Incubate 2-3 hours at 37°C.



- Preserve DNA at -20°C or 4°C.
- **Quantification of total obtained DNA** (1 AU<sub>260 nm</sub> = 50 µg/ml (ng/µl)).
- Dilute sample (1/25 or 1/15, or less depending on the sample amount) in a total volume of 60 µl to achieve the minimum threshold required to read the absorbance in quartz cuvettes.
- Read at 260 nm (DNA wavelength) and at 280 nm (proteins wavelength).
- Determine the ratio 260 nm/280 nm to assess the purity of the sample (ideally free of proteins). A ratio from 1.8-1.9 units is accepted.

The concentration of total DNA is expressed in ng/µl. A summary of the procedure is represented (**Figure 27**).

Note: In case of microvolume devices for spectrophotometric DNA quantification (Nanodrop-like), the volume of required sample is limited up to 1-2 µl.



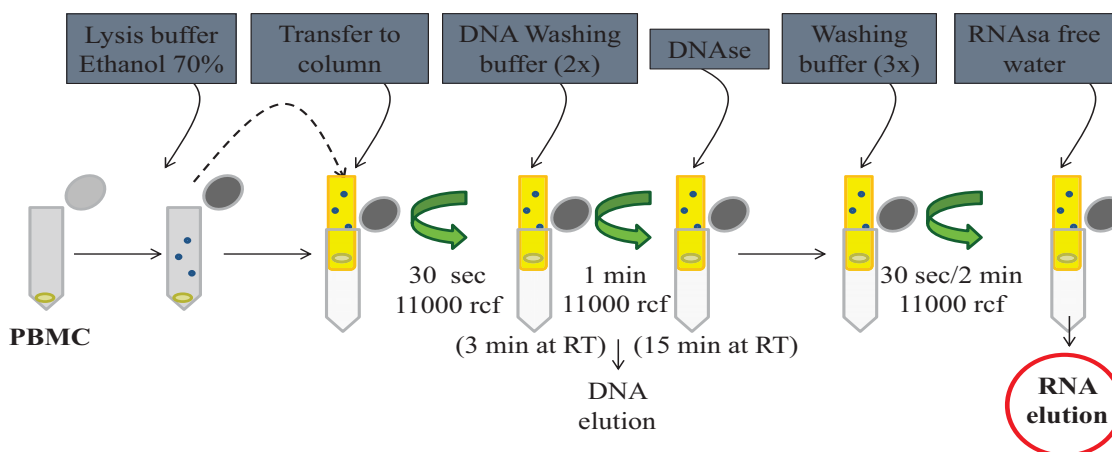
**Figure 27.** Phenol-chloroform procedure for total DNA isolation. SDS: sodium dodecyl sulphate, WCLB: white cell lysis buffer, EDTA: ethylenediaminetetraacetic acid, rpm: revolutions per minute.

## 7. RNA EXTRACTION

- **Extraction of RNA** (Macherey Nagel, Nucleospin RNA II ®, Cultek, Düren, Germany)
- Disinfect the working area with Ethanol 70-96° C and maintain the samples frozen until the beginning of the procedure.
- Reconstitute DNase. Add 540 µl H<sub>2</sub>O at the DNase (kit) and mix gently (by inversion, not vortex). Aliquote (e. g. 200 µl) the remainder DNase and keep it at -20°C for future extractions.
- Add β-Mercaptoethanol in lysis buffer (1:100).
- Add 300 µl lysis buffer (with β-Mercaptoethanol already added) to each sample.
- Vortex vigorously (approximately for 1 minute).
- Add 300 µl of 70% ethanol and mix with pipette or vortex 2 x 5 seconds.
- Prepare microcolumns in a tube rack and transfer the content from the eppendorfs to the columns.
- Centrifuge 30 seconds at 11,000 rcf.
- Prepare DNase: 10 µl of reconstituted rDNase + 90 µl Buffer for rDNase (for each sample).
- 1st DNA washing: 500 µl buffer RNA/DNA set. Centrifuge 1 minute at 11,000 rcf.
- 2nd DNA washing: 500 µl buffer RNA/DNA set. Centrifuge 1 minute at 11,000 rcf.
- Dry the membrane for 3 minutes at room temperature.
- Collect the elution which corresponds to DNA and recycle the collector.
- Add 95 µl DNA reaction mixture (= 10 µl reconstituted DNase in H<sub>2</sub>O + 90 µl of buffer for DNase) on the membrane and leave it 15 minutes at room temperature.
- Add 200 µl RA2 and centrifuge 30 seconds at 11,000 rcf. Discard the elution.
- Add 600 µl RA3 and centrifuge 30 seconds at 11,000 rcf. Discard the elution.
- Add 250 µl RA3 and centrifuge 2 minutes 11,000 rcf. Discard the elution.
- **Collect elution:**
- Add 30 µl of RNase free water on the filter. Centrifuge 1 minute at 11,000 rcf. Substitute the collector by a sterilized eppendorf where RNA elution will be collected.
- Add 20-30 µl of RNase free water and centrifuge 1 minute at 11,000 rcf.

Note: RNA is very unstable and sensitive to degradation. A summary of the overall procedure is represented (**Figure 28**).

- Preserve at  $-80^{\circ}\text{C}$ .
- Quantification of RNA ( $1 \text{ AU}_{260 \text{ nm}} = 40 \mu\text{g/ml}$  ( $\text{ng}/\mu\text{l}$ )).
- **Retrotranscription of RNA** from RNA to complementary DNA (cDNA) (RT-PCR) (GeneAmp PCR System 2400, Germany).
- . Final volume:  $20 \mu\text{l}$ .
- . For each reaction:  $10 \mu\text{l}$  Master Mix (TaqMan reverse transcriptase, Applied Biosystems, Ca, USA):
  - $2 \mu\text{l}$  Buffer.
  - $0.8 \mu\text{l}$  dNTPs.
  - $2 \mu\text{l}$  Random Primers.
  - $1 \mu\text{l}$  Transcriptase.
  - $1 \mu\text{l}$  inhibitors RNase.
  - $3.2 \mu\text{l}$  water (nuclease-free).Total =  $10 \mu\text{l}$  Master Mix.
- +  $10 \mu\text{l}$  of the diluted sample ( $0.5 \mu\text{g}$  of RNA).
- . Thermocycler program:
  - 10 minutes at  $25^{\circ}\text{C}$ .
  - 120 minutes at  $37^{\circ}\text{C}$ .
  - 5 seconds at  $85^{\circ}\text{C}$ .
  - $4^{\circ}\text{C}$ .



**Figure 28.** Total RNA isolation procedure. PBMC: peripheral blood mononuclear cells, sec: seconds, min: minutes, rcf: relative centrifugal force, RT: room temperature.

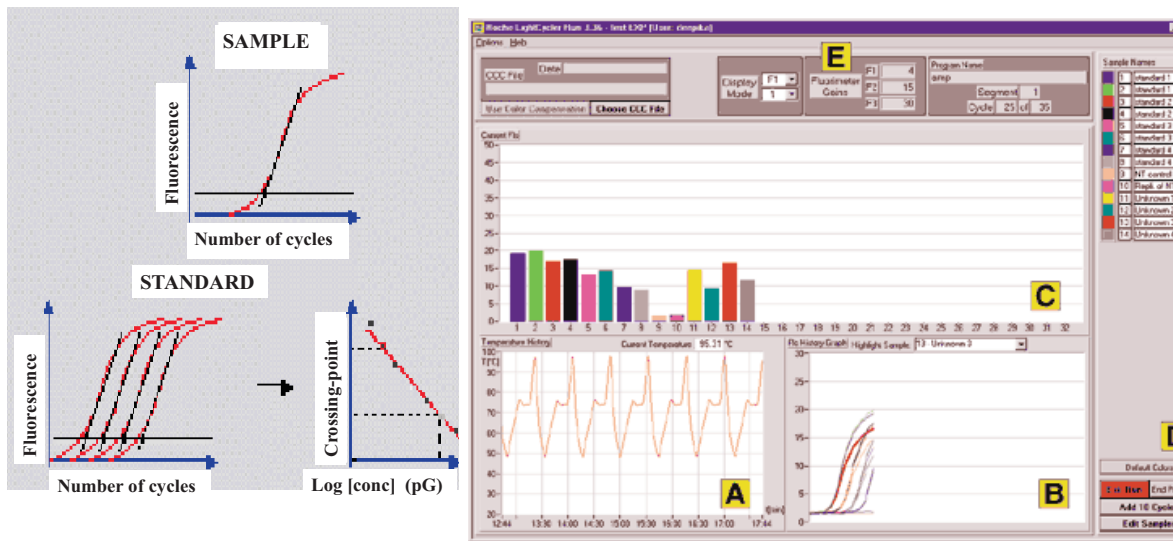
## **8. MITOCHONDRIAL DNA AND MITOCHONDRIAL RNA QUANTIFICATION**

For each extract of total DNA and RNA, it is necessary to perform a quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals®, Germany) to determine the mtDNA and mtRNA content, respectively. In the former, the primers for the DNA strand are used, while in the latter, the procedure is carried out from cDNA. In both cases, a 200 bp fragment of the highly conserved mitochondrial ND2 gene and a fragment of the 18SrRNA or RNA polymerase II b housekeeping nuclear genes are amplified separately, and the results are expressed as the ratios mtND2/n18SrRNA or mtND2/RNA pol Iib.

Quantification of ND2 is performed using the forward 5'- GCCCTAGAAATAAACATGCTA-3' and the reverse 5'- GGGCTATTCCTAGTTTTATT- 3' sequences and for the amplification of the 18SrRNA gene the forward 5'- ACGGACCAGAGCGAAAGCAT- 3' and the reverse 5'- GGACATCTAAGGGCATCACAGAC-3' primers are used. The nuclear housekeeping RNA polymerase II b is amplified using the forward 5'- CACCACGTCCAATGACAT-3' and the reverse 5' GTGCGGCTGCTTCCATAA-3' primers. Mitochondrial or nuclear genes are amplified separately in duplicates or triplicates.

The PCR reactions for mitochondrial gene amplification contain: 3mM MgCl<sub>2</sub>, 0.25 pmol/μl of each primer, 2 ng of DNA in 20 μl of final volume. The PCR reactions for the nuclear gene contain: 2 mM MgCl<sub>2</sub>, 0.3 pmol/μl of each primer and 2 ng of DNA in 20 μl of final volume.

The PCR amplification program consists of a single enzyme denaturalization-activation step of 10 minutes at 95°C, followed by 35 cycles in case of 18SrRNA and 29 cycles in case of ND2 gene. Each cycle consists of a denaturalization step (e.g. 95°C), an annealing step (e.g. 53°C), and an extension step (e.g. 72°C). The fluorescent product is detected at the last step. This method uses an unspecific double-stranded DNA dye (SYBR Green I) to monitor the product formation of dimers at real time (**Figure 29**). After the amplification, a melting curve is acquired to determine the specificity of the PCR products.



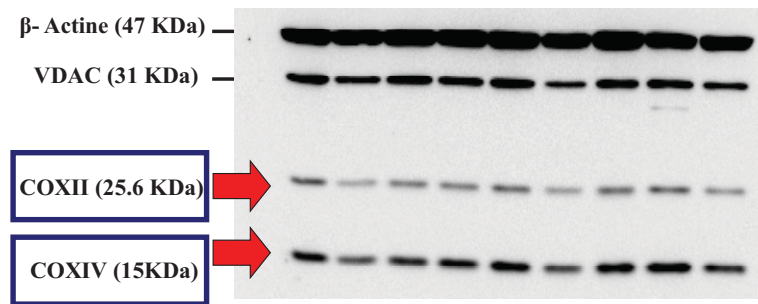
**Figure 29.** On the left, graphs showing the crossing points of both samples and standards (number of the cycle in which the fluorescence is detectable). On the right, screen of the Lightcycler program showing the fluorescence of each unknown samples and known standards (upper right), as well as the repetitive temperature cycles (bottom left) and the curve of fluorescence (bottom right).

## 9. MITOCHONDRIAL PROTEIN SUBUNITS QUANTIFICATION

The quantification of the protein subunit content of the CIV of the MRC (COXII and COXIV) is carried out by western blot. The COXII subunit is mitochondrially encoded, whereas COXIV is nuclear-encoded. The comparison or relative protein content on COXII or COXIV subunit illustrates mitochondrial or nuclear protein synthesis.

The preparation of samples, the electrophoresis gel, the transfer to the membrane, the blocking, the revealing and the quantification procedures are performed as in the VDAC quantification protocol, except the overnight incubation which is carried out with the corresponding COXII (25.6 kDa) and COXIV (15 kDa) primary antibodies (1/800), together with the  $\beta$ -Actine (1/30,000) as the loading sample control (47 kDa) and the VDAC (31 kDa, 1/800) as the mitochondrial loading control. An example of an immunoblot is provided (**Figure 30**).

The results are expressed as the ratios COXII/ $\beta$ -Actin, COXIV/ $\beta$ -Actin (total protein content), COXII/VDAC, COXIV/VDAC (mitochondrial protein content) and COXII/COXIV (mitochondrial vs. nuclear protein content).



**Figure 30.** Western blot showing the structural  $\beta$ -Actin cytoplasmic protein content as a total protein loading marker, the structural porin voltage dependent anion channel (VDAC) as a mitochondrial loading marker, the mitochondrially encoded subunit II of cytochrome c oxidase (COXII) and the nuclear encoded subunit IV of cytochrome c oxidase (COXIV). kDa: kiloDalton.

## **10. ENZYMATIC ACTIVITIES OF THE MITOCHONDRIAL RESPIRATORY CHAIN**

All the enzymatic activities are measured spectrophotometrically at 37°C and the absolute results are expressed as nmol/min·mg protein. However, it is possible to normalize the absolute enzymatic activities by the mitochondrial mass, estimated by citrate synthase activity. The different protocols for the measurement of the MRC complexes are detailed as follows.

### **10.1. COMPLEX II**

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#### **Principle**

Complex II of the MRC transfers electrons from the succinate to the ubiquinone. Its activity can be determined by monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP) measuring the decrease of the absorbance of the oxidized DCPIP at 600 nm. The ubiquinone (Coenzyme Q) is a very hydrophobic natural acceptor that is substituted by decylubiquinone, which is more hydrophilic.

Baseline has to be subtracted to the activity that is initiated by the addition of the decylubiquinone to eliminate the unspecific electron transfer to the DCPIP.

## Method

### Preparation of the reaction mixture:

- . In a 15 ml tube, prepare reaction mixture enough for the amount of programmed samples:
- 50  $\mu\text{l}$  of 500 mM phosphate buffer KP pH 7.5 (composition in the reaction mixture: 25 mM).
- 100  $\mu\text{l}$  of 200 mM succinate (composition in the reaction mixture: 20 mM).
- 10  $\mu\text{l}$  of 5mM DCPIP (composition in the reaction mixture: 50  $\mu\text{M}$ ).
- 100  $\mu\text{l}$  de 10 mM KCN (composition in the reaction mixture: 1 mM).
- 40  $\mu\text{l}$  de 50 mg/ml BSA (composition in the reaction mixture: 2mg/ml).
- 676  $\mu\text{l}$  of distilled  $\text{H}_2\text{O}$ .
- . In a cubette, add 976  $\mu\text{l}$  of the mixture and 20  $\mu\text{l}$  of sample.

### Assay

- Read in the spectrophotometer at 37°C, 600 nm of wavelength. Initial calibration performed on air.
- Incubate the cubettes at 37°C during 5 minutes.
- Read baseline during 3 minutes.
- Start the reaction by adding 4  $\mu\text{l}$  of 25 mM decylubiquinone kept at room temperature (composition in the reaction mixture: 100  $\mu\text{M}$ ).

### Calculations

Subtract baseline (without decylubiquinone); (Abs/min) Specific= (Abs/min) Total- (Abs/min) Baseline.

Extinction coefficient of DCPIP  $\Sigma = 19.2 \text{ mM}^{-1}\text{cm}^{-1}$

Units: nmols/min·mg protein.

## 10.2. COMPLEX II+III

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### Principle

Combined enzymatic activity of the complexes II and III transfers electrons from the succinate to the cytochrome c. This activity can be determined following the increase of the absorbance of the reduced cytochrome c at 550 nm. The following oxidation of the cytochrome c is inhibited by the potassium cyanide (KCN) added to the reaction mixture to avoid simultaneous unspecific CIV measurement.

### Method

Preparation of the reaction mixture:

. In a 15 ml tube prepare enough reaction mixture depending of the programmed samples (there will be 2 assays for each case, one with sample and one without sample for unspecific activity calculation):

- 40  $\mu$ l of 500 mM phosphate buffer KP pH 7.5 (concentration in the reaction mixture: 20 mM).
- 40  $\mu$ l 50 mg/ml BSA (concentration in the reaction mixture: 2 mg/ml).
- 100  $\mu$ l 200 mM succinate (concentration in the reaction mixture: 20 mM).
- 100  $\mu$ l 10 mM KCN (concentration in the reaction mixture: 1 mM).
- 600  $\mu$ l of distilled H<sub>2</sub>O.

. Add 880  $\mu$ l of the reaction mixture in a cubette and 20  $\mu$ l of sample and mix.

### Assay

- Read at 37°C in the spectrophotometer, at 550 nm of wavelength. Initial calibration performed on air.
- Incubate the cubette at 37°C during 5 minutes in the spectrophotometer.
- Start the reaction by adding 100  $\mu$ l of 1mM cytochrome c kept at room temperature.

### Calculations

Specific enzymatic activity of the complex II+III is calculated in nanomols/min mg protein.

Extinction coefficient of the cytochrome c is  $\Sigma = 18.5 \text{ mM}^{-1} \text{ cm}^{-1}$



Specific activity (abs/min)= Total activity (abs/min) (with sample) – Unspecific activity (abs/min) (without sample).

### 10.3. COMPLEX III

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#### Principle

Complex III of the respiratory chain transfers electrons from ubiquinol (reduced form of the ubiquinone or coenzyme Q10) to the cytochrome c. Its reduction can be monitored following the increase of the absorbance at 550 nm, (due to the increase in reduced cytochrome c content).

To evaluate the activity of the III, it is required to perform 2 assays in parallel, either in presence or in absence of antimycin A, the inhibitor of complex III. The specific activity of this complex is the activity which is sensitive to the antimycin A and is calculated by the subtraction of the insensitive activity from the total ubiquinol cytochrome c oxide-reductase activity.

#### Method

##### Preparation of the reaction mixture:

In a 50 ml Falcon prepare enough reaction mixture depending on the programmed samples (there will be 2 assays for each sample, either with or without inhibitor):

- 440 µl of 500 mM phosphate buffer KP pH 7.5 (Concentration in the reaction mixture: 100 mM).
- 110 µl 1 mM cytochrome c (Concentration in the reaction mixture: 50 µM).
- 11 µl 50 mM EDTA (dilution 1/10 stock 0.5M) (Concentration in the reaction mixture: 250 µM).
- 220 µl 10 mM KCN (Concentration in the reaction mixture: 1 mM).
- 1353 µl of distilled H<sub>2</sub>O.
- Add 2134 µl of reaction mixture and 33 µl of sample and mix in a tube.
- Add 5 µl of antimycin 2.5 mg/ml in one of the cubettes and 5 µl of ethanol to the other.
- Transfer 985 µl of the mixture to the 2 cubettes and mix.

#### Assay

- Read at 37°C in the spectrophotometer, at 550 nm of wavelength. Initial calibration performed on air.

- Incubate the 2 cubettes at 37°C during 5 minutes in the spectrophotometer.
- Start the reaction by adding 10 µl of 21.3 mM decylubiquinol (kept at 37°C) (Concentration in the reaction mixture: 213 µM).
- Both cubettes containing the same sample should be read in parallel simultaneously.

#### Calculations

Specific activity of complex III is calculated in nmols/min·mg protein by subtracting the unspecific activity to the total enzymatic activity:

Specific (Abs/min) = total (Abs/min) / without antimycin - (Abs/min) / with antimycin

Extinction coefficient of cytochrome c is  $\Sigma = 18.5 \text{ mM}^{-1} \text{ cm}^{-1}$

#### Reduction of decylubiquinone

Preparation: heat a block or a bath at 110°C and a thermoblock at 37°C.

- Boil 1 ml of water in an eppendorf with a hole in the lid at 110°C (bath or plate).
- Thaw an aliquot of 88 µl of decylubiquinone (in a 1.5 ml tube) at room temperature. Each tube of 88 µl is used for 2 samples (4 cubettes).
- Weigh at least 230 mg of dithionite in an eppendorf (2-3 tubes).
- Dilute the dithionite that has been weighed with the boiling water (1µl/1mg) and mix with a pipette.
- Shake with the finger (no vortex). Make a hole in the lid and leave it at 110°C during 3-4 minutes until it is dissolved without stopping the boiling point. It is not always completely dissolved, in that case, make a spin (10 seconds) in the centrifuge and use the supernatant.
- Add 15 µl of the upper part of the 100% dithionite solution to the 88 µl of decylubiquinone at room temperature and mix with a pipette (no vortex).
- Leave it at 37°C without shaking and in darkness for 30 minutes (without handling it anymore. It will turn into a white-transparent colour). Use the transparent central part within the next 30 minutes (it is possible to leave it, maximum, for 1 hour and 30 minutes).
- 6 cubettes may be used simultaneously. If the decrease is too fast following a non lineal curve, it is necessary to repeat the assay by diluting the sample with mannitol buffer.

## 10.4 COMPLEX IV

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### Principle

The complex IV transfers electrons from the reduced cytochrome c to the oxygen and this activity can be measured by monitoring the decrease of the absorbance of the reduced cytochrome c at 550 nm.

### Reaction medium composition:

- Phosphate buffer 50 mM pH 7.0.
- 100  $\mu$ M reduced cytochrome c.

### Preparation of reduced cytochrome c

- Initial solution: Prepare 14 ml of 100  $\mu$ M cytochrome c in 50 mM phosphate buffer KP pH 7,0 through the formula  $0.930 \text{ ml buffer} \times \text{mg of cytochrome c}$  (weight 13-14 mg of cytochrome).
- 100% oxidized total solution: Take 1 ml of initial solution and add some grains (spatula) of potassium hexacyanoferrate (III) (the colour of the solution will turn into dark red-brown).
- 100 % reduced total solution: Take 2 ml of initial solution and add some grains (spatula) of sodium dithionite (the colour of the solution will turn into a pink salmon).
- Autozero on air and reach the absorbance of the 100% oxidized solution at 550 nm in the spectrophotometer. The absorbance should be around 0.7 in the 100  $\mu$ M solution.
- Autozero with the 100% oxidized solution and read the absorbance at 550 nm of the 100% reduced solution. This value will be considered the 100% of reduction.

### Preparation of the initial solution with the reduced solution

- Transfer an aliquot (50-100  $\mu$ l) of the 100% reduced solution into the initial solution of the cytochrome c and read the new absorbance.
- Continue adding aliquots until reach an absorbance between 90- 95% of the absorbance of the 100% reduced solution.

### Assay:

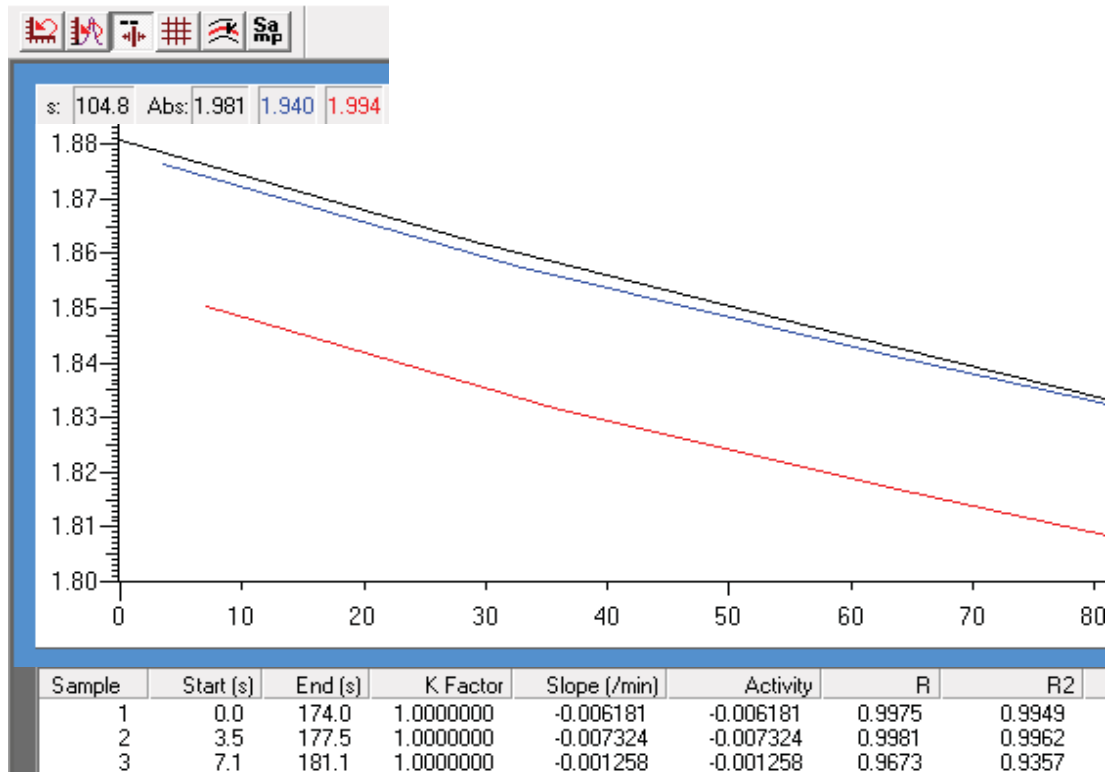
- Read in the spectrophotometer at 37°C, 550 nm of wavelength (**Figure 31**). Initial calibration performed on air.
- Incubate the cubettes containing 980  $\mu$ l of the reduced initial solution at 37°C, during 5 minutes.

- Start the reaction by adding 20  $\mu\text{l}$  of the sample.

Calculations:

Extinction coefficient for the cytochrome c is  $\epsilon = 18.5 \text{ mM}^{-1} \text{ cm}^{-1}$

Specific activity of the complex IV is calculated in nmols/min-mg protein.



**Figure 31.** Graphic showing the decrease of the absorbance of reduced cytochrome c at 550 nm.

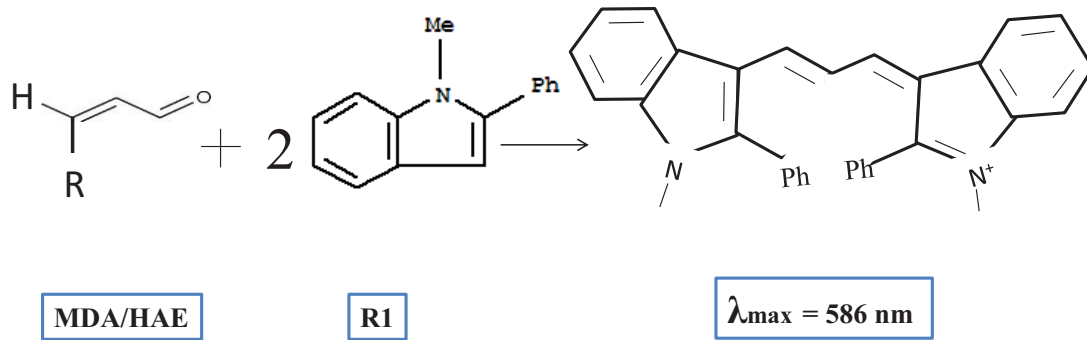
## 11. OXIDATIVE STRESS: QUANTIFICATION OF LIPID PEROXIDATION

### Principle

The assay (OxisResearch<sup>TM</sup> Bioxytech® LPO-586) is based on the reaction of a chromogenic agent, N-metil-2-phenylindol (R1), with the malondialdehyde (MDA) and 4-hydroxialkenals (HAE) at 45°C. A molecule, either MDA or HAE, reacts with two molecules of the R1 to yield a stable chromophore with the maximal absorbance at 586 nm (**Figure 32**). For a simultaneous determination of MDA and HAE, methanesulfonic acid (MSA) is used as the acid solvent. On

the contrary, the procedure in which HCl is used will only detect MDA, since the HAE do not form a chromophore with reagent R1 under those conditions.

The overall reaction is:



**Figure 32.** The reaction of malondialdehyde (MDA) and/or 4-hydroxyalkenals (HAE) with two molecules of n-metil-2-phenylindol (R1) results in the formation of the stable chromophore with maximal absorbance at 586 nm.

#### Assay

- Prepare 0-200  $\mu\text{M}$  standards of 1,1,3,3- tetramethoxypropane (TMOP) provided (this compound is equivalent to and will generate MDA).
- Add 20  $\mu\text{l}$  of sample in eppendorf tubes.
- Add 65  $\mu\text{l}$  of diluted R1 and vortex.
- Add 15  $\mu\text{l}$  12N MSA (or HCl) and vortex.
- Incubate at 45  $^{\circ}\text{C}$  during 60 minutes.
- Centrifuge at 15,000 rcf for 10 minutes and take the clear supernatant.
- Transfer the supernatant to the wells in a plate and read the absorbance at 586 nm.

The results are expressed as the concentration of MDA and HAE ( $\mu\text{M}$ ) normalized by the cellular protein content ( $\mu\text{M}/\text{mg}$  protein).

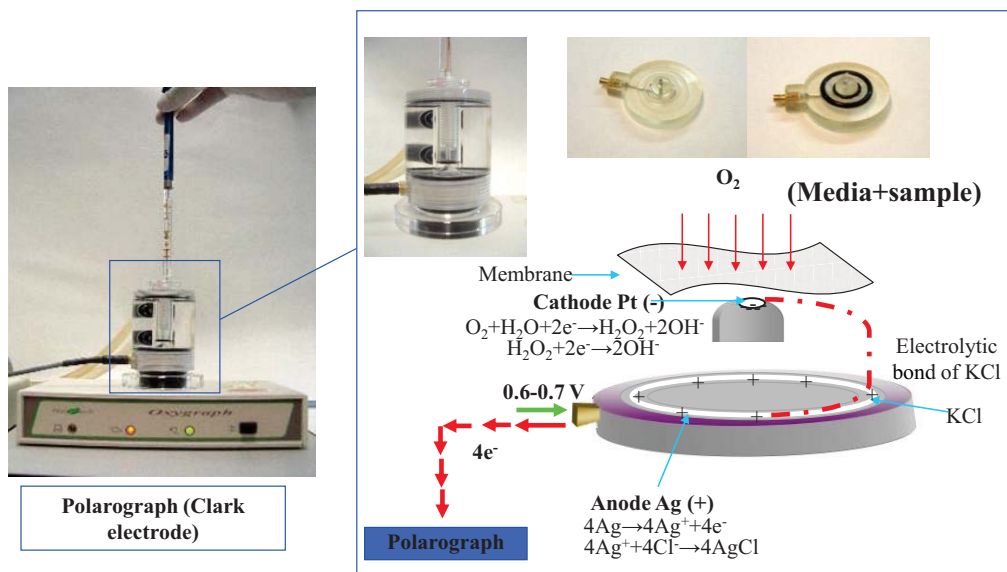
## **12. MITOCHONDRIAL OXIDATIVE ACTIVITIES**

The oxygen consumption is measured by polarography in fresh samples. This procedure is carried out by a Clark polarograph consisting of two thermostated chambers at 37  $^{\circ}\text{C}$  (**Figure**

33). Under each microcubette there is an electrode covered by a Teflon membrane, permeable to oxygen. This membrane is in contact with the sample and detects the reduction of oxygen concentration over time, as a consequence of the naturally occurring cellular oxygen consumption through the mitochondria. It is possible to either determine the oxidative activities of intact cells through the oxidation of endogen substrates within them (Cellox), or to analyze isolate complexes by the addition of specific substrates and inhibitors. Thus measuring, for instance, specific CI stimulation through pyruvate-malate oxidation (PMox), glutamate-malate oxidation (GMox), specific CII stimulation through succinate oxidation (Sox), glycerol-3-phosphate oxidation (G3Pox), specific CII stimulation through succinate oxidation (Sox), glycerol-3-phosphate oxidation (G3Pox), specific CIV stimulation through ascorbate oxidation (ATox) and  $\beta$ -oxidation activity stimulation through acetyl carnitine oxidation.

The results are expressed by nmols of consumed oxygen/minute·mg of protein.

### Oxidative activities in fresh cells/Mitochondria



**Figure 33.** On the left hand side, one of the two chambers containing a microcubette, in the moment of the addition of a given substrate through the Hamilton syringe. On the right hand side, an electrode located at the base of the chamber, consisting of an argent anode and a platinum cathode, covered with a Teflon membrane, which is permeable to oxygen and which contacts directly to the sample. When the electric voltage is applied, the cathode is negatively charged and the anode is positively charged and the oxygen from the cubette diffuses through the membrane and it is reduced. The generated electrons flow through an electrolytic bond (potassium chloride, KCl) to the anode and contact to the argent generating an electric current

which is directly proportional to the oxygen concentration within the cubette and this is registered by the polarograph, being possible to measure the decrease of oxygen derived from mitochondrial respiration.

### **13. APOPTOSIS**

The assessment of the apoptosis enables the measurement of early or advanced stages of programmed cell death. The former can be measured through the analysis of the membrane potential and the latter can be determined through the quantification of caspase-3 content.

#### **13.1. MITOCHONDRIAL MEMBRANE POTENTIAL MEASUREMENT \_\_\_\_\_**

##### Principle

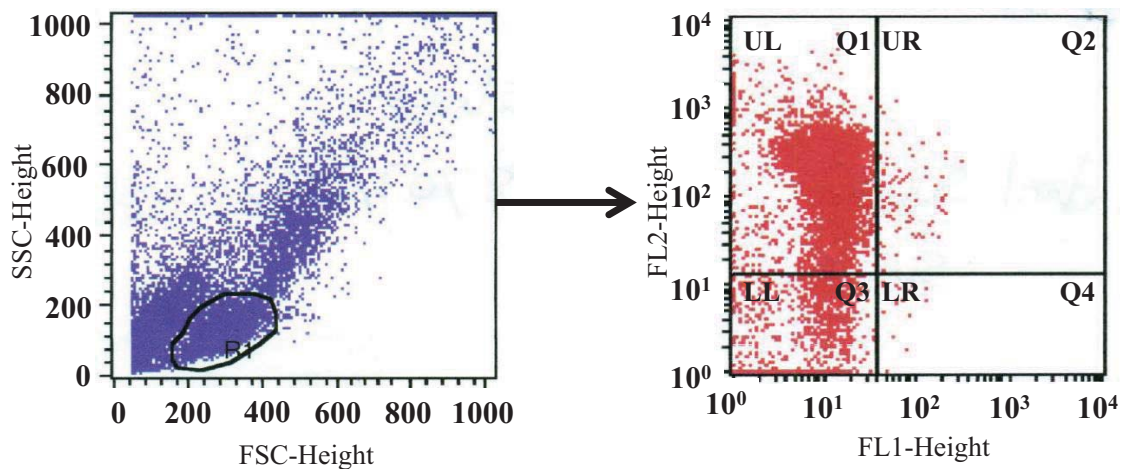
The measurement of the membrane potential is carried out by flow cytometry by means of the JC1 fluorochrome. The JC1 changes the emission wave length depending on the mitochondrial membrane potential status. Thus, JC1 forms aggregates and emits orange spectrum of light (FL2) when the membrane potential remains intact (-180 mV) and turns into monomers which emit green light (FL1) when mitochondrial membrane potential is disrupted.

The results are expressed as the percentage of cells containing reversibly or irreversibly depolarized mitochondria with respect the total cells.

The procedure to assess the membrane potential is detailed.

- Add ~ 3 µl PBMC to 3 ml of complete media.
- Place the cellular suspension on a Neubauer chamber to count.
- Use  $0.75 \times 10^6$  cells for each condition (in each cytometer tubes):
  - . Blank= negative control → not dyed and not depolarized sample.
  - . JC1= unknown sample → dyed sample.
  - . Valinomycin + JC1= Positive control → Depolarized and dyed sample.
- Centrifuge 7 minutes at 250-300 rcf.
- Discard supernatant and dissolve in 1 ml of complete media.
- . In the positive control:
  - Add 0.5 µl of intermedia solution of valinomycin.
  - Incubate at room temperature for 10 minutes (in the dark).

- Add 1 ml PBS.
- Centrifuge 7 minutes at 250-300 rcf.
- Discard supernatant and dissolve with 1 ml of PBS.
- . In the unknown sample as well as in the positive control.
- Prepare intermediate solution of JC1= 1.25 mg/ml of JC1 in dimethyl formamide DMFA from stock solution= 2.5 mg/ml (in the dark).
- Add 2  $\mu$ l of intermediate solution JC1 in each tube.
- Incubate 10 minutes at room temperature and in the dark.
- . In all tubes (blank, unknown sample and positive control):
- Centrifuge 7 minutes at 250-300 rcf.
- Discard supernatant.
- Dissolve in 400  $\mu$ l of PBS.
- Analyze in FACScan/FACScalibur (**Figure 34**).



**Figure 34.** Measurement of mitochondrial membrane potential levels through JC1. UL: Upper left quadrant, Q1: Quadrant 1 showing those cells with polarized mitochondria. UR: Upper right, Q2: Quadrant 2 showing cells with reversibly depolarized mitochondria. LL: Lower left, Q3: Quadrant 3 showing unspecific fluorescence. LR: Lower right, Q4: Quadrant 4 showing cells containing irreversibly depolarized mitochondria.



### **13.2. QUANTIFICATION OF ACTIVE/CLEAVED CASPASE-3 CONTENT**\_\_\_\_\_

The quantification of the caspase-3 content is performed through western blot. The procedure is the same as in the quantification of VDAC methodology. However, the main differences are that the primary antibody to use overnight is against cleaved and active caspase-3 and that the second antibody is anti-rabbit.

## RESULTS



**Mitochondrial assessment in  
asymptomatic HIV-infected  
paediatric patients on HAART**

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Antiviral Therapy 2011;16(5):719-724



## Original article

# Mitochondrial assessment in asymptomatic HIV-infected paediatric patients on HAART

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**Background:** HAART can cause mitochondrial DNA (mtDNA) depletion, which may lead to mitochondrial dysfunction. We aimed to determine whether mtDNA and mitochondrial function abnormalities are present in peripheral blood mononuclear cells from asymptomatic HIV-infected children.

**Methods:** A cross-sectional study in peripheral blood mononuclear cells was performed in 47 asymptomatic (free from any HIV- or AIDS-related active condition or HAART-related toxicity), HIV-infected, HAART-treated children and adolescents and 27 uninfected healthy paediatric patients. We measured mtDNA and mitochondrial RNA (mRNA) content by quantitative real-time PCR. Mitochondrial respiratory chain enzymatic activity of complex-IV (CIV) and mitochondrial mass (estimated by citrate synthase) were measured spectrophotometrically, and CIV protein subunit content was measured with western blot analysis.

**Results:** A reduction in mtDNA levels was observed in HIV-infected children compared with controls (mean  $\pm$ SEM 4.47  $\pm$ 0.31 and 5.82  $\pm$ 0.48, respectively; 23% depletion;  $P=0.018$ ), whereas similar levels of mRNA, CIV protein subunit content and enzymatic activity were found in the two groups. These findings remained unaltered after considering mitochondrial abundance. Among HIV-infected children, mtDNA levels did not correlate with viral load, CD4<sup>+</sup> T-cell counts or lactataemia at the time of assessment. No differences were observed when current or past use of individual antiretroviral drugs or HAART regimens were taken into account.

**Conclusions:** Depletion in mtDNA from asymptomatic HIV-infected children did not lead to differences in mRNA levels or mitochondrially-encoded CIV proteins, nor to CIV dysfunction. This may be explained by homeostatic-compensatory mechanisms at the transcription level or by the mild depletion we observed.

## Introduction

HAART has improved morbidity and mortality from HIV infection and AIDS, including among paediatric patients [1]. HAART-related toxicities are increasingly being reported in HIV-infected children as well [2,3]. In children, HAART generally includes two or more nucleoside reverse transcriptase inhibitors (NRTIs), which inhibit both HIV transcriptase and DNA polymerase- $\gamma$  [4–6]. As DNA polymerase- $\gamma$  is the only enzyme responsible for mitochondrial DNA (mtDNA) replication and mtDNA repair, the presence of mtDNA depletions, multiple deletions and point mutations has been described in HIV-infected patients receiving NRTIs [4,5]. Since mtDNA codifies for 13 proteins of the oxidative phosphorylation

system responsible for cellular respiration and aerobic energy production, a reduced content of protein subunits in some complexes of the mitochondrial respiratory chain (MRC) and abnormalities in MRC activities have also been observed in adults [7]. Our understanding of the complexity of HAART-related mitochondrial toxicity has increased with the recent description of compensatory homeostatic mechanisms [8] and alternative pathogenic pathways different from DNA polymerase- $\gamma$  inhibition [9–11]. Furthermore, protease inhibitors (PIs) [12] and non-nucleoside reverse transcriptase inhibitors (NNRTIs) [13] have been associated with some effects on mitochondrial-dependent apoptotic pathways.

Mitochondrial toxicities have been scarcely studied in the paediatric population. HAART-associated mitochondrial toxicity in children has varied clinical presentations, comparable to those affecting the HIV-infected adult, but also comparable to children with congenital mitochondrial diseases. The fact that children will be exposed to antiretrovirals (ARVs) for an ever-increasing length of time throughout postnatal growth and development should not be forgotten. A few studies, with small numbers of patients and no healthy controls, have quantified mtDNA content in HAART-treated HIV-infected children, and they have failed to demonstrate depletion [14,15], without yielding further insight into the pathogenesis underlying these findings. In this study, we aim to reveal whether there is HAART-related depletion of mitochondrial genome in a large series of asymptomatic, HIV-infected, HAART-treated, paediatric patients and, if it is present, to determine whether it is reflected at the transcriptional, translational or functional levels.

## Methods

A cross-sectional study was conducted in a series of consecutive asymptomatic HIV-infected HAART-treated children and adolescents ( $n=47$ ) belonging to a larger cohort of HIV-infected paediatric patients followed-up in a tertiary-care paediatric centre in Barcelona, Spain. At inclusion, being free from any HIV- or AIDS-related clinical condition and HAART-related toxicity was required; the latter included any clinical sign or symptom consistent with hyperlactataemia, myopathy, neuropathy or fat redistribution, which were assessed by means of clinical interview, patients and parents self-report, and physical exam. A sex- and age-matched control group of HIV-uninfected healthy paediatric patients referred for presurgical routine analysis was used ( $n=27$ ). Local ethics committee approval and informed consent from parents or legal guardians were obtained at inclusion.

Peripheral blood mononuclear cells (PBMCs; lymphocytes and monocytes) were obtained from 5–10 ml of venous blood. PBMCs were isolated by centrifugation, using a Ficoll's gradient (Histopaque 1077; Sigma Diagnostics, St Louis, MO, USA) [16]. Less than 200 platelets were counted for each PBMC. Isolated PBMCs were cryopreserved at  $-80^{\circ}\text{C}$ . Total protein content of cells was measured using Bradford's reagent method at 595 nm [17]. Mitochondrial mass (MM) was estimated by citrate synthase (CS) activity, using an enzyme located in the mitochondrial matrix that acts in Krebs cycle [18,19], and by western blot analysis, assessed as the ratio between the mitochondrial voltage-dependent anion channel (VDAC) and the cytoplasmatic  $\beta$ -actin protein [8].

As a representative of replication efficiency, we quantified mtDNA content by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals®, Mannheim, Germany), separately analysing a fragment of the highly conserved ND2 mitochondrial-encoded (mtND2) gene and a sequence of the housekeeping nuclear-encoded 18S rRNA gene (n18S). Extensive methodology is reported elsewhere [7]. The results were expressed as the ratio between mtND2 gene and n18S gene [20].

As a representative of transcriptional efficiency, mitochondrial RNA (mtRNA) was extracted by means of an affinity column-based procedure (RNA II, Nucleospin®; Cultek, Madrid, Spain) and reversely transcribed (GeneAmp PCR System 2400; Perkin Elmer, Rodgau, Germany) using random primers (TaqMan reverse transcriptase; Applied Biosystems, Foster City, CA, USA) in a total reaction volume of 20  $\mu\text{l}$ . For each extract, mtRNA was assessed by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I). Quantification of ND2 mtRNA was performed using the forward 5'-GCCCTAGAAATAAACATGCTA-3' and the reverse 5'-GGGCTATTCCTAGTTTTATT-3' sequences. The results were expressed as the ratio between mtND2 RNA and n18S RNA.

As a representative of translational efficiency, cytochrome c oxidase (CIV or COX) subunits II (COXII; mitochondrial-encoded) and IV (COXIV; nuclear-encoded) were quantified by western blot analysis, and expressed as the ratio of COXII/COXIV [8].

As a representative of functional efficiency, MRC enzymatic activity was evaluated using the methodology of Rustin *et al.* [21], slightly modified for mitochondrial CIV measurement in minute amounts of biological sample [22]. Enzymatic activities were expressed as absolute values in nmol/min/mg. All absolute measurements were corrected by MM in order to exclude any influence of mitochondrial proliferation or reduction in such values. This correction was performed by dividing mtDNA content, mtRNA content and CIV activity by CS activity, and COXII/COXIV ratio by VDAC.

## Statistical analysis

Mitochondrial assessment results were expressed as mean  $\pm$ SEM, and those of HIV-infected children were expressed as percentages of control values, which were arbitrarily assigned to 100%. The Kolmogorov–Smirnov test was performed to ascertain the normal distribution of data. Groups with normal data were compared using the Student's *t*-test on unrelated samples. Non-normally distributed parameters were analysed with the non-parametric Mann–Whitney U test. Linear regression analysis was used to uncover any relationship between quantitative variables. Statistical significance was considered when a *P*-value was  $<0.05$ .

Statistical analysis was performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

## Results

Baseline characteristics of the 47 asymptomatic HIV-infected HAART-treated children included in the study are summarized in Table 1. At the time of assessment, none of the patients in our study showed clinically significant signs or symptoms consistent with HAART-related toxicities or other active conditions. MM, estimated by means of CS activity and VDAC content (Figure 1), remained similar in HIV-infected children and healthy controls ( $n=27$ , 52% females, 89% Caucasian race, median age 12.3 years).

The mtDNA content was 23% lower in HIV-infected HAART-treated children with respect to healthy controls (mean mtND2/n18s gene ratios  $\pm$ SEM  $4.47 \pm 0.31$  versus  $5.82 \pm 0.48$ ;  $P=0.018$ ); conversely, mtRNA, mitochondrial protein content and CIV activity were not significantly different between groups (Figure 2). When all these parameters were corrected by MM, nearly identical results were found (Figure 3): mtDNA depletion with indemnity of all other mitochondrial downstream parameters.

Regarding the mtDNA depletion found among HIV-infected HAART-treated children, mtDNA amount was not associated with gender, age or lactate levels (normal range 0.55–1.77 mmol/l), CD4<sup>+</sup> T-cell counts, and HIV plasmatic viral load at the time of assessment. Receiving an ARV combination containing didanosine, zidovudine or stavudine, or being allocated on a PI-based regimen (lopinavir/ritonavir  $n=10$  or fosamprenavir/ritonavir  $n=8$ ) did not influence the degree of mtDNA depletion. Finally, no differences were observed in mitochondrial parameters when current therapeutic regimens were taken into account (time period on HAART regimen, individual use of each of the NRTIs and tenofovir, and use of the following combinations: zidovudine plus lamivudine/emtricitabine, abacavir plus lamivudine/emtricitabine and tenofovir plus lamivudine/emtricitabine; CM *et al.*, data not shown).

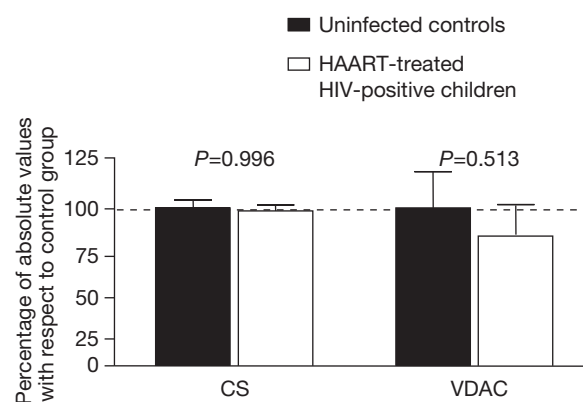
After excluding those patients not vertically infected ( $n=3$ ), 27 out of 44 children (61%) had been treated with suboptimal NRTI monotherapy or bitherapy before HAART implementation. These patients were younger at HIV infection diagnosis (mean values 0.78 versus 3.05 years;  $P=0.028$ ), had been on ARV (13.01 versus 6.21 years;  $P=0.006$ ) and on HAART (9.09 versus 6.21 years;  $P<0.001$ ) treatment longer, and had developed AIDS more often (52% versus 15%;  $P=0.009$ ) than children who were initially treated with a HAART regimen. Again, no differences in mtDNA content or mitochondrial function parameters were observed between these two groups. Overall, median

**Table 1.** Baseline characteristics of the HIV-infected patients at the time of mitochondrial function and mitochondrial DNA content assessment

Patient characteristic ( $n=47$ )	Value
Female gender, $n$ (%)	28 (59.6)
Caucasian, $n$ (%)	37 (78.7)
Vertically-transmitted HIV infection, $n$ (%)	44 (93.6)
Age at HIV infection diagnosis, years	4.5 (0–14.4)
AIDS, $n$ (%)	17 (36.2)
Age, years	13.3 (0.9–19.3)
CD4 <sup>+</sup> T-cell count, cells/mm <sup>3</sup>	840 (182–4,346)
CD4 <sup>+</sup> T-cell percentage	34 (13–53)
HIV viral load <400 copies/ml, $n$ (%)	41 (87.2)
Lactate levels, mmol/l	1.0 (0.5–2.5)
<b>Previous ARV treatment</b>	
NRTIs suboptimal therapy, $n$ (%)	27 (57.4)
Cumulative time on any ARV, years	10.8 (0.3–17.1)
Cumulative time on HAART, years	8.8 (0.3–10.8)
Number of HAART regimens	4 (1–11)
<b>Current type of HAART</b>	
PI-based regimen, $n$ (%)	26 (55.3)
NNRTI-based regimen, $n$ (%)	18 (38.3)
Triple-NRTI regimen, $n$ (%)	3 (6.4)
<b>Nucleoside/nucleotide backbone</b>	
Lamivudine or emtricitabine, $n$ (%)	38 (80.8)
Tenofovir, $n$ (%)	22 (46.8)
Zidovudine, $n$ (%)	12 (25.5)
Abacavir, $n$ (%)	11 (23.4)
Stavudine, $n$ (%)	8 (17.0)
Didanosine, $n$ (%)	2 (4.3)

All results expressed as median (range), except where stated otherwise. ARV, antiretroviral; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

**Figure 1.** Percentage of mitochondrial mass of the patients with respect to the control group



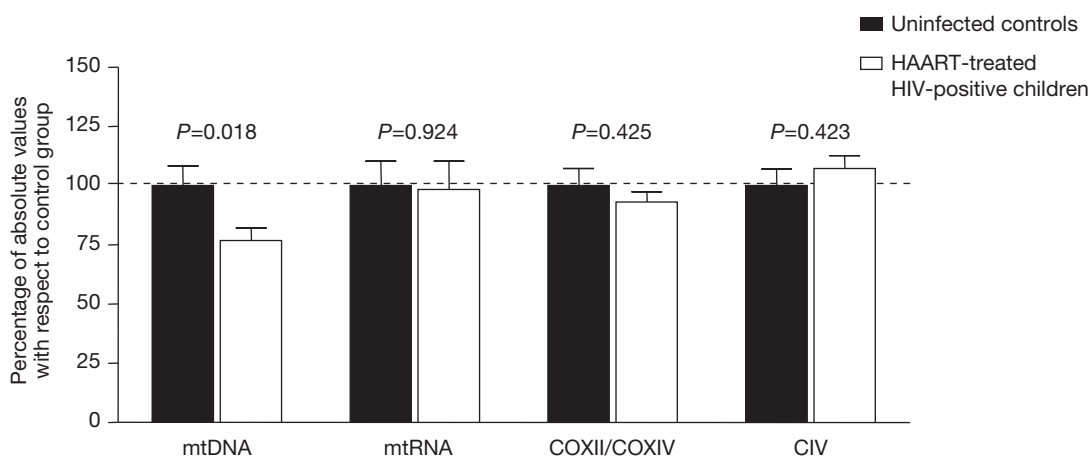
The dotted line represents 100%. CS, citrate synthase; VDAC, voltage-dependent anion channel.



cumulative time on any ARV treatment and on HAART were 10.8 and 8.8 years, respectively; median (range) cumulative time (time on each separate drug in the class added together) for the patients on NRTIs, PIs and NNRTIs were 218 (8–350), 53 (0–116) and 25 (0–116) months, respectively. Neither the cumulative

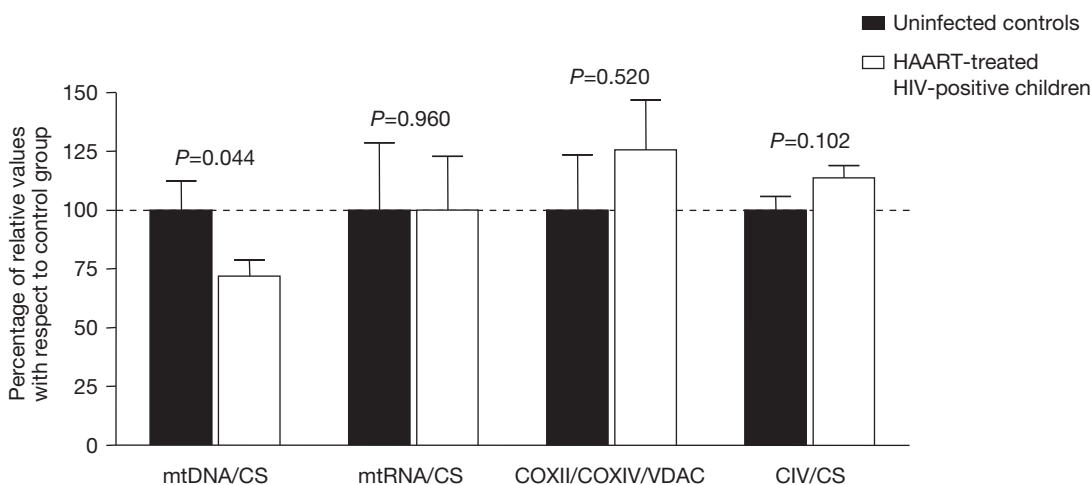
time (on ARV families and on individual drugs including all NRTIs, tenofovir, efavirenz, nevirapine, lopinavir/ritonavir and nelfinavir) nor the exposure (yes/no) to each of these ARV families and drugs were significantly correlated with mtDNA content (CM *et al.*, data not shown).

**Figure 2.** Percentage of absolute values of mitochondrial DNA and RNA, mitochondrial protein content, and complex IV activity of patients with respect to control group



Mitochondrial protein content expressed as the ratio between cytochrome c oxidase subunits II (COXII) and IV (COXIV). The dotted line represents 100%. CIV, complex IV; mtDNA, mitochondrial DNA; mitochondrial RNA.

**Figure 3.** Percentage of relative values of mitochondrial DNA and RNA, mitochondrial protein content, and complex IV activity, normalized by mitochondrial mass of the patients with respect to the control group



Mitochondrial protein content expressed as the ratio between cytochrome c oxidase subunits II [COXII] and IV [COXIV]. Mitochondrial mass represented by citrate synthase (CS) or voltage-dependent anion channel (VDAC). The dotted line represents 100%. CIV, complex IV; mtDNA, mitochondrial DNA; mitochondrial RNA.

## Discussion

To date, three studies have investigated mtDNA levels in the PBMCs of HIV-infected children. Saitoh *et al.* [23], in a 104-week follow-up of 31 patients on stable HAART and suppression of viral replication, identified didanosine as the only NRTI associated with mtDNA suppression in PBMCs before and during HAART. Another study showed no difference in PBMC mitochondrial function or mtDNA content between HIV-infected children with ( $n=6$ ) or without ( $n=12$ ) lipodystrophy [14]. Recently, mtDNA evolution was investigated in a group of 18 HIV-infected children who were randomized either to continue on a stavudine-containing HAART regimen (arm A,  $n=9$ ) or to switch to tenofovir (Arm B,  $n=9$ ); no changes were noted in PBMC mtDNA levels after 18 months of follow-up [15]. To our knowledge, our study is the first one to investigate not only mtDNA content, but also mtRNA, mitochondrial protein subunits content and mitochondrial function in a large series of asymptomatic, HIV-infected, HAART-treated patients in the paediatric age. As opposed to previous reports, which describe relevant variability in the mtDNA content and which do not show any mtDNA depletion in HIV-infected children [14,15], we found a 23% decrease in mtDNA in PBMCs of asymptomatic, HIV-infected, HAART-treated children when compared with HIV-negative healthy controls. This difference may be due to the greater number of patients recruited in this study.

Mitochondrial DNA depletion was not reflected in a decrease of mtRNA amount or in the content of protein subunits, nor, consequently, in function. Therefore, although statistically significant, mtDNA depletion appears to have no current clinical or cellular consequences in the patients we present. Whether these alterations in mtDNA content will become clinically significant in the presence of precipitating factors or persist into adulthood remains unknown.

The fact that mtDNA depletion was not associated with unaltered transcriptional, translational and functional efficiency may be explained by different hypothesis. It is possible that changes in mtDNA and mitochondrial function in PBMCs are not good markers of mitochondrial toxicity in organ-specific tissue, especially in those tissues more dependent on oxidative phosphorylation, such as muscle [24]. By contrast, we previously hypothesized that, in HIV-infected adults, up-regulatory transcriptional or post-transcriptional mechanisms could compensate for mtDNA depletion as well [8]. Supporting the latter view, the differences in mtDNA were not reflected in mtRNA content in our study. Furthermore, since we only observed mild mtDNA depletion (23%), another explanation for the normal mtRNA amount, protein subunit content and

function is that only mtDNA defects involving >80% of the genetic material are able to induce MRC failure and cellular dysfunction, potentially leading to clinically significant symptoms [25]. Compensatory mechanisms, especially an increase in the transcriptional rate, are probably sufficient to maintain mitochondrial function.

Several studies in adults have described decreased mtDNA levels in HIV-infected ARV-naive patients, confirming the role that HIV infection by itself plays in mitochondrial dysfunction. Saitoh *et al.* [23], in a longitudinal study of 31 children, showed a negative correlation between mtDNA and intracellular HIV DNA levels in PBMCs in early stages of HAART, and afterwards they found that didanosine-containing HAART regimens led to smaller increases in mtDNA over time when compared with other regimens, demonstrating that the beneficial effect of decreasing viraemia in mtDNA replication may be masked by the negative effect of some NRTIs on mitochondria. By contrast, not only the use of NRTIs, but also exposure to PIs and NNRTIs has been recently associated with mitochondrial damage [12,13]. In our study, current or past use of ARV families and drugs, clinical characteristics, and immunological or virological parameters were not associated with differences in mtDNA content. The cross-sectional study that we present does not allow us to draw causal conclusions, but both long-term exposure to HAART and complete viral suppression at the time of assessment in most patients suggest that the depletion in mtDNA that we observed is attributable to the long-term use of ARV rather than to a harmful effect of HIV. Accordingly, we recently reported that planned interruption of HAART led to a partial restoration of mtDNA levels and a significant decrease in lactate values in a series of 13 asymptomatic vertically HIV-infected children [26]. The small sample size and the heterogeneity in the characteristics of the patients studied are major limitations of our study and hampered identification of the risk factors of mitochondrial damage that several authors have previously described. As an example, only eight and two children, respectively, were receiving stavudine or didanosine, the most harmful NRTIs, at the time of assessment, but 34 out of 47 of the patients had previously received one or the other of those drugs for a mean cumulative time of 91 months. Finally, excluding those patients with symptomatic HAART-related toxicities probably represents a selection bias in our study that may depict the mildest form of the toxicity spectrum, since some of these adverse effects have been associated with mitochondrial dysfunction in HIV-infected adult patients [7,11,24].

In summary, we detected mtDNA depletion in PBMCs from asymptomatic HIV-infected children receiving HAART. This depletion was not reflected in differences in mtRNA or in structural or functional enzymatic

activities of MRC. These results are consistent with the large body of evidence available on the pathogenesis of HIV-related and ARV-related mitochondrial toxicity in the HIV-infected adult patient. In the investigation of mitochondrial toxicity, PBMCs remain, despite their well-known limitations, the tissue of choice with regard to ease of sampling and analysis, especially among children. Further investigation is needed to better define mitochondrial toxicity pathways in HIV-infected paediatric patients who develop adverse effects, but also to determine the long-term clinical significance of these findings in asymptomatic children.

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## Disclosure statement

The authors declare no competing interests.

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**Study of oxidative, enzymatic  
mitochondrial respiratory chain  
function and apoptosis in perinatally  
HIV-infected pediatric patients**

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CHAIN FUNCTION AND APOPTOSIS IN PERINATALLY HIV- INFECTED  
PEDIATRIC PATIENTS**

**Running head:** Mitochondrial toxicity in HIV+ pediatric patients

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## **ABSTRACT**

**Background.** Mitochondrial toxicity in perinatally HIV-infected pediatric patients has been scarcely investigated. There is left to know about HIV or antiretroviral mediated mitochondrial damage in this population group, specifically, regarding oxygen consumption and apoptosis approach. We aimed to elucidate whether a given mitochondrial DNA depletion is reflected at downstream levels, to gain insight pathology of HIV and HAART in perinatally HIV-infected pediatric patients.

**Methods.** We studied 10 healthy controls and 20 perinatally HIV-infected pediatric patients (10 under antiretroviral treatment and 10 off treatment). We determined mitochondrial mass, subunits II and IV of complex IV, global and specific mitochondrial enzymatic and oxidative activities and apoptosis from peripheral blood mononuclear cells.

**Results.** Global oxygen consumption was significantly compromised in HIV-infected untreated patients, in whom apoptosis showed a trend to increase. The overall CI-III-IV activity of the mitochondrial respiratory chain was significantly decreased in HIV-infected treated patients.

**Conclusions.** These findings suggest the pathogenic role of HIV and HAART in mitochondrial dysfunction in vertical infection. The abnormalities in mitochondrial genome may be downstream reflected through a global alteration of the mitochondrial respiratory chain. Mitochondrial impairment associated with HIV and HAART was generalized rather than localized in our perinatally HIV-infected cohorts.

A dramatic decrease has been observed in the rates of HIV mother-to-child transmission following the implementation of several prophylactic measures in the past 15 years [1]. These measures include the use of antiretrovirals in HIV-infected women during pregnancy and delivery as well as in newborns during the neonatal period, the use of elective caesarean section as the standard method of delivery and avoidance of breastfeeding. Nonetheless, mother-to-child transmission of HIV remains a major health problem, with a yearly estimate of 370,000 new pediatric infections, mostly in developing countries. Currently, every infant under the age of 12 months with newly diagnosed HIV infection is expected to receive highly



active antiretroviral treatment (HAART) regardless of the clinical, immunological or virological status. According to current guidelines, these infants will be exposed to HAART throughout their lives. Mitochondrial toxicity by different pathogenic routes has been extensively documented in adults with HIV and HAART, and it has been suggested that this mitochondrial toxicity underlies several secondary effects, such as hyperlactatemia/lactic acidosis, pancreatitis, myopathies, neuropathies and lipodystrophy [2]. Mitochondrial DNA (mtDNA) depletion caused by nucleoside-reverse-transcriptase-inhibitors leads to mitochondrial alterations in respiratory function and metabolic and energetic cell failure, and is thought to play a central role in the pathogenesis of these side effects in adults [3]. Additionally, HIV itself is able to induce apoptosis [4] and secondarily affect mitochondrial function in adults [5].

However, the specific role of both the virus and HAART on mitochondrial damage in HIV-infected perinatally-infected pediatric patients remains unknown. Our group previously reported a decrease in the amount of mtDNA in a series of asymptomatic HIV-infected pediatric patients (6). In the present study, we aimed to elucidate whether this genetic mitochondrial abnormality is also present downstream by studying mitochondrial enzymatic activities related to oxygen consumption in a subpopulation of the previous study, in which a mtDNA depletion was observed. Only a few studies have been carried out on this issue and these have usually focused on one aspect of mitochondrial biogenesis [7] providing controversial information about mtDNA content in HIV-infected treated children [8,9] without taking the role of apoptosis into account. In addition, there is no information on parameters of mitochondrial oxidative with polarographic studies in perinatally HIV-infected pediatric patients. In the present study we assessed different aspects of mitochondrial biogenesis to gain insight into the mitochondrial pathology caused by HAART and HIV in vertically-infected pediatric patients on and off HAART.

We performed a cross-sectional, single-center, observational study. The study was approved by the local Ethical Committee and the parents of the patients provided signed informed consent before inclusion. Patients were classified according to two categories: uninfected healthy controls (n=10) and HIV-infected pediatric patients (n=20). The latter group was split into patients off HAART (for a minimum period of 3 years, n=10), hereafter referred to as untreated group, and HAART-treated patients (n=10). Both controls and patients were consecutively included in parallel in order to avoid technical variability confounders.



Untreated HIV-infected patients had undergone planned treatment interruption mostly because of treatment fatigue. At the time of treatment interruption all were free from any active HIV-related clinical condition, showed plasma HIV-RNA levels (CA HIV Monitor; Roche, Basel, Switzerland) below 50 copies/mL, and maintained a CD4 cell count or percentage (flow cytometry, FACSCalibur; BD Biosciences, San Jose, CA, USA) within the CDC Immunological Category 1 (>350 cells/mm<sup>3</sup> for patients aged over 12 or >25% for children aged 12 years or less) for at least the preceding two years.

Peripheral blood mononuclear cells (PBMC) of the patients were obtained from 20 ml of peripheral venous blood by a Ficoll gradient. Mitochondrial mass was evaluated by citrate synthase (CS) activity measurement by means of spectrophotometry [10].

#### *Mitochondrial respiratory chain study*

To evaluate translation efficiency mitochondrially-encoded cytochrome-c-oxidase (COX) subunit II and nuclear-encoded subunit IV (COXII and COXIV) content of the mitochondrial respiratory chain (MRC) complex (C) IV (CIV) were measured by Western blot and expressed as COXII/COXIV ratio [11]. Functional efficiency was determined by spectrophotometric measurement of mitochondrial CII, CII-III, glycerol-3-phosphate dehydrogenase (G3PDH), G3PDH-CIII, CIV and global CI-III-IV enzymatic activities at 37° C, as reported elsewhere [12]. Mitochondrial respiration was evaluated by polarographic measurement of oxidative activities of fresh PBMC using a Clark oxygen electrode at 37°C (Hansatech Instruments Limited ®, Norfolk, UK). Under these conditions we determined the oxygen consumption rate of specific MRC complexes I, II, III, through succinate oxidation (Sox), G3PDH oxidation (G3Pox), piruvate-malate oxidation (PMox), glutamate malate oxidation (GMox) using the corresponding specific substrates and inhibitors. Lastly, the global endogenous oxygen consumption in intact cells (Cellox) in the absence of substrates and inhibitors was determined [10].

#### *Apoptosis study*

Apoptosis was assessed by two different methods: by measurement of the loss of mitochondrial membrane potential of fresh PBMC, indicative of early apoptosis development, by flow cytometry through JC-1 fluorescence quantification [13], and by determination of caspase-3 (Casp-3) content, suggestive of advanced apoptosis development, through Western blot analysis [11].

### *Statistical analysis*

The normal distribution of the variables was ascertained with the Kolmogorov-Smirnov test, and comparisons between groups were performed using the unrelated Student's t-test or non-parametric Mann-Whitney test, as needed. Statistical significance was accepted with a *p*-value less than 0.05.

Twenty HIV-vertically-infected Caucasian patients were included in the study (10 females; median age: 14.8 years). At the time of mitochondrial function assessment, 10 were receiving a protease inhibitor-based HAART regimen (median time on this regimen: 25.7 months, range: 2.0-64.0 months), while the remainder were untreated for several years before inclusion (range: 3.1-7.1 years). As expected, the HIV plasma viral load was significantly higher among untreated patients (median values: 4.5 log versus 1.9 log RNA-HIV copies/ml; *p*=.008), whereas CD4 cell counts remained similar between groups and within normal values in all cases (median values: 731 versus 826 cells/mm<sup>3</sup>; *p*=NS). Normal plasma lactate levels were also observed in both groups (median value: 1.14 mmol/l, range: 0.74-1.61 mmol/l), and none of the patients presented symptoms consistent with mitochondrial toxicity. A sex- and age-matched control group of 10 healthy patients and young adults was used.

Mitochondrial content was very similar in both groups with respect to uninfected controls (103.0 ± 8.2 % for untreated patients, 91.2 ± 5.4 % for treated patients, *p*=NS for both).

On analysis of MRC (Fig. 1), the translational COXII/COXIV ratio was preserved in HIV-infected patients, irrespectively of whether they were receiving HAART or not. Similarly, the functional efficiency of mitochondrial COX (enzymatic activity of CIV) as well as the remaining MRC complexes (CII, CII-III, G3PDH, G3PDH-CIII) were unaffected, although general CI-III-IV activity was significantly reduced by about 75% in HAART-treated HIV-infected patients with respect to controls (1.52 ± 0.38 vs. 6.38 ± 1.53). This reduction was not statistically significant in untreated patients (3.15 ± 0.82) (Table 1). The respiration capacity by stimulation of specific MRC complexes I, II, III was similar in controls and cases (Table 1). However, the global oxygen consumption (of all complexes) in intact non-stimulated cells was significantly reduced by about 50% in HIV-infected untreated patients compared to the control group (0.76 ± 0.01 vs. 1.59 ± 0.15; *p*=.014), with this decline not being statistically significant in HIV-infected patients on HAART (1.06 ± 0.24, *p*=NS).

On the other hand, apoptosis, measured by either caspase-3 quantification or by mitochondrial membrane potential assessment, did not show any significant changes between groups (Fig. 1), although a trend was found to greater activation in untreated patients.

The specific role of both virus and HAART on mitochondrial damage in HIV-infected perinatally-infected pediatric patients has not been well established. Only a few studies have focused on one aspect of mitochondrial biogenesis [7] and there is some controversial information about mtDNA in HIV-infected children [8,9]. Our group previously reported mtDNA depletion in asymptomatic HIV-infected pediatric patients (6) and, in the present study, we aimed to assess whether this genetic mitochondrial abnormality is also present at a functional level. In the present study, no significant differences were found in mitochondrial protein synthesis or in the specific function of most MRC complexes between asymptomatic perinatally HIV-infected pediatric patients off/on HAART with respect to uninfected controls, since all groups consistently showed very similar experimental approaches. Conversely, a significant decrease was observed in the global CI-III-IV enzymatic activity of MRC in HAART-treated patients, as well as in the global endogenous mitochondrial respiration in intact cells from untreated HIV-infected patients.

To date, there are no data available on polarographic studies related to oxygen consumption in this population group. The decrease observed in mitochondrial respiration does not seem to be related to any intrinsic dysfunction of a specific MRC complex as mitochondrial protein synthesis and function in each isolated complex were preserved in our patients. Alternatively, the trend towards an increase in apoptosis observed in the untreated group may indicate that apoptotic activation of untreated HIV patients could underlie the suboptimal cell respiration in this group. Previous studies have reported the apoptotic and deleterious role of HIV associated with mitochondrial dysfunction in adults [4,5].

The global CI-III-IV enzymatic activity of MRC was significantly reduced in the HAART-treated group, although the enzymatic activities of each isolated complex remained unaltered, suggesting a lack of a specific interaction of HAART with any of the complexes and a deleterious and generalized effect caused by HAART.

Although the significance was not achieved in CI-III-IV activity in the untreated group or in oxygen consumption in the HAART-treated group compared to uninfected controls, it is of

note that all HIV-infected patients, either on or off HAART, showed a decrease in global CI-III-IV activity and global endogenous oxygen consumption.

We found that mitochondrial abnormalities in PBMC of vertically HIV-infected young patients were not detected through isolated mitochondrial studies but rather were revealed with general MRC assessment. These findings suggest the presence of moderate and diffuse mitochondrial impairment due to both HIV and HAART rather than a specific profound and localized damage in any of the complexes of the electron transport chain. Whatever the cause, the characterization of mitochondrial function to detect early mitochondrial disturbances before relevant clinical manifestations occur is essential.

One limitation of our study is the small sample size and heterogeneity in the characteristics of the patients, although both are common methodological limitations of studies involving HIV-infected pediatric patients. Additionally, the large blood volume necessary to perform all the scheduled experiments in fresh samples also precluded the inclusion of a larger number of patients. Nevertheless, we present the first polarographic studies of mitochondrial respiration parameters in perinatally HIV-infected patients and our results seem to support the pathogenic role of HIV and HAART in mitochondrial dysfunction in vertical infection downstream through a global alteration of the MRC. In summary, our results suggest that mitochondrial impairment associated with HIV and HAART was generalized rather than localized in our perinatally HIV-infected cohorts.

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**Table 1:** Enzymatic and oxidative activities of MRC:

	<b>Controls</b>	<b>Untreated</b>	<b>HAART</b>	<b>p</b>
CII	26.11±3.62	18.37±14.85	22.28±6.11	NS
CII-III	16.02±3.57	17.63±13.90	20.12±3.07	NS
G3PDH	45.93±3.42	30.41±19.42	32.90±6.36	NS
G3PDH-CIII	16.06±2.25	13.82±8.84	19.44±2.19	NS
CI-III-IV	6.38±1.53	3.15±0.82	1.52±0.38	*
Sox	2.48±0.93	0.45±0.08	0.58±0.18	NS
G3Pox	1.58±0.54	1.42±0.21	1.70±0.61	NS
PMox	1.56±0.16	1.08±0.22	1.31±0.24	NS
GMox	1.36±0.12	1.00±0.07	1.12±0.17	NS
Cellox	1.58±0.14	0.76±0.014	1.06±0.24	**

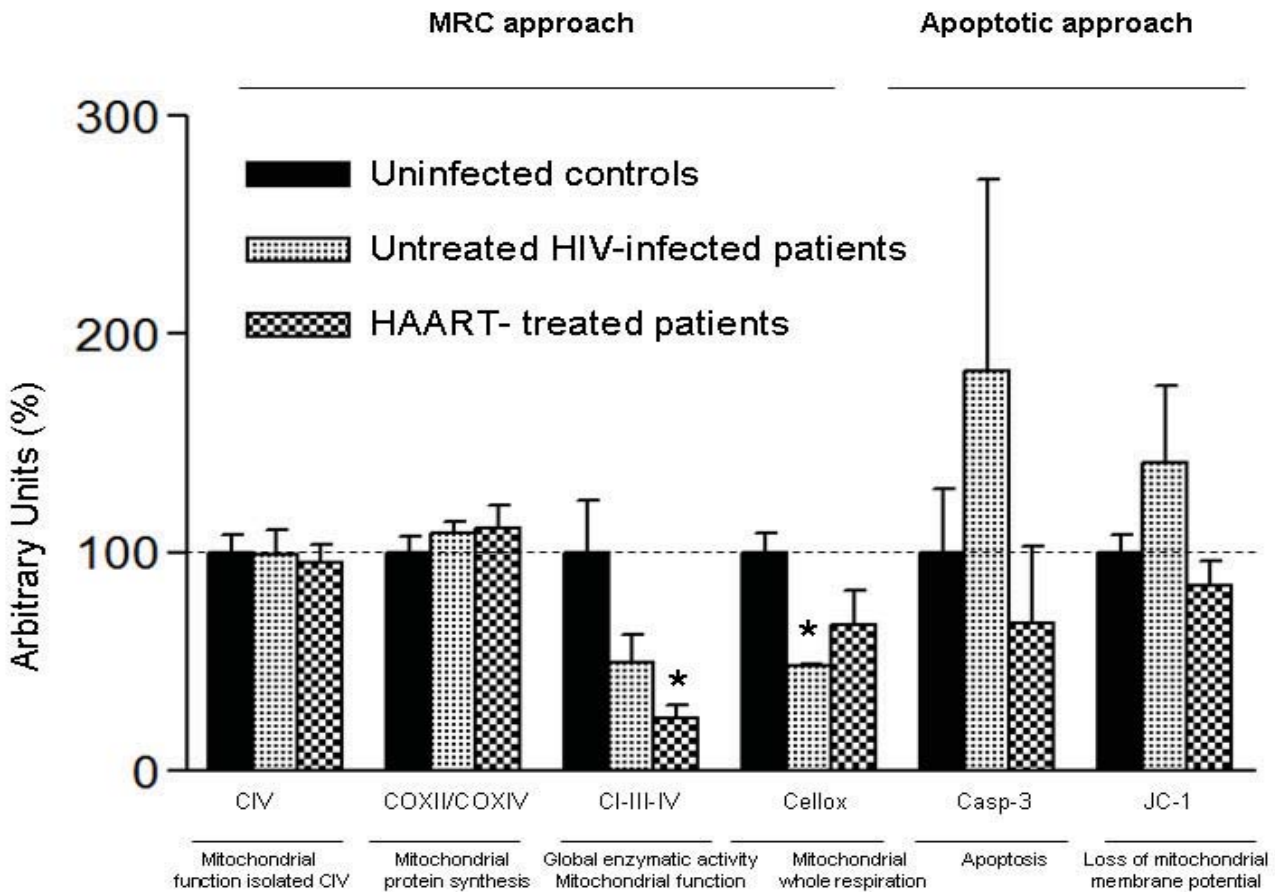
(\*) Significant differences between controls and HAART-treated patients (p=0.02)

(\*\*) Significant differences between controls and untreated patients (p=0.014).

CII, Complex II; CII-CIII, Complex II-III; G3PDH, Glycerol-3-phosphate dehydrogenase; G3PDH-CIII, Glycerol-3-phosphate dehydrogenase- Complex III, CI-III-IV, Complex I-III-IV; Sox, Succinate oxidation; G3Pox, Glycerol-3-phosphate oxidation; PMox, Piruvate oxidation; GMox, Glutamate oxidation; Cellox, Global endogenous oxidation of the cell; HAART, Highly active antiretroviral therapy.



**Figure 1. Mitochondrial and apoptotic parameters**



Results are expressed as mean percentage of the parameters with respect to controls, represented by the line (100%) (mean±SEM). CIV: cytochrome-c-oxidase or complex IV; COXII and COXIV: cytochrome-c-oxidase subunits II and IV; CI-III-IV: Complex I-Complex III-Complex IV global enzymatic activity; Cellox: cell oxidation; Casp-3: caspase 3; JC-1: loss of mitochondrial membrane potential. (\*) p<0.05.





**Mitochondrial impact of human  
immunodeficiency virus and  
antiretrovirals on infected pediatric  
patients with or without  
lipodystrophy**

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undifferentiated AM from April to December in a Lyme disease-endemic region. We found that the LM prevalence in this situation was at least 13.3%. Because of inconsistent *B. burgdorferi* serologic studies by treating clinicians, this was likely an underestimate of the actual prevalence. We conclude that clinicians should evaluate children for LM when children with undifferentiated AM with CSF pleocytosis present in Lyme disease-endemic regions between April and December. Failure to consider LM as a diagnosis may result in delayed antibiotic treatment and subsequent resolution of symptoms. Evaluation for LM includes clinical decision models to risk-stratify children based on presenting features,<sup>9</sup> 2-tier serum *B. burgdorferi* testing, and infectious disease consultation to guide CSF *B. burgdorferi* testing, treatment, and follow-up.

Positive CSF ELISA provided evidence that the LM cases represented active central nervous system infections, as opposed to serologic positivity from previous *B. burgdorferi* infection. CSF ELISA was positive in 6 of the 7 LM patients in whom sufficient CSF was available. The single negative CSF ELISA occurred in a child with short duration of illness (4 days), and it is likely that CSF antibodies were absent because intrathecal antibody response to *B. burgdorferi* begins in the second week after onset of neurologic symptoms.<sup>10</sup>

A limitation of this study is that it did not require serum *B. burgdorferi* testing, resulting in a possible underestimation of LM prevalence. We attempted to minimize this limitation by detecting additional cases of LM using CSF ELISA. We also emphasize that the LM prevalence is an underestimate.

We also considered biases that could overestimate LM prevalence. First, selection bias may have occurred when clinicians did not perform *B. burgdorferi* serology and insufficient CSF was available for *B. burgdorferi* studies. Six children were excluded for this reason. If all of these children were negative for LM, the study prevalence would still be 12.1% (95% CI: 5.7%–23.0%). Second, an overestimate could occur if children with serologic positivity from previous *B. burgdorferi* infections were misclassified as LM. To minimize misclassification, we demonstrated the presence of concurrent intrathecal anti-*B. burgdorferi* antibodies, suggesting active central nervous system infection. We could not prove that intrathecal antibodies did not come from passive diffusion from the serum, but there was no previous history of LM in any of the confirmed or probable cases of LM. It is also possible that the 33 missed participants had a different prevalence of LM than the participants who were successfully enrolled. Because “misses” were based on time of the day, we do not expect that this population would have a clinically distinct Lyme disease distribution.

## CONCLUSION

The LM prevalence in children with CSF pleocytosis and undifferentiated AM is at least 13.3% in a Lyme disease-endemic region from April to December. Clinicians should evaluate children for LM in this scenario.

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## MITOCHONDRIAL IMPACT OF HUMAN IMMUNODEFICIENCY VIRUS AND ANTIRETROVIRALS ON INFECTED PEDIATRIC PATIENTS WITH OR WITHOUT LIPODYSTROPHY

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**Abstract:** We determined the mitochondrial status of a group of HIV-infected children, some with body fat abnormalities (BFA). We included 24 controls, 16 HIV-infected untreated, 26 HIV-infected treated, 6 BFA-untreated, and 21 BFA-treated patients. Genetic, translational, and functional mitochondrial values were measured. As compared with controls, mitochondrial DNA depletion and a reduction in functionality were found in BFA groups.

**Key Words:** antiretrovirals, children, lipodystrophy, HIV, mitochondrial toxicity

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Mitochondrial alterations have been widely associated with HIV infection<sup>1</sup> and with antiretroviral (ARV) drugs<sup>2</sup> in adulthood, even in asymptomatic patients.<sup>3</sup> Although nucleoside reverse transcription inhibitors (NRTI) inhibit DNA polymerase

gamma leading to mitochondrial dysfunction,<sup>4</sup> non-nucleoside reverse transcription inhibitors and protease inhibitors (PIs) have also been described to cause mitochondrial apoptosis,<sup>2</sup> although to a lesser extent. The combination of ARV drugs, known as highly active antiretroviral therapy (HAART), leads to a variety of important adverse secondary effects, including body fat abnormalities (BFA). Changes in body fat content and distribution prevalence rates in HIV-infected children range from 18% to 33%.<sup>5</sup> BFA etiology and pathophysiology have not been completely elucidated. Both HIV and host-dependent factors are thought to be involved in this through premature aging, immune activation, and adipose tissue inflammation.<sup>6</sup> Although some studies have not demonstrated the evidence of ARV class-specific effects,<sup>7</sup> other studies support that BFA is an adverse event linked to ARV, specially NRTI and PI, which have been related to lipoatrophy and lipohypertrophy, respectively. All drug classes would potentially alter adipogenesis and adipocyte differentiation, but the main mechanism proposed to contribute to BFA development is NRTI capacity to inhibit DNA polymerase  $\gamma$ , first suggested by Brinkman in 1999.<sup>8</sup> PIs can alter lipid and carbohydrate metabolism and have also been related to lipoatrophy through their proapoptotic and lipolytic effects in adipocytes.<sup>9</sup>

To date, very few studies have focused on mitochondrial function in HIV-infected children. Saitoh et al<sup>10</sup> observed a mitochondrial DNA (mtDNA) depletion in the peripheral blood mononuclear cells (PBMC) of children receiving didanosine. Rosso et al<sup>11</sup> did not identify mitochondrial benefits after switching from stavudine to tenofovir, a less toxic drug. Only one study has assessed possible associations between mitochondrial toxicity and BFA in children.<sup>12</sup> Mitochondrial function and apoptosis in PBMC were studied in a group of HIV-infected children on HAART with and without BFA, as compared with a control group of uninfected children, and no differences were observed. Because a group of untreated HIV-infected children was lacking, an independent effect of HIV on mitochondria could not be assessed.

We hypothesized that HIV-infected children may show an HIV- and HAART-related mitochondrial dysfunction that may be more important in patients developing BFA. This impairment could be present at different molecular levels (genetic, translational, biochemical, or functional) and, therefore, the character of the lesion may be diffuse rather than localized.

## MATERIALS AND METHODS

We designed a cross-sectional study in 69 HIV-infected children followed up in a tertiary care pediatric center in Barcelona, Spain and 24 uninfected healthy controls. HIV-infected patients were classified into 4 groups: 16 without BFA and off HAART (BFA-HAART-), 26 without BFA and on HAART (BFA-HAART+), 6 untreated patients presenting BFA (BFA+HAART-), and 21 patients with BFA on HAART (BFA+HAART+). Simultaneously, we also classified HIV-infected patients according to HIV viral load (CA HIV Monitor; Roche, Basel, Switzerland; limit <50 copies/mL), either undetectable ( $n = 39$ ) or not ( $n = 30$ ), to assess the independent effect of HIV on mitochondrial function. BFA was assessed by physical examination at the time of assessment and was defined as the presence or not of lipoatrophy (peripheral fat wasting in limbs, face, or buttocks), lipohypertrophy (central fat accumulation on neck, breasts, or abdomen), or a mixed pattern. Informed consent and local ethics committee approval were obtained.

**Collection of Samples.** We collected 5 to 10 mL of venous peripheral blood and isolated their PBMC by a Ficoll density gradient centrifugation (Histopaque-1077; Sigma Diagnostics, St Louis, MO).

We determined the total protein content of PBMC by Bradford reagent method, and all PBMC values were normalized to protein amount.<sup>13</sup>

To characterize the presence of mitochondrial lesion at any level, we measured 9 mitochondrial features related to different aspects of mitochondrial status.

**Mitochondrial Content.** We estimated mitochondrial mass (MM) through the quantification of the porin content, also known as voltage-dependent anion channel protein (Calbiochem Anti-Porin 31HL; Darmstadt, Germany) by Western blot<sup>14</sup> and through the estimation of citrate synthase (CS) activity (enzyme commission (EC) number 4.1.3.7) by spectrophotometry at 412 nm.<sup>14</sup>

**Mitochondrial DNA Quantification.** We quantified the amount of mtDNA, extracted by standard phenol-chloroform procedure, through quantitative real-time polymerase chain reaction (PCR; LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany) by 2 separate amplifications of the mitochondrial gene ND2 and the housekeeping nuclear gene 18SrRNA and the results were expressed as the ratio ND2/18SrRNA.<sup>14</sup>

**Cytochrome c Oxidase Subunits II and IV of Complex IV.** We measured protein subunits content of complex IV (CIV) of mitochondrial respiratory chain (MRC); COXII, which is entirely encoded, transcribed, and translated in mitochondria and COXIV, which is nuclear encoded, and transcribed and translated in cytoplasm by Western blot analysis. Details of the experiment are extensively reported elsewhere.<sup>14</sup>

**Enzymatic Activities Measurement.** We measured mitochondrial enzymatic activities glycerol-3-phosphate-dehydrogenase (G3PDH) (EC 1.1.1.8), succinate-ubiquinone reductase or complex II (CII) (EC 1.3.99.1), CII-ubiquinol-cytochrome c reductase or complex III (CIII) (EC 1.10.2.2), cytochrome c oxidase or CIV (EC 1.9.3.1), and the overall CI-III-IV enzymatic activity of the MRC.<sup>14</sup> Enzymatic activities were expressed as nanomoles of oxidized substrate per minute and per milligram of protein. The enzymatic activities were normalized per mitochondrion by dividing absolute enzymatic activities per CS activity.

**Statistical Analysis.** Statistical analyses were performed by Statistical Package for the Social Sciences version 15.0 for windows (SPSS, Inc., Chicago, IL). Results were expressed by means and standard error means. Normality of data was ascertained by Kolmogorov-Smirnov test. Comparisons between groups were carried out by using k-unrelated samples Kruskal-Wallis and 2-unrelated samples Mann-Whitney tests in case of nonparametric distribution. In case of normal data, parametric unrelated samples Student  $t$  test was used.  $P < 0.05$  was considered statistically significant.

## RESULTS

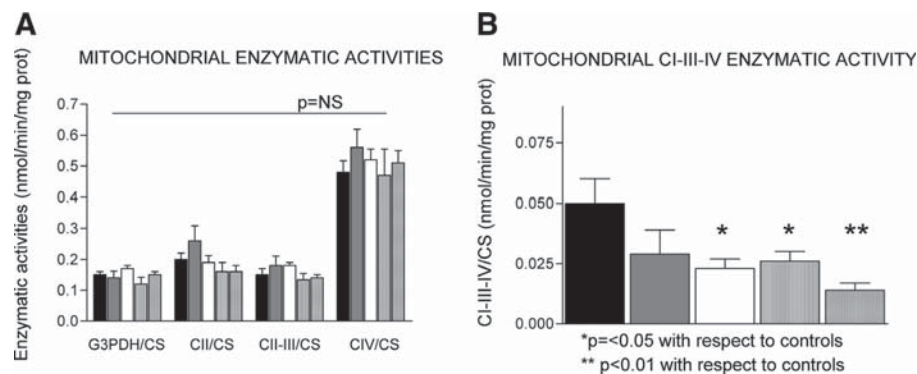
Characteristics of the patients included in the study were summarized in Table, Supplemental Digital Content 1, <http://links.lww.com/INF/A883>, which includes clinical and epidemiological data of the patients.

As expected, untreated patients showed higher viral loads than patients on HAART ( $4.18 \pm 0.26$  RNA HIV log vs.  $0.68 \pm 0.19$ ;  $P < 0.001$ ), but no differences in CD4<sup>+</sup> T-cell counts were observed.

MM was maintained in all groups, measured either by CS or voltage-dependent anion channel (Fig., Supplemental Digital Content 2, <http://links.lww.com/INF/A884>, illustrates mitochondrial content estimated by both the methods).

No significant correlation between mtDNA and lactate levels, CD4<sup>+</sup> T-cell counts, and the type of ARV therapy was found (data not shown).

A depletion in mtDNA was found in symptomatic BFA+ groups, either untreated or treated, with respect to



**FIGURE 1.** A, Mitochondrial enzymatic activities of MRC normalized per MM. G3PDH indicates glycerol-3-phosphate-dehydrogenase; CII, complex II; CII-III, complex II-III; CIV, complex IV or cytochrome c oxidase; CS, citrate synthase. B, Global mitochondrial enzymatic activity normalized per MM. CI-III-IV indicates complex I-III-IV; CS, citrate synthase.

controls ( $2.91 \pm 0.43$ ,  $4.10 \pm 0.41$  vs.  $5.62 \pm 0.50$ ;  $P = 0.014$  and  $P = 0.024$ , respectively) (Fig., Supplemental Digital Content 3, <http://links.lww.com/INF/A885>, represents mtDNA amount in all the groups). Both untreated and treated HIV-infected children without BFA showed lower levels of mtDNA, but significant differences with respect to healthy controls were not attained ( $4.11 \pm 0.36$ ,  $4.78 \pm 0.44$  vs.  $5.62 \pm 0.50$ ;  $P = NS$ ). When patients affected with BFA were classified according to viral load (detectable or not), the depletion in mtDNA remained significant in both the groups with respect to controls ( $3.81 \pm 0.42$ ,  $3.89 \pm 0.49$  vs.  $5.77 \pm 0.49$ ;  $P = 0.010$  and  $P = 0.012$ , respectively; data not shown).

Expression of COXII and COXIV subunits of CIV (ratio COXII/COXIV) showed similar results between all groups (Fig., Supplemental Digital Content 4, <http://links.lww.com/INF/A886>, illustrates subunits COXII and COXIV content).

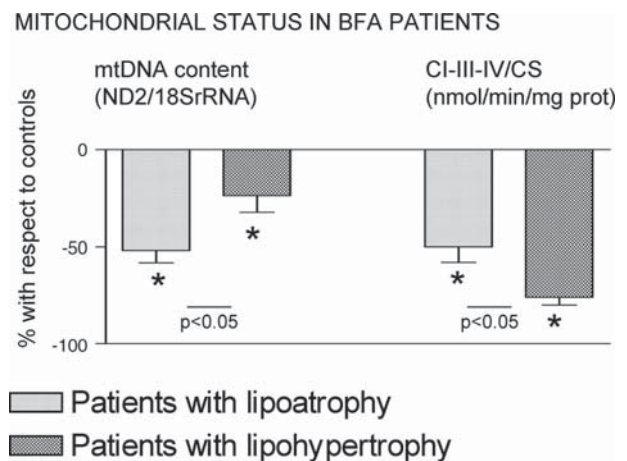
The measurement of isolated mitochondrial complexes G3PDH-CIII, CII, CII-III, and CIV of MRC (normalized per MM by dividing absolute values per CS activity) remained similar in all groups (Fig. 1A).

However, the assessment of overall functionality of MRC through the measurement of CI-III-IV enzymatic activity showed at least a 50% reduction of the activity in 3 groups with respect to healthy patients ( $0.023 \pm 0.004$  in BFA-HAART+,  $0.026 \pm 0.004$  in BFA+HAART-, and  $0.014 \pm 0.003$  in BFA+HAART+ vs.  $0.05 \pm 0.01$ ;  $P = 0.025$ ,  $P = 0.041$ , and  $P = 0.004$ , respectively) (Fig. 1B). Global CI-III-IV/CS activity of BFA-affected patients was also decreased both in patients with detectable and undetectable viral loads with respect to controls ( $0.020 \pm 0.004$ ,  $0.03 \pm 0.012$  vs.  $0.05 \pm 0.01$ ;  $P = 0.036$  and  $P = 0.012$ , respectively).

Among BFA+ patients, those affected with lipoatrophy presented lower mtDNA levels than lipohypertrophic ones ( $3.19 \pm 0.43$ ,  $4.29 \pm 0.47$ ;  $P = 0.018$ ), and both the groups presented a decrease in CI-III-IV/CS enzymatic activity that was 2-fold higher in children with lipohypertrophy ( $0.025 \pm 0.004$ ,  $0.012 \pm 0.002$ ;  $P = 0.034$ ) (Fig. 2).

## DISCUSSION

Data associating BFA with mitochondrial toxicity in adults are controversial. In some studies, mtDNA depletion has been observed in fat and PBMC from adult patients with BFA.<sup>15</sup> In other studies, fat metabolism and mitochondrial function were normal or even increased in this population.<sup>16</sup> In the present study, HIV-infected children showed lower mtDNA levels and a reduc-



**FIGURE 2.** mtDNA and mitochondrial function in patients with body fat changes presenting lipoatrophy and lipohypertrophy. Bars represent percentage of decrease of mitochondrial values in HIV patients with respect to mean values of uninfected controls (arbitrarily assigned 0%). BFA indicates body fat changes; mtDNA, mitochondrial DNA; CI-III-IV, complex I-III-IV; CS, citrate synthase.

tion in mitochondrial functionality, as assessed per CI-III-IV/CS enzymatic activity, with respect to healthy controls. These findings were more pronounced in patients affected with BFA, regardless of the use of HAART. Even in the presence of mtDNA depletion, no further decrease of subunits COXII and COXIV from MRC CIV was detected. Thus, there was not a deficit in the translational pathway from mitochondrial genome to subunit II or from nuclear genome to subunit IV of MRC CIV. Therefore, the CI-III-IV dysfunction we describe could rather be due to an independent pathway different from mitochondrial genetics or due to a dysfunction of the MRC, not related to any alteration in CIV.

Of note, mitochondrial parameters were differently affected in children presenting with lipohypertrophy (higher CI-III-IV dysfunction) and lipoatrophy (higher mtDNA depletion), suggesting different pathogenic pathways in these different presentations of BFA. To our knowledge, this has not been previously reported, but



the fact that most patients were receiving a PI-based regimen at the time of assessment suggests that these findings are more likely related to genetic variability rather than to class-specific toxicity. To date, only one study has evaluated the association of mitochondrial toxicity and BFA in children.<sup>12</sup> Cossarizza et al did not find any differences in PBMC mtDNA copies, mitochondrial functionality, or in the trend to undergo apoptosis in HIV-infected children with or without BFA and also in uninfected controls. In our series, we included a group of untreated patients, either presenting BFA or not, and we observed that the presence of genetic and functional mitochondrial damage arises in symptomatic BFA+ patients regardless of the administration of therapy. Mitochondrial alterations were found in both BFA+HAART- and BFA+HAART+ groups of children, at both genetic and functional levels. In patients with long-term undetectable viral load after HAART implementation, the mitochondrial dysfunction should probably be ascribed to ARV-related toxicity, whereas in the patients who are off HAART, the HIV-related mitochondrial damage should also be taken into account.

Similar previous findings of mitochondrial genetic and functional impairment in adipose tissue from HIV-infected adults corroborate the potential mitochondrial basis for BFA development.<sup>17</sup> Accordingly, Milazzo et al<sup>18</sup> have recently observed a protective role of antioxidant supplementation on mitochondrial function in HIV-infected patients presenting lipoatrophy.

As opposite to Cossarizza et al,<sup>12</sup> our findings suggest that the use of PBMC may be an adequate indicator for the assessment of the mitochondrial toxicity that remains at the etiopathogenic basis of BFA in HIV-infected children. Many other studies have previously validated the use of PBMC model, as a noninvasive approach, especially important in children, in the assessment of HIV- and HAART-mediated mitochondrial toxicity.<sup>1,10,14,19,20</sup> Small numbers, the heterogeneity in patients characteristics, and the cross-sectional design are obvious methodologic limitations of our study; in fact, we were not able to identify any of the acquired or inherited factors that have been associated with the different patterns of BFA, such as the use of stavudine, AIDS, nadir CD4 counts, or IL-1beta (+3954C/T) polymorphism.<sup>21</sup>

According to our findings, HIV and HAART exert similar mitochondrial toxicity. It is important to keep attention at the secondary events resulting from ARV drugs. It is remarkable fact that mitochondrial toxicity derived from ARV drugs seems to be reversible, once the toxic agent is interrupted.<sup>19</sup>

Our results indicate that mitochondrial function is more severely affected in BFA+ patients than in HIV-treated patients without BFA, suggesting a mitochondrial etiology for BFA. Further studies of mitochondrial function and evolution on children over time, considering the different HAART regimens, should be addressed to ascertain more secure ARV regimens and reduce BFA development.

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## INTERFERON-GAMMA RELEASE ASSAY PERFORMANCE FOR DIAGNOSING TUBERCULOSIS DISEASE IN 0- TO 5-YEAR-OLD CHILDREN

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 Albert Faye, MD, PhD,† and Ghislaine Sterkers, MD, PhD\*

**Abstract:** QuantiFERON-TB Gold In-Tube performance was evaluated in 19 French immunocompetent children (0.29–5.36 years; median: 1.52) with active tuberculosis. The rate of indeterminate results was 0/19 and the rates of positivity were 6/10 and 9/9 in <2 and 2- to 5-year-old children, respectively. QuantiFERON-TB Gold In-Tube in association with

**Evolution of mitochondrial DNA  
content after planned interruption of  
HAART in HIV-infected pediatric  
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AIDS Research and Human Retroviruses 2009;  
26(9):1015-1018





## Evolution of Mitochondrial DNA Content After Planned Interruption of HAART in HIV-Infected Pediatric Patients

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### Abstract

HAART-related long-term toxicities, many of them ascribed to mitochondrial (mt) toxicity of the nucleoside analogues, are being increasingly reported in HIV-infected children. HIV infection can also cause mt damage. Case series include 13 vertically HIV-infected pediatric patients (9 girls, median age 10.5 years) with optimal long-term response to a first-line HAART regimen who underwent planned treatment interruption (PTI). MtDNA content from peripheral blood mononuclear cells was assessed by means of a real-time PCR technique at PTI and 12 months later and expressed as an mtDNA/nuclear DNA ratio, together with lactate levels. At PTI, patients had remained a median time of 4.7 years on HAART and 4.3 years with complete suppression of viral replication. The main reason leading to PTI was treatment fatigue. One month after PTI, HIV plasmatic viral load had increased to 4.8 log copies/ml and stabilized thereafter. During the 12-month study period, all children remained free from any HIV-related clinical event. A progressive and significant decrease in median CD4 cell counts and percentages was observed 12 months after PTI. One year after PTI, the median mtDNA/nuclear DNA ratios had increased from 0.76 to 1.08 ( $p = 0.002$ ) and lactate levels had decreased (from 1.12 to 0.73 mmol/liter;  $p = 0.019$ ). Changes in mtDNA did not correlate with changes in lactate levels. No relationship was found between the evolution in mt toxicity markers and the rest of the clinical, immunological, and virological variables. In this series, PTI led to a partial restoration of mtDNA levels and a significant decrease in lactate values.

**T**HE IMPLEMENTATION OF HIGHLY ACTIVE ANTIRETROVIRAL (ARV) THERAPY (HAART) has significantly improved morbidity and mortality from HIV infection/AIDS in the pediatric population.<sup>1</sup> Nucleoside analogue reverse transcriptase inhibitors (NRTIs) remain the backbone of most HAART regimens, especially among children, for whom many of the newest drugs have not yet been licensed. NRTIs also inhibit DNA polymerase gamma, which can lead to mitochondrial (mt) DNA (mtDNA) depletion, and to mt dysfunction. HAART-related long-term toxicities are being increasingly reported in vertically HIV-infected pediatric patients.<sup>2,3</sup> Many of these have been ascribed to direct mt toxicity of the NRTIs. Further investigation on the pathogenesis, clinical manifestations, and therapeutics of these toxicities in children is mandatory, as these patients shall be exposed to ARV for an ever-increasing length of time

throughout postnatal growth and development. We report our experience with HAART interruption in a series of HIV-infected children and its effects on biological markers of mt toxicity.

This was a prospective case series of perinatally HIV-infected pediatric patients followed up in a third-level pediatric hospital in Barcelona (Spain) who underwent planned treatment interruption (PTI). At the time of PTI, all patients fulfilled the following requirements: to be on a first-line HAART, freedom from any active HIV-related clinical condition, undetectable plasmatic HIV-RNA (limit of 50 copies/ml; CA HIV Monitor; Roche, Basel, Switzerland), and a maintained immune situation (flow cytometry, FACSCalibur; BD Biosciences, San Jose, CA) within CDC Category 1 ( $>350$  cells/mm<sup>3</sup> for adolescents or  $>25\%$  for children aged 12 years or less) for at least the past 2 years. ARV treatment

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reinitiation was planned in case any HIV-related laboratory (grade 3 or 4 anemia, thrombocytopenia, leukopenia, or elevation of liver enzymes according to the Division of Acquired Immunodeficiency Syndrome toxicity grades<sup>4</sup>) or clinical event, or a marked decrease in CD4 cells (<350 cells/mm<sup>3</sup> for adolescents or <20% for children aged 12 years or less) was observed. Informed consent was obtained from parents or legal guardians. The study was approved by the Ethics Committee of the Hospital Sant Joan de Déu. There was a close clinical and biological follow-up after PTI was performed, with monthly controls during the first 6 months and every 2–3 months thereafter.

Peripheral blood mononuclear cell (PBMC) samples were obtained by standard methods (density gradient centrifugation in a dextran medium) and cryopreserved at –80°C. MtDNA content from cryopreserved PBMCs was assessed at the time of PTI (baseline) and 12 months later. Plasmatic lactate levels (normal values: 0.55–1.77 mmol/liter) were also obtained at these two time points; given the physiological variability and lack of specificity when determining lactate concentrations, previously reported strict validation criteria were used.<sup>5</sup> DNA extraction from PBMCs was performed by the standard phenol-chloroform isolation protocol.

Total DNA was quantified by spectrophotometry and 2 ng of each sample was amplified by real-time polymerase chain reaction (rtPCR) using LightCycler technology and SYBR Green I dye (Roche, Indianapolis, IN). DNA quantification by PCR is based on the ability of fluorescent SYBR Green I to bind double-stranded DNA. Both DNA and SYBR Green fluorescence duplicate exponentially in each PCR cycle. Fluorescence intensity can be measured all along the amplification process and is proportional to the final DNA amount and, therefore, to the initial DNA concentration. To assess mtDNA content the rtPCR was carried out separately to amplify a sequence of a highly conserved mt ND2 gene and a fragment of the nuclear-coded housekeeping 18S rRNA gene. MtDNA content was expressed as the ratio of ND2 mtDNA with respect 18S rRNA nDNA.

The one-sample Kolmogorov–Smirnov test was used to assess that continuous data followed a normal distribution. Changes for mtDNA, lactate, CD4 cell percentage, and viral load were evaluated by means of a paired *t*-test. Statistical significance was set at 0.05.

From June 2001 to June 2006, 13 patients underwent PTI and completed a 12-month period off therapy (9 girls, median age at PTI: 10.5 years; age range: 3.9–16.0 years; Table 1). At the time of PTI, patients had remained on HAART a median time of 4.7 years and 4.3 years with complete suppression of viral replication. The main reason leading to PTI was treatment fatigue.

During the 12-month study period, all children remained free from any HIV-related clinical event. As expected, an increase in RNA-HIV plasmatic load up to a median value of 4.8 log copies/ml (range 2.9–5.6; *p* < 0.0001) was observed in all cases 1 month after PTI; HIV viremia stabilized thereafter (median values 12 months after PTI: 4.5 log, range: 3.2–5.1; *p* = 0.32 when compared to the initial blip 1 month after PTI). A progressive and significant decrease in median CD4 cell counts and percentages was observed 12 months after PTI (from 858 to 504 cells/mm<sup>3</sup>, *p* = 0.002; and from 36.0% to 28.0%, *p* < 0.0001), with the following slopes of decline: –30 CD4 cells/mm<sup>3</sup> per month and –0.8% per month.

Baseline median ND2 mtDNA/18S rRNA nDNA ratios and lactate levels were as follows: 0.76 (range: 0.09–1.79) and 1.12 mmol/liter (range: 0.67–3.00 mmol/liter); no differences in mtDNA ratios or lactate levels were observed between children who received didanosine (ddI, *n* = 8) or stavudine (d4T, *n* = 10) or ddI plus d4T (*n* = 7) and those who did not at the time of PTI. No other association was observed between mt toxicity markers at baseline and background characteristics of the patients (sex, age, CDC clinical category, duration of therapy, nadir CD4 cell count or percentage, highest plasma HIV-RNA value, and HAART regimen).

One year after PTI, ND2 mtDNA/18S rRNA nDNA ratios had increased in seven patients and had decreased in six patients, with variations ranging from –0.59 to 2.57 (paired

TABLE 1. BASELINE CLINICAL AND IMMUNOLOGICAL CHARACTERISTICS OF PATIENTS UNDERGOING PLANNED INTERRUPTION OF HAART<sup>a</sup>

Gender and CDC clinical category	Nadir CD4 cells		At PTI			12-month evolution	
	/mm <sup>3</sup>	%	HAART regimen	Main reason	Age (years)	Δ LA	Δ mtDNA
1 M A	882	21	d4T-ddI-NFV	tx fatigue	10.5	–0.5	2.57
2 F B	792	22	d4T-ddI-NFV	Hepatic toxicity	8.4	–1.34	1.21
3 F A	1127	23	d4T-ddI-NFV → NVP	tx fatigue	12	–0.73	0.77
4 F C	300	21	d4T-NVP-NFV	tx fatigue	13.2	–0.04	1.69
5 M A	736	23	ddI-d4T-NFV → NVP	Psychiatric tx	14.3	–0.45	–0.36
6 F C	2496	32	ABC-ddI-d4T-NFV	tx fatigue	5.5	–0.42	1.45
7 F B	310	21	d4T-3TC-EFV	Central obesity	12.7	–0.16	–0.38
8 F A	882	18	ZDV-3TC-RTV → NVP	tx fatigue	7	–0.28	–0.59
9 M B	756	26	d4T-ddI-NFV	tx fatigue	12.6	–0.21	0.77
10 M B	442	13	3TC-ZDV-NVP	Facial lipoatrophy	16	–0.51	0.85
11 F A	226	14	d4T-ddI-NVP-NFV	tx fatigue	4.9	–0.2	–0.26
12 F A	1100	25	d4T-3TC-NFV	tx fatigue	3.9	0.41	–0.01
13 F A	1200	31	AZT-ddI-NVP	tx fatigue	4.1	–1.94	–0.06

<sup>a</sup>M, male; F, female; PTI, planned treatment interruption; d4T, stavudine; ddI, didanosine; NFV, nelfinavir; NVP, nevirapine; ABC, abacavir; 3TC, lamivudine; EFV, efavirenz; ZDV, zidovudine; RTV, ritonavir; tx, treatment; LA, lactate in mmol/liter; mtDNA, mitochondrial DNA.

## HAART INTERRUPTION AND MITOCHONDRIA IN CHILDREN

3

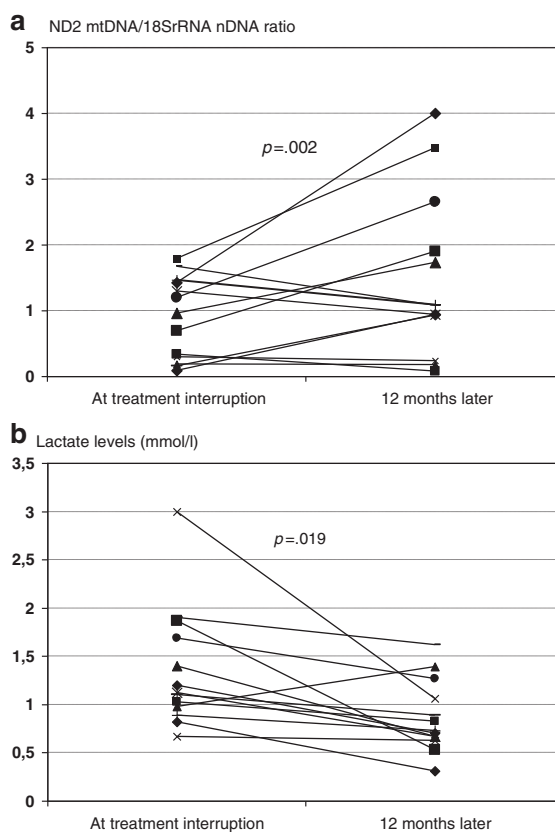
**F1** ▶ *t*-test,  $p = 0.002$ ; Fig. 1a); overall, a median net increase up to 1.08 (range: 0.08–4.00) was observed. A significant decrease in median lactate levels was also noted (median value 0.73 mmol/liter, range: 0.31–1.62; paired *t*-test,  $p = 0.019$ ); lactatemia decreased in all but one patient, with changes ranging from  $-1.94$  to  $0.41$  mmol/liter (Fig. 1b). Changes in ND2 mtDNA/18S rRNA nDNA ratios did not correlate with changes in lactate levels. Again, no relationship was found between the evolution in mt toxicity markers and the rest of the clinical, immunological, and virological variables (data not shown).

After the SMART trial,<sup>6</sup> treatment interruptions in the adult patient are strongly discouraged. The natural history of vertically transmitted HIV infection is different from that of the adult and, therefore, response to PTIs in children may as well be different. To pose minimal risk to patients, only children fulfilling very stringent requirements were selected to undergo PTI in our study (i.e., age over 2 years, when the risk of progression to AIDS diminishes, to be on a first-line HAART, and long-term normal CD4 cell counts and complete suppression of viral replication). The clinical, immunological, and virological evolution we observe is very similar to that reported by the only clinical trial on PTIs in HIV-infected children published to date<sup>7</sup> and warrants the need to further investigate this therapeutic strategy in this population.

To date, few studies have focused on mt function markers in HIV-infected children. In a 2-year follow-up including 80 HIV-infected pediatric patients, we reported the calculated incidence of asymptomatic hyperlactatemia to be 8.7 per 100 patient-years; a younger age at the beginning of HAART was the only risk factor for developing hyperlactatemia by logistic regression analysis.<sup>5</sup> Similar figures have been observed by other authors, both in children<sup>8</sup> and adult patients.<sup>9</sup> ARV-related mt dysfunction in the pediatric age group is rarely symptomatic. In addition to some cases of lactic acidosis,<sup>10,11</sup> mt toxicity in children usually manifests with neurological symptoms. Of note, ARV-related mt toxicity also affects HIV-uninfected infants who were exposed perinatally to ARV, in whom neurological syndromes similar to those classically described in inherited mt diseases have been reported.<sup>12,13</sup>

Only three small studies have investigated mtDNA levels in the PBMCs of HIV-infected children. Saitoh *et al.*,<sup>14</sup> in a 104-week follow-up of 31 patients on stable HAART and suppression of viral replication, identified ddI as the only NRTI associated with mtDNA suppression in PBMCs before and during HAART. Another study showed no difference in mt function or content between HIV-infected children with ( $n = 6$ ) or without lipodystrophy ( $n = 12$ ).<sup>15</sup>

Recently, the metabolic and mt function evolution was investigated in a group of 18 patients who were randomized either to continue on a d4T-containing HAART regimen (arm A,  $n = 9$ ) or to switch to tenofovir (arm B,  $n = 9$ ); although a significant decrease was observed in plasma HDL-cholesterol in arm B, no other changes were noted in metabolic parameters or mtDNA levels after a 18-month follow-up.<sup>16</sup> To our knowledge, the effects of HAART interruption on mt function in children have not been investigated to date. In our small series, a 12-month treatment interruption led to a partial restoration of mtDNA levels in PBMCs and to a decrease in plasmatic lactatemia. Whether these changes leads to clinical improvements in the long term remains uncertain. Likewise, it is unknown if similar changes in mtDNA also occurred in



**FIG. 1.** Twelve-month evolution in ND2 mtDNA/18S rRNA nDNA ratios (a) and lactate levels (b) in the 13 patients undergoing planned HAART interruption.

other tissues known to be affected by clinically relevant ARV-related mt toxicity, such as fat.<sup>17</sup>

The deleterious effect of HIV infection on mtDNA content was demonstrated early by Côté and colleagues,<sup>18</sup> as was its partial restoration after treatment interruption.<sup>18,19</sup> We have also reported a decrease both in mtDNA content and in mt respiratory chain enzyme activities of complexes partially encoded by mtDNA in HIV-infected adult patients who were never treated with ARV when compared with healthy controls.<sup>20</sup> Other authors have even described an inverse correlation between HIV plasmatic viral load and mtDNA content in PBMCs.<sup>21</sup> In our study, despite a global and significant increase in mtDNA levels, these decreased in almost half of the patients following HIV viremia rebound after PTI, although these decreases were of smaller magnitude. Our results suggest that mtDNA loss in HIV-infected children is both due to the use of NRTIs and to the deleterious effects of HIV on mitochondria, although persistent mt damage cannot be discarded.

We were not able to show the association between ddI or d4T and mt toxicity reported by other authors,<sup>22</sup> probably because of the small numbers and because most of the patients were receiving either of those drugs or both at the time of treatment interruption. Moreover, our preliminary results

should be taken with caution as we could not completely decontaminate blood samples of platelets, which contain mtDNA but not nuclear DNA<sup>23</sup>; proper elimination of platelets after density gradient centrifugation requires large volumes of whole blood, and these are often not available in children. Considering the growing number of children who will be exposed to these agents in the following years, in our opinion, continuous investigation of ARV-related toxicity in the pediatric age is warranted.

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**Mitochondrial evolution in HIV-  
infected children receiving first or  
second generation nucleoside  
analogues**

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**MITOCHONDRIAL EVOLUTION IN HIV-INFECTED CHILDREN RECEIVING  
FIRST OR SECOND GENERATION NUCLEOSIDE ANALOGUES**

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**Running head:** Toxicity over time in HIV-infected children



## ABSTRACT

**Background:** Highly-active-antiretroviral-therapy (HAART) and HIV-related mitochondrial toxicity lead to several adverse effects and have become a major issue, especially in children. The main goal in the treatment of HIV-infected children is to maximize cost-effectiveness, while minimizing toxicity. We aimed to study the evolution of mitochondrial parameters over time in children receiving different types ARV schedules.

**Methods:** Over a 2-year period we followed the immunovirological and mitochondrial status of 28 HIV-infected children receiving HAART including either first-generation nucleoside-reverse-transcriptase-inhibitors (1gNRTIs; didanosine, zidovudine or stavudine; n=15) or second-generation NRTIs (2gNRTIs; the remaining drugs; n=13), and compared these subjects with a group of untreated HIV-infected patients (n=10) and with uninfected controls (n=27). We measured T lymphocyte CD4<sup>+</sup> content (flow-cytometry), viral load (rt-PCR), and lactate levels (spectrophotometry); we assessed mtDNA content (rt-PCR), mitochondrial protein levels (Western blot), oxidative stress, mitochondrial mass, and electron transport chain function (spectrophotometry) in blood-mononuclear cells.

**Results:** At the second time-point, lactate levels were significantly higher in children on 1gNRTIs compared with those receiving 2gNRTIs (1.28±0.08 vs. 1.00±0.07 mmol/l; p=0.022). MtDNA content was similar among all HIV-infected groups and significantly lower than in healthy controls at baseline. Oxidative stress tended to increase over time in all the groups with no differences among them. However, a significant decrease in cytochrome-c-oxidase activity was found over time in HIV-infected patients; this decline was greater in the 1gNRTIs group.

**Conclusions:** HIV-infection and the use of 1gNRTIs caused greater mitochondrial damage than 2gNRTIs over time. The higher lactate levels and the significant decrease observed in cytochrome-c-oxidase activity argue against the use of 1gNRTIs in HIV-infected children when an alternative is available, in accordance with international recommendations.

**Key words:** Children, HIV, Mitochondrial toxicity, Therapeutic strategies.

## INTRODUCTION

Highly active antiretroviral (ARV) therapy (HAART) reduces mortality and morbidity by HIV infection and AIDS in both adults and children. Nonetheless, HAART may lead to adverse events, which have become a major issue, especially in HIV-infected children. It has been proposed that many of these adverse events have a mitochondrial basis. Currently, the main goal in the treatment of HIV infection is to reduce the risk of virological failure, while maximizing cost-effectiveness and minimizing toxicity. Although a wide range of new drugs is available (1), few data have been reported on mitochondrial toxicity in children, and thus further investigations are needed.

It has been previously reported that HIV is responsible for mitochondrial DNA (mtDNA) depletion in adults (2), and, subsequently it was shown that this effect was reflected in mitochondrial dysfunction (3).

Many studies have looked at how nucleoside reverse transcriptase inhibitors (NRTIs) trigger mitochondrial impairment through the inhibition of the gamma-polymerase ( $\gamma$ -pol) enzyme, causing mtDNA depletion (4,5) which, in turn, may lead to mitochondrial failure (6). There has been less examination of the effects in perinatally HIV-infected paediatric patients, for whom this issue has special relevance since they constitute the first generation that will receive ARV treatment throughout their lives.

As in adult patients, HAART-related mitochondrial effects in children has been reported by our group (7). Rosso et al. studied, for the first time, the mitochondrial effects observed after switching from mitochondrial-toxic drugs to less toxic compounds in children over a period of 18 months, finding no significant changes in mtDNA content (8). In fact, the mitochondrial toxicity of NRTIs may be different depending on the specific drug; accordingly, a ranking of toxicities *in vitro* has been described in the literature, from the most toxic to the least toxic drugs, as follows: zalcitabine (ddC) > didanosine (ddI) > stavudine (d4T) > zidovudine (ZDV) > lamivudine (3TC)/emtricitabine (FTC) > abacavir (ABC) = tenofovir (TDF) (9) (10). The use of ddC (no longer administered) and ddI has been related to pancreatitis (11,12); d4T has been associated with lipodystrophy (13,14), hyperlactatemia and lactic acidosis (15), and ZDV has been linked to myopathy (16). In addition, 3TC/FTC and ABC present a lower affinity for  $\gamma$ -pol (17), and are thus considered to be the least mitochondrial-toxic compounds, together with TDF.

We aimed to study the evolution of mitochondrial parameters along time in a series of paediatric patients receiving different types of ARV therapy, and we hypothesized that

children receiving a HAART regimen based on highly mitochondrial toxic NRTIs (first generation NRTIs, 1gNRTIs: ddI, ZDV and d4T) develop more mitochondrial toxicity than those on an ARV combination that does not include these, but rather second generation NRTIs, 2gNRTIs: ABC, 3TC/FTC and TDF. The results in these patients were compared with 2 control groups of children, one consisting of HIV-infected but untreated patients, in whom HIV is the only detrimental agent for mitochondria, and one group of uninfected healthy controls providing reference values of normality.

The main objective of this study was to provide more information related to the evolution of mitochondrial markers in HIV-infected paediatric patients undergoing different ARV schedules and to elucidate whether a given ARV therapy is safer than others from the mitochondrial point of view, while also remaining effective.

## METHODS

A longitudinal study was conducted over 2 years. The immunovirological and mitochondrial status of 28 vertically-HIV-infected children (64% girls, median age  $\pm$  SEM at baseline, 11.1  $\pm$  0.7 years) on HAART that either included 1gNRTIs (n=15) or 2gNRTIs (n=13) was assessed and compared to the status of an untreated group of HIV-infected children (no ARV group; n=10) and to the values of normality in a group of healthy uninfected children (n=27). The HAART regimes of the 1gNRTIs and 2gNRTIs groups were maintained during the study period. Informed consent to participate in the study was obtained from parents or legal guardians, and approval from the local ethics committee was given.

T-lymphocyte CD4<sup>+</sup> content was analysed by flow-cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA). RNA-HIV viral load was quantified by quantitative real-time PCR (CA HIV Monitor; Roche, Basel, Switzerland; limit <50 copies/ml) and plasmatic lactate levels (a surrogate biomarker of mitochondrial lesion, mmol/l) were measured with a spectrophotometric procedure (Cobas Fara II Analyzer, Roche).

Peripheral blood mononuclear cells (PBMCs) were isolated from 5-10 ml of venous blood with a Ficoll gradient (Histopaque 1077; Sigma Diagnostics, St Louis, MO, USA) (18) for mitochondrial studies.

Mitochondrial mass was estimated by the measurement of citrate synthase (CS) enzymatic activity with spectrophotometry at 412 nm (Hitachi 2900, Hitachi Instruments, Inc., San Jose, CA, USA), as previously reported (19). Results were expressed as nmols of reduced substrate per minute and per milligram of cell protein (nmols/min/mg protein).

We assessed mtDNA content by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemical, Mannheim, Germany) through the amplification of a fragment of the highly conserved mitochondrial gene ND2 and a sequence within the housekeeping 18SrRNA nuclear gene, as reported elsewhere (19-21). The results of mtDNA content were expressed with respect to nuclear DNA content as the ratio ND2/18SrRNA, and normalized by CS.

Mitochondrial protein levels of mtDNA-encoded subunit II (COXII) and nuclear DNA-encoded subunit IV (COXIV) of CIV were quantified by Western blot, using the porin voltage-dependent anion carrier protein (VDAC) as a marker of mitochondrial protein loading, and  $\beta$ -actin as a marker of overall cell protein loading, as previously reported (22).

Oxidative stress was assessed by lipid peroxidation analysis with the measurement of malondialdehyde (MDA) and hydroxialkenal (HAK) content at 586 nm (22) and results were normalized per total protein content ( $\mu$ M MDA and HAK/mg protein).

Measurement of the enzymatic activity (also in nmol/min/mg of protein) of the mitochondrial respiratory chain (MRC) complexes was performed by means of spectrophotometry (Hitachi 2900, Hitachi Instruments, Inc., San Jose, CA, USA) and the results were referred to overall cell protein and normalized by mitochondrial mass, estimated by CS activity. Since isolated complex I and V activities cannot be measured in whole cells due to the absence of activation of the former with decylubiquinone and due to an oligomycin-insensitive ATPase activity of the latter (23), we determined the following: cytochrome-c-oxidase activity or complex IV (CIV) at 550 nm according to Rustin et al. (24), slightly modified for minute amounts of biological samples (19, 25); complex II-III (CII-III) at 550 nm; glycerol-3-phosphate dehydrogenase- complex III (G3PDH-CIII) at 550 nm; complex II (CII) at 600 nm; and isolated glycerol3-phosphate dehydrogenase activity (G3PDH) at 600 nm.

Statistical analysis was carried out with the SPSS 18.0 program. The results were expressed as mean  $\pm$  standard error mean (SEM). Normality of values was confirmed with the Kolmogorov Smirnov test. For cross-sectional analysis, the Mann-Whitney test was carried out, and for longitudinal analysis the Wilcoxon test was used.

## RESULTS

The clinical, immunovirological and mitochondrial characteristics of the patients and controls at baseline are shown in **Table 1**.

All patients on HAART showed significantly lower plasmatic viral loads ( $p < 0.001$ ) at both time-points when compared to children not receiving therapy; CD4+ T cell percentages remained within normal limits in all HIV-infected patients but were higher in treated patients. The evolution of immunovirological parameters (with respect to baseline) of the patients included in the study is shown in **Figure 1**.

Lactate levels, which constitute a plasmatic biomarker of mitochondrial dysfunction, were similar in all groups at baseline (see **Table 1**), but were higher in the 1gNRTIs group with respect to HIV-infected untreated controls ( $1.28 \pm 0.08$  vs.  $0.85 \pm 0.08$  mmol/l;  $p < 0.001$ ) and also with respect to the 2gNRTIs group ( $1.00 \pm 0.07$  mmol/l;  $p = 0.000$ ) at the second time-point, after 2 years (see **Figure 2a**).

The molecular mitochondrial parameters (mtDNA and the ratio of mitochondrial protein levels) are represented in **Figure 2b**. Mitochondrial DNA content was similar in all HIV-infected groups at baseline (see **Table 1**) and 2 years later ( $0.04 \pm 0.008$  in untreated,  $0.04 \pm 0.010$  in 2gNRTIs,  $0.04 \pm 0.009$  in 1gNRTIs;  $p = \text{NS}$ ), and remained unchanged over time within the different groups of HIV-infected patients (see **Figure 2b**). When compared to healthy controls, mtDNA was significantly depleted at baseline in all HIV-infected patients (see **Table 1**), but remained significantly depleted only in the untreated group through follow-up ( $0.04 \pm 0.008$  mmol/l in untreated group,  $0.05 \pm 0.006$  in healthy controls;  $p = 0.048$ ). These results were consistently similar in relative and absolute values (referring to mitochondrial mass or to nuclear DNA).

Mitochondrial protein levels of subunits COXII and COXIV were decreased in all HIV-infected patients with respect to control reference values at baseline (see **Table 1**) and after 2 years. However, significant changes in these parameters were not observed within groups along time (data not shown). Furthermore, the ratio of the mtDNA-encoded subunit COXII and the nuclear DNA-encoded COXIV remained unaltered along time (see **Figure 2b**). An example of an immunoblot is provided (see **Figure 3**).

The oxidative stress, estimated by lipid peroxidation measurement, tended to increase in all groups of HIV-infected children over time, although this change was not statistically significant (see **Figure 2c**).

Mitochondrial mass was similar at baseline and after 2 years in all groups (see **Figure 4**).

Enzymatic activities of MRC were similar referred to overall cell protein and to mitochondrial mass. At baseline, MRC enzymatic activities (nmols/ minute/ mg protein) relativized by CS (nmols/ minute/ mg protein), were similar for all HIV-infected groups. However, at the second time-point, after 2 years, G3PDH-CIII/CS activity was significantly lower in the untreated group with respect to the 2gNRTIs and 1gNRTIs groups ( $0.064 \pm 0.01$ ,  $0.13 \pm 0.02$ ,  $0.14 \pm 0.02$ , respectively;  $p= 0.012$  and  $p= 0.015$ ) (see *Figure 4*).

Mitochondrial function was preserved over time except for CIV/CS activity, which significantly decreased in untreated patients and in the 1gNRTIs groups ( $0.256 \pm 0.023$  and  $0.292 \pm 0.036$ , respectively) with respect to baseline ( $0.519 \pm 0.06$  and  $0.512 \pm 0.036$ ;  $p= 0.015$  and  $p= 0.006$ , respectively) (see *Figure 4*). Absolute CIV enzymatic activity per overall cell protein was significantly decreased in all HIV-infected groups with respect to baseline and with respect to uninfected controls. At baseline results on absolute CIV enzymatic activity were:  $52.20 \pm 5.62$  for untreated patients,  $57.58 \pm 5.64$  for 2gNRTIs group and  $65.06 \pm 5.84$  in 1gNRTIs group. At the second time point, the CIV enzymatic activity was significantly decreased by 26.58% in untreated patients ( $38.33 \pm 13.29$ ), by 33.54% in 2gNRTIs group ( $38.27 \pm 4.93$ ) and by 48.31% in 1gNRTIs group ( $33.60 \pm 4.48$ ) ( $p<0.05$  for all).

## DISCUSSION

Although a wide range of new drugs is available (1) for the treatment of HIV infection and AIDS, there is scarce data on mitochondrial toxicity in children.

We studied the evolution of mitochondrial parameters in a series of paediatric patients undergoing different ARV schedules to provide more information about these mitochondrial markers over time in HIV-infected children and to elucidate whether a given ARV therapy is safer than others from the mitochondrial point of view, while also remaining effective.

The fact that after 2 years of treatment lactate levels significantly increased in the 1gNRTIs group with respect to the untreated group and to the 2gNRTIs is an indicator of a mitochondrial alteration in the former.

Mitochondrial DNA content was decreased in all HIV-infected groups with respect to controls at baseline. Thus, HIV and HAART-induced mtDNA depletion was present in the untreated and treated groups, respectively. There was a slight, albeit not significant, increase in this parameter over time, and after 2 years, mtDNA content remained significantly lower with respect to the control reference values in the untreated group. Nevertheless, in patients treated



with either 1gNRTIs or 2gNRTIs, no significant mtDNA depletion was detected with respect to control values as described by Rosso et al (8). Indeed, HIV infection-related phenomena, regardless of treatment, can cause mtDNA depletion (2,3). At the second time-point in our series we found that HIV was more harmful than HAART regarding mtDNA depletion. Many proteins encoded by HIV genome are apoptogenic, such as Env, gp120, gp41, Vpr, Nef or Tat. Most of these induce mitochondrial apoptosis through the depolarization of the mitochondrial membrane potential, or the release of material within the mitochondria to the cytosol, such as cytochrome c or the apoptotic inducing factor (26-28). The apoptotic process, derived from HIV-infection, is mainly associated with mitochondrial abnormalities, such as mtDNA depletion, mitochondrial dysfunction, and increase of oxidative stress (29). Of note, there has recently been shown to be an increase in plasmatic mtDNA released from damaged or dead cells which, in turn, may explain an inflammatory response in the organism (30).

According to the results observed in mtDNA content in all the HIV-infected groups, the results of protein levels of the mtDNA-encoded subunit II (COXII) and the ratio COXII/COXIV of CIV were significantly lower at both time-points with respect to the control reference values, suggesting a decrease in the translational capacity of the organelle.

Although oxidative stress did not significantly increase over time, it tended to be higher in all HIV-infected patients with respect to baseline. Some studies have reported that HIV (31) as well as HAART (6) can induce oxidative stress. Further studies are required to elucidate whether oxidative stress will continue to increase in our patients over a longer follow-up period.

Mitochondrial mass, estimated by CS activity, remained stable in all the groups over time, leading to the use of this parameter as a normalizing factor for the assessment of mtDNA content and the enzymatic activities of the MRC.

As expected, after the mtDNA depletion observed in untreated HIV-infected children at the second time-point, HIV-infection irrespective of ARV drugs also damaged G3PDHCIII/CS enzymatic activity, which was significantly lower in the untreated group compared to both treated groups. Along this line, in the literature it has been described how HIV triggers mitochondrial impairment, not only at a genetic but also at a functional level in adults (3). In contrast, this enzymatic activity was not compromised in the groups treated with NRTIs, suggesting that these ARVs do not alter MRC function at this point.

All the enzymatic activities were preserved over time, except for cytochrome c oxidase activity. CIV/CS activity significantly dropped in the untreated and 1gNRTIs groups with respect to baseline, suggesting that HIV-infection and 1gNRTIs, respectively, triggered

mitochondrial dysfunction of cytochrome c oxidase activity. It is remarkable that this alteration was not found in the group receiving 2gNRTIs in which almost normal CIV/CS activity (75%) was preserved in comparison with healthy controls. Absolute CIV enzymatic activity dropped in all HIV-infected groups over time; this decline was greater (a half per cent) in 1gNRTIs group. These results support the idea that a therapy including 2gNRTIs, other than ddI, ZDV or d4T, could preserve the mitochondria from significant alterations in the functionality of the MRC over time.

Some limitations of our study are the sample size and the lack of a longitudinal assessment of the healthy controls, due in both cases to the complexity of the recruitment of such samples in the paediatric age. Further, there is a lack of a direct clinical repercussion, although it is possible that clinical manifestations might arise in the future.

In conclusion, our findings support the contention that HIV-infection and the use of 1gNRTIs cause higher mitochondrial damage than the use of 2gNRTIs over time in perinatally HIV-infected children. Current recommendations strongly encourage the early start of HAART in these children in the first year of life, regardless of their clinical or immunological status. Likewise, HAART changes are often required in paediatric patients, usually because of toxicity or resistance. According to our results, the use of 1gNRTIs should only be considered in the HIV-infected child when 2gNRTIs are no longer an option for the patient.

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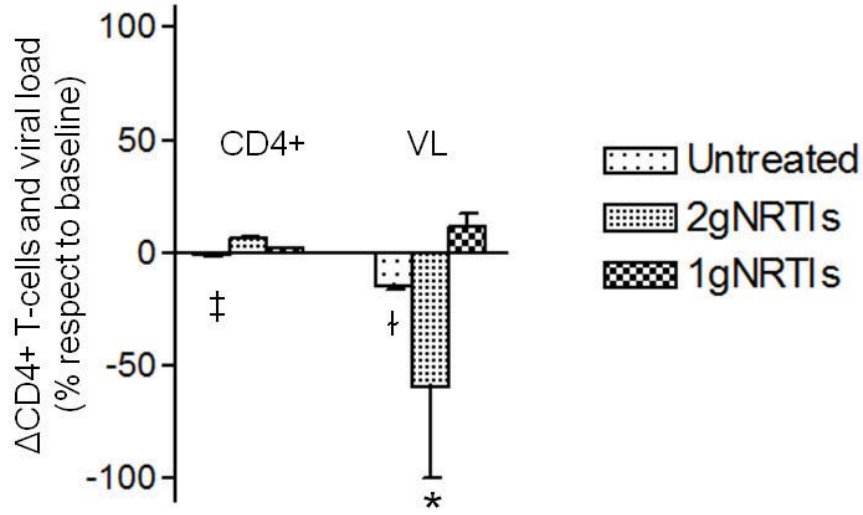
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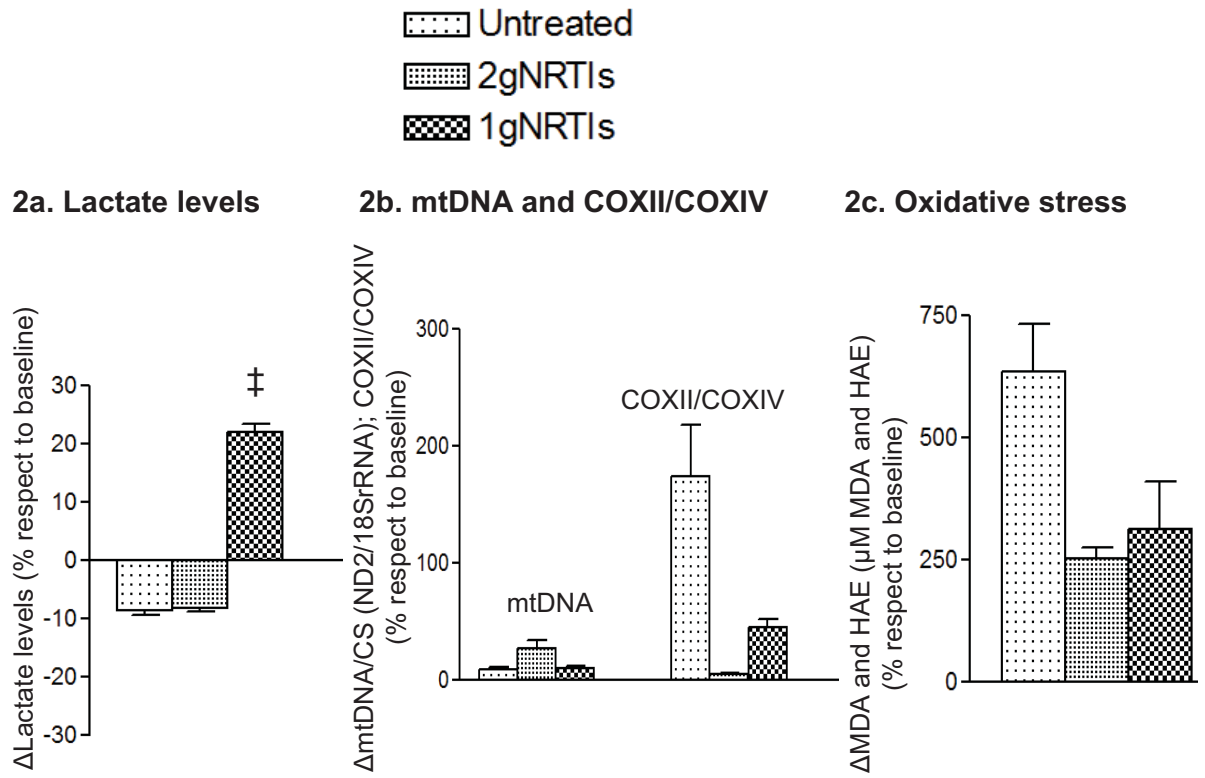
**Table 1.** Clinical, immunovirological and mitochondrial data of patients and controls at baseline. All data are expressed as mean  $\pm$  SEM, except when stated otherwise.

	<b>1gNRTIs</b>	<b>2gNRTIs</b>	<b>Untreated</b>	<b>Uninfected</b>
<b>Clinical data</b>				
Number of subjects (n)	15	13	10	27
Age (years)	9.53 $\pm$ 1.39	13.13 $\pm$ 0.91	13.20 $\pm$ 1.44	9.96 $\pm$ 0.82 (*)
Gender (girls, %)	53	77	30	33
Time on HAART (years)	9.53 $\pm$ 1.39	13.13 $\pm$ 0.91	13.20 $\pm$ 1.44	-
<b>Immunovirological data</b>				
CD4+ T-cell percentages	32.76 $\pm$ 2.51	33.33 $\pm$ 2.73	26.00 $\pm$ 1.78 (ř )	-
Viral load (log RNA-HIV, copies/ml)	0.84 $\pm$ 0.39	0.81 $\pm$ 0.8	4.46 $\pm$ 0.24 (ř)	-
<b>Mitochondrial data</b>				
Lactate levels (mmol/l)	1.05 $\pm$ 0.13	1.09 $\pm$ 0.13	0.93 $\pm$ 0. 1	-
mtDNA/nDNA (ND2/18SrRNA)	4.25 $\pm$ 0.61	3.49 $\pm$ 0.61	3.26 $\pm$ 0.90	5.82 $\pm$ 0.48 (**)
mtDNA/CS (ND2/18SrRNA)	0.04 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.05 $\pm$ 0.01 (**)
COXII/ $\beta$ -Actin	0.038 $\pm$ 0.006	0.065 $\pm$ 0.018	0.07 $\pm$ 0.029	2.90 $\pm$ 0,80 (‡)
COXIV/ $\beta$ -Actin	0.14 $\pm$ 0.03	0.26 $\pm$ 0.08	0.34 $\pm$ 0.16	2,29 $\pm$ 0.533 (‡)
COXII/CS	0.0003 $\pm$ 0.00008	0.0006 $\pm$ 0.00018	0.0006 $\pm$ 0.00025	0.03 $\pm$ 0.012 (‡)
COXIV/CS	0.0013 $\pm$ 0.00038	0.0026 $\pm$ 0.00084	0.003 $\pm$ 0.0014	0.0234 $\pm$ 0.006 (‡)
COXII/COXIV subunits	0.36 $\pm$ 0.06	0.39 $\pm$ 0.08	0.23 $\pm$ 0.03	1.08 $\pm$ 0.08 (‡)
MDA and HAE ( $\mu$ M/mg protein)	0.57 $\pm$ 0.14	0.57 $\pm$ 0.05	0.43 $\pm$ 0.07	0.59 $\pm$ 0.09
CS (nmol/min/mg protein)	127.87 $\pm$ 8.43	118.77 $\pm$ 8.64	107.33 $\pm$ 11.47	118.20 $\pm$ 6.10
CII/CS	0.15 $\pm$ 0.02	0.15 $\pm$ 0.02	0.25 $\pm$ 0.07	0.21 $\pm$ 0.02
CII-III/CS	0.13 $\pm$ 0.02	0.15 $\pm$ 0.02	0.15 $\pm$ 0.03	0.15 $\pm$ 0.02
G3PDH/CS	0.33 $\pm$ 0.04	0.32 $\pm$ 0.04	0.35 $\pm$ 0.46	0.43 $\pm$ 0.03
G3PDH-CIII/CS	0.14 $\pm$ 0.01	0.16 $\pm$ 0.02	0.14 $\pm$ 0.019	0.15 $\pm$ 0.01
CIV/CS	0.51 $\pm$ 0.04	0.51 $\pm$ 0.05	0.51 $\pm$ 0.06	0.45 $\pm$ 0.03

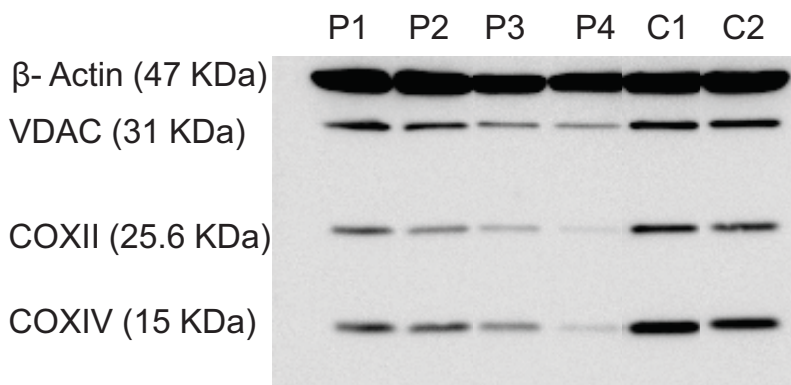
**Figure 1. Immunovirological parameters.**  
CD4+ T-cells and plasmatic viral load



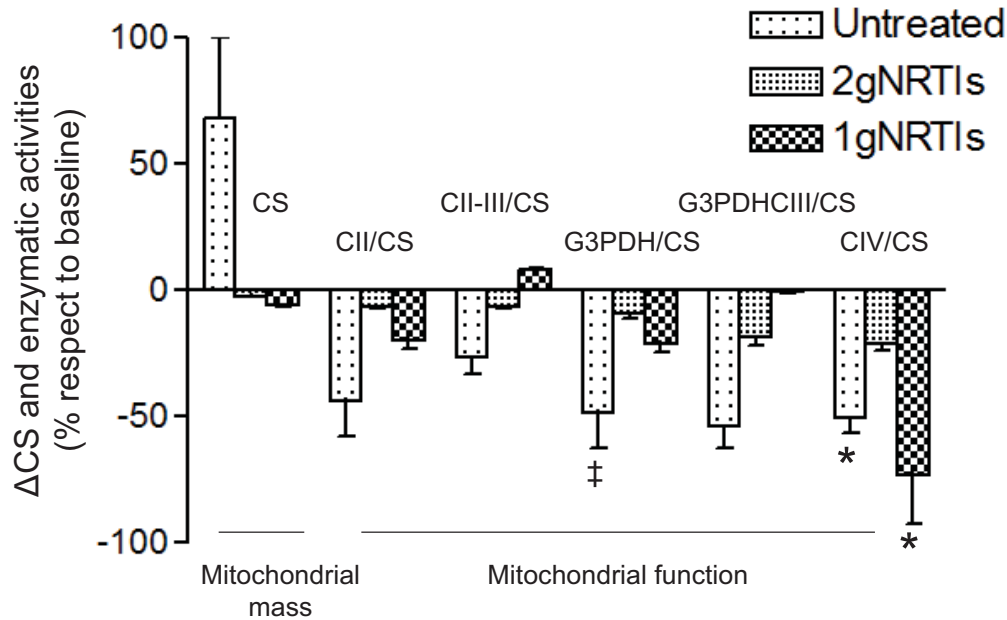
**Figure 2. Mitochondrial parameters.**



**Figure 3. Immunoblot analysis**



**Figure 4. Mitochondrial parameters. Mitochondrial mass and enzymatic activities**



CAPTIONS

**Table 1.** (\*) Significant differences in age of uninfected controls with respect to untreated group ( $p < 0.05$ ).

(†) CD4+ T-cell percentages in untreated group vs. 2gNRTIs group ( $26.00 \pm 1.78$ ,  $33.33 \pm 2.73$  respectively,  $p < 0.05$ ).

(‡) Viral load (HIV RNA copies/ml) in untreated group vs. 2gNRTIs group and vs. 1gNRTIs group ( $4.46 \pm 0.24$ ,  $0.81 \pm 0.38$ ,  $0.84 \pm 0.39$ , respectively;  $p < 0.001$  both).

(\*\*) Significant depletion in all the groups of HIV-infected children (2gNRTIs, 1gNRTIs and untreated groups) with respect to uninfected controls ( $p < 0.005$  all).

(‡) Significant decrease in mitochondrial protein levels COXII and COXIV compared to overall cell protein ( $\beta$ - Actin) or to mitochondrial mass (CS) and in the ratio COXII/COXIV in all groups of HIV-infected patients (2gNRTIs, 1gNRTIs and untreated groups) with respect to uninfected controls ( $p < 0.001$  in all cases).

**Figure 1.** Immunovirological parameters. Differences between groups of HIV-infected patients in CD4+ T- cell percentages and HIV plasmatic viral load (HIV RNA copies/ml).

Untreated: untreated group; NRTIs: nucleoside reverse transcriptase inhibitors; 1gNRTIs: first generation NRTIs: didanosine (ddI), zidovudine (ZDV) and stavudine (d4T); 2gNRTIs: second generation NRTIs: all other drugs. The 0 axis represents the baseline of the 3 HIV-infected groups and the columns represent the increase or decrease of the parameters with respect to baseline. Bars represent mean  $\pm$  SEM.

(\*) Viral load (log HIV RNA copies/ml) significantly decreased in the 2gNRTIs group compared to baseline (from  $0.81 \pm 0.38$  to  $0.33 \pm 0.22$ ;  $p < 0.05$ ).

(‡) At the second time-point, CD4<sup>+</sup> T- cells (%) were significantly lower in the untreated group vs. the 2gNRTIs and 1gNRTIs groups ( $25.70 \pm 1.70$ ,  $35.53 \pm 2.35$ ,  $33.53 \pm 1.31$ ;  $p < 0.05$  both).

(†) Despite a decrease of almost one log at the second time-point, the viral load (log HIV RNA copies/ml) was still higher in the untreated group when compared with the 2gNRTIs and 1gNRTIs groups: ( $3.81 \pm 0.51$ ,  $0.33 \pm 0.22$ ,  $0.94 \pm 0.45$ ;  $p < 0.05$  both).

**Figure 2a.** Lactate levels (mmol/l). Untreated: untreated group; NRTIs: nucleoside reverse transcriptase inhibitors; 1gNRTIs: first generation NRTIs: didanosine (ddI), zidovudine (ZDV) and stavudine (d4T); 2gNRTIs: second generation NRTIs: the remaining drugs. The 0 axis represents the baseline of the 3 HIV-infected groups and the columns represent the increase or decrease of the parameters with respect to baseline. Bars represent mean  $\pm$  SEM.

(‡) After 2 years, at the second time-point, lactate levels were significantly higher in the 1gNRTIs group with respect to the untreated group and to the 2gNRTIs group ( $1.28 \pm 0.083$ ,  $0.85 \pm 0.081$  and  $1.00 \pm 0.071$ ;  $p < 0.05$  both).

**Figure 2b.** Mitochondrial DNA content and mitochondrial protein levels over time in the 3 groups of HIV-infected children. MtDNA: mitochondrial DNA; CS: citrate synthase enzymatic activity; ND2: mitochondrial ND2 gene; 18SrRNA: housekeeping nuclear gene; COXII: subunit II of cytochrome c oxidase or complex IV; COXIV: subunit IV of cytochrome c oxidase or complex IV.

**Figure 2c.** Evolution of oxidative stress measurements over time in the 3 groups of HIV-infected children. MDA: malondialdehyde; HAE: hydroxialkenals;

**Figure 3.** Example of immunoblot analysis of cytochrome c oxidase subunits II (COXII) and IV (COXIV), VDAC: Voltage dependent anion channel and overall cell protein  $\beta$ -Actin. P: Patient; C: Control.



**Figure 4.** Mitochondrial mass and enzymatic activities relativized by CS (citrate synthase) over time in the 3 groups of HIV-infected children. CS, citrate synthase enzymatic activity (nmol oxidized substrate/min/mg of protein); CII: succinate- coenzyme Q reductase; CII-III: complex II-III or CII- cytochrome c reductase; G3PDH: glycerol- 3- phosphate dehydrogenase; G3PDHCIII: G3PDH- cytochrome reductase; CIV: complex IV or cytochrome c oxidase activity. Untreated: untreated group; NRTIs: nucleoside reverse transcriptase inhibitors; 1gNRTIs: first generation NRTIs: didanosine (ddI), zidovudine (ZDV) and stavudine (d4T); 2gNRTIs: second generation NRTIs: the remaining drugs. The 0 axis represents the baseline of the 3 HIV-infected groups and the columns represent the increase or decrease of the parameters with respect to baseline. Bars represent mean  $\pm$  SEM.

(\*) CIV/CS activity significantly decreased in untreated and in the 1gNRTIs groups ( $0.256 \pm 0.023$  and  $0.292 \pm 0.036$ ) with respect to baseline ( $0.519 \pm 0.06$  and  $0.512 \pm 0.036$ ;  $p=0.015$  and  $p= 0.006$ , respectively).

(‡) At the second time-point, G3PDH-CIII/CS activity significantly decreased in untreated group with respect to the 2gNRTIs and 1gNRTIs groups ( $0.064 \pm 0.010$ ,  $0.13 \pm 0.02$ ,  $0.14 \pm 0.02$ ;  $p= 0.012$  and  $p= 0.015$ , respectively).

## DISCUSSION



In adults it has been widely reported the presence of anomalies in mitochondrial parameters, as a consequence of a mitochondrial toxicity derived from the HIV and ARV (HAART). Albeit mitochondrial toxicity has been extensively studied in adults, it has been scarcely determined and remains still unclear in perinatally HIV-infected children. Indeed, although approaches on that issue are increasing, to date, few studies have investigated mitochondrial role of HIV-infected children. The fact that children will be exposed to ARV for a lifelong period entails the necessity of a deeper approach in the study of the potential toxicities in this population group.

As a general conclusion, we can state that all the studies included in this Thesis show an evidence of mitochondrial abnormalities present in HIV-infected children, either off or on HAART, suggesting a detrimental effect in mitochondria derived from both the virus and the treatment; as well as described in adults (Cotê H 2002) (Miró Ò 2004). Additionally, the findings gathered from this Thesis lead us to consider the use of PBMC as a proper study model, able to detect anomalies and differences in mitochondrial parameters of the different immunovirological or therapeutic groups. The use of PBMC represents a non invasive study model which has been widely accepted and validated for the scientific community. In the investigation of the mitochondrial toxicity, PBMC remain, despite their well-known limitations, the tissue of choice with regard to ease of sampling and analysis, especially among children. Particular limitations of each study are detailed extensively throughout each one, although they do not preclude our general conclusions stated above.

As a summary of all the studies carried out, to elucidate first whether there was an association between HIV and/or ARV and the mitochondrial toxicity in children, we aimed to study the mitochondrial functionalism in HIV-infected pediatric patients under ARV and lacking of clinical manifestations. Once we found a depletion in the mitochondrial genome, albeit no dysfunction in cytochrome c oxidase was found, we analyzed the remainder MRC complexes of asymptomatic patients, in order to confirm whether the depletion was downstream reflected in a general mitochondrial affectation. We observed a dysfunction of the global activity of the MRC and a dysfunction of the global oxygen consumption. Once confirmed the affectation in the mitochondrial genome (and downstream) of asymptomatic pediatric patients, we further

analyzed if this affectation was higher in those patients presenting clinical manifestations, such as body fat abnormalities (lipodystrophy). Indeed, we observed a major affectation in this group of patients. Once confirmed the association between infection, ARV and mitochondrial toxicity, either in asymptomatic or in symptomatic pediatric patients, we wondered whether, a withdrawal of the toxic agent, the treatment, was followed of a recovery of the mitochondrial impairment. In this case we observed an increase of the mtDNA content and a recovery of the lactate levels. As not in all cases it is possible to carry out an ARV treatment interruption, we analyzed whether there was a different mitochondrial toxicity under different ARV schedules including first or second generation NRTI. We found a different intensity damage confirming that those ARV previously described in vitro as the most toxics for mitochondria, indeed were associated with a higher toxicity in vivo in our series.

In the first study included in this Thesis a depletion of mitochondrial genome is described in asymptomatic HIV-infected children receiving ARV treatment. Such findings lead us to confirm that, as in adults, there is a mitochondrial toxicity associated to HIV infection and ARV treatment in children, and furthermore this toxicity is plausible and uncovered even in children lacking clinical symptomatology. In this study, the presence of mitochondrial genome depletion was not accompanied by any decrease in the transcription, the mitochondrial protein synthesis of cytochrome oxidase (and therefore the translational capacity), or in the function of the specific enzymatic complex of the MRC. In summary, we detected a depletion of mitochondrial genome in PBMC from asymptomatic HIV-infected children receiving HAART, which was not reflected in differences in transcription, structural or functional activities. These results were consistent with the large body of evidence available on the pathogenesis of HIV-related mitochondrial toxicity in the HIV-infected adult patient (Coté H 2002) (Miró Ò 2004).

In the second study of the analysis of the remaining enzymatic activities of MRC, any alteration of the mentioned activities was found when they were measured in isolated conditions, suggesting some possible homeostatic mechanisms. On the other hand, the mild level of depletion found may also explain the normal functionality in the transcriptional and translational processes, as it has been reported that only mitochondrial genome defects

involving more than 80 % of the genetic material are able to induce MRC failure and cellular dysfunction, potentially leading to significant clinical symptoms (White AJ 2001). However, in the global assessment of the MRC, a dysfunction was detected. Such global dysfunction was reflected in a significant endogenous oxygen consumption deficiency in the untreated group and in a significant decrease of the overall enzymatic activity of the MRC in the treated group. Both groups tended to have a general decrease in both functional parameters. According to the generalized essence of the lesion in a global assessment of the MRC, the oxygen consumption was conserved when it was assessed in isolated conditions, although the endogenous oxygen consumption of the cell, in a general assessment, without the addition of substrates and inhibitors, turned out to be altered. In the same way, the enzymatic activities of specific complexes of MRC were preserved in isolated conditions, although the global enzymatic activity was found altered. Such findings could be due to artefactual oxygen consumption or enzymatic activities in case of artificial MRC electron feeding with respect to natural and spontaneous cell consumption of endogenous substrates.

The existence of HIV and HAART-related mitochondrial toxicity in asymptomatic children, lead us to assume that in the pediatric patients manifesting an evident clinical symptomatology, this mitochondrial toxicity will be present as well and, moreover, it is expected to be more important. HAART associated mitochondrial toxicity in children has varied clinical presentations, comparable to children with congenital mitochondrial diseases. Indeed, in our third study, in which a series of symptomatic pediatric patients manifesting abnormal body fat distribution were included, we observed that the depletion of mitochondrial genome was more evident than in asymptomatic patients, as well as the generalized dysfunction of the MRC. The fact that this mitochondrial lesion is accompanied by clinical manifestations has also been previously observed in HIV-infected adult patients developing hyperlactatemia (Garrabou G 2009). Our findings suggest the possibility of taking into account the measurement of mitochondrial parameters as a reliable biomarker, not only for an assessment of mitochondrial toxicity, but also as a factor associated to the potential clinical manifestation of secondary effects derived from the infection and from the treatment in the pediatric patient. However, differences between groups had not always been apparent in previous reports. For instance, in a previous study any differences in mitochondrial genome or

mitochondrial function were found in PBMC from HIV-infected children, either presenting or not presenting lipodystrophy, probably due to the sample size (Cossarizza A 2002).

Four main strategies to mitigate the toxic effects linked to the ARV therapies have been tested in HIV-infected adults:

(a) Dose reduction, as previously described in some studies in which a partial immunological and mitochondrial recovery was observed after reducing didanosine or stavudine doses in a series of adult patients on didanosine and tenofovir-based regimens (Negredo E 2008) (Milinkovic A 2007).

(b) Interruption of therapy, as described in a study in which a mild improvement of mitochondrial function after 3 years of ARV treatment interruption was observed (Negredo E 2010).

(c) NRTI- sparing strategy, as reported in a study in which an increase of mitochondrial genome copies and cytochrome oxidase activity was found in a group of HIV-infected adults after NRTI withdrawal (Negredo E 2009).

(d) Substitution from a harmful agent to a lesser toxic compound from the mitochondrial point of view, following the different capacity of the different NRTI to inhibit DNA pol  $\gamma$ , as previously reported in vitro. The corresponding in vitro studied hierarchy was described as follows: ddC > ddI > d4T > AZT > 3TC > ABC = TDF (Lim SE 2001) (Kakuda TN 2000). It is of note that ddC is not currently available to administration due to its high toxicity.

Once the mitochondrial lesion associated to ARV has been established and confirmed in children, and following the second strategy to mitigate the toxic effects derived from the ARV therapies, we aimed to elucidate whether this mitochondrial affectation could be reversible in case of withdraw the exposition to the detrimental toxic agent. In our small series, presented in the fourth study, a 12-month treatment interruption led to a partial restoration of mtDNA levels in PBMCs and to a decrease in plasmatic lactatemia. Whether these changes lead to clinical improvements in the long term assessment remains uncertain. In our study, despite a global and significant increase in mitochondrial genome content levels, these decreased in almost half of the patients following HIV viremia rebound after PTI, although these decreases were of smaller magnitude. Our results suggest that loss of mitochondrial genome copy

number in HIV-infected children is due to both the use of NRTI and to the deleterious effects of HIV on mitochondria, as previously described in adults (Coté H 2002), although persistent mitochondrial damage cannot be discarded.

The importance of studying the mitochondrial toxicities derived from HIV and from ARV in children is not delimited to a cross-sectional research, as longitudinal approach plays a key role in order to investigate the immunovirological and mitochondrial status, as well as the potential secondary adverse effects over time, specially, relevant for a therapeutic approach which becomes chronic. In this sense, we carried out our fifth study and following the fourth mentioned strategy of minimize mitochondrial toxicity derived from ARV therapy, a longitudinal assessment in HIV-infected children receiving either first or second generation of NRTI, was conducted. The mitochondrial status from both groups was compared with two control groups: a group of uninfected children and a group of untreated HIV-infected children. A depletion in the mitochondrial genome was found in all HIV-infected groups at baseline. The depletion was maintained over time in untreated HIV-infected children at the second time-point. HIV itself also damaged a specific enzymatic activity at the second time point. Accordingly, in the literature it has been described that HIV itself triggers mitochondrial impairment, not only at a genetic but also at a functional level in adults (Coté 2002) (Miró Ò 2004). All the enzymatic activities were preserved over time, except for cytochrome oxidase activity, which significantly dropped in the untreated and in first generation nucleoside groups (those including ddI, ZDV or d4T), with respect to baseline. Our findings support the idea that a therapy including second generation nucleosides, other than ddI, ZDV or d4T, could preserve the mitochondria from significant alterations in the functionality of the respiratory chain over time. In our study it was confirmed that the ranking of toxicities reported *in vitro* was also found *in vivo* in our series of pediatric patients. Current recommendations strongly encourage the early start of HAART in these children in the first year of life, regardless of the clinical or immunological status. Likewise, HAART changes are often required in pediatric patients, usually because of toxicity or resistance. According to our findings, the use of first generation nucleosides should only be considered in the HIV-infected child when second generation nucleosides are no longer an option for the patient.

Currently, the main goal in the treatment of HIV infection is to reduce the risk of virological failure, while maximizing cost-effectiveness and minimizing toxicity. Although a wide range



of new drugs is available (Imaz A 2011), most of them are not still frequently used in children, and this situation entails a lack of data reporting on mitochondrial toxicity in children regarding new generation drugs. Therefore, further investigations are needed on that issue.

Some limitations of our studies are the sample size, the reduced sample volumes available in children and the lack of a longitudinal assessment of the healthy controls due to, in all cases, the complexity of the recruitment of such samples at the pediatric age.

In conclusion, our findings support the contention that HIV and HAART cause mitochondrial impairment also at the pediatric age, that this affectation includes depletion of the mitochondrial genome and it might be downstream reflected in a global dysfunction of the MRC, that mitochondrial impairment is present either in asymptomatic or symptomatic patients presenting lipodystrophy, that it is possible a recovery of some mitochondrial and clinical abnormalities after an interruption of the therapy and, finally, that the use of first generation NRTI, associated with high mitochondrial toxicity in vitro, causes higher mitochondrial damage, in vivo and over time, than the use of second generation NRTI.

FINAL CONCLUSIONS



The final conclusions derived from the studies exposed in the present Thesis are listed below:

**General conclusion:** As well as described in adults, mitochondrial toxicity, derived from HIV and ARV, is also present in pediatric patients.

**Conclusion 1:** Abnormalities in mitochondrial parameters are present in asymptomatic HIV-infected pediatric patients.

**Conclusion 2:** Even with a decrease in the mitochondrial genome copy number of asymptomatic patients, the functionality of some complexes remains unaltered, probably through the presence of homeostatic mechanisms. However, there is a global dysfunction of the respiratory chain and an affection of oxygen consumption suggesting a diffuse, rather than a localized, lesion of the organelle.

**Conclusion 3:** The pediatric patients manifesting body fat abnormalities, as lipoatrophy or lipohypertrophy, present higher levels of depletion and mitochondrial dysfunction than asymptomatic children.

**Conclusion 4:** After a 12-month planned treatment interruption of ARV therapy the levels of the depleted mitochondrial genome and the lactate levels are recovered in HIV-infected children.

**Conclusion 5:** Mitochondrial impairment varies depending on the type of HAART regimen over time in children. The intensity of mitochondrial toxicity after a 2-year-following-up is higher in children receiving first generation NRTI than those under second generation NRTI. Thus, confirming that those NRTI, previously associated to a high mitochondrial toxicity in vitro, are also related to a higher toxicity in vivo.



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## APPENDIX



The studies carried out during the last years, some of them presented in this Thesis, have been exposed in poster and oral communications of many national and international meetings:

Conference: 4th IAS Conference on HIV Pathogenesis, Treatment and Prevention. Sydney, Australia. 22-25th July, 2007.

Title: Mitochondrial impairment in mononuclear cells of hyperlactatemic patients on HAART.

Authors: G Garrabou, S López, **C Morén**, V Rodríguez, A Milinkovic, E Martínez, J Riba, J Casademont, F Cardellach, JM Gatell, Ò Miró.

Conference: II Annual meeting CIBERER. 19-21st November, 2008. València, Spain.

Title: MtDNA depletion in HIV pediatric patients.

Authors: **C Morén**, G Garrabou, N Rovira, A Noguera, M Nicolàs, S Hernández, J Casademont, F Cardellach, C Fortuny, Ò Miró.

Conference: 9th International Congress on Drug Therapy in HIV Infection, Glasgow, United Kingdom. 9-13th November, 2008.

Title: Mitochondrial impairment in HIV-infected children

Authors: **C Morén**, G Garrabou, N Rovira, A Noguera, M Nicolàs, F Cardellach, Ò Miró, C Fortuny.

Conference: 9th International Congress on Drug Therapy in HIV Infection, Glasgow, United Kingdom. 9-13th November, 2008.

Title: Highly active antiretroviral treatment (HAART) interruption leads to an increase in mitochondrial DNA content in HIV-infected children.

Authors: **C Morén**, G Garrabou, N Rovira, A Noguera, M Nicolàs, F Cardellach, Ò Miró, C Fortuny.

Conference: 9th International Congress on Drug Therapy in HIV Infection, Glasgow, United Kingdom. 9-13th November, 2008.

Title: Mitochondrial effects of 3 years of CD4-guided HIV treatment interruption.

Authors: G Garrabou, E Negredo, **C Morén**, J Romeu, B Rodríguez-Santiago, M Nicolàs, Ò Miró, F Cardellach, J Puig, N Pérez-Álvarez, R López-Blánquez, L Ruiz, R Bellido, C Miranda, B Clotet.

Conference: 9th International Congress on Drug Therapy in HIV Infection, Glasgow, United Kingdom. 9-13th November, 2008.

Title: Mitochondrial toxicity of antiretrovirals in non-HIV-infected patients.

Authors: G Garrabou, E Pedrol, E Deig, **C Morén**, M Nicolàs, P García, I Vidal, F Cardellach, Ò Miró.

Conference: 10th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV. London, United Kingdom. 6-8th November, 2008.

Title: Mitochondrial dysfunction in HIV-infected children receiving or not antiretroviral therapy.

Authors: **C Morén**, G Garrabou, A Noguera, M Nicolàs, F Cardellach, C Fortuny, Ò Miró.

Journal supplement: Antiretroviral Therapy 2008, 13 Suppl 4, A70 (P-64).

Conference: 10th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV. London, United Kingdom. 6-8th November, 2008.

Title: Antiretroviral treatment interruption followed by an increase in mitochondrial DNA content in HIV-infected children.

Authors: **C Morén**, G Garrabou, N Rovira, A Noguera, M Nicolàs, F Cardellach, Ò Miró, C. Fortuny.

Journal supplement: Antiretroviral Therapy 2008, 13 Suppl 4, A70 (P-65).

Conference: 10th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV. London, United Kingdom 6-8th November, 2008.

Title: Effects of a 144-week-long CD4-guided HIV treatment interruption on mitochondria

Authors: G Garrabou, E Negredo, **C Morén**, J Romeu, B Rodríguez-Santiago, M Nicolàs, Ò Miró, F Cardellach, J Puig, N Pérez-Álvarez, R López-Blánquez, L Ruiz, R Bellido, C Miranda, B Clotet.

Journal supplement: Antiretroviral Therapy 2008, 13 Suppl 4, A69.

Conference: 10th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV. London, United Kingdom. 6-8th November, 2008.

Title: Mitochondrial damage induced by the highly active antiretroviral treatment in non-HIV-infected patients.

Authors: G Garrabou, E Pedrol, E Deig, **C Morén**, M Nicolàs, P García, I Vidal, F Cardellach, Ò Miró.

Journal supplement: Antiretroviral Therapy 2008, 13 Suppl 4, A69.

Conference: CROI, 15th conference on Retroviruses and Opportunistic Infections. 3-6th February, 2008. Boston, Massachusetts, USA.

Title: Partial Restoration of Mitochondrial DNA Content in Perinatally HIV-infected Pediatric Patients after Planned Interruption of HAART.

Authors: A Noguera, **C Morén**, E Corrales, E Sánchez, G Garrabou, V Rodríguez, O Miró, C Fortuny.

Conference: IV Congreso de la Sociedad Española de Infectología Pediátrica. 6- 8th March, 2008. Marbella, Spain.

Title: Aumento significativo en los niveles de ADN mitocondrial en células mononucleares de sangre periférica tras interrupción de TARGA en pacientes pediátricos infectados por el VIH.

Authors: C Fortuny, A Noguera, **C Morén**, M Simó, E Sánchez, G Garrabou, V Rodríguez, Ò Miró.

Conference: EUROMIT. 11- 14<sup>th</sup> June, 2008. Stockholm, Sweden.

Title: Mitochondrial DNA amount restoration after planned antiretroviral interruption in HIV-infected children.

Authors: **C Morén**, N Rovira, A Noguera, G Garrabou, V Rodríguez, F Cardellach, Ò Miró, C Fortuny.

Conference: EUROMIT. 11- 14<sup>th</sup> June, 2008. Stockholm, Sweden.

Title: Mitochondrial damage in peripheral blood mononuclear cells in HIV-infected pediatric patients.

Authors: **C Morén**, E Molina, A Noguera, G Garrabou, V Rodríguez, F Cardellach, C Fortuny, Ò Miró.

Conference: EUROMIT. 11- 14th June, 2008. Stockholm, Sweden.

Title: Mitochondrial lesion in acute carbon monoxide poisoning: effects of normobaric or hyperbaric oxygen treatment.

Authors: G Garrabou, **C Morén**, V Rodríguez, JM Inoriza, MJ Martí, G Oliu, Ò Miró, J Casademont, F Cardellach.

Conference: EUROMIT. 11- 14<sup>th</sup> June, 2008. Stockholm, Sweden.

Title: Mitochondrial effects of mehtylene chloride substance abuse.

Authors: G Garrabou, **C Morén**, V Rodríguez, JC Trullàs, S Mondón, V Navarro, J Jou, S Nogué, Ò Miró, J Casademont, F Cardellach.

Conference: EUROMIT. 11- 14<sup>th</sup> June, 2008. Stockholm, Sweden.

Title: Oocyte mitochondrial DNA content implication on HIV-infected antiretroviral-treated women fertility outcome.

Authors: G Garrabou, **C Morén**, V Rodríguez, O Coll, M Durban, S López, R Vidal, A Suy, S Hernández, J Casademont, F Cardellach, D Mataro, Ò Miró.

Conference: EUROMIT. 11- 14th June, 2008. Stockholm, Sweden.

Title: Mitochondrial effects on sepsis.

Authors: G Garrabou, **C Morén**, S López, V Rodríguez, F Cardellach, Ò Miró, J Casademont.

Conference: XVII International AIDS Society (IAS) Conference. 3-8<sup>th</sup> August, 2008. Mexico DF, Mexico.

Title: Lopinavir- ritonavir plus Nevirapine as a strategy to improve nucleoside-related mitochondrial toxicity in chronically treated HIV-infected patients: 48 week follow-up of MULTINEKA study.

Authors: E Negro, Ò Miró, G Garrabou, **C Morén**, F Cardellach, C Estany, B Rodríguez-Santiago, A Masabeu, L Force, P Barrufet, J Cucurull, P Domingo, CA Villaverde, N Pérez-Álvarez and B Clotet.

Conference: 27th Annual Meeting of the European Society for Paediatric Infectious Diseases. 9-13th June, 2009. Brussels, Belgium.

Title: HIV and HAART-mediated mitochondrial function in children.

Authors: A Noguera, **C Morén**, G Garrabou, N Rovira, M Nicolàs, F Cardellach, Ò Miró, C Fortuny.

Conference: Mitochondrial Medicine. 24-27th June, 2009. Vienna- Virginia, USA.

Title: Mitochondrial damage in acute carbon monoxide poisoning: the effect of oxygen treatment.

Authors: G Garrabou, JM Inoriza, **C Morén**, M Nicolàs, G Oliu, Ò Miró, MJ Martí, F Cardellach.

Conference: Mitochondrial Medicine. 24-27th June, 2009. Vienna- Virginia, USA.

Title: Sepsis effects on mitochondria.

Authors: G Garrabou, **C Morén**, S López, M Nicolàs, F Cardellach, Ò Miró, J Casademont.

Conference: Mitochondrial Medicine. 24-27th June, 2009. Vienna- Virginia, USA.

Title: Methylene chloride effects on mitochondria.

Authors: G Garrabou, **C Morén**, M Nicolàs, JC Trullàs, S Mondón, V Navarro, J Jou, S Nogué, Ò Miró, J Casademont, F Cardellach.

Conference: Mitochondrial Medicine 2009. 24-27th June, 2009. Vienna- Virginia (USA).

Title: HIV and antiretroviral-mediated mitochondrial DNA depletion in children.

Authors: **C Morén**, N Rovira, A Noguera, G Garrabou, M Nicolàs, F Cardellach, Ò Miró, C Fortuny.

Conference: 11th International Workshop on Adverse Drug Reactions and Co-morbidities in HIV. 26-28<sup>th</sup> October, 2009. Philadelphia, PA, USA.

Title: Increased mitochondrial toxicity correlates adverse perinatal outcome in HIV-infected women on antiretroviral treatment and their newborn.

Authors: G Garrabou, S Hernández, **C Morén**, M Nicolàs, M López, S López, A Gonce, Ò Miró, O Coll.

Journal supplement: Antiretroviral Therapy 2009, 14 Suppl 2, A65.

Conference: 11th International Workshop on Adverse Drug Reactions and Co-morbidities in HIV. 26-28<sup>th</sup> October, 2009. Philadelphia, PA, USA.

Title: HIV and antiretroviral-mediated depletion in children.

Authors: **C Morén**, A Noguera, G Garrabou, N Rovira, M Nicolàs, F Cardellach, Ò Miró, C Fortuny.

Journal supplement: Antiretroviral Therapy 2009, 14 Suppl 2, A64

Conference: 49th Annual Meeting of the Society of Toxicology (SOT). 7-11th March, 2010. Salt Lake City, Utah, USA.

Title: Mitochondrial DNA depletion in HIV-infected children.

Authors: **C Morén**, A Noguera, G Garrabou, N Rovira, M Nicolàs, F Cardellach, E Martínez, E Sánchez, Ò Miró, C Fortuny.

Conference: III Reunión CIBERER. 29-30th October, 2010. Madrid, Spain.

Title: Efectos mitocondriales derivados del abuso de cloruro de metileno.

Authors: G Garrabou, **C Morén**, M Nicolàs, JC Trullàs, S Mondón, V Navarro, J Jou, JM Grau, J Casademont, S Nogué, Ò Miró, F Cardellach.

Conference: 49th Annual Meeting of the Society of Toxicology (SOT). 7-11th March, 2010. Salt Lake City, Utah, USA.

Title: Mitochondrial toxicity of tobacco in smoking pregnant mothers and their newborn.

Authors: F Cardellach, S Hernández, G Garrabou, **C Morén**, M Nicolàs, O Coll, Ò Miró.

Conference: 49th Annual Meeting of the Society of Toxicology (SOT). 7-11th March, 2010. Salt Lake City, Utah, USA.

Title: Mitochondrial and neuropathic toxicity of the antibiotic linezolid.

Authors: G Garrabou, A Soriano, T Pinós, D Pacheu-Grau, J Casanova-Mollà J, **C Morén**, E García-Arumí, E Ruiz-Pesini, M Morales, M Nicolàs, JA Martínez, JM Grau, Ò Miró Ò, J Montoya, AL Andreu, J Mensa, F Cardellach.

Conference: XII European Congress of Perinatal Medicine. 26-29th May, 2010. Granada, Spain.

Title: Mitochondrial toxicity in smoking pregnant women and infants exposed in utero to tobacco

Authors: S Hernández, G Garrabou, M López, **C Morén**, M Nicolàs, O Coll, Ò Miró, E Gratacós, F Cardellach.

Conference: 4th World Congress on Controversies in Neurology- 4-7th November, 2010. Berlín (Germany).

Title: Subclinical small fiber neuropathy related to long treatment with linezolid: the possible implication of mitochondrial failure.

Authors: J Casanova-Molla, M Morales, G Garrabou, **C Morén**, M Nicolàs, F Cardellach, A Soriano, J Valls-Solé.

Conference: IV Reunión Científica Anual CIBERER. 28-29th October, 2010

Title: Mitochondrial function in healthy infants perinatally exposed to HIV and antiretrovirals. **POSTER AWARDED.**

Authors: **C Morén**, N Rovira, A Noguera-Julian, G Garrabou, M Nicolàs, F Cardellach, Ò Miró, C Fortuny.

Conference: 18th Conference on Retroviruses and Opportunistic Infections (CROI). 27<sup>th</sup> February- 2<sup>nd</sup> March, 2011. Boston, USA.

Title: Higher Mitochondrial DNA Content Compensates for Lower Mitochondrial-encoded Complex IV Activity in Antiretroviral-exposed Healthy Infants

Authors: A Noguera-Julian, N Rovira, **C Morén**, G Garrabou, M Nicolás, F Cardellach, E Sánchez, Ò Miró, C Fortuny.

Conference: 50th Society of Toxicology (SOT) Conference. 6-10<sup>th</sup> March, 2011. Washington DC, USA.

Title: Evolution of mitochondrial function in healthy infants in utero exposed to HIV and antiretrovirals.

Authors: **C Morén**, N Rovira, A Noguera, G Garrabou, M Nicolàs, F Cardellach, Ò Miró, C Fortuny.

Conference: Jornadas Nacionales VIH- Mitocondria. 3rd December, 2008. Hotel Catalonia, Barcelona, Spain.

Title: Toxicidad Mitocondrial en Pacientes Pediátricos VIH+.

Oral presentation

Conference: Jornadas Científicas Mitolab-CM y CIBERER “La Mitocondria en la Patología”. 29-31st October, 2008. Residencia La Cristalera, Miraflores de la Sierra, Madrid.

Title: Toxicidad mitocondrial en pacientes pediátricos VIH+.

Oral presentation by **C Morén**.

Conference: Mitochondrial Medicine, 2009, Capitoll Hill. 24-27<sup>th</sup> June, 2009. Vienna, Virginia, USA.

Title: Mitochondrial toxicity in HIV-infected children.

Oral presentation by **C Morén**.



Some other studies not included in this Thesis have been published in different scientific journals:

Title: Mitochondrial DNA depletion in oocytes of HIV-infected antiretroviral-treated infertile women.

Authors: S López, O Coll, M Durban, S Hernández, R Vidal, A Suy, **C Morén**, J Casademont, F Cardellach, D Mataró, Ò Miró, G Garrabou.

Journal: Antivir Ther 2008; 13(6):833-888

Title: Partial immunological and mitochondrial recovery after reducing didanosine doses in patients on didanosine and tenofovir-based regimens.

Authors: E Negredo, G Garrabou, J Puig, S López, **C Morén**, R Bellido, R Ayen, F Cardellach, Ò Miró, B Clotet.

Journal: Antiviral Therapy 2008; 13(6):231-240.

Title: Genetic and functional mitochondrial assessment of HIV-infected patients developing HAART-related hyperlactatemia.

Authors: G Garrabou, **C Morén**, JM Gallego-Escudero, A Milinkovic, F Villarroya, E Negredo, M Giralt, F Vidal, E Pedrol, E Martínez, F Cardellach, JM Gatell, Ò Miró.

Journal: Journal of Acquired Immune Deficiency Syndrome 2009; 52(4):443-451.

Title: Improvement of mitochondrial toxicity in patients receiving a nucleoside reverse-transcriptase inhibitor-sparing strategy: results from the Multicenter Study with Nevirapine and Kaletra (MULTINEKA).

Authors: E Negredo, Ò Miró, B Rodríguez-Santiago, G Garrabou, C Estany, A Masabeu, L Force, P Barrufet, J Cucurull, P Domingo, C Alonso-Villaverde, A Bonjoch, **C Morén**, Pérez-Alvarez N, Clotet B; MULTINEKA Study Group.

Journal: Clin Infect Dis. 2009; 49(6):892-900.

Title: Mild improvement in mitochondrial function after a 3-year antiretroviral treatment interruption despite persistent impairment of mitochondrial DNA.

Authors: E Negredo, J Romeu, B Rodríguez-Santiago, Ò Miró, G Garrabou, J Puig, N Pérez-Álvarez, **C Morén**, L Ruiz, R Bellido, C Miranda, B Clotet.

Journal: Current HIV Research 2010; 8(5):379-385.

Title: Mitochondrial injury in human acute carbon monoxide poisoning: the effect of oxygen treatment.

Authors: G Garrabou, JM Inoriza, **C Morén**, G Oliu, Ò Miró, MJ Martí, F Cardellach.  
Journal: Journal of Environmental Science Health C Environmental Carcinogenesis  
Ecotoxicology 2011; 29(1):32-51.

Title: Hyperbaric oxygen therapy of carbon monoxide poisoning.  
Authors: G Garrabou, JM Inoriza, **C Morén**, G Oliu, Ò Miró, MJ Martí, F Cardellach.  
Journal: Intensive Care Medicine 2011; 37(10):1711-1712.

Title: Mitochondrial damage in adipose tissue of untreated HIV-infected patients.  
Authors: G Garrabou, S López, **C Morén**, E Martínez, J Fontdevila, F Cardellach, JM Gatell, Ò Miró.  
Journal: AIDS 2011; 25(2):165-170.

Title: Sepsis effects on mitochondria.  
Authors: G Garrabou, **C Morén**, S López, E Tobías, F Cardellach, Ò Miró, J Casademont.  
Journal: Journal of Infectious Diseases 2011. In press.

Title: Perinatal outcomes, mitochondrial toxicity and apoptosis in HIV-infected and treated pregnant women and in utero-exposed newborn.  
Authors: S Hernández, **C Morén**, M López, O Coll, F Cardellach, E Gratacós, Ò Miró, G Garrabou.  
Journal: AIDS 2011. In press.

Two chapter books have been written while the period of this Thesis:

Book: "*Infección VIH: Tratamiento antirretroviral y mitocondria*".  
Chapter 5: "*VIH-Mitocondria. Mecanismos de Interacción*". HIV-infection: antiretroviral treatment and mitochondria. HIV-Mitochondria. Interaction mechanisms.  
Authors: **C Morén** and Ò Miró.  
ISBN: 978-84-691-1475-9

Book: Mitochondrial pathophysiology. Editors: Susana Cadenas and Francesc Palau.  
Editorial: Transworld Research Network  
Chapter 12: Secondary mitochondriopathies (pages 249-279)  
Authors: G Garrabou, **C Morén**, M Nicolàs, JM Grau, J Casademont, Ò Miró, F Cardellach  
ISBN: 978-81-7895-514-8



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## MITOCHONDRIAL DNA DEPLETION IN OOCYTES OF HIV-INFECTED ANTIRETROVIRAL-TREATED INFERTILE WOMEN

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**Objective:** HIV-infected women under highly active antiretroviral therapy (HAART) undergoing *in vitro* fertilization (IVF) have a lower pregnancy rate than non-infected controls, which depends on oocyte-related factors. We hypothesize that mitochondrial toxicity caused by antiretrovirals could be the underlying mechanism of such disturbance.

**Methods:** We have studied 16 and 19 previously frozen-thawed oocytes obtained after oocyte retrieval IVF cycles from 8 and 14 infertile HIV-infected and uninfected women, respectively, matched by age. At inclusion, HIV-positive women had been infected for more than 13 years and had received HAART for more than 9 years, including at least one NRTI. All of them had undetectable HIV-viral load and a good immunological status.

Mitochondrial DNA (mtDNA) content was determined by quantitative real-time PCR in each individual oocyte.

**Results:** HIV-infected infertile women on HAART showed significant oocyte mtDNA depletion when comparing with uninfected controls (32% mtDNA decrease;  $p < 0.05$ ). This oocyte mtDNA depletion was even greater on those HIV-infected women who failed to become pregnant when comparing with controls (39% mtDNA decrease;  $p = 0.03$ ). No significant correlation was found between mtDNA oocyte content and cumulative doses of antiretrovirals or the immunological status of HIV-patients.

**Conclusions:** Oocytes from infertile HIV-infected HAART-treated women show decreased mtDNA content and this could explain their poor reproductive outcome.

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### INTRODUCTION

The ability of highly active antiretroviral therapy (HAART) to decrease viral load up to undetectable levels and to increase CD4<sup>+</sup> T lymphocytes has drastically reduced mortality and morbidity among HIV-infected patients

[1,2]. As a consequence, HIV-infection has become a chronic disease.

Many adverse effects have been described and associated with long-term use of antiretrovirals. Most of them (miopathy, lipodystrophy, lactic acidosis, polyneuropathy or pancreatitis) have been related to mitochondrial toxicity caused by

nucleoside analogues reverse transcriptase inhibitors (NRTIs) [3-7].

The main underlying mechanism described for mitochondrial toxicity is the capacity of NRTIs of inhibiting the mtDNA synthesis by both direct inhibition of the human DNA polymerase- $\gamma$  (DNA pol- $\gamma$ , the only polymerase responsible for the mtDNA replication) and by acting as chain terminators of the growing DNA strand. Consequently, NRTIs lead to the generation of abnormal mitochondria, with decreased number of mtDNA molecules per organelle (depletion), as well as an increase of mutations (deletions and/or point mutations) in the mtDNA genome [8-11]. MtDNA is a 16.6-kb double-stranded molecule which only encodes for 13 proteins of the mitochondrial respiratory chain associated with the oxidative phosphorylation process, 2 mitochondrial ribosomal RNA (mt rRNA) and 22 mitochondrial transfer RNA (mt tRNA). In this context, a depletion of the mtDNA levels can finally lead to mitochondrial dysfunction and energetic cell impairment.

According to epidemiological data, HIV-infected women have a lower spontaneous fertility rate than uninfected women [12-14]. Recently, we reported the same observation when these women underwent *in vitro* fertilization (IVF) with their own oocytes. Furthermore, no significant reduction in the pregnancy rate was found when healthy donated oocytes were used. These results suggest that the reduced pregnancy rate observed among HIV-infected women on HAART could be attributed to the oocyte [15]. Although the underlying mechanism of this decreased fertility is unknown, it has been shown that sufficient energy production from mitochondria seems to

be relevant in oocytes viability and in the development of embryos, so any mitochondrial defect in oocytes could eventually lead to cell dysfunction and infertility. In this sense, it is especially noteworthy the fact that *a priori*, the toxic effects of NRTIs can affect any cell containing mitochondria, since they have not been specifically designed to enter a particular cellular type, and that oocytes are post-mitotic cells, with no ability to remove damaged mtDNA. We hypothesize that mtDNA content is depleted in oocytes of infertile HIV-infected women under HAART treatment.

## PATIENTS AND METHODS

A total of 8 HIV-infected women all under HAART and 14 controls (non-infected women) undergoing *in vitro* fertilization (IVF) for infertility treatment at 'Clinica Eugin' matched by age and IVF indication were included. All women provided informed consent to participate in the study. Duration of, number of previous ART-cycles, and total dose of FSH for ovarian hyperstimulation were similar in both groups.

A total of 16 oocytes non-suitable from 8 HIV-infected women and 19 non suitable oocytes from 14 HIV-negative women were obtained after ovarian hyperstimulation performed using recombinant FSH or human menopausal gonadotrophin and hypophysary suppression was obtained using either GnRH agonists or antagonists in a short-stimulation protocol. Oocytes were recovered 36 h after the administration of recombinant HCG.

During an IVF cycle, a proportion of the retrieved oocytes are immature, either at the metaphase-I (absence of both a germinal vesicle and a first polar body) or at the germinal vesicle

(GV) stage. On day 1 after sperm insemination a proportion of metaphase II (mature) oocytes are not fertilized (NF). All of them are defined as non-suitable for assisted reproduction and therefore, the study did not limit the likelihood of achieving a pregnancy.

All the women from both groups had viable embryos, which were transferred.

Human oocytes can only be assessed during an IVF cycle. Given that mature oocytes have to be used for fertility purposes, the assessment of non-suitable oocytes and non-fertilized oocytes from the same cycle from both infected and uninfected women is the best model available, although it may have some limitations. Nevertheless, it is currently the only ethically acceptable approach.

The mean age of women included in the study was  $36\pm 2$  years in the HIV group and  $37,4\pm 4$  in the control group. At the time of the IVF cycle, all the HIV-infected women had undetectable HIV viral load and a good immunological status. The mean time after HIV diagnosis was more than 13 years ( $153\pm 57$  months). All patients were on HAART (mean duration of  $101\pm 58$  months), which included NRTIS in all cases. These clinical and epidemiological characteristics of all the patients are summarized in Table 1.

Oocytes were obtained after a standard IVF cycle (hormonal-stimulated poliovulation and ultrasound guided oocyte retrieval) and treated with hyaluronidase to remove cumulus cells. Mature oocytes were inseminated by intracytoplasmic sperm injection (ICSI) and observation of non-fertilization was performed on day 1 (18-20 hours after ICSI). Selected oocytes were individually rinsed in 0.5 ml of PBS 1x (Dulbecco's Phosphate Buffered Saline

Solution without Calcium and Magnesium Salts; Irvine-Acientific, USA) and placed into a DNase/RNase free cryotube which were frozen in liquid nitrogen.

#### **DNA isolation and mtDNA quantification**

Lysis of each individual oocyte was done in the appropriate buffer containing 125  $\mu$ l of proteinase K solution (2 mg/ml proteinase K - Roche Diagnostics GmbH, Mannheim, Germany-, SDS 1% and 2 mM EDTA pH 8.0), 50  $\mu$ l of SDS 10% and 750  $\mu$ l of lysis solution (10 mM Tris-HCl pH 7.5, 400 mM NaCl, 2 mM EDTA pH 8.0) and incubated for 60 min at 55°C and for 10 min at 100°C. After lysis, we added to each sample 1  $\mu$ l of pellet paint (Pellet Paint<sup>®</sup> Co-Precipitant, Novagen, Merck KgaA, Darmstadt, Germany) and 10  $\mu$ g of glycogen (Roche Applied Science, Mannheim, Germany) as carriers. Total DNA from each sample was isolated by standard phenol-chloroform procedure adapted to microvolumes (1:1 phenol:chloroform:isoamyl alcohol 25:24:1, Sigma-Aldrich, Inc., Sant Louis, MO) and then precipitated with 0.1 volumes of 3 M sodium acetate pH 5.3 and 2.5 volumes of absolute ethanol previously kept at -20°C. The pellet of DNA was dissolved in 40  $\mu$ l of TE 10:1 buffer (Tris 10 mM / EDTA 1 mM; pH 7.5) and homogenized at 37°C for 2 hours. For each DNA extract, the highly conserved mitochondrial ND2 gene was quantified by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals<sup>®</sup>, Germany) in duplicate using the sequences of the primers and the conditions of the PCR reaction previously reported [16]. The results for the mtDNA content were expressed as picograms (pg) of the

ND2 gene. The mtDNA content of controls was considered to be 100%.

**Statistical analysis**

Clinical and epidemiological characteristics of all women included in the study were expressed as mean ± SD ( quantitative data ) and percentages (qualitative data). One-way ANOVA was used to detect significant differences between groups. When ANOVA was significant and comparisons included three groups, the Bonferroni post-hoc analysis was used to uncover between which pair of groups the difference laid. Linear regression analysis

was performed to establish any association among mtDNA content and cumulated doses of antiretrovirals or immunovirological parameters. In all cases, p values less than 0.05 were considered statistically significant.

**RESULTS**

Clinical and epidemiological characteristics of HIV-infertile women and controls included in the study are detailed in table 1.

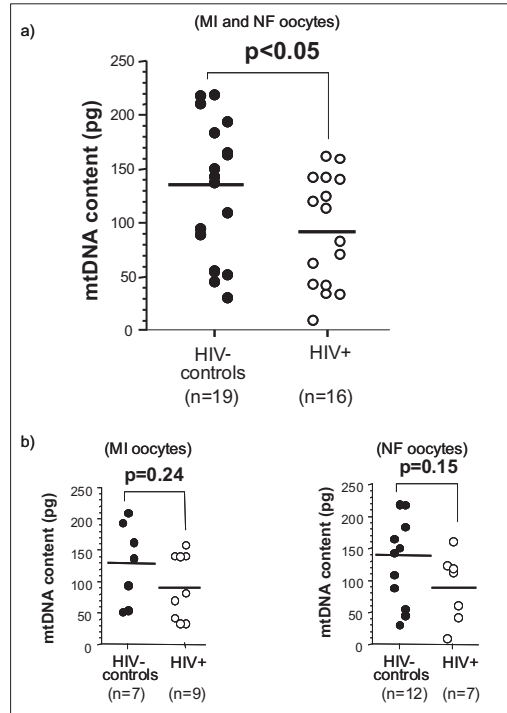
**Table 1:** Clinical and epidemiological characteristics of infertile women included in the study.

	<b>Cases</b>	<b>Controls</b>
<b>Patients (n)</b>	8	14
<b>Age (years ± SD)</b>	36 ± 2	37 ± 4
<b>CD4 (cells/mm<sup>3</sup> ±SD)</b>	781 ± 298	
<b>Viral load &lt;200 (%)</b>	100	
<b>Months (± SD) from HIV diagnosis</b>	153 ± 57	
<b>Months (± SD) on HAART</b>	101 ± 58	
<b>Months (± SD) on NRTIs</b>	100 ± 59	
<b>Patients on NRTIs at the time of IVF (%)</b>	100	
<b>Total oocytes analysed (n)</b>	16	19

Infertile HIV-infected women on HAART had 32% lower oocyte mtDNA content than infertile controls (92±51 vs 136±71, respectively; p<0.05) (Figure 1-a). When mtDNA content was analyzed stratifying by the type of oocytes (MI or NF), differences between infected and uninfected women were no longer statistically significant (Figure 1-b). Nevertheless, as shown in Figure 1-b, a consistent trend persisted in the

two types of oocytes of infected patients, with 27% depletion for MI oocytes and 36% depletion for NF oocytes when comparing with controls (94±52 vs 129±64 for MI oocytes; p=NS, and 90±53 vs 140±77 for NF oocytes; p=NS). Lack of statistical differences could be attributable to the small number of oocytes analyzed in each subgroup.

**Figure 1:** Oocyte mitochondrial DNA (mtDNA) content (expressed as picograms of the ND2 gene) for controls (uninfected women) and cases (HIV-infected women), a) when analysed both MI and NF oocytes all together, and b) when analysed according to the type of oocyte (MI or NF). (MI: metaphase I; NF: non-fertilized).



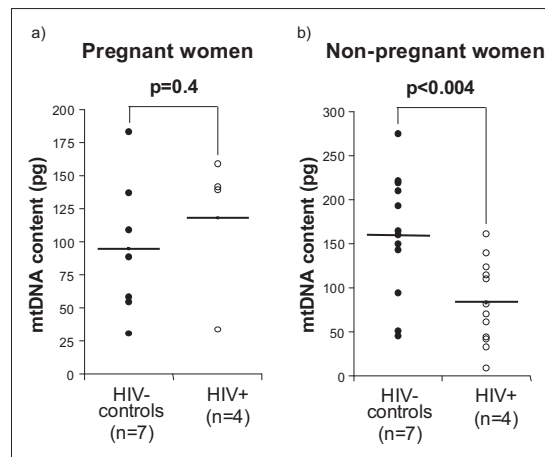
Mitochondrial DNA levels were evaluated according to pregnancy outcome of the same IVF cycle. Pregnancy was established if at least one intrauterine sac revealed by ultrasonography approximately 5 weeks after embryo transfer. Pregnancy rate per cycle was 25% (4/16) among infertile HIV-infected women on HAART and 37% (7/19) in the control group. Among HIV-infected women, oocytes from a non-pregnancy (n=12) had 30% depletion of mtDNA content with respect to oocytes from a pregnancy cycle (n=4) ( $83 \pm 48$  vs  $119 \pm 57$ , respectively;  $p=0.237$ ). When comparing with the control group (n=19; 100%), oocytes from a non-pregnancy (n=12) showed 39% decrease of the mtDNA levels ( $p=0.03$ ), while oocytes from a

pregnancy cycle (n=4) only showed 13% decrease of the mtDNA content ( $p=0.653$ ). In addition, when comparing HIV-infected women oocytes from a non-pregnancy (n=12) and uninfected control oocytes from a non-pregnancy (n=12) the former showed 48% decrease of the mtDNA ( $83 \pm 48$  vs  $161 \pm 70$ , respectively;  $p=0.004$ ) (Figure 2). However, no differences were found when comparing HIV+ and HIV- oocytes from a successful pregnancy ( $119 \pm 57$  vs  $94 \pm 53$ , respectively;  $p=NS$ ) (Figure 2).

No significant correlation was found between oocyte mtDNA content and cumulated antiretroviral doses or the immunovirological status of HIV-patients (data not shown).



**Figure 2:** Oocyte mitochondrial DNA (mtDNA) content (expressed as picograms of the ND2 gene) for controls (uninfected women) and cases (HIV-infected women) according to the pregnancy outcome of the same *in vitro* fertilization (IVF) cycle: a) oocyte mtDNA content analysed in pregnant women, and b) oocyte mtDNA content analysed in non-pregnant women.



## DISCUSSION

The oocyte is the largest human cell (300 times bigger in average than the rest of somatic cells) and contains large amount of mitochondria that represent at least the 23% of the ooplasm [17]. Mitochondria are double-membrane intracellular organelles and the main source of the high-energy phosphate molecule adenosine triphosphate, which is essential for all active intracellular processes [18]. Oocytes are packed with mitochondria, and disorders of mitochondrial function may cause reproductive failure. Mitochondrial DNA copy number per mature human oocyte is about 100.000-600.000 molecules, compared with the 500-10.000 molecules for the rest of the cells [19-20]. Furthermore, the oocyte approximately contains a single mtDNA molecule per mitochondrion to avoid heteroplasmy segregate through the maternal lineage [21], and differs from the 2-10 mtDNA copies for the rest of human somatic mitochondria [17]. This fact makes oocyte

mitochondria especially vulnerable to mtDNA depletion and oocyte especially sensitive to mtDNA depleting factors (drugs, toxins, infections, etc.).

Mitochondrial dysfunction has been associated with reproductive outcome since their functionalism influences the viability of both sperm and oocytes. Accordingly, low mtDNA content in both male [22] and female gametes [17, 23, 24] has been associated with infertility. In addition, mutations in the mtDNA genome have been also described in spermatozoa with declined motility and fertility [25]. Other investigations relate the generation of abnormal sperm mtDNA molecules (with multiple mtDNA deletions) to long-term antiretrovirals intake in HIV-infected patients [26]. Moreover, it has been suggested that HIV infection and NRTIs have negative effects on semen parameters (semen volume, percentage of progressive motile spermatozoa, total sperm count, polynuclear cell count, pH, spermatozoa anomalies), compromising male fertility.

In the oocyte, mitochondria contribute to fertilization and embryonic development. Cohen et al. reported that ooplasm transfer (including mitochondria) from a young donor oocyte partially restores the reproductive capacity in oopausal oocytes [27]. Both Reynier et al. [23] and Santos et al. [17] established an association between the mtDNA content and the probability of oocyte fertilization. The latest suggested that the mtDNA content could be an oocyte quality and fertility marker. Another study suggests that low mtDNA content is associated with the impaired oocyte quality observed in ovarian insufficiency [24]. These studies suggest that mitochondria are critical to fertilization outcome and embryonic development [27]. Nevertheless, there are no published studies assessing mtDNA levels in oocytes of HIV-infected women.

Epidemiological and clinical data suggest that HIV-infected women have a lower spontaneous pregnancy rate. We recently described that infertile HIV-infected women on HAART undergoing *in vitro* fertilization (IVF) had lower pregnancy than uninfected women (16.2% vs 39.2%). However, the effect of HIV infection was not observed in women undergoing oocyte donation (36% vs 45.1%).

This is the first study that addresses the underlying mechanism that could explain a low pregnancy rate in HIV-infected women. Our data suggest that oocytes from infertile HIV-infected women on HAART have decreased mtDNA levels compared with infertile uninfected controls.

Two possible mechanisms could explain the mtDNA depletion found in our study. One of them is related to the secondary effects of antiretrovirals and oocyte characteristics. HAART combinations against HIV, specifically

those containing NRTIs, may cause mitochondrial toxicity. *A priori*, the toxic effects of NRTIs can affect any cell containing mitochondria, since they have not been specifically designed to enter a particular cellular type. Therefore, oocytes may be exposed to cumulated therapeutic doses of these drugs. In addition, oocytes are post-mitotic cells with no ability to eliminate damaged mitochondria and with high dependence on the oxidative phosphorylation system. Moreover, oocytes contain large number of mitochondria with only one molecule of mtDNA per organelle, so they are more sensitive to mtDNA depleting factors. In this scenario, oocytes would be especially prone to decrease the mtDNA levels. On the other hand, HIV may induce unspecific mitochondrial damage in other tissues. HIV may cause disruption of the mitochondria-mediated apoptotic mechanisms and thus, may indirectly deplete oocyte mtDNA levels [28]. We did not find an association between mtDNA content and the length of antiretroviral therapy and immunovirological status of patients and, therefore, we cannot further demonstrate the contribution of each mechanism. However, we believe that HIV should not have a direct impact on the human oocyte because no receptors for HIV have been described on either the cumulus cells or on the surface of the oocyte.

According to the above mentioned, the most plausible hypothesis is that the underlying mechanism that cause oocyte mtDNA depletion and reduced fertility among HIV-infected women on HAART is the antiretroviral therapy. However, we can not give a firm conclusion, since all HIV-infected women included in our study were on HAART. In this context, it is

currently not possible to discriminate whether these results are attributable to HIV-infection or to HAART. No data are available on mtDNA content of oocytes from HIV-infected non-HAART-treated women.

Interestingly, among HIV-infected women, mtDNA content in the non-viable oocytes from women that did not become pregnant was lower than among women who achieved a pregnancy, although differences were not significant ( $83\pm 48$  vs  $119\pm 57$ , respectively;  $p=0.237$ ). However, this mtDNA depletion was significant when comparing HIV-infected women that did not become pregnant with uninfected controls (39% depletion;  $p=0.03$ ). Lack of statistical significance for the first comparison could be attributed to the small size of patients in the subgroups analysed. These results could explain the low pregnancy rate after IVF observed among HIV-infected women.

Human oocytes can only be assessed during an IVF cycle. Given that mature oocytes will be used for fertility purposes, the assessment of non-viable oocytes and non-fertilized oocytes from the same cycle from both infected and uninfected women is the best model available.

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According to our results, the lower fertility rate observed in IVF cycles among HIV-infected women under antiretroviral therapy may be explained by oocyte mitochondrial impairment secondary to mitochondrial toxicity of HAART. However, we can not rule out the potential effects that HIV could indirectly have on fertilization outcome and on mtDNA content through other unknown biological causes. Further studies are required to confirm these results.

## ACKNOWLEDGEMENTS

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## Original article

# Partial immunological and mitochondrial recovery after reducing didanosine doses in patients on didanosine and tenofovir-based regimens

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**Background:** Tenofovir disoproxil fumarate (TDF) has a safe toxicity profile; however, administration together with didanosine (ddl) increases ddl levels causing mitochondrial damage and CD4<sup>+</sup> T-cell decline. We assessed whether a simple reduction of the ddl dose in patients receiving ddl (400 mg/day) and TDF could revert this side effect.

**Methods:** Immunological and mitochondrial changes were analysed in 20 patients at baseline, after 14 months of receiving ddl (400 mg/day), TDF (300 mg/day) and nevirapine (NVP; 400 mg/day) and 14 months after a ddl dose reduction to 250 mg/day. Immunological analyses measured CD4<sup>+</sup> and CD8<sup>+</sup> T-cell counts and mitochondrial studies in peripheral blood mononuclear cells assessed mitochondrial DNA content by quantitative real-time PCR, cytochrome *c* oxidase (COX) activity by spectrophotometry and mitochondrial protein synthesis (COX-II versus  $\beta$ -actin or COX-IV expression) by western blot.

**Results:** Treatment with TDF, ddl (400 mg/day) and NVP for 14 months produced significant decreases in mitochondrial parameters and CD4<sup>+</sup> T-cell counts. The reduction in ddl dose resulted in mitochondrial DNA recovery; however, the remaining mitochondrial parameters remained significantly decreased. Levels of CD4<sup>+</sup> T-cells were partially restored in 35% of patients. Subjects presenting a significant reduction in CD4<sup>+</sup> T-cells during the high ddl dose period showed greater mitochondrial impairment in this stage and better mitochondrial and immunological recovery after drug reduction.

**Conclusions:** Administration of high ddl doses together with TDF was associated with mitochondrial damage, which may explain the observed CD4<sup>+</sup> T-cell decay. A reduction of the ddl dose led to mitochondrial DNA recovery, but was not sufficient to recover baseline CD4<sup>+</sup> T-cell counts. Other mitochondrial toxicity in addition to DNA  $\gamma$ -polymerase inhibition could be responsible for CD4<sup>+</sup> T-cell toxicity.

## Introduction

Mitochondrial dysfunction has been proposed as the etiopathogenic mechanism of many adverse events induced by nucleoside reverse transcriptase inhibitors (NRTIs) [1,2]. Inhibition by nucleoside analogues of DNA polymerase  $\gamma$  (DNA pol- $\gamma$ ), the only enzyme responsible for mitochondrial DNA (mtDNA) replication, leads to a reduction in mtDNA production (mtDNA copy number) and quality by increasing the number of point mutations and deletions [3–5]. The mtDNA encodes for components of the mitochondrial

oxidative phosphorylation (OXPHOS) system, such as subunits I, II and III of the cytochrome *c* oxidase complex (COX-I, COX-II and COX-III) and, consequently, nucleoside analogues can induce the dysfunction of OXPHOS complexes like COX. Nonetheless, alternative mechanisms of mitochondrial damage other than DNA pol- $\gamma$  inhibition have also been described for NRTIs, such as mitochondrial transcription impairment [6,7], inhibition of the transport of energetic substrates (succinate or



ADP/ATP), depletion of mitochondrial carnitine content or direct inhibition of COX activity [8–10].

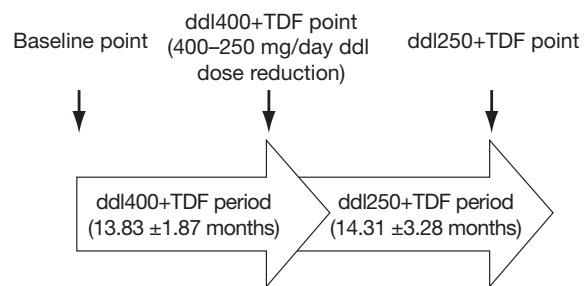
Pancreatitis, lipoatrophy, peripheral neuropathy, lactic acidemia, steatosis and myopathy are some of the mitochondrial-related toxicities observed. These secondary effects have prompted the modification of highly active antiretroviral therapy (HAART) schedules, including the substitution of highly mitochondrial toxic drugs with other antiretroviral combinations comprising less toxic nucleoside analogues.

One of these 'new' HAART schedules comprises the combination of didanosine (ddI) and tenofovir disoproxil fumarate (TDF) – the only nucleotide analogue marketed and which has been reported to be free of adverse effects on mitochondria [11–13]. However, our group has described a surprising and marked mitochondrial toxicity [14] and a risk of dramatic CD4<sup>+</sup> T-cell decline [15] related to the combination of these two adenosine analogues with ddI doses of 400 mg/day. These findings are supported by the results of other investigators [16]. The reduction in ddI degradation, which occurs when purine nucleoside phosphorylase (PNP) is inhibited by phosphorylated metabolites of tenofovir [17], could explain the marked elevation of plasma and intracellular levels of ddI during co-administration with TDF [18–20]. The maintenance of increased ddI levels achieved in regimens that include high doses of ddI together with TDF could favour ddI-driven mitochondrial damage, despite the favourable mitochondrial toxicity profile of TDF [14].

Pancreatitis can occur in many well-known classical primary mitochondrial diseases and increased pancreatic toxic effects have been described with the combined use of ddI and TDF [21,22]. Moreover, as mentioned earlier, our group and others have also reported a risk of CD4<sup>+</sup> T-cell decline with the use of this antiretroviral combination [15,16], but to our knowledge no study has established a possible mitochondrial basis for this deleterious effect.

Pharmacokinetic studies support the need to reduce ddI doses with the administration of this combination. It is currently recommended to prescribe 250 mg of ddI for subjects weighing >60 kg who also receive TDF. In patients receiving this dosage, plasma and intracellular concentrations of ddI diminish to levels similar to those achieved when ddI is administered without TDF [15,23,24]. However, changes in mitochondrial function after ddI dose reduction on administration together with TDF have not been described. The objective of the present study was to investigate the correlation between immunological and mitochondrial changes, measured in peripheral blood mononuclear cells (PBMCs), in patients receiving a ddI+TDF regimen, before and after reducing ddI from 400 mg to 250 mg.

Figure 1. Study design: time points and study periods



ddl, didanosine; TDF, tenofovir disoproxil fumarate.

## Methods

### Study design and participants

We selected all HIV-infected subjects from our clinical unit who had received ddI (400 mg/day), TDF (300 mg/day) and NVP (400 mg/day) for at least 1 year, and who remained virologically suppressed for at least 12 months after reducing the ddI dose to 250 mg/day. Patients requiring changes in some component of their antiretroviral regimen, other than a ddI dose reduction, were excluded from the study as were those presenting viral failure while using these regimens or receiving concomitant therapies with immune suppressors (hydroxyurea, interferon and ribavirin) or immune modulators (interleukin-2). Other exclusion criteria were changes or discontinuations of therapy, lack of adherence or a follow-up of less than 3 months after the switch. A total of 20 patients met the inclusion criteria.

Mitochondrial and immunological analysis were performed in all 20 patients at three time points: immediately before starting the ddI 400 mg/day plus TDF-based regimen (baseline point); after at least 1 year of this HAART regimen and immediately before the ddI reduction (ddl400+TDF point); and after at least 12 months of having reduced ddI to 250 mg/day (ddl250+TDF point) (Figure 1). In between these time points we analysed changes during two periods of time: the minimum 12-month period while receiving ddI at 400 mg/day plus TDF and NVP between the baseline point and the ddl400+TDF point (ddl400+TDF period); and during the minimum 1-year period of ddI at 250 mg/day plus TDF and NVP between the ddl400+TDF point and ddl250+TDF point (ddl250+TDF period) (Figure 1).

Patients were divided into two groups depending on CD4<sup>+</sup> T-cell evolution during the ddl400+TDF period: those presenting a significant decay >50 cells/mm<sup>3</sup> (CD4<sup>+</sup> T-cell declined group) and those maintaining or even increasing the T-cell count (CD4<sup>+</sup> T-cell preserved group).

## Data collection

Epidemiological data and antiretroviral history were recorded retrospectively.

### *Immunological analyses*

To assess changes in immunological parameters the absolute and perceptual number of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells were collected at baseline and at the ddI400+TDF and ddI250+TDF time points.

### *Mitochondrial parameters*

At the same three study points of the follow up (see Figure 1), 20 ml of venous peripheral blood were obtained for the isolation of PBMCs with Ficoll density gradient centrifugation. Different mitochondrial parameters were measured in these cells.

The mtDNA content was measured by quantitative real-time PCR. A fragment of the mitochondrial-encoded ND2 gene was amplified separately and levels monitored with respect to a fragment of the nuclear-encoded housekeeping 18S rRNA gene: the result was expressed as the ND2/18S rRNA ratio [25].

Cytochrome *c* oxidase (COX) or mitochondrial respiratory chain (MRC) complex IV (EC 1.9.3.1) enzymatic activity were measured by spectrophotometry and expressed as nmols/min/mg protein [26,27].

Mitochondrial protein synthesis was measured by western blot quantification of COX-II subunit abundance (mitochondrially encoded, transcribed and translated) with respect to the amount of two nuclear-encoded proteins, either mitochondrially (COX-IV subunit) or cytoplasmatically located ( $\beta$ -actin) [28] and expressed as COX-II/COX-IV or COX-II/ $\beta$ -actin ratios.

## Study endpoints

### *Immunological endpoints*

Changes in the immunological parameters were analysed during three study time points – the ddI400+TDF period, the ddI250+TDF period, and between baseline and the ddI250+TDF point – to determine changes before and after exposure to the whole combination.

### *Mitochondrial endpoints*

Changes in mitochondrial parameters were investigated during the ddI400+TDF and ddI250+TDF periods. Variations during the ddI400+TDF period have already been partially published by our group [14]. The current manuscript includes complementary information regarding this period, including the analysis of mitochondrial protein synthesis (COX-II expression study) and mitochondrial parameter changes depending on CD4<sup>+</sup> T-cell evolution, in order to correlate mitochondrial dysfunction with immunological deterioration. All these studies were also performed during the ddI250+TDF period and have not been previously published.

## Statistical analysis

Qualitative variables are expressed as a percentage of change or as a percentage of the remaining content with respect to the baseline measurement, which was considered 100%. Quantitative variables are expressed as mean ( $\pm$ SD). For these quantitative variables, differences for each immunological or mitochondrial parameter between time-points for all studied patients (Figures 2 and 3) or for all patients in the same group (Figures 4 and 5) were assessed using the paired-samples t-test for comparison of the mean of repeated measures. By contrast, differences between groups on the same time-point (Figures 4 and 5) were assessed using the independent-samples t-test for the comparison of the mean for independent measures. Associations between quantitative variables were assessed by means of linear regression (Figures 4 and 5). In all cases, *P*-values are quoted in the text and the respective figures and were considered statistically significant when *P*-values were <0.05.

## Results

A total of 20 HIV-infected men with a mean age of  $37 \pm 6$  years were included in the study. At inclusion, all patients had an undetectable viral load and a mean CD4<sup>+</sup> T-cell count of  $733 \pm 243$  cells/mm<sup>3</sup>. All were antiretroviral-experienced individuals with a mean time of exposure of  $68 \pm 43$  months to NRTIs,  $42 \pm 18$  months specifically to ddI and  $9 \pm 13$  months to a combination including stavudine (d4T)+ddI. The epidemiological characteristics and antiretroviral history of the patients are described in Table 1.

### The ddI400+TDF period

#### *Immunological outcome*

Considering all patients included, the mean absolute CD4<sup>+</sup> T-cell count decreased during the ddI400+TDF period from  $733 \pm 243$  to  $626 \pm 213$  cells/mm<sup>3</sup> (*P*=0.05; Figure 2) and the mean percentage of change in CD4<sup>+</sup> T-cell count was a significant -15%.

The CD4<sup>+</sup> T-cell declined group was composed of half of the subjects (10/20) who showed a significant -36% decline in CD4<sup>+</sup> T-cell count (*P*=0.001) compared with the CD4<sup>+</sup> T-cell preserved group (10/20) who presented a mean increase of 15% in CD4<sup>+</sup> T-cell count. The statistical analysis was considered not significant [NS] when *P*-values were >0.05.

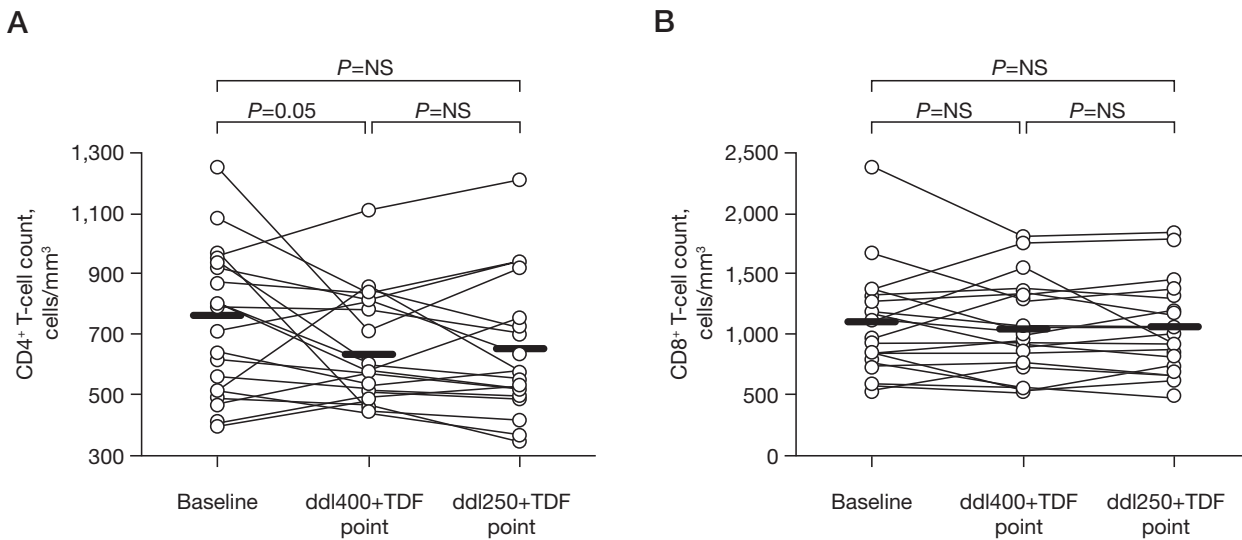
CD8<sup>+</sup> T-cell count did not vary significantly during this period (from  $1,084 \pm 434$  to  $1,046 \pm 395$  at the ddI400+TDF point; *P*=NS).

#### *Mitochondrial outcome*

A general decline in all mitochondrial parameters was observed during this period (Figure 3). With respect to baseline (considered as 100%), treatment with



Figure 2. Immunological parameters measured throughout the study for all patients



(A) CD4<sup>+</sup> T-cell count and (B) CD8<sup>+</sup> T-cell count. ddl, didanosine; NS, not significant ( $P>0.05$ ); TDF, tenofovir disoproxil fumarate.

TDF+ddI400+NVP was associated with a significant decrease in mtDNA (63% content;  $P=0.001$ ), COX activity (53%;  $P=0.01$ ) and mitochondrial protein synthesis (measured by the COX-II/ $\beta$ -actin or COX-II/COX-IV ratio, 32% and 69% expression, respectively;  $P=0.006$  in both cases).

Mitochondrial parameter status differed depending on CD4<sup>+</sup> T-cell evolution throughout the ddi400+TDF period (Figure 4). Mitochondrial impairment was higher in the CD4<sup>+</sup> T-cell declined group who showed a significant reduction in mtDNA content, COX activity, COX-II/ $\beta$ -actin and COX-II/COX-IV ratio (51% [ $P=0.007$ ], 37% [ $P=0.020$ ], 19% [ $P=0.013$ ] and 57% [ $P=0.041$ ] content, respectively). In the CD4<sup>+</sup> T-cell preserved group only the decrease in the COX-II/COX-IV ratio achieved statistical significance (76% expression;  $P=0.016$ ).

Nonetheless, when we compared mitochondrial parameters between the two groups of patients at the ddi400+TDF point, only COX activity was found to be significantly decreased in the CD4<sup>+</sup> T-cell declined group (37% versus 76% COX activity;  $P=0.039$ ).

When we analysed mitochondrial changes with respect to CD4<sup>+</sup> T-cell count change, COX activity again showed a direct (positive) and significant association with CD4<sup>+</sup> T-cell evolution (Pearson's correlation significance of  $P=0.004$ ).

#### The ddi250+TDF period

##### Immunological outcome

Considering all the subjects included, the mean CD4<sup>+</sup> T-cell count at the ddi250+TDF point did not vary

from the ddi400+TDF point ( $626 \pm 213$  cells/mm<sup>3</sup> to  $638 \pm 223$  cells/mm<sup>3</sup>;  $P=0.696$ ). Only 35% of the total number of patients included (7/20) recovered  $>50$  CD4<sup>+</sup> T-cells/mm<sup>3</sup> during the ddi250+TDF period and only the same percentage of individuals (35% or 7/20) achieved CD4<sup>+</sup> T-cell counts similar to those at baseline (established as a difference  $<50$  cells/mm<sup>3</sup> compared with original counts).

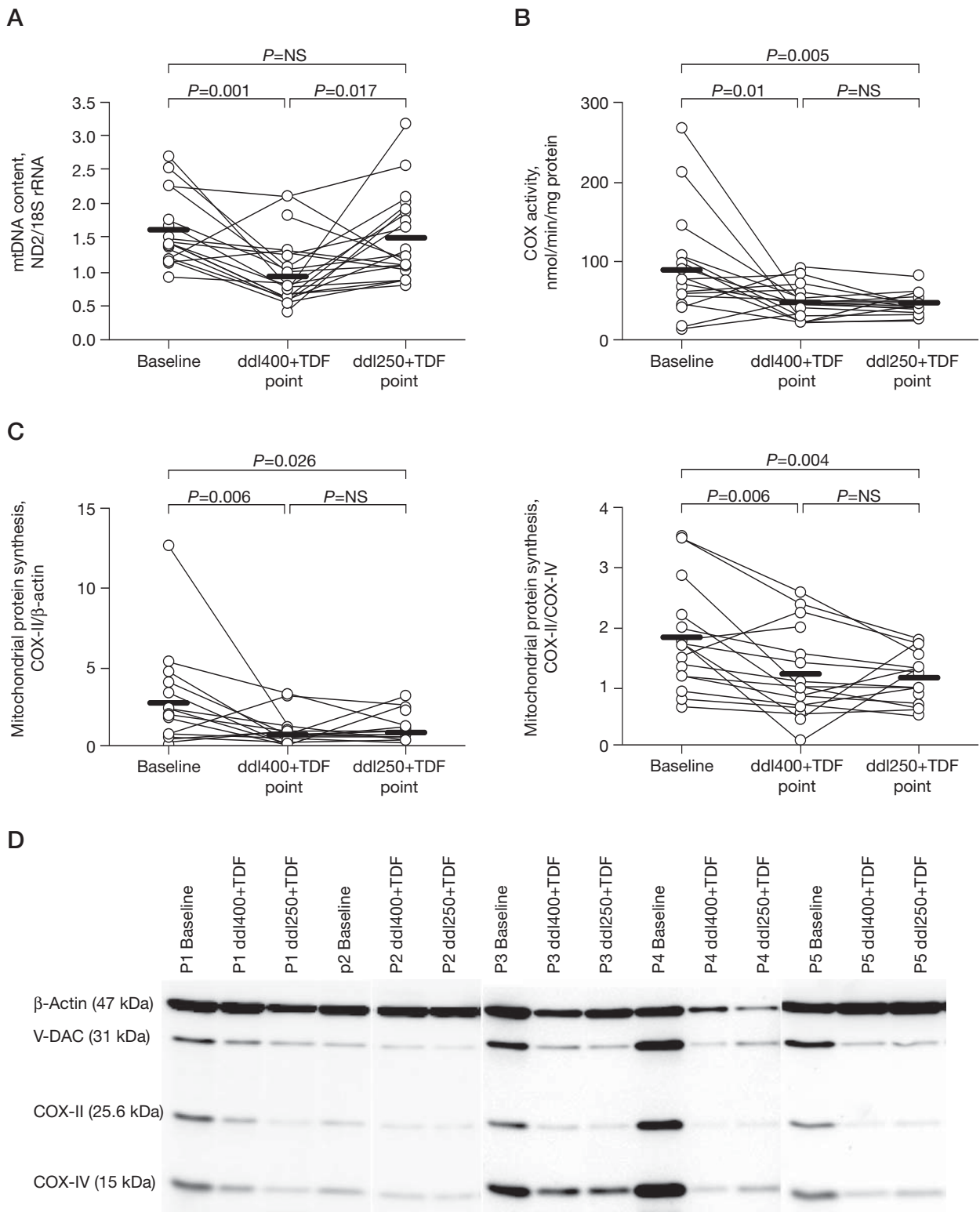
When we considered the CD4<sup>+</sup> T-cell declined group, the mean CD4<sup>+</sup> T-cell value at the ddi250+TDF point increased almost to statistical significance (from  $558 \pm 199$  cells/mm<sup>3</sup> to  $619 \pm 197$ ;  $P=0.1$ ) compared with the CD4<sup>+</sup> T-cell preserved group of patients ( $693 \pm 215$  versus  $657 \pm 255$ ;  $P=0.4$ ). Among the CD4<sup>+</sup> T-cell declined group, 50% of patients recovered  $>50$  CD4<sup>+</sup> T-cells/mm<sup>3</sup> during the ddi250+TDF period (5/10) and 20% achieved baseline CD4<sup>+</sup> T-cell counts (1/5).

The mean CD8<sup>+</sup> T-cell count at the ddi250+TDF point was  $1,030 \pm 387$  cells/mm<sup>3</sup>, with no differences with respect to the two previous points ( $P=0.7$  and  $P=0.315$ , respectively).

##### Mitochondrial outcome

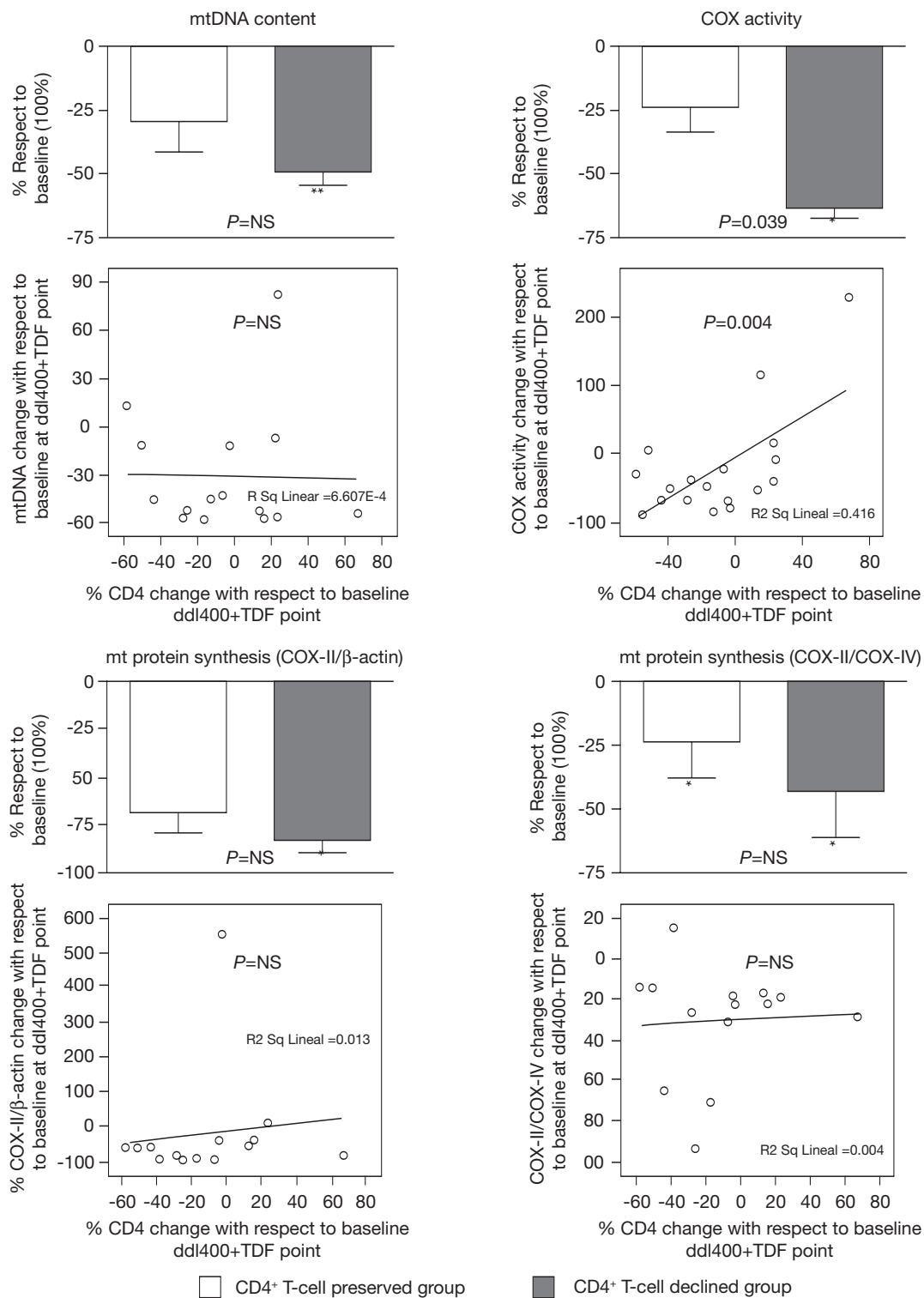
At the ddi250+TDF point, the mtDNA rose to 93% of its original value ( $P=0.017$  compared with the ddi400+TDF point and  $P=NS$  with respect to baseline). Conversely, COX activity and mitochondrial protein synthesis maintained similar values to those observed at the ddi400+TDF point (-4% for COX activity, +4% for COX-II/ $\beta$ -actin content and -3% for COX-II/COX-IV quantity;  $P=NS$  in all cases). Consequently, except for mtDNA content, mitochondrial parameters

Figure 3. Changes in mitochondrial parameters for all the patients included in the study



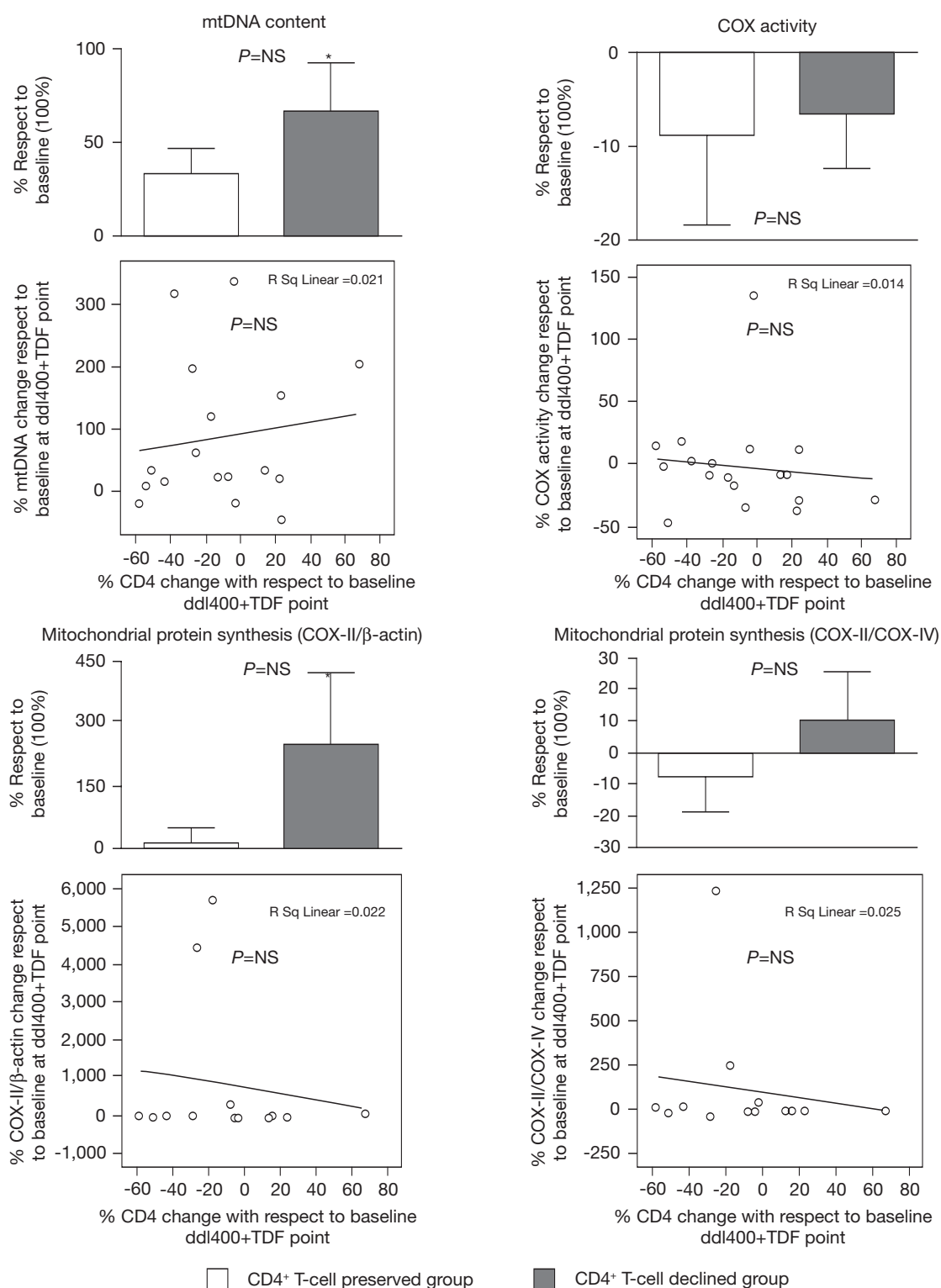
(A) Mitochondrial DNA (mtDNA) content, (B) cytochrome c oxidase (COX) activity, and (C) mitochondrial protein synthesis (mitochondrial-encoded COX-II subunit expression compared with nuclear-encoded and mitochondrially located COX-IV or nuclear-encoded and cytoplasmically located  $\beta$ -actin content) for all patients included in the study. (D) Western blot depicting expression of four proteins ( $\beta$ -actin, V-DAC, COX-II and COX-IV) at the three different time points (baseline, ddl400+TDF and ddl250+TDF). P1, P2, P3 refer to patients 1, 2, 3 and so on. ddl, didanosine; NS, not significant; TDF, tenofovir disoproxil fumarate; V-DAC, voltage-dependent anion channel.

**Figure 4.** Mitochondrial parameters measured during the ddl400+TDF period for the CD4<sup>+</sup> T-cell declined and CD4<sup>+</sup> T-cell preserved groups of patients



Percentage of mitochondrial change with respect to baseline (100%) depending on development or not of CD4<sup>+</sup> T-cell decay (upper panels) and relationship between changes in CD4<sup>+</sup> T-cell count and changes in mitochondrial parameters (lower panels). The differences between the ddl400+TDF point and baseline measurement for each group are indicated below each bar (\* $P < 0.05$ ; \*\* $P < 0.001$ ) and differences between the two groups of patients at the ddl400+TDF point are shown at the bottom of each upper panel. In lower panels data fitting to the linear regression model are shown by R2 Sq lineal coefficients and association between parameters by the statistical significance  $P$ -value. COX, cytochrome c oxidase; ddl, didanosine; mtDNA, mitochondrial DNA; NS, not significant ( $P > 0.05$ ); TDF, tenofovir disoproxil fumarate.

Figure 5. Mitochondrial parameters during the ddl250+TDF period for the CD4<sup>+</sup> T-cell declined and CD4<sup>+</sup> T-cell preserved groups of patients



Percentage mitochondrial change with respect to baseline (100%) depending on development or not of CD4<sup>+</sup> T-cell decay (upper panels). Relationship between changes in CD4<sup>+</sup> T-cell count and changes in mitochondrial parameters (lower panels). The differences between the ddl400+TDF point and ddl250+TDF point for each group are indicated at the bottom of each bar (\* $P<0.05$ ) and differences between the two groups of patients at the ddl250+TDF point is shown at the bottom of each upper panel. In lower panels data fitting to the linear regression model are shown by R2 Sq linear coefficients and association between parameters by the statistical significance  $P$ -value. COX, cytochrome c oxidase; ddl, didanosine; mtDNA, mitochondrial DNA; NS, not significant ( $P>0.05$ ); TDF, tenofovir disoproxil fumarate.

remained significantly or almost significantly decreased at the ddI250+TDF point with respect to baseline measurement (49% COX activity,  $P=0.005$ ; 37% COX-II/ $\beta$ -actin content,  $P=0.026$ ; and 66% COX-II/COX-IV quantity,  $P=0.004$ ; Figure 3).

Again mitochondrial evolution was different in patients belonging to the CD4<sup>+</sup> T-cell declined group or CD4<sup>+</sup> T-cell preserved group (Figure 5). Mitochondrial parameters showed a stronger trend towards amelioration for the CD4<sup>+</sup> T-cell declined group of patients, although statistical significance was only achieved by mtDNA content.

## Discussion

A reduction in ddI dose from 400 mg to 250 mg per day has been recommended when using TDF in the same HAART schedule [19,23,24] as the use of both analogues at high ddI dosage has been shown to be associated with serious toxic effects [15,16,21], such as a decline in CD4<sup>+</sup> T-cells [15,16]. Many authors have demonstrated that after this intervention both increased plasma and intracellular levels of ddI become similar to those observed when ddI is used without TDF [18,23,24]. At the same time, a decreased rate of related toxicities has also been reported when recommended doses of ddI are used together with TDF [29,30]. However, it has not been assessed whether the simple reduction of ddI doses is enough to recover CD4<sup>+</sup> T-cells and mitochondrial parameters in patients who have shown a significant decline when using a previous HAART combination including ddI at 400 mg/day and TDF.

Our current findings confirm the previously reported immunological and mitochondrial toxicity of ddI at 400 mg/day plus TDF-based regimens [14–16], not only on genetic or enzymatic mitochondrial measurements but also on mitochondrial protein synthesis. Of patients on a mean 14-month course of this antiretroviral combination, 50% showed significant CD4<sup>+</sup> T-cell decay while conserving CD8<sup>+</sup> T-cell values, excluding virological causes of immunological failure. All the mitochondrial parameters evaluated showed an important decrease during this antiretroviral treatment period, especially in patients showing a significant CD4<sup>+</sup> T-cell decline. Mitochondrial and CD4<sup>+</sup> T-cell evolution were consequently matched during this period. Patients presenting a greater CD4<sup>+</sup> T-cell depletion also showed increased mitochondrial lesion and mitochondrial function was better preserved in patients who conserved CD4<sup>+</sup> T-cell counts, suggesting that the underlying mechanism of CD4<sup>+</sup> T-cell damage may be mitochondrial toxicity of nucleoside analogues.

Our results also demonstrate a certain improvement in CD4<sup>+</sup> T-cell count after 14 months of ddI dose

**Table 1.** Antiretroviral history and baseline epidemiological, virological and immunological data of patients included in the study

Characteristic	Value
Age, years ( $\pm$ SD)	37 ( $\pm$ 6)
Male gender, n %	20 (100)
CD4 <sup>+</sup> T-cell count, cells/mm <sup>3</sup> ( $\pm$ SD)	733 ( $\pm$ 243)
HIV RNA <50 copies/ml, n (%)	20 (100)
Patients on ddI+d4T at baseline, n (%)	7 (35)
Patients on ddI+other NRTIs at baseline, n (%)	15 (75)
Time on treatment at baseline	
Receiving NRTIs, months ( $\pm$ SD)	68 ( $\pm$ 43)
Receiving ddI, months ( $\pm$ SD)	42 ( $\pm$ 18)
Receiving ddI+d4T, months ( $\pm$ SD)	9 ( $\pm$ 13)

ddI, didanosine; NRTI, nucleoside reverse transcriptase inhibitor, d4T, stavudine.

reduction from 400 to 250 mg/day in patients presenting a relevant CD4<sup>+</sup> T-cell decline while receiving ddI at 400 mg/day plus TDF and NVP, but they did not achieve total recovery. Among the patients included, only 50% of those showing a previous significant reduction in CD4<sup>+</sup> T-cell count during the period on high ddI dose recovered >50 cells/mm<sup>3</sup> after at least 1 year of ddI dose reduction, and only 20% achieved baseline CD4<sup>+</sup> T-cell values. These immunological results are further supported by mitochondrial data. Although a total recovery of mtDNA was seen after ddI dose modification, the remaining mitochondrial parameters did not improve, suggesting that mechanisms other than DNA pol- $\gamma$  inhibition (classically believed as being responsible for NRTI-mediated mitochondrial toxicity) could be responsible for maintaining mitochondrial damage and T-cell CD4<sup>+</sup> cytotoxicity.

The existence of alternative antiretroviral-mediated mechanisms to regulate or alter mitochondrial gene expression, independently of the quantity of mtDNA, has been previously reported, both *in vivo* [6,28] and *in vitro* [31] and at the transcriptional and translational level. In the present study, mitochondrial protein expression correlated with mtDNA content decrease during the ddI400+TDF period. This data could indicate the absence of upregulatory mechanisms to compensate for mtDNA depletion [28] and suggests the presence of extensive mitochondrial damage throughout the different stages of the mitochondrial protein homeostasis system, which finally affects mitochondrial function at a biochemical level.

The etiopathogenic mechanism of cytotoxicity and CD4<sup>+</sup> T-cell decline in HIV-infected patients receiving full doses of ddI plus TDF is currently better understood. It is most likely that mitochondrial dysfunction induced by some antiretroviral drugs, other than the mitochondrial-driven apoptosis

caused by HIV itself, plays an important role [32,33]. Antiretroviral-mediated mitochondrial toxicity could affect different types of body cells (if not all), but HIV tropism preferentially chooses immunological cells, including CD4<sup>+</sup> T-cells. We demonstrate a direct positive correlation between mitochondrial damage and CD4<sup>+</sup> T-cell toxicity in these patients: those presenting a negative effect on CD4<sup>+</sup> T-cell counts during the nucleoside combination showed a significantly higher decrease in all the PBMC mitochondrial parameters measured than those who did not. Therefore, the cytotoxic effect on CD4<sup>+</sup> T-cells could be directly related to the mitochondrial toxicity of the antiretrovirals, although HIV-induced damage could preferentially and specifically locate this deleterious effect in CD4<sup>+</sup> T-cells. A similar combined mechanism has been proposed in adipocytes as the etiopathogenic basis of lipodystrophy [34]. The shorter CD4<sup>+</sup> T-cell life and increased cell replacement rate with respect to CD8<sup>+</sup> T-cells could also make CD4<sup>+</sup> T-cells more vulnerable to toxicity. Different mitochondrial effects have been previously reported among CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations [35]. This finding should motivate clinicians to replace those antiretrovirals with a high mitochondrial toxicity profile with less toxic drugs, to avoid or delay long-term toxicities, which may otherwise be difficult to revert.

An interesting finding was that immunological or mitochondrial changes did not appear immediately after drug intervention. Likewise, the decline in CD4<sup>+</sup> T-cells and mitochondrial parameters was not detected until at least 24 weeks on ddI400+TDF [15]. Indeed, many subjects continued presenting a CD4<sup>+</sup> T-cell decline during the first weeks after ddI dose reduction. These findings support the theory that this phenomenon might be attributed to an accumulative toxicity [15]. The long-term intracellular half-life of tenofovir diphosphate, which can be greater than 4 weeks [18], and of ddI [36] may explain the slow CD4<sup>+</sup> T-cell recovery over time after reduction of the ddI dose or ddI substitution [37]. Indeed, most mitochondrial parameters require a lengthy time to change status after toxic disruption [35,38,39], and if mitochondrial toxicity was the basis of CD4<sup>+</sup> T-cell decline this would explain the slow change in immunological parameters. It is necessary to perform complete mitochondrial toxicity studies based on measuring different mitochondrial parameters to better understand mitochondrial toxicity mechanisms.

In conclusion, ddI dose reduction can help to improve CD4<sup>+</sup> T-cell count and mitochondrial function in NRTI-experienced HIV-infected patients receiving ddI and TDF-based regimens; however, this intervention does not allow a total recovery of immunological or mitochondrial parameters, as previously reported using other interventions to reduce nucleoside-related mitochondrial

damage [40]. Patients starting an antiretroviral treatment at recommended ddI doses together with TDF, who have never received high doses of ddI and TDF before, should also be studied. A long-term follow up is necessary to ascertain whether this recommended combination is totally free of adverse mitochondrial and immunological effects. Mitochondrial and CD4<sup>+</sup> T-cell toxicity of the ddI plus TDF combination has been demonstrated to be a long-term effect, and the delay in toxic manifestations could disguise the toxic effects of this schedule, even at low ddI doses.

Finally, new antiretroviral drugs or HAART combinations should not only take antiviral efficiency into consideration, but should also consider the lack of side effects for mitochondria and cell toxicity to avoid long-term treatment of chronic toxicities that become difficult to revert.

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## Disclosure statement

The authors declare no conflicts of interest.

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# Genetic and Functional Mitochondrial Assessment of HIV-Infected Patients Developing HAART-Related Hyperlactatemia

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**Background:** Mitochondrial damage of HIV and antiretrovirals, especially nucleoside-analogue interference on mitochondrial DNA (mtDNA) replication, is reported to underlay highly active

antiretroviral therapy (HAART)-related hyperlactatemia, but scarce approaches have been performed to correlate clinical manifestations and mitochondrial abnormalities.

**Methods:** We obtained lymphocytes and monocytes of 26 HIV-infected and treated patients who developed hyperlactatemia and after recovery, 28 nonhyperlactatemic HIV subjects on HAART, 31 naive individuals, and 20 uninfected controls. Mitochondrial replication and transcription analysis were performed by quantitative real-time PCR, mitochondrial translation quantification by western blot and mitochondrial enzymatic activities by spectrophotometry.

**Results:** Mitochondrial parameters decreased during hyperlactatemia and improved at recovery. Mitochondrial replication and transcription species were reduced ( $P = 0.16$  and  $P = 0.71$ ), but the most significant decay was observed on mitochondrial protein content ( $P < 0.05$ ) and mitochondrial complexes III and IV activities ( $P < 0.01$  and  $P < 0.001$ ). During hyperlactatemia lactate level correlated complexes III and IV function ( $P < 0.05$ ). After recovery mitochondrial parameters achieved values of nonhyperlactatemic HIV individuals, which were lower than ranges of naive subjects and uninfected controls.

**Conclusions:** HIV and HAART-related hyperlactatemia is associated with a general mitochondrial impairment which reverts after recovery. Mitochondrial biochemistry show a better correlation with lactate levels than mitochondrial genetics suggesting that mitochondrial function could be a better marker of hyperlactatemia development than mtDNA content.

**Key Words:** HIV, HAART, hyperlactatemia, mitochondria, mitochondrial toxicity, mitochondrial function/dysfunction

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## INTRODUCTION

Lai et al<sup>1</sup> reported in 1991 for the first time a case of severe lactic acidosis and fulminant hepatic failure in an HIV-infected patient on didanosine (ddI) antiretroviral monotherapy. Since then, the introduction of the highly active antiretroviral therapy (HAART) has increased the risk of suffering from secondary effects and several additional reports have confirmed that patients who receive some nucleoside analogues reverse transcriptase inhibitors (NRTIs),



particularly ddI and stavudine (d4T), are at increased risk for developing hyperlactatemia and lactic acidosis,<sup>2,3</sup> a condition thought to be amongst the most serious adverse effects attributed to HIV and antiretroviral drugs.

Increased blood lactate levels may be associated with a multitude of accompanying and unspecific symptoms including fatigue, weakness, abdominal pain, weight loss, tachycardia, or dyspnea. The clinical presentation of hyperlactatemia is strikingly variable and the severity of symptoms usually shows a good positive correlation with plasma lactate levels. Cohort studies report that 15%–20% of persons/yr who receive a stable HAART regimen develop asymptomatic hyperlactatemia<sup>3</sup>; these patients show blood lactate levels usually between 2 and 5 mmol/L. Otherwise, 1% of patients/yr develop symptomatic hyperlactatemia, which is usually associated with plasma lactate levels above 5 mmol/L and one or more of the above mentioned manifestations.<sup>3</sup> Finally, 0.4–1% of patients on HAART/yr present lactic acidosis (blood pH imbalance), which is often associated with higher lactate levels and severe symptomatology, which can lead to death in up to 50% of cases through fulminant hepatic failure.<sup>2,3</sup> The prevalence and incidence of hyperlactatemic-related disorders is increasing in developing countries (because of growing access to antiretrovirals) and shows a trend towards reduction in the developed world because of physicians awareness and available routine lactate measures, although unfortunately it is still a quite frequent and life-threatening event.

Mitochondrial toxicity of antiretroviral drugs, particularly NRTIs, has been postulated to be responsible for the etiopathogenesis of many secondary effects of HAART,<sup>4,5</sup> including hyperlactatemia. Moreover, HIV itself could extend the mitochondrial adverse effects of antiretrovirals by modulating inflammatory and/or apoptotic cellular mechanisms.<sup>6,7</sup> Mitochondria are the center of energy supply in nearly all body cells by coupling ATP synthesis to oxygen consumption through the oxidative phosphorylation (OXPHOS) system. Specifically, hyperlactatemia can be the result of hypoxic atmospheres or mitochondrial dysfunction which drives energy production out of the mitochondria through anaerobic metabolism and lactic acid generation, which acidifies blood through conversion into lactate and consequent proton release. Blood lactate concentration is the result of lactate production through anaerobic glycolysis (in all body cells but especially on skeletal muscle, liver, nervous and lymphoid system) and its plasmatic clearance by gluconeogenic pathways (mainly on the liver and secondary on the kidney). NRTIs inhibit the unique enzyme responsible for mitochondrial DNA (mtDNA) replication (DNA polymerase  $\gamma$ ),<sup>8–10</sup> increase the number of mtDNA mutations and reduce the number of entire mitochondrial genomes. Because mtDNA encodes for 13 proteins of the OXPHOS system responsible for aerobic energy production, important mtDNA depletions can lead to mitochondrial dysfunction moving energy production towards anaerobic metabolism and lactate production. But the complexity of HAART-induced mitochondrial toxicity is however increasing with the description of alternative mechanisms for mitochondrial lesion by NRTIs in absence of mtDNA depletion<sup>11–15</sup> and homeostatic mechanisms able to compensate severe mtDNA depletion and preserve mitochondrial function.<sup>16</sup>

The hypothetic connection between antiretroviral-mediated mitochondrial toxicity and hyperlactatemia was first reported on liver<sup>17–19</sup> and skeletal muscle<sup>17,19–23</sup> as mtDNA depletion and/or OXPHOS system dysfunction. These investigations included however a small number of patients, used invasive approaches and, in most of them, the exploration of mitochondria was partial and limited to mtDNA quantification. Additionally, a correlation between mitochondrial parameters and blood lactate levels was lacking. We herein present the replicational, transcriptional, translational, and biochemical mitochondrial analysis of 26 HIV-infected patients under HAART who developed hyperlactatemia with different degree of clinical severity and lactate levels, both during the hyperlactatemic episode and after clinical recovery, to better assess mitochondrial basis of HAART-related hyperlactatemia. We used a noninvasive method since we studied peripheral blood mononuclear cells (PBMCs). These results have been compared with the values found in nonhyperlactatemic HIV-infected patients on HAART (treated), infected but untreated HIV individuals (naive) and noninfected volunteers (healthy controls).

## PATIENTS AND METHODS

### Patients

We studied genetic and biochemical PBMC mitochondrial parameters of 26 consecutive HIV-infected patients on HAART undergoing an hyperlactatemic episode (lactate levels above 2 mmol/L) and after clinical recovery. Patients were recruited during 3 years on the Infectious Diseases department of 4 different Catalan hospitals (Hospital Clinic of Barcelona, Hospital Germans Trias i Pujol of Badalona and Hospital Joan XXIII and Hospital of Sant Pau i Santa Tecla from Tarragona) because of increased lactate levels, sometimes accompanied by clinical symptomatology, or because they presented severe accompanying symptomatology with moderate to high lactate values. Patients were categorized in 3 different clinical forms, according to clinical presentation: 13 were asymptomatic, 8 were symptomatic, and 5 had lactic acidosis. We considered as symptoms of hyperlactatemia: fatigue, weakness, abdominal pain, weight loss, tachycardia, and/or dyspnea, after other causes of disease were conveniently discarded. Lactate levels, immunovirologic parameters and one sample of peripheral blood for mitochondrial studies were obtained on admission and after the clinical recovery of hyperlactatemia. Clinical and antiretroviral histories were obtained from the patients' medical records to be correlated with mitochondrial toxicity results. An extensive work-up was performed to exclude other causes of hyperlactatemia. In most of cases hyperlactatemic episode prompted antiretroviral treatment withdrawal that was exclusively restarted, most of times after changing its composition, after clinical recovery and lactate normalization or because of severe immunovirologic reasons.

We compared the results of these hyperlactatemic patients with respect to 3 control groups of subjects that were consecutively collected during the same period of time in the same participant hospitals: 28 nonhyperlactatemic HIV subjects on HAART (treated), 31 HIV-infected but untreated individuals (naive), and 20 uninfected controls (healthy).

Clinical and epidemiological characteristics of included patients and controls are summarized on Table 1. Control group inclusion was made trying to match individual characteristics with those of the hyperlactatemic group of subjects. Treated nonhyperlactatemic patients were matched by sex, age, and time on HIV infection, and time on HAART and time on d-drug treatment (ddI and/or d4T administration) with the hyperlactatemic patients. Naive subjects were matched by sex with hyperlactatemic individuals, but presented statistical significant differences ( $P < 0.05$ ) with respect to age and time on HIV infection. Healthy volunteers presented differences in terms of age and sex with respect the rest of studied groups. All those differences found among our study groups represent, in most of cases, those found in the general population.

All individuals were informed and signed written consent to be included in this protocol that was approved by the Ethical Committee of the Hospital Clinic of Barcelona.

To avoid confounders of mitochondrial toxicity, those patients taking other potentially toxic drugs for mitochondria (ie, aminoglycosides, linezolid, or statins) were excluded from the study, and those subjects with familiar history of mitochondrial disease.

**Samples**

Mononuclear cells (lymphocytes and monocytes) were isolated by Ficoll density gradient centrifugation<sup>24</sup> and we confirmed a platelet count below 25 per PBMC in all patients coming from the different groups suggestive of negligible platelet contamination.

Protein content was measured according to the Bradford protein-dye binding-based method.<sup>25</sup> Samples were frozen at  $-80^{\circ}\text{C}$  until mitochondrial analysis.

**Mitochondrial Studies**

**mtDNA Quantification**

Total DNA was obtained by the standard phenol-chloroform extraction procedure. A fragment of the mitochondrial-encoded *ND2* gene and the nuclear-encoded *18S rRNA* gene were amplified in duplicate and separately by quantitative real-time PCR using Lightcycler Roche thermocycler (Roche Diagnostics, Mannheim, Germany), as

previously reported.<sup>26,27</sup> The relative content of mtDNA was expressed as the ratio between mtDNA and nDNA amount (*ND2* mtDNA/*18S rRNA* nDNA content).

**Mitochondrial RNA Quantification**

Total RNA was obtained by an affinity column-based procedure (Rneasy; Qiagen Sciences, Germantown, MD). RNA was reverse-transcribed to cDNA using random hexamer primers and the real-time PCR reaction used to quantify relative mitochondrial cDNA content was performed using Applied Biosystems technology in an ABI PRISM 7700 sequence detection system (Applied Biosystems Inc., Foster City, CA). Quantification of the mitochondrial encoded cytochrome *c* oxidase subunit-II (COX-II) mRNA and the nuclear-encoded housekeeping 18S rRNA were performed using the amplification conditions and the primers previously reported.<sup>27</sup> The relative content of mitochondrial RNA (mtRNA) was expressed as the ratio between mtRNA and nuclear RNA (nRNA) amount (COX-II mtRNA/18S rRNA nRNA content).

**Mitochondrial Protein Synthesis**

We assessed mitochondrial protein synthesis of the COX-II subunit (mitochondrially encoded, transcribed, and translated) by western blot immunoanalysis.<sup>16,27</sup> This expression was normalized by the content on the mitochondrially located COX-IV subunit (nuclear-encoded and cytoplasmically transcribed and translated) to establish the relative mitochondrial protein expression amount (mtCOX-II/nCOX-IV protein abundance).

**Mitochondrial OXPHOS Complexes II, III, and IV (COX) Enzyme Activity**

All mitochondrial enzymatic activities were measured spectrophotometrically according to the Rustin et al<sup>28</sup> methodology, slightly modified for complex IV measurement in minute amounts of biological samples.<sup>29</sup> OXPHOS complex II is completely encoded, transcribed and translated by cytoplasmic machinery, whereas CIII and CIV (COX) complexes are partially encoded, transcribed and translated by mitochondrial means. Specific enzymatic activities were expressed in absolute values as nanomols of synthesized substrate or

**TABLE 1.** Clinical and Epidemiological Characteristics of Included Patients and Controls

	Hyperlactatemic	Treated	Naive	Healthy
Number of subjects (n)	26	28	31	20
Sex (% men)	84.61	83.33	80.64	40 (*)
Age (yrs ± SEM)	46.50 ± 1.86	49.17 ± 2.42	36.33 ± 1.37 (*)	62.80 ± 4.32 (*)
Time infected (mo ± SEM)	118.89 ± 12.90	101.14 ± 14.13	55.26 ± 16.14 (*)	—
Time on HAART (mo ± SEM)	77.54 ± 7.37	62.17 ± 9.66	—	—
At inclusion				
Patients on ddI (%)	25	47	—	—
Patients on d4T (%)	20.8	45	—	—
Patients on ddI + d4T (%)	37.5	6	—	—
Patients without ddI or d4T (%)	16.7	2	—	—

Results are expressed in percentages or as mean value ± SEM. Hyperlactatemic: HIV-infected patients on HAART developing a hyperlactatemic episode; treated: nonhyperlactatemic HIV-infected subjects on HAART; naive: HIV-infected but untreated individuals; healthy: uninfected controls.

\*Significant differences ( $P < 0.05$ ) with respect hyperlactatemic-patient's values.

consumed product per minute and milligram of measured protein (nmol/min/mg protein).

**Statistical Analysis**

The main outcome was the assessment of genetic or biochemical PBMC's mitochondrial parameter change of HIV-infected patients on HAART undergoing a hyperlactatemic crisis (lactate levels over 2 mmol/L) and after clinical recovery and lactate normalization. As mitochondrial genetics parameters we considered mtDNA and mtRNA content and mitochondrial protein synthesis amount. As mitochondrial biochemical parameters we considered those enzymatic activities which take part of the mitochondrial respiratory chain (complexes II, III, and IV).

Additionally, mitochondrial results of hyperlactatemic patients during and after the episode were compared with respect to 3 control groups: nonhyperlactatemic HIV subjects on HAART (treated), HIV-infected untreated individuals (naive), and uninfected volunteers (healthy).

Results were expressed as mean ± standard error of the mean (SEM) or as percentage with respect to healthy controls, the latter were arbitrarily assigned as 100%. We ascertained the normal distribution of mitochondrial and clinical parameters using the Kolmogorov–Smirnov analysis. Parametric *T*-test for independent or paired normal-distributed measures (as needed) were used to search for differences and regression analysis was used to find relationship between quantitative parameters. Otherwise, for nonnormal-distributed parameters, the nonparametric test Mann–Whitney was used to search for independent sample differences, Wilcoxon paired rank test for paired comparisons and Spearman's rank coefficient to search for parameter correlation.

A *P* value of less than 0.05 was considered significant.

**RESULTS**

**Lactate Levels**

Patients suffering from hyperlactatemia on admission had a mean blood lactate value of 3.7 ± 0.6 mmol/L (normal range: 0.8–2 mmol/L). Awareness of early clinical suspicious of hyperlactatemia and routine lactate measurement screening on current HIV clinical management made the lactate level of our

patients to be lower with respect to that found in the past or in previous reports,<sup>1–3</sup> and most of our patients were, because of that, asymptomatic (13 of the 26 included). All hyperlactatemic patients were reassessed 9.7 ± 1.2 months later, when blood lactate levels were normalized (1.9 ± 0.1, *P* = 0.01 compared with the baseline values) and they achieved clinical recovery.

**Mitochondrial Analyses During Hyperlactatemia and After Clinic Recovery**

Hyperlactatemic patients presented all mitochondrial parameters decreased during the hyperlactatemic episode and with respect clinical recovery and lactate normalization (Figs. 1–4). The mitochondrial parameters which showed a most significant improvement after the hyperlactatemic episode resolution were the mitochondrial protein synthesis (*P* < 0.05) and the OXPHOS enzymatic activities of respiratory complexes III and IV (*P* < 0.01 and *P* < 0.001, respectively, Figs. 1, 3, and 4).

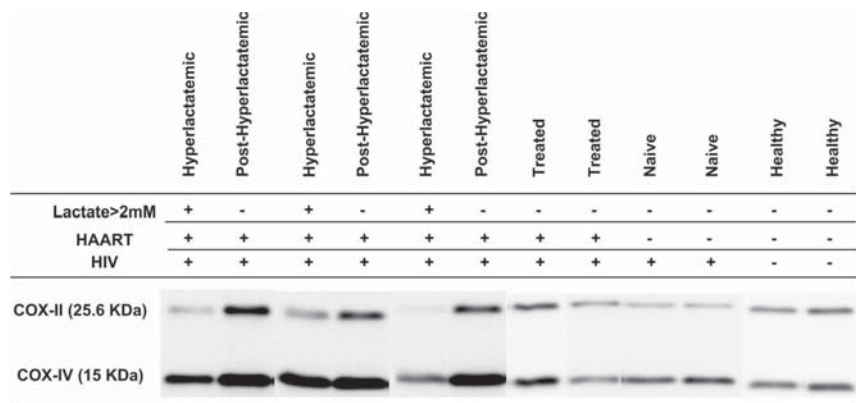
**Mitochondrial Analyses of Hyperlactatemic Patients Compared With Control Groups**

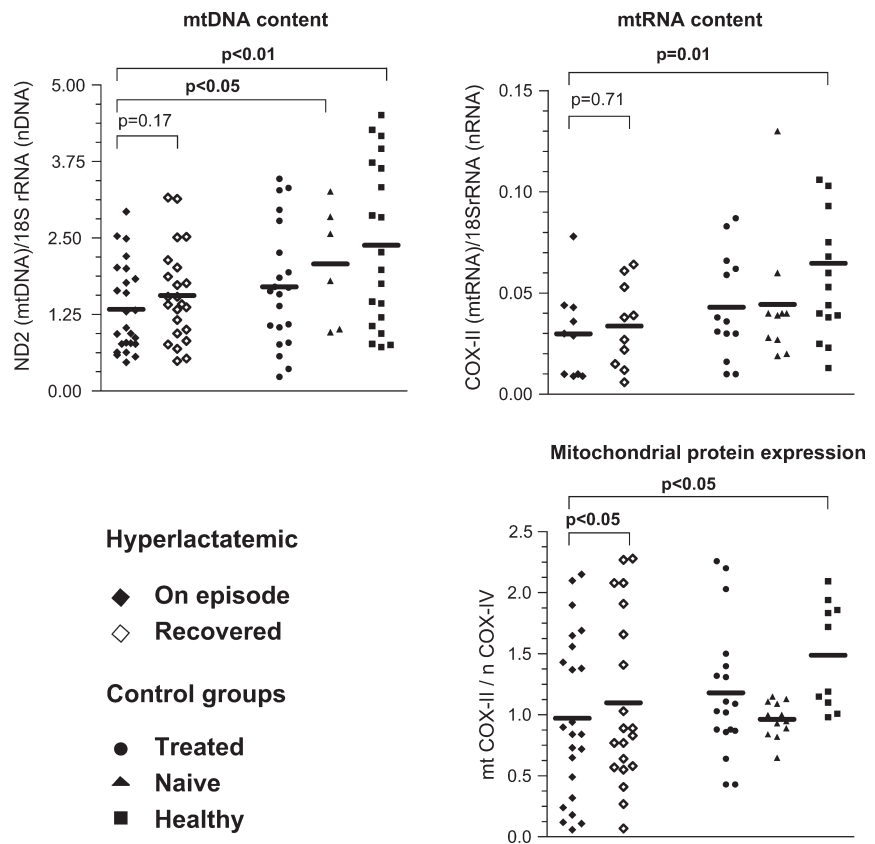
During hyperlactatemia, mtDNA was decreased with respect to the 3 control groups; the comparisons achieved statistical significance with respect to HIV-naive patients (*P* < 0.05) and healthy persons (*P* < 0.01; Fig. 2). This decrease was accompanied by a diminished amount on mitochondrial transcription (mtRNA) and translation species amount (mitochondrial proteins; as shown in Figs. 1 and 2), although the statistical significance was only achieved when hyperlactatemic values were compared with healthy people (*P* = 0.01 and *P* < 0.05, respectively).

The unique parameter which remained nearly equal in all groups was OXPHOS complex II enzymatic activity (Fig. 3). Complex II is not mtDNA encoded rather is entirely encoded by nuclear DNA and entirely transcribed and translated on cytoplasmic ribosomes, and consequently, is supposed to be conserved. Conversely, OXPHOS complexes III and IV function was significantly decreased compared with HIV naive (*P* < 0.05 and *P* < 0.01, respectively) and healthy controls (*P* < 0.01 and *P* < 0.05, respectively; Fig. 3).

If we consider the values found on healthy people as 100%, patients developing hyperlactatemia had a remaining content of 52% of mtDNA, 46% of mtRNA amount, 63% of

**FIGURE 1.** Mitochondrial protein expression measured by western blot immunoanalysis of relative mitochondrial-encoded COX-II to nuclear-encoded COX-IV amount on peripheral blood mononuclear cells of studied patients and controls. Hyperlactatemic or posthyperlactatemic: HIV-infected patients on HAART developing a hyperlactatemic episode or after recovery; treated: nonhyperlactatemic HIV-infected subjects on HAART; naive: HIV infected but untreated individuals; healthy: uninfected controls.





**FIGURE 2.** Mitochondrial genetic parameters during and after the hyperlactatemic episode with respect to 3 different control groups: HAART-treated HIV-infected patients with normal lactate (treated), HIV-infected and untreated patients (naive) and HIV-uninfected volunteers (healthy). Statistical differences between hyperlactatemics during and after the episode have been added (in bold when they are significant), and only those significant differences between hyperlactatemics and the rest of groups.

mitochondrial protein expression quantity and 49% and 69%, respectively, of OXPHOS complex III and IV enzymatic activities (Fig. 4).

### Correlation Between Mitochondrial and Clinic Parameters

The comparison for all these mitochondrial parameters according to the subtype of hyperlactatemia (asymptomatic, symptomatic, or lactic acidosis) did not render statistical differences (data not shown), maybe because of the reduced statistical power of so small groups. But when we assessed the relationship between mitochondrial disturbances and the severity of hyperlactatemia measured as blood lactate levels we found that such a relationship existed only for the enzymatic activity of OXPHOS complexes III and IV, which were negatively correlated with lactate concentration ( $P < 0.05$  in both cases; Fig. 5), whereas mitochondrial genetic parameters did not ( $P = 0.99$  for mtDNA,  $P = 0.41$  for mtRNA and  $P = 0.38$  for mitochondrial protein expression; data not shown).

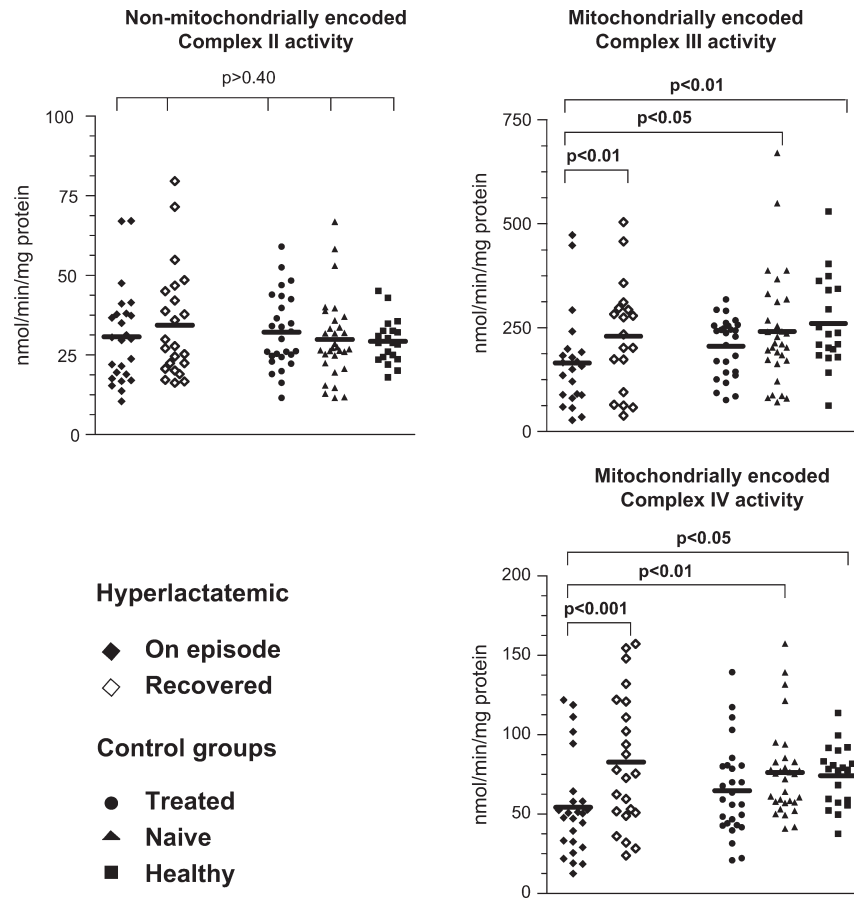
### DISCUSSION

We found that HAART-related hyperlactatemia is associated with a decrease in all mitochondrial parameters assessed with respect to control values of healthy people and,

in some cases (mtDNA and OXPHOS complexes III and IV), also with respect to naive patients. Nonetheless, although mitochondrial parameters were lower than in HIV-infected patients on HAART with normal lactate, none of these differences achieved statistical significance. Interestingly, although all mitochondrial parameters trend to increase after recovery of the hyperlactatemic episode, only mitochondrial translation and OXPHOS complexes III and IV enzymatic activities significantly increase. In addition, although PBMC have been demonstrated a reliable and noninvasive model to perform mitochondrial studies in hyperlactatemic patients, it is foreseeable we can not discard that mitochondrial deficits are bigger in more energy-dependent tissues or those target centers of lactate homeostasis (liver and muscle). Overall, we believe that our findings support the mitochondrial basis for HIV and HAART-related hyperlactatemia.

Mitochondrial toxicity of antiretroviral drugs has been associated mainly with NRTIs use due to its capacity to inhibit mtDNA replication.<sup>4,5</sup> Among dideoxynucleoside analogues, d4T seems to be the most powerful inducer of hyperlactatemia,<sup>19,22,23,30,31</sup> albeit toxic effects of other d-drugs has not been discarded. Most of our hyperlactatemic patients were taking d4T, but most remarkable is the great amount of these hyperlactatemic subjects that were receiving d4T in combination with ddI in comparison to those treated patients who did



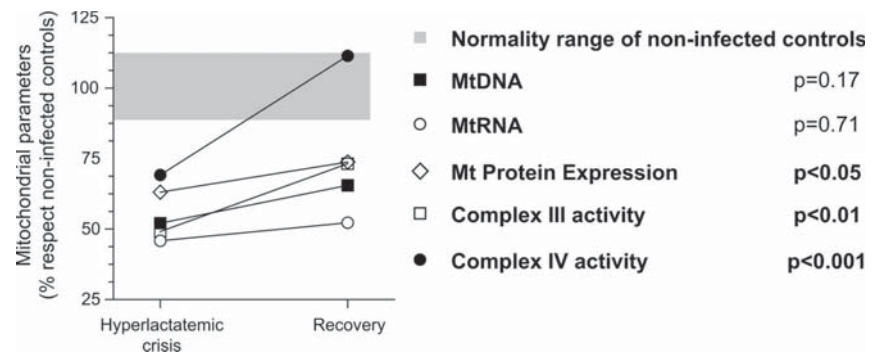


**FIGURE 3.** Mitochondrial biochemical parameters during and after the hyperlactatemic episode with respect 3 different control groups: HAART-treated HIV-infected patients with normal lactate (treated), HIV-infected and untreated patients (naive), and HIV-uninfected volunteers (healthy). Statistical differences between hyperlactatemics during and after the episode have been added (in bold when they are significant), and only those significant differences between hyperlactatemics and the rest of groups.

not developed the hyperlactatemic disorder. Current guidelines strongly discourage concomitant administration of d4T and ddI, but most of the studied hyperlactatemic patients were included in 2004, when such antiretroviral combination was quite common.

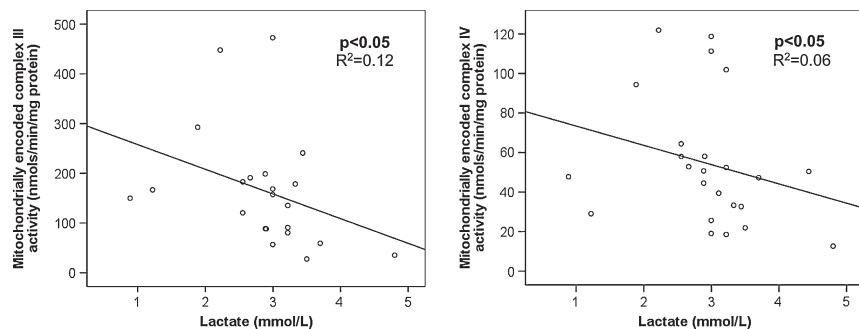
As mtDNA encodes for mitochondrial OXPHOS components, NRTIs-induced mtDNA depletion would lead to

mitochondrial function impairment. This hypothesis is supported by our data; the finding of mtDNA depletion during hyperlactatemia is associated with a downstream decay of mitochondrial transcription, translation and function. A striking feature of our study is that during the hyperlactatemic episode, mitochondrial biochemistry abnormalities better correlated with blood lactate levels than mitochondrial



**FIGURE 4.** Mitochondrial parameters during and after the hyperlactatemic episode expressed as percentages with respect noninfected controls (healthy, arbitrarily assigned 100%). mt protein expression, mitochondrial protein expression.

**FIGURE 5.** Relationship between mitochondrial-encoded biochemical parameters and lactate levels during the hyperlactatemic crisis.



genetics. In agreement with this finding, when blood lactate normalized and clinical recovery was achieved, mitochondrial-encoded OXPHOS complex III and IV enzymatic activities, together with the mitochondrial protein expression, significantly recovered, whereas mitochondrial replication and transcription species amount did not.

The mitochondrial hypothesis of HAART toxicity launched by Brinkman and colleagues in 1998<sup>8</sup> has gained complexity during the last recent years. First, reports show that, even in the absence of mtDNA depletion, NRTIs are able to cause mitochondrial lesion independent to DNA polymerase  $\gamma$  inhibition.<sup>11–15</sup> Second, severe mtDNA depletion induced by NRTIs has been reported to be compensated by mitochondrial transcriptional or translational upregulatory homeostatic mechanisms.<sup>16</sup> These mechanisms could maintain mitochondrial function on adverse circumstances. However, during hyperlactatemia, transcription and translation intermediates were decreased, suggesting lack of upregulatory response. Third, assessment of the mitochondrial function has become essential because it is the expected consequence of the genetic lesion and is, ultimately, the responsible of clinical symptoms. Using this overall approach, it has been shown that the change from a highly mitochondriotoxic HAART to other drug schedules with lower toxic potential for mitochondria is first accompanied with a recovery of mitochondrial functions, even if not net changes in mtDNA content are observed.<sup>32,33</sup> This finding suggests that mitochondrial functional recovery antedates the improvement of the genetic lesion or, possibly, that the improvement achieves only one part of the mechanisms disrupted. Both are possible explanations for recovery of mitochondrial function after hyperlactatemia in the absence of substantial mtDNA improvement. But finally we can not discard that mtDNA content of hyperlactatemic patients before the crisis could be so close to the threshold limit value which supports mitochondrial function that the small decay occurred during the hyperlactatemic episode, even not statistically significant, could cross this critical value leading to impaired mitochondrial function. Whatever the explanation is, mitochondrial dysfunction is the final determinant to drive energy production out of the mitochondria towards the cytoplasmatic anaerobic glycolytic pathway responsible of lactate production. All studied patients presented such an increase in blood lactate levels and decreased mitochondrial parameters, but each one of them

presented one or more of these parameters especially altered. Consequently with other toxic or genetic mitochondrial diseases that correlate with increased lactate levels but have different mitochondrial parameter impairment etiology, mitochondrial dysfunction of HIV and antiretroviral-induced hyperlactatemia could stand at different genetic, biochemical or synthetic mitochondrial levels, and increased lactate production would just be the common consequence of final mitochondrial impairment.

Although HAART-related hyperlactatemia can be developed in uninfected patients exclusively exposed to antiretroviral therapy,<sup>34</sup> in chronically treated patients' scenario we can not forget the HIV and mitochondria interactions. HIV is able to cause mitochondrial diffuse genetic<sup>5,7</sup> and functional<sup>7,35</sup> lesion by itself that could be mediated by indirect inflammatory or apoptotic mechanisms. The mitochondrial damage present in HIV-infected patients on HAART that underwent an hyperlactatemic crisis could be due to the summatory effect of both HIV-induced damage (also present in naive patients) and mitochondrial toxicity of antiretroviral drugs (also found in nonhyperlactatemic asymptomatic subjects). All these additive adverse effects on mitochondrial function could not be exclusively related to interference of mtDNA replication.

At the present time we can not completely eradicate HIV infection, but we can minimize HIV secondary effects, like mitochondrial lesion, by reducing viral load through antiretroviral administration.<sup>36</sup> Current guidelines advice the beginning of antiretroviral therapy before it did in the past and one of the benefits of early HIV suppression could be avoiding HIV-induced mitochondrial damage. But we have to take care choosing which drugs to use, at which doses and which antiretroviral combinations can be administered together, because the management of all these parameters will also determinate accumulative and chronic mitochondrial damage and future development of adverse clinical events with mitochondrial basis, like hyperlactatemia. Clinicians must be aware of any early sign or symptom of coming toxicities and therapy change could be welcome not only after an hyperlactatemic crisis, but also previously to its development. Although we demonstrate that mitochondrial recovery is possible after an hyperlactatemic episode, it is essential to prevent secondary effects of HAART better than managing them. Once hyperlactatemia is developed, early management

of all disturbances and normalization of lactate and acidemia, will help in mitochondrial and clinical recovery achievement.

Currently available information about HAART-related adverse event etiology has moved antiretroviral guidelines to less potent mitotoxic drug administration, which has fortunately reduced associated mitochondrial damage and derived adverse events, like hyperlactatemia. In developed countries these strategies consist on reducing antiretroviral doses, changing HAART-schedules to nucleoside-sparing regimens or guiding patients to structured-treatment interruptions,<sup>33,37–40</sup> but scarce work has been done to evaluate strategies which actively reverts mitochondrial induced damage, even in the context of concomitant antiretroviral administration, as mitochondrial drug therapy.<sup>41</sup> Further studies should be addressed to assess how to prevent or correct mitochondrial function in HIV-infected and HAART-treated symptomatic or asymptomatic patients but also to find premature toxicity markers that would allow us avoiding adverse effects of chronic HIV infection and treatment. The performance of mitochondrial assays in noninvasive and easy-obtaining samples (as mononuclear cells) based on measuring functional parameters and non-exclusively limited on measuring mtDNA content could be a useful tool for these screenings.

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# Improvement of Mitochondrial Toxicity in Patients Receiving a Nucleoside Reverse-Transcriptase Inhibitor–Sparing Strategy: Results from the Multicenter Study with Nevirapine and Kaletra (MULTINEKA)

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**Background.** Nucleoside reverse-transcriptase inhibitor (NRTI)-related mitochondrial toxicity has been suggested as a key factor in the induction of antiretroviral-related lipoatrophy. This study aimed to evaluate in vivo the effects of NRTI withdrawal on mitochondrial parameters and body fat distribution.

**Methods.** A multicenter, prospective, randomized trial assessed the efficacy and tolerability of switching to lopinavir-ritonavir plus nevirapine (nevirapine group;  $n = 34$ ), compared with lopinavir-ritonavir plus 2 NRTIs (control group;  $n = 33$ ) in a group of human immunodeficiency virus-infected adults with virological suppression. A subset of 35 individuals (20 from the nevirapine group and 15 from the control group) were evaluated for changes in the mitochondrial DNA (mtDNA) to nuclear DNA ratio and cytochrome c oxidase (COX) activity after NRTI withdrawal. Dual-energy X-ray absorptiometry (DEXA) scans were used to objectively quantify fat redistribution over time.

**Results.** The nevirapine group experienced a progressive increase in mtDNA content (a 40% increase at week 48;  $P = .039$  for comparison between groups) and in the COX activity (26% and 32% at weeks 24 and 48, respectively;  $P = .01$  and  $P = .09$  for comparison between groups, respectively). There were no statistically significant between-group differences in DEXA scans at week 48, although a higher fat increase in extremities was observed in the nevirapine group. No virologic failures occurred in either treatment arm.

**Conclusions.** Switching to a nucleoside-sparing regimen of nevirapine and lopinavir-ritonavir maintained full antiviral efficacy and led to an improvement in mitochondrial parameters, which suggests a reversion of nucleoside-associated mitochondrial toxicity. Although DEXA scans performed during the study only revealed slight changes in fat redistribution, a longer follow-up period may show a positive correlation between reduced mitochondrial toxicity and a clinical improvement of lipodystrophy.

Lipodystrophy syndrome remains one of the most important and relatively common adverse effects of long-term highly active antiretroviral therapy (HAART) and

often leads to treatment withdrawal and psychological and social upset. Studies assessing the relative contribution of pharmacological treatment to fat redistribution have suggested a predominant role for mitochondrial toxicity in lipoatrophy and have identified the use of specific nucleoside analogues as a statistically significant risk factor for peripheral lipoatrophy [1, 2].

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These observations have become of paramount importance after the good results observed with viral suppression maintenance for human immunodeficiency virus (HIV)-infected patients using nucleoside reverse-transcriptase inhibitor (NRTI)-sparing regimens, which may be an option to avoid mitochondrial toxicity in these patients [3–5]. In addition, the use of protease inhibitors (PIs) has been frequently associated with insulin resistance, dyslipidemia, and central lipohypertrophy, which is an observation that further supports the use of a nonnucleoside reverse-transcriptase inhibitor (NNRTI), such as nevirapine, which has been shown to have a protective role in cases of lipid abnormalities [6, 7].

The Nevirapine-Kaletra (NEKA) study was a randomized, open-label pilot study that compared the efficacy and safety of a nucleoside-sparing simplification approach that used nevirapine at a dosage of 200 mg twice daily in addition to a standard dose of lopinavir-ritonavir with that of a conventional triple-drug HAART regimen of lopinavir-ritonavir and 2 NRTIs in a group of antiretroviral-experienced patients with long-lasting viral suppression [8]. The results of this pilot study showed that dual therapy with nevirapine plus lopinavir-ritonavir at standard dosage was as potent and safe as triple standard-of-care HAART at 48 weeks of follow-up [8]. It further supported the assessment of this approach in a larger multicenter, randomized trial evaluating key safety aspects, such as the possible reduction in mitochondrial toxicity with use of an NRTI-sparing approach and consequent improvement in lipodystrophy. Some studies have demonstrated recovery of fat loss in extremities after nucleoside withdrawal [3–5], but to date, few data have been reported assessing changes in mitochondrial parameters with use of this strategy. The study also assessed the improvement in lopinavir-ritonavir-associated metabolic abnormalities as a result of the better lipid profile attributed to nevirapine.

## PATIENTS AND METHODS

**Study design and participants.** The Multicenter Study with Nevirapine and Kaletra (MULTINEKA) was a prospective multicenter study with a randomized, open design and a follow-up period of 48 weeks (ClinicalTrials.gov NCT00335686). The main objective was to determine the changes in mitochondrial parameters among patients who received the NRTI-sparing regimen of nevirapine plus lopinavir-ritonavir.

Eligible patients were adults ( $\geq 18$  years of age) with long-term HIV infection who had been undergoing treatment with a HAART regimen that included 2 NRTIs combined with a PI or an NNRTI (eg, efavirenz or nevirapine) for at least 6 months. All patients were stable, had presented with an undetectable plasma viral load ( $< 80$  copies/mL) for at least 6 months before screening (at least 2 measurements within a 6-month period), and presented with liver function test results (transaminase

enzymes)  $< 5$  times greater than normal values. Exclusion criteria included opportunistic infection and/or neoplasm within the previous 6 months; clinical suspicion of or documented resistance to study drugs; poor treatment adherence; pregnancy, breast-feeding, and/or refusal of contraception during the study follow-up period; known allergic drug hypersensitivity to study drugs and similar drugs; and enrollment in other clinical trials. Individuals with a known history of mitochondrial disease, as well as those receiving toxic drugs for mitochondria (eg, aminoglycosides and statins) were also excluded from the study.

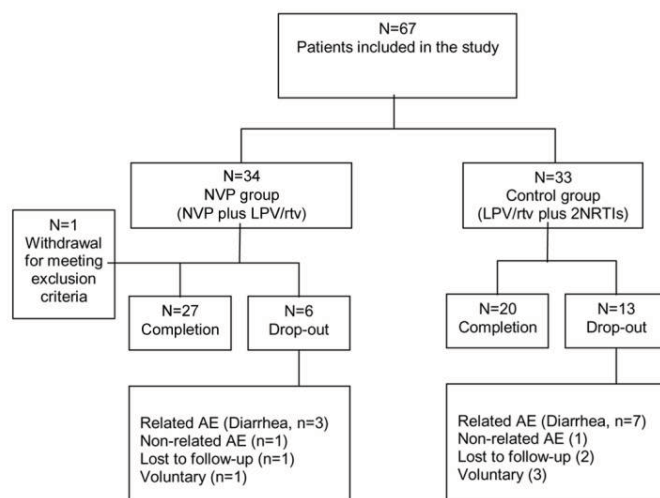
Study participants were recruited from HIV units from 13 Spanish tertiary care hospitals. The study was approved by the ethics committees of each participating hospital and by the Spanish Health Authorities, and all patients gave written informed consent.

Patients enrolled in the study were randomized to either substitute their current PI or NNRTI for lopinavir-ritonavir (lopinavir 400 mg/ritonavir 100 mg capsules administered twice per day) (Kaletra; Abbott) and continue therapy with 2 NRTIs (the control group) or to switch to 200 mg of nevirapine administered twice per day (Viramune; Boehringer Ingelheim) plus twice-daily lopinavir-ritonavir (the nevirapine group). Patients without previous exposure to nevirapine received a daily dose of 200 mg for the first 2 weeks, which was increased to 200 mg of nevirapine twice daily thereafter.

All study patients were included in the clinical (including changes in fat distribution), virologic, and immunologic end points, whereas only the 35 patients from the centers in Catalonia entered the mitochondrial study (main end point), because some of the tests required fresh blood analysis.

**End points.** The primary end point of the study was the *in vivo* analysis of changes in mitochondrial DNA (mtDNA) content by means of the ratio of mtDNA to nuclear DNA (nDNA) and changes in mitochondrial respiratory chain complex IV (cytochrome c oxidase [COX] IV) enzyme activity as markers of mitochondrial toxicity after switching from an NRTI to the NNRTI nevirapine.

Secondary end points of the study included the following: (1) evaluation of the ability of the lopinavir-ritonavir plus nevirapine combination to stop lipodystrophy or achieve a reversion of the process, as measured by changes in dual-energy X-ray absorptiometry (DEXA) and self-reported questionnaires of body fat changes; (2) assessment of the hypolipemiant effects of nevirapine on lopinavir-ritonavir-associated dyslipidemia as measured by changes in lipid profile; (3) appraisal of the safety and tolerability of the lopinavir-ritonavir and nevirapine combination throughout the 48-week follow-up period, determined by the percentage of patients with adverse events and interruption of therapy as a result of toxicity or other reasons; and (4) evaluation of the efficacy of the lopinavir-ritonavir plus nevirapine simplified treatment combination in maintaining



**Figure 1.** Trial outcome and reasons for treatment discontinuation. AE, adverse event; LPV/rvt: lopinavir-ritonavir; NRTI: nucleoside reverse transcriptase inhibitor; NVP, nevirapine.

viral suppression (plasma HIV RNA level, <50 copies/mL) and progression towards immunological recovery of patients.

**Interventions and follow-up.** All patients were assessed at baseline and at weeks 4, 12, 24, 36, and 48 of follow-up. The results of hematological analysis, clinical chemistry, and lipid profiles (total cholesterol levels, high-density lipoprotein [HDL] and low-density lipoprotein [LDL] cholesterol levels, and triglyceride levels, measured by the Friedewald equation) were evaluated at every visit. HIV RNA quantification and CD4<sup>+</sup> and CD8<sup>+</sup> T cell counts were performed at baseline and every 12 weeks thereafter. Samples were available for genotypic analysis for those patients who experienced virologic failure during the study. DEXA scans and self-reported questionnaires regarding body fat changes were evaluated at baseline and every 24 weeks thereafter during the follow-up period.

**Mitochondrial DNA study.** Peripheral blood mononuclear cells (PBMCs) were obtained at baseline and at weeks 24 and 48 with use of Ficoll's density gradient centrifugation [9] from patients who participated in the mitochondrial study. The aforementioned PBMC isolation method removes most of the platelets and reduces the risk of possible sample contamination that may influence the quantitation of mtDNA.

The relative quantification of mtDNA was performed essentially as described elsewhere [11], but small modifications were made to adapt the protocol to the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) [10]. Primer and probe sequences were mtND2-F (5'-CATCTTTGCA-GGCACACTC-ATC), mtND2-R (5'-TGTTTAGAACTGGAATAAAAGCTA-GCA), and mtND2-P (5'-AGCGCTAAGCTCGCACTGATTT-TTACCTGA) for the mitochondrial gene and 18S-F (5'-AGTGGAGCCTGCGGCTTAAT), 18S-R (5'-ACCCACGG-

AATCGAGAAAGAG), and 18S-P (5'-CCGGACACGGACAG-GATT-GACAGAT) for the nuclear gene. A DNA reference sample was always included in each polymerase chain reaction (PCR) performed throughout the study to assess the interexperiment variation.

Relative quantification of mtDNA content was determined using the comparative cycle threshold (CT) ( $2^{-\Delta\Delta CT}$ ) method after verifying that the efficiency of both PCR amplification products was similar [12]. The  $\Delta CT$  value was obtained by subtracting the mean nDNA reference CT value from the average CT value of mtDNA. The average  $\Delta CT$  of the control (lopinavir-ritonavir plus NRTIs) baseline group was used as the calibrator. This study showed mtDNA content results as fold changes, calculated according to the formula  $2^{-\Delta\Delta CT}$ , where  $\Delta\Delta CT$  was the difference between  $\Delta CT$  and the  $\Delta CT$  calibrator value.

**Mitochondrial function study.** The COX enzyme activity was spectrophotometrically measured according to the methodology previously described by Rustin et al [13] and slightly modified for minute amounts of biological samples [14]. The specific COX activity was expressed in absolute values as nanomoles per minute per milligram of protein.

**Statistical analysis.** The analyses were done per protocol or using the on-treatment approach for all variables in the study. Intention-to-treat analyses, which considered missing values to equal treatment failure, were also performed for the measurement of efficacy.

Variables with a normal distribution were described as mean ( $\pm$  standard deviation) and compared with use of the independent-sample *t* test or paired-sample *t* test; otherwise, median values (interquartile range) and nonparametric tests (Mann-

**Table 1. Baseline Epidemiologic, Clinical, Metabolic, and Mitochondrial Characteristics of the Study Patients**

Variable	Mitochondrial substudy			All patients		
	Nevirapine group (n = 20)	Control group (n = 15)	P	Nevirapine group (n = 33)	Control group (n = 33)	P
Sex, %			>.99			>.99
Male	89	92		85	85	
Female	11	8		15	15	
Age, median years (IQR)	40 (37.4–36.3)	39 (37–42.8)	.478	42.3 (37.3;–47.3)	42.1 (38.5–46.7)	.893
Risk, %			.057			.187
MSM	78.9	30.8		60.6	36.4	
Heterosexual sex	10.5	30.8		15.2	15.2	
Drug use	5.3	23.1		21.2	39.4	
Other	5.3	15.4		3.0	9.1	
Baseline CD4 <sup>+</sup> T cell count, median cells/mm <sup>3</sup> (IQR)	483 (351–559)	457 (405–587)	.857	452 (303–596)	471 (385–722)	.443
Duration of HAART, median years (IQR)	9.16 (4.48–11.64)	6.66 (3.81–9.82)	.196	8.03 (4.03–10.53)	7.32 (5.11–10.38)	.990
NRTI, %						
Didanosine	52.6	50	.881	51.5	39.4	.323
Didanosine and stavudine	5.3	14.3	.561	9.1	15.2	.708
Zidovudine	21.1	14.3	>.99	30.3	24.2	.580
NNRTI, %						
PI	57.9	57.1	.966	57.6	57.6	>.99
NNRTI	57.9	50	.653	48.5	45.5	.805
mtDNA relative amount <sup>a</sup> , mean value (±SD)	–2.357 (0.86)	–2.509 (0.9)	.635	...	...	...
COX activity, mean nmol/min/mg protein (±SD)	98.89 (13.16)	130.21 (25.08)	.280	...	...	...

**NOTE.** COX, cytochrome c oxidase; IQR, interquartile range; MSM, men who have sex with men; mtDNA, mitochondrial DNA; NNRTI, nonnucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; PI, protease inhibitor; SD, standard deviation.

<sup>a</sup> mtDNA relative content at baseline was calculated by subtracting the nuclear DNA measurement from the mtDNA amount.

Whitney *U* test or Kruskal-Wallis one-way analysis of variance test) were used. Categorical variables were assessed by means of absolute frequencies and percentages and the  $\chi^2$  test or Fisher's exact test, as appropriate. Statistical analyses were performed using SPSS software, version 15.0 (SPSS), with univariate 2-tailed significance levels of 5%.

## RESULTS

**Study population.** A total of 67 subjects were recruited and randomly assigned to receive nevirapine plus lopinavir-ritonavir (nevirapine group; *n* = 34) or to maintain their current treatment with 2 NRTIs, substituting only the PI or NNRTI for lopinavir-ritonavir (control group; *n* = 33). One patient from the nevirapine group was excluded from the final analyses (figure 1). Thirty-five patients from the Catalonian centers entered the mitochondrial study. There were no statistically significant differences in baseline characteristics between the treatment arms (table 1).

**Mitochondrial toxicity.** There were no statistically significant differences in mtDNA content and COX activity between groups at baseline (table 1). The nevirapine group showed a progressive and stable increase in mtDNA content throughout the follow-up period (a 40% increase), which achieved statistical significance versus the control group at week 48 (*P* = .039).

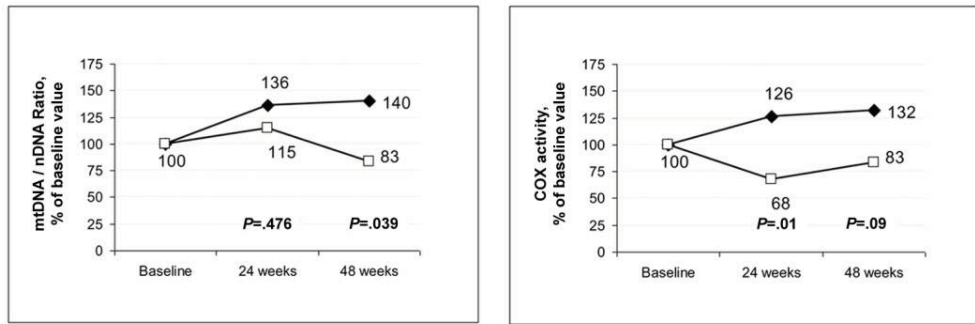
Regarding the COX activity, the nevirapine group showed

an improvement during the follow-up period (26% and 32% increases at weeks 24 and 48, respectively), whereas in the control group, COX activity was impaired. Differences between groups reached statistical significance at week 24 (*P* = .01; *P* = .09 at week 48) (figure 2).

**Lipodystrophy assessment.** DEXA scans showed no statistically significant changes in fat distribution in any group at week 48 with respect to baseline values (*P* = .24 for the nevirapine group; *P* = .94 for the control group). No differences were seen between groups at week 48 (*P* = .97).

Despite this, a lasting increase in fat on the upper and lower limbs was observed throughout the entire follow-up period, mostly in the nevirapine group (from 13.2% at baseline to 17.7% at week 48 in the upper limbs and from 11.1% to 14.1% in the lower limbs), whereas the control group mainly showed a fat increase in the trunk area (from 20.6% to 22.6% at week 48), resulting throughout the study in a progressive increase in the trunk-to-extremity fat ratio, compared with the nevirapine group (figure 3). Based on the questionnaire on subjective body evaluation, no significant changes in body fat distribution were detected at week 48 from baseline in either arm (*P* > .99; *P* = .317 between groups).

**Lipid profile.** Evolution of total cholesterol and LDL cholesterol fraction were similar in both study groups throughout the 48-week follow-up period. The nevirapine group, however, showed a consistent increase in HDL cholesterol levels (change



**Figure 2.** Changes in mitochondrial parameters with respect to baseline values. *P* values indicate differences between groups. Full squares represent the nevirapine group, and empty squares represent the control group.

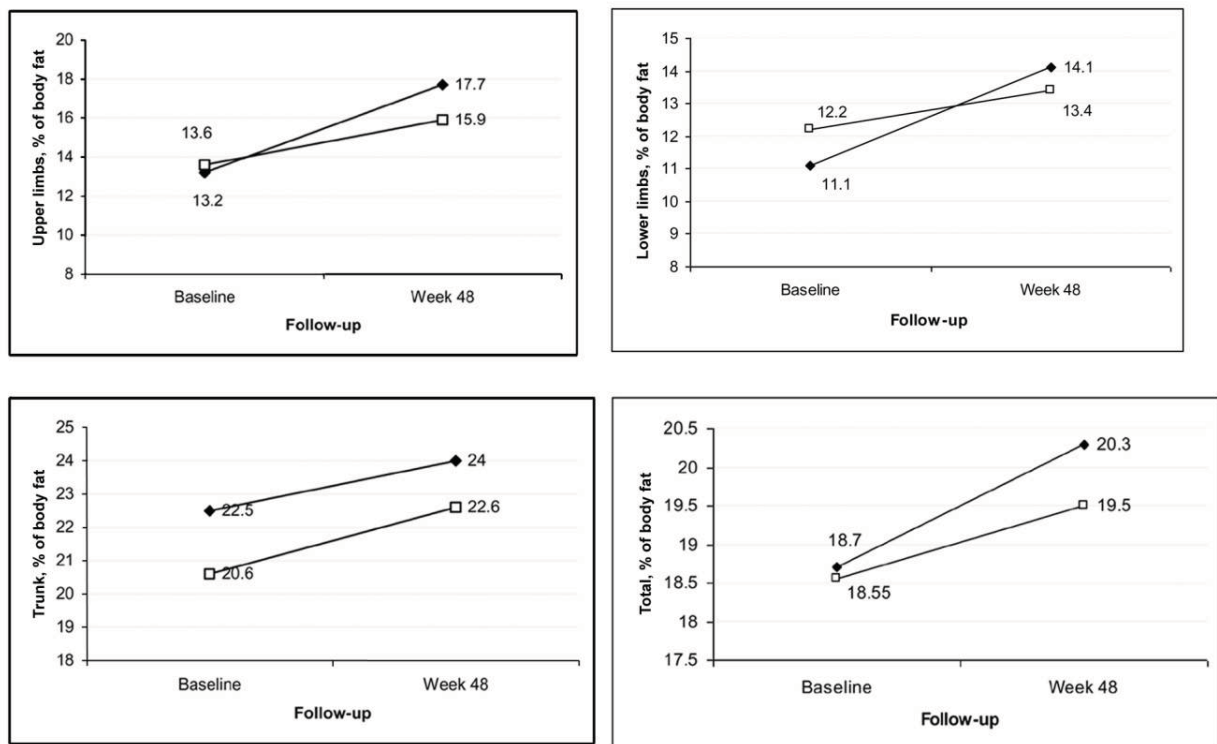
from baseline at week 48, *P* = .012) and a lower increase in triglycerides versus the control group (table 2).

**Clinical evolution and safety profile.** During the follow-up period, a total of 42 adverse events were detected. Of these, 25 were treatment-related adverse events (12 in the nevirapine group and 13 in the control group); 4 were rated as severe (grades III and IV), including 1 in the nevirapine arm and 3 in the control arm. Gastrointestinal complaints—mainly diarrhea, vomiting, and abdominal disturbances—were the most frequently observed adverse events. They mainly appeared dur-

ing the first 12 weeks of therapy, were usually mild (grade I or II), and were transient in most patients.

Six (17.6%) of the patients from the nevirapine arm and 13 (39.4%) of the patients from the control arm discontinued treatment (*P* = .05 for comparison between groups). Of these, 3 (50%) and 7 (53.8%), respectively, discontinued the treatment as a result of diarrhea (table 3). Seventy percent of treatment discontinuation occurred within the first 24 weeks of follow-up; the remaining 21% occurred at week 36.

In the group of patients who withdrew from the study, only



**Figure 3.** Evolution of fat redistribution. Comparison of median percentage of body fat change for upper limbs, lower limbs, trunk, and total throughout the follow-up period for both study groups. Full squares represent the nevirapine group, and empty squares represent the control group.



**Table 2. Lipid Profiles in Both Study Groups through the Study Follow-Up Period**

Variable	Nevirapine group, median mg/dL (IQR)			Control group, median mg/dL (IQR)		
	Baseline	Week 48	<i>P</i>	Baseline	Week 48	<i>P</i>
Total cholesterol level	205 (185–226)	214 (192–233)	.163	196 (174–216)	209 (178–232)	>.99
LDL cholesterol level	129 (110–148)	130 (110–142)	.829	122 (99–134)	111 (98–134)	.307
HDL cholesterol level	47 (39–53)	50 (44–68)	.012	46 (39–51)	46 (42.5–63)	.170
Triglyceride level	109 (96–221)	131 (121–172)	.443	136 (85–184)	175 (109–209)	.057

**NOTE.** HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein.

1 from the nevirapine group and 3 from the control group belonged to the mitochondrial substudy; the rest of participants in this substudy completed the follow-up.

Mean transaminase levels did not show any significant change from baseline, and no patient developed grades III or IV liver toxicity or acute clinical hepatitis during follow-up (data not shown).

**Virologic and immunologic assessments.** All patients had viral suppression (viral load, <50 copies/mL) at 48 weeks of follow-up. For the intention-to-treat analyses (in which treatment discontinuation was regarded as treatment failure), 27 (81.8%) of the patients from the nevirapine arm and 20 (60.6%) from the control arm remained stable, with an undetectable plasma viral load at the end of the study ( $P = .05$  for comparison between groups). Median CD4<sup>+</sup> T cell count remained unchanged in both treatment arms (from 452 cells/mm<sup>3</sup> [range, 303–596 cells/mm<sup>3</sup>] to 476 cells/mm<sup>3</sup> [335–607 cells/mm<sup>3</sup>] in the nevirapine group and from 471 cells/mm<sup>3</sup> [range, 385–722 cells/mm<sup>3</sup>] to 556 cells/mm<sup>3</sup> [range, 338–850 cells/mm<sup>3</sup>] in the control group;  $P = .082$  and  $P = .088$ , respectively) with no statistically significant differences at week 48 between groups.

## DISCUSSION

The results of this larger multicenter study reinforce our previous results from the NEKA study [8] and show dual antiretroviral therapy with nevirapine plus standard dose lopinavir-ritonavir to be as potent and safe as standard care regimens after 48 weeks of treatment in antiretroviral-experienced subjects with long-lasting viral suppression. The current study also shows that substituting NRTI drugs with the NNRTI nevirapine leads to a progressive and maintained increase in mtDNA con-

tent throughout the entire follow-up period and, thus, to an improvement in mitochondrial function, as well as an increase in HDL-cholesterol, which are effects that were not observed in the control group.

The specific etiology of peripheral lipoatrophy and some other clinical and metabolic consequences of antiretroviral therapy are now clearer. Regarding the possible etiology of lipoatrophy, current evidence supports a multifactorial mechanism with a predominantly drug-related adverse effect, mainly described as NRTI-related mitochondrial DNA polymerase gamma inhibition; it is also a possible immune reconstitution or proinflammatory cytokine-mediated phenomenon [15]. The suggestion that NRTI-induced mitochondrial toxicity is a key factor in the induction of HAART-related lipoatrophy, remains one of the strongest, as it is supported by increasing evidence showing that NRTIs strongly affect mitochondria in the adipose tissue [16]. In fact, it has been suggested that the toxic effects of NRTIs on human adipose cells could not only limit themselves to a lipoatrophic effect but also extend to an opposite lipohypertrophic effect depending on the oxygen availability. Furthermore, the lipoatrophic and lipohypertrophic phenotype characteristic of lipodystrophy could be a differential consequence of NRTI effects, depending on the metabolic status of the targeted adipose tissues [17]. Although our study design does not allow us to fully identify the causes of mitochondrial dysfunction in patients with HIV infection, these results suggest that NRTIs and their affinity for uptake by mitochondrial DNA polymerase gamma could play an important role in the process and that, despite other possible causes of mitochondrial dysfunction, the mitochondrial function is improved in patients

**Table 3. Total and Treatment-Related Adverse Events**

Variable	Nevirapine group ( <i>n</i> = 33)	Control group ( <i>n</i> = 33)	Total ( <i>n</i> = 66)
Total adverse events	24	18	42
Treatment-related adverse events	12	13	25
Grades III or IV	1 <sup>a</sup>	3 <sup>b</sup>	4

<sup>a</sup> Dyslipemia.

<sup>b</sup> Ascites requiring hospital admission, splenectomy requiring hospital admission, and diarrhea.

treated with a simplified regimen combining lopinavir-ritonavir and nevirapine and excluding NRTIs.

Mitochondrial toxicity becomes apparent particularly during medium-term to long-term therapy with NRTIs, and in addition to lipodystrophy, it may also lead to a wide range of severe adverse events in HIV-infected patients, including lactic acidosis, hepatic steatosis, neuropathy, myopathy, cardiomyopathy, and pancreatitis [18]. The measurement of mitochondrial DNA and its function in PBMCs may be of value for the early prevention of these toxicities in treated patients with HIV infection. A significant number of studies have shown the association between the use of NRTIs and mtDNA depletion, as well as the association between low mtDNA levels and subsequent development of lipodystrophy in patients with HIV infection [2, 19–33]. On the other hand, it has also been suggested that mtDNA content in peripheral blood cells may not be an accurate biomarker of mitochondrial toxicity in lipotrophic adipose tissue because of a poor correlation between this parameter and any other mitochondrial parameter, as well as because of the preservation of mtDNA-dependent mitochondrial functions despite severe mtDNA depletion [34–37]. All of these discrepancies between studies further underline the complexities involved in the relationship between the lipodystrophic syndrome and mitochondrial function and show some of the difficulties involved in their evaluation [38]. Thus, the current study includes not only the mtDNA determination but also the study of the COX enzyme activity to evaluate the mitochondrial function.

Although there was a trend toward positive fat redistribution in nevirapine-treated patients who were evaluated by DEXA scan that showed a progressive fat increase in upper and lower limbs over time, our results did not achieve statistical significance for these parameters, and these patients did not eventually reveal any evident fat change. Most probably, this lack of statistical significance could be attributable to some study limitations, such as the small number of patients or the limited period of follow-up. Our data does not support slow recovery of adipose tissue in spite of NRTI withdrawal and mitochondrial lesion improvement. This could be attributable to a variety of factors. First, there could be a differential capacity to revert mitochondrial toxicity in adipose tissue (the target tissue for lipodystrophy, which could be still damaged) with respect to the mononuclear cells that we used as a study model (which usually have a faster turnover). Second, it could be attributable to a general mitochondrial improvement in all body cells and mitochondria, but fat recovery could take longer. A longer follow-up of the patients who underwent the treatment switch would have helped us to address this question. Finally, the effects of HIV infection per se on mitochondrial function and lipodystrophy development cannot be forgotten. HIV has been reported to induce mitochondrial damage in the absence of

antiretroviral treatment by modulating inflammatory and/or apoptotic cellular mechanisms [39–41]. This could lead to chronic depletion of mtDNA content and deterioration of mitochondrial function, despite the fact that all of the patients maintained virologic suppression throughout the study. In addition to the effect of HIV on mitochondria, the immune response to HIV infection would increase proinflammatory cytokines, such as tumor necrosis factor- $\alpha$ , all of which would contribute to lipodystrophy syndrome, independently of antiretroviral therapy and/or NRTI withdrawal.

In conclusion, in antiretroviral-experienced subjects with long-lasting viral suppression treated with NRTIs, switching to a nucleoside-sparing regimen using nevirapine and lopinavir-ritonavir retained the entire antiviral efficacy and led to an improvement in mtDNA/nDNA ratio and COX activity, suggesting a reversion of nucleoside-associated mitochondrial toxicity. Although this improvement in mitochondrial function did not translate into significant changes in fat redistribution during the study, a longer follow-up may show a positive correlation between this reduced mitochondrial toxicity and a clinical improvement of lipodystrophy.

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**MILD IMPROVEMENT OF MITOCHONDRIAL FUNCTION AFTER 3 YEARS OF  
ANTIRETROVIRAL TREATMENT INTERRUPTION IN SPITE OF MITOCHONDRIAL DNA  
IMPAIRMENT**

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**Objective:** To evaluate whether a prolonged antiretroviral treatment interruption could reverse mitochondrial toxicity, a prospectively defined sub-study of the TIBET study (a CD4 cell-guided antiretroviral treatment interruption trial) was performed.

**Patients and Methods:** We included those patients from the TIBET study who were followed for  $\geq 96$  weeks and whose peripheral blood mononuclear cells (PBMCs) had been collected at baseline and throughout the study period. Out of the total number of 201 patients included in the TIBET study, 38 patients were selected for the mitochondrial sub-study; 18 patients had discontinued antiretroviral therapy for  $\geq 96$  weeks and 20 had maintained therapy. Mitochondrial DNA (mtDNA) was measured in PBMCs by real-time polymerase chain reaction and mitochondrial function was estimated through a cytochrome c oxidase and citrate synthase (COX/CS) ratio.

**Results:** Whereas mtDNA content showed a similar progressive decrease throughout the study period in both study arms, the COX/CS ratio significantly improved in patients who interrupted antiretroviral therapy in comparison with those who did not. The univariate and multivariate analysis performed showed that only CD4+ T-cell value at the time of initiation of antiretroviral therapy and time with viral suppression before the study were associated with the change of the COX/CS ratio.

**Conclusions:** Mitochondrial function improved during a prolonged antiretroviral treatment interruption in spite of a decrease in mtDNA levels in PBMCs. The absence of correlation between mitochondrial parameters suggests the existence of a mitochondrial transcriptional or translational upregulation mechanism or the reversion of a mitochondrial toxicity through a DNA polymerase gamma-independent way.

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## INTRODUCTION

It is well-known that the use of more intensive antiretroviral therapies in HIV-infected patients has

allowed to reduce substantially the morbidity and mortality rates of these patients in the previous

years [1]. Current guidelines for the management of HIV-infected patients recommend the combination of at least three agents of which usually two of them are nucleoside reverse transcriptase inhibitors (NRTIs), and the third one could be a protease inhibitor (PI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), or even another NRTI [2]. Despite of the positive effects of antiretroviral drugs on HIV infection, their long-term use have revealed the appearance of a wide variety of serious adverse effects, many of which have been directly related with the mitochondrial function in association with NRTIs [3]. In fact, the administration of nucleosides is probably the factor with higher detrimental effect on mitochondrial function, although other factors, such as the virus itself, can play also a complementary role [4].

Previous studies have showed that NRTIs affect the function of DNA polymerase beta and gamma among the 5 cellular enzymes that repair and/or replicate DNA. The effects of DNA polymerase beta on nuclear DNA (nDNA) reparation are redundant, but it is the effect on DNA polymerase gamma, the unique enzyme that replicates mitochondrial DNA (mtDNA), which lead to mtDNA depletion and/or mutation, insufficient energy generation and subsequent cellular dysfunction [3]. The clinical presentation of mitochondrial dysfunction generally occur after several months of NRTI treatment and could be very heterogeneous as it will depend on the organ that is specifically affected [5]. The most dramatic clinical presentation of mitochondrial toxicity is acute lactic acidosis with hepatic failure. The estimated incidence is around 1.3 per 1000 person-years and is fatal in most cases [6]. Other clinical presentations of mitochondrial toxicities could include lipoatrophy, peripheral neuropathy, myopathy, nephrotoxicity and pancreatitis, although the incidences reported in literature vary widely [7].

Some options to manage mitochondrial toxicity and its clinical manifestations have already been proposed. Supplements such as acetyl-carnitine, riboflavin, thiamine and coenzyme-Q are able to give some benefit in these situations [8-11]. Other options include the substitution of the probable causative NRTIs by an alternative less toxic nucleoside analogue [12-15] or NRTI-sparing approaches in which a PI is combined with a NNRTI or monotherapies with boosted PIs [16], as well as the uridine supplementation [17].

Finally, the complete interruption of the antiretroviral regimen may be even a better strategy to resolve, or at least to minimize, not only mitochondrial toxicity but also other antiretroviral-related toxicities. This is especially true in the case of PI- or NNRTI-associated adverse events, such as gastrointestinal disorders, hypersensitivities, hepatotoxicity or even lipid abnormalities. However, controversial data has been published regarding the reversibility of mitochondrial-related toxicity during antiretroviral treatment interruptions among HIV-infected patients [18, 19].

In order to address these concerns, we performed a prospectively defined sub-study of the TIBET study [20], a CD4 cell-guided antiretroviral treatment interruption trial, to evaluate in chronically HIV-1 infected patients the effect of prolonged antiretroviral therapy interruptions on mitochondrial parameters.

## **METHODS**

### ***Study design***

This was a prospectively defined mitochondrial sub-study of the TIBET study [20], a trial designed to investigate the impact of CD4-guided treatment interruption on clinical, virological and immunological parameters.

The TIBET study was a randomized, open-label trial that was performed from May 2001 to January

2002 in five hospitals of Spain and Italy. Participants were randomized to interrupt all drugs simultaneously (CD4 guided-treatment interruption [GTI] arm) or to continue the current antiretroviral treatment (control arm). Patients allocated to the GTI arm restarted antiretroviral treatment if any of the following situations were observed: an AIDS-defining illness, a severe or prolonged acute retroviral syndrome occurred, CD4 cell counts  $\leq 350/\mu\text{l}$  and/or plasma HIV-RNA level  $\geq 5.0 \log_{10}$  copies/ml.

The study was approved by the Institutional Review Board of each participating site and all patients provided their written informed consent.

### **Objectives**

The primary objective of this sub-study was to evaluate the effect of prolonged antiretroviral therapy interruption on mitochondrial parameters in chronically HIV-1 infected patients.

Secondary objectives included to identify those factors which may have an impact on mitochondrial function and to assess whether clinical prospective and retrospective covariates had any effect on mtDNA and mitochondrial function.

### **Selection of patients**

Apart from the selection criteria defined in the TIBET study (i.e. basically being on intensive antiretroviral therapies for more than one year without interruptions, absolute CD4 cell counts  $\geq 500/\mu\text{l}$  during at least six months and never below  $50/\mu\text{l}$ , HIV-RNA  $< 400$  copies/ml during at least one year and  $< 50$  copies/ml at study entry, and no previous opportunistic infections), patients had to stay in the TIBET study for at least 96 weeks and had to have peripheral blood mononuclear cells (PBMC) stored throughout the study period to participate in the mitochondrial sub-study.

### **Study procedures**

In the mitochondrial sub-study, biochemical data was determined in PBMCs stored at baseline, at

week 96 and at week 144. Briefly, 10-20 ml of whole blood was collected in ethylenediaminetetraacetic acid-Vacutainer tubes (BD, Madrid, Spain) and processed immediately after collection for plasma and PBMC isolation. Plasma was recovered after centrifugation at  $1200 \times g$  for 10 min and immediately cryopreserved and stored at  $-80^{\circ}\text{C}$  until used. PBMC were obtained by separation on Ficoll-Hypaque (Atom Reactiva, Barcelona, Spain) density gradient and cryopreserved in a mixture of DMSO/FCS (10:90) in liquid nitrogen for further determinations.

### **Mitochondrial DNA analysis**

Total DNA was obtained by a standard phenol-chloroform extraction procedure from an aliquot of PBMCs and used for genetic studies. Quantification of mitochondrial NADH dehydrogenase subunit 2 (ND2) and nuclear ribosomal RNA 18S reference genes was performed in a similar strategy as previously described [21]. Amplification and detection for each gene were carried out in separated reactions using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Primer and probe sequences were ND2-F (5'-CATCTTTGCAGGCA-CACTCATC), ND2-R (5'-TGGTTAGAACTGGA-ATAAAAGCTAGCA), ND2-P (5'-AGCGCTA-AGCTCGCACTGATTTTTTACCTGA) for the mitochondrial gene and 18S-F (5'-AGTGGAGCC-TGCGGCTTAAT), 18S-R (5'-ACCCACGGAAT-CGAGAAAGAG), 18S-P (5'-CCGGACACGGA-CAGGATTGACAGAT) for the nuclear gene.

Before analysing GTI and control subjects, amplification with optimised PCR conditions of the two genes was performed with a 6-fold serial DNA dilution to measure the PCR efficiency for both genes. Following these considerations, DNA of GTI and control groups was analysed in triplicates and a DNA reference sample was always included in each



performed PCR throughout the study to assess the inter-experiment variation.

As the efficiency value remained similar for mtDNA and nuclear genes when analysing both amplification curves generated from DNA dilutions, the use of the comparative CT method ( $2^{-\Delta\Delta CT}$ ) was allowed for ratio calculation and comparisons between groups [22].

#### ***Citrate Synthase (CS) and Cytochrome C Oxidase (COX) enzymatic activity***

The quantity of mitochondria was assessed by the spectrophotometric measurement of CS activity (EC: 4.1.3.7), a mitochondrial matrix enzyme of the Krebs' cycle, which remains highly constant in mitochondria and is considered to be a reliable marker of mitochondrial content (expressed as nmol/min/mg protein) [23-26]. COX, also known as mitochondrial complex IV, is an essential enzyme in the mitochondrial respiratory chain. COX activity was measured spectrophotometrically (EC 1.9.3.1) according to the Rustin et al methodology [25], slightly modified for minute amounts of biological samples [27], and expressed in absolute values (nmol/min/mg protein), as well as in relative values per mitochondria by dividing absolute COX values by mitochondrial mass (CS activity), COX/CS ratio.

#### ***Statistical analysis***

Statistical summaries for the main retrospective and baseline variables before antiretroviral interruption, as well as a pictorial and tabular description of mitochondrial function (COX activity) and mtDNA measurements were prepared. The statistical significance of the longitudinal changes and group differences in mitochondrial function and mtDNA measurements were assessed by means of Wilcoxon, Mann-Whitney, Chi-square or Fisher's exact tests for all the patients and distinguishing

between study arms. A linear mixed model with a random intercept was used for each patient to study the relationship between the COX/CS ratio and the plasma viral load, absolute CD4<sup>+</sup> T-cell counts and the retrospective and baseline variables while off-treatment. The equation for the suggested model indicated that the average value of the COX/CS for a given patient "i" at time "t" was linearly related to the considered covariates [ $COX/CS_i(t) = (\text{Intercept} + b_i) + \alpha \text{Covariate } i + \beta t + \epsilon_i(t)$ ], where the random effect  $b_i$  identified individual characteristics, and in particular showed how the specific patient differed from the average subject with respect to course of disease. It was assumed that  $b_i$  followed a multivariate normal distribution centred at zero and with unstructured variance-covariance for model flexibility. The variable  $t$  was expressed in weeks and only took values 0, 96 and 144;  $\epsilon_i(t)$  was the within-patient measurement error and was assumed to follow a multivariate normal distribution centred at zero. An F-statistic was used to test the hypotheses about the fixed  $\alpha$  and  $\beta$  effects. The model specification was validated by the Akaike Information Criterion (AIC). A model adding a quadratic term of  $t$  was also considered.

The data consisted of all the COX/CS measurements from the moment that a patient interrupted antiretroviral therapy until therapy was restarted. For each patient at least two and at the most three measurements were available.

Statistical analyses were performed with the use of SPSS software (version 11.5, SPSS, Inc., Chicago, Illinois, USA) and S-PLUS 2000 (version 2000 Professional Release 3, SPSS, Inc., Chicago, Illinois, USA).

## RESULTS

### Baseline characteristics

From 201 chronically HIV-1 infected patients included in the TIBET study, 38 (19%) stayed at least 96 weeks and had PBMC stored throughout the study period; so they were selected to participate in the mitochondrial sub-study. Of them, 18 patients belonged to the GTI arm and 20 patients to the control arm. In fact, 30 out of 38 patients (79%) remained in the study until week 144 (17 in the GTI arm and 13 in control arm). Baseline characteristics of these patients are described in Table 1. No differences in baseline epidemiological or HIV-related parameters were observed between both study arms.

### Mitochondrial DNA analysis (2- $\Delta\Delta\text{CT}$ )

MtDNA content showed a progressive decrease when each study arm was independently analysed throughout the follow-up period (Figure 1). From baseline to week 96, both study arms showed a similar decay in mtDNA (15% in GTI arm and 17% in control arm;  $p=0.76$  between both arms). The median value of mtDNA [interquartile range] in GTI arm decreased from 1.00 [1.00-1.00, reference value] at baseline to 0.85 [0.59-1.01] at week 96 ( $p=0.10$ ). In the control arm, mtDNA decreased to 0.83 [0.56-1.07] at week 96 ( $p=0.03$ ) (Figure 1).

Surprisingly, the percentage of change from baseline to week 144 seemed to be higher in the GTI arm (51%) than in the control arm (34%,  $p=0.73$  between both study arms). Median values

### Mitochondrial function analysis

No statistically significant differences between GTI and control arm were observed in the baseline mitochondrial function (median [interquartile range] COX/CS ratio) (0.31 [0.23-0.66] versus 0.56 [0.33-0.68] respectively;  $p=0.18$ ).

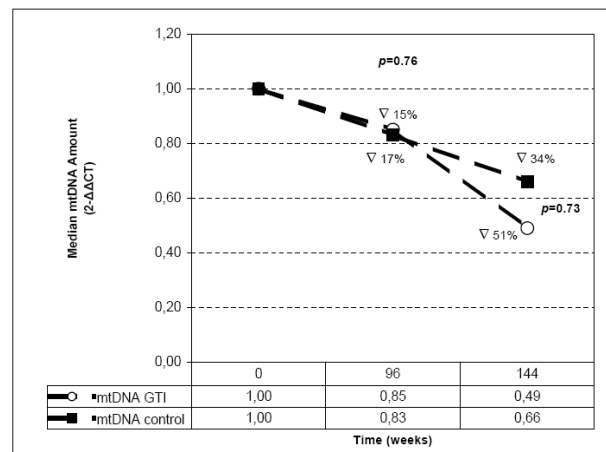
Throughout the follow-up period, the COX/CS ratio did not vary in the control arm. Considering the baseline value as 1 (100%), the percentage of

Characteristic	Control arm n = 20	GTI arm n = 18	P value
Age, years	38 (32; 42)	38 (34; 41)	0.704
Male, n (%)	13 (65)	14 (78)	0.264
Route of HIV transmission, n (%)			
Men's sex with men	10 (50)	11 (61)	
Injecting drug users	6 (30)	4 (22)	0.771
Heterosexual sex	4 (20)	3 (17)	
Time with HIV infection, months	87.8 (50.6; 110.7)	66.9 (38.5; 90.0)	0.143
Time on ART, months	47.7 (41.6; 55.9)	46.3 (40.3; 56.0)	0.264
Type of previous ART, n (%)			
ddl	5 (25)	3 (17)	0.380
d4T	11 (55)	11 (61)	0.552
Baseline CD4 cell count, percentage	41 (33; 48)	32 (29; 40)	0.245
Time on VL<50 copies/ml, months	29.4 (25.7; 41.5)	31.5 (17.2; 37.9)	0.413

GTI: Guided-treatment interruption arm; IQR: Interquartile range; ART: Antiretroviral treatment; VL: Viral load  
All data has been expressed as median and IQR except when was a percentage

decreased from 1.00 [1.00-1.00, reference value] to 0.49 [0.21-1.26] ( $p=0.31$ ) in the GTI arm and to 0.66 [0.33-0.79] ( $p=0.01$ ) in the control arm (Figure 1).

Figure 1. Analysis of mitochondrial DNA (mtDNA, 2- $\Delta\Delta\text{CT}$ ).



change was 2% at week 96 and 2% at week 144 with respect baseline values ( $p=0.71$  and  $p=0.11$  respectively) (Figure 2).

In contrast, we observed a significant increase in the COX/CS ratio in the GTI arm at week 96 (130%,  $p=0.28$ ) and at week 144 (80%,  $p=0.50$ ) from baseline (Figure 2).



No statistically significant differences were observed in the change of COX/CS ratio between both arms at week 144 ( $p=0.42$ ), although a trend was observed at week 96 ( $p=0.06$ ).

### Modelling analysis

Previous modelling, the time effect on the COX/CS ratio was assessed. Different baseline factors were included in the fixed part of the model. The univariate approach revealed that CD4+ T-cell percentage at baseline and the time with undetectable viral load previous to study entry affected the expected mean value of COX/CS ratio. Higher baseline CD4+ T-lymphocyte values and longer time with undetectable viral load previous to study entry were associated with greater improvement of COX/CS ratio.

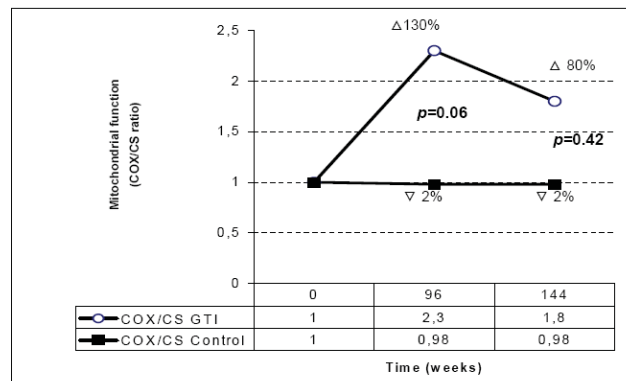
The multivariate model was constructed including all the covariates that reached a  $p$  value of less than 0.10 in the univariate model. The multivariate model showed that both, the percentage of CD4+ T-cell count at the time of beginning antiretroviral therapy and the time with viral suppression before treatment interruption were associated with the change of the COX/CS ratio. Complete data of these models is shown in Table 2.

According to this data, the expected value of mitochondria function marker for a patient who was 32 months with undetectable viral load and who had 32% of CD4+ T-cells previous to antiretroviral

### Discussion

We have prospectively evaluated the effect of a prolonged antiretroviral therapy interruption on mitochondrial parameters such as mtDNA content and mitochondrial function (COX/CS ratio) in chronically HIV-1 infected patients. Our results indicate that, whereas mtDNA content showed a similar progressive decrease throughout the study period in both study arms, the mitochondrial

Figure 2. Changes in mitochondrial function (Delta COX/CS ratio)



therapy would be 0.477 units. The effect of 96 weeks without treatment would represent an increase of 0.144 units.

The mean number of COX/CS units increased by 0.2 with each increase of 10% CD4 T-lymphocytes previous to antiretroviral therapy. The effect of being six additional months with undetectable viral load before treatment interruption increased the expected value of COX/CS by 0.1 units. Furthermore, every six months without treatment, the COX/CS ratio increased 0.04 units.

Table 2. Multivariate model for COX/CS dynamics during HIV treatment discontinuation.

Covariates	Coefficient	SE (Coefficient)	$p$ value
Intercept	-0,5444	0,3246	0,10
Week	0,0015	0,0010	0,13
CD4 percentage Pre-HAART	0,0169	0,0078	0,05
Days undetectable VL	0,0005	0,0002	0,01

$b_0 \sim N(0, 0.000005)$ ;  $e_j \sim N(0, 0.384539)$

function significantly improved only in the GTI arm in comparison with the control arm.

Currently, there is an important concern regarding which is the optimal method to diagnose and evaluate nucleoside-induced mitochondrial toxicity. Although many authors recognize that the gold standard would be to perform a liver or a muscle biopsy [18, 19], other investigators have tested the suitability of adipose tissue to clearly show changes in mtDNA or mtRNA [28-31]. However, none of

those methods seems to be practical for routine screening and follow-up. It has been speculated that the quantification of mtDNA in PBMCs [18, 19, 32], could be a reliable predictor of mitochondrial toxicity. Cote et al concluded that mtDNA levels in PBMCs were significantly decreased in patients with symptomatic, nucleoside-related hyperlactatemia and that this effect could be reversed after antiretroviral therapy discontinuation [18]. Mussini et al showed in a platelet-free highly purified lymphocyte population that mtDNA content increased in CD8<sup>+</sup> T lymphocytes after six months of treatment interruption, although it did not change in CD4<sup>+</sup> T lymphocytes [32]. Nonetheless, other authors have found opposite results. In a prospective evaluation of mtDNA in PBMCs as a marker of toxicity in 157 consecutively recruited HIV-positive patients, a similar decrease of mtDNA was observed in both, naïve and antiretroviral treated patients [19]. These findings suggest a direct mitochondrial toxicity of the virus itself, but they did not find any link between the mtDNA/nDNA ratio in PBMCs and any clinical symptoms or lactate level. Consequently, some authors have indicated the necessity of analysing the mitochondrial function, together with the quantification of mtDNA, in order to achieve a more deep knowledge of mitochondrial damage and changes [13]. This should be also specially useful considering that mitochondrial toxicity of antiretroviral drugs has been demonstrated in the absence of mtDNA depletion [31, 33-36]. In the present work, the study of quantification of mtDNA content has been completed with the enzymatic analysis of mitochondrial function in order to obtain a more deep and reliable vision of the real mitochondrial status.

Based on our data, we observed a similar progressive decrease of mtDNA content in both study arms. An explanation for these findings

would be the role of HIV itself on mitochondrial damage, i.e. the re-emergence of HIV in all of these patients who interrupted therapy after a long-term viral suppression. In fact, the in vitro and in vivo association between HIV itself and mitochondrial damage has already been described [4, 18, 37]. Unfortunately, we were unable to demonstrate in our study the influence of HIV itself on mitochondrial parameters. This was due to the low viral replication rate observed along with the fact that none of the participants in the GTI arm needed to reinitiate treatment during the follow-up period of the study according to the protocol guidelines. Nonetheless, we observed a significant recovery of mitochondrial function after two or three years of antiretroviral treatment interruption in comparison with the control arm, although no statistical differences were observed between both arms, probably due to the wide range of COX/CS ratio values.

The absence of correlation between mitochondrial parameters such as mtDNA and mitochondrial function can be explained, in our opinion, at least by two different hypothesis. One could be the existence of an upregulatory mechanism, in which an increase of the mitochondrial transcriptional (that synthesise RNA from DNA) or of the post-transcriptional (which translates RNA into proteins) rates could compensate the acute mtDNA depletion after antiretroviral therapy interruption and virus re-emergence. This mechanism has been suggested before in the context of antiretroviral-mediated damage [36]. The other possibility could be that the antiretroviral treatment interruption favours the reversibility of a previously antiretroviral-induced mitochondrial damage, independently of DNA polymerase gamma function, that would have directly affected mitochondrial function. Then, treatment interruption would improve mitochondrial function without being able to revert

those mitochondrial lesions which depend on DNA polymerase activity. Recently, previous reports have found improvements in all functional mitochondrial parameters except for mtDNA content when high-mitochondrial-toxic antiretroviral therapy was reduced [13, 38]. These data suggest the existence of alternative mitochondrial toxicity mechanisms to DNA polymerase gamma inhibition, which could respond independently to mtDNA content. Conversely, in the control arm, the additive mitochondrial toxicity of the continued antiretroviral therapy during the study period would explain the more stable loss of mtDNA content throughout the 3 years of follow-up and the lack of a homeostatic mechanism that compensates mtDNA depletion.

Apart from that, the modelling analysis of our study showed that only the value of CD4<sup>+</sup> T-cell count at the time of beginning antiretroviral therapy and time with undetectable viral load before the study could influence in some way the COX/CS ratio at baseline; those patients who presented higher CD4 values and had registered longer time with viral suppression had a higher COX/CS ratio. As well, the time without therapy increased the mean value of the ratio throughout the study. No other epidemiological factors nor HIV-related factors were associated with the change of the mitochondrial function.

The design of the study did not contemplate the possibility of recording clinical changes in fat distribution or other mitochondrial-related toxicities. Therefore, the association between the variation of mitochondrial parameters and more relevant clinical changes remain to be elucidated. Indeed, we should have evaluated the real benefits obtained with the prolonged antiretroviral therapy

interruption since they could not compensate the risks associated with the deleterious virological and immunological outcome along with the quicker clinical progression described recently [39].

In summary, two main conclusions may be obtained from this study. The first one is that the mitochondrial function is improved during a prolonged antiretroviral treatment interruption in spite of the progressive decrease of mtDNA content in PBMCs, probably related to the re-emergence of the virus. The second conclusion, which is derived from the first, is to highlight the real usefulness of mtDNA quantification in PBMCs if there is not a certain correlation with mitochondrial function. The existence of mitochondrial homeostatic mechanisms to preserve mitochondrial function albeit genetic disturbances or alternative antiretroviral toxicity mechanisms to DNA polymerase gamma inhibition could explain the lack of correlation. Other questions like which is the best strategy to achieve a recovery of mitochondrial toxicity, whether this recovery could be total or partial, or the real influence of mitochondrial changes in more relevant clinical features like fat distribution remain to be answered.

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#### **Transparency declaration**

The authors do not have any financial conflicts of interest and the founders have not played any decision-making role in the research.

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# Mitochondrial Injury in Human Acute Carbon Monoxide Poisoning: The Effect of Oxygen Treatment

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The best oxygen therapy for acute carbon monoxide poisoning (ACOP) remains unestablished. Reported mitochondrial complex IV (mtCIV) inhibition, together with carboxyhaemoglobin (COHb)-induced hypoxia, may influence acute clinical symptoms and outcome. To “mitochondrially” evaluate treatment efficacy, we correlated intoxication severity and symptoms with mitochondrial function (mtCIV activity) and oxidative stress (lipid peroxidation) in 60 poisoned patients and determined ACOP recovery depending on either normobaric or hyperbaric oxygen therapy along a 3-month follow-up. In the present article we positively evaluate mtCIV as a good marker of ACOP recovery, treatment effectiveness, and late neurological syndrome development, which advocates

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**Standard and non-standard abbreviations:** carbon monoxide (CO), carboxyhaemoglobin (COHb), acute carbon monoxide poisoning (ACOP), moderate and severe acute carbon monoxide poisoned patients (MCOP and SCOP), normobaric and hyperbaric oxygen (NBO and HBO), severe acute carbon monoxide intoxicated patients treated with one or two hyperbaric oxygen sessions (SHBO1 or SHBO2), moderate acute carbon monoxide poisoned patients treated with normobaric oxygen or one session of hyperbaric oxygen (MNBO or MHBO), late neurological syndrome (LNS), mitochondrial complex IV (mtCIV) or cytochrome c oxidase (COX), citrate synthase (CS), malondialdehyde (MDA) and 4-hydroxyalkenal (HAE), reactive oxygen species (ROS), peripheral blood mononuclear cells (PMBC) and standard error of the mean (SEM).

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for hyperbaric oxygen therapy as the treatment of choice. However, we discourage its usefulness as a severity marker because of its excessive sensitivity. We additionally evaluate oxidative stress role and prognostic factors for neurological sequelae development.

*Keywords:* Mitochondrial toxicity; carbon monoxide (CO); hyperbaric oxygen treatment (HBO); normobaric oxygen treatment (NBO); cytochrome c oxidase (COX) or mitochondrial complex IV (mtCIV); oxidative stress (lipid peroxidation); late neurological syndrome (LNS)

## 1. INTRODUCTION

Carbon monoxide (CO) is a colorless, odorless, tasteless toxic gas produced by incomplete combustion in fuel-burning devices such as motor vehicles, gas-powered furnaces, and portable generators. Acute carbon monoxide poisoning (ACOP) is the most frequent cause of intoxication by suffocating gases and is the greatest cause of death by occupational inhalation [1–6]. Unintentional CO exposure accounts for an estimated 15,000 emergency department visits and 500 unintentional deaths in the United States each year [7], and similar ratios have been reported in other countries. Symptoms of ACOP are generally attributed to tissular hypoxia due to carboxyhaemoglobin (COHb) formation [8, 9], which is generated when CO expels oxygen out of haemoglobin because of its higher affinity for this toxic gas. Carboxyhaemoglobin levels depend on the intensity and duration of CO exposition, and they are used in clinical practice to establish ACOP severity. However, although COHb levels confirm poisoning exposure, they do not necessarily correlate with acute symptoms or future outcome [10–12]. In addition, some clinical symptoms may appear as late sequelae several weeks after COHb normalization, while others may develop without COHb formation [13], suggesting the existence of secondary damage mechanisms independent of COHb-induced hypoxia.

Carbon monoxide prevents oxygen-binding to other physiologic proteins containing iron-porphyrin groups such as myoglobin, cytochrome p450, and mitochondrial respiratory chain complex IV (cytochrome c oxidase or mtCIV). Inhibition of mtCIV has been proposed as an alternative mechanism of persistent injury independent of COHb-induced hypoxia [12, 14–18]. Mitochondrial complex IV is the last component of the electron transport chain located in the inner-membrane of the mitochondria, which guides oxygen reduction into water. This complex participates in the oxidative phosphorylation system responsible for energy supply but is also a main center for oxidative stress production, especially when its impairment drives electron flow from oxygen reduction to reactive oxygen species (ROS) generation. Experimental models have demonstrated CO binding to mtCIV cytochrome aa<sub>3</sub> subunit [17]. In studies performed in human peripheral blood mononuclear cells (PMBC) of ACOP

patients we have demonstrated *in vivo* CO inhibition of mtCIV activity and consequent impairment of cellular respiration [14–16].

Treatment of ACOP consists in oxygen administration to promote CO dissociation of haemoglobin. Normobaric oxygen (NBO) treatment consists of pure oxygen administration at standard pressure (one atmosphere), and hyperbaric oxygen (HBO) therapy involves the administration of pure oxygen at high pressure (1.4 or more atmospheres). Both interventions, especially the latter, increase the CO-haemoglobin dissociation rate [19], oxygen transport into tissues, and hypoxia resolution [20]. The only meta-analysis performed in this field abrogates for HBO to minimize the incidence of late neurological syndrome (LNS) development, although this finding is not statistically significant [21]. Therefore, to date, the oxygen therapy of choice for ACOP treatment remains unestablished [19, 22–25].

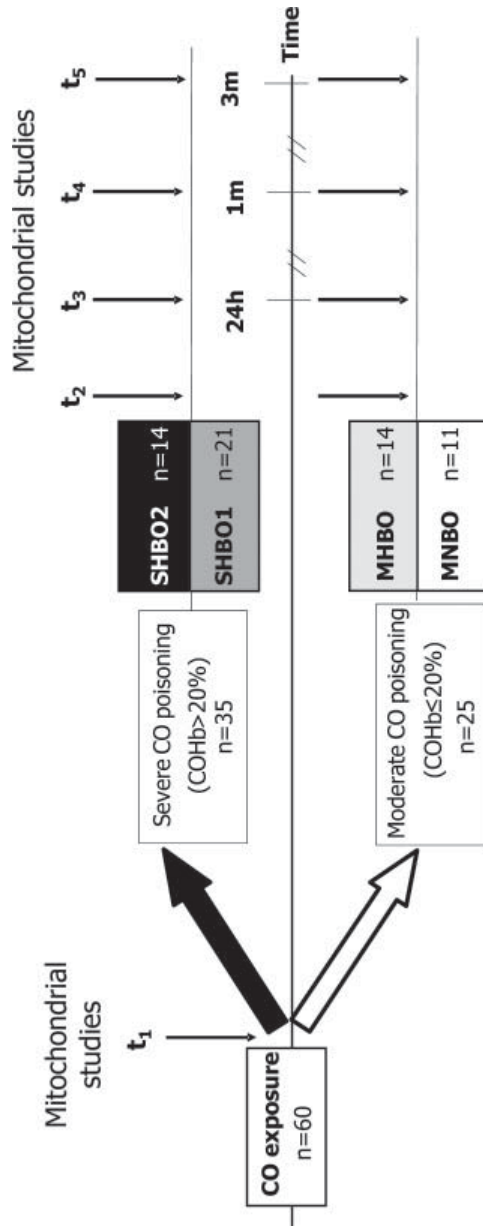
Regardless of the choice of therapy, it is assumed that oxygen treatment rapidly decreases COHb. However, since normalization of mtCIV activity takes longer [14–16], we hypothesized that inhibition of mtCIV, the final determinant of cellular oxygen consumption, could be a sensitive marker of LNS development and the assessment of ACOP treatment efficacy.

The objectives of the present study were to evaluate the usefulness of mtCIV activity and oxidative stress as markers of ACOP severity and outcome, to evaluate the best oxygen treatment depending on mitochondrial recovery, and to establish prognostic factors and the mitochondrial basis for LNS development.

## 2. METHODS

### 2.1. Patients

From May 2005 to January 2008, 60 consecutive subjects were attended in the emergency department of the Hospital of Palamos (Girona, Spain) because of ACOP, and all of them were included in the present study after signing the written informed consent approved by the Ethical Committee of our hospitals. Inclusion criteria for poisoned patients were age  $\geq 18$  years and clinical suspicious of ACOP (headache, asthenia, dizziness, nausea, confusion or loss of consciousness, among others) confirmed by COHb levels above 5%. Patients, on admission, were classified according to their clinical severity and COHb levels into two groups: those subjects with COHb levels between 5%–20% were considered as moderate ACOP and those individuals with COHb content above 20% or the presence of severe neurological or cardiac manifestations (arrhythmia, ischemia, loss of consciousness, or coma) or with a base deficit of  $-2$  units, independent of COHb levels, were classified as severe intoxicated patients (Figure 1). Oxygen treatments currently available in clinical practice were administered to the included patients according to severity group assignment.



**Figure 1:** Study design. CO: Carbon monoxide; COHb: Carboxyhaemoglobin; SHBO1 and SHBO2: Severe acute CO poisoned subjects (ACOP) treated with 1 or 2 hyperbaric oxygen sessions; MHBO and MNBO: Moderate ACOP patients treated with hyperbaric (MHBO) or normobaric oxygen (MNBO); h: Hours; m: Months;  $t_1, t_2, t_3, t_4, t_5$ : Mitochondrial time-point studies 1 to 5.

Severe ACOP patients ( $n = 35$ ) were randomly distributed to receive either one (SHBO1,  $n = 21$ ) or two (SHBO2,  $n = 14$ ) HBO sessions and moderate ACOP subjects ( $n = 25$ ) were randomized to receive either NBO (MNBO,  $n = 11$ ) or one HBO session (MHBO,  $n = 14$ ). Normobaric oxygen therapy consisted in 12 hours of pure oxygen administration at standard pressure by means of a reservoir face mask while HBO involved the administration of a table 5 US Navy oxygen treatment [26] in a multiplace hyperbaric chamber. In the SHBO2 group, HBO sessions were separated by 6 hours. Thirty non-smoker healthy individuals, matched by age and gender with poisoned patients, were consecutively included in parallel to patient admission. Control subjects were volunteer staff of the hospital and emergency patients with no diagnosis of disease. Exclusion criteria for both patients and controls were the presence of previous familiar or personal history of mitochondrial disease, treatment with mitochondrial toxic drugs, smoking habit, or ongoing pregnancy.

## 2.2. Methods

We registered symptoms, COHb levels, mtCIV activity, mitochondrial mass (through citrate synthase -CS- activity), and oxidative stress levels (lipid peroxidation) before ( $t_1$ ) and immediately after oxygen treatment ( $t_2$ ), as well as 24 hours ( $t_3$ ), 1 month ( $t_4$ ), and 3 months ( $t_5$ ) after therapy. The usual time interval between  $t_1$  and  $t_2$  was 12 hours.

## 2.3. Clinic Information

Extensive clinical data were collected through detailed questionnaires at each visit along the follow-up ( $t_1$ – $t_5$ ). Epidemiological data, information about CO-exposure, blood analyses, and signs and symptoms were systematically recorded in these questionnaires. Clinic physiologic information consisted in the evaluation of 26 parameters divided into 4 categories (general, respiratory, cardiac, and neurological data) designed to detect acute symptoms and clinical outcome (Table 1).

On detection of LNS, consisting of neurologic impairment affecting superior cognitive functions which usually appear one to three weeks after CO-intoxication and treatment administration, mitochondrial analyses and symptoms were registered between the established time-points.

## 2.4. COHb

It was measured in blood samples along the follow-up using the Radiometer CO-oximeter OSM3. First COHb measure was assessed on patient admission, after a short period of normobaric oxygen administration previous to hospital arrival. Elapsed time on normobaric oxygen treatment previous to first

**Table 1:** Acute clinical symptoms (t1) and outcome (t5) of moderate or severe acute CO-intoxicated patients (ACOP) with respect to poisoned individuals presenting late neurological symptoms (LNS). Results are expressed as number of patients with respect to the total (or in percentages) for each category. Prognostic factors for LNS development have been calculated considering all moderate and severe intoxicated subjects with standard outcome and have been expressed as odds ratio (OR) with 95% confidence interval (95% CI)

	t1			t5			LNS Prognostic symptoms Significance OR(95%CI)
	Moderate ACOP	Severe ACOP	ACOP with LNS	Moderate ACOP	Severe ACOP	ACOP with LNS	
Nausea/Vomiting Skin lesions	5/24 (20.83) 0	13/31 (41.93) 0	3/5 (60) 1/5 (20)	0 0	1/24 (4.17) 0	0 0	p = NS p < 0.0005 22.6 (1.3–381.8)
Asthenia/Fatigue	11/24 (45.83)	19/31 (61.29)	5/5 (100)	0	1/24 (4.17)	4/5 (80)	p < 0.05 1.8 (1.1–3.1)
Weakness	1/24 (4.17)	3/30 (10)	2/5 (40)	0	1/24 (4.17)	1/5 (20)	p < 0.001 5.4 (1.8–15.9)
Respiratory auscultation abnormalities	0	1/30 (3.33)	<b>Respiratory symptoms</b> 0	0	0	0	p = NS
Cardiac auscultation abnormalities	1/24 (4.17)	2/30 (6.67)	<b>Cardiac symptoms</b> 1/5 (20)	0	1/24 (4.17)	0	p = NS
Chest pain/Ischemic symptoms	0	2/30 (6.67)	0	0	0	0	p = NS
Arrhythmia	0	0	0	0	0	0	p = NS

(Continued on next page)

**Table 1:** Acute clinical symptoms (†1) and outcome (†5) of moderate or severe acute CO-intoxicated patients (ACOP) with respect to poisoned individuals presenting late neurological symptoms (LNS). Results are expressed as number of patients with respect to the total (or in percentages) for each category. Prognostic factors for LNS development have been calculated considering all moderate and severe intoxicated subjects with standard outcome and have been expressed as odds ratio (OR) with 95% confidence interval (95% CI). (Continued)

	†1			†5			LNS Prognostic symptoms Significance OR(95%CI)
	Moderate ACOP	Severe ACOP	ACOP with LNS	Moderate ACOP	Severe ACOP	ACOP with LNS	
	<b>Neurological symptoms</b>						
Loss of Consciousness	0	19/30 (63.33)	4/5 (80)	0	0	0	p < 0.01 2.3 (1.2-4.1)
Confusion	4/24 (16.67)	18/30 (60)	4/5 (80)	0	0	1/5 (20)	p < 0.05 2.0 (1.1-3.5)
Seizures	0	2/30 (6.67)	0	0	0	0	p = NS
Amnesia	0	5/30 (16.67)	2/5 (40)	0	0	1/5 (20)	p < 0.005 4.3 (1.6-11.6)
Headache	22/24 (91.67)	29/30 (96.67)	4/5 (80)	0	3/24 (12.5)	3/5 (60)	p = NS
Dizziness	10/24 (41.67)	13/30 (43.33)	1/5 (20)	0	0	0	p < 0.05 0.5 (0.2-0.9)
Visual impairment	0	0	1/5 (20)	0	0	0	p < 0.0005 22.2 (1.3-375)
Abnormal pupilar reflex	0	0	0	0	0	0	p = NS
Sensitivity abnormalities							p = NS
Tactile	1/24 (4.17)	2/30 (6.67)	0	0	0	0	p = NS
Vibratory	0	0	0	0	0	0	p = NS
Osteotendinous reflexes alteration	0	2/29 (6.9)	1/5 (20)	0	0	0	p < 0.05
Babinski reflex	0	2/30 (6.67)	1/5 (20)	0	0	0	5.4 (1.2-24) p < 0.05
Cerebelous function impairment							5.4 (1.2-24) p = NS
Finger-nose	1/24 (4.17)	0	1/5 (20)	0	0	1/5 (20)	p < 0.005 10.8 (1.4-82.7)
Knee-Wrinkle	0	0	1/5 (20)	0	0	1/5 (20)	p < 0.0005 22.2 (1.3-375)
Prono-supination maneuver	0	0	1/5 (20)	0	0	1/5 (20)	p < 0.0005 22.2 (1.3-375)
Rom berg	1/24 (4.17)	3/30 (10)	1/5 (20)	0	0	1/5 (20)	p = NS
Standard walk	2/24 (8.33)	2/30 (6.67)	1/5 (20)	0	0	1/5 (20)	p = NS
Tandem walk	2/24 (8.33)	5/30 (16.67)	1/5 (20)	0	0	1/5 (20)	p = NS

**Table 2:** Elapsed time on normobaric oxygen treatment previous to first carboxyhaemoglobin measurement (NBO-CO<sub>Hb</sub>), time between CO poisoning and hospital admission (CO-Hospital) or between CO intoxication and specific oxygen treatment assignment (CO-SpecO<sub>2</sub>Therapy) for the different oxygen treatments

Treatment Groups	Elapsed time (minutes) between:		
	NBO-CO <sub>Hb</sub>	CO-Hospital	CO-SpecO <sub>2</sub> Therapy
SHBO2	28 ± 19	131 ± 29	207 ± 31
SHBO1	11 ± 5	<b>60 ± 6*</b>	<b>101 ± 13**</b>
MHBO	57 ± 24	138 ± 22	135 ± 22
MNBO	30 ± 30	124 ± 27	128 ± 30

SHBO1 and SHBO2: Severe poisoned patients treated with 1 or 2 hyperbaric oxygen sessions; MNBO and MHBO: Moderate intoxicated individuals treated with normobaric or hyperbaric oxygen. \* $p < 0.001$  between SHBO1 and the rest of treatment groups; \*\* $p < 0.05$  between SHBO1 and SHBO2 groups.

CO<sub>Hb</sub>, time between CO poisoning and hospital admission, or between CO intoxication and specific oxygen treatment assignment have been summarized in Table 2.

## 2.5. Samples

Mitochondrial analyses were carried out in monocytes and lymphocytes isolated from 20 mL of peripheral blood by Ficoll gradient centrifugation [27] in the Hospital of Palamos (Girona, Spain), and frozen samples were transported at  $-80^{\circ}\text{C}$  to the Hospital Clinic of Barcelona (Barcelona, Spain) for mitochondrial studies.

## 2.6. Mitochondrial Parameters

Protein content was measured by the Bradford protein-dye-binding-based method [28] and mtCIV-specific enzymatic activity (EC 1.9.3.1) was quantified spectrophotometrically according to the methodology of Rustin and collaborators [29], slightly modified for mtCIV measurement in minute amounts of sample [30]. Mitochondrial CIV activity was expressed both in absolute values as nanomols of oxidized substrate per minute and milligram of protein (nmols/min.mg protein) as well as in relative units to mitochondrial content by dividing absolute mtCIV activity by CS activity (EC: 4.1.3.7; nmols/min.mg protein; mtCIV/CS). Citrate synthase is a Krebs' cycle enzyme that remains highly constant in the mitochondrial matrix and is widely considered as a reliable marker of mitochondrial content [29, 31–33].



Lipid peroxidation levels are an indicator of oxidative damage of ROS on cellular lipid compounds [34]. Lipid peroxidation was quantified using the Oxys Research kit of Deltaclone through the spectrophotometric measurement of malondialdehyde (MDA) and 4-hydroxyalkenal (HAE), both products of fatty acid peroxide decomposition, normalized by protein content ( $\mu\text{M MDA} + \text{HAE}/\text{mg protein}$ ).

## 2.7. Statistical Analysis

We assessed the normal distribution of parameters using the Kolmogorov-Smirnov test. Different statistical analyses were performed to determine (i) differences for an intention-to-treat principle depending on group assignment (severe vs. moderate intoxication) using the independent sample T-test; (ii) inter- or intra-group treatment differences (among MNBO, MHBO, SHBO1, and SHBO2 patients) using the analysis of variance for repeated measures and consequent Student's t-test confirmation; (iii) correlation between mitochondrial and/or clinic parameters using Pearson linear regression analysis; and (iv) prognostic factors for LNS development using contingency tables and Fisher's test for odds ratio quantification.

Results were expressed as mean and standard error of the mean (SEM) accompanied by 95% confidence intervals as percentages with respect to baseline (arbitrarily considered as 100%) or as odds ratio with 95% confidence intervals. In all cases a  $p$  value of less than 0.05 was considered to be statistically significant.

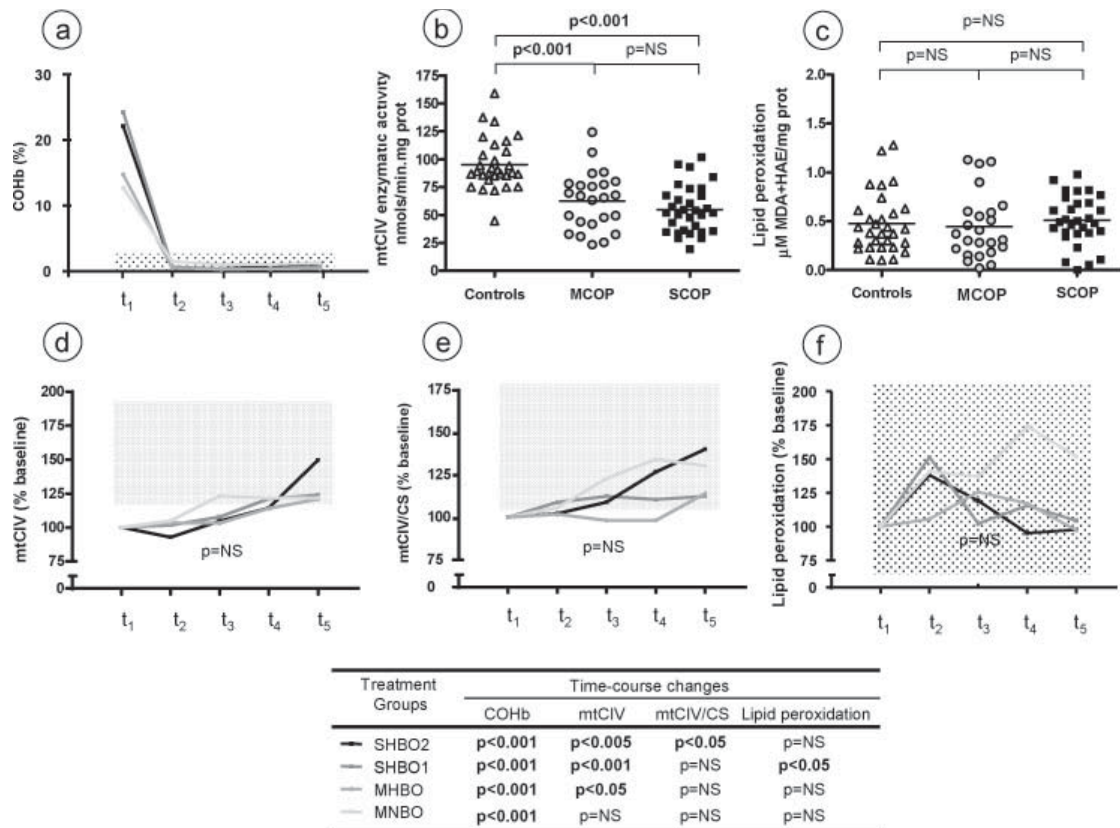
## 3. RESULTS

### 3.1. Carboxyhaemoglobin (COHb) Measurements

First, COHb measure was assessed on patient admission after a short period of normobaric oxygen administration previous to hospital arrival, which was similar for all patients. However, elapsed time between ACOP, hospital admission and first COHb determination was shorter for SOHB1 patients with respect to the rest of the treatments groups ( $p < 0.001$ ), and the time between CO poisoning and specific oxygen treatment assignment was also shorter for the SHBO1 group compared to the SOHB2 poisoned patients (Table 2).

Pre-treatment COHb levels differed between severe and moderate ACOP subjects according to severity ( $22.94 \pm 1.37$  [20.15–25.74] vs.  $12.45 \pm 1.05$  [10.28–14.62], respectively,  $p < 0.001$ ). All oxygen treatments were similarly effective in normalizing COHb values immediately after therapy ( $t_1$  vs.  $t_2$ ,  $p < 0.001$ , Figure 2a).





**Figure 2:** Carboxyhaemoglobin (COHb) and mitochondrial parameters. (a) Time-course COHb levels; (b) pre-treatment ( $t_1$ ) absolute mitochondrial complex IV (mtCIV) activity and (c) lipid peroxidation levels; (d) Absolute time-course mtCIV activity or (e) relative to mitochondrial content (mtCIV/CS); and (f) time-course lipid peroxidation levels. MCOP and SCOP: Moderate and severe acute CO poisoned patients (ACOP); SHBO1 and SHBO2: Severe ACOP individuals treated with 1 or 2 hyperbaric oxygen sessions; MNBO and MHBO: moderate ACOP subjects treated with normobaric oxygen (MNBO) or with 1 session of hyperbaric oxygen (MHBO);  $p = NS$ : non-statistically significant differences.  $t_1, t_2, t_3, t_4, t_5$ : Mitochondrial time-point studies 1 to 5.

In time-course graphs mitochondrial values are expressed as percentages with respect to baseline (arbitrarily considered 100%) and shadowed areas represent control ranges (mean  $\pm$  1 standard deviation). In the graphs  $p$  denotes time-point differences among groups. In the legend  $p$  denotes time-course statistical changes for each treatment group.

### 3.2. Mitochondrial Studies

Mitochondrial complex IV activity was decreased on admission ( $t_1$ ) in all intoxicated patients. Both severe and moderate poisoned subjects showed a significant decrease in mtCIV activity compared to controls ( $54.80 \pm 3.79$  [47.06–62.53] and  $62.64 \pm 5.27$  [51.74–73.55] vs.  $95.29 \pm 4.20$  [86.70–103.89], respectively,  $p < 0.001$ ), but no significant differences were found between the two groups of poisoned patients according to intoxication severity (Figure 2b). Pre-treatment lipid peroxidation levels ( $t_1$ ) remained unaltered in severe and moderate poisoned subjects compared to controls ( $0.51 \pm 0.05$  [0.42–0.70] and  $0.44 \pm 0.07$  [0.30–0.58] vs.  $0.47 \pm 0.06$  [0.36–0.59], respectively) (Figure 2c).

We did not find differences in mtCIV enzymatic activity before and immediately after treatment measurements ( $t_1$  vs.  $t_2$ ) in any treatment group. We also did not find significant differences in mtCIV function immediately after treatment ( $t_2$ ) between severe and moderate intoxicated groups ( $59.20 \pm 4.53$  [49.91–68.50] vs.  $63.08 \pm 5.20$  [52.32–73.83], respectively), or between one or two hyperbaric oxygen sessions in severe poisoned subjects (SHBO1 vs. SHBO2;  $58.11 \pm 6.61$  [43.71–72.53] vs.  $55.89 \pm 7.74$  [38.02–73.76]), or between normobaric oxygen and one hyperbaric oxygen session in moderate intoxicated patients (MNBO vs. MHBO;  $59.55 \pm 7.22$  [42.47–76.63] vs.  $74.22 \pm 4.93$  [62.15–86.30]) (Figure 2d). However, when considering the complete time-course mtCIV follow-up ( $t_1$  to  $t_5$ ), absolute mtCIV recovery was observed in all treatment groups, but was only found to be statistically significant in poisoned individuals under hyperbaric oxygen (SHBO2:  $p < 0.005$ ; SHBO1:  $p < 0.001$ ; MHBO:  $p < 0.05$ , Figure 2d). Note in Figure 2d that hyperbaric oxygen-treated groups significantly achieved mtCIV recovery at different time intervals depending on the choice of therapy: after 24 hours ( $t_1$  vs.  $t_3$ ) in the SHBO1 group ( $57.18 \pm 4.94$  [46.42–67.94] vs.  $61.64 \pm 6.50$  [47.46–75.81],  $p < 0.05$ ) and after three months ( $t_1$  vs.  $t_5$ ) in both SHBO2 and MHBO groups ( $60.18 \pm 8.52$  [40.54–79.83] vs.  $90.01 \pm 14.58$  [56.35–123.63],  $p < 0.05$ ;  $71.80 \pm 4.64$  [60.46–83.14] vs.  $86.82 \pm 6.56$  [70.76–102.87],  $p < 0.005$ ).

Recovery of mtCIV function related to mitochondrial content (mtCIV/CS) was also observed in all treatment groups, being exclusively significant in severe intoxicated subjects who received two sessions of hyperbaric oxygen (SHBO2,  $p < 0.05$ ) (Figure 2e).

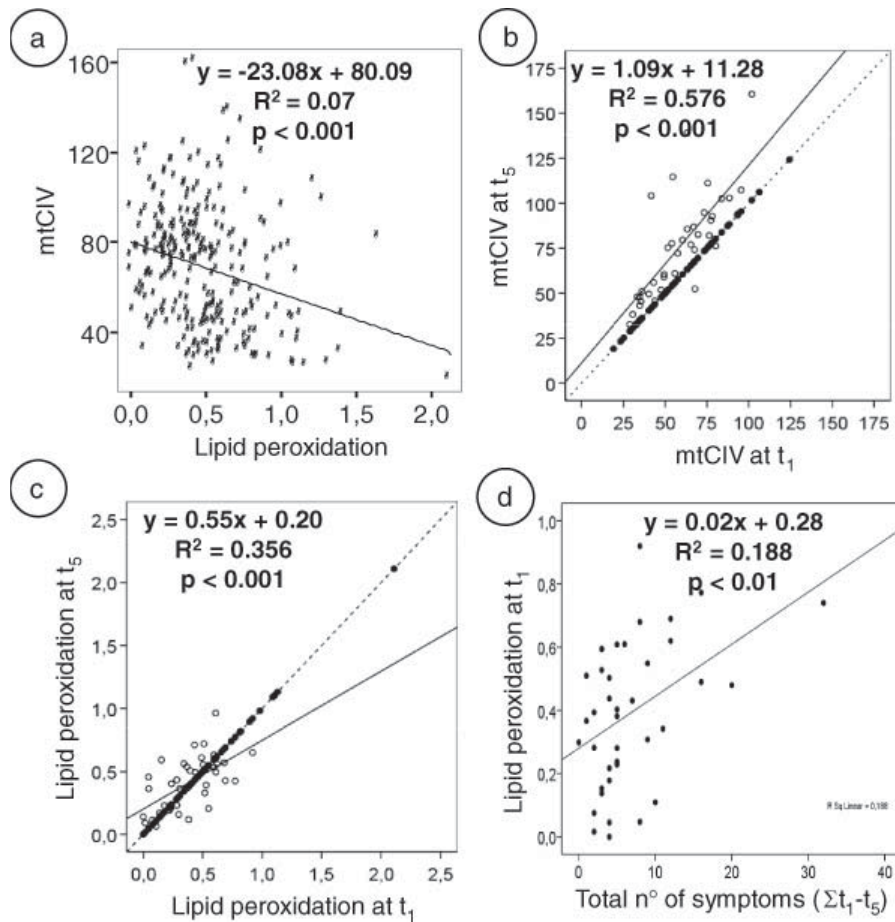
Lipid peroxidation levels showed a trend to an increase after oxygen treatment, especially in the SHBO1 group ( $t_1$  vs.  $t_2$ ;  $0.45 \pm 0.06$  [0.32–0.57] vs.  $0.68 \pm 0.11$  [0.42–0.93],  $p < 0.05$ ), normalizing 24 hours after therapy ( $t_2$  vs.  $t_3$ ;  $0.68 \pm 0.11$  [0.42–0.93] vs.  $0.46 \pm 0.07$  [0.31–0.61],  $p < 0.05$ ). Nonetheless, these levels were always within control ranges (Figure 2f).

Interestingly, mtCIV activity and lipid peroxidation levels were negatively correlated (Figure 3a,  $p < 0.001$ ), and initial and end time-point values for each of these parameters showed a positive correlation (Figures 3b and 3c,  $p < 0.001$ ).

Differences between  $t_1$  and  $t_5$  mtCIV measurements for all treatment groups consisted of a mean increase in mtCIV of 32% at the end of the follow up with respect to pre-treatment values (Figure 3b). Additionally, lipid peroxidation level tended to reduce in all treatment groups at the end of the follow up with respect to pre-treatment time (Figure 3c).

### 3.3. Relation Between Mitochondrial Studies and Clinical Data

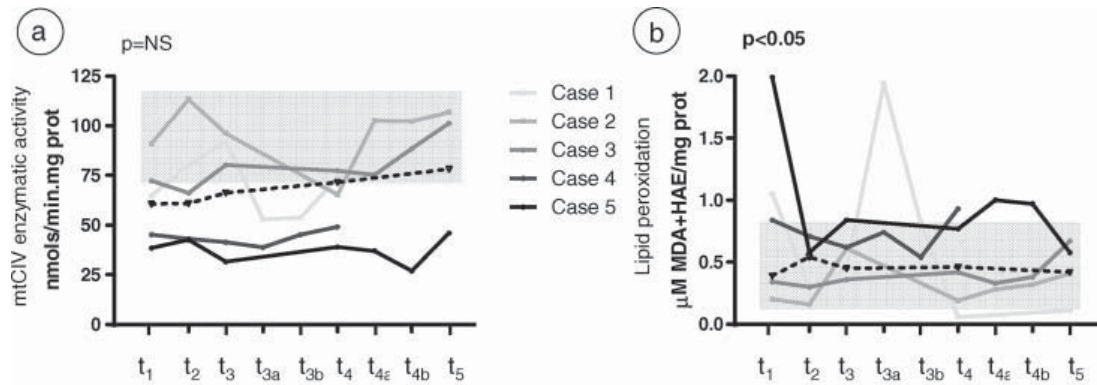
All patients showed acute clinical symptoms of ACOP, most being non-severe. The most frequent manifestations were headache (92.7%), asthenia



**Figure 3:** Correlation between mitochondrial parameters and clinical symptoms. (a) Mitochondrial CIV and lipid peroxidation correlation. Graphs (b) and (c) show mitochondrial complex IV (mtCIV) enzymatic activity and lipid peroxidation levels, respectively. Full points and the dotted line represent non-modified pre-treatment mitochondrial parameters with respect to inclusion ( $t_1$ ,  $t_1$ ) and empty points and the continuous line symbolize evolution of mitochondrial parameters from  $t_1$  to  $t_5$  ( $t_1$ ,  $t_5$ ). (d) Linear regression analysis between pre-treatment ( $t_1$ ) lipid peroxidation levels and total number of clinical manifestations along the follow-up ( $t_1$  to  $t_5$ ).

(54.5%), dizziness (41.8%), confusion (40%), loss of consciousness (34.5%), and nausea (32.7%), which were especially present in severe intoxicated patients (see Table 1 for detailed analysis). All these acute manifestations quickly disappeared after oxygen treatment, parallel to COHb normalization. Acute symptoms did not correlate with mitochondrial parameters. However, the total number of clinical manifestations along the follow-up positively correlated with pre-treatment lipid peroxidation levels (Figure 3d).

None of CO poisoned subjects suffered from acute anoxic encephalopathy on admission. However, some weeks after intoxication, 5 patients (8.3%) developed different type and severity level of late neurologic impairing, affecting superior cognitive functions as speech, orientation, or memory (LNS; Figure 4). Four were severe poisoned individuals with loss of consciousness during



**(c)**

	Case 1	Case 2	Case 3	Case 4	Case 5
<b>General Clinical data</b>					
Severity and Treatment	SHBO2	SHBO1	SHBO1	SHBO1	MHBO
Nausea/Vomiting			x----	x----	x----
Skin lesions	xx---				
Asthenia/Fatigue	xxxxx	xxxxx	xxxxx	xxx--	x-xxx
Weakness	xxxx-		x----		
<b>Respiratory symptoms</b>					
Respiratory auscultation abnormalities					
<b>Cardiac symptoms</b>					
Cardiac auscultation abnormalities	xxxx-				
Chest pain/Ischemic symptoms					
Arrhythmia					
<b>Neurologic symptoms</b>					
Loss of Consciousness	x----	x----	x----	x----	
Confusion	xx---	x----	x----	x----	---xx
Seizures					
Amnesia	xx---			-xx--	x-xxx
Headache	x----	x-xxx	x--xx	xxx--	--xxx
Dizziness			x----		--x--
Visual impairment				x----	
Abnormal pupliar reflex	x----				
<b>Sensitivity abnormalities</b>					
Tactile					
Vibratory					--x--
Osteotendinous reflexes alteration	x----				
Babinski reflex	x----				
<b>Cerebelous function impairment</b>					
Finger-nose	xxxxx				
Knee-Wrinkle	xxxxx				
Prono-supination maneuver	xxxxx				
Romberg	xxxxx				
Standard walk	xxxxx				
Tandem walk	xxxxx				--x--



intoxication, mostly treated with just one hyperbaric oxygen session (3 SHBO1: 1 SHBO2). The fifth was a moderate intoxicated subject treated with hyperbaric oxygen who presented mild acute symptoms (MHBO). Some acute neurological symptoms were common to ACOP individuals with standard recovery, although more prevalent (Table 1). Some of these acute manifestations did not completely revert after oxygen therapy (cases 1 and 4), re-appear several weeks later (case 5), and, in some subjects (cases 2, 3, and 5), did not completely disappear during the follow up (Figure 4c).

In our study, prognostic symptoms on admission for LNS development consisted of increased prevalence of skin lesions, asthenia, weakness, loss of consciousness, confusion, amnesia, visual impairment, osteotendinous reflexes alteration, Babinski reflex and cerebelous function impairment (finger-nose, knee-wrinkle and pronosupination maneuver), and decreased prevalence of dizziness (see Table 1 for detailed odds ratio analysis). It is of note the small size of our sample ( $n = 5$ ).

Pre-treatment mtCIV activity in patients with LNS was similar to intoxicated subjects with a standard outcome ( $59.76 \pm 10.85$  [29.63–89.89] vs.  $58.32 \pm 3.14$  [51.61–64.11], respectively,  $p = \text{NS}$ , Figure 4a), although lipid peroxidation levels were significantly increased in the former ( $0.88 \pm 0.32$  [0.01–1.76] vs.  $0.52 \pm 0.05$  [0.42–0.62], respectively,  $p < 0.05$ , Figure 4b). All 5 patients presenting LNS showed abnormal mitochondrial recovery compared to the remaining poisoned subjects: mtCIV did not ameliorate or its impairment punctually preceded or accompanied the clinical episode and lipid peroxidation level tended to increase, compared to controls and intoxicated individuals without neurological sequelae.

## 4. DISCUSSION

The present study establishes that mitochondrial damage can be a useful marker of ACOP outcome and that the different treatment efficacies can have

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**Figure 4:** Mitochondrial and clinic outcome of 5 acute CO-intoxicated patients (ACOP) with late neurologic syndrome (LNS); case 4 did not finish follow-up. (a) Mitochondrial complex IV (mtCIV) activity; (b) Lipid peroxidation levels; and (c) Clinical evolution. Shaded areas in the graphs represent control values (mean  $\pm$  1 standard deviation) and  $p$  value denotes time-point pre-treatment differences between the 5 ACOP subjects presenting LNS with respect to ACOP individuals with standard outcome (represented by discontinuous line).  $p = \text{NS}$ : non-statistically significant differences;  $t_1, t_2, t_3, t_{3a}, t_{3b}, t_4, t_{4a}, t_{4b}, t_5$ : Mitochondrial time-point studies 1 to 5; SHBO1 and SHBO2: Severe ACOP patients treated with 1 or 2 hyperbaric oxygen sessions; MHBO: Moderate ACOP subjects treated with hyperbaric oxygen. In the table, empty squares indicate asymptomatic patients along the follow-up. Cross signs (x) and hyphens (–) indicate presence or absence of symptoms in a given patient in a given time-point.

variable effect on mitochondrial recovery. Additionally, we have evaluated prognostic factors for LNS development, including mitochondrial impairment.

Several plasmatic markers have been proposed to evaluate severity and outcome of CO poisoned patients. Partial pressure of CO, oxygen or carbon dioxide, venous oxygen saturation, base deficit, sodium bicarbonate, lactate, or COHb content have been proposed, as well as creatine kinase or troponin levels to assess organ-specific impairment in intoxicated patients. Among them, increased lactate levels after CO exposition have been associated to the manifestation of adverse outcome [35]. However, lactate is an unspecific marker of CO poisoning and has also been involved in the development of many other disorders and intoxications. Consequently, COHb level is the current marker of tissular hypoxia for ACOP, despite its inaccuracy when correlating with acute symptoms and future outcome [10–12].

This study clearly establishes that CO inhibited *in vivo* mtCIV function in 60 ACOP patients corroborating our previous results obtained from isolated cases of clinical ACOP [14–16], which previously validated the usefulness of the PBMC model in CO-intoxication.

Carbon monoxide immediately inhibits mtCIV activity, independent of COHb levels and intoxication severity, demonstrating that mitochondria are very sensitive to CO. Mitochondrial CIV sensitivity and time-extended recovery, even when COHb levels restore, indicate that mtCIV could be more sensitive to CO than haemoglobin, perhaps due to stronger or prolonged binding to CO. Accordingly, mitochondria rather than or in addition to hypoxia, could contribute to explain early and late-development of COHb-independent symptoms. Early mtCIV CO-sensitivity could justify some ACOP symptoms that appear without COHb formation [10–13] while mtCIV time-extended inhibition could explain sequelae that appear weeks after COHb normalization. Unfortunately, early saturation of mtCIV by CO could limit its use as a marker of severity, explaining the lack of differences in pre-treatment mtCIV activity between severe and moderate ACOP subjects and the absence of a correlation with COHb or acute clinical manifestations.

On the other hand, although CI and CIII mitochondrial respiratory chain complexes are the main source of ROS, mtCIV finally reduces oxygen into water, and its dysfunction can also increase ROS production. In our series oxidative stress did not accompany ACOP, limiting again its usefulness as a severity marker for CO intoxication. Interestingly, these findings corroborate our previous studies performed in tobacco smokers. CO, one of the toxic compounds present in tobacco smoke, has been demonstrated to inhibit mtCIV activity in *in vitro* models [36] and in both chronic [37–39] and acute [40] smokers. However, oxidative stress was only found to be increased in chronic addict

smokers, probably due to time exposure accumulative ROS production, or antioxidant impairment, which could be similar in ACOP subjects. Positive correlation between mtCIV dysfunction and increased ROS production (present in CO poisoned patients) is an evidence of mitochondrial-derived oxidative lesion, which causes cellular damage and may underlie the development of several neurological primary diseases [41–43] and secondary lesions caused by some intoxications [44]. Neuron metabolism is typically oxidative and, additionally, does not have active antioxidant system against oxidative stress [42, 45]. Thus, in case of CO-mediated oxidative stress exposure, neurons are especially sensitive to oxidative lesion, which could be the basis for late memory impairment [46]. Although oxidative damage in lymphocytes and monocytes was always within control ranges in acute CO intoxication (similar to results found in acute tobacco exposure), it could be increased in neurons. This hypothesis would explain the positive correlation between oxidative stress and the prevalence of neurologic (and other) symptoms.

Oxygen is the first-line therapy in ACOP. However, dose, optimal pressure, length of administration, and number of hyperbaric oxygen sessions has not been clearly established in clinical trials [12, 19–25, 47]. Oxygen immediately decreases COHb content, normalizing oxygen transport into surrounding tissues and restoring the previous normoxic status [19, 20]. Despite this, mtCIV activity remains still decreased, which confirms the previous reported slow-movement recovery of this parameter [14–16] and could explain the lack of mtCIV differences at the immediate post-treatment stage among the different treatment groups. Differences on mtCIV recovery were found 3 months after therapy assignment, suggesting that oxygen treatment not only promotes CO-haemoglobin dissociation but also induces mtCIV-CO separation, according to Brown and Piantadosi findings [48–50]. We also demonstrate that the effectiveness in recovering mtCIV function depends on the oxygen treatment administered, since hyperbaric oxygen was more effective than normobaric treatment in moderate ACOP, and, in severe poisoning, one session of hyperbaric oxygen was effective enough to restore mtCIV activity, although two sessions allowed a stronger beneficial effect. The choice of HBO has been supported by clinical data [21, 51, 52] and by recently published results in experimental models [53, 54]. It is of note that oxygen therapy induced a non-significant increase in oxidative stress in some ACOP patients, despite returning to baseline after 24 hours, probably suggesting null future clinical consequences.

Dissociation of CO from mtCIV promoted by oxygen has been proposed as the main mechanism for mitochondrial recovery. However, the increase in mitochondrial mass could also play a secondary role. Although controversial, CO-mediated increase of mitochondrial mass has been previously proposed [55] and may explain why recovery of mtCIV function in HBO groups is lesser when

normalized by mitochondrial content, since absolute increase in mtCIV function would be partially due to CO-mtCIV separation and partially caused by an increase in mitochondrial number.

All ACOP patients showed acute clinical symptoms, especially severe CO-exposed subjects. Nonetheless, all these manifestations quickly disappeared after oxygen treatment and maintained a time-course correlation with mitochondrial recovery. However, five patients developed LNS accompanied by increased oxidative stress levels on admission and abnormal mtCIV recovery, which suggest a mitochondrial role in clinical outcome and LNS etiopathogenesis. However, it is difficult to separate these mitochondrial-derived effects from that induced by hypoxia. These patients presented increased prevalence of acute symptoms on admission, which could warn for future LNS development. A special clinical attention should be addressed to poisoned patients presenting prognostic factors for LNS development (either mitochondrial or clinical), although our results should be taken with caution due to the small size of our sample.

We assume that short treatment with normobaric oxygen previous to specific treatment assignment, similar in all patients, should not affect (or should affect the same way) all assessed COHb levels and mitochondrial results. However, one limitation of our study could be that the elapsed time between acute CO intoxication, hospital admission and first COHb assessment was shorter in the SHBO1 group of patients with respect to other treatment groups. Similarly, elapsed time between acute poisoning and specific oxygen treatment assignment was also shorter for SHBO1 patients with respect to SHBO2 subjects.

In summary, ACOP inhibits mtCIV function and hyperbaric oxygen is a better therapeutic option than normobaric oxygen to reverse mitochondrial abnormalities independently of the severity of intoxication; it is of note that one single hyperbaric oxygen session seems to be effective enough to promote mitochondrial recovery. Mitochondrial CIV activity could be a good marker for ACOP treatment effectiveness and, together with derived oxidative stress and hypoxia, could contribute to the development of CO-mediated LNS. Further studies should elucidate if prolonged mitochondrial injury in neurons underlies sequelae and whether mitochondrial therapeutic agents such as antioxidants could prevent or reverse mitochondrial damage, especially on those ACOP patients presenting prognostic factors for LNS development.

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# Hyperbaric oxygen therapy for carbon monoxide poisoning

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## Hyperbaric oxygen therapy for carbon monoxide poisoning

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Dear Editor,  
Annane et al.'s article on carbon monoxide (CO) poisoning remarks on the lack of clinical benefits of hyperbaric oxygen therapy based on the results of two randomized trials comparing treatment with normobaric (NBO) versus hyperbaric (HBO) oxygen or one versus two sessions of HBO therapy [1]. We recently published a similar study on CO-poisoned patients trying to establish

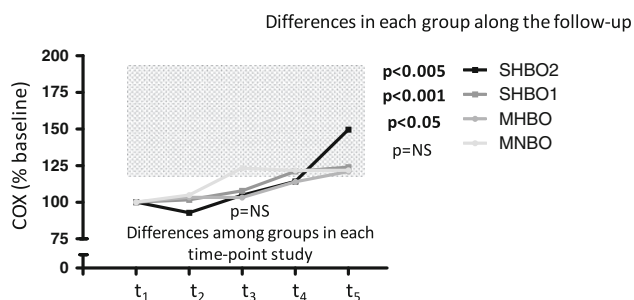
the therapy of choice for CO intoxication based, in addition to clinical symptoms, on mitochondrial functionality [2]. It is well known that CO inhibits mitochondrial cytochrome c oxidase (COX) function, and such inhibition could participate in acute symptoms and clinical outcome of CO poisoning, classically attributed to carboxyhemoglobin (COHb)-mediated hypoxia [3–5].

We studied COX activity in 35 severely CO-intoxicated patients (mean COHb  $23 \pm 1.4\%$ ) randomly treated with one ( $n = 21$ ) or two ( $n = 14$ ) HBO sessions [2]. After intoxication, COX activity was decreased by 42.5% compared with 30 healthy individuals matched by age and sex ( $54.80$  versus  $95.29$  nmol/min/mg protein) and progressively recovered after treatment, independently of the number of sessions administered (one versus two HBO sessions; Fig. 1). Our mitochondrial results in severely poisoned patients were in accordance with the lack of clinical benefits of one versus two HBO sessions reported by Annane and collaborators [1, 2].

We also studied COX function in 25 moderately CO-intoxicated patients

(mean COHb  $12.4 \pm 1.0$ ) randomly treated with NBO ( $n = 11$ ) or HBO ( $n = 14$ ) [2]. After intoxication, COX activity was decreased by 34.3% compared with age- and sex-matched controls ( $62.64$  versus  $95.29$  nmol/min/mg protein) and exclusively recovered its function in HBO-treated subjects (Fig. 1). Our mitochondrial results in moderately poisoned patients recommend usage of HBO with respect to NBO, in comparison with Annane's clinical results reporting lack of beneficial effects of HBO therapy [1, 2].

Molecular data in CO poisoning suggest a role for COX inhibition. Its slow recovery in the persistence of symptoms and delayed sequelae recommends HBO therapy, probably one single session, independently of intoxication severity [2]. Neither our findings nor those reported by Annane et al. support the usefulness of multiple-HBO-session therapy. However, both findings highlight divergences on the usefulness of HBO which may be due to distinct molecular or clinical effects of CO poisoning, different types of patients included in each study, different conditions of HBO administration, or differences in elapsed time before COHb assessment or before treatment randomization [1, 2]. Thus, the ideal treatment for CO poisoning remains controversial.



**Fig. 1** Cytochrome c oxidase (COX) activity measured in peripheral blood mononuclear cells following standard procedures [2–5] in severely carbon monoxide-poisoned patients treated with one or two hyperbaric oxygen sessions (SHBO1 or SHBO2) or moderately intoxicated subjects treated with hyperbaric or normobaric oxygen (MHBO or MNBO). Shaded area represents values of healthy controls (mean  $\pm$  standard deviation). t<sub>1</sub>, immediately after poisoning; t<sub>2</sub>, immediately after oxygen treatment; t<sub>3</sub>, t<sub>4</sub>, and t<sub>5</sub>, 24 h, 1 month, and 3 months after therapy. Statistical significance for changes on time-course COX activity were calculated by the analysis of variance (ANOVA) for repeated measures as follows: differences within groups by one-way ANOVA ( $p$  values quoted at left-hand side of the legend) and comparison among groups by two-way ANOVA (not significant;  $p$  value quoted on the graph)

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# Mitochondrial damage in adipose tissue of untreated HIV-infected patients

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**Objective:** Antiretrovirals, especially thymidine-analogue nucleoside reverse transcriptase inhibitors (tNRTIs), may cause the mitochondrial damage in adipose tissue that has been associated with lipodystrophy development. HIV itself may damage blood cell mitochondria. However, the viral capacity to induce adipose tissue mitochondrial lesion is still a matter of doubt. We aimed to assess whether untreated HIV infection was associated with adipose tissue mitochondrial abnormalities.

**Design:** Single-site, cross-sectional, controlled observational and exploratory study without intervention.

**Methods:** We included 24 uninfected controls and 18 HIV-infected patients with undetectable viral load and no clinical signs of lipodystrophy stratified as antiretroviral naive ( $n = 11$ ) or at least 6-month antiviral-treated with a double NRTI combination, including lamivudine plus one tNRTI ( $n = 7$ ). Subcutaneous adipose tissue was homogenated to determine mtDNA content by rtPCR and mitochondrial function per mitochondria through the spectrophotometric measurement of cytochrome c oxidase activity normalized by citrate synthase amount (COX/citrate synthase). Differences in mitochondrial parameters among groups were sought to determine the contribution of HIV and antiretrovirals to mitochondrial alterations.

**Results:** Compared with uninfected controls (arbitrarily assigned 100%), naive individuals presented a marked decrease in adipose tissue mtDNA content and COX/citrate synthase function (62 and 75% remaining content/activity,  $P < 0.001$  and  $P < 0.05$ ). Antiretrovirals did not increase this impairment (69 and 70% remaining content/activity,  $P < 0.05$  compared to controls and  $P =$  not significant compared to naives). Additionally, molecular and functional mitochondrial parameters were positively correlated ( $P < 0.05$ ).

**Conclusion:** In nonlipodystrophic HIV-infected naive patients, viral infection is associated with adipose tissue mtDNA decrease and mitochondrial dysfunction independently of antiretroviral treatment. © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins

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**Keywords:** adipose tissue, antiretroviral treatment, HIV, mitochondria, mitochondrial DNA, mitochondrial function/dysfunction, mitochondrial toxicity

## Introduction

Although clinical evidence of lipodystrophy is currently decreasing due to safer antiretroviral treatment (ART), lipodystrophy is still one of the most frequent adverse effects in HIV-infected patients on therapy.

Lipodystrophy etiology has not been completely elucidated, although ART is classically considered to play a main role. Some protease inhibitors and nonnucleoside reverse transcriptase inhibitors (non-NRTI) can trigger apoptosis that could underlie loss of peripheral adipose tissue [1,2]. Drugs belonging to protease inhibitor, NRTI, and non-NRTIs families could alter adipogenesis and adipocyte differentiation, which may contribute to lipodystrophy development [2–4]. Additionally, NRTIs could inhibit mitochondrial DNA (mtDNA) polymerase  $\gamma$ , the enzyme responsible for mtDNA synthesis and repair, thereby causing mtDNA depletion, which can lead to mitochondrial dysfunction and cell death [5]. This latter toxic antiretroviral effect, particularly attributed to thymidine analogues (tNRTIs), has been suggested to play a key role in lipodystrophy development [6].

Nonetheless, HIV and host-dependent factors (genetics and environment) are also thought to be involved [7]. Although adipocytes may be potentially infected by HIV, there is controversial evidence [8,9]. However, indirect effects of HIV infection, even under effective ART, have been reported in lipodystrophy adipose tissue to contribute to lipodystrophy [10,11]. These indirect effects include premature aging, immune activation, and inflammation through macrophage infiltration across adipose tissue and pro-inflammatory adipokine and cytokine secretion [11,12]. Additionally, HIV-infected and activated adipose tissue-resident macrophages could release viral proteins, which may contribute to adipocyte lesion [11].

Additionally, mtDNA depletion and mitochondrial impairment have been found in peripheral blood mononuclear cells (PBMCs) of ART-naïve patients, suggesting that HIV itself could cause mitochondrial lesion [13–15].

Classical studies have blamed NRTI therapies of mtDNA depletion and mitochondrial dysfunction found in adipose tissue of lipodystrophy [16–21] or nonlipodystrophy HIV patients [22–26]. However, the role of HIV in mitochondrial adipose tissue lesion is still a matter of doubt [10].

We hypothesized that adipose tissue mtDNA depletion and consequent dysfunction might be detected in

antiretroviral-naïve patients and that this impairment could be compensated in patients under effective ART without lipodystrophy.

## Methods

### Patients

We performed a single-site, cross-sectional, controlled observational exploratory study without intervention. Patients were consecutively included on their routine clinical visits in the Hospital Clinic of Barcelona (Barcelona, Spain), after signing the informed consent approved by the Ethical Committee of our hospital. We included 24 healthy volunteers and 18 asymptomatic HIV-infected adults matched by age and sex. Clinical data were recruited on admission. The inclusion criteria for HIV patients were diagnosis of HIV infection, age at least 18 years, and no clinical evidence of lipodystrophy [21]. HIV patients were stratified into two subgroups: 11 antiretroviral-naïve and seven on ART, to assess HIV and antiretroviral isolated or combined effects on mitochondrial adipose tissue lesion in non-lipodystrophy patients. Inclusion criteria for treated patients were undetectable viral load ( $< 50$  copies/ml) and receipt of stable double NRTI ART for at least 6 consecutive months prior to study entry consisting of lamivudine (3TC) and one tNRTI [stavudine (d4T) or zidovudine (ZDV)]. Double NRTI-treated patients with thymidine analogues were included to assess mitochondrial effects of ART classically considered harmful for mitochondria. Exclusion criteria were familial history of mitochondrial disease, treatment with mitochondrial toxic drugs (including tobacco and alcohol), opportunistic infections, or neoplasia.

### Sample

Approximately, 80 mm<sup>3</sup> (50 mg) of subcutaneous adipose tissue (SAT) was obtained by punch biopsy from the periumbilical region under local anesthesia. When it was not possible, SAT was obtained from the arm, respecting patient and clinician advice. Obtention of white adipose tissue was always confirmed by anatomopathological means. All biopsies were immediately frozen at  $-80^{\circ}\text{C}$  until analysis.

### Mitochondrial parameters

Mitochondrial parameters were measured in 10% (w/v) adipose tissue homogenates after skin, connective tissue, and blood removal from the biopsy.

Mitochondrial DNA was measured in 300–600 ng of total DNA extracted by phenol-chloroform. Only those samples showing a purity absorbance cut-off between 1.7 and 1.9 (260/280 nm ratio) were analyzed in triplicates. Quantitative rtPCR (efficiency replication  $2^{\text{p}}$  between 1.8 and 2) allowed mtDNA quantification by expressing the highly conserved mitochondrially encoded ND2 gene with respect to the nuclear-encoded housekeeping 18SrRNA gene (mtDNA/nuclear DNA; ND2/18SrRNA) [14]. In order to minimize interassay and intra-assay variability, samples from the three different kinds of patients were set together in the same rtPCR cycle and analyzed using the same internal curve of standard. PCR sensibility yielded between 3 and 30 000 ng for the amplified product. Positive and negative controls were systematically used and melting temperature test allowed specificity analysis for the amplified product of each sample.

Mitochondrial function was measured in duplicate by spectrophotometric quantification of cytochrome *c* oxidase activity (COX or mitochondrial complex IV; EC1.9.3.1) normalized by mitochondrial mass through citrate synthase measurement (citrate synthase; EC4.1.3.7, widely considered a reliable mitochondrial mass marker) and expressed as the COX/citrate synthase ratio [14]. Specific absorbance of spectrophotometric analyses was systematically monitored and, in order to minimize interassay and intra-assay variability, enzymatic activities of the three different kinds of patients were set together in the same cycle of analysis and performed using identical reagents.

### Statistical analysis

Clinical and epidemiological parameters are expressed as median values and 95% confidence interval of the mean and experimental results as percentages compared to median values of uninfected individuals (arbitrarily assigned 100%).

The minimum sample size to detect mitochondrial differences was estimated based on previous experimental PBMC results obtained in similar population groups [14].

Differences in mitochondrial parameters between groups and correlation between quantitative parameters were analyzed using nonparametric statistical tests, with level of significance set at 0.05.

## Results

Clinical and epidemiological characteristics of participants are summarized in Table 1. They were white, predominantly men (84–100%, depending on the group), with median age ranging from 37 to 47 years, depending on group assignment. Naive individuals had been recently HIV-diagnosed (median time 18 months after first seropositive HIV testing). Treated individuals, who had been HIV-infected and HAART-treated for a median times of 120 and 40 months, respectively, received at least 6 months of ART consisting of 3TC and one tNRTI (d4T/ZDV) prior to study entry. As expected, CD4<sup>+</sup> T-cell count and viral load were significantly increased and reduced, respectively, in HIV-treated patients compared to naive individuals because of therapeutic ART activity.

Compared with uninfected controls (assigned 100%), naive patients showed a marked decrease in SAT mtDNA content (62% remaining mtDNA,  $P < 0.001$ , Fig. 1a) and COX function (75% remaining COX/citrate synthase,  $P < 0.05$ , Fig. 1b). With respect to uninfected controls, HIV-treated individuals also showed a remarkable reduction in SAT mtDNA content (69% remaining mtDNA,  $P < 0.05$ , Fig. 1a) and COX function (70% remaining COX/citrate synthase,  $P < 0.05$ , Fig. 1b). However, mitochondrial parameters between both groups of infected individuals were similar irrespective of ART administration.

**Table 1. Clinical and epidemiological characteristics of individuals included in the study.**

	Uninfected ( <i>n</i> = 24)	HIV-Naive ( <i>n</i> = 11)	HIV-Treated ( <i>n</i> = 7)	<i>P</i> value <sup>a</sup>
Age (years) (median ± 95% CI of the mean)	47 [33–56]	37 [32–43]	44 [25–55]	NS
Male sex (%)	84	100	86	NS
Time on HIV (mon) (median ± 95% CI of the mean) <sup>b</sup>	–	18 [12–69]	120 [36–173]	NS
ART duration (mon) (median ± 95% CI of the mean)	–	–	40 [24–47]	–
2 NRTI regimen: 3TC+T-analogue (d4T/ZDV) ( <i>n</i> ) <sup>c</sup>	–	–	7	–
CD4 <sup>+</sup> T-cell count (cells/μl) (median ± 95% CI of the mean)	–	178 [79–435]	491 [242–810]	$P < 0.05$
Viral load (Log <sub>10</sub> copies RNA/μl) (median ± 95% CI of the mean) <sup>d</sup>	–	5.2 [3.7–5.9]	1.7 [1.1–3.4]	$P < 0.001$

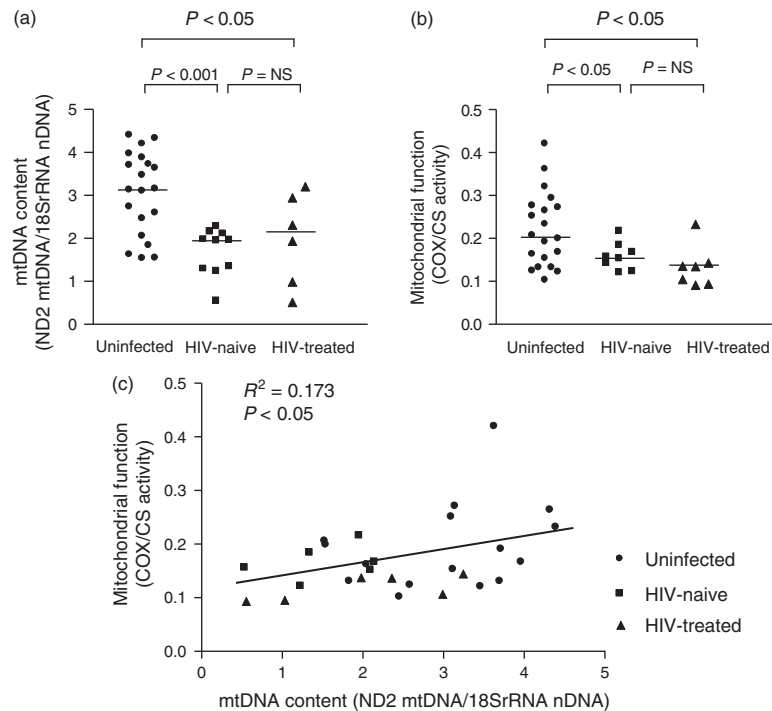
95% CI of the mean, 95% confidence interval of the mean; mon, months; NS, nonsignificant; y, years.

<sup>a</sup> $P < 0.05$  was considered to be statistically significant.

<sup>b</sup>Determined since the time after first seropositive HIV testing.

<sup>c</sup>NRTI, nucleoside analogue reverse transcriptase inhibitor; 3TC, lamivudine; T-analogue, thymidine analogue (d4T, stavudine or ZDV, zidovudine).

<sup>d</sup>Viral load < 50 copies/ml was considered to be 49 copies/ml for statistical analysis.



**Fig. 1. Mitochondrial parameters of patients included in the study.** Adipose tissue mitochondrial DNA content (a); mitochondrial function measured as cytochrome c oxidase activity related to mitochondrial mass (COX/citrate synthase relative units) (b); and correlation between both mitochondrial parameters (c). In (a) and (b), horizontal lines represent median values for each parameter and study group. NS, nonsignificant.

In HIV-infected patients, viral load negatively correlated with the levels of mtDNA and COX/citrate synthase function, being exclusively significant for COX/citrate synthase activity ( $P < 0.05$ ).

Additionally, both molecular and functional mitochondrial parameters (mtDNA content and COX/citrate synthase activity) were positively correlated ( $P < 0.05$ , Fig. 1c).

## Discussion

As an exploratory study, the number of included patients was small. The reduced sample size was due to the invasiveness of the approach, which required adipose tissue biopsy collection. Unfortunately, this is a common characteristic of many other studies in the literature [17–20,25,26]. However, despite the reduced number of cases, we were able to obtain conclusive results for the testing of our hypothesis. Other limitation of our study could be the different location of SAT. We did not use visceral adipose tissue or SAT susceptible to containing brown adipocytes (present on the buffalo hump depots of lipodystrophy patients). We analyzed periumbilical SAT of nonlipodystrophy individuals, unless clinician or patient advice asked for an alternative location. In those few cases, SAT was obtained from the arm. Therefore, we think that dual

SAT location should not affect our findings because both kinds of samples are constituted by white adipocytes and were always present in all the groups of patients analyzed.

Our results indicate that mtDNA depletion and associated mitochondrial dysfunction are present in SAT of naive patients, even just after 18 months of infection. Such finding corroborates our hypothesis that HIV itself may play a role in adipose tissue mitochondrial lesion, classically considered a characteristic feature of lipodystrophy. However, most of the studies performed on adipose tissue did not find mitochondrial differences between seronegative and naive [18,20,24,25] or treated HIV patients [25,26], except when tNRTI-treated individuals were individually compared. These studies mainly concluded that ART led to adipose tissue mitochondrial impairment [18–20,22–26] and demonstrated mitochondrial benefits of tNRTI interruption or switching to less mitotoxic schedules [7,17,27].

Based on our findings, mitochondrial damage in fat of naive individuals suggests that the virus itself may contribute to the mitochondrial dysfunction present in the target tissue of lipodystrophy, which is indeed confirmed by the negative correlation found between viral load and SAT mitochondrial function. These results corroborate previous findings of altered mitochondrial function in adipocytes [10,11] and PBMC [13,14] of naive patients. According to the present

findings, HIV could be contributing to the mitochondrial impairment found on adipose tissue of lipodystrophy patients, together with ART. That fact could explain rare cases of lipodystrophy in naive individuals and, above all, would explain reported association between decreased number of CD4<sup>+</sup> T lymphocytes and higher risk of lipodystrophy [28]. Such finding abrogates for the current established guidelines of early treatment management of HIV infection to avoid deleterious effects triggered by the virus and to prevent reservoirs constitution.

We did not observe additive mitochondrial toxicity when considering dual presence of HIV and ART, though considering tNRTI drugs with well known mitochondrial toxicity. This may be explained because ART-negative effects exerted against mitochondria may be balanced by ART-positive mitochondrial effects decreasing viral load and its derived mitotoxic capacity, thereby leading to similar levels of mitochondrial lesion in naive and treated patients. Such finding is in agreement with results of clinical trials reporting SAT increase in HIV individuals after tNRTI introduction [29,30]. However, we do not know future consequences of HIV-induced mitochondrial dysfunction compensated by ART into lipodystrophy development.

Both molecular and functional mitochondrial parameters were positively correlated confirming the strong dependence of mitochondrial function on mitochondrial genome [5,14]. A similar correlation was observed by Hammond *et al.* [24] in adipose tissue of HIV-treated patients concomitant with evidence of cellular toxicity. Homeostatic transcriptional and translational upregulatory mechanisms can compensate HIV and ART-induced mtDNA depletion in PBMCs [31]. However, observed adipose tissue mitochondrial dysfunction in naive and treated patients suggests that such upregulatory mechanism designed to preserve mitochondrial function may be weaker in adipocytes. This would explain adipocyte susceptibility to become damaged by mitotoxic agents and physiologic vulnerability of individuals to lipodystrophy. Additionally, the idea of a complex scenario involving other modulating factors (inflammation, oxidative stress, apoptosis, alteration of adipogenesis and adipocyte differentiation or adipokine and cytokine levels) is gaining in strength and may help to explain lipodystrophy and different regional fat behavior (visceral versus subcutaneous) [1–4,10–12]. Probably, all these factors condition lipodystrophy. Nonetheless, without HIV or ART mitotoxic activity, lipodystrophy would not probably be developed.

In summary, we have demonstrated that uncontrolled HIV infection is associated with mitochondrial abnormalities in SAT. These effects are partially compensated by ART, even when such treatment contains tNRTIs.

Clinical implications of these findings in the context of currently administered ART are unknown and would deserve prospective evaluation in longitudinal studies to assess mitochondrial effects of ART introduction in adipose tissue of HIV individuals.

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None of the abovementioned authors has any financial, consultant, institutional, and other relationship that might lead to bias or a conflict of interest for the present manuscript.

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# The Effects of Sepsis on Mitochondria

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**Background.** Sepsis is associated with mitochondrial dysfunction and impaired oxygen consumption, which may condition clinical outcome independent of tissue oxygenation. However, mitochondrial role in sepsis severity remains unknown. We aimed to characterize mitochondrial function in sepsis, establish its origin and cellular consequences, and determine its correlation with clinical symptoms and outcome.

**Methods.** Different markers of mitochondrial activity, nitrosative and oxidative stress, apoptosis, and inflammation were measured in peripheral blood mononuclear cells (PBMCs) and plasma of 19 septic patients and 20 controls. Plasma capacity to induce mitochondrial dysfunction was assessed in muscle mitochondria from 5 healthy individuals incubated with plasma of septic patients or controls.

**Results.** Despite unaltered mitochondrial mass and protein synthesis, enzymatic mitochondrial complexes I, III, and IV and oxygen consumption were significantly inhibited in sepsis. Septic plasma tended to reduce oxygen consumption of healthy mitochondria and showed significantly increased amounts of extracellular mitochondrial DNA and inflammatory cytokines, especially in patients presenting adverse outcome. Active nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) was also significantly increased, together with nitric oxide, oxidative stress and apoptosis. Additionally, sepsis severity significantly correlated with complex I inhibition, NFκB activation and intercellular adhesion molecule expression.

**Conclusions.** A plasmatic factor such as nitric oxide, increased in inflammation and able to induce mitochondrial dysfunction, oxidative stress and apoptosis, may be responsible for cell damage in sepsis. Together with bacterial infection, leakage of mitochondrial DNA from damaged cells into circulation could contribute to systemic inflammatory response syndrome. Mitochondrial dysfunction and inflammation correlate with sepsis severity and outcome, becoming targets for supporting therapies.

Sepsis is the systemic inflammatory response syndrome (SIRS) secondary to bacterial infection [1]. It is a complex defense mechanism triggered by microbial antigens and mediated through cytokines and cells [2]. It is characterized by activation of the inflammation and coagulation systems, which can lead to generalized hypoperfusion, multiorgan failure, and death [3]. The

risk factors currently associated with favorable or unfavorable outcome are poorly understood.

Cells of septic patients seem to be unable to maintain intermediate metabolism and, consequently, develop an energetic failure that may lead to cell death and threaten the patient's life [4]. Oxidative metabolism is highly energetic compared with anaerobic glycolysis, and thus the viability of high energy-dependent cells (especially neurons, myocytes, or hepatocytes) depends directly on continuous oxygen delivery. Many factors may limit the arrival of oxygen into the tissue of septic patients: pulmonary damage, reduction in preload and cardiac output due to increased permeability and vessel dilatation, decrease in left ventricular function due to impaired myocardial contractibility, and increased microthrombi formation due to increased erythrocyte deformation and local formation of platelets and leukocytes, among others.

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Liquid expansion, transfusions, and inotropic agents designed to improve oxygen arrival into tissues reportedly enhance the outcome of septic patients, albeit only in early stages [5]. Indeed, oxygen administration in advanced phases of the disease has been described as detrimental [6]. Direct measures of partial oxygen pressure in blood of subjects in septic shock have demonstrated that the oxygen amount is preserved, even increased, suggesting that oxygen consumption, but not oxygen availability, could be impaired [7, 8]. The hypothesis supporting the inability of septic cells to use the oxygen available has been called cytopathic hypoxia [9]. Different experimental models support an underlying cytopathic hypoxia by mitochondrial dysfunction. First, pyruvate dehydrogenase would be inhibited, leading to increased lactate production [10] and reduced mitochondrial energetic activity. A second mechanism would involve the activation of poly (adenosine diphosphate-ribose)polymerase-1 enzyme through DNA breakage (apoptosis), inflammation mediators [11], and reactive oxygen species (ROS), especially peroxynitrite (ONOO<sup>-</sup>) [12], which is considered a highly cytotoxic radical. The activation of this enzyme would reduce nicotinamide adenine dinucleotide, oxidized/nicotinamide adenine dinucleotide, reduced (NADH) content, leading to inhibition of mitochondrial respiratory chain (MRC) complex I (CI). Finally, endotoxins have been shown to promote inducible nitric oxide synthase activity and, consequently, increase the amount of nitric oxide (NO<sup>·</sup>) that directly binds to and limits MRC complex IV (CIV) [13]. Mitochondrial CIV is responsible for final oxygen reduction into water and, its inhibition could lead to mitochondrial dysfunction and oxidative energetic failure, even in an oxygen-rich atmosphere. Additionally, NO<sup>·</sup> can inhibit the mitochondrial adenosine triphosphate mitochondrial adenosine triphosphate/proton synthase (ATPase)/H<sup>+</sup> synthase responsible for energetic cell production [14]. Moreover, interaction of NO<sup>·</sup> with O<sub>2</sub><sup>-</sup> generates ONOO<sup>-</sup> that is able to inhibit mitochondrial CI [15]. Adequate control of all these mechanisms could be more relevant for cell survival than correct tissular perfusion and oxygen delivery into tissues.

Most of these findings have been reported in experimental models. In humans there is little evidence of mitochondrial impairment in sepsis. One study performed in quadriceps muscle tissue of severe septic patients established a relationship between NO<sup>·</sup> production, antioxidant depletion, adenosine triphosphate decrease, and MRC dysfunction, especially mediated by CI impairment [16]. Other studies have found differences on comparing respiratory and leg muscles [17, 18]. Nonetheless, perfusion is not guaranteed in muscle studies because of the septic process, and thus the results reported could be partially due to hypoxia, particularly in the development of shock. Additionally, muscle biopsy constitutes an invasive approach, making longitudinal follow-up in patients with unstable clinical conditions difficult.

Mitochondrial studies in peripheral blood mononuclear cells (PBMCs), particularly after setting the methodological conditions

for mitochondrial CI activity measurement [19], are currently a good alternative to study mitochondrial dysfunction [20]. Circulating cells are not affected by an eventual hypoxemia and, because they participate in immune response, may exemplify the immune dysfunction underlying septic SIRS.

It has recently been shown that leakage of mitochondrial DNA (mtDNA) or mitochondrial peptides into the bloodstream in different diseases (trauma, cancer, or human immunodeficiency virus [HIV] chronic inflammation) [21–23] can activate the immune system and may contribute to SIRS and compromise organ function.

The aim of this study was to assess mitochondrial function in circulating PBMCs of patients in the initial stages of sepsis, in the absence of shock. As secondary objectives, we aimed to characterize the levels of plasmatic mtDNA, inflammatory cytokines, nitric oxide, oxidative stress, and apoptosis in these patients and determine their origin. The hypothesis is that disarrangement in these metabolic pathways may contribute to or be the result of a cell energetic failure that correlates with clinical manifestations and septic outcome.

## MATERIAL AND METHODS

### Patients

From March 2005 to November 2009, we included 19 patients consecutively admitted to the Hospital Clinic of Barcelona for infectious SIRS. Inclusion criteria for septic patients were the presence of an identifiable site of infection and evidence of SIRS without septic shock, as manifested by 2 of the following signs: temperature >38°C or <36°C, heart rate >90 beats/min, respiratory rate >20 breaths/min or partial pressure of carbon dioxide in the blood (PaCO<sub>2</sub>) <32 mmHg, and white blood cell count >12 × 10<sup>9</sup> cells/L, <4 × 10<sup>9</sup> cells/L, or >10% of immature forms.

Twenty healthy volunteers, matched by age and sex with septic patients, were included as controls for blood analysis. Additionally, for the isolation of mitochondria, 5 additional healthy volunteers undergoing hip replacement surgery were included for skeletal muscle collection.

Exclusion criteria for both patients and controls were the presence of a family history of primary mitochondrial disease, treatment with potential mitochondrial drugs [24], and septic shock. All individuals or their next of kin, in patients with altered consciousness, gave informed consent to be included in this protocol, which had been previously approved by the ethical committee of our hospital.

### Samples

#### Plasma

Plasma was collected in ethylenediaminetetraacetic acid tubes from 20 mL of whole peripheral blood by centrifugation at 1500 g for 15 minutes and immediately stored at -80°C until

analysis. Plasma was used for the quantification of NO<sup>-</sup>, mtDNA, and cytokine levels and the screening of potential inducers of mitochondrial dysfunction.

#### **Peripheral Blood Mononuclear Cells**

Once plasma was collected, PBMCs were isolated by Ficoll density gradient centrifugation [25]. PBMCs were used to measure mitochondrial, apoptotic, and oxidative stress markers; fresh cells were used to measure mitochondrial membrane potential and oxygen consumption, and frozen cells were used for the remaining measures.

#### **Isolation of Mitochondria**

Mitochondria were isolated from skeletal muscle of healthy controls according to the methodology of [26], and mitochondria were incubated with either septic or control plasma in order to search for potential plasmatic inducers of mitochondrial dysfunction able to impair oxygen consumption of healthy mitochondria.

#### **Protein Content**

All PBMCs or skeletal muscle parameters were normalized to protein content measured according to the Bradford protein-dye binding-based method [27].

### **Mitochondrial Markers**

#### **Mitochondrial Protein Synthesis**

Mitochondrial protein synthesis was assessed by Western blot immunoquantification of the mitochondrial-encoded cytochrome c oxidase (COX) II subunit and the nuclear-encoded COX-IV subunit, both components of the mitochondrial CIV, normalized by the amount of  $\beta$ -actin protein to establish the relative COX-II or COX-IV abundance per overall cell protein [28].

#### **Global and Specific MRC CI-Stimulated Oxygen Consumption**

Global and specific MRC CI-stimulated oxygen consumption was measured by polarography using a thermostated Clark electrode at 37°C. Different oxidative activities were measured: the first was performed in intact cells as indicative of spontaneous and endogenous substrate consumption, and the second was undertaken in digitonin-permeabilized cells using specific electron donors and inhibitors of CI to specifically quantify CI-stimulated oxygen consumption. Oxidative activities were expressed as nanomoles of oxygen per minute and milligram of cellular protein [26, 29].

#### **Enzymatic Activity of MRC Complexes I, III and IV**

The enzymatic activity of MRC complexes I, III, and IV were measured by thermostated spectrophotometry at 37°C according to the methodology of Rustin et al [26], slightly modified for CIV measurement in minute amounts of biological samples [30]. Measurement of CI activity required previous treatment of cells with triton and digitonin detergents to obtain specific mitochondrial NADH-dehydrogenase activity [19]. Enzymatic activities were expressed as nanomoles of consumed substrate or generated product per minute and milligram of protein [19, 26, 30].

#### **Mitochondrial Content**

Mitochondrial content was estimated by thermostated spectrophotometry at 37°C with the measurement of citrate synthase (CS) activity [28] because CS is a Krebs cycle enzyme widely considered as a reliable marker of mitochondrial amount [26, 31–33].

#### **Plasmatic Mitochondrial Dysfunction Inducer Analysis**

Skeletal muscle mitochondria from healthy controls were incubated for 30 minutes at 37°C with plasma either from septic patients or healthy volunteers. After incubation we measured CI- or CIV-stimulated oxygen consumption by polarography to search for eventual plasmatic inducers of mitochondrial dysfunction.

### **Apoptotic Markers**

#### **Mitochondrial Membrane Potential**

Flow cytometry was used to measure mitochondrial membrane potential by JC-1 staining [34]. The results were expressed as the percentage of PBMCs with depolarized mitochondria compared with the total amount of PBMCs analyzed and were interpreted as a marker of early apoptosis.

#### **Caspase 3**

The activation of this protein was determined by Western blot immunoquantification of cleaved caspase 3 proapoptotic protein normalized by the content of  $\beta$ -actin protein as a cell loading control. Chemoluminescence results were expressed as caspase 3/ $\beta$ -actin relative content and were interpreted as a marker of advanced apoptosis.

### **Inflammatory Markers**

#### **Plasmatic mtDNA Content**

Extracellular mtDNA was isolated from 400  $\mu$ L of filtered plasma (using a 0.22- $\mu$ m filter pore to exclude circulating bacteria) through the QIAamp DNA Blood Mini Kit (Qiagen), as described elsewhere [21–23, 35]. A 235-base pair fragment of the mitochondrial-encoded *ND2* gene was quantified by SYBER-green and Roche rtPCR Technology [28] after testing specificity through melting temperature analysis and checking for no sequence homology with DNA found in any bacterial species published on the basic local alignment search tool. Plasmatic mtDNA content was expressed as the absolute amount of *ND2* gene per milliliter of plasma [35].

#### **Plasmatic Cytokines**

Plasmatic cytokines were assessed with Luminex technology, allowing simultaneous measurement through internal curve quantification of either plasmatic interleukin (IL) 6, tumor necrosis factor (TNF)  $\alpha$  and monocyte chemotactic protein 1 (MCP-1) molecules or intercellular adhesion molecule (I-CAM) and vascular cell adhesion protein (V-CAM) cytokines.

#### **Nuclear Factor Kappa-Light-Chain Enhancer of Activated B Cells**

Nuclear factor kappa-light-chain enhancer of activated B cells (NF $\kappa$ B) was assessed by Western blot immunoquantification of



phosphorylated and active NF $\kappa$ B protein expression normalized by the content of total NF $\kappa$ B protein as a cell loading control. Chemoluminescence results were expressed as phosphorylated/total NF $\kappa$ B relative content and were interpreted as a marker of inflammation, cellular stress, and survival.

### Nitrosative and Oxidative Stress Markers

#### Plasmatic NO $\cdot$

Plasmatic NO $\cdot$  was indirectly measured by the spectrophotometric measurement at 540 nm of plasmatic nitrate and nitrite amount (both products of NO $\cdot$  metabolism) using the Griess assay adapted to plasma samples [36] in the Lipid-LPL Unit of the University of Barcelona (Titertek Multiskan PLUS MKII) [37].

#### Lipid Peroxidation

Lipid peroxidation was measured as an indicator of oxidative damage of ROS in cellular lipid compounds using the Oxys Research kit of Deltaclon by spectrophotometric measurement of malondialdehyde (MDA) and 4-hydroxyalkenal (HAE), both products derived from fatty acid peroxide decomposition, normalized by protein content and expressed as the concentration of MDA and HAE (in micromoles per liter) per milligram of cell protein [38].

#### Statistical Analysis

Mitochondrial, inflammatory, apoptotic, nitrosative, and oxidative stress markers were compared between septic and healthy subjects. The results were expressed as means  $\pm$  standard errors

of the mean or as percentages of decrease or increase with respect to healthy controls. Differences between groups were determined with the nonparametric Mann–Whitney test for independent measures and correlation between quantitative parameters with the nonparametric Spearman analysis. The cutoff for significance ( $P$  value) was set at .05.

## RESULTS

The main characteristics of the septic patients at inclusion are shown in Table 1. The patients included were predominantly female (15/19) with a mean age of 64 years. The primary sites of infection were thoracic (6/19), urinary (5/19), abdominal (3/19), and skin (3/19). At inclusion, the patients were in the initial stages of SIRS and presented a mean simplified acute physiology score (SAPS II) of 45.5 and a sequential organ failure assessment (SOFA) value of 7.6, corresponding to moderate to severe sepsis.

Mitochondrial PBMC markers showed unaltered protein expression for MRC subunits compared with controls, considering either mitochondrial-encoded (COX-II) or nuclear-encoded (COX-IV) proteins. The number of mitochondria in PBMCs of septic patients was also not modified (data not shown). On the contrary, PBMCs of septic patients compared with healthy subjects showed a reduction of 32% in CI function ( $P < .05$ ), a decrease of 42% in CIII activity ( $P < .05$ ), and an inhibition of

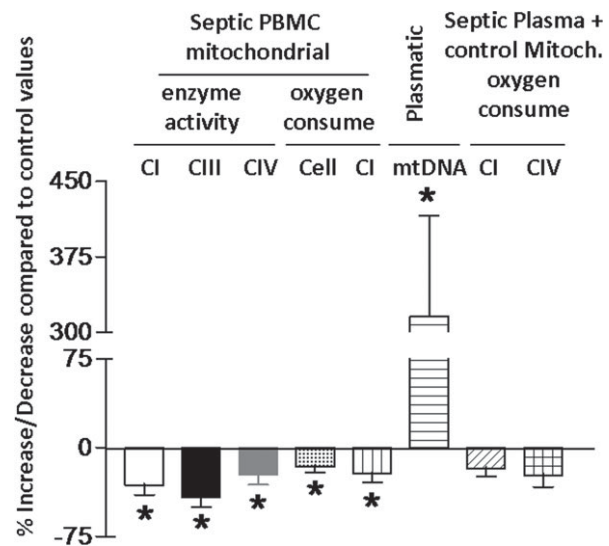
**Table 1. Clinical and Epidemiological Characteristics of the Patients Included in the Study**

Characteristic	Value
Age, mean years $\pm$ SD	64 $\pm$ 18
Sex, No. female/No. male	15/4
Site of infection, No.	
Thoracic	6
Urinary	5
Abdominal	3
Skin	3
Other	2
SAPS II in first 24 h <sup>a</sup>	45.5 $\pm$ 13.3
SOFA <sup>a</sup>	7.6 $\pm$ 3.2
Sepsis outcome, No. (%)	
Death <sup>b</sup>	6 (32)
Survival	13 (68)

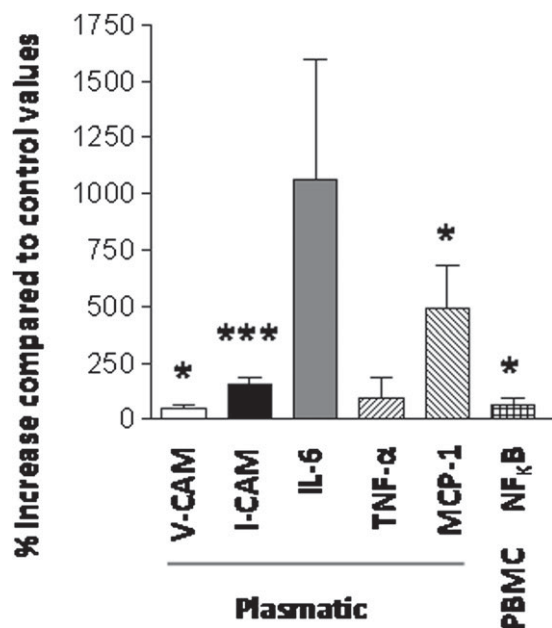
Abbreviations: SAPS II, simplified acute physiology score; SD, standard deviation; SOFA, sequential organ failure assessment.

<sup>a</sup> Both the SAPS II and SOFA scales classify sepsis severity from null (0 score) to severe forms of the disease (maximum score is 60 for the SAPS II scale and 10 for the SOFA). Individual values in the SAPS II and SOFA scales were calculated for each patient, at study inclusion, specifically punctuating all physiological measures indicative of sepsis development included in both measures.

<sup>b</sup> Patients who died had comorbidities with either lymphoma, myeloblastic syndrome, metastatic lung cancer, Chronic obstructive lung disease stage IV, or Alzheimer dementia.



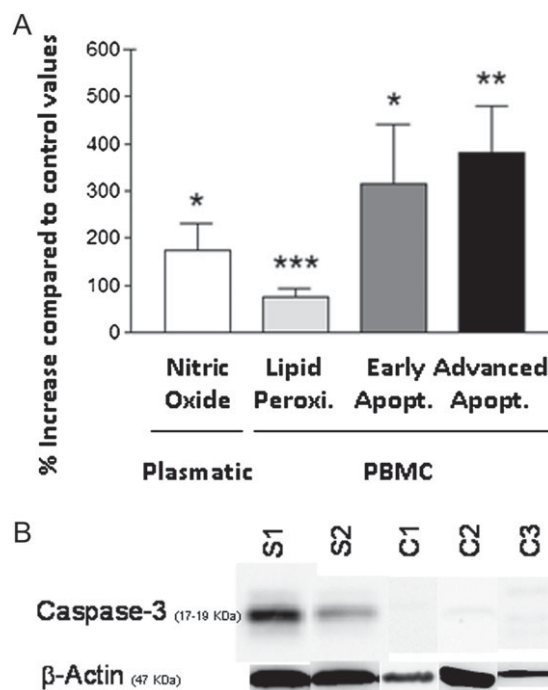
**Figure 1.** Mitochondrial markers in peripheral blood mononuclear cells (PBMCs) of septic patients and in mitochondria of healthy individuals incubated with plasma of septic patients, with respect to controls. Bars represent the percentage of increase/decrease in septic patients with respect to controls. Abbreviations: Cell, spontaneous cell (oxygen consumption); CI, mitochondrial respiratory chain complex I; CIII, mitochondrial respiratory chain complex III; CIV, mitochondrial respiratory chain complex IV; mitochon, mitochondria; mtDNA: mitochondrial DNA. \* $P < .05$  between cases and controls.



**Figure 2.** Inflammatory markers of plasmatic cytokine expression and peripheral blood mononuclear cells (PBMCs) NF $\kappa$ B activation in septic patients with respect to controls. Bars represent the percentage of increase in septic patients with respect to controls. Abbreviations: I-CAM, intercellular adhesion molecule; IL-6, interleukin 6; MCP-1, monocyte chemotactic protein 1; NF $\kappa$ B, nuclear factor kappa-light-chain enhancer of activated B cells; TNF $\alpha$ , tumor necrosis factor $\alpha$ ; V-CAM, vascular cell adhesion protein. \*  $P < .05$ ; \*\*\* $P < .001$  between cases and controls.

mitochondrial CIV of 23% ( $P < .05$ ) (Figure 1). Consequently, the capacity of PBMCs of septic patients to consume oxygen was also significantly impaired for either endogenous substrates (spontaneous cell oxygen consumption, 16% decrease;  $P < .05$ ) or CI-stimulated oxygen consumption (22% decrease;  $P < .05$ ) (Figure 1). Additionally, plasma of septic patients incubated with mitochondria from healthy individuals tended to decrease the oxidative capacity of control mitochondria to consume oxygen, although not significantly (18% decrease in CI-stimulated oxygen consumption and 23% decrease in CIV-stimulated respiration, not significant [NS]) (Figure 1).

Inflammatory markers increased in plasma of septic patients compared with controls. The specific increase in proinflammatory cytokines was 46% for V-CAM ( $P < .05$ ), 155% for I-CAM ( $P < .001$ ), 1062% for IL-6 (NS), 98% for TNF- $\alpha$  (NS) and 494% for MCP-1 ( $P < .05$ ) (Figure 2). Moreover, the amount of circulating free mtDNA was significantly increased in plasma of septic patients with respect to controls (316% increase;  $P < .05$ ). Plasmatic mtDNA content significantly and positively correlated with V-CAM and TNF- $\alpha$  expression ( $P < .05$ ; data not shown). Additionally, phosphorylated and transcriptional active NF $\kappa$ B increased 68% in PBMCs of septic patients compared with healthy volunteers ( $P < .05$ ) (Figure 2). Nitrosative and oxidative stress markers (nitric oxide amount and lipid peroxidation levels) were

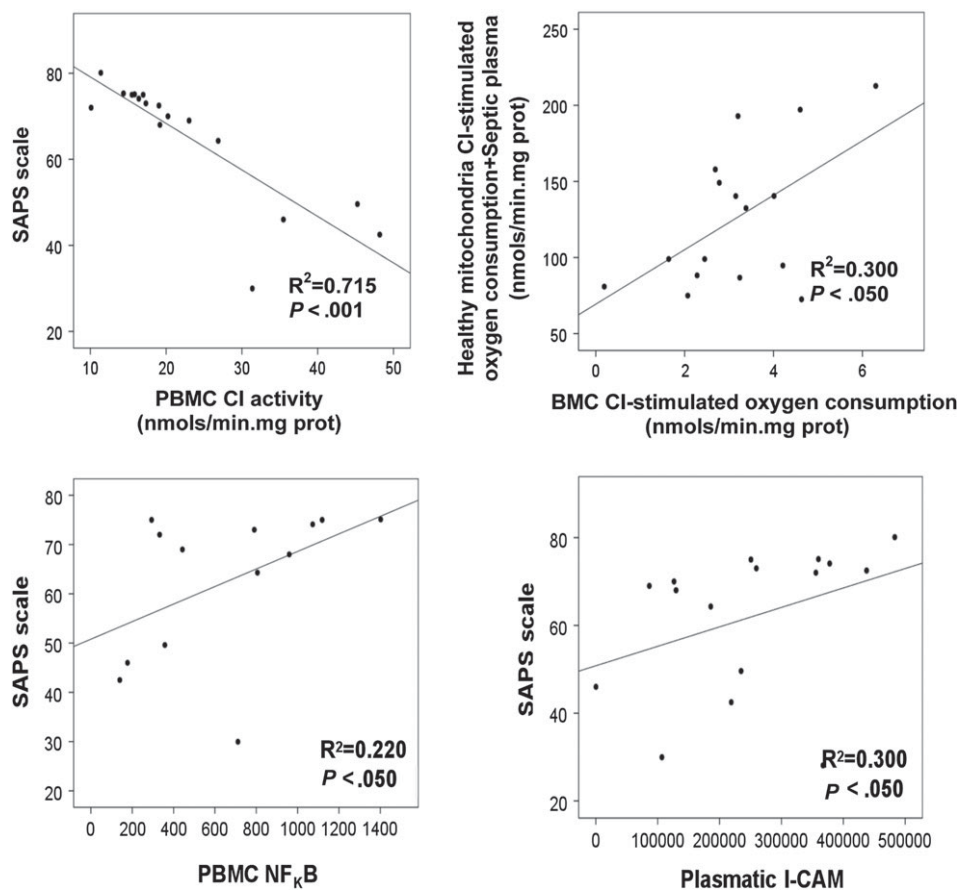


**Figure 3.** Nitrosative, oxidative, and apoptotic markers in plasma and peripheral blood mononuclear cells (PBMCs) of septic patients with respect to controls. *A*, Plasmatic nitric oxide levels and PBMC oxidative stress and early (mitochondria membrane potential) or advanced (active caspase 3/ $\beta$ -actin) apoptosis development. Bars represent the percentage of increase in septic patients with respect to controls. Apopt., apoptosis; Lipid Peroxi., lipid peroxidation. \* $P < .05$ ; \*\* $P < .005$ ; \*\*\* $P < .001$  between cases and controls. *B*, Western blot analysis of advanced apoptosis (active caspase 3 amount/ $\beta$ -actin) in PBMCs of septic patients. Note increased active caspase 3 in PBMCs of septic patients with respect to controls for similar amounts of  $\beta$ -actin signal.

also increased 174% and 76%, respectively, in PBMCs of septic subjects compared with controls ( $P < .05$  and  $P < .001$ ) (Figure 3A).

All apoptotic markers were also significantly increased. The percentage of cells with depolarized mitochondria, indicative of early apoptosis, increased 315% in septic PBMCs compared with controls ( $P < .05$ ) and the expression of active (cleaved) caspase 3 protein with respect to  $\beta$ -actin amount, indicative of advanced apoptosis, increased 380% ( $P < .005$ ) (Figure 3A and 3B).

Sepsis severity measured using the SAPS scale negatively correlated with PBMC CI enzymatic function ( $R^2 = 0.715$ ;  $P < .001$ ) (Figure 4). Additionally, CI-stimulated oxygen consumption in PBMCs of septic patients and mitochondria from control individuals incubated with septic plasma were positively correlated ( $R^2 = 0.300$ ;  $P < .05$ ) (Figure 4). Furthermore, sepsis severity was positively correlated with NF $\kappa$ B activation and I-CAM expression (both  $P < .05$ ) (Figure 4). Finally, V-CAM, MCP-1, and IL-6 cytokine expression and lipid peroxidation were abnormally increased in septic patients presenting a fatal



**Figure 4.** Correlations among mitochondrial or inflammatory markers and clinical parameters.

outcome, compared with patients with a favorable disease evolution ( $P < .05$ ; all cases) (Figure 5).

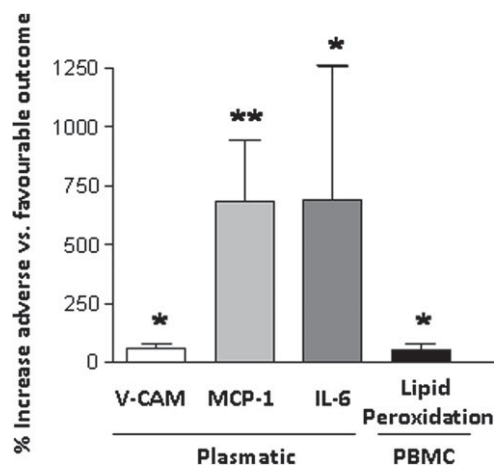
## DISCUSSION

Our findings support the presence of general mitochondrial dysfunction in patients in early stages of sepsis, in the absence of shock or multiple organ failure. Such mitochondrial impairment was first theoretically suggested because of clinical evidence of impaired oxygen extraction in sepsis, despite apparent correct tissular perfusion. Mitochondrial dysfunction could be interpreted as a normal physiological response to metabolic changes occurring in sepsis because metabolic slow-down has been suggested to play a protective role [39]. However, here we describe a multitude of simultaneous disarrangements that suggest a primary and pathological role for mitochondrial dysfunction.

In PBMCs of septic patients we found that mitochondrial protein synthesis was unaltered, despite a previous report showing altered protein expression in patients with sepsis-induced multiple organ failure [40]. The mitochondrial number was also not altered in our series of septic patients. However, we found a global mitochondrial dysfunction affecting different MRC

enzymes (CI, CIII, and CIV) and oxygen consumption (spontaneous or CI stimulated) responsible for energy supply. Mitochondrial dysfunction of CI and CIV has been reported elsewhere in muscle tissue of patients with septic shock and multiorgan failure [16, 40]. Our results confirm that mitochondrial dysfunction is present in the PBMCs of septic patients regardless of the absence of these clinical manifestations. Consequently, mitochondrial dysfunction seems to be a primary event in sepsis, whereas mitochondrial protein synthesis deregulation may be exclusively attributed to advanced stages of sepsis in which multiorgan failure is frequent.

Mitochondria are the respiratory and energetic centers of cells. However, mitochondrial dysfunction enhances ROS production, especially when CI and CIII are impaired. ROS are highly unstable molecules that attack cellular structures, including nucleic acids, lipids, carbohydrates, and proteins, causing oxidative stress damage that can lead cells to apoptosis. Although impaired oxidative metabolism has been suggested elsewhere to play a role in sepsis through increased superoxide dismutase activity [40], our study is the first to correlate increased oxidative stress with an unfavorable outcome. Additionally, apoptotic and  $\text{NF}_\kappa\text{B}$  levels were also increased in cells of septic patients.



**Figure 5.** Markers of plasmatic inflammation and peripheral blood mononuclear cells (PBMCs) oxidative stress depending on sepsis outcome. Bars represent the percentage of increase in septic patients with fatal outcome with respect to septic patients presenting a favorable evolution. Abbreviations: I-CAM, intercellular adhesion molecule; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein 1; V-CAM: vascular cell adhesion protein. \* $P < .05$  between septic patients with a favorable or fatal septic outcome; \*\*  $P < .005$ .

Apoptosis, especially when cell death involves immune system effectors, may be particularly relevant in septic patients that require effective function of their defense cells.

Our results suggest that, in the context of sepsis, mitochondrial dysfunction may increase oxidative stress, which could, in turn, enhance apoptosis. All of these physiopathological processes may be linked and may be primarily caused by mitochondrial dysfunction, as suggested elsewhere [41]. However, the specific mechanisms that underlie mitochondrial dysfunction and derived oxidative and cell damage have only been theoretically suggested.

Other studies have demonstrated that mitochondrial dysfunction is inducible in healthy cultured cells with plasma of septic patients [42]. We have confirmed this finding in isolated mitochondria with CI- and CIV-stimulated oxygen consumption deficiency. Nonetheless, our results were not statistically significant, perhaps owing to the small sample size or to inappropriate incubation conditions. However, we show, for the first time, a positive correlation between the mitochondrial lesion induced by septic plasma in mitochondria from healthy controls and the mitochondrial damage present in PBMCs from the same septic patients. This finding confirms the existence of a plasmatic agent able to induce the systemic mitochondrial lesion present in cells of septic patients and potentially responsible for interference in ROS metabolism and, consequently, for triggering apoptosis.

We found abnormally increased amounts of  $\text{NO}\cdot$  and cytokines in the plasma of septic patients. These cell messengers are produced as a consequence of bacterial infection; additionally,  $\text{NO}\cdot$  is a mediator of inflammatory response secondary to infection.  $\text{NO}\cdot$  is formed by constitutive and inducible NO synthases in response

to inflammation, and although vital for cell life in standard circumstances, an increase in  $\text{NO}\cdot$  concentrations over certain physiological ranges may induce oxidative stress and mitochondrial dysfunction. Inhibition of CIV has been suggested to be caused by direct  $\text{NO}\cdot$  binding to the CIV core. Moreover,  $\text{NO}\cdot$  interaction with  $\text{O}_2^-$  leads to the formation of  $\text{ONOO}\cdot$ , which has also been suggested to bind and inhibit mitochondrial CI. We observed inhibited CIV function in PBMCs of septic patients and, additionally, for the first time, we observed that the inhibition of CI was directly related to sepsis severity determined with the SAPS scale. Consequently, mitochondrial dysfunction may be responsible, at least in part, for the clinical symptoms and evolution of sepsis, which seem to be caused by some plasmatic factor similar to  $\text{NO}\cdot$  presenting dual mitotoxic and cytotoxic effects. Our study corroborates in blood cells (not susceptible to hypoperfusion) the results of Bradley and Singer performed in muscle tissue [39], suggesting that exacerbated immunological response in sepsis may enhance  $\text{NO}\cdot$  above physiological ranges [43], causing the derived mitochondrial, oxidative, and cell lesions. Thus, the study and potential treatment of mitochondrial dysfunction and  $\text{NO}\cdot$  metabolism could be of interest in the clinical management of sepsis.

Furthermore, extracellular free mtDNA levels were increased in septic patients, suggesting that immune activation and SIRS may not be exclusively restricted to infectious pathogens but also secondarily caused by circulating mitochondrial antigens. Such mitochondrial compounds (especially mtDNA or formyl peptides), also called damage-associated molecular patterns (DAMPs), were first described to cause inflammatory responses to injury in cases of trauma, some cancers, or chronic HIV inflammation [21–23] due to analog immune activation to that caused by microbial pathogen-associated molecular patterns (PAMPs). In sepsis, mitochondrial DAMPs could probably be released into the bloodstream by leakage from cells damaged through mitochondrial, apoptotic, or inflammatory means.

Patients presenting severe acute symptoms and adverse clinical outcome showed increased levels of mitochondrial dysfunction (CI activity), oxidative stress, and inflammatory response (I-CAM, V-CAM, MCP-1, IL-6, and  $\text{NF}\kappa\text{B}$  levels). This observation was reported elsewhere for an increase in IL-6 [44] and other proinflammatory cytokines [45], suggesting that all these mechanisms of physiological response to bacterial infection could influence the development of sepsis and patient survival and are, together with  $\text{NO}\cdot$ , prognostic factors of disease progression. Further studies interfering in the  $\text{NF}\kappa\text{B}$  transduction pathway or ROS metabolism could help discard alternative mechanistic explanations.

A limitation of the present study is that PBMCs may be poorly representative of postmitotic tissues more directly related to mortality and multiorgan failure in sepsis, such as heart, liver, brain, or muscle. PBMCs are immune system effectors that



become activated during infection, and their eventual adaptation to energy supply may differ from cells that exclusively rely on mitochondria to meet their energetic demands. On the other hand, PBMCs are easy to obtain, do not require invasive approaches, are not susceptible to the hypoperfusion found in muscle biopsies of septic patients, and have been validated elsewhere in the study of many other mitochondrial disorders.

If the inability of septic patients to consume oxygen is due not to hypoxia (lack of oxygen transport into tissues) but rather to energetic and metabolic failure of mitochondria (cytopathic hypoxia and consequent oxidative damage that could lead cell to apoptosis), clinical measures aimed at preserving organ and tissue function should be modified to potentiate mitochondrial function, in addition to providing adequate perfusion and oxygenation.

## Notes

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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# Perinatal outcomes, mitochondrial toxicity and apoptosis in HIV-treated pregnant women and in-utero-exposed newborn

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**Objective:** Highly active antiretroviral therapy (HAART) has decreased the risk of HIV mother-to-child transmission. However, HIV and HAART have been associated with adverse perinatal outcome. HAART has been associated with mitochondrial dysfunction in nonpregnant adults, and HIV, additionally, to apoptosis. We determined whether mitochondrial toxicity and apoptosis are present in HIV-pregnant women and their newborns and could be the basis of adverse pregnancy outcome.

**Design:** Single-site, cross-sectional, controlled observational study without intervention.

**Methods:** We studied mitochondrial and apoptotic parameters in mononuclear cells from maternal peripheral blood and infant cord blood at delivery in 27 HIV-infected and treated pregnant women, and 35 uninfected controls and their infants, to correlate clinical outcome with experimental findings: mitochondrial number (CS), mtDNA content (ND2/18SrRNA), mitochondrial protein synthesis (COX-II/V-DAC), mitochondrial function (enzymatic activities) and apoptotic rate (caspase-3/ $\beta$ -actin).

**Results:** Global adverse perinatal outcome, preterm births and small newborn for gestational age were significantly increased in HIV pregnancies [odds ratio (OR) 7.33, 5.77 and 9.71]. Mitochondrial number was unaltered. The remaining mitochondrial parameters were reduced in HIV mothers and their newborn; especially newborn mtDNA levels, maternal and fetal mitochondrial protein synthesis and maternal glycerol-3-phosphate + complex III function (38.6, 25.8, 13.6 and 31.2% reduced, respectively,  $P < 0.05$ ). All materno-fetal mitochondrial parameters significantly correlated, except mtDNA content. Apoptosis was exclusively increased in infected pregnant women, but not in their newborn. However, adverse perinatal outcome did not correlate mitochondrial or apoptotic findings.

**Conclusions:** Transplacental HAART toxicity may cause subclinical mitochondrial damage in HIV-pregnant women and their newborn. Trends to increased maternal apoptosis may be due to maternal-restricted HIV infection. However, we could not demonstrate mitochondrial or apoptotic implication in adverse perinatal outcome.

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**Keywords:** antiretroviral treatment, apoptosis, HIV, infants, mitochondrial DNA, mitochondrial toxicity, pregnancy

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## Introduction

Antiretroviral therapy (ART) during pregnancy is critical to prevent mother-to-child transmission (MTCT) of human immunodeficiency virus (HIV) infection and to delay disease progression [1]. Widespread use of antiretrovirals (ARVs) has been accepted for the prevention of MTCT, despite the lack of safety data related to human pregnancies [2–4].

The potential clinical risks associated with ARV exposure in HIV-pregnant women, fetus and infants have been reported by observational studies with varying strengths of evidence and conflicting results [5–9]. ARVs have been associated with adverse pregnancy outcomes such as preeclampsia, fetal death, preterm birth and low birth weight, although controversial results have been published [10–15].

Several physiopathological mechanisms have been proposed to explain the adverse clinical effects in HIV pregnancies. The negative mitochondrial and clinical effects of nucleoside reverse transcriptase inhibitors (NRTIs) used in highly active antiretroviral therapy (HAART) have been firmly established in HIV-infected nonpregnant adults [16–18]. These negative effects depend on the capacity of the NRTI to inhibit DNA  $\gamma$ -polymerase, the only enzyme devoted to the replication (and to a lesser extent, the repair) of the mitochondrial DNA (mtDNA) genome, thus leading to a decrease in mtDNA copy number and quality, which, in turn, may finally cause mitochondrial dysfunction [16].

Such mitochondrial abnormalities may be magnified by the effects of HIV itself, since it has been demonstrated to cause diffuse mitochondrial alterations, either *in vivo* or *in vitro*, probably through the activation of apoptotic mechanisms triggered by HIV proteins [19–21]. Additionally, increased apoptosis rates have also been demonstrated after *in-vitro* exposure to NRTI and in tissue from patients using ARV drugs [22].

The known patterns of mitochondrial and apoptotic toxicity in HIV patients receiving NRTI therapy suggest that some children, though HIV-uninfected, may be at risk of developing sequels from *in-utero* HIV and NRTI exposure [5–15,23–32]. Mitochondrial toxicity along pregnancy has been studied in animal models including uninfected pregnant monkeys exposed to ART. This model demonstrates the association between *in-utero* NRTI exposure and fetal tissue mtDNA depletion, altered mitochondrial respiratory chain (MRC) function and mitochondrial dysmorphology [23].

Blanche *et al.* [24] associated, for the first time, mitochondrial dysfunction with the manifestation of perinatal hyperlactaemia and neurological and developmental sequels in NRTI-exposed children from HIV

mothers. Although few abnormal clinical findings are usually found in NRTI-exposed infants, the incidence of mitochondrial dysfunction is increased in these children by 26% [25]. However, conflicting experimental results have been reported in the few studies currently available analysing mitochondrial toxicity in cord blood mononuclear cells (CBMCs), peripheral blood mononuclear cells (PBMCs) or placenta of asymptomatic ARV-exposed newborn [24–32]. Consequently, no concern has been raised about the obstetric and perinatal safety of NRTIs in human pregnancies. Some studies have shown mtDNA depletion compared to controls [26–29], whereas others have reported no significant changes [25,30] and the remaining have even described increased content [31,32]. Additionally, most of these studies did not assess mitochondrial number, function, translation efficiency or apoptosis development, which would bring light into the cell consequences of mtDNA depletion.

Considering the controversial results regarding mtDNA depletion in newborn from HIV-infected mothers and the lack of assessment of alternative markers of mitochondrial lesion we measured the real impact of mitochondrial dysfunction on CBMCs of ARV-exposed HIV-uninfected children. Additionally, to our knowledge, no study has evaluated mitochondrial function and apoptosis in HIV-pregnant women or has correlated these data with pregnancy outcome.

We hypothesized that mitochondrial toxicity and apoptosis caused by HIV and ART could be the underlying pathophysiological mechanism of adverse perinatal outcome in HIV pregnancy. To address this question we compared mitochondrial and apoptotic parameters of HIV-infected women and their children with respect to healthy controls to correlate experimental results with immunovirological, therapeutic and obstetric data.

## Patients and methods

### Design

We performed a single-site, cross-sectional, controlled observational study without intervention.

### Study population

Sixty-two pregnant women were prospectively and consecutively included in the present study during their routine prenatal care at first trimester of gestation, in the Materno-Fetal Medicine Department of the Hospital Clinic of Barcelona (Barcelona, Spain).

Mitochondrial and apoptotic studies of 27 asymptomatic HIV-1-infected pregnant women, 35 uninfected pregnant controls and their newborns were performed at delivery.

Controls and cases were matched by age and parity. The inclusion criteria for pregnant women were: age at least 18 years, single pregnancy and delivery after at least 22 weeks of gestation and, in case of HIV-infected patients, previous diagnosis of HIV-1 infection.

All individuals were informed and signed written consent that was obtained for inclusion in this protocol and approved by the Ethical Committee of our hospital.

In order to avoid confounders of mitochondrial toxicity, patients taking other potentially toxic drugs for mitochondria were excluded from the study as were patients with familiar history of mitochondrial disease.

An electronic database was created to collect epidemiological, obstetric, immunovirological and therapeutic data, perinatal outcome and experimental results.

Maternal epidemiological and obstetric parameters included information on maternal age, race, parity, illicit substance abuse and mode of delivery.

Immunovirological parameters for HIV-1-infected women consisted in measuring CD4<sup>+</sup> T-cell count (by flow cytometry) and plasmatic viral load by HIV-1 RNA copy quantification (Amplicor HIV Monitor, Roche Diagnostic Systems, Branchburg, NJ, USA) along pregnancy.

Antiretroviral therapy was administered to all HIV-infected pregnant women following international guidelines. HIV women were stratified into different categories of therapeutic care according to the use of ARV during pregnancy. HAART always consisted of a double-NRTI schedule and either one protease inhibitor or one non-NRTI drug (NNRTI).

Information regarding perinatal outcome for both HIV-infected pregnant women and controls included: gestational diabetes mellitus, preeclampsia (new onset of hypertension of >140 mmHg systolic or >90 mmHg diastolic pressure and >300 mg proteins/24 h of urine), fetal death (>22 weeks of pregnancy), gestational age at delivery, preterm birth (<37 weeks of gestation), birth weight, newborn small for gestational age (<10th percentile), 5-min Apgar score below 7, neonatal admission to intensive care unit and global adverse perinatal outcome.

Finally, experimental data included the collection of maternal and fetal mitochondrial and apoptotic measures.

### Sample collection and processing

Immediately after delivery, 20 ml of peripheral blood was collected from women by antecubital vein puncture and 20 ml of cord blood was collected from their infants in EDTA tubes. This method of infant blood extraction was

designed to prevent invasive sample collection and increase study participation. In both samples mononuclear cells were isolated by Ficoll density gradient centrifugation, divided into aliquots and stored at  $-80^{\circ}\text{C}$  until analysis.

Platelet count was confirmed below 25 per lymphocyte/monocyte, suggesting negligible platelet contamination for the mtDNA depletion assay.

Protein content was measured according to the Bradford protein-dye binding-based method [33].

### Mitochondrial studies in maternal peripheral blood mononuclear cells and newborn cord blood mononuclear cells

#### *Mitochondrial DNA quantification*

To evaluate mtDNA depletion, total DNA was obtained by standard phenol-chloroform extraction procedure. A fragment of the mitochondrial-encoded *ND2* gene and the nuclear-encoded *18S rRNA* gene were amplified in triplicate and separately by quantitative rtPCR using the Roche Lightcycler thermocycler [18]. The relative content of mtDNA was expressed as the ratio between mitochondrial to nuclear DNA amount (*ND2* mtDNA/*18S rRNA* nDNA content).

#### *Mitochondrial protein synthesis*

To assess mitochondrial translation efficiency, we performed Western blot analysis of 20  $\mu\text{g}$  of total cell protein through 7/13% SDS-PAGE electrophoresis and posterior immunoquantification of the mitochondrial-encoded and located COX-II subunit (25.6 kDa) with respect to the nuclear-encoded and mitochondrially located voltage-dependent anion channel protein (V-DAC; 31 kDa) to establish the relative COX-II abundance per mitochondria (COX-II/V-DAC) [18].

#### *Mitochondrial respiratory chain complex II, glycerol-3-phosphate dehydrogenase + complex III (G3Pdh + III) and complex IV (COX) enzyme activity*

To evaluate mitochondrial function, all mitochondrial enzymatic activities were measured by spectrophotometry according to the Rustin *et al.* [34] and Miró *et al.* [35] methodology. Enzymatic activity of MRC complex II was used as the control parameter supposedly unaffected by ARV because it is completely encoded, transcribed and translated by cytoplasmic machinery independent of mitochondria, whereas MRC complexes CIII and CIV are partially encoded, transcribed and translated by mitochondrial means. Specific enzymatic activities were expressed in absolute values as nanomols of synthesized substrate or consumed product per minute and milligram of protein (nmols/min/mg protein).

#### *Mitochondrial mass*

To assess mitochondrial number we performed the spectrophotometric measurement of citrate synthase

AQ2 activity (CS; EC 4.1.3.7), a mitochondrial enzyme of the Krebs's cycle widely considered as a reliable marker of mitochondrial content [34].

### Apoptotic studies

To evaluate apoptotic cell death we performed Western blot analysis of 20 µg of total cell protein by 7/13% SDS-PAGE electrophoresis and posterior immunoanalysis of active (cleaved) caspase-3 pro-apoptotic protein expression (17–19 KDa) normalized by the content on β-actin protein signal (47 KDa) as a cell loading control. Chemiluminescence results were expressed as caspase-3/β-actin relative content and were interpreted as a marker of advanced apoptotic events.

### Statistical analysis

Clinical and epidemiological parameters were expressed as means and range interval and experimental results as means and standard error of the mean (SEM) or as a percentage of increase/decrease with respect to healthy controls.

Adverse perinatal outcome, mitochondrial and apoptotic results of HIV-infected women and newborn were compared to those of uninfected women and their children to assess the presence of obstetric/perinatal problems and mitochondrial or apoptotic damage due to HIV infection and ART. Additionally, different correlations were sought between: molecular and functional mitochondrial parameters (to ascertain dependence of proper mitochondrial function on mitochondrial genome depletion); mother-to-child mitochondrial or apoptotic parameters (to determine maternal influence on newborn cellular status); and clinical and experimental data (to assess mitochondrial or apoptotic basis of obstetric problems and perinatal outcome).

Differences between cases and controls and correlations among quantitative parameters were assessed using nonparametric tests: Mann–Whitney analysis to search for independent sample differences, Chi-square test to calculate odds ratio (OR) values [OR; 95% confidence interval (CI); significance] and the Spearman's rank coefficient for parameter correlation ( $R^2$  and significance). The level of significance was set at 0.05 for all the statistical tests.

## Results

### Clinical data

The clinical and epidemiological characteristics of the study participants and immunovirological and therapeutic data of HIV mothers are summarized in Table 1.

All pregnant women were mainly of Caucasian origin, with an age ranging from 25 to 42 years. Nonsignificant

differences were observed in maternal epidemiological data between HIV-positive and HIV-negative women.

For HIV participants, the mean time of HIV infection and HAART treatment prior to delivery was 84 and 48 months, respectively.

Most HIV women were under HAART before pregnancy (85%) and only four cases (15%) were naive for ART and started HAART at the second trimester of gestation. HAART was given to all patients at the time of delivery to avoid MTCT and consisted of two NRTI and either one protease inhibitor or one NNRTI (see Table 1 for percentages of treatment assignment).

At delivery, all women had received at least 6 months of double-NRTI treatment and the mean CD4<sup>+</sup> T-cell count was 560 cells/ml with an undetectable viral load.

### Perinatal outcomes

The obstetric and neonatal outcomes of the study cohort are detailed in Table 2.

All infants were HIV-uninfected with no clinical symptoms compatible with mitochondrial toxicity. Gestational diabetes mellitus, gestational age at delivery and neonatal intensive care unit admission tended to be increased, albeit not significantly, among HIV pregnancies, together with the reduction of birth weight at delivery.

Additionally, global adverse perinatal outcomes were significantly more frequent among HIV-positive pregnancies (11/27 vs. 3/35, OR 7.33; 95% CI 1.8–30.1;  $P=0.048$ ). The overall prematurity rate was also significantly increased for newborns to HIV mothers (7/27 vs. 2/35, OR 5.77; 95% CI 1.1–30.6;  $P=0.034$ ) with 57% (4/7) of the premature deliveries being at less than 35 weeks, 50% of which (2/4) were at less than 32 weeks. Finally, the number of infants who were small for gestational age was also significantly increased among HIV pregnancies (6/27 vs. 1/35, OR 9.71; 95% CI 1.1–86.5;  $P=0.036$ ).

### Mitochondrial and apoptotic maternal peripheral blood mononuclear cell analysis

Compared with uninfected controls, HIV-pregnant women showed a marked but nonsignificant trend to a decreased mtDNA content of 26.3% ( $P=NS$ , and Table 1a).

HIV-pregnant women also exhibited a significant decrease in the mitochondrial protein synthesis rate (25.9% COX-II/V-DAC content reduction,  $P=0.042$ ; and Table 1a and b) and all MRC enzyme activities (18.5% for CIV and 31.2% for G3Pdh + CIII function diminution;  $P=NS$  and  $P=0.049$ , respectively; and Table 1a). The only mitochondrial enzymatic activity

**Mitotoxicity and apoptosis in HIV pregnancy Hernández et al.****Table 1. Clinic, epidemiologic, immunovirologic and therapeutic characteristics of HIV-infected and uninfected pregnant women.**

	HIV-positive (n = 27)	HIV-negative (n = 35)	P
Maternal age at delivery <sup>a</sup>	34.7 (27–42)	33.7 (25–41)	NS
HCV infection [N (%)]	3 (11.1)	1 (2.6)	NS
Illicit drug use [N (%)]	0 (0)	0 (0)	–
Alcohol use [N (%)]	0 (0)	0 (0)	–
HIV RNA copies per ml at delivery <sup>a</sup>	62.3 (49–250)	–	–
CD4 <sup>+</sup> T-cell count per ml at delivery <sup>a</sup>	560.2 (97–1242)	–	–
NRTI prior pregnancy (months) <sup>a</sup>	48 (0–106)	–	–
NNRTI prior pregnancy (months) <sup>a</sup>	3 (0–86)	–	–
PI prior pregnancy (months) <sup>a</sup>	12 (0–97)	–	–
Time from diagnosis of HIV infection to delivery (months) <sup>a</sup>	84 (4–228)	–	–
HAART second and third trimesters			
2NRTI + 1PI [No. (%)]	4 (15)	–	–
HAART all trimesters			
2 NRTI + 1 PI [No. (%)]	15 (55.5)	–	–
2 NRTI + 1 NNRTI [N (%)]	8 (29.5)	–	–

HCV, hepatitis C virus; HIV, human immunodeficiency virus; NRTI, nucleoside-analogue reverse transcriptase inhibitor; NNRTI, non-NRTI reverse transcriptase inhibitor; NS, not significant; PI, protease inhibitors; RNA, ribonucleic acid.

<sup>a</sup>Data are presented as means and range interval.

which was preserved in HIV women was MRC complex II (increased 13.7% compared to controls,  $P = NS$ ), used as a control parameter because it is independent to mtDNA depletion.

No differences were found in mitochondrial mass (CS activity) between cases and controls and, consequently, mtDNA content, mitochondrial protein synthesis or MRC enzymatic activities maintained the same trends when normalized to mitochondrial mass (data not shown).

HIV-infected pregnant women presented a marked, albeit nonsignificantly increased apoptotic rate of 100% compared with healthy controls ( $P = NS$ ; and Table 1a and b).

Maternal mtDNA levels and mitochondrial function (G3Pdh + CIII activity) were positively and significantly correlated (Fig. 2).

No significant correlation was, however, found between maternal experimental (mitochondrial or apoptotic) parameters and clinical results (including perinatal

outcome, immunological status, ART or maternal age), except on considering time on NRTI treatment before pregnancy. Women exposed to NRTI for over 100 months presented a lower mtDNA content with respect to those under-exposed (88 vs. 55% remaining mtDNA content compared to controls,  $P = 0.048$ ).

### Mitochondrial and apoptotic newborn cord blood mononuclear cell analysis

Infants born from HIV women exposed *in utero* to HIV and ART showed a significant decrease in mtDNA content of 38.6% compared with uninfected controls ( $P = 0.006$ ; and Table 1a).

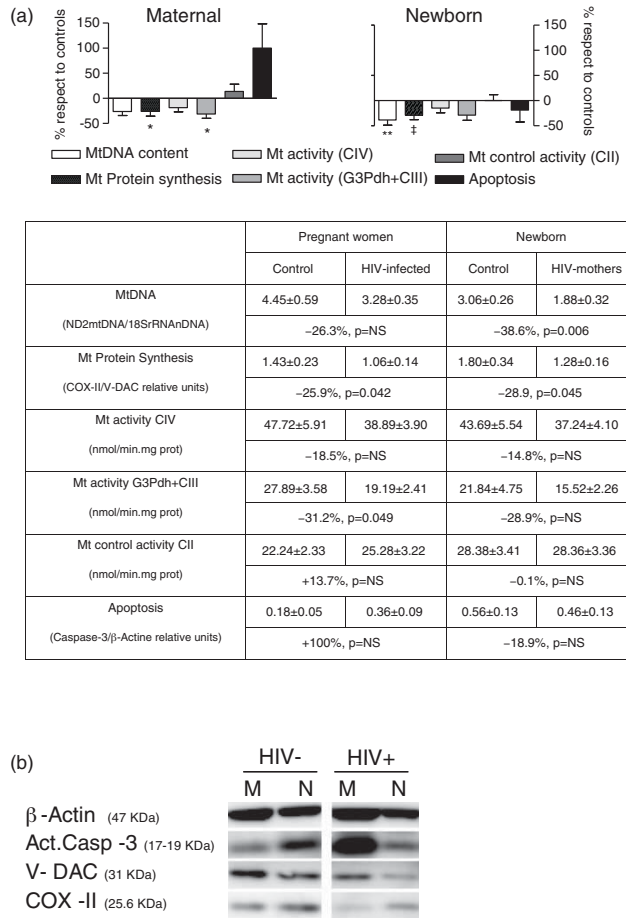
Similar to their mothers, infants born to HIV women also exhibited a significant decrease in mitochondrial protein synthesis (28.9% COX-II/V-DAC content reduction,  $P = 0.045$ ; and Table 1a and b) and a trend towards a decrease in all MRC enzyme activities compared to controls (14.8% for CIV and 28.9% for G3Pdh + CIII activity diminution,  $P = NS$  in both cases; and Table 1a). According to its control function, the only mitochondrial enzymatic activity which remained unchanged between

**Table 2. Obstetric and neonatal outcomes of the study cohorts.**

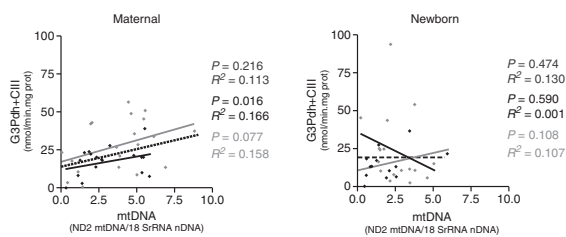
	HIV-positive (n = 27)	HIV-negative (n = 35)	OR [95% CI]
Gestational diabetes mellitus [N (%)]	2 (7.4)	1 (2.5)	2.72 [0.2–31.7]
Preeclampsia [N (%)]	0 (0)	0 (0)	–
Fetal death [N (%)]	0 (0)	0 (0)	–
Gestational age at delivery (weeks) <sup>a</sup>	37.5 (32.2–41.2)	39.2 (38.5–40.4)	–
Preterm birth (<37 weeks of gestation) <sup>a</sup>	7 (25.9)	2 (5.7)	5.77 [1.1–30.6]
Birth weight (g) <sup>a</sup>	2879 (1940–4040)	3165 (3130–3350)	–
Small for gestational age (<10th percentile) [N (%)]	6 (22.2)	1 (2.8)	9.71 [1.1–86.5]
5-min Apgar score <7 [N (%)]	0 (0)	0 (0)	–
Neonatal intensive care unit admission [N (%)]	3 (11.1)	1 (2.8)	4.25 [0.4–43.4]
Global adverse perinatal outcome [N (%)]	11 (40.7)	3 (8.5)	7.33 [1.8–30.1]

95% CI, 95% confidence interval of the mean; No., number; NS, not significant; OR, odds ratio.

<sup>a</sup>Data are presented as means and range interval.



**Fig. 1. Mitochondrial and apoptotic parameters.** (a) Percentage of increase/decrease of mitochondrial parameters in HIV participants with respect to mean values of uninfected controls. MtDNA, mitochondrial DNA; Mt Protein synthesis, mitochondrial protein synthesis (COX-II/V-DAC); CIV, G3Pdh + CIII or CII enzymatic activity, mitochondrial respiratory chain complex IV, glycerol-3-phosphate dehydrogenase and complex III or complex II enzymatic function, respectively; apoptosis, caspase cleavage and activation (caspase-3/β-Actine). (\*)  $P = 0.042$ ; (●)  $P = 0.049$ ; (‡)  $P = 0.006$ ; (‡) 0.045. (b) Western blot for mitochondrial protein synthesis and apoptosis quantification. HIV±: human immunodeficiency virus infected/not-infected pregnant-women; M/N: mothers/newborn.



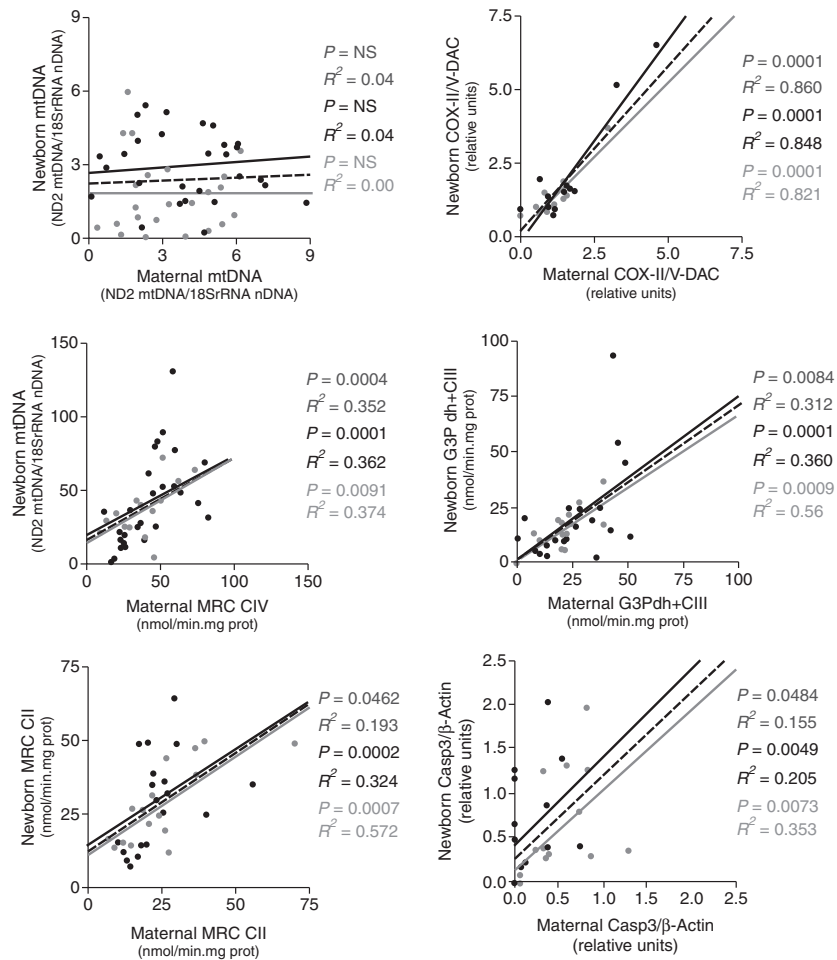
**Fig. 2. Correlation between genetic and functional mitochondrial parameters.** Association between genetic and functional parameters is shown in the graph for HIV-pregnant women or newborn from HIV pregnancies (black line and significance), for control women and newborn (grey line and significance) and for all included women or children (discontinued line and significance in bold).

cases and controls was complex II (0.1% decreased in HIV and HAART-exposed infants,  $P = NS$ ).

Again, no differences were found in mitochondrial mass (CS activity) between cases and controls and, consequently, mtDNA content, mitochondrial protein synthesis or MRC function preserved identical trends when normalized to mitochondrial mass (data not shown).

Contrarily to their mothers, however, newborn mtDNA levels and mitochondrial function (G3Pdh + CIII activity) were not correlated (Fig. 2) and seronegative infants born from HIV women presented an unaltered apoptotic rate (18.9% decreased) with respect to controls ( $P = NS$ ; and Table 1a and b).





**Fig. 3. Correlation between materno-fetal mitochondrial or apoptotic parameters.** Association between quantitative materno-fetal parameters are shown in the graphs for HIV patients (grey line and significance), uninfected controls (black line and significance) or for both patients and controls (discontinued line and significance in bold). MtDNA, mitochondrial DNA; Mt protein synthesis, mitochondrial protein synthesis (COX-II/V-DAC); MRC CIV, G3Pdh + CIII or CII enzymatic activity, mitochondrial respiratory chain complex IV, glycerol-3-phosphate dehydrogenase and complex III or complex II enzymatic function; Casp3/β-actin, apoptotic development (caspase-3/β-actin).

Additionally, there was a positive and significant correlation between all maternal and fetal mitochondrial and apoptotic parameters except in mtDNA content, either considering exclusively HIV cases, controls or both (Fig. 3).

No significant correlation was, however, found between newborn mitochondrial parameters and maternal immunological status, ART regimen, age or adverse perinatal outcome.

## Discussion

Animal models have shown evidence of mitochondrial toxicity from in-utero NRTI exposure [23]. However,

clinical evidence of mitochondrial toxicity has largely been lacking in HIV mothers and their infants and there is conflicting evidence regarding the association of in-utero HIV and ART exposure with mortality and morbidity due to mitochondrial dysfunction [24–32]. We observed a reduced mtDNA content and derived protein synthesis and MRC function in both PBMC of HIV-pregnant women at delivery receiving ART as well as in CBMC of their in-utero-exposed newborns. Although not all mitochondrial parameters showed statistically significant differences between cases and controls, all measures were reduced in HIV-pregnant women and their newborn, suggesting NRTI or HIV-mediated mitochondrial lesion. Our results are in concordance with the studies reporting mtDNA depletion in newborn from HIV-infected and treated mothers [26–29]. However, none of these

studies parallelly evaluated mitochondrial number, mtDNA-encoded MRC protein expression and mitochondrial function in HIV and HAART-exposed newborn. The present work strengthens the mitochondrial toxicity hypothesis by confirming that fetal mtDNA depletion is not an isolated finding and subclinical mitochondrial lesion is present in several markers of mitochondrial function downstream from mitochondrial genetics.

Although previously reported in nonpregnant adults [16–18], this is the first time that similar results have been reported in HIV-infected and treated pregnant women. Mitochondrial lesion in oocytes of HIV-infected and treated women has previously been suggested to play an etiopathological role in the infertility of these women [17] and could, potentially, be the cause of their associated obstetric problems.

Whether mitochondrial lesion in both infected mothers and their newborn is due to HIV or ART is still a matter of doubt because, in our study, all HIV-pregnant women received ART prior delivery following international guidelines. Interestingly, we found increased mtDNA depletion in HIV-pregnant women with NRTI administration extended over 100 months, suggesting NRTI-mediated injury. However, HIV implication in mitochondrial lesion can not be discarded, especially because apoptosis was increased in HIV-infected women. HIV-derived mitochondrial toxicity has been reported to be indirectly caused by the activation of apoptotic pathways [19–21]. To our knowledge, apoptotic studies have never previously been performed in HIV mothers and their infants. We observed that apoptotic events tend to be increased in HIV-pregnant women but remain unaltered in their newborn. This finding, although not significant, suggests that HIV itself could trigger the apoptotic phenomenon exclusively in HIV-infected pregnant women, whereas their infants, which are uninfected because of the therapeutic activity of HAART along pregnancy, show an unaltered apoptotic rate. Further studies are required to elucidate if apoptotic lesion is also present in naive pregnant women and in HIV-infected newborns. If apoptotic lesions are increased in these populations, obstetric problems of HIV pregnancies could be attributed, at least in part, to apoptotic means, thereby confirming the prevention of not only MTCT of HIV infection but also HIV-mediated apoptosis in newborn from HIV-infected women with the use of HAART.

Despite studying maternal and newborn mitochondrial and apoptotic parameters in different kinds of tissues (PBMCs and CBMCs, respectively), all were positively and significantly correlated, except for mtDNA content. We attribute this concordance to the maternal heritage of mitochondria organelles, exclusively provided by the oocyte along the fecundation process [17].

In our opinion, different theories may explain materno-fetal correlation of mitochondrial parameters and strengthen the hypothesis of NRTI-mediated mitochondrial toxicity, in detriment of HIV-induced mitochondrial damage. First, mitochondrial injury in maternal blood cells could limit its transplacental function once maternal blood is in the fetus. Second, mitochondrial toxicity may be nonexclusively restricted to maternal cells and could also be present in tissues more related to fetal development (including placenta) [29]. Finally, NRTIs cross the placenta [36], becoming direct fetal-damaging agents.

Interestingly, maternal mtDNA levels and function positively correlated, demonstrating the dependence of mitochondrial functionalism on mitochondrial genetics. However, mtDNA levels and function were independent parameters in their newborn, with no trend towards association, especially in infant born from HIV pregnancies. This finding may explain why maternal and fetal mtDNA levels were the only parameters not associated between mother and child; mtDNA levels seem to be independently regulated in maternal and newborn compartments, perhaps due to distinct biological needs or capacities of response to toxic exposure. In our HIV cohort, global adverse perinatal outcome, preterm birth and frequency of small for gestational age newborns were significantly increased. However, in these women and children, mitochondrial and/or apoptotic compromise was not increased compared to uninfected controls or the remaining HIV cohort. Although all these adverse outcomes are unspecific of HIV and HAART and could be associated with other pathological situations, with respect to HIV pregnancies, our findings failed to demonstrate that mitochondria or apoptosis disturbances played a role in infant outcome. This assertion does not rule out the possibility that mitochondrial dysfunction in newborns could contribute to the future development of metabolic problems in adulthood, independently of its subclinical perinatal consequences [37].

The study may have some limitations. First, the analysis of mononuclear cells could show different mitochondrial and apoptotic results compared to those present in other tissues directly related to pregnancy development. Second, the small size of our sample could hinder the testing of our hypothesis (mitochondrial or apoptotic basis of adverse perinatal outcome), independently of its veracity. Third, lack of pregnant HIV women naïve for HAART or HIV-infected children could limit the assessment of HIV-implication in adverse perinatal outcome. Fourth, lack of symptomatic newborn of mitochondrial toxicity at delivery could reduce the presence of molecular lesion in our infant population. Fifth, although all treated women had received at least 6 months of double-NRTI schedules, previous and current treatment options were not completely uniform and the results could therefore differ in alternative treatment interventions. Finally, we cannot discard the possibility that adverse effects of HIV or

HAART, alternative to mitochondrial dysfunction or apoptosis, may play a role.

Despite these limitations, we can, however, conclude that ART administration along pregnancy may cause the transplacental subclinical mitochondrial lesions observed in both pregnant women and their in-utero-exposed newborn. This finding is confirmed by materno-fetal correlated mitochondrial protein synthesis and enzymatic function. On the contrary, ARV may prevent newborn apoptotic lesions, thereby leading to beneficial effects independent of MTCT prevention.

In conclusion, no relationship was found between mitochondrial and apoptotic abnormalities and adverse perinatal outcome. However, mitochondrial toxicity associated with NRTI administration and newborn morbidity should be further investigated in tissues directly related to pregnancy in larger cohorts of patients including naïve pregnant HIV women or women under NRTI-sparing regimens, as well as HIV-infected newborn and newborn with symptomatic manifestation of mitochondrial or apoptotic lesion.

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## Conflicts of interest

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# Infección por VIH.

*Tratamiento antirretroviral  
y mitocondria*

*Coordinadores:*

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# Mitocondria y VIH: Mecanismos de interacción

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**ACTUALMENTE** está bien documentado que el VIH produce un efecto lesivo generalizado sobre la mitocondria. Se cree que esta lesión no se alcanza a través de un mecanismo específico sino mediante una alteración a distintos niveles, especialmente, por la modificación de los procesos reguladores de la apoptosis.

## Inducción de la apoptosis

La apoptosis es un proceso fisiológico de muerte celular programada, necesario en la embriogénesis, morfogénesis de órganos y homeostasis de los tejidos y que mantiene intacto el tejido adyacente. Hay diversos factores que pueden causar un aumento patológico de la apoptosis, como son los radicales libres, desequilibrios en la relación de proteínas proapoptóticas y antiapoptóticas, el factor de necrosis tumoral (TNF), infuljos de calcio o la presencia de agentes infecciosos, como es el caso del VIH.

Existe una vía intrínseca de la apoptosis, mediada por señales intracelulares en situaciones de estrés celular, que está regulada por las mitocondrias y una vía extrínseca, mediada por receptores celulares. (**Figura 1**).

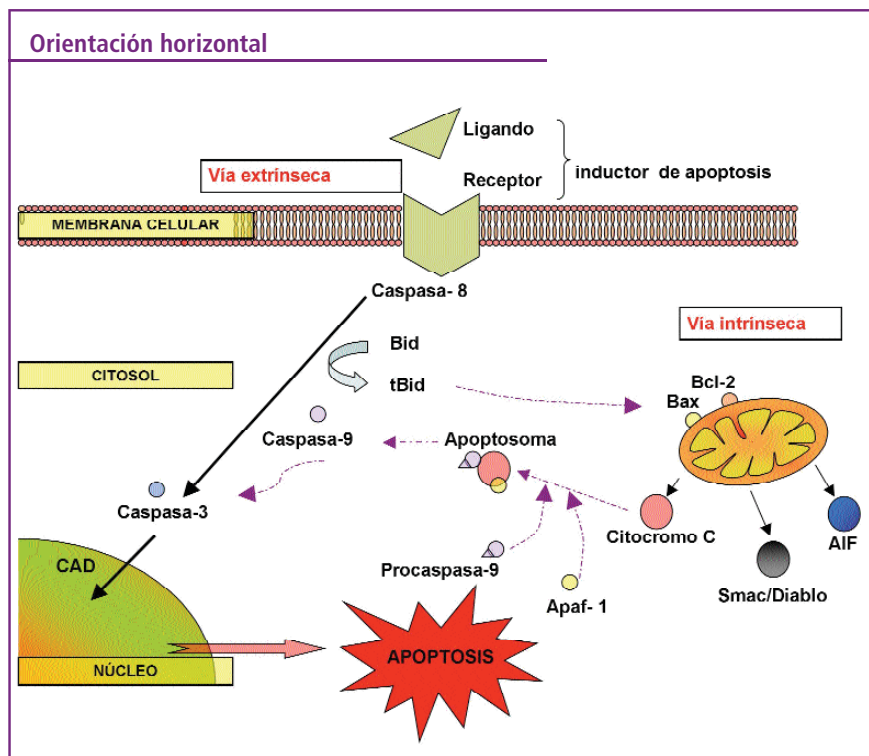


FIGURA 1.

**Vía extrínseca.** Unión del ligando inductor de apoptosis a su receptor. Activación de la caspasa-8 y desencadenamiento de una cascada de reacciones catalizadas por caspasas (cisteína proteasas celulares). Acción de la proteína ADNasa activada por caspasa (CAD) que degrada el ADN e induce la muerte celular. La caspasa-8 también activa por proteólisis la proteína Bid en la forma Bid truncada (tBid) que interacciona en la mitocondria con la proteína Bax u otros miembros proapoptóticos de la familia Bcl-2. A continuación la vía converge con la vía intrínseca y la mitocondria actúa como intensificador del proceso. **Vía intrínseca.** Una señal intracelular produce desequilibrios en las proteínas de la familia Bcl-2. Se liberan componentes proapoptóticos: factor de inducción de apoptosis (AIF), que activa reacciones apoptóticas independientes de las caspasas, el segundo activador de caspasas derivado de la mitocondria (SMAC) o su forma análoga en ratón (DIABLO), el citocromo C, el cual se une al factor activador de proteasas apoptóticas (APAF-1) y a la procaspasa-9 formando el apoptosoma<sup>24</sup>. Éste activa la caspasa-9 y el proceso continúa con la reacción en cascada de las caspasas, según lo explicado anteriormente.

## Infección por VIH. Tratamiento antirretroviral y mitocondria

El VIH induce apoptosis mitocondrial en neuronas<sup>1,2,3</sup> y miocardiocitos<sup>1</sup>, adipocitos<sup>4,5,6</sup>, músculo esquelético<sup>7</sup>, células endoteliales<sup>8</sup> y, especialmente, en las células T CD4, en las cuales prolifera y causa una alteración en la respuesta inmune del huésped,<sup>1,9,10</sup> lo cual se traduce en una reducción progresiva del número de linfocitos T CD4 y, por tanto, una inmunodeficiencia y un incremento en la susceptibilidad a infecciones oportunistas<sup>9</sup>.

En el espacio intermembranoso mitocondrial se encuentran diversos componentes potencialmente letales, como el citocromo c, el factor de inducción de la apoptosis (AIF), activadores de nucleasas, etc. La permeabilización de la membrana mitocondrial externa implica la liberación de estos componentes al citosol y el desencadenamiento de la vía apoptótica mitocondrial. Además, estos factores inducen la activación de vías apoptóticas dependientes (citocromo c) o independientes de las caspasas (AIF, endonucleasa G).

La membrana mitocondrial externa es el lugar de acción de unas proteínas reguladoras de la apoptosis que pertenecen a la familia Bcl-2. Las proteínas proapoptóticas (Bax, Bak, t-Bid, Bim, Bad y Bik) facilitan

la permeabilización de la membrana mitocondrial, mientras que las antiapoptóticas (bcl-2) mantienen la función de barrera de las membranas. Cuando se inician señales, intracelulares o extracelulares, de muerte celular se produce un desequilibrio de estas proteínas proapoptóticas y antiapoptóticas a favor de las primeras. Esto provoca una permeabilización en la membrana, mediante la formación de poros de transición (PTP, *permeability transition pore*) y la pérdida del gradiente electroquímico requerido para la fosforilación oxidativa. Así se favorece el proceso apoptótico.

De esta manera, las células T CD4+ son eliminadas específicamente por el VIH mediante la inducción de la vía apoptótica mitocondrial.

### Proteínas virales que inducen apoptosis

Las alteraciones mitocondriales asociadas a la infección por el VIH se relacionan con la activación de vías apoptóticas inducidas por el virus. En el año 2000 Ferri et al<sup>11</sup> describieron la existencia de algunas de las proteínas apoptogénicas codificadas por el virus. (**Figura 2**).

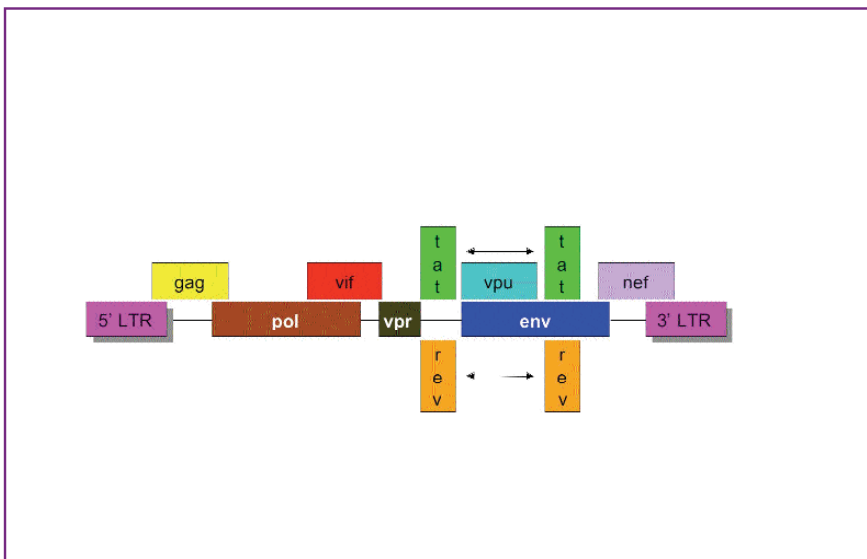


FIGURA 2.

Genoma del VIH. Se constituye de tres genes estructurales (gag, pol y env), presentes en todos los retrovirus y que codifican componentes proteicos y estructurales de la partícula vírica y seis genes accesorios (vif, vpr, tat, vpu, rev, nef), que controlan la infectividad y la expresión vírica.

“El VIH produce un efecto lesivo generalizado sobre la mitocondria.”

## Mitocondria y VIH: Mecanismos de interacción

El VIH-1 puede actuar como inductor apoptótico desde el interior de la célula infectada. Vpr, Tat y la proteasa viral (Pr) participan en este proceso.<sup>9</sup>

La infección por VIH causa muerte celular tanto en células infectadas como en no infectadas (proceso conocido como "Bystander Killing").<sup>1,9,12</sup>

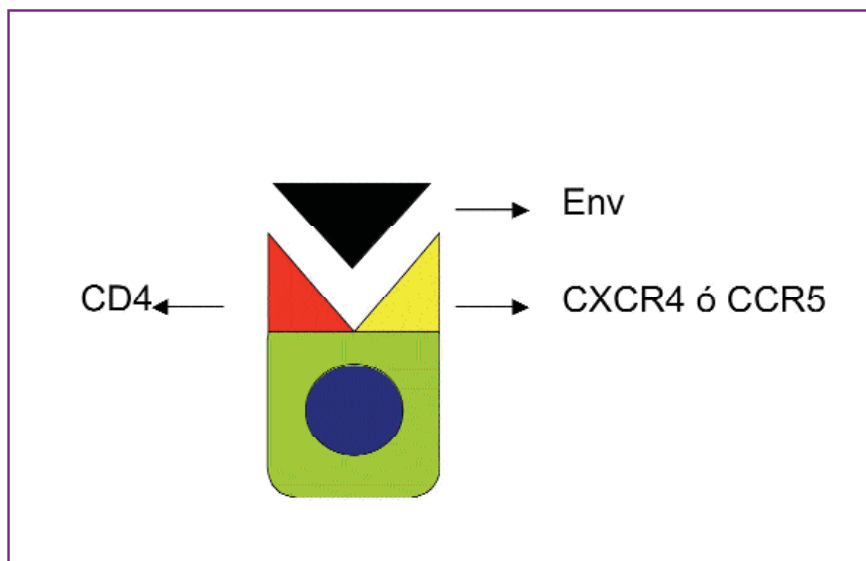
Las células infectadas por el virus expresan en su superficie el complejo gp120-gp41 que forma la envoltura lipídica del virus, la más externa (Env). Estas células tienen la capacidad de destruir aquellas no infectadas que expresen el receptor CD4 y el correceptor CXCR4 para Env de variante linfotrópicas (que infectan células T CD4+ principalmente) o el correceptor CCR5 para las variantes Env monocitotrópicas (infectan principalmente las células T CD4+, monocitos, macrófagos y células dendríticas).<sup>1,13</sup> **(Figura 3).**

La muerte de las células no infectadas se debe a la activación de respuestas inmunológicas por parte de los linfocitos T, como estrategia del organismo ante la presencia del virus. Estas respuestas incluyen la sobreproducción de receptores de muerte celular, como el APO-1 (también conocido como Fas o CD95), o el receptor factor de necrosis tumoral y sus respectivos ligandos. La muerte de células no infectadas también puede producirse por la acción de proteínas accesorias del VIH, descritas a continuación.

La proteína Tat del VIH es secretada por las células infectadas y colabora en los procesos de infección de las células no infectadas. Esta proteína puede translocarse a través de la membrana plasmática e inducir apoptosis en una amplia variedad de células. Interacciona específicamente con los microtúbulos y provoca la translocación de la proteína proapoptótica Bim desde los microtúbulos a la mitocondria. Esto promueve la permeabilización de la membrana y, por tanto, la vía apoptótica mitocondrial<sup>14</sup>. Además, Tat reduce la expresión del enzima mitocondrial superóxido dismutasa<sup>15</sup>, e incrementa la expresión de la caspasa-8<sup>16</sup>, y del ligando Fas de muerte celular en las células T<sup>17</sup>, disminuye la producción de Bcl-2<sup>18</sup> y aumenta los niveles de calcio<sup>19</sup>.

La proteasa del VIH degrada proteínas existentes en la célula huésped. Bcl-2 se inactiva por proteólisis<sup>20</sup>, se activa la caspasa-8<sup>21</sup> y se forma la proteína proapoptótica tBid, la cual se transloca a la mitocondria y estimula la permeabilización de la membrana mitocondrial.

La proteína viral R (Vpr), producida en las fases tardías de la infección<sup>22</sup>, presenta un dominio mitocondriotóxico e induce apoptosis durante la infección. Se asocia con las mitocondrias por la unión con el translocador de nucleótidos de adenina (ANT) presente en la membrana mitocondrial interna. ANT, junto con el canal aniónico dependiente de voltaje (VDAC), forma un complejo multiproteico que constituye el poro de transición. Esto



**FIGURA 3.**

Infección de una célula no infectada. Unión de Env (gp41-gp120) de la célula infectada al receptor CD4 y su correceptor (CXCR4 o CCR5) de células no infectadas. Este proceso culmina con la transmisión de señales letales y la permeabilización de la membrana mitocondrial.

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induce la permeabilización de la membrana mitocondrial interna. A continuación se produce la pérdida del potencial de membrana, la liberación del citocromo c y la activación de la apoptosis.<sup>23,24</sup> Vpr destruye linfocitos<sup>25</sup>, monocitos<sup>26</sup> y neuronas<sup>27</sup>.

Además, las citoquinas liberadas en respuesta a la infección pueden inducir apoptosis mitocondrial, sobre todo, en tejidos resistentes a la infección como son el nervio, el adipocito, o el músculo esquelético.

En la **Figura 4**<sup>28</sup> se muestra un incremento de la apoptosis en el músculo esquelético de pacientes infectados por el VIH y que no han recibido terapia antirretroviral.

En conclusión, múltiples proteínas codificadas por el VIH confluyen a nivel mitocondrial causando toxicidad. Este hecho se pone de manifiesto, por ejemplo, por la existencia de una fracción de linfocitos con un bajo potencial de membrana<sup>29</sup> o por la sobreproducción de radicales libres en las mitocondrias<sup>1</sup>, lo cual representa una evidencia clínica de la apoptosis mitocondrial derivada del VIH.

### Parámetros mitocondriales que resultan afectados a causa de la infección por VIH

Además de los mecanismos apoptóticos inducidos por el VIH en su interacción con la mitocondria, se ha comprobado la afectación de ciertos parámetros

mitocondriales. Los efectos mitocondriales asociados a la infección por VIH se observan en forma de lesiones en el ADN mitocondrial (ADNmt), especialmente en forma de depleción<sup>30</sup>, aunque también mutaciones puntuales y deleciones únicas o múltiples<sup>31</sup>, y deben entenderse como una consecuencia, principalmente, de las alteraciones en la homeostasis apoptótica comentada anteriormente.

El primer estudio que aportó datos sobre la existencia de un daño en el ADN mitocondrial asociado a la infección de VIH fue presentado en el 2002 por Côté et al. En él se comprobó la disminución del ADNmt en linfocitos de sangre periférica en pacientes infectados por VIH que no habían recibido terapia antirretroviral con respecto a sujetos no infectados<sup>30</sup>. Esta disminución en el ADNmt se correlaciona directamente con la cantidad de linfocitos CD4 en sangre periférica e inversamente con la carga viral. Así pues, una mayor carga viral promueve una reducción de linfocitos CD4 y de ADNmt en estas células. Este hecho coincide con un estudio anterior in vitro en el que se observaron anomalías mitocondriales, signos de necrosis y apoptosis inducidos por la infección de VIH-1 en las células CD4<sup>+</sup><sup>32</sup>.

Las depleciones en el ADNmt de células mononucleadas de sangre periférica (linfocitos y monocitos), así como la coexistencia con alteraciones de otros parámetros mitocondriales, causadas por infección del VIH se han confirmado posteriormente<sup>33</sup>.

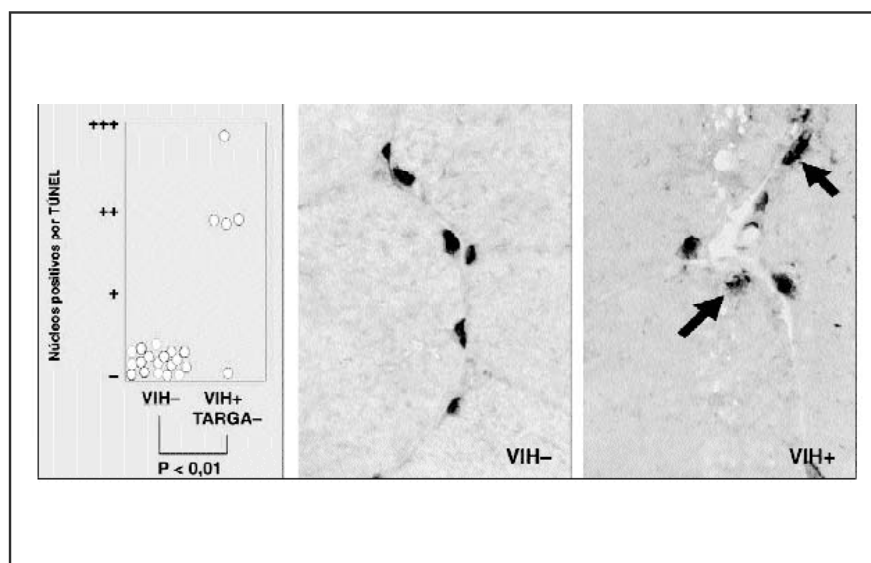


FIGURA 4.

Sección transversal de músculo esquelético de un paciente VIH- (centro) y VIH+ (derecha) estudiados mediante la reacción inmunohistoquímica TUNEL. Esta técnica visualiza fragmentos rotos de ADN nuclear, consecuencia final del proceso apoptótico. Los pacientes VIH+ presentan un mayor porcentaje de núcleos positivos en el TUNEL (derecha, flechas), lo cual es significativamente superior a lo que se observa en pacientes VIH- (izquierda). (Reproducido con permiso del autor.<sup>28</sup>)

## Mitocondria y VIH: Mecanismos de interacción

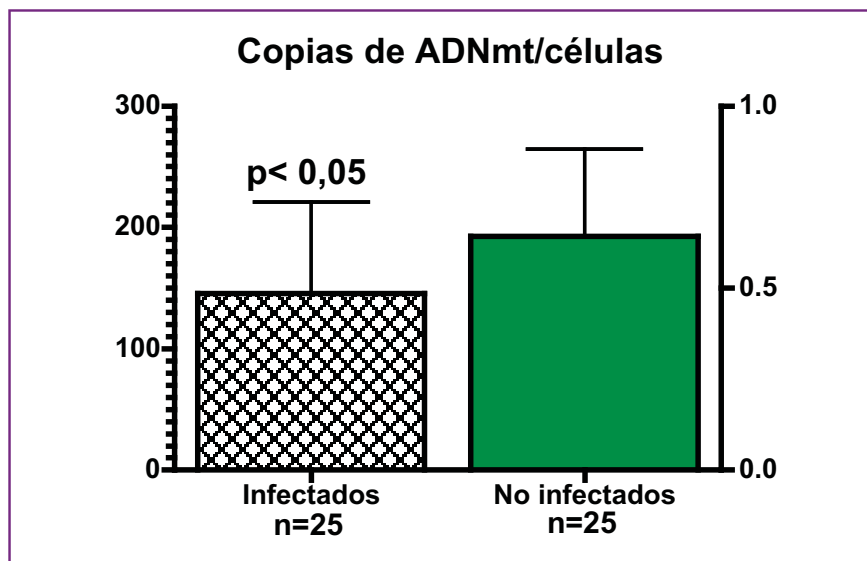
En la **Figura 5** se muestra una reducción significativa (del 23%),  $p < 0.05$  del contenido de ADNmt en los pacientes infectados por el VIH.

Otros parámetros mitocondriales resultaron afectados en este estudio por la presencia de VIH. De esta manera, se vieron reducidos los complejos II (reducción de la actividad, 41%,  $p < 0,001$ ), III (reducción, 38%,  $p < 0,001$ ) y IV (reducción, 19%,  $p < 0,001$ ) de la cadena respiratoria mitocondrial, así como la actividad glicerol-3-fosfato deshidrogenasa (reducción, 22%,  $p < 0,001$ ) y el aumento de la peroxidación lipídica en las membranas de las células mononucleares de sangre periférica.

El hecho de que estas disfunciones mitocondriales afecten tanto a proteínas codificadas como no codificadas por el ADNmt induce a pensar que es un mecanismo lesivo inespecífico el que causa dicho daño mitocondrial. Probablemente, la activación de la apoptosis es una buena explicación para justificar estas lesiones mitocondriales.

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**FIGURA 5.** Cuantificación del ADN mitocondrial en células mononucleadas de sangre periférica que muestra un menor contenido en los pacientes infectados respecto a los no infectados por el VIH (Modificado de Miró, Ò. 2004)



## Infección por VIH. Tratamiento antirretroviral y mitocondria

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## **ABSTRACT**

Primary mitochondriopathies are those caused by mutations in genes encoding for respiratory chain proteins. Secondary mitochondrial diseases encompass the remaining mitochondrial disturbances; those caused by genetic alterations in genes not related to respiratory chain subunits or those of non genetic origin which lead to mitochondrial dysfunction by altering biochemical or functional pathways. Secondary mitochondrial diseases include mitochondrial disorders caused by contact with toxic agents, in which individual mitochondrial or nuclear genetic context may play a role.

Despite presenting diverse aetiology, primary and secondary mitochondrial disorders usually present common symptoms.

The objective of this work is to review the characteristics of mitochondrial lesion and the mechanisms of toxicity that certain agents which damage mitochondria present, many used daily in clinical practice.

These toxic compounds usually exert their damaging capacity by impairing a specific genetic, biochemical or molecular mitochondrial pathway, but in cases of chronic abuse most of these toxic agents finally lead to general mitochondrial dysfunction which can compromise cellular and tissue viability and, in some cases, the life of the patient. Mitochondrial recovery is remarkable and most of the mitochondrial lesion and the clinical effects caused by these agents usually disappear with discontinuation of exposure to the toxin. However, when toxic exposure cannot be disrupted, clinicians must manage secondary effects due to mitochondrial toxicity such as myopathy, hyperlactatemia, lactic acidosis, pancreatitis, neuropathy or lipodystrophy. Mitochondrial therapies designed to revert mitochondrial-induced damage are currently being developed and, as yet, are not available in routine clinical practice. Treatment of secondary mitochondriopathies is currently exclusively symptomatic and supportive.

Understanding the aetiology of toxic mitochondriopathies and approaches to prevent mitochondrial-induced damage are both fundamental to prevent symptoms and avoid serious clinical consequences of mitochondrial diseases which, otherwise, may become difficult to reverse.

## 1. INTRODUCTION

Mitochondrial function is essential for energetic and metabolic cell survival. Mitochondrial impairment can lead to the development of mitochondrial diseases, also known as mitochondriopathies, which may affect different levels of mitochondrial function. These disorders present some special characteristics. First, mitochondria are maternally inherited organelles because the oocyte is the only gamete that provides all the mitochondria of the future embryo in the fertilisation process. Second, mitochondrial components are partially encoded by the nuclear and the mitochondrial genome and, consequently, mitochondrial diseases can be caused by impairment in both genetic compartments as well as by intergenomic communication deficiencies. Third, there are multiple numbers of copies of the mitochondrial genome in each mitochondrion and mitochondrial genomes are randomly distributed in these organelles when mitochondria divide. Thus, different mitochondrial genomes can coexist in the same mitochondria or cell causing the phenomenon known as heteroplasma (coexistence of wild type and mutated mitochondrial genome molecules). Fourth, manifestation of mitochondrial disease requires the existence of a certain number of mutated molecules, a phenomenon known as the threshold effect. Fifth, these mitochondrial-mutated genomes can be preferentially present in different tissues. Finally, the disorder also depends on the nuclear context that is strikingly variable and characteristic of each individual. All these reasons confer a special inheritance to mitochondrial diseases that can be translated into a wide spectrum of clinical manifestations [1].

Mitochondriopathies can be classified into primary or secondary disorders depending on their aetiology. Primary mitochondriopathies are those caused by mutations in genes encoding respiratory chain proteins [2-7]. Mutations can be present in nuclear or mitochondrial genes, but the affected gene always encodes a protein of the mitochondrial respiratory chain (MRC). Primary mitochondrial diseases are usually due to genetically-inherited mutations which are maternally transmitted if of mitochondrial origin. Secondary mitochondrial diseases encompass the remaining mitochondrial disturbances including those caused by genetic alterations in genes not related to MRC subunits or those of non genetic origin which lead to mitochondrial dysfunction by altering biochemical or functional pathways. Secondary mitochondrial diseases involve those due to disturbances in intergenomic communication between the nucleus and the

mitochondria, alterations in components involved in mitochondrial biogenesis or mtDNA replication, transcription or translation processes, as well as alterations of biochemical and functional cellular pathways that can lead to mitochondrial dysfunction, among others. Some of these diseases can be caused by contact with toxic agents able to damage mitochondria. Mitochondrial damage induced by these agents is universal. However, certain mitochondrial or nuclear genetics settings can predispose individuals to preferentially develop clinical symptoms following identical toxic exposure.

The nature of the toxic compound determines the aetiology of the mitochondrial lesion (genetic, structural or functional). The clinical symptoms of secondary mitochondrialopathies are usually similar to those of primary disorders and are often key for clinical suspicion of a mitochondrial basis for these toxic disorders.

Toxic agents can exert mitochondrial lesion at different levels of mitochondrial function, depending on their nature and the particular mitochondrial function they impair. The basic levels at which mitochondrial function is usually affected by these compounds are mtDNA replication, transcription or translation, MRC enzymatic function, oxygen consumption, reactive oxygen species (ROS) generation, membrane potential depolarisation or mitochondrial biogenesis, among others.

The toxic substance can initially exert isolated and specific mitochondrial damage that affects only one of these levels of function. However, if the contact with the toxic agent is not disrupted, the isolated effect can ultimately lead to a general mitochondrial dysfunction with severe consequences.

In secondary mitochondrialopathies, several factors, many of which are common to primary disorders such as divergence in impaired mitochondrial or nuclear-encoded function, heteroplasmy, threshold effect, affected tissue or individual genetic context, also determine the clinical manifestations and disease severity. Mitochondrial toxic disorders present a wide spectrum of manifestations ranging from almost asymptomatic individuals to severe cases in which the disease can have fatal consequences, and clinical divergence can frequently be present even in the context of the same aetiological factor.

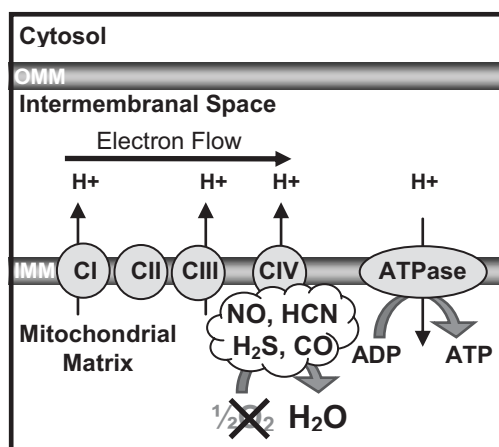
The knowledge on mitochondrial disorders has dramatically increased and, in the last years, some 'mitochondrial treatments' (such as vitamins, enzymatic cofactors or antioxidants) have been proposed to minimise their consequences [1, 8, 9].

## 2. MITOCHONDRIAL TOXIC COMPOUNDS

Nature and damage mechanisms differ among the different mitotoxic compounds. They can be chemical compounds (i.e: toxic gases), biological agents (i.e: retroviruses), pharmaceutical drugs commonly used in clinical practice (antivirals, neuroleptics, antibiotics, hypolipemiant, anaesthetics, sedatives, antiinflammatories, antineoplastics, antidiabetics, antiarrhythmics, antimalarics and fungicide drugs), pesticides (herbicides, acaricides or insecticides) and abuse drugs (i.e: tobacco or alcohol). These agents present different toxicity mechanisms and damage capacity, some of which are currently being established.

### 2.1. Toxic gases

The mitotoxic agent can frequently be a natural compound present in the environment. This is the case of some lethal gases such as nitric oxide, cyanide, hydrogen sulphide or carbon monoxide, some of which are responsible for severe intoxications which may lead to death. The severity of symptoms and appearance of late sequelae depend on the intensity and duration of the exposure. Interestingly, all these toxic gases present the same mitotoxic pathophysiologic mechanism of damage through the inhibition of mitochondrial complex IV (mtCIV) function.



**Figure 1:** Mechanism of nitric oxide (NO), cyanide (CN), hydrogen sulphide (H<sub>2</sub>S) and carbon monoxide (CO) damage of mitochondrial function by the inhibition of mitochondrial complex IV (mtCIV) function. OMM: outer mitochondrial membrane; IMM: inner mitochondrial membrane; CI, II, III and IV: complexes I, II, III and IV of

the mitochondrial respiratory chain; ADP: adenosine diphosphate; ATP: adenosine triphosphate; OH<sup>-</sup>: hydroxyl anion; H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide; O<sub>2</sub><sup>-</sup>: superoxide anion.

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**2.1.1. Nitric oxide** (NO) is both a natural compound with vital physiologic functions including signalling molecule such as neurotransmission or vasodilatation and a product of the chemical industry, power plants, automobile engines and cigarette smoke as an air pollutant. Nitric oxide is a messenger molecule involved in many physiological and pathological processes within the mammalian body presenting both beneficial and detrimental functions. For instance, appropriate levels of NO production are important for protecting tissues and organs from ischemic damage and are also necessary for the maintenance of blood flow, kidney and reproductive function or peristalsis. However, sustained levels of NO production or chronic gas exposure result in direct tissue toxicity, leading to the development of carcinomas and inflammatory conditions. Nitric oxide is naturally produced by NO synthases through arginin, oxygen and NADPH consumption and is metabolised to peroxynitrite (NOO<sup>-</sup>) in combination with oxygen. Nitric oxide directly binds and inhibits mtCIV function and the resultant peroxynitrite has been reported to inhibit MRC complex I activity through impairment of poly (ADP-ribose) polymerase-1 (PARP-1) function and a decrease in NAD/NADH content [10]. Therefore NO content must be controlled within narrow physiologic ranges since external intoxication or conditions that increase the physiologic quantity of NO can produce severe clinical consequences.

**2.1.2. Cyanide** group (CN) is the toxic molecule present in inorganic gas salts such as hydrogen cyanide or HCN, inorganic solid salts including potassium cyanide or KCN as well as in organic nitriles. However, organic compounds are harmless because they cannot release the damaging CN group. Cyanide also presents a strong ability to bind iron ions in haemoproteins, especially the mtCIV enzymatic complex of the MRC. Mitochondrial CIV is the final enzyme responsible for oxygen reduction in water and CN binding to mtCIV block cellular respiration being one of the few mitotoxic gases responsible for lactic acidosis production [11, 12]. This natural poisoning primarily alters consciousness with early onset of severe alteration of vital functions and intoxication occurs within seconds or minutes after exposure. Cyanide intoxication treatment consists of concomitant administration of antidotes together with oxygen but consequences may be lethal because of its rapid development.

**2.1.3. Hydrogen sulphide** ( $H_2S$ ) is a soluble gas formed in non-enriched aerobic atmospheres in the presence of organic materials and sulphates. Most  $H_2S$  is of natural origin and only around 10% of its content is anthropogenically produced in industries. Intoxication by  $H_2S$  can be produced by inhalation as well as by ingestion or transdermic absorption of contaminated substances. Hydrogen sulphide is a mitochondrial poison. Its damaging action is based on binding and inhibiting the function of iron containing proteins such as mtCIV, but it also has a high affinity for haemoglobin. In mitochondria,  $H_2S$  binding to mtCIV prevents the mitochondrial utilization of oxygen with an uncoupling of oxidative phosphorylation. Mitochondrial toxicity of  $H_2S$  involves ROS formation and mitochondrial depolarisation [13]. In addition,  $H_2S$  binds to haemoglobin in red blood cells interfering with oxygen transport [14-16]. Although the severity of intoxication and outcome depend on the toxic dose,  $H_2S$  poisoning rapidly causes loss of consciousness and death.

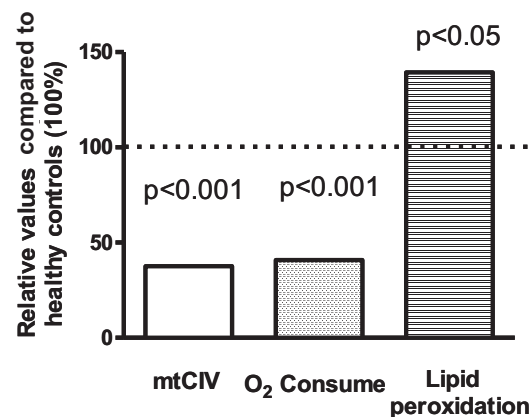
**2.1.4. Carbon monoxide** (CO) is a toxic gas generated on abnormal combustion when the oxidation of more complex organic compounds in an atmosphere lacking oxygen (usually in closed areas) preferentially forms CO in spite of the otherwise commonly produced carbon dioxide ( $CO_2$ ) [17]. Nitric oxide, CN and  $H_2S$  are dangerous and potentially lethal gases; however they are not frequently responsible for intoxications. On the other hand, CO constitutes the most frequent cause of death by asphyxiant gases and the most frequent cause of occupational death by inhalation [18, 19]. Carbon monoxide presents a high affinity for haemoglobin, the erythrocyte molecule responsible for oxygen transport into tissues. This association is responsible for carboxyhaemoglobin (COHb) generation and tissular hypoxia development without lactic acid formation [10]. Symptoms of acute CO poisoning (ACOP; including cephalaea, asthenia, vertigen, confusion, palpitations, nausea, vomiting, loss of consciousness, arrhythmia, ischaemia and coma [20, 21]) have been classically attributed to COHb-mediated tissular hypoxia [12, 22]. COHb levels are therefore usually determined in clinical practice in order to establish the severity of intoxication [23]. However, some symptoms appear without COHb formation [23] or even some weeks after COHb resolution, indicating the inaccuracy of COHb as a marker of severity or adverse outcome [18] and suggesting the existence of alternative injury mechanisms independent of hypoxia. In addition to haemoglobin, CO presents affinity for other iron-containing proteins such as myoglobin, cytochrome p450 and mtCIV, the



impairment of which may modulate clinical symptoms. It has been demonstrated that CO binding to subunit cytochrome aa3 of mtCIV prevents mtCIV function in experimental models [24] and our group has shown that CO also inhibits human mtCIV in ACOP [25-27].

Treatment of ACOP consists of pure oxygen administration, either at standard (normobaric therapy) or at higher pressure (hyperbaric treatment). In environmental conditions oxygen therapy reduces COHb separation from 4 hours to 45 or 23 minutes, depending on the use of normobaric or hyperbaric treatment [28]. Oxygen treatment restores oxygen transport into tissues [29] and resolves cellular hypoxia [30], although the specific oxygen treatment of choice for CO intoxication remains to be established [31].

Cellular respiration does not however exclusively depend on oxygen supply because CO directly inhibits mtCIV function [7, 23, 25, 30] and this inhibition could be more prolonged. We have observed that mtCIV function remains inhibited in ACOP patients even 10 days after COHb normalisation [25]. Persistence of cellular inhibition can cause deficiencies in the oxidative phosphorylation system and increase ROS formation [23, 25, 32] that may in turn, damage mitochondria again.



**Figure 2:** Mitochondrial complex IV activity (mtCIV), oxygen consumption (O<sub>2</sub> consume) and oxidative stress levels (lipid peroxidation of membranes) in acute CO intoxicated patients compared to controls (arbitrarily assigned 100%). Extended information is available in the articles by Miro et al. published in *Toxicol Lett* 1999; 110: 219-223 and *Med Clin (Barc)* 2004; 122: 401.

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All this mitochondrial damage could be the basis of the symptoms and sequelae of ACOP and could be a useful marker for clinic evolution.

It is of note that some chemical products such as **methylene chloride**, a common dissolvent present in many compounds including hair lacquers, can lead to dangerous clinical effects by generating CO once metabolised in the liver [33, 34].

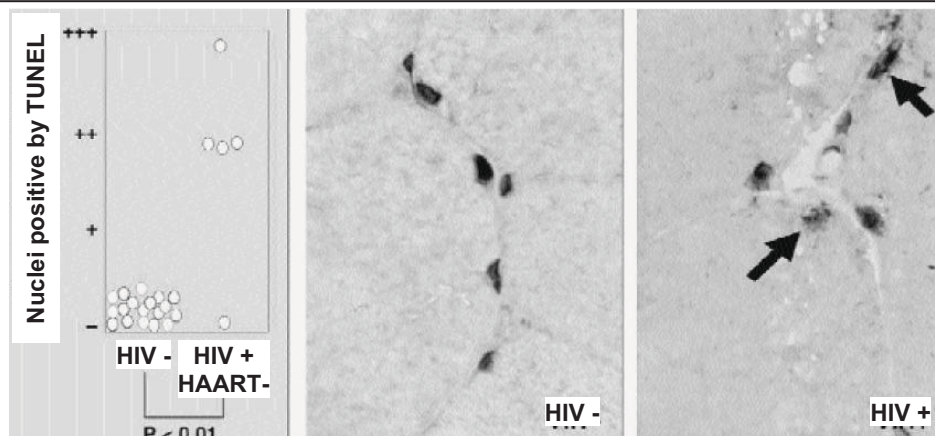
The mitochondrial toxicity induced by the CO present in tobacco smoke is discussed below.

## 2.2. Biological agents

Some biologic agents such as viruses have been reported to cause mitochondrial damage which, in some cases, may be the basis for some clinic manifestations. This is the case of the human immunodeficiency virus (HIV) and the hepatitis C virus (HCV) whose mitochondrial damage has been associated with adverse secondary effects of the infection including myopathy or hyperlactatemia.

**2.2.1. HIV** infection has been reported to cause a decrease in the number of mitochondrial genome copies per cell [7, 35-37], a phenomenon known as depletion. We demonstrated that such depletion is accompanied by altered mitochondrial function [7, 36, 37]. HIV-induced mitochondrial lesion is generalised and affects different parts of mitochondrial function, including those non-mitochondrially encoded such as MRC complex II activity.

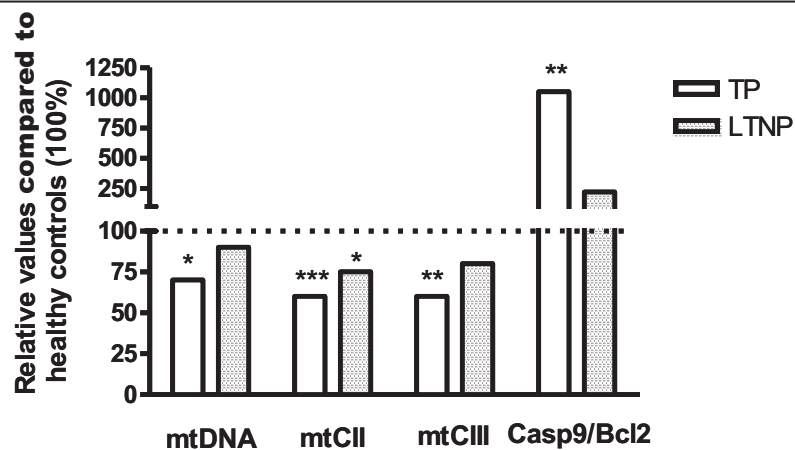
HIV has also been reported to induce apoptosis (Figure 3).



**Figure 3:** Transversal section of skeletal muscle of an HIV-infected patient (right panel) compared to that of a non-infected individual (middle panel) analysed by immunohistochemistry TUNEL reaction (deoxyribonucleotidyl-transferase-mediated-dUTP-biotin nick-end labelling) useful to detect nuclear fragmented nuclei as a result of

the apoptotic process. The presence of positive TUNEL nuclei (right panel; black arrows) is increased in those HIV-infected individuals (left panel). Extended information is available in the book chapter by Casademont et al, published in the book *New Frontiers in Mitochondrial Biogenesis and Disease* 2005, 37, Editor Francesc Villarroya, Research Signpost.

We have demonstrated that mitochondrial damage and mitochondrial-triggered apoptosis levels differ depending on whether HIV-infected patients have a standard progression of the infection (typical progressors) or a self-controlled disease (long-term non-progressive infection) [37]. Long-term non-progressor HIV-infected patients have an intermediate level of mitochondrial damage and mitochondrial-driven apoptosis compared to uninfected individuals (who have not) and HIV-infected patients presenting a standard progression of the infection (showing an increase in both rates) [37].



**Figure 4:** Mitochondrial DNA content (mtDNA), mitochondrial complex II and III activity (mtCII and mtCIII) and mitochondrial-driven apoptosis levels (Caspase 9 normalised to Bcl2 or Casp9/Bcl2 quantity) in HIV-infected patients with respect to controls (arbitrarily assigned 100%). HIV-infected patients have been divided as typical progressors (TP, white bars) and long-term non-progressors (LTNP, dotted bars). One, two and three asterisks represent significant differences with respect to controls ( $p < 0.01$ ,  $p < 0.05$  and  $p < 0.01$ , respectively). Extended information is available in the article by Peraire et al, published in *Curr HIV Res* 2007, 5, 467.

HIV-induced mitochondrial lesion has been reported to be due to the viral activation of cellular apoptotic pathways [36-38]. Some viral proteins such as Vpr, Tat or Pr can

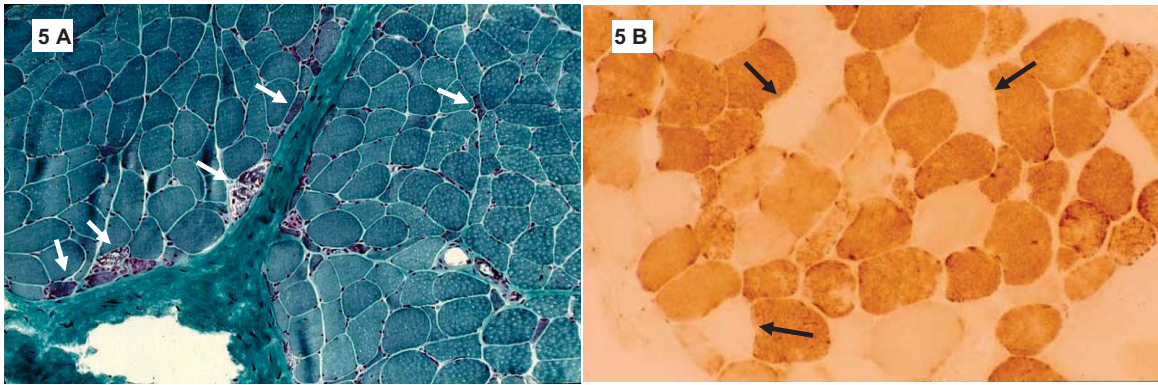
trigger intrinsic apoptosis and some cytokines produced against the infection can mediate extrinsic apoptosis in infected and uninfected cells of HIV-infected individuals [39, 40]. Apoptotic pathways converge into mitochondria to amplify death signals and interfere with normal mitochondrial function causing general mitochondrial impairment.

**2.2.2. HCV** infection has also been reported to cause mtDNA depletion [41]. Thus, mitochondrial toxicity is more evident in HIV/HCV co-infected patients presenting an increased risk of derived chronic consequences including hyperlactatemia or liver failure [42, 43].

### **2.3. Drugs**

Many pharmaceutical drugs frequently used in clinical practice can exert mitochondrial lesion. This is the case of some antiretrovirals (especially the nucleoside analogue reverse transcriptase inhibitors or NRTI), antipsychotics (especially the first generation of neuroleptic drugs, such as haloperidol), antibiotics (including chloramphenicol, aminoglycosides or linezolid), hypolipemiant, anaesthetics and sedatives, antiinflammatories, antineoplastics, antidiabetics, antiarrhythmics, antimalarical and fungicide drugs. The mitochondrial toxicity of some of these drugs could underlie some secondary effects associated with different pharmacologic treatments.

**2.3.1. Antivirals** used to treat HIV or HCV infection have also been reported to induce mitochondrial lesion which may be the basis for adverse effects associated with viral therapy, including myopathy, lipodystrophy, hyperlactatemia, lactic acidosis, polyneuropathy or pancreatitis [7, 44-50]. Antiviral-induced mitochondrial toxicity is added to the mitochondrial damage caused by the virus itself and together both generate severe clinic consequences in chronically infected and treated patients. Muscle mitochondrial toxicity due to antiretroviral treatment can be easily identified on routine histochemistry sections by the presence of the so-called 'zidovudine (or AZT)-fibres', quite similar to classic ragged-red fibres but smaller and more ragged [44] (Figure 5A), that were first described in patients on antiretroviral treatment with this drug (AZT). Furthermore, early mitochondrial toxicity can be suspected by the presence of COX negative fibres (Figure 5 B).

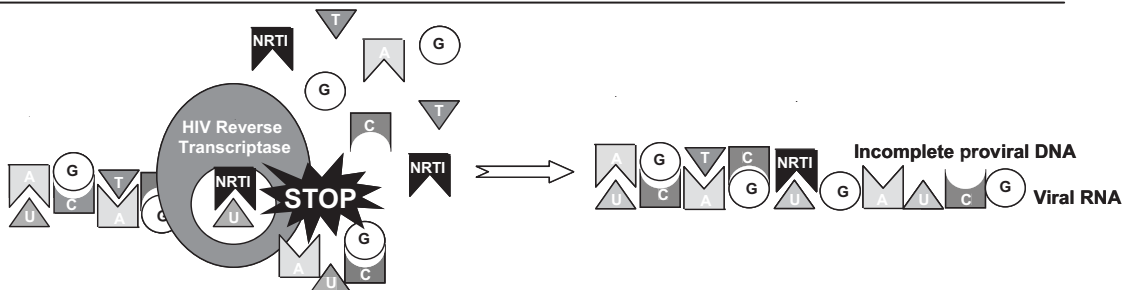


**Figure 5:** Mitochondrial toxicity induced by a zidovudine (AZT)-based regimen in frozen sections of skeletal muscle using (a) Gomori's trichrome staining or (b) Cytochrome c oxidase (COX) reaction. Note the presence of ragged-red cells (4a; white arrows), which are smaller and more ragged than the classical cells, and negative COX fibres (4b; black arrows) are seen in early AZT toxicity in contrast to normal type I and type II fibres.

Mitochondrial toxicity of antiretrovirals has mainly been attributed to NRTI and, to a lesser extent, to protease inhibitors.

### 2.3.1.1. Nucleoside analogue reverse transcriptase inhibitors

(NRTI) exert their therapeutic activity of blocking viral cycle by inhibiting the reverse transcriptase (RT) enzyme of the virus. Viral RT is present in all retroviruses to reverse transcribe the ssRNA of the virus into the proviral dsDNA that are able to integrate into the host genome. The antiviral efficacy of drugs responsible for inhibiting viral RT is very high and these drugs are therefore included in most therapeutic regimens used to treat HIV or HCV infection. There are different classes of RT inhibitors, although NRTI are the most common. They are synthetic compounds similar to physiologic (wild type) nucleosides except for a structural modification which prevents elongation of the genetic strand produced by the RT.

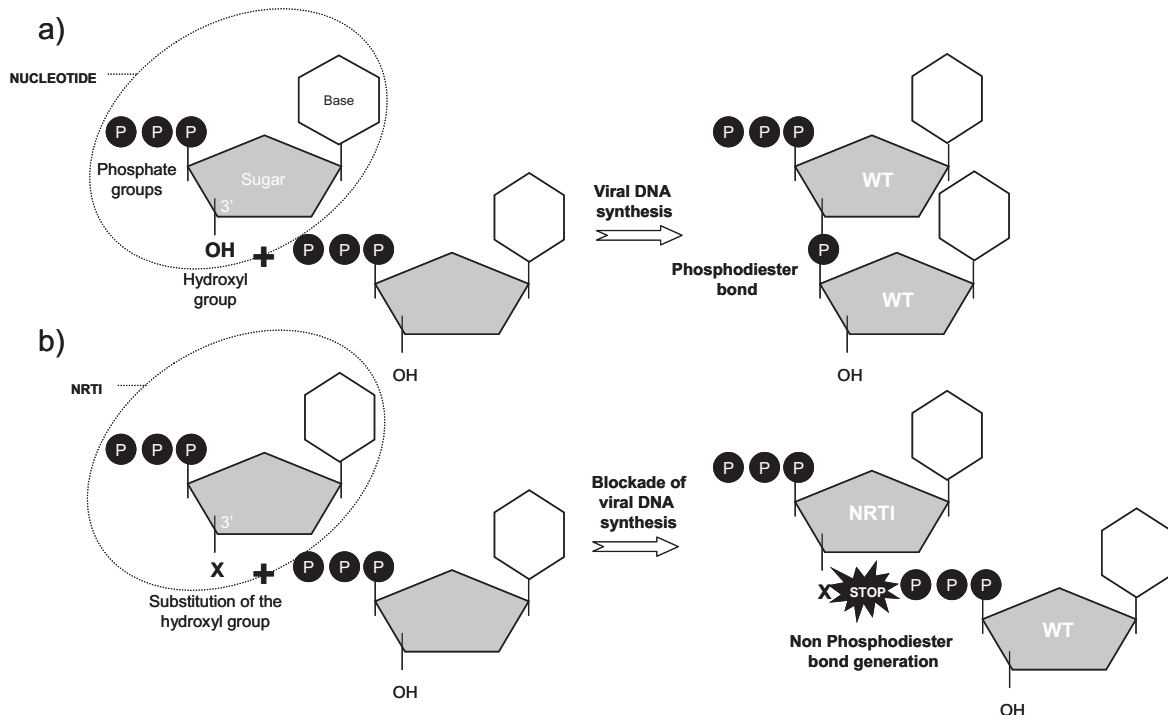


**Figure 6:** Blockade of viral retrotranscription by nucleoside analogue reverse-transcriptase inhibitors (NRTI). DNA: deoxyribonucleic acid; RNA: Ribonucleic acid; Deoxynucleotides (DNA nucleotides): A (Adenine), G (Guanine), T (Thymine) and C (Cytosine); Ribonucleotides (RNA nucleotides): A (Adenine), U (Uracil), T (Thymine) and C (Cytosine).

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Structural modification of NRTI consists in the substitution of the hydroxyl group at the 3' position of the deoxyribose by alternative residues. Therefore, once the NRTI is incorporated in the strand on elongation, 3' residue of NRTI cannot create the phosphodiester bond with the 5' phosphate group of the next nucleotide causing the blockade of the RT enzyme, strand elongation and the viral cycle.

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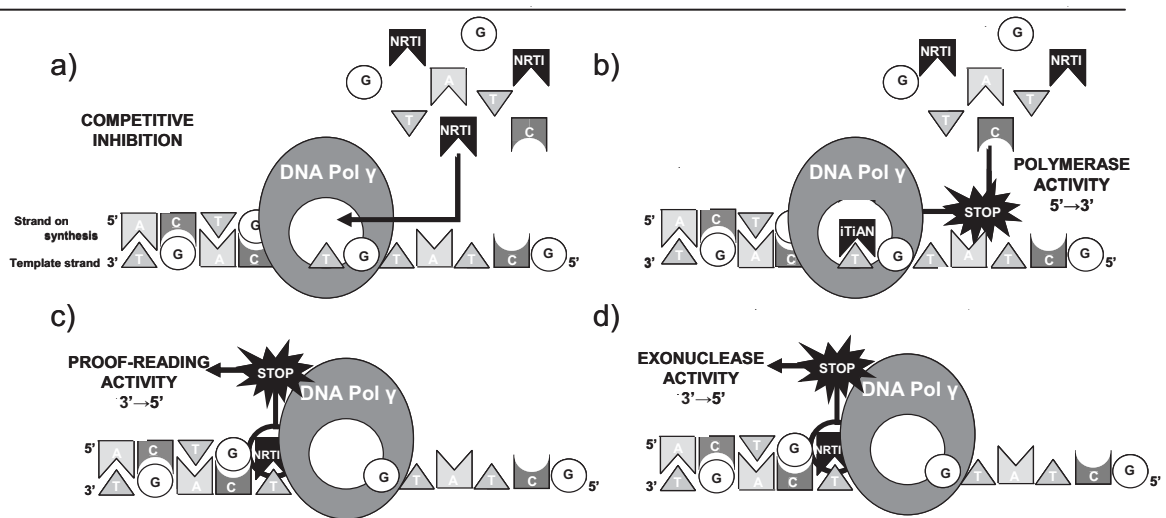
**Figure 7:** Structure and synthesis mechanism of deoxyribonucleic acid (DNA) using (a) wild-type (WT or physiologic) nucleotides or (b) nucleoside analogue reverse transcriptase inhibitors (NRTI). OH: Hydroxyl group; P: Phosphate group.

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Different NRTI have been designed for each physiologic nucleoside (adenosine, thymidine, cytidine and guanosine). Nucleosides and nucleotide-analogues (NRTI) must be phosphorylated three times by cellular kinases to be incorporated as nucleotides or nucleotide-analogues, respectively, into the DNA strand.



Mitochondrial toxicity and the secondary effects of NRTI have been reported to be caused by inhibition of mitochondrial replication [51]. These drugs have no effect on the nuclear genome but are incorporated into the viral and the mitochondrial genetic material due to inhibition of viral RT as well as cellular DNA polymerases ( $\beta$  and  $\gamma$ ). Polymerase  $\beta$  repairs mistakes produced during nuclear replication but its activity is redundant to polymerase  $\epsilon$  and thus, its inhibition has non severe consequences. However, inhibition of mitochondrial polymerase  $\gamma$  (DNApol $\gamma$ ), the only enzyme responsible for mtDNA replication and repair, has more serious derived effects. Inhibition of DNApol $\gamma$  by NRTI is produced by different mechanisms summarised in figure 8.



**Figure 8:** Blockage of mitochondrial genome replication by nucleoside analogue reverse transcriptase inhibitors (NRTI). First, (a) they block DNA polymerase  $\gamma$  (DNApol $\gamma$ ) by exerting a competitive inhibition of the enzyme; Second, (b) they act as premature strand terminators of mtDNA on synthesis by inhibiting the polymerase activity of DNApol $\gamma$  generating mitochondrial incomplete genomes with deletions; Third, they increase the punctual mutation rate by inhibition of the proof-reading capacity of DNApol $\gamma$  that recognises (c) and repairs (d), respectively, mismatch mistakes. Deoxynucleotides (DNA nucleotides): A (Adenine), G (Guanine), T (Thymine) and C (Cytosine).

Inhibition of DNApol $\gamma$  decreases the quantity and quality of mtDNA by increasing the number of point mutations, deletions and depletion rate which can lead to mitochondrial dysfunction. Inhibition of DNApol $\gamma$  is responsible for associated mtDNA depletion and consequent decreased synthesis of the 13 protein subunits of the MRC encoded by

mtDNA. It may lead to energetic and metabolic mitochondrial dysfunction and cell impairment that could be the aetiopathogenic basis for many secondary effects associated with NRTI administration [51-53]. The capacity of NRTI to inhibit DNAPoly was established *in vitro* by Kakuda and collaborators: ddC (zalcitabine) > dDI (didanosine) > d4T (stavudine) >>> AZT (zidovudine) > 3TC (lamivudine) > ABC (abacavir) = TDF (tenofovir) [54]. This scale is proportional to NRTI capacity to exert mitochondrial lesion and secondary effects if NRTI only exerted mitochondrial damage through DNAPoly inhibition. However, NRTI can cause mitochondrial impairment by alternative damaging mechanisms independent of DNAPoly. First, NRTI have been reported to promote an imbalance in physiologic nucleotide pools and synthetic mitochondrial and cytoplasmic pathways leading to mtDNA depletion [55]. Second, NRTI can cause mitochondrial lesion without depleting mtDNA content. Zidovudine (AZT) has been shown to inhibit mitochondrial transport of energetic substrates (such as succinate or ADP/ATP), to directly inhibit mtCIV activity or to diminish mitochondrial carnitine content [50, 56, 57]. All NRTI have indeed been reported to potentially inhibit mitochondrial transcription *in vivo* [58] and *in vitro* [59].

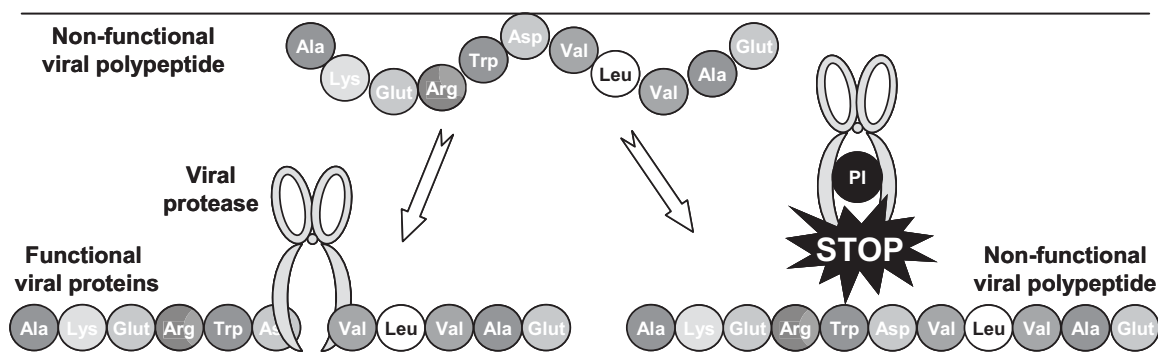
On the other hand, in severe NRTI-induced mtDNA depletion, transcriptional and translational upregulation mechanisms have been reported to homeostatically compensate mtDNA depletion and support mitochondrial function [60]. Sometimes, therefore, NRTI-mediated depletion does not have any effect on mitochondrial function and, contrarily, sometimes mitochondrial dysfunction may not be accompanied by mtDNA depletion.

Apoptotic effects have also been described for chronic administration of NRTI by an increase in mitochondrial ROS production and a decrease in ATP levels. Oxidative stress and energetic deprivation triggers intrinsic apoptosis [61-65], especially negative when it is the responsible for the depletion of CD4<sup>+</sup> T-lymphocytes of HIV-infected patients (as preferentially infected cells by the virus and the most important defense cells against the infection).

**2.3.1.2. Protease inhibitors (PI)** exert their antiviral therapeutic activity by blocking the viral cycle through the inhibition of the aspartyl-protease of the virus responsible for generating the new structural and enzymatic protein units necessary for the synthesis of new viral particles. Viral proteins are translated by host



machinery generating large polypeptide units that need to be proteolysed by the viral protease into small protein subunits for virion assembling. Protease inhibitors block viral protein processing.



**Figure 9:** Blockade of viral cycle by protease inhibitor (PI) antiviral drugs through inhibition of the protease of the virus. Ala: Alanine; Lys: Lysine; Glut: Glutamate; Arg: Arginine; Trp: Tryptophan; Asp: Aspartate; Val: Valine; Leu: Leucine.

Together with NRTI, PI are the most frequently administered antiviral drugs. However, their usage has been associated with several secondary manifestations and different adverse effects have been attributed to PI, including those at a metabolic, mitochondrial and apoptotic level.

Homology between cellular and viral proteins is responsible for the lipid and carbohydrate metabolic disturbances caused by PI which could, in turn, be responsible for the metabolic syndrome associated with these antiviral drugs.

However, to date, there is no consensus about the mitochondrial and apoptotic effects of PI. The anti-apoptotic and mitochondrial protection benefits reported by PI are based on the PI-mediated capacity to prevent mitochondria depolarisation and mitochondrial triggered apoptosis development [66]. Contrarily, other authors have reported that higher doses of PI depolarise mitochondria with the consequent development of apoptosis [67].

The mitotoxicity of PI has been described for PI ritonavir through glycoprotein-p inhibition [68] (which expels NRTI out of the cell) and cytochrome p450 inhibition [69] (responsible for some antiviral detoxification). Concomitant administration of PI with NRTI, contained in most antiviral regimens, increases the mitochondrial toxicity of NRTI.

Some authors have reported an anti-apoptotic effect for PI [70-74] because of their capacity to modulate proteasome activity and antigen presentation that decreases

immune activation and the derived apoptosis rate [71], and because they directly mediate a decrease in pro-apoptotic protein expression [72-74]. Other authors have described pro-apoptotic effects for PI [75-77] because of adipocyte [75, 76], T-lymphocyte [67, 77] or endothelial [78] PI-mediated cell death that has prompted the idea of using PI as antineoplastic drugs [79-81].

However, most authors accept a dual model of PI-mediated anti and pro-apoptotic behavior and beneficial and deleterious effects of PI with respect to mitochondria depending on drug dose [67, 72, 73]. Most of the data on the pro-mitochondrial and anti-apoptotic effects of PI have been obtained *in vitro* using drug concentrations under physiologic values [66, 72-74]. Contrarily, most studies using PI concentrations in plasma have reported mitotoxic and pro-apoptotic properties for these drugs [64].

Albeit controversial, all experts agree that in case that PI exerted mitotoxic effects, they would be fewer than that mediated by NRTI. We have indeed demonstrated that in case that PI caused apoptosis, it would be lesser than that induced by HIV itself [82].

**2.3.2. Neuroleptics or antipsychotics** are commonly used in psychiatric patients for the treatment of schizophrenia-related disorders, although paranoia and organic or functional psychosis are also frequently treated with antipsychotic drugs.

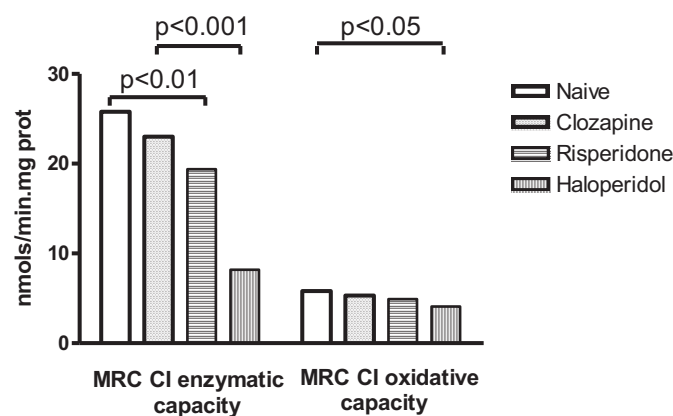
The first (classic) antipsychotic drugs (chlorpromazine and haloperidol) were discovered in the mid-90s. However, only a few years later extrapyramidal disorders were associated with neuroleptic administration [83]. Since then several new antipsychotic drugs have been described and commercialised.

Neuroleptic drugs exert their therapeutic activity by blockade of cerebral dopaminergic D<sub>2</sub> receptors. However, antipsychotics also block peripheral dopaminergic receptors and, in some cases, central receptor neurotransmitters different from dopamine, such as those of serotonin. These secondary effects of neuroleptics may only partially explain some of the associated manifestations in both the peripheral and central nervous systems. One of the most serious manifestations are the extrapyramidal effects of medication consisting in movement disorders.

Antipsychotics can be functionally and therapeutically divided into classical (such as haloperidol) or atypical drugs (including clozapine and risperidone). Atypical neuroleptics are characterised by parallel blockade of D<sub>2</sub> dopaminergic and 5HT<sub>2</sub> serotonergic receptors, by presenting activity on both negative and positive symptoms

of schizophrenia and refractory periods and, with respect to secondary manifestations, by causing minimum or null extrapyramidal disorders [84].

The development of extrapyramidal movement disorders in patients under neuroleptic treatment has been associated with different risk factors such as the time on treatment and patient age [85, 86], polymorphisms in cytochrome p450 responsible for antipsychotic detoxification [87] and the different number and sensitivity of dopamine receptors [88]. However, these risk factors cannot completely explain the individual susceptibility to develop extrapyramidal movement disorders. Alternative mechanisms of drug toxicity have therefore been proposed and the mitochondrial damage that causes antipsychotic inhibition of MRC complex I activity may help to explain unresolved questions by modulating the appearance of extrapyramidal movement disorders [7, 89-91]. Our group assessed mitochondrial toxicity in asymptomatic schizophrenic patients under treatment with different antipsychotics and confirmed mitochondrial toxicity of neuroleptics in MRC complex I function, either in its enzymatic and oxidative capacities. Nonetheless CI function was demonstrated to be different for the distinct antipsychotic drugs [92]. Antipsychotic drugs showed a scale-grade mitochondrial toxicity inhibiting MRC complex I function similar to the frequency of prevalence of extrapyramidal movement disorders caused by these drugs (haloperidol > risperidone > clozapine).



**Figure 10:** Mitochondrial respiratory chain complex I (MRC CI) enzymatic and oxidative activities in different naive or treated (with clozapine, risperidone or haloperidol) schizophrenic patients. Extended information is available in the article by Casademont et al, published in the *J Clin Psychopharmacol* 2007; 27: 284.

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We also demonstrated that only schizophrenic patients treated with haloperidol, showing the greatest CI dysfunction, presented increased oxidative stress (lipid peroxidation), which could, in turn, be produced by MRC CI impairment [92].

The hypothesis of mitochondrial toxicity induced by neuroleptic drugs could be related to monoamine oxidase function. Monoamine oxidase enzyme is responsible for dopamine synthesis, among others. Neuroleptics block dopamine receptors interfering with dopamine metabolism and turnover pathways leading to an increase in the oxidative stress caused by monoamine oxidase [93]. Mitochondria, and specifically MRC CI and CIII enzymes, are the main centre for ROS production and are thus, especially sensitive to ROS damage [94] that could, in turn, impair CI function generating more ROS in a sort of catastrophic vicious cycle [95] that could be responsible for the development of the secondary manifestation.

Because extrapyramidal manifestations are sometimes non-reversible, despite antipsychotic discontinuation, some authors have proposed a permanent alteration in mtDNA [96] as the aetiology of the secondary effect, which has not been confirmed by other authors [97].

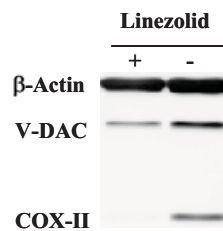
**2.3.3. Antibiotics** are used to treat bacterial infections. They are commonly classified depending on their nature or the antibacterial activity they exert.

The endosymbiotic origin of mitochondria postulates that mitochondria are ancient aerobic prokaryotes ( $\alpha$ -proteobacterias) that were evolutionally absorbed by the primitive anaerobic cell to obtain the ability to consume oxygen [98]. Because of bacterial origin, mitochondria share a high homology with some prokaryotes and, consequently, some antibiotics can exert severe mitochondrial lesion that can lead to severe secondary effects of medication.

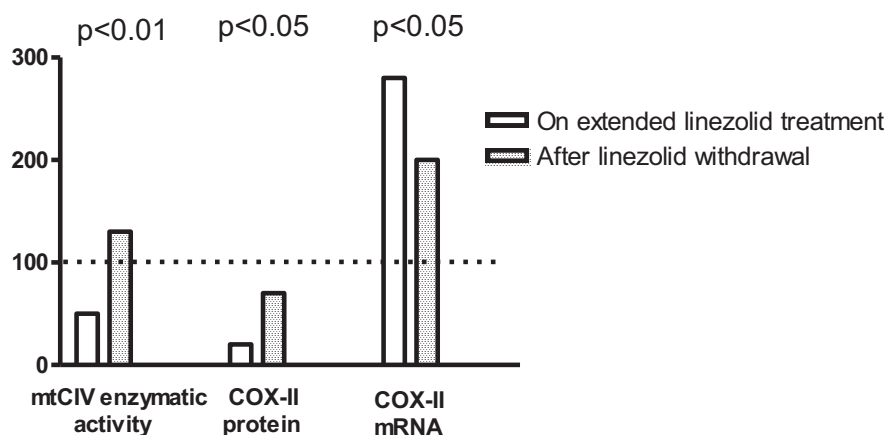
Some antibiotics present the side effect of blockade of MRC complexes leading, in most of cases, to the blockade of cellular respiration. This is the case of **piERICIDINE a**, **ANTIMICYNE a** and **OLIGOMYCINE**. Piercidine inhibits MRC complex I (specifically the electron transfer between NADH and ubiquinone), antimicyne a blocks MRC complex III (specifically electron transfer between cytochromes b and c) and oligomycine prevents MRC complex V (ATPsynthase) function.

Other antibiotics such as **VALINOMICYNE** and **GRAMICIDINE** exert an uncoupler effect on oxidative phosphorylation by preventing proto-motive ATP synthesis from the energy stored as the electrochemical gradient across the mitochondrial inner membrane.

Most of these antibiotics are not currently administered in clinical practice. However, some antibiotics that exert their therapeutic activity by inhibiting bacterial translation are frequently administered and have been demonstrated to be dangerous for mitochondria. This is because such antibiotics are also able to inhibit mitochondrial protein synthesis leading to consequent functional energetic and metabolic organelle failure. Mitochondrial protein synthesis inhibition is due to the high homology shared between both prokaryote and mitochondrial ribosomes. Some of these antibiotics (**chloramphenicol, tetracycline, erythromycin, eperezolid, linezolid** and **aminoglycosides**) present well-known mitochondrial toxicity. They block bacterial but also mitochondrial protein synthesis by preventing ribosome formation (and translation initiation) or peptidyl transferase activity. As a result there is no synthesis of bacterial proteins (leading to a decrease in bacterial growth) but there is also a reduction of mitochondrial proteins synthesis (leading to mitochondrial dysfunction and the manifestation of secondary effects) [99].

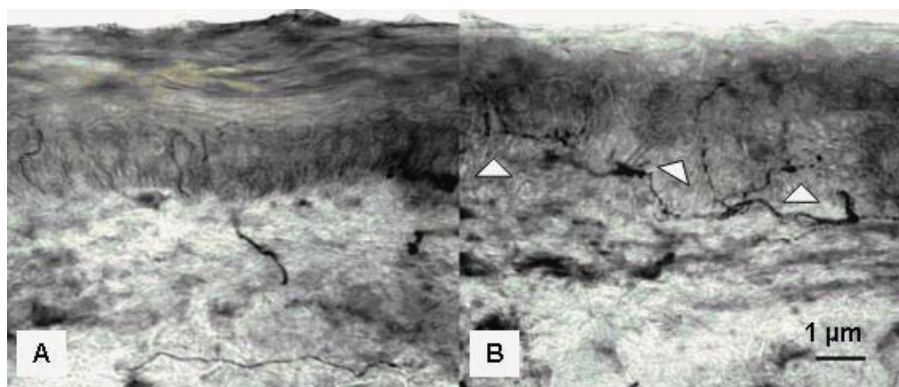


**Figure 11:** Amounts of mitochondrial protein before (-) or after (+) extended linezolid administration.  $\beta$ -Actine as cell loading control; V-DAC (voltage dependent anion channel) as mitochondrial amount control; COX-II (subunit II of the cytochrome c oxidase) as mitochondrial encoded and synthesized protein. Note COX-II decrease after linezolid administration (+). Extended information is available in the article by Garrabou et al, published in *Antimicrob Agents Chemother* 2007; 51: 962.



**Figure 12:** Mitochondrial complex IV (mtCIV) activity and mitochondrial protein and transcript expression (of mtCIV subunit II) in patients on extended linezolid treatment (white bars) or after drug withdrawal (black bars). Note the reduction in mtCIV protein and activity despite increased mitochondrial transcription. Mitochondrial RNA levels increase, probably, due to a homeostatic intent to preserve the mitochondrial protein rate unaltered. Extended information is available in the article by Garrabou et al, published in *Antimicrob Agents Chemother* 2007; 51: 962.

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**Figure 13:** Epidermal nerve fibers immunostained with the panaxonal marker PGP 9.5 in a skin biopsy performed at distal leg before (A), and after 30-days treatment with linezolid (B). Morphological abnormalities (small axonal swellings and fragmented fibers) are pointed by head-arrows in a subclinical small fiber neuropathy. By courtesy of Dr. Jordi Casanova-Molla and Mercedes Morales from the Neurology Department of the Hospital Clinic of Barcelona.

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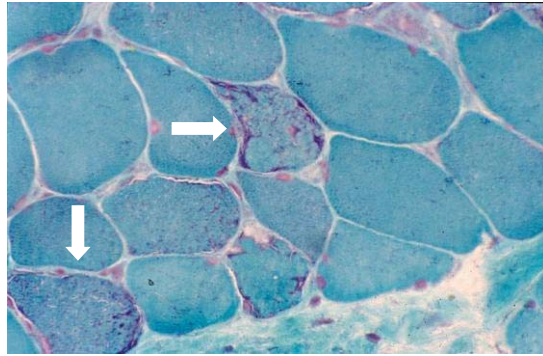
The adverse effects of these antibiotics responsible for mitochondrial synthesis inhibition show a wide range of variability. The most frequent secondary effects of erythromycin consist in gastrointestinal, hepatobiliar and dermatologic disorders. Tetracycline treatment can be associated with dizziness, cephalgia and dermatologic alterations. Chloramphenicol can cause bone marrow suppression and aplastic anaemia, conditions which may be accompanied by hyperlactatemia, peripheral neuropathy, lactic acidosis or optic nerve damage with extended treatment with linezolid or eperezolid. The mitochondrial toxicity of aminoglycosides consists in nephrotoxicity, neuromuscular blockade but also ototoxicity. Aminoglycoside-induced toxicity and derived hypoacusia are not universal and are mainly developed in subjects presenting the mtDNA mutation A1555G [100] and are not reversible after treatment disruption. It

has become one of the most prevalent causes of late-developing non-syndromic deafness and it is caused by damage of the cochlear and vestibular sensitive neurons of the VIII cranial pair. Eighty percent of individuals with the mitochondrial A1555G mutation may develop deafness along their lives. However, treatment of these patients with aminoglycosides causes immediate loss of audition in all of cases.

**2.3.4. Hypolipemiant**s therapeutic activity consists in decreasing the blood amount of lipids and thus are used to correct dyslipidemias. There are different kinds of hypolipemiant agents.

**2.3.4.1.** Anticholesterolemics are specifically used to reduce cholesterol, with **statins** being the most frequently administered. Statins decrease cholesterol levels by blocking its synthesis through competitive inhibition of the enzyme HMG CoA reductase. Cholesterol, and the remaining fatty acids, are initially synthesised from acetyl coenzyme a (CoA) in the mitochondria. The degradation process (through  $\beta$ -oxidation) undertakes just the opposite pathway to generate CoA, again in the mitochondria. Statin blockade of cholesterol synthesis has been associated with a reduction in mitochondrial Coenzyme Q content (mobile electron transfer located between MRC complexes I and II with respect to III) that can be clinically accompanied by the development of a myopathic syndrome (rise in creatine kinase, severe muscular weakness, rhabdomyolysis and myoglobinuria) which can, in some cases, lead to death. Fortunately, the frequency of statin-induced myopathy is quite reduced (about 1% of patients on treatment per year). However, certain drug combinations can increase its incidence. This is the case of concomitant administration of statins with the immunosuppressor cyclosporine, in renal or cardiac transplanted patients, in which myopathy frequency increases up to 30% [101]. An alternative pharmacokinetic interaction that increases statin-induced myopathy is the combination of statins with the antihypertensive treatment propranolol. Furthermore, the association of statins with other hypolipemiant agents such as fibrates notoriously increases the risk of the development of myopathy, especially when the patient is over 70 and kidney function is reduced [102]. In some cases muscle biopsy shows evident mitochondrial abnormalities (Figure 14).





**Figure 14:** Gomori trichrome staining in a frozen section of skeletal muscle from a patient with symptomatic statin toxicity. Observe classical ragged-red fibers (white arrows).

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**2.3.4.2. Fibrates**, used to treat hyperlipidemia, have also been associated with mitochondrial damage, especially if concomitantly administered with statins. Individual fibrates induce mitochondrial dysfunction via different molecular mechanisms to finally impair cell respiration. Fenofibrate, per example, predominantly acts by inhibition of the MRC complex I enzymatic activity [103].

**2.3.5. Anaesthetics and sedatives** are commonly used in clinical practice to lessen sensitivity or the awareness of pain or consciousness, frequently to undergo surgery.

**2.3.5.1. General anaesthetics** have been reported to inhibit mitochondrial function in experimental and in human models, demonstrating a decreased capacity to consume oxygen [104]. General anaesthetic inhalants such as halothane, isoflurane and sevoflurane have been shown to inhibit MRC NADH:ubiquinone oxidoreductase (complex I) of cardiac mitochondria [105]. Mitochondrial-like myopathies have been described for intravenous anaesthetics as in the severe, albeit rare, *propofol infusion syndrome*, which is often lethal and is characterised by metabolic acidosis, acute cardiomyopathy and skeletal myopathy. This syndrome has been reported to be due to the failure of free fatty acid metabolism due to inhibition of the entry of free fatty acid into the mitochondria and interference at specific sites in the MRC [106].

**2.3.5.2. Barbiturates** (such as amital), used as sedatives, anaesthetics, ansiolytics, hypnotics and anticonvulsants have also been described to



block initial MRC electron transport between MRC complex I (Fe-S centre) and ubiquinone. However, MRC electron transfer from MRC II to the remaining MRC is preserved.

**2.3.5.3.** Multiple mitochondrial effects have been described for **benzodiazepines**, which present a mitochondrial receptor that has been implicated in steroid-synthesis and permeability transition pore opening and apoptosis development.

**2.3.5.4. Local anaesthetic** drugs have been described to cause mitochondrial damage through uncoupling oxidative phosphorylation and inhibiting mitochondrial ATPase and different MRC complexes [107].

**2.3.5.5.** Concomitant administration of the **neuromuscular relaxant drug** pancuronium and antiinflammatory steroids in patients receiving mechanical ventilation has been related to the development of toxic myopathy [101].

**2.3.6. Nonsteroidal antiinflammatory drugs** (NSAID) such as aspirin or ibuprofen are among the most prescribed drugs around the world because of their pharmacotherapeutic activity in releasing inflammation and pain through cyclooxygenase inhibition and a consequent decrease in prostaglandine production.

Mitochondrial damaging mechanisms induced by NSAID have been studied in depth. They have been described to cause an uncoupling of the oxidative phosphorylation pathway, a increase in resting state respiration, a decrease in ATP synthesis and mitochondrial membrane potential, inhibition of adenine nucleotide translocase and to cause an alteration in mitochondrial lipid metabolic pathways (through inhibition of  $\beta$ -oxidation) [107].

All this mitochondrial damage, together with cyclooxygenase inhibition and inflammatory tissue-reaction, has been proposed to be responsible for gastrointestinal damage (ulcers, gastrointestinal perforation and bleeding) associated with NSAID administration [107]. Reye's syndrome, a childhood disorder characterized by liver disease and encephalopathy, was also initially associated with aspirin administration and potential mitochondrial damage. The association of Reye's syndrome with aspirin is currently weakening as is the implication of mitochondrial damage in this syndrome.

**2.3.7.** The therapeutic activity of some **antineoplastics** and **chemotherapies** is based on inducing damaged cell death through apoptosis by interfering with mitochondrial pathways. This is the case of flutamide, tamoxifen and doxorubicine, which are chemotherapy drugs used for prostate, breast and soft-tissue

cancer treatment, respectively, that have been reported to interfere with mitochondrial respiration, uncoupling of the oxidative phosphorylation system and, specifically, the electron transference from MRC complex I by a redox cycling mechanism [108-110].

**2.3.8. Antidiabetic** drugs such as metformine have also been related to mitochondrial damage through specific MRC impairment. For instance, metformine specifically inhibits MRC complex I activity [111].

**2.3.9. Antiarrhythmic** and **antimalaric drugs** such as quinidine have also been described as mitotoxic agents. Quinidine has been particularly associated with ATP-synthase inhibition [112].

**2.3.10. Fungicide** agents including mixotiazol (responsible for MRC complex III blockade), sodium azyde (which inhibits mtCIV function) or rutamicine (also used as an antibiotic; responsible for ATP-synthase inhibition), can also seriously impair mitochondrial function leading to cell death.

## **2.4. Chemical substances: pesticides**

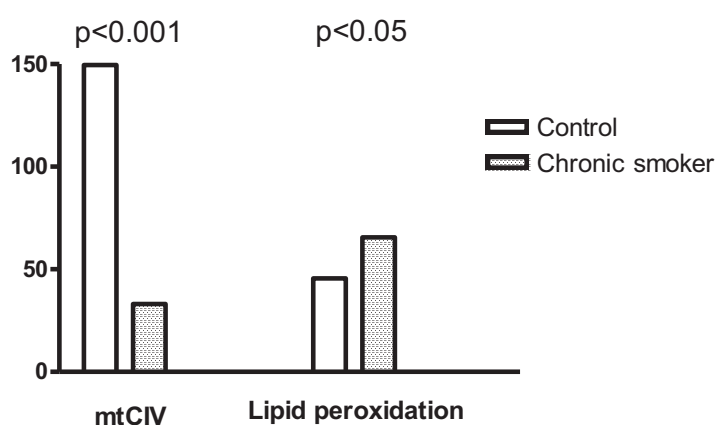
Some pesticides (herbicides, insecticides or acaricides) can seriously damage mitochondria. These compounds may be the cause of clinical symptoms by acute intoxication but usually produce clinic manifestations after prolonged low-dose chronic exposure such as that produced by occupational contact. At present, many neurodegenerative disorders, especially Parkinson's disease, are being associated with the toxic effect of certain of these agents on neurons, and many of these toxic agents exert damage in mitochondrial activity.

Different mechanisms damaging mitochondria have been described among pesticides, but the inhibition of the MRC complex I enzymatic activity is one of the most common mitotoxic capacities among the most frequently used compounds. The commonly used insecticides **rotenone** and **pyridaben** and the frequently widespread acaricides **fenazaquin** and **fenpyroximate** share the common capacity of blocking MRC complex I function. Among them pyridaben exerts the strongest inhibition of MRC complex I in neuroblastoma exposed cells and, together with rotenone, are able to increase the amount of ROS and cause derived oxidative damage in neurons, being frequently associated with neurodegenerative disorders.

Herbicides such as **paraquat** and **glyphosphate** have been described to cause diffuse but severe mitochondrial lesions that increase the rate of apoptosis, in the case of paraquat, probably mediated through increased ROS production [113].

## 2.5. Abuse drugs

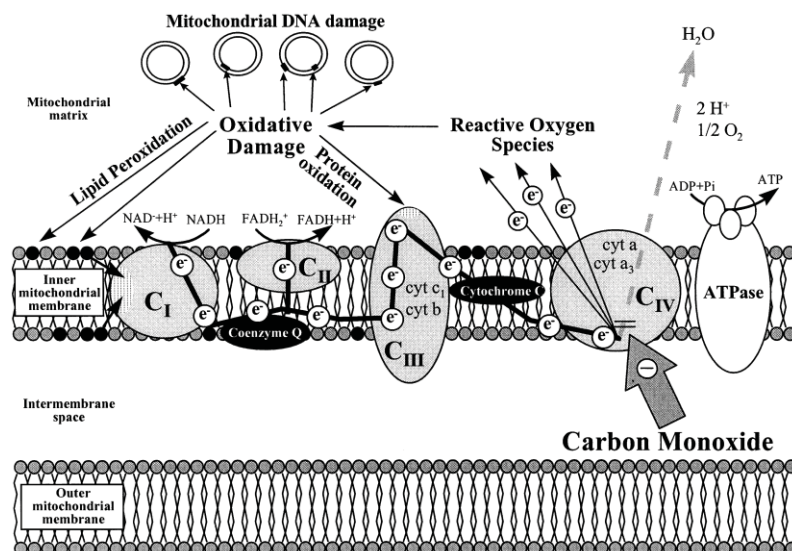
**2.5.1. Tobacco smoke** contains thousands of toxic compounds. Among them our group has proposed CO as mainly responsible for the mitochondrial disturbances observed both in chronic smokers [32, 114, 115] and non-smokers exposed to tobacco smoke [111]. These *in vivo* analyses reported the inhibition of mtCIV in these subjects, characteristic of CO contact, both in chronic [32, 114, 115] and acute [116] smokers; whereas an increase in oxidative stress damage (lipid peroxidation of membranes) is only present in chronic individuals in a sort of additive accumulative effect.



**Figure 15:** Mitochondrial complex IV enzymatic activity (mtCIV) and oxidative stress (lipid peroxidation) damage in chronic tobacco smokers. Extracted from the article by Miro et al. published in *Toxicol Lett* 1999; 110: 219-223.

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*In vitro* analyses performed by our group studying the direct and isolated effect of CO on healthy mitochondria showed dose-dependent identical results with respect to tobacco-induced mitochondrial damage in smoker subjects. These findings confirm the mitochondrial damage produced by CO in tobacco smokers [117].



**Figure 16:** Carbon monoxide-derived mitochondrial effects. Reprinted from the article by Miro et al. published in *Toxicol Lett* 1999; 110: 219-223, with permission from Elsevier.

**2.5.2. Alcohol-induced oxidative stress** is linked to the metabolism of ethanol involving both the microsomal and mitochondrial systems. Ethanol metabolism is directly involved in the production of ROS and reactive nitrogen species generating an environment favorable to oxidative stress. Ethanol intake results in the depletion of glutathione levels and decreases antioxidant activity. It elevates malondialdehyde, hydroxyethyl radical, and hydroxynonenal protein adducts [118].

However, the oxidative damage of ethanol is reduced and even reversed when ethanol is administered together with polyphenols (in case of wine or beer). This may explain why many studies performed in alcoholic subjects have failed to find mitochondrial alterations [119, 120].

### **3. TREATMENT OF MITOCHONDRIOPATHIES**

The first therapeutic measure to assume when secondary mitochondrial toxicity is suspected, either asymptomatic or accompanied by clinical symptoms, is to discontinue the contact with the toxic compound. However, this is sometimes not possible, for instance, because mitochondrial toxicity is the adverse effect of an imperative therapeutic regimen. If toxic discontinuation is not possible, treatment of secondary mitochondrial diseases becomes necessary. However, to date, the treatments of

mitochondriopathies are exclusively symptomatic and supportive. Symptomatic treatment of mitochondriopathies is based on administering drugs, blood transfusions, haemodialysis, invasive measures, surgery, dietary measures, and physiotherapy [121]. Drug treatment may be classified as specific (treatment of epilepsy, headache, dementia, dystonia, extrapyramidal symptoms, Parkinson syndrome, stroke-like episodes or non-neurological manifestations), non-specific (antioxidants, electron donors/acceptors, alternative energy sources, cofactors), or restrictive (avoidance of drugs known to be toxic for mitochondrial functions) [121]. In the case of symptomatic hyperlactatemia and lactic acidosis as secondary mitochondriopathies induced by both HIV and antiretroviral treatment, concomitant administration of mitochondrial non-specific drugs (L-carnitine, B6 and C vitamins, thiamine and hydroxycobalamine) has been associated with faster and better amelioration of affected patients [8]. The information currently available on the mitotoxic aetiology of adverse events associated to HIV infection and antiviral treatment has led to antiretroviral guidelines for restrictive strategies based on administering less potent mitotoxic regimens (reduction in antiretroviral doses, a change in antiviral schedules to nucleoside-sparing therapies or the implementation of structured-treatment interruptions) [122-125].

In secondary mitochondrial disturbances caused by toxic drug administration, clinicians should be aware of which drug to choose, the doses and combinations of both. Physicians must be aware of early signs or symptoms of toxicity with a subsequent change in therapy not only after the manifestation of toxicity but also prior to its development. Although mitochondrial recovery is possible in some cases after discontinuation of the toxin, it is preferable to prevent the secondary effects of therapies rather than manage them and, once the secondary effect has developed, early management will help to achieve mitochondrial and clinical recovery.

Blood transfusions and haemodialysis help in the clearance process of the metabolites that have been stored in the blood as well as in the renovation of the blood cell units responsible for metabolite detoxification. If anaemia is present, transfusions will help to maintain the erythrocyte count.

Invasive measures include surgery for the implantation of devices able to maintain impaired organ function. For instance, in the case of heart diseases, pacemakers, portable defibrillators or stent therapies will help to prevent heart failure [121].

For the treatment of some manifestations secondary to mitochondrial disease, including diabetes, hyperlipidemia or epilepsy, dietary measures can be offered (such as ketogenic diet or anaplerotic diet) [121].

Treatment of mitochondrial diseases should be individualized because of the peculiarities of mitochondriopathies.

Despite limited possibilities, symptomatic treatment should be provided to patients with mitochondriopathies, since it may have a significant impact on the course and outcome of the disease.

Further studies should be addressed to seek the bases of mitochondriopathies and to assess how to prevent or correct mitochondrial function and determine markers of premature toxicity that will allow the adverse effects of chronic secondary mitochondrial diseases to be avoided.

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