Mitochondrial profile and amyloidogenic molecules in sporadic inclusion body miositis

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Thesis presented by

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For the Degree of Philosophy Doctor by the University of Barcelona

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Barcelona, 2017

Als meus pares

“Tu mente responderá más preguntas si aprendes a relajarte y a esperar la respuesta”

William S. Burroughs

“El cerebro es un universo en constante expansión y movimiento. Vamos en una cárcel racional que navega dentro de un loco.”

Alejandro Jodorowsky

DIRECTOR'S REPORT

The thesis presented by the PhD-student Marc Catalán is a compendium of two publications. Marc Catalán has participated actively in the experimental process, data analysis and interpretation of the results and also in the drafting of both manuscripts. The Fundació Cellex financially supported the research not only in laboratory expenses but with the incorporation of Marc to the Muscle Research and Mitochondrial function Laboratory.

The two publications included are the core of this thesis and should not be used for other thesis.

Publication 1. *Mitochondrial DNA disturbances and deregulated expression of OXPHOS and mitochondrial fusion proteins in sporadic inclusion body myositis.*
Marc Catalán-García, Glòria Garrabou, Constanza Morén, Mariona Guitart-Mampel, Adriana Hernando, Àngels Díaz-Ramos, Ingrid González-Casacuberta, Diana-Luz Juárez, Maria Bañó, Jennifer Enrich-Bengoa, Sonia Emperador, JC. Milisenda, P. Moreno, Ester Tobías, Antonio Zorzano, Julio Montoya, Francesc Cardellach, Josep Maria Grau.

Clinical Science. IF: 5.016 (Q1). 2014 IF 5.60 D1; 2015 IF 4.99 Q1

Marc Catalán has contributed in the patient inclusion, sample processing, molecular experiments, data analysis and statistics, interpretation of results, manuscript draft and revision.

Publication 2. *BACE-1, PS-1 and sAPPβ levels are increased in plasma from sporadic inclusion body myositis patients: surrogate biomarkers among inflammatory myopathies.*
Marc Catalán-García, Glòria Garrabou, Constanza Morén, Mariona Guitart-Mampel, Ingrid González-Casacuberta, Adriana Hernando, Jose Gallego-Escuredo, Dèlia Yubero, Francesc Villarroya, Raquel Montero, Albert Selva O'Callahan, Francesc Cardellach, Josep Maria Grau.

Molecular Medicine. IF: 3.530 (Q1). 2014 IF 4.51 Q1; 2015 IF 3.53 Q2

Marc Catalán has contributed in the study design, patient inclusion, sample processing, molecular experiments, data analysis and statistics, interpretation of results, manuscript draft and revision.

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CONTENTS

1. LIST OF ABBREVIATIONS

Aβ: amyloid-β
AD: alzheimer's disease
AIF: apoptotic inducing factor
APAF1: apoptotic protease activator factor 1
APP: amyloid precursor protein
ARF: ADP ribosilation factor
ATF: activating transcription factor
ATP: adenosine triphosphate
BACE1: beta-secretase 1
CAD: caspase activated DNAse protein
CK: creatin kinase
CN1A: cytosolic 5' nucleotidase 1A
CoQ: Coenzyme Q
COX: cytochrome C oxidase
CS: citrate synthase
CytC: cytochrome C
DM: dermatomyositis
Drp1: dynamin-related protein 1
EMG: electromyography
ENMC: European neuromuscular centre
ER: endoplasmic reticulum
FAD: flavin adenine dinucleotide
HAE: hydroxyalkenal
hIBM: hereditary inclusion body myiositis
IBMFRS: inclusion body myositis functional rating scale
IL-1: interleukin 1
IMM: inner mitochondrial membrane
IVIG: immunoglobulin therapy
kDa: kilodalton
MHC: major histocompatibility complex
MiD49: mitochondrial dynamics protein 49
MiD51: mitochondrial dynamics protein 51
MDA: malondialdehyde
MDV: mitochondria derived vesicles
MFF: mitochondrial fusion factor
Mfn1: mitofusin 1
Mfn2: mitofusin 2
MHC: major histocompatibility complex
MRC: mitochondrial respiratory chain
mRNA: messenger ribonucleic acid
mtDNA: mitochondrial deoxyribonucleic acid
NADH: nicotidamide adenine dinucleotide
NAM: necrotizing autoimmune myositis
OMM: outer mitochondrial membrane
OPA1: optic atrophy 1
OXPHOS: oxidative phosphorilation
PARL: presenilins-associated rhomboid like protein
PBMC: peripheral blood mononuclear cells
PM: polymyositis
PS1: presenilin 1
QMT: quantitative muscle testing
ROS: reactive oxygen species
RRF: ragged red fiber
rRNA: ribosomal sibonucleic acid
sAPPβ: amyloid precursor protein (β)
SDH: succinate dehydrogenase
SMAC: second mitochondria-derived activator
sIBM: sporadic inclusion body myositis
SOD1: superoxide dismutase
TGF-β: transforming growth factor β
TNF-α: tumour necrosis factor alpha
tRNA: transference ribonucleic acid
Ubb+1: Ubiquitin-B +1
UPR: unfolded protein response
VDAC1: voltage-dependant anion channel 1

2. INTRODUCTION

2.1. The disease: Sporadic Inclusion Body Myositis (sIBM)

2.2.1.- INTRODUCTION

Inflammatory myopathies constitute a group of potentially treatable myopathies in children and adults. Regarding their distinct clinicopathological features, they can be classified in four main subtypes: dermatomyositis (DM), polymyositis (PM), necrotizing autoimmune myositis (NAM) and sporadic inclusion body myositis (sIBM). The common features are proximal muscle wasting and increasing difficulty with tasks requiring these muscles such as lifting objects or climbing steps among others. In addition, neck extensors and pharyngeal muscles could be affected in all forms of inflammatory myopathies, causing dropped head or dysphagia, respectively. The identification of the current subtype is fundamental, because each one has a different prognosis and response to therapies. Typical differential features of the DM consist in skin manifestations such as periorbital heliotrope rash with edema or erythrematous rash. Regarding PM, is still a diagnosis of exclusion and most of the patients diagnosed as PM receive the final diagnoses of autoimmune disease associated myositis, sIBM, NAM or "inflammatory" dystrophy. It is defined as a subacute proximal myopathy in adults who do not have rash, familiar history of neuromuscular disease, clinical phenotype of IBM, involvement of facial and extraocular muscles and exposure to myotoxic drugs. NAM can start acutely or subacutely causing progressive weakness with serum high creatine kinase levels. This condition may occur after viral infections, in association with cancer, in patients under statins treatment or in patients with any autoimmune disease. Some patients with NAM present antibodies against signal reconition particle (SRP) or against 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR)(1, 2).

sIBM is the most common form of inflammatory myopathy in individuals over the age of 50 years and the most important myopathy associated with aging (3). The aetiology of this progressive degenerative disorder of the skeletal muscle remains unknown. Distinctive pathological features of sIBM were first described in 1967 (4), and the name of the disease entity derives from a 1971 case report that in retrospect did not have sIBM (5). However, the first clinical and pathological series was not published until 1978 (6). sIBM should be distinguished from hereditary inclusion body myositis (hIBM) in which histological and ultra-structural findings resemble those of sIBM with two clear exception: the absence of inflammation at pathological level (7) and the recognition of an hereditary pattern of segregation through the familial lineage.

2.1.2.-EPIDEMIOLOGY

As recent studies have shown, a considerable global variability in sIBM prevalence exists, which is estimated at 4.5 - 9.5 per million people but raises to 22-35 per million for subjects over 50 years old. This geographic variability has been studied recently and sets sIBM prevalence to 1.0 per million people in Turkey (8), 4.7 per million in the Netherlands (9), 9.8 per million in Japan (10) or 50 per million in South Australia (11), for example. These prevalence rates are likely to be an under-estimate of the real frequency of the disease due to its insidious nature and delays in diagnosis as well as a high rate of initial misdiagnosis.

The improvement of the diagnosis algorithms developed in the last years together with population ageing will also raise these numbers up in the coming years. *Mastaglia et al.* proposed that variations in prevalence may be related to population frequencies of the HLA-DRB1* 03:01 risk allele which is known to be strongly associated with the disease in European, North American and Australian population (12), but further research should further establish the behaviour of its distribution and frequency.

3.- CLINICAL FEATURES

The typical disease phenotype is present in the majority of cases and includes slowly progressive weakness and wasting of proximal and distal muscles being the quadriceps and forearm and deep finger flexors typically affected muscles. The pattern of weakness is usually asymmetric. First signs for patients are falls and difficulty walking or climbing stairs due to the quadriceps muscle wasting. Other clinical manifestations appearing as the disease progresses are dropped head and camptocormia due to selective atrophy and weakness of paraspinal muscles. It has recently been described that some patients could present the Beevor's sign. Dysphagia is also a common feature, affecting 60% of the patients. It also has been recently reported that exists a high incidence of sleep disorder breathing (SDB) due to weakness of oropharyngeal muscles (13). Clinical course is always chronic or very chronic (Fig. 1). There have been many studies about how to measure the clinical course and rate of deterioration of sIBM patients, but the IBM-Net Project conclude that quantitative muscle testing (QMT) and the 10-point IBM Functional Rating Scale (IBM-FRS) are the best indicators of disease progression (14, 15). Although life expectancy is not significantly reduced in sIBM compared to the general population, the quality of living decreases as disability and morbidity increases in the later stages of the disease. The most disabling features are the confinement of the patient in a wheelchair, as well as the severe dysphagia and respiratory function impairment.

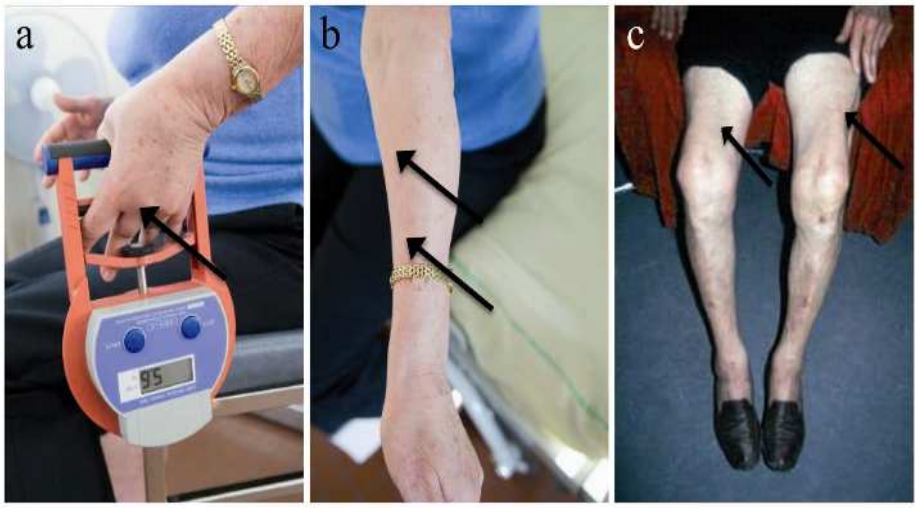

Fig. 1 Clinical features of sIBM. **a** Finger flexors weakness (*arrow* maximum obtained by this patient: 5.6 kg, normal >21 kg for a woman at 70 years old), and **b** atrophy (*arrows*). **c** Quadriceps atrophy (*arrows*) for a man at 75 years old

Fig1. Clinical features of sIBM, from 1-3 Acta Neuropathol (2015) 129:611–624 DOI 10.1007/s00401-015-1384-5.

2.1.4.- DIAGNOSIS

As the diagnosis of sIBM is more evident in the advanced stages of the disease, the real challenge is to establish an early diagnosis in the initial course of the disease when potential disease modifying therapies are more likely to be effective. The latest diagnostic criteria that has been proposed for use in patients is the 2011 European Neuromuscular centre (ENMC) guidelines (16). sIBM is often diagnosed as PM or other diseases. In a patient complaining of falls due to weakness at the knees and feet with atrophic thighs and without paresthesias or cramps the most plausible diagnosis is sIBM. However, muscle biopsy remains the definitive diagnostic procedure for sIBM. Nevertheless, the emphasis in the importance of specific clinical features is becoming stronger, such as long finger flexor weakness and clinical-pathological correlations (16, 17)(Fig. 2). At histopathological level, the combination of inflammatory changes (CD8+ T-cell infiltrate, and expression in muscle of MHC-I antigens), degenerative features (rimmed vacuoles and intra-cellular protein aggregates) and mitochondrial changes (COX-/SDH+ fibers and ragged-red-fibers or RRF) is highly specific for Sibm (Fig. 3). The current challenge in sIBM diagnosis is that it is uncommon to find all these pathologic features together in a muscle biopsy. In particular, in early stages of the disease the inflammatory changes tend to be more prominent and the degenerative changes ted to be less evident.

Diagnostic criteria proposed for sIBM.

Clinical features:
- Duration of illness >6 months
- Age at onset > 30 years
- Slowly progressive muscle weakness and atrophy: selective pattern with early involvement of quadriceps femoris and finger flexors (frequently not symmetric)
- Dysphagia

Laboratory features:
- Serum CK levels might be high but can be normal
- EMG: myopathic or mixed patterns, with both short and long duration motor unit potentials and spontaneous activity

Muscle biopsy:
- Myofiber necrosis and regeneration
- Endomysial mononuclear cell infiltrate (in variable degree)
- Mononuclear cell-invasion of non-necrotic fibers (mainly CD8)
- MHC class I expression in otherwise morphologically healthy muscle fibers
- Vacuolated muscle fibers (rimmed vacuoles)
- Ubiquitin-positive inclusions and amyloid deposits in muscle fibers
- Nuclear and/or cytoplasmic filamentous inclusions of 16–20 nm on electron microscopy
- COX-negative fibers

Fig2. Diagnostic criteria proposed for sIBM, from Autoimmunity Reviews 13 (2014) 363-366 (CK: creatin kinase, EMG: electromyography, MHC: major histocompatibility complex, COX: cytochrome c oxidase.)

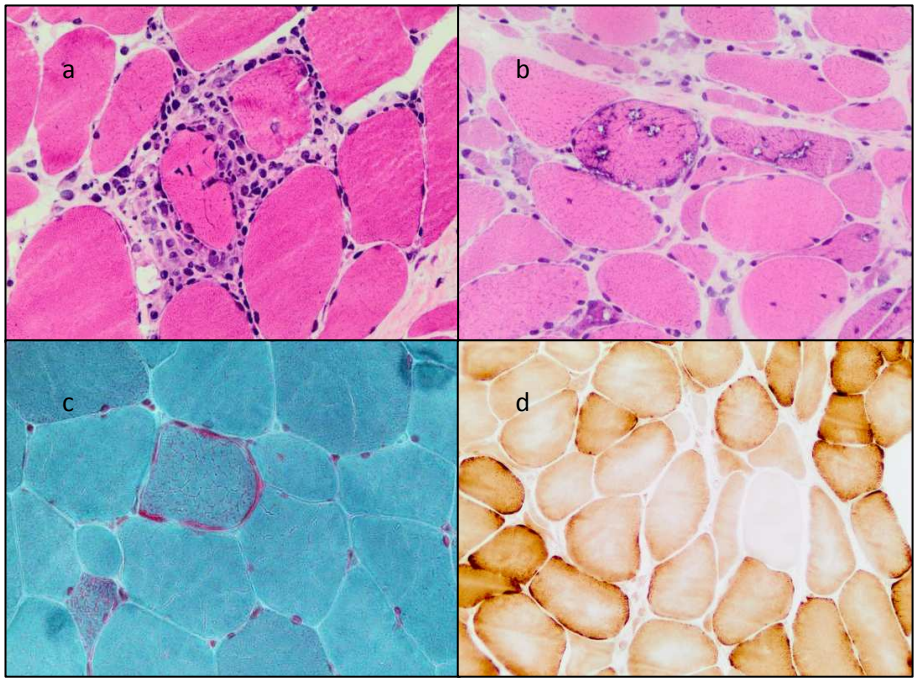

Fig3. sIBM features at histopathological level: a. T-cell infiltrate b. rimmed vacuoles c. ragged-red fibers d. Cytochrome c oxidase negative fibers.

2.1.5.- PATHOGENESIS

Although many different theories of the aetiology if sIBM have been proposed, the major event that triggers the sIBM development is still unclear. Among all the pathological processes, many authors recently considered the myodegenerative changes to be primary (18), being the immunologic response and the mitochondrial alterations collateral and secondary damage to this muscle degeneration. However, which one of these processes occurs first and has the dominant role is still debated (19). In addition, there is an absence of genetically recognized cause, thus the information about the key mechanisms involved in this disease is poor. In this section we will focus on describing the three main pathological events in sIBM pathogenesis:

-Immunopathology

Inflammation in sIBM is commonly more severe in early stages of the disease. This feature is represented mainly by a T-cell infiltrate in muscle fibers, as well as the expression of cytokines and chemokines and also the expression of MHC-I in the sarcolemma of the muscle fibers. There are evidences of a clonal expansion of these T-cells, but no study has identified yet an auto antigen that T cells are directed to in sIBM (20). This CD8+ T-cell infiltrate commonly can be detected surrounding myofibers, but rarely invading them. Nevertheless, some current models propose the cytotoxic T-cell invasion of myofibers as the final pathway by which the immune system injures sIBM muscle. This clonal expansion of T-cells and the induction of cytokines could also cause the upregulation of MHC-I (21), which could be seen in morphologically healthy muscle fibers and in those invaded by T-cells (22, 23). Apart from this, the upregulation of MHC-I might also be a result of the endoplasmic reticulum (ER) overload response, which has a key role in sIBM pathogenesis (24, 25). The

accumulation of misfolded proteins causes the activation of highly specific signalling pathways namely the unfolded protein response (UPR). This response, through the upregulation of chaperones and the overload response, lead to an increase in the transcription of cytokines, MHC-I and amyloid precursor protein (APP) (26).

In addition, an autoantibody that represented the first serological biomarker for sIBM has been recently described.These anti-Mup 44 antibodies are targeted to cytosolic 5'-nucleotidase 1A (cN1A), and were found in 33% of sIBM patients, being poorly detected in patients with DM (4.2%), PM (4.5%) or patients with other neuromuscular disorders (3.2%) (27). Lately, *Lloyd et al* found that this autoantibody was not only specific for sIBM, but was also present in several autoimmune diseases (28).

-Myodegeneration

Apart from the evidence of inflammatory findings in sIBM muscle tissue, muscle degenerative features such as myofiber necrosis, vacuolated muscle fibers (rimmed vacuoles), ubiquitin-positive inclusions with amyloid deposits and nuclear and/or cytoplasmic filamentous inclusions coexist with the inflammatory response (7). Some authors suggest that abnormal accumulation of amyloid precursor protein and amyloid β (Aβ) are key upstream pathogenic events in the vacuolar degeneration and atrophy of muscle fibers (29). Besides the amyloid β accumulation, there are many other proteins that accumulate in muscle from sIBM patients including the apoptotic regulators Bcl-2, Bcl-x and Bax, phosporylated tau, ubiquitin and also the mutated form (ubb^{+1}), parkin, presenilin 1 (PS-1), superoxide dismutase (SOD1) and many more (29).

The mechanism leading to the abnormal protein accumulation and muscle degeneration is still unclear, but it is well known that other mechanisms are impaired, including the failure of calcium homeostasis, ER stress and proteasome inhibition (30). *Askanas et al.* demonstrate that the muscle from sIBM patients present an activation of the UPR caused by the abnormal accumulation of proteins in the ER that leads to ER stress. The activation of UPR causes the increase of the activating transcription factor 4 (ATF4) protein, cleavage of activating transcription factor 6 (ATF6) and the increase of the spliced form of X-box binding protein 1 and increased mRNA of ER degradation-enhancing α-mannosidase-like protein. In addition, they demonstrate that these features are not present in muscle from patients of the hereditary form of the disease (31). They also demonstrate the upregulation of the chaperone-mediated autophagy (32), the proteasome inhibition and decreased lysosomal degradation in muscles from sIBM patients (33), key processes for the understanding of the abnormal protein accumulation and, ultimately, the muscle degeneration.

-Mitochondrial Alterations

Mitochondrial abnormalities such as mitochondrial DNA (mtDNA) deletions and the presence of COX deficient muscle fibers have been reported in sIBM patients in the last 20 years (34-37). In addition, the presence of RRF and SDH+ fibers are common features in muscle from sIBM patients, showing typical diagnostic clues of primary mitochondrial disorders also present in sIBM patients (38). However, there are few studies that deepen in mitochondrial impairment of sIBM patients at molecular level. *Rygiel et al.* recently

identifies the presence of respiratory-deficient fibers with down regulated expression of complex I and mtDNA rearrangements. They also found a strong correlation between then incidence of T-cell infiltrate and the abundance of respiratory-deficient fibers, and that those fibers were more likely to be atrophic compared to normal fibers (39).

There is scarce information about the mitochondrial respiratory chain (MRC) function in those patients but *Joshi et al.* found that three out of nine sIBM patients had decreased activity of MRC complex IV also known as COX (40). Although these results are promising, further research in this area is needed to deepen in how MRC is affected in sIBM patients.

2.1.6.- THERAPY

The classic therapy that has been proposed for sIBM treatment is the immunosuppressive and immunomodulatory therapies, but the response from the patients to this therapy is poor and, at best, only a temporary improvement or period of stabilisation, followed by the disease progress (41). Therapeutic options for sIBM treatment can be separated in two categories: nonpharmacologic and pharmacologic treatment options.

- Nonpharmacologic treatment options:

- Exercise: There is a duality regarding exercise for sIBM treatment. Although there is a strong belief that exercise has to be avoided in this condition due to the hypothetic muscle breakdown caused by overwork, many authors recently have reported in small studies that mild home exercise could improve the muscle strength (42). Other studies prescribing exercise regime in sIBM patients reported improvements in strength, function and aerobic capacity (43, 44). Although there is no large randomized-controlled trial evaluating the effect of exercise in sIBM patients at date, these data suggest that mild exercise supervised by a trained physical therapist could be a possible tangible treatment for patients to temporarily reduce the progression of sIBM over time (45).

- Orthoses: As instability and frequent falls are common in sIBM patients due to knee collapse and foot drop, the use of orthoses may have potential to improve sIBM patient's mobility. Studies evaluating the use of these mechanisms in those patients are scarce; however *Bernhardt et al.* evaluate the use of a specific stance control orthoses unilaterally in a small cohort of sIBM patients. Although most of them reported more stability and fewer falls, and the braces were well-tolerated, they resulted in slower walking speed which may not be suitable for all patients (46, 47).

- Pharmacological treatment options

- Immunosupressants: One of the most important features in sIBM muscles is inflammation, and many immunosuppressant drugs have been tested in smaller and larger sIBM cohorts to unsuccessfully improve its symptoms (48). Prednisone administration showed improvement in a case report, but studies with larger cohorts demonstrate that prednisone administration and prednisone with other immunosupressant drug does not improve disease progression and even caused decrement in muscle strength (48, 49). Although some pilot studies with mycophenylate, cyclosporine-A and tacrolimus showed

potential for sIBM treatment, larger trials reported no long-term positive effect in this disease (50, 51). The administration of intravenous immunoglobulin therapy (IVIG) had similar results than the previous treatments. Although a recent follow-up confirmed that could be short-benefit in some patients (52), there is general consensus that long-term treatment is not justified. Other therapies targeting the immune system in sIBM patients have been tested with similar results, including T-cell depletion using alemtuzumab, anti-lymphocyte globulin or tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) blocking agents (53-56).

- Other treatments: Alternative therapeutic strategies apart from immunomodulation have been studied due to the failure of previous therapies and also due to new insights into the pathogenesis of sIBM (31, 57). Arimoclomol is a heat shock protein enhancer in muscle and has demonstrated its cytoprotective effect, its safety and its potential to increase muscle strength in preliminary studies (58). Myostatine is a protein that inhibits the muscle development. Myostatine inhibitors like follistatin and bimagrumab also have promising results in improving the muscle condition in sIBM, and both of them are at this moment being tested in clinical trials (NCT01925209 and NCT01519349, respectively). Bimagrumab is a human monoclonal antibody developed by Novartis to treat pathological muscle loss and weakness. Bimagrumab infusion before the 6-min walk test succeeds in improving its performance. Other alternative treatments are being tested and, for example, an intramuscular injection of follistatin, a protein involved in the binding and bioneutralization of members of the transforming growth factor β (TGF-β) superfamily, through adeno-associated virus vector in the quadriceps is being tested in trials for muscle improvement.

2.2. The Mitochondrion

2.2.1.- DEFINITION, HISTORY AND THE ENDOSYMBIOTIC THEORY

The mitochondrion is a cell organelle of maternal inheritance involved in crucial processes for cell survival: energy and heat production, cell respiration or calcium homeostasis, among others. However, under pathological conditions mitochondria is the centre that triggers reactive species production (ROS) and apoptosis. This organelle is located in the cytoplasm of most eukaryotic cells and measures 0.5-1 µm of diameter and 10 µm long. Mitochondrion produces energy by the formation of adenosine triphosphate (ATP) molecules. This ATP synthesis is coupled to cell respiration in a process called oxidative phosphorilation (OXPHOS).

We cannot really assign the discovery of the mitochondria to a single person, but there were many important researchers and events that were significant in mitochondria discovery: Kolliker describe in 1880 the presence of grains in insect cells, and call them *sarcomeres* (this could be probably the first observation of mitochondria). They were also observed by Flemming and called *fila* at that moment in 1882. The term mitochondrion was keyed by Benda C. in 1889 and, since then, many investigators like Kingsbury, Warburg or Lehninger contributed to the characterization of the morphology and function of the mitochondrion.

The endosymbiotic theory is widely accepted by the scientific community as the origin of mitochondria. In 1975, *Margulis et al.* reformulated an ancient hypothesis about mitochondrial origin and exposed the following: 2,500 million years ago, a primitive eukaryotic cell phagocyted another prokaryotic cell without digesting it. A symbiotic relationship was produced between both cells, one of them provided energy in ATP form and the other offered a stable media rich in nutrients. This theory is reinforced by the fact that the mitochondrion structure is very similar to a prokaryotic cell.

2.2.2.- STRUCTURE

The structure of the mitochondrion is characterized by the presence of two membranes: the outer membrane and the inner membrane. The existence of these two membranes generates two main mitochondrial spaces which are key for mitochondrial function: the inter-membrane space between the two membranes, and the mitochondrial matrix inside the inner membrane, which is highly folded to integrate mitochondrial cristae (Fig. 4).

Mitochondrial structure is not static along time, these organelles are subjected to both internal cristae remodelling and constant fusion and fission processes within different organelles of the mitochondrial network. Fusion and fission events entail mitochondrial dynamics that helps to maintain the correct mitochondrial function and health.

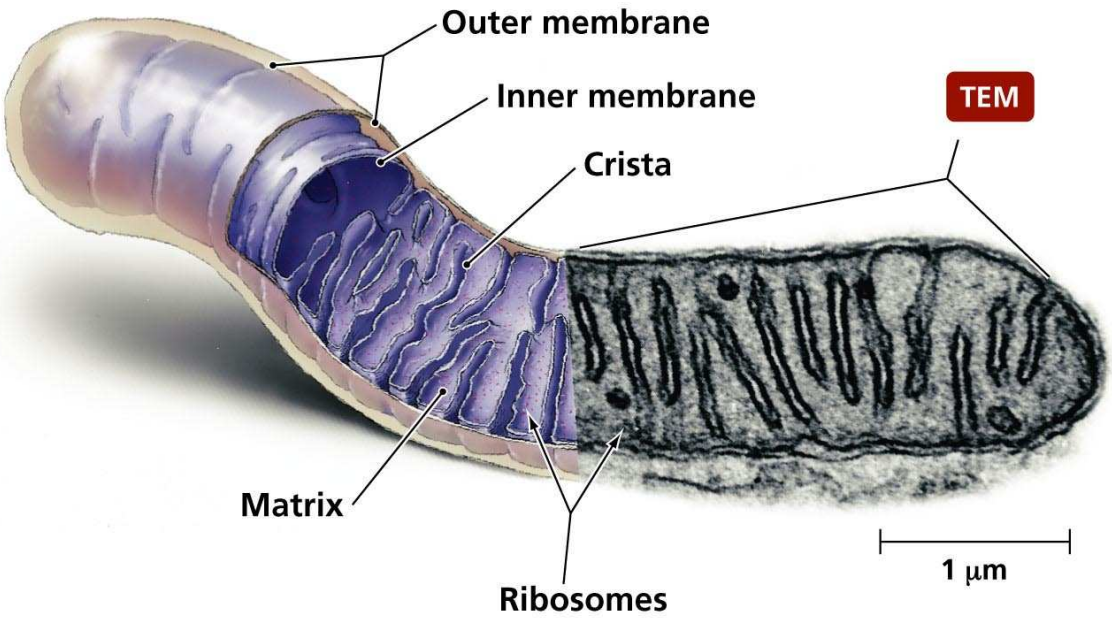

Fig4: Mitochondrial Structure. Right side is a real Transmission Electron Microscopy (TEM) image of a mitochondrion. Credits of the image to Pearson Education©, Inc.

- Outer mitochondrial membrane (OMM)

This lipid bilayer membrane separates the cytoplasm and the inter-membrane space and contains specific proteins called porins or voltage dependent anion channels (VDAC). These proteins form transmembrane channels that confer to the membrane permeability for molecules as large as 20 Å and up to 20kDa.

- Inner mitochondrial membrane (IMM)

Unlike the outer membrane, the inner membrane is impermeable for the majority of molecules and ions. However, it also has membrane transport proteins and translocases like adenine nucleotide translocase (ANT) or phosphate translocase. Is in the highly folded inner membrane where the OXPHOS system is located.

The OXPHOS system is constituted by the MRC (which is formed by complexes I to IV, two mobile electron carriers [coenzyme Q (CoQ) and cytochrom C (CytC)]) and a fifth complex (the ATP-proton synthase or complex V) (59). The electrons flow through the MRC by redox reactions that end at the final acceptor of electrons, at complex IV, where the oxygen in reduced into H_2O. The energy released in these reactions is used to export protons (H^+) from the mitochondrial matrix to the intermembrane space through complexes I, III and IV, against gradient. At the same time, the ATP-proton synthase returns these protons to the mitochondrial matrix forward gradient and uses the release of this energy to phosphorylate ADP molecules into ATP.

Specifically, complex I (NADH-ubiquinone oxidoreductase) oxidizes nicotidamide adenine dinucleotide (NADH) and transfers 2 electrons to CoQ. This reaction is accompanied by the transfer of 4 protons from the matrix to the intermembrane space as well. Complex II

(succinate-ubiquinone oxidoreductase) transfers 2 electrons to CoQ like the complex I, but in this case this reaction is not accompanied by transference of protons between the matrix and the intermembrane space. Succinate is the substrate of this complex. CoQ is a mobile electron carrier and its function is to transport electrons from complexes I and II to complex III. Complex III (Ubiquinol-cytochrome C oxidoreductase) transfers 2 electrons from the reduced form of CoQ ($CoQH_2$) to another electron carrier, cytochrome c (Cytc). This reaction also pumps 4 protons to the intermembrane space similar to the reaction that takes place in complex I. Cytc is a mobile electron carrier located in the external face of the mitochondrial inner membrane that transfers electrons from complex III to complex IV. Complex IV (COX) transfers 4 electrons to 2 molecules of oxygen reducing them into 2 molecules of water. In this process, Cyt c is oxidized as well and 2 protons are transferred to the intermembrane space upon reoxidization of Cyt c. Finally, the complex V (ATP-synthase) is the responsible for the synthesis of ATP from ADP and inorganic phosphate using the proton gradient that the MRC generates (Fig. 5).

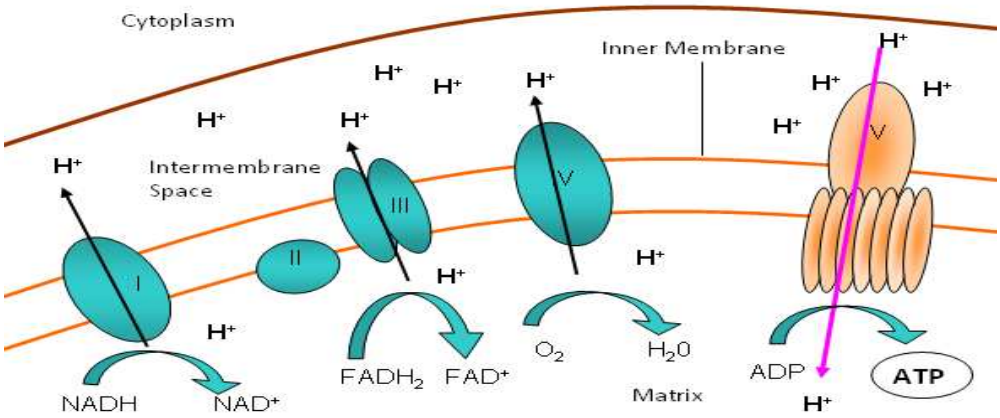

Fig5: Mitochondrial respiratory chain complexes (I-V). Protons accumulate in the intermembrane space due to the effect of complexes I-IV on reducting NADH, $FADH_2$ and O_2. Compex V uses this proton gradient to phosphorylate ADP to ATP.

- The intermembrane space

This space delimited between the two mitochondrial membranes (MIM and MOM) is where the protons accumulate as a result of the proton pumping through the enzymatic complexes I, III and IV of the MRC. Numerous apoptotic effectors (Arf, Cytc, endonuclese G, Smac/Diablo…) are herein located.

- The mitochondrial matrix

Mitochondrial matrix is delimited by the inner membrane and contains the double stranded mtDNA. In the mitochondrial matrix also takes place important metabolic pathways crucial for the cell survival such as Krebs cycle and the β-oxidation of the fatty acids.

- Mitochondrial dynamics

Mitochondria, far from being static organelles, are reticular organelles that have high plasticity subject to cristae remodelling and constantly undergoing fusion and fission as well as movement through the cytoskeleton (60). These are the main processes utilized by the mitochondria to maintain their homeostasis. By undergoing constant fission and fusion, mitochondria repairs damaged components by segregating damaged mitochondria and exchanging material with healthy mitochondria by fusion process. The altered or abnormal mitochondria can be enveloped by autophagosomes to trigger their degradation in the lysosome via mitophagy (61-64) and also portions of mitochondria can form mitochondria derived-vesicles (MDV) under oxidative stress conditions that fuse with lysosomes to degrade oxidized mitochondrial proteins (65).

Mitochondrial fusion in mammals is mediated by mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and optic atrophy 1 (OPA1) proteins, among others. While both mitofusins are responsible for the fusion of the MOM, OPA1 is responsible for the fusion of the MIM. Mitofusins 1 and 2 are highly conserved GTPases homologues that play a role in mitochondrial fusion, however a functional difference of these two proteins remains to be clarified (66, 67). OPA1 is also a dynamin-related GTPase and mitochondrial intermembrane protease Yme1 cleaves OPA1 under normal conditions to generate short and long forms of OPA1 (S-OPA1 and L-OPA1)(68) responsible of protein activation/inactivation. The processing of OPA1 is complex. It is known that presenilin-associated-rhomboid-like (PARL) and paraplegin induce alternative splicing and alternative processing of OPA1 that generates eight OPA1 isoforms. In addition to mitochondrial dynamics, OPA1 has other functions that had recently been proposed by many authors. Strong evidences suggest that OPA1 is required in maintaining respiratory chain integrity (69, 70). OPA1 also exerts a role in controlling apoptosis, a processing that involves OMM fragmentation and IMM dismantling. The release of OPA1 itself conduces to an irremediable major cristae disorganization (71).

Mitochondrial fission is mainly mediated by dynamin-related protein 1 (Drp1) which is also a large GTPase (60). Drp1 is a cytosolic protein and can be recruited to the OMM to constrict mitochondria and eventually divide a mitochondrion in two separate organelles. It is also known that Drp1 interacts with at least four receptor proteins: fission 1 (Fis1), mitochondria fission factor (MFF), and mitochondrial dynamics protein 49 and 51 (MID49 and MID51). Interactions of Drp1 with MFF, MID49 and MID51 have been demonstrated to play a major role in fission regulation, unlike Drp1-Fis1 interaction, which does not seem to have a major role in regulate this mitochondrial fission (72-74).

Finally, the movement of mitochondria through the cytoskeleton is also important for its distribution and turnover. This movement is promoted by kinesins that transport the mitochondria in the plus-end (anterograde) direction and dyneins that perform the same function but in the minus-end (retrograde) direction (75). There are many molecules that interact in the mitochondrion-kinesin union, for example the adapter protein Milton interacts with the OMM protein Miro. Loss of MFN2 alone is enough to cause a defect in mitochondrial transport (60, 76).

2.2.3.- MITOCHONDRIAL GENOME

Mitochondria are the only organelle that has its own genetic material. This (mtDNA) can be found in the mitochondrial matrix in a variable number of copies. This DNA is double stranded circular and covalently closed molecule that in humans has 16.569 base pairs. This mtDNA contains 37 genes that codify for 2 ribosomal RNA (rRNA), 22 transfer RNA (tRNA) and 13 messenger RNA (mRNA) that are translated into 13 proteins involved in the structure and function of MRC complexes I, III, IV and V. Some polymorphisms of this mtDNA generate a variation in the mitochondrial genome known as haplogroups, which have been useful to study human migrations over the history.

Nuclear DNA is diploid (each cell only contains at least 2 copies of each gene) but mtDNA is poliploid and it can be found from 2 to 10 number of copies per mitochondrion. Given that each cell contains from 100-1,000 mitochondria (depending on the cell type), the number of copies of mtDNA per cell moves from 200 to 10,000, approximately.

Mitochondrial DNA is replicated, transcribed and translated in the mitochondrial matrix and in coordination with nuclear DNA, as nuclear-encoded enzymes are imported to the mitochondrion to catalyze these processes. γ-DNA polymerase is the only responsible for mtDNA replication and, in a lesser extent, repair. This protein, together with the rest of proteins required for mitochondrial function and biogenesis (counted as thousands) are encoded in the nuclear DNA and further transcribed and translated into the cytoplasm to be imported into the mitochondria. Thus, the intergenomic communication between the nucleus and the mitochondria is essential for bioenergetic cell supply.

2.2.4.- OXIDATIVE STRESS AND APOPTOSIS

Mitochondria are one of the main responsible for the formation of ROS and consequently the presence of oxidative stress. These ROS are generated in the mitochondrial respiration, but under stress conditions or mitochondrial malfunctioning, there is an increase in the production of these ROS.

These compounds are extremely reactive, and they will react to lipids, proteins and even genetic material, mainly where they are produced, in the mitochondria. This creates a kind of “vicious circle” in which mitochondrial structures are being degraded by the presence of the ROS, which causes an increase in the production of these extremely reactive molecules (77-80).

Mitochondria also have a major role in one of the most important processes that involves cell homeostasis: apoptosis. This programmed cell death can be triggered by an external agent (extrinsic pathway) or by an internal stimulus as a defense mechanism against potential tumor cells, natural senescence, cell stress and aging (intrinsic pathway).

Mitochondria participates in both pathways with the release of pro-apoptotic factors such as apoptotic inducing factor (AIF), second mitochondria derived activator (SMAC), (CytC), apoptotic proteases activator factor (APAF-1) and the procaspase-9. The formation of the

apoptosome activates caspase-9 and the reaction cascade continues until a caspase activated DNAse protein (CAD) cleavages DNA leading to cell death.

2.3. Amyloidogenesis and sIBM

2.3.1.- β- AMYLOID ACCUMULATION AND ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a chronic neurodegenerative disease that is the cause of 60% to 70% cases of dementia. The accumulation in brain of Aβ, an abnormal proteolytic byproduct of the transmembrane protein APP, is one of the main characteristic features of AD.

The APP could be potentially processed by different enzymes in two different pathways: a non- amyloidogenic pathway and an amyloidogenic pathway, the latter related to disease due to abnormal Aβ accumulation (Fig. 6). In the non amyloidogenic pathway, APP is cleaved by the enzyme α-secretase. The products from this cleavage do not accumulate as β-amyloid aggregates. However, the APP protein can also be cleaved by the β-secretase (BACE-1) protein at the N-termini. This results in a sAPPβ fragment and another fragment that is cleaved at the C-termini by the γ-secretase (presenilin-1 or PS-1). The result from this enzymatic reaction is the Aβ peptide that has the capacity to oligomerize and form β-amyloid aggregates.

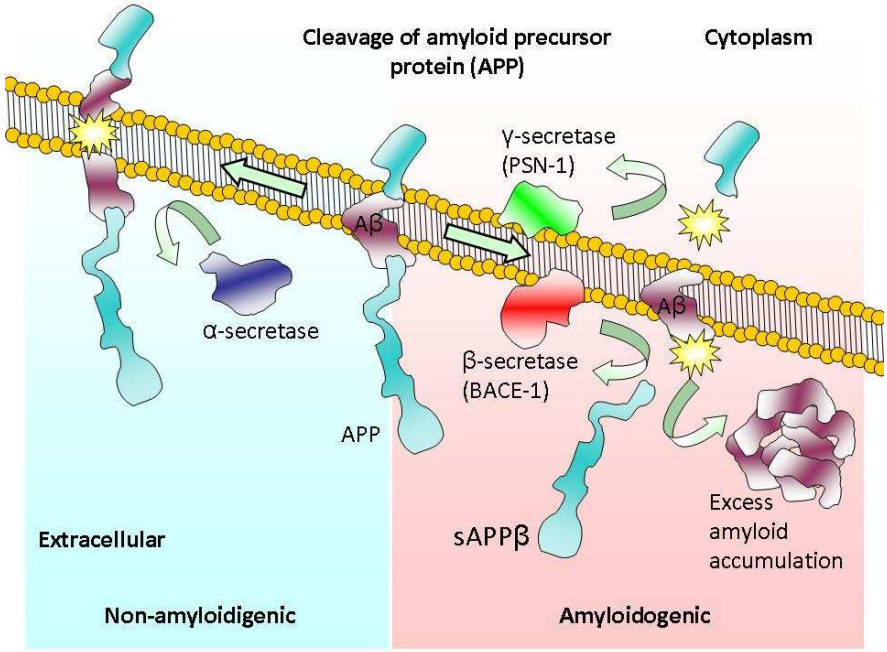

Figure 6: Non-amyloidogenic and amyloidogenic cleavage of the amyloid precursor protein (APP). Both pathways are physiologic, but an increase in the amyloidogenic pathway mediated by β-secretase (BACE-1) and presenilin-1 (PS-1) leads to amyloid-β oligomerization and the accumulation of amyloid-β plaque.

2.3.2.- RELATIONSHIP BETWEEN sIBM AND ALZHEIMER'S DISEASE

The relationship between sIBM and AD has recently been discussed due to remarkable pathogenic similarities between these two conditions. The main common feature of both diseases is the pattern of protein accumulation in the target tissue of the disease (brain in

AD and muscle in sIBM), where can be found Aβ, phosphorylated tau, and more than 15 other AD characteristic proteins (18, 29, 81) many of them in common to sIBM depots. This suggests that both diseases might share certain pathogenic mechanisms and that the knowledge in one disease might help to elucidate the other. The respective cascade of events in each disease leading to common pathologic aspects is not well understood, however some mechanisms and events have been proposed to contribute to both sIBM and AD pathogenesis: cellular aging, protein misfolding and aggregation, proteasome inhibition, mitochondrial abnormalities as well as oxidative and ER- stress (81-85). Although there are cases where both diseases coexist in the same patient (86, 87), a large epidemiological comorbidity study is still needed.

Amyloid deposits in muscle from sIBM patients are the most relevant feature that this disease shares with AD. This feature was first described by *Mendell et al.* who observed amyloidogenic birefringent green deposits after Congo red staining in muscle from sIBM patients (88). Shortly after, Askanas et al. demonstrated that inclusions within vacuolated fibres were immunoreactive with anti-β-amyloid antibodies, and also that Aβ protein reactivity was localized near to cytoplasmic tubulofilaments by immunogold electron microscopy (89, 90). The oligomeric fragment on Aβ, Aβ42 is also increased in plasma from sIBM patients compared to PM patients and healthy controls (91). However, this increase is also noticed in DM patients, limiting the value of this potential diagnostic or prognostic biomarker. The main difference in the accumulation of Aβ between sIBM and AD is that, in sIBM, the accumulation of Aβ is intracellular in muscle fibers while, in central nervous system, we find the Aβ extracellularly (92).

To unveil the mechanisms of this Aβ accumulation, many researchers had tried to understand the physiology of the amyloid deposits by studying protein degradation pathways such as 26S proteasome activity and autophagy. Regarding 26S proteasome, its subunits co-localize in sIBM with phosphorylated tau, ubiquitin and Aβ deposits. Even both subunits (19Sand 20S) are upregulated in sIBM, the main proteolytic activities of the 20S were dramatically reduced (30). In addition, and as the main responsible for the removal of protein aggregates in cell is autophagy, Nogalska et al. demonstrated the impairment of the autophagy in sIBM through the detection of autophagy induction markers and, at the same time, a significant decrease of lysosomal enzyme activities of cathepsins D and B (93). Together, these findings suggest that there is abnormal protein degradation in these patients that could lead to the protein accumulation observed.

Although the understanding of protein clearance mechanisms are of high relevance, the process by which APP is processed to generate Aβ and its subsequent accumulation is key and has been also intensively studied in relation to its importance in the pathogenesis of AD and also in sIBM. The APP has been demonstrated to be increased in sIBM at transcript level (94). In addition, in sIBM, it has been demonstrated that BACE-1 accumulates in form of strongly immunoreactive plaque-like inclusions within the vacuolated muscle fibers. It also colocalize with β-amyloid, suggesting its participation in APP processing and Aβ generation and accumulation there (95, 96). It has also been demonstrated that there is an increase of BACE-1 mRNA transcripts (and also an mRNA increase of BACE-1-AS, a novel non-coding BACE-antisense) in sIBM patients, and that the experimental induction of ER stress provokes the same increase in transcript levels of BACE-1 and BACE-1-AS.

This finding in sIBM is consistent with the fact that BACE-1 activity and protein levels in the frontal cortex of AD brain are substantially increased (97, 98). Also, there is a positive correlation between the amount of BACE-1 and the amount of Aβ in the brain of AD patients (97).

Not only BACE-1, but γ-secretase (with active complex PS1 that catalyzes the final step of the reaction) is needed for the formation of Aβ and consequent accumulation in muscle tissue. In sIBM muscle fibers have been recently demonstrated the increase of γ-secretase components, the increase of mRNAs of these components and also the increase of γ-secretase activity (99). All these data together present a strong evidence of the upregulated machinery for Aβ production in sIBM muscle fibers.

The relationship between Aβ accumulation and sIBM muscle degeneration is still unclear. It is also very difficult to elucidate if inflammation or degeneration are the initial triggers for sIBM. Expression of the mRNA of the APP consistently correlated with inflammation and enhanced mRNA levels of chemokines and IFN-γ (100). Inflammation seems to have an important role in amyloid-β accumulation, and *in vitro* experiments have been performed to explore this issue. Exposure to interleukin 1β IL-1β caused up-regulation of APP with subsequent intracellular aggregation of amyloid-β (100).

There is an interesting relationship for sIBM pathogenesis of Aβ with myostatin. Myostatin is a negative regulator of muscle growth during development and muscle mass in adulthood. Askanas et al. has demonstrated that myostatin precursor accumulates and associates with aggregates containing Aβ in sIBM muscle fibers. Not only this, but also they demonstrated that expression of myostatin precursors and myostatin dimer are also increased, and that myostatin precursor protein physically associates with Aβ, identifying Aβ as its novel binding partner (101, 102). Taking into account that myostatin inhibitors are one of the most promising therapeutical options that clinical trials are testing for sIBM treatment, the relevance of amyloid-β in sIBM pathogenesis is beyond any doubt.

In the following two published articles there are some answers to the raised questions.

3.HYPOTHESIS AND OBJECTIVES

The hypothesis of this work is that sIBM patients will manifest mitochondrial alterations at molecular level in muscle, according to previous histological studies. We hypothesize that the abnormal features of the disease may be not only confined to muscle but will also be present in other body tissues like PBMC, requiring less invasive approaches. In addition, molecules related to inflammation, mitochondrial dysfunction and degeneration (histological hallmark features of the disease) will be altered in plasma, and its measurement could be useful as diagnostic/prognostic biomarkers.

The main objective of the present doctoral thesis is exploring pathological features of sIBM (inflammation, mitochondrial abnormalities and degeneration) at molecular level to improve the knowledge in disease aetiology and investigate potential diagnostic/prognostic biomarkers.

The concrete objectives of this thesis are:

1.- To assess the molecular mitochondrial phenotype in muscle, the target tissue of the disease, from a sIBM patient cohort compared with healthy controls.

2.- To evaluate the same biochemical parameters in PBMC, in order to assess if these alterations could extend to other body tissues that could constitute a less invasive approach.

3.- To study the presence of inflammatory, mitochondrial and amyloidogenic related molecules in plasma from sIBM patients compared to patients with other inflammatory myopathies, to evaluate if such molecules could be useful as diagnostic/prognostic biomarkers.

4. ARTICLES AND STUDIES

Publication 1

Title:

Mitochondrial DNA disturbances and deregulated expression of oxidative phosphorylation and mitochondrial fusion proteins in sporadic inclusion body myositis

Authors:

Marc Catalán-García, Glòria Garrabou, Constanza Morén, Mariona Guitart-Mampel, Adriana Hernando, Àngels Díaz-Ramos, Ingrid González-Casacuberta, Diana-Luz Juárez, Maria Bañó, Jennifer Enrich-Bengoa, Sonia Emperador, JC. Milisenda, P. Moreno, Ester Tobías, Antonio Zorzano, Julio Montoya, Francesc Cardellach, Josep Maria Grau.

Reference:

Clin Sci (Lond). 2016 Oct 1;130(19):1741-51.

Clinical Science (2016) **130**, 1741–1751 doi: 10.1042/CS20160080

Original Paper

Mitochondrial DNA disturbances and deregulated expression of oxidative phosphorylation and mitochondrial fusion proteins in sporadic inclusion body myositis

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Abstract

Sporadic inclusion body myositis (sIBM) is one of the most common myopathies in elderly people. Mitochondrial abnormalities at the histological level are present in these patients. We hypothesize that mitochondrial dysfunction may play a role in disease aetiology. We took the following measurements of muscle and peripheral blood mononuclear cells (PBMCs) from 30 sIBM patients and 38 age- and gender-paired controls: mitochondrial DNA (mtDNA) deletions, amount of mtDNA and mtRNA, mitochondrial protein synthesis, mitochondrial respiratory chain (MRC) complex I and IV enzymatic activity, mitochondrial mass, oxidative stress and mitochondrial dynamics (mitofusin 2 and optic atrophy 1 levels). Depletion of mtDNA was present in muscle from sIBM patients and PBMCs showed deregulated expression of mitochondrial proteins in oxidative phosphorylation. MRC complex IV/citrate synthase activity was significantly decreased in both tissues and mitochondrial dynamics were affected in muscle. Depletion of mtDNA was significantly more severe in patients with mtDNA deletions, which also presented deregulation of mitochondrial fusion proteins. Imbalance in mitochondrial dynamics in muscle was associated with increased mitochondrial genetic disturbances (both depletion and deletions), demonstrating that proper mitochondrial turnover is essential for mitochondrial homoeostasis and muscle function in these patients.

Key words: deletions, depletion, mitochondria, mitofusin-2, OPA1, sporadic inclusion body myositis.

INTRODUCTION

Sporadic inclusion body myositis (sIBM) is the most common inflammatory myopathy in individuals aged >50 years [1] and one of the most important myopathies associated with ageing [2]. With a male:female ratio of 3:1, it is a rare disease (ORPHA611) and its prevalence varies from 4.7 per million in the Netherlands to 14.9 per million in western Australia [3]. These rates continue to increase, probably due to improved diagnostic protocols and increased ageing of the population. This myopathy is a devastating condition, causing slow, progressive muscle weakness and wasting, with quadriceps and finger flexors being the muscles most typically affected. Nevertheless, the clinical presentation varies considerably. This muscle weakness usually leads to falls and difficulty in standing. Around 60% of the patients also present with dysphagia. With the wide spectrum of clinical

Abbreviations: CK, creatine kinase; COX, cytochrome c oxidase; CS, citrate synthase; H&E, haematoxylin and eosin; HAE, hydroxyalkenal; IBMFRS, Inclusion Body Myositis Functional Rating Scale; MDA, malondialdehyde; MFN2, mitofusin 2; MRC, mitochondrial respiratory chain; NSE, non-specific esterase; OPA1, optic atrophy 1; ORO, Oil Red O; PAS-D, periodic acid–Schiff diastase stain; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcriptase PCR; ROS, reactive oxygen species; SDH, succinate dehydrogenase; sIBM, sporadic inclusion body myositis; TRC, tyrosine-rich crystalloids; VDAC1, voltage-dependent anion channel 1.

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manifestations in sIBM, muscle biopsy continues to be the gold standard for diagnosis [4].

The histological features observed in muscle biopsies of sIBM patients include: (i) inflammatory changes with predominant CD8+ T-cell infiltrates and the expression of MHC-I antigens by non-necrotic muscle fibres, (ii) different degrees of degenerative changes in muscle fibres and the presence of rimmed vacuoles composed mainly of β-amyloid, phosphorylated tau and caveolin proteins, among others, and (iii) mitochondrial abnormalities characterized by the presence of ragged-red fibres, cytochrome *c* oxidase (COX)-negative and succinate dehydrogenase (SDH)-positive muscle cells [5,6], all of which are widely associated with mitochondrial dysfunction and ageing [1,2,7]. However, the distribution of these histological features is not homogeneous, and they may not be simultaneously present, particularly in the early stages of the disease. For all these reasons, and due to its slow progression, diagnosis may be delayed for 5–10 years.

Mitochondria are the cell's powerhouse. They are responsible for most of the energy supply, metabolic reactions, calcium homoeostasis and cell respiration. However, under pathological conditions, mitochondria are the main centres for reactive oxygen species (ROS) production and for triggering apoptosis. Mitochondria are essential for cell bioenergetics, especially in highly energetic tissues such as muscle, in which mitochondrial dysfunction may become evident much earlier. Molecular mitochondrial alterations have recently been found in sIBM by down-regulated expression of mitochondrial respiratory chain (MRC) complex I subunits and mitochondrial DNA (mtDNA) variations [7]. In addition, mtDNA deletions have also been reported in sIBM patients [6,8], but not correlated with abnormal mitochondrial function or dynamics. Despite mitochondrial abnormalities having classically been described at a histological level, mitochondrial dysfunction in sIBM patients has scarcely been assessed at a molecular level.

Mitochondrial dynamics is a recently discovered mechanism responsible for mitochondrial turnover and renewal [9]. Mitochondria constitute a complex network that is constantly undergoing fusion and fission processes to exchange genetic and structural components, which include mtDNA and MRC machinery. Deregulation of mitochondrial dynamics has been associated with disease, but has not previously been described in sIBM patients [10–16].

Although the pathological features of sIBM have been widely described, its aetiology remains unknown. It has been proposed that its development could be due to a complex interaction of environmental agents, accelerated ageing and genetic susceptibility [2,17]. As muscle tissue has a high dependence on ATP to exert its function, mitochondrial alterations in sIBM could be one of the factors involved in triggering muscle weakness and degeneration, because these alterations have been related to muscle disease by many authors [18–20]. For an in-depth evaluation of the pathological characteristics of the disease, we describe the mitochondrial phenotype at a genetic, molecular and functional level in sIBM patients.

To determine potential disease biomarkers we assessed abnormal mitochondrial fingerprints, in the target tissue of the disease (muscle) and with less invasive approaches using peripheral blood mononuclear cells (PBMCs). This PBMC model is widely feasible for the evaluation of mitochondrial function [21–23]. Thus, we designed the present study to evaluate mitochondrial dysfunction in both muscle and PBMCs of sIBM patients to correlate dysfunction severity with genetic and molecular mitochondrial alterations, and determine their potential association with the deregulation of mitochondrial dynamics.

MATERIALS AND METHODS

Study design

We performed a single-site, cross-sectional, case–control, observational study.

Study population, diagnosis, clinical data and sample collection

A total of 30 patients with sIBM who had attended the Internal Medicine Department of the Hospital Clinic of Barcelona (Spain) over the last 20 years were prospectively and consecutively included. These patients were age and gender paired with 38 controls. All muscle biopsies were performed for diagnostic purposes indicated for muscle weakness or raised creatine kinase (CK) levels. Surgical muscle biopsies were obtained by trained physicians, and the samples were processed routinely in the laboratory as described elsewhere [24]. For histological studies fresh muscle samples were frozen in cooled isopentane, sectioned by cryotome at $-30\,^{\circ}C$ and stained with different reagents for diagnostic purposes: haematoxylin and eosin (H&E), tyrosine-rich crystalloids (TRCs), non-specific esterase (NSE), periodic acid–Schiff diastase stain (PAS-D), Oil Red O (ORO), acid and alkaline phosphatase, NADH, COX, SDH and ATPase at pH 4.3, 4.6 and 9.4, as reported elsewhere [24]. In addition some immunohistochemistry reactions such as class I antigens from the MHC as well as p62 were performed. The same expert pathologist (J.M.G.) read all the samples. Leftover biopsy material from both sIBM patients and individuals with no histological myopathy was included as patient and control samples, respectively. Histological data were collected to study inflammation and the presence of ragged-red fibres, COX-negative and SDH-positive fibres, as well as the number of vacuolated muscle cells. Diagnosis of sIBM was considered as definite or probable according to the criteria of the European Neuromuscular Centre [1,25]. Samples from 23 muscle biopsies from sIBM patients and 18 from controls were included following these criteria. In parallel, blood samples were collected from 14 sIBM patients and 20 control individuals free of muscle disease. Exclusion criteria were: age <40 years, muscle disease in the case of controls, mitochondrial disease, and family history of hereditary mitochondrial pathology, HIV infection or drug abuse. All individuals were informed, and signed written consent was obtained for inclusion in this study, which was approved by the Ethical Committee of our hospital, following the Declaration of Helsinki. All sIBM patients answered the Inclusion Body Myositis Functional Rating Scale (IBMFRS) test, which is a validated, disease-specific test to assess disease severity.

A database was created to collect epidemiological, clinical and histological data, which were further complemented with experimental results.

Leftover samples from mandatory muscle biopsy diagnosis were included in optimal cutting temperature compound (OCT) and immediately frozen and stored at −80 °C until homogenization (5 % w/v) with mannitol to perform experimental studies.

Around 20 ml of peripheral blood was also obtained by antecubital vein puncture and collected in EDTA tubes. PBMCs were isolated using Ficoll density gradient centrifugation, divided into aliquots and stored at −80 °C until analysis.

Mitochondrial genetic studies

Total DNA from muscle biopsies and PBMCs was obtained by the standard phenol–chloroform extraction procedure. The assessment of mtDNA deletions was performed in muscle samples by long-range PCR using Phusion High-Fidelity PCR Master Mix with GC Buffer (F-532L, ThermoFisher Scientific) and the following primers: forward 5′-TTAGCAAGGGAACTACTCCCA-3′ and reverse 5′-CGGATACAGTTCACTTTAGCTACCCCCAAGTG-3′. The methodology of this procedure was performed as previously described [26–28]. The PCR products were electrophoresed in a 0.8 % agarose gel with ethidium bromide to detect different sizes in the mitochondrial genome (see Supplementary Figure S1).

To evaluate mtDNA content, fragments of the mitochondrially encoded 12S rRNA gene and the nuclear-encoded RNase-P gene were amplified separately in triplicate by quantitative reverse transcriptase PCR (RT-PCR) using Applied Biosystems technology [29]. The relative mtDNA content was expressed as the ratio mtDNA 12S rRNA:nDNA RNase-P.

Mitochondrial transcript quantification

To evaluate mtRNA content in both muscle and PBMCs, total RNA was first extracted using the TriPure procedure and immediately retro-transcribed into cDNA using random hexamer primers, following previously described methodology [30]. The mtRNA:nRNA content was then measured by RT-PCR as previously described for mtDNA and expressed in the same units (mtRNA 12S rRNA:nRNA RNase-P).

Mitochondrial protein quantification

Mitochondrial protein quantification was assessed in muscle and PBMCs by the quantification of two mitochondrial complex IV protein subunits: COX-II (encoded by the mitochondrial genome) and COX-IV (encoded by the nuclear genome). These proteins were assessed through immunoblotting using SDS/7/13% PAGE and immunodetection using previously described reagents and antibodies [22]. Mitochondrial values normalized to nuclear-encoded COX proteins were expressed as the COX-II:COX-IV ratio, and absolute values of COX-II and COX-IV were normalized with a loading control (α-tubulin for muscle samples and β-actin for PBMC samples) and expressed as the COX-II:α-tubulin and COX-IV:α-tubulin ratios in muscle or the COX-II:β-actin and COX-IV:β-actin ratios in PBMCs. COX-II and COX-IV values were also normalized by mitochondrial mass [through constitutive voltage-dependent anion channel 1 (VDAC1) expression] and expressed as COX-II:VDAC1 and COXIV:VDAC1 ratios in both tissues (see Supplementary Figure S2) [31].

MRC complex I and complex IV enzyme activity

MRC complex I (EC 1.6.5.3) and IV (COX – EC 1.9.3.1) enzyme activities were measured spectrophotometrically according to the methodologies of Spinazzi et al. [32] in both muscle homogenates and PBMCs. Specific enzymatic activities were expressed in absolute values as nanomoles of substrate consumed or product generated per minute per milligram of cell protein (nmol/min per mg protein) or in relative values with respect to mitochondrial content normalized by citrate synthase (CS) activity (COX:CS).

MRC complex assembly

Total oxidative phosphorylation rodent WB Antibody Cocktail (MS604, Abcam) was used to detect one subunit from each MRC complex (I–V): NDFUFB8 from complex I, SDHB from complex II, UQCRC2 from complex III, MTCO1 from complex IV and ATP5A from complex V. These subunits are labile when the respiratory complex to which they belong is not assembled and a decrease in this subunit would manifest this phenomenon. To normalize the levels of expression of these five proteins from the MRC we analysed using the SYPRO technique for the total protein content and the VDAC1 levels for the mitochondrial mass in the same membrane.

Mitochondrial mass analysis

Mitochondrial mass was analysed in both muscle and PBMCs by spectrophotometric measurement of CS (EC 4.1.3.7) enzyme activity and, in parallel, by the measurement of VDAC1 protein levels by Western blotting. CS is a mitochondrial enzyme of the citrate cycle widely considered to be a reliable marker of mitochondrial content, and VDAC1 [22] is an outer membrane anion channel also considered to be a mitochondrial mass marker [22]. CS activity was expressed as nmol/min per mg of protein and VDAC1 expression was normalized by α-tubulin in muscle and by β-actin in PBMCs, both of which are validated proteins for assessing total cell loading mass and expressed as the VDAC1:α-tubulin ratio in muscle or the VDAC-1:β-actin ratio in PBMCs.

Oxidative stress assay

Lipid peroxidation (an indicator of oxidative damage produced by ROS in cellular lipid compounds) was quantified in muscle and PBMCs using the Oxys Research kit (Deltaclon) through the spectrophotometric measurement of malondialdehyde (MDA) and 4-hydroxyalkenal (HAE), both products of fatty acid peroxide decomposition and normalized by protein content (μM MDA + HAE per mg of protein) [33].

Expression of mitochondrial fusion proteins

Mitochondrial dynamics were assessed in muscle tissue by the evaluation of both optic atrophy 1 (OPA1) and mitofusin 2 (MFN2) expression at two different levels: transcript and protein quantification. For the assessment of transcript levels of OPA1 and MFN2, mRNA, total RNA extracted by TriPure was reverse

transcribed with the SuperScript III reverse transcriptase kit (Invitrogen). Quantitative PCRs were performed with ABI Prism 7900HT real-time PCR equipment (Applied Biosystems) using materials and reagents previously described [34,35]. All measurements were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA content. Protein levels of OPA1 and MFN2 were assessed in parallel using Western blotting on SDS/7/8% PAGE and posterior immunodetection using a BD antibody (612606) and an Abcam antibody (ab56889), respectively. In the case of OPA1, results were expressed as the ratio between the long and the short forms of the protein (OPA1 long:short), because this ratio decreases in abnormal mitochondrial dynamic states. In the case of MFN2, absolute values were normalized by total cell loading mass and expressed as the ratio MFN2:α-tubulin.

Statistical analysis

Statistical analysis was performed using the SPSS, version 20.00 to search for differences depending on group assignment (patients vs controls or presence vs absence of mtDNA deletions) using the non-parametric Mann–Whitney U-test for independent samples. Results were expressed as means $\pm$ S.E.M.S or as a percentage of increase/decrease with respect to controls that were arbitrarily assigned as 0%. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Clinical and histological data

According to the design of the study, no statistical differences were observed with regard to age and gender between sIBM patients and controls. Clinical, epidemiological, histological and mtDNA deletion data of both patients and controls are summarized in Table 1 and histological data examples are compiled in Figure 1. At the time of study inclusion the sIBM patient cohort scored 26.61 $\pm$ 1.19 out of 40 with the IBMFRS test, indicating moderate-to-advanced sIBM clinical severity.

MtDNA deletions

When assessing for the presence of mtDNA deletions in muscle from sIBM patients, 57% of the sIBM cohort presented multiple mtDNA deletions, whereas the rest showed no alterations in this parameter (see Supplementary Figure S1).

MtDNA content

MtDNA content was significantly decreased by 36% in muscle from sIBM patients compared with controls (641.6 $\pm$ 141.2 vs 979.7 $\pm$ 111.3, respectively, $P < 0.05$) (Figure 2). Similarly, mtDNA was decreased, albeit not significantly, by 14.5% in PBMCs (Figure 2).

Mitochondrial transcript quantification

No variation was found in the levels of mtRNA in either muscle or PBMCs (Figure 2) of sIBM patients compared with controls.

Table 1 Clinical, epidemiological and histological data of patients with sIBM and controls included in the present study

Muscle biopsies	sIBM (n=23)	Controls (n=18)
Clinical and epidemiological data		
Age (years)[a]	68.64 $\pm$ 2.87	63.22 $\pm$ 2.55
Male:female ratio	0.64	0.38
IBMFRS score[a]	26.61 $\pm$ 1.19/40	–
mtDNA deletions (%)[b]	57	–
Histological data (%)		
Rimmed vacuoles[b]	61.20	–
Ragged-red fibres[b]	60	–
COX-negative fibres[b]	36.36	–
SDH-positive fibres[b]	44	–
MHC-I expression[b]	57.14	–
Inflammation[b]	67.74	–
PBMC samples	**sIBM (n=14)**	**Controls (n=20)**
Clinical and epidemiological data		
Age (years)[a]	68.43 $\pm$ 3.28	68.55 $\pm$ 1.41
Male:female ratio	1	1.85
IBMFRS score[a]	25.85 $\pm$ 1.31/40	–

[a] Data presented as means $\pm$ S.E.M.s.
[b] Data presented as percentage.

Mitochondrial protein quantification

As shown in Figure 2, muscle from sIBM patients presented an increase of 25% in the COX-II:COX-IV ratio with respect to controls, suggesting increased expression of mitochondrial versus nuclear COX subunits (see Supplementary Figure S2). It is interesting that in PBMCs this ratio showed a statistically significant decrease of 45.46% (0.42 $\pm$ 0.05 vs 0.77 $\pm$ 0.09, $P < 0.05$; Figure 2). In addition, in PBMCs COX-IV protein levels normalized by total cell mass showed a strong trend to be increased by 67.85% in sIBM patients compared with controls (0.47 $\pm$ 0.08 vs 0.28 $\pm$ 0.03, $P = 0.064$; Figure 2), suggesting increased nuclear versus mitochondrial COX protein expression. This abnormality became statistically significant when COX-IV values were normalized by mitochondrial mass (COX-IV:VDAC-1), which showed a 172.72% increase in sIBM patients compared with controls (1.80 $\pm$ 0.35 vs 0.66 $\pm$ 0.01, $P < 0.05$; Figure 2).

Mitochondrial complex I and IV activity

Measurements of respiratory chain activities showed no differences in complex I activity in muscle of patients and controls, in either absolute or relative units (data not shown). However, complex IV activity was abnormally decreased in both muscle and PBMCs of sIBM patients with respect to controls (Figure 2). Thus, taking into account absolute values, a decrease of 13.55% was found in muscle (Figure 2). These differences became statistically significant in muscle when complex IV activity was normalized to mitochondrial mass (CS), by showing a 30.31% decrease in sIBM patients compared with controls (0.26 $\pm$ 0.02 vs 0.33 $\pm$ 0.03, $P < 0.05$; Figure 2). On analysing this parameter in PBMCs, complex IV activity was also significantly decreased by 31.4% in absolute values in patients compared with controls (24.32 $\pm$ 3.1 vs 35.45 $\pm$ 3.4, $P < 0.05$; Figure 2) and by 28.21%

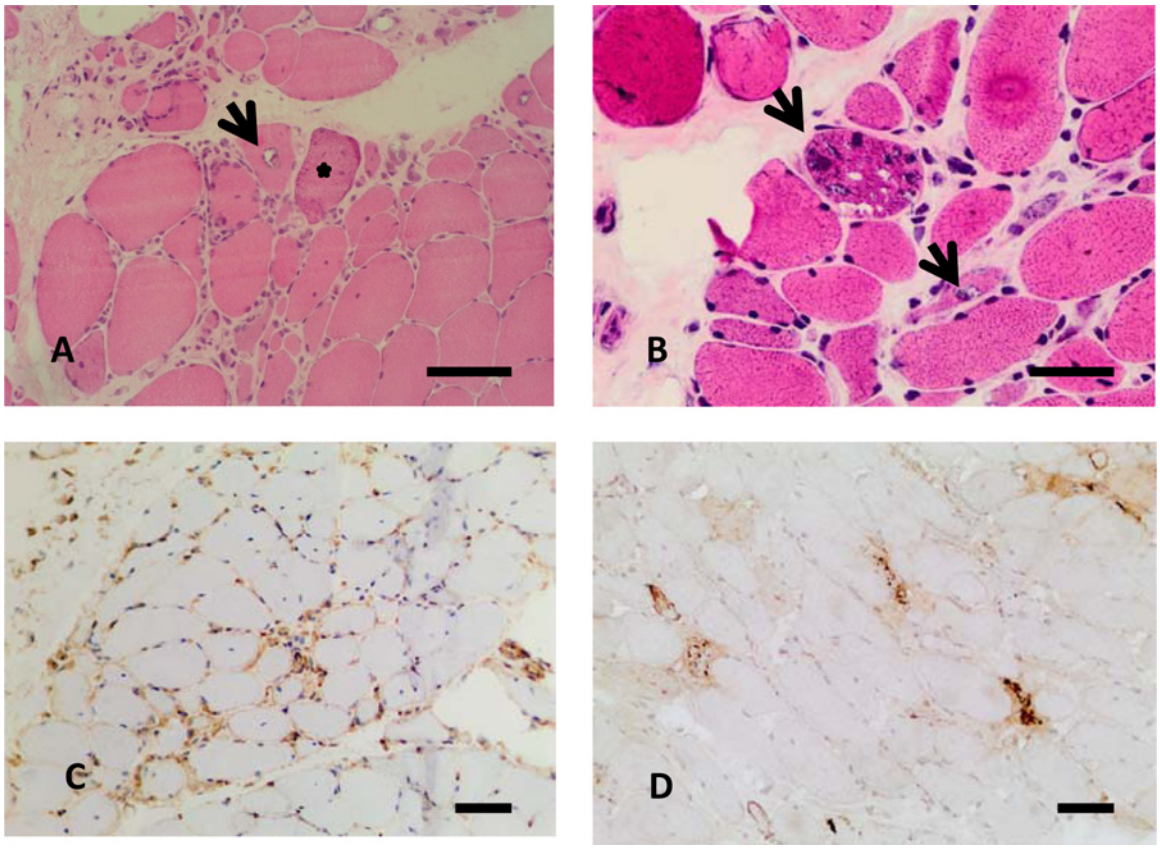

Figure 1 **Images from the histological staining of sIBM muscle tissue**
(**A**, **B**) H&E in frozen muscle tissue: marked variability in fibre size. Some inflammatory cells are observed in the endomysium, together with rimmed vacuoles (arrows) and a typical ragged-red fibre (star). (**C**) Almost universal sarcolemmal positivity of class I antigen from the MHC, a typical feature in sIBM. (**D**) Positivity of p62 in rimmed vacuoles, suggestive of muscle degeneration. Scale bar=50 μm.

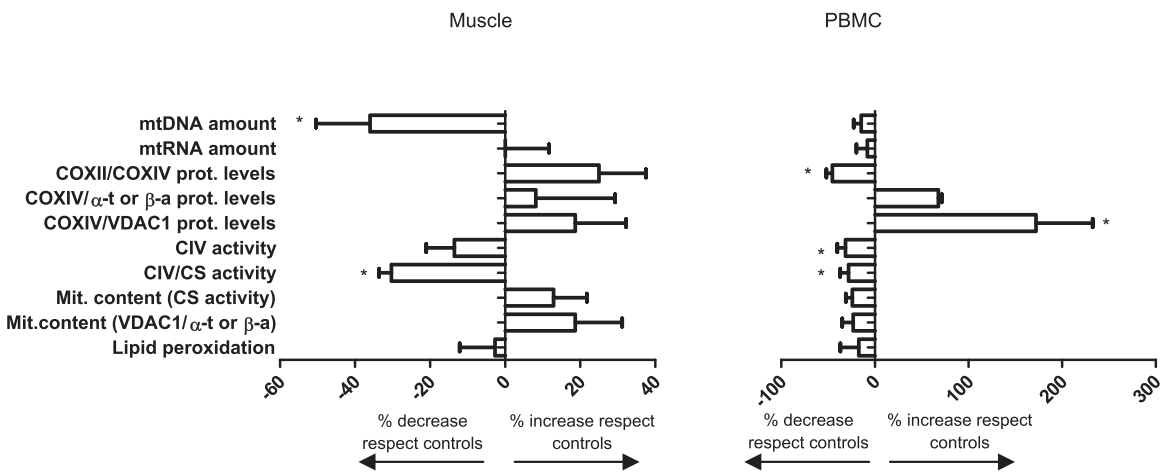

Figure 2 **Mitochondrial parameters in muscle and PBMCs from sIBM patients compared with controls**
Mitochondrial parameters in muscle tissue and PBMCs from patients with sIBM expressed as percentage of increase/decrease compared with controls. A significant decrease in mtDNA amount and complex IV activity normalized to mitochondrial content can be observed in muscle, and also a significant decrease in COX-II:COX-IV ratio, complex IV absolute activity and complex IV activity normalized to mitochondrial content can be observed in PBMCs. In this muscle tissue, a significant increase in COX-IV subunit normalized to mitochondrial content can be observed as well as other non-significantly altered parameters. α-t, α-tubulin; β-a, β-actin; C-IV, MRC complex IV. * : $p < 0.05$.

when this parameter was normalized to the mitochondrial mass (0.28 ± 0.1 vs 0.39 ± 0.1, $P < 0.05$; Figure 2).

MRC complexes assembly

The analysis of the subunit levels for each complex of the MRC (I–V) that correlates with the proper assembly of the respiratory chain complexes did not show differences between sIBM patients and controls, normalized to either total protein or VDAC1 content. It is interesting that the complex V subunit presented a trend towards a decrease in sIBM patients, but this difference did not reach statistical significance (Figure 3).

Mitochondrial mass content

Mitochondrial mass, assessed by both the quantification of CS enzyme activity and VDAC1 protein content, was increased in muscle of sIBM patients compared with controls (12.9% in CS activity and 18.7% in VDAC1:α-tubulin protein levels; see Figure 2) and decreased in PBMCs of sIBM patients compared with controls (by 24.18% in CS activity and 23.26% in VDAC1:β-actin protein content; see Figure 2). However, none of these results was statistically significant.

Oxidative stress levels

No differences were found in lipid peroxidation, an indicator of oxidative damage produced by ROS in lipid compounds, between sIBM patients and controls in muscle or PBMCs (see Figure 2).

Quantification of mitochondrial dynamics

Transcript levels of OPA1 showed a significant 37% reduction in muscle of sIBM patients compared with controls (0.59 ± 0.11 vs 0.94 ± 0.19, $P < 0.05$; Figure 4). OPA1 long:short protein isoform ratios, although not significant, were decreased by 45% in muscle of sIBM patients compared with controls (0.55 ± 0.17 vs 1 ± 0.16, $P < 0.083$; Figure 4). In the case of MFN2 transcripts, muscle of sIBM patients showed a 31% reduction. With regard to MFN2 protein expression, a strong trend towards a decrease was observed in sIBM patients, with 54% compared with controls (1.93 ± 0.3 vs 4.17 ± 0.1, $P = 0.096$; Figure 4). The differences in MFN2 levels in either transcript or protein content were not statistically significant.

Correlation of mitochondrial parameters with regard to the presence/absence of mtDNA deletions

Last, the sIBM cohort was divided into two groups on the basis of the presence or absence of mtDNA deletions for further exploration of mitochondrial homoeostasis, depending on this condition with respect to controls. The mtDNA content was slightly decreased in sIBM patients with mtDNA deletions compared with those without (Figure 5). These differences became statistically significant in sIBM patients with deletions with respect to the control group (498.50 ± 178.70 vs 979.70 ± 111.38, $P < 0.05$). MFN2 levels showed a similar pattern to those of mtDNA content, showing the lowest content in sIBM patients with mtDNA deletions and the highest in controls (1.16 ± 0.24 vs 4.17 ± 0.96, $P < 0.05$), with sIBM patients without mtDNA deletions presenting an intermediate value of expression (Figure 5).

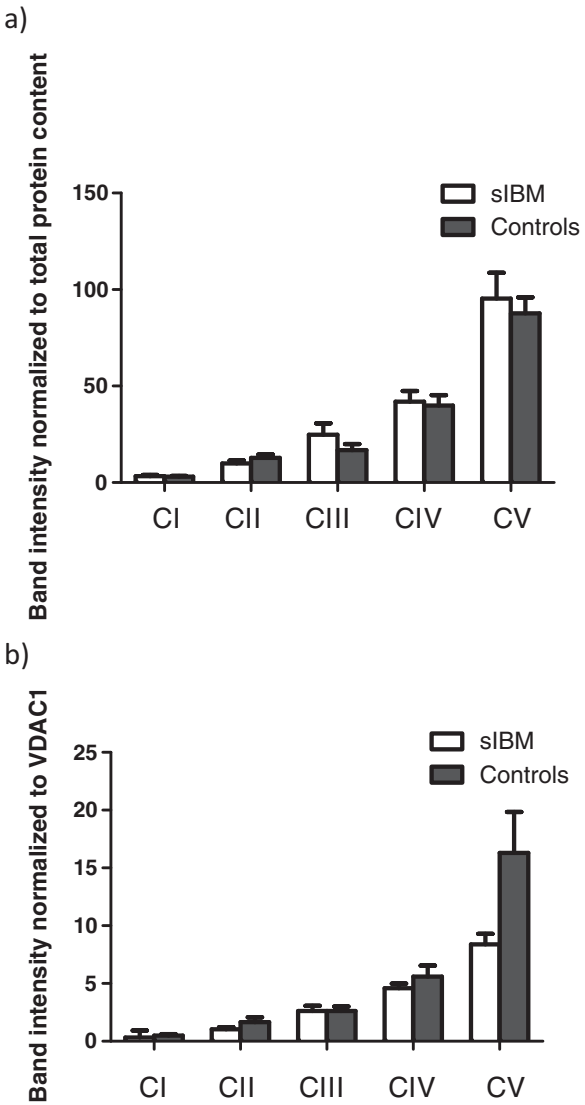

Figure 3 MRC complex assembly through the measurement of protein expression level of labile subunits of each complex in muscle tissue from sIBM patients compared with controls

No significant differences with regard to levels of specific subunits for each MRC complex were found between muscle from sIBM patients and muscle from controls, normalized to (**a**) total protein content and (**b**) the nuclear-encoded mitochondrial protein VDAC1. A trend towards increase is observed in the complex V subunit when normalized to VDAC1 which, although not significant, may give us an idea of complex V disassembly. This multiplex Western blotting measures the protein expression level of a subunit of each MRC complex (I–V), labile when the complex is not properly assembled; the following were measured: NDFUFB8 from complex I (CI; 20 kDa), SDHB from complex II (CII; 30 kDa), UQCRC2 from complex III (CIII; 48 kDa), MTCO1 from complex IV (CIV; 40 kDa) and ATP5A from complex V (CV; 55 kDa).

DISCUSSION

Mitochondrial abnormalities in muscle from sIBM patients are accepted in the scientific community as a result of histological evidence [7,36]. However, little research has been performed to characterize potential functional or molecular mitochondrially

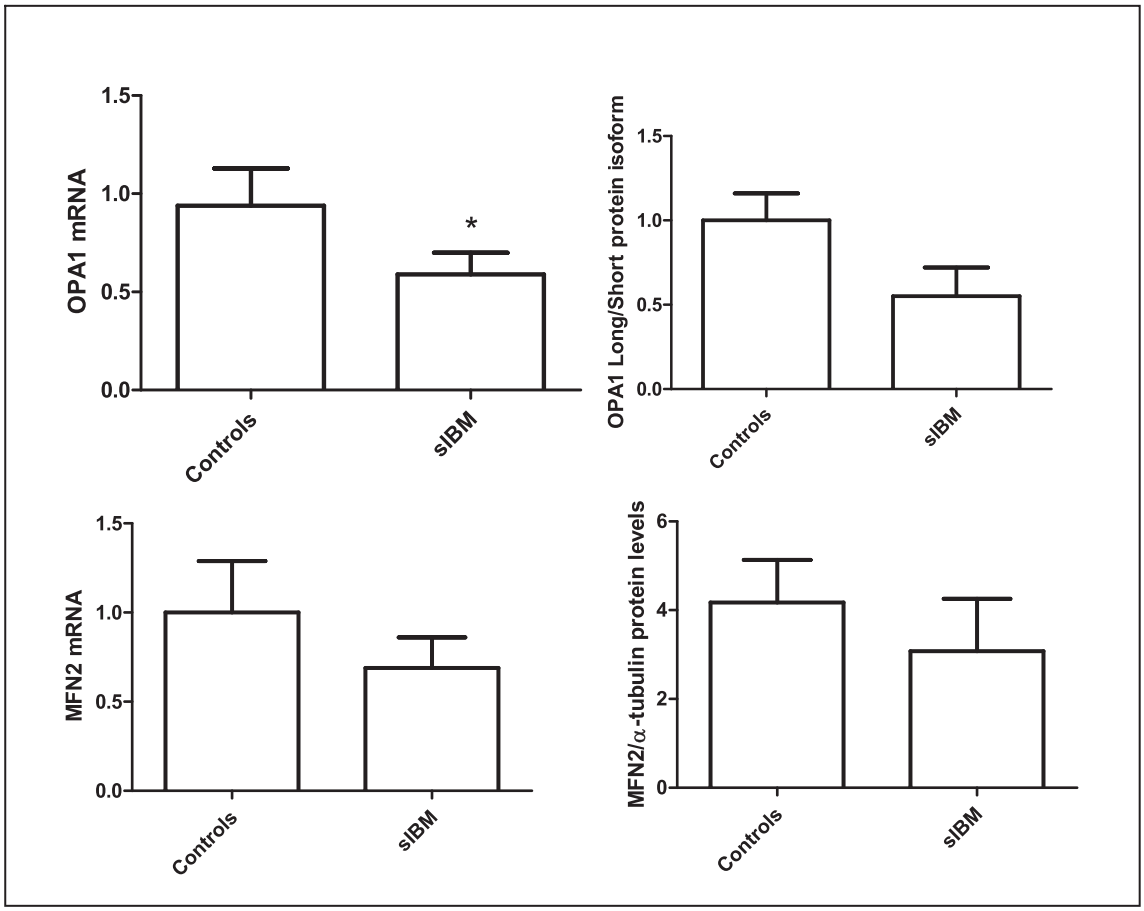

Figure 4 Mitochondrial dynamics in muscle from sIBM patients

MFN2 and OPA1 were assessed in muscle from sIBM patients at the transcript and protein levels. Significant differences were found when OPA1 mRNA levels were compared between the two groups. The other parameters showed a non-significant trend to be decreased in the sIBM respective controls. * : $p < 0.05$.

disrupted pathways [6–8]. In the present study, we evaluated mitochondrial dysfunction at a genetic and molecular level, and studied its potential association with mitochondrial dynamic deregulation. We compared mitochondrial status in muscle and PBMCs from sIBM patients with that of controls, first to evaluate its involvement in disease aetiology and, second, to determine whether mitochondrial alterations are confined to muscle tissue (the target tissue of the disease) or whether these alterations extend to other body tissues, which may allow less invasive approaches and potential follow-up as a putative biomarker.

With regard to experimental data, sIBM patients showed a decrease in mtDNA content. This mtDNA depletion was statistically significant in muscle from sIBM patients, because it is post-mitotic tissue that is prone to store mitochondrial deficiencies. On the other hand, mtDNA depletion was only mildly manifested in PBMCs, probably due to their higher renewal capacity and shorter mean lifespan. Although mtDNA depletion was found in both tissues, no significant changes were observed in mtRNA levels of either muscle or PBMCs [37].

On analysing MRC function, no differences were found in complex I activity in muscle from sIBM patients compared with controls. Otherwise, MRC complex IV deficiency was present in both tissues (muscle and PBMCs) of sIBM patients. Absolute values of complex IV activity in muscle showed a trend towards a decrease, but, on normalizing these absolute values by mitochondrial mass, complex IV deficiency became significant. These phenomena may be due to the increase in mitochondrial biogenesis detected in muscle of sIBM patients as compensating for mitochondrial dysfunction. This increase in muscular mitochondrial content, characteristic of primary mitochondrial diseases [38], is observed in parallel in sIBM patients as an increase in CS activity and VDAC1:β-actin protein levels, as well as by the presence of ragged-red fibres. Similarly, mitochondrial protein synthesis tended to increase, albeit not significantly, in muscle from sIBM patients (COX-II:COX-IV), probably as a consequence of the trend of this homoeostatic muscle to increase mitochondrial biogenesis. As mentioned previously, PBMCs present a shorter mean lifespan, thereby hindering the development of homoeostatic mechanisms such as increasing mitochondrial content to deal with mitochondrial dysfunction. Thus, in PBMCs, complex IV activity was found to be significantly decreased in both absolute and relative values to mitochondrial

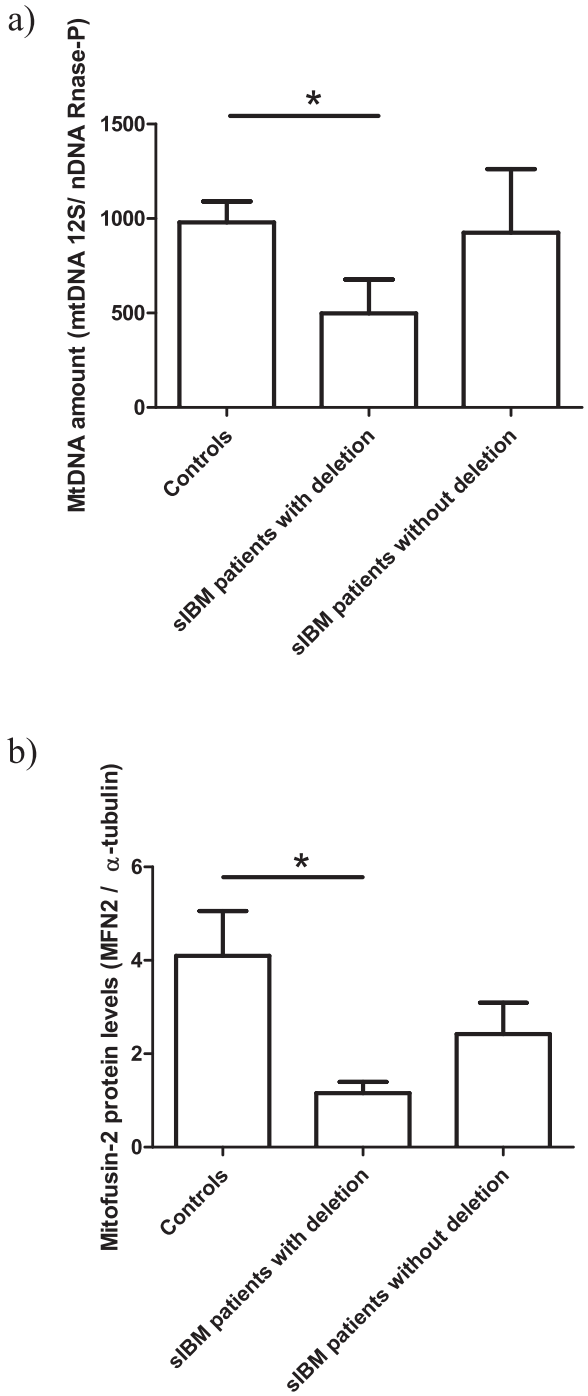

Figure 5 MtDNA amount and MFN2 protein levels in muscle of sIBM patients with and without mtDNA deletions and controls

Comparison of (**a**) mtDNA amount and (**b**) MFN2 levels among patients with sIBM with and without mtDNA deletions and controls. The patients with sIBM and mtDNA deletions showed significantly reduced muscle levels of mtDNA and MFN2 protein levels with respect to the controls. The patients with sIBM and no mtDNA deletions presented an intermediate value, suggesting the implication of mtDNA deletions in other mitochondrial alterations or the involvement of mitochondrial dynamics in mtDNA homoeostasis. * : $p < 0.05$.

mass, which additionally showed trends towards a decrease, according to poor homoeostatic compensation. Unlike in muscle, in PBMC nuclear-encoded protein subunits of COX (COX-IV) were slightly increased to compensate for COX dysfunction. The trends towards up-regulation of mitochondrially encoded proteins in muscle or nuclear-encoded proteins in PBMCs, to compensate for MRC dysfunction, may depend on the levels of endoplasmic reticular stress of both cell types, which has been reported to be altered in muscle [39,40].

Mitochondrial dysfunction in sIBM patients is not caused by abnormal assembly of MRC complexes, at least as assessed through the measurement of protein levels of specific subunits that are labile in cases of complex instability. However, there was a strong trend of the complex V subunit, when normalized to mitochondrial mass, to be decreased. This decrease disappeared when the complex V subunit content was normalized to total cell protein. This could be explained by the slight increase in mitochondrial mass that underlies sIBM. Further analysis of complex V activity could be useful to elucidate whether this complex is altered in this disease.

Oxidative stress does not seem to play a role in sIBM pathogenesis, because lipid peroxidation levels are not increased in muscle or PBMCs from sIBM patients, at least in the present study cohort.

Overall, these data describe an altered mitochondrial phenotype in muscle and PBMCs of sIBM patients, with both tissues presenting a different lesion profile, probably due to different compensatory mechanisms related to the disease. It is interesting, regardless of the homoeostatic attempt to preserve mitochondrial function, that COX enzymatic deficiency is present in both tissues as a common pathogenic parallelism. Thus, it seems that mitochondrial dysfunction in sIBM is not confined only to the target tissue of the disease, muscle, but may also extend to other body tissues. Whether COX/CS activity could be used as a prognostic or severity biomarker of the disease should be assessed in further studies. Alternatively, PBMC mitochondrial alterations may be interpreted as a deregulation of the immune system, non-specific for sIBM, as stated before for other diseases with inflammatory components [41–45]. Regardless of the interpretation, mitochondrial impairment is present in both PBMCs and muscle tissue of sIBM patients.

Mitochondrial dynamics are the mechanism for this organelle recycling and turnover. Imbalance of proper ratios of fusion and fission events has recently been associated with disease [13,46,47]. Mitochondrial dynamics were severely altered in muscle of sIBM patients as demonstrated by the decreased expression of OPA1 and MFN2 transcript and protein levels. Deregulation of the mitochondrial fusion:fission ratio leads to abnormal mitochondrial turnover, which is essential for the recycling of damaged and dysfunctional mitochondria [48], such as those characteristic of sIBM patients.

It is interesting that reduced expression of MFN2 in sIBM patients is associated with abnormal mitochondrial genetics (both deletions and depletion). Previous studies have reported the strong dependence of mtDNA maintenance on proper mitochondrial dynamics in physiological conditions [10]. This is the first study to report this association in the context of sIBM disease.

Whether mitochondrial genetic and molecular alterations lead to abnormal mitochondrial dynamics or, on the contrary, to an

imbalance in mitochondrial dynamics underlying the mitochondrial genetic and molecular disturbances is still a matter of debate. In any case, both adverse conditions seem to go hand in hand with the development of sIBM disease.

In summary, in the present study we demonstrate that mitochondria are impaired in sIBM, not only at histological but also at genetic, molecular and functional levels, confirming the involvement of mitochondria in the aetiology of this disease. The lesion is characterized by different patterns in both muscle and PBMCs of sIBM patients, demonstrating that mitochondrial injury is not exclusive to the target tissue of the disease but is also present in peripheral tissues. It is interesting that mitochondrial COX activity normalized by mitochondrial mass is altered in both tissues, highlighting the relevance of this parameter, and the need for further studies on this as a putative candidate diagnostic tool or therapeutic target. In addition, deregulation in mitochondrial dynamics seems to be crucial for mtDNA stability, not only in healthy conditions [10] but also in sIBM disease. Deregulation of mitochondrial dynamics in muscle leads to increased genetic alterations such as mtDNA depletion and multiple deletions, demonstrating that mitochondrial turnover is essential for proper mitochondrial health and, furthermore, for muscle function. Although further investigation is needed to demonstrate whether mitochondrial alterations are primary or secondary in sIBM, the present study provides more in-depth understanding of the role of mitochondria in this disease. It also provides knowledge related to the mechanisms implicated in order to help develop new diagnostic tools or therapeutic targets for this disease, in the absence of non-invasive diagnostic tools or effective therapy.

CLINICAL PERSPECTIVES

- The better understanding of how mitochondria is in sIBM would be useful at clinical level at different levels: to confirm that mitochondrial changes seen in the muscle biopsy of sIBM patients is a solid marker to help diagnose this disease.
- This information could be useful to develop new therapeutical strategies to treat muscle dysfunction in sIBM patients.
- The knowledge about the pathogenic mechanisms of the disease allow us to elaborate a more accurate description of the disease for the patient, as they could be able to know about the disease they're suffering.

AUTHOR CONTRIBUTION

J.M. Grau, along with F. Cardellach and G. Garrabou, conceived the study and supervised the data collection and analysis. J.M. Grau was responsible for the diagnosis and inclusion of all patients, performed the IBMFRS test and collected all the clinical data with the help of C. Morén. M. Catalán-García was responsible for the experimental analysis of the amounts of mtDNA and mtRNA (with the help of M. Bañó and J. Enrich-Bengoa), for the analysis of the MRC complex IV activity and CS activity (with the help of A. Hernando), for the analysis of the oxidative stress and mitochondrial protein synthesis (with the help of M. Guitart-Mampel and I. González-Casacuberta), and for the analysis of mitochondrial mass by Western blotting (with the help of D.-L. Juárez). MtDNA deletions were assessed by our collaborators S. Emperador and J. Montoya, and mitochondrial dynamics-related proteins were analysed through collaboration with A. Díaz-Ramos and A. Zorzano. E. Tobías provided technical assistance for reactive preparation. M. Catalán-García created a database to collect all clinical and experimental parameters and performed the statistical analysis of the data, under the supervision of G. Garrabou and C. Morén. All authors, especially G. Garrabou, J.M. Grau, J. Montoya and A. Zorzano, participated in the revision of the manuscript, adding new concepts of high relevance.

ACKNOWLEDGEMENTS

We would like to specially thank Fundació CELLEX for funding this project and to the extraordinary generosity of sIBM patients and families.

FUNDING

This study has been funded by Instituto de Salud Carlos III (ISCIII) [PI14/00005, PI15/00903, PI15/00817 and PIE1400061] and Fondo Europeo de Desarrollo Regional (FEDER), Suports a Grups de Recerca de la Generalitat de Catalunya [SGR 2014/376], La Marató [87/C/2015), Fundació CELLEX and CIBER of Rare Diseases (CIBERER, an initiative of ISCIII).

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Received 3 February 2016/5 July 2016; accepted 13 July 2016

Accepted Manuscript online 13 July 2016, doi: 10.1042/CS20160080

Publication 2

Title:

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Reference:

Molecular Medicine. 2015 Nov 3. doi: 10.2119/molmed.2015.00168.

Molecular Medicine

BACE-1, PS-1 and sAPPβ Levels Are Increased in Plasma from Sporadic Inclusion Body Myositis Patients: Surrogate Biomarkers among Inflammatory Myopathies

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Sporadic inclusion body myositis (sIBM) is a rare disease that is difficult to diagnose. Muscle biopsy provides three prominent pathological findings: inflammation, mitochondrial abnormalities and fibber degeneration, represented by the accumulation of protein depots constituted by β-amyloid peptide, among others. We aim to perform a screening in plasma of circulating molecules related to the putative etiopathogenesis of sIBM to determine potential surrogate biomarkers for diagnosis. Plasma from 21 sIBM patients and 20 age- and gender-paired healthy controls were collected and stored at –80°C. An additional population of patients with non-sIBM inflammatory myopathies was also included (nine patients with dermatomyositis and five with polymyositis). Circulating levels of inflammatory cytokines (interleukin (IL)-6 and tumor necrosis factor (TNF)-α), mitochondrial-related molecules (free plasmatic mitochondrial DNA (mtDNA), fibroblast growth factor-21 (FGF-21) and coenzyme-Q10 (CoQ)) and amyloidogenic-related molecules (beta-secretase-1 (BACE-1), presenilin-1 (PS-1), and soluble Aβ precursor protein (sAPPβ)) were assessed with magnetic bead–based assays, real-time polymerase chain reaction, enzyme-linked immunosorbent assay (ELISA) and high-pressure liquid chromatography (HPLC). Despite remarkable trends toward altered plasmatic expression of inflammatory and mitochondrial molecules (increased IL-6, TNF-α, circulating mtDNA and FGF-21 levels and decreased content in CoQ), only amyloidogenic degenerative markers including BACE-1, PS-1 and sAPPβ levels were significantly increased in plasma from sIBM patients compared with controls and other patients with non-sIBM inflammatory myopathies ($p < 0.05$). Inflammatory, mitochondrial and amyloidogenic degeneration markers are altered in plasma of sIBM patients confirming their etiopathological implication in the disease. Sensitivity and specificity analysis show that BACE-1, PS-1 and sAPPβ represent a good predictive noninvasive tool for the diagnosis of sIBM, especially in distinguishing this disease from polymyositis.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2015.00168

INTRODUCTION

Although sporadic inclusion body myositis (sIBM) is considered a rare disease (ORPHA611), it is the most common myopathy in individuals over 50 years (1). This disease belongs to the group of inflammatory myopathies, together with dermatomyositis (DM) and polymyositis (PM). Its prevalence (4.5–35 per million) varies among countries and ethnic groups, although several studies have suggested that this value could be underestimated. Clinically, sIBM is characterized by insidious weakness in proximal and distal muscles, especially in the quadriceps and finger flexors. Neck flexors and extensors are frequently affected, and also dysphagia is present in up to 60% of patients with sIBM. The clinical progression is slow and often leads to severe disability (2–6).

The first diagnostic criteria for sIBM were proposed by Calabrese *et al.* (7) in 1987, but so far, muscle biopsy is

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Submitted July 8, 2015; Accepted for publication October 27, 2015; Published Online (www.molmed.org) November 3, 2015.

still essential for diagnosis. Although the pathogenesis of sIBM is not well known, inflammatory, mitochondrial and degenerative pathogenic mechanisms have been described. Diagnosis is confirmed by characteristic findings on muscle biopsy demonstrating endomysial mononuclear cell infiltrates, rimmed vacuoles, amyloid deposits and mononuclear cell invasion of non-necrotic fibers. Although the presence of β-amyloid deposits in muscle of sIBM patients is classically accepted by the scientific community (8), some controversy arose regarding the methodology used to detect this molecule (9). The presence of increased β-amyloid peptide has also been described in plasma by Abdo *et al.* (10), confirming its implication in the disease. Recent studies also reported the presence of the TDP-43 protein in these inclusion bodies, which may be more specific in sIBM than β-amyloid peptide (11). In addition, COX (cytochrome c oxidase) negative and SDH (succinate dehydrogenase) positive fibers are present in most of the cases.

Because of the slow progression of the disease and the diagnostic difficulties, the diagnosis is often delayed or misdiagnosed commonly as PM (1,3,12). Salajegheh *et al.* reported the existence of circulating autoantibodies against a 43-kDa muscle protein called CN1A, highly specific to IBM (13,14), although recent investigations found this autoantibody in other autoimmune diseases (15). Apart from this report, there is no information about noninvasive circulating diagnostic biomarkers in sIBM.

Inflammatory processes, as well as mitochondrial dysfunction and degeneration, are pathologic processes that are widely known to play a role in sIBM. These pathological features found in muscle of sIBM patients may involve different molecules that might be altered in this disease. Regarding inflammation, it has been demonstrated that myoblasts produce interleukin (IL)-6 and tumor necrosis factor (TNF)-α in response to inflammatory stimuli of T-lymphocytes (16), and T-lymphocyte infiltrates are commonly present in PM and in sIBM (17). In relation to mitochondrial and inflammatory lesions, it was also recently reported that circulating mitochondrial DNA (mtDNA) in plasma released by injured cells causes a powerful innate immune response that triggers inflammation through the recognition of damage-associated molecular patterns (DAMPs) by toll-like receptors (TLRs) (18,19). Since sIBM presents both mitochondrial and cell damage accompanied by chronic inflammation, this parameter may also be potentially involved in the development of sIBM.

Parallelly, fibroblast growth factor-21 (FGF-21) was reported to be a plasmatic biomarker for mitochondrial muscle disease (20), which would increase as a protective compensatory mechanism in response to mitochondrial damage. Additionally, coenzyme-Q10 (CoQ) is widely known as a key molecule in mitochondrial respiratory chain function. The plasma levels of this coenzyme are reportedly related to myopathy, especially with statin-induced myopathies (21). In addition, CoQ10 seems to be related to the production of tau-aggregation present in Alzheimer's disease (AD) as well as in sIBM biopsies (22). However, to our knowledge, this is the first time that these mitochondrial markers have been quantified in the plasma of sIBM patients.

With regard to amyloidogenic protein deposition leading to cell degeneration, many studies have recently described parallelisms between sIBM and AD (23,24). These studies suggest that sIBM and AD may share a common etiology. In fact, amyloid-β deposition and the presence of phosphorylated tau protein have been detected in both brain tissue and muscle biopsy from patients with AD and sIBM disorders, respectively (25). These amyloid-β depositions are caused by the amyloidogenic processing of amyloid precursor protein (APP) by β-secretase-1 (BACE-1) and γ-secretase (presenilin-1 [PS-1]) leading to cell injury. Non-amyloidogenic processing of APP by the α-secretase does not cause amyloid-β depositions. Wu *et al.* (26) have proposed the measurement of plasma BACE-1 activity as a potential biomarker for AD, and Nogalska *et al.* (27) found increased BACE-1 mRNA levels in sIBM muscle fibers. Another product of this amyloidogenic processing of APP is sAPPβ. This fragment is released when BACE-1 cleaves the APP protein, releasing soluble Aβ precursor protein (sAPPβ) and the amyloid-β fragment. Although sAPPβ does not oligomerize and causes depositions, its presence indicates that APP has been cleaved by BACE-1, and a fragment of amyloid-β has also been released. Thus, increased levels of plasma sAPPβ indicate a higher amount of amyloidogenic particles that will lead to an increased amount of inclusion bodies (Figure 1). This molecule is used to monitor AD patients to demonstrate the efficacy of new therapeutic drugs (28). However, as far as we know, none of these amyloidogenic degeneration molecules have ever been measured in plasma of sIBM patients.

Because information about circulating biomarkers in sIBM is scarce, the aim of the present study was to evaluate if inflammatory, mitochondrial and degenerative circulating molecules potentially involved in the etiopathogenesis of sIBM may be altered in plasma of sIBM patients and if they may be useful as diagnostic tools.

MATERIALS AND METHODS

Study Design

We performed a multicenter, cross-sectional, case-controlled, observational study.

Study Population

The respective cases of sIBM, DM and PM were diagnosed by clinical and pathological tests in the Department of Pathology and Internal Medicine of the Hospital Clínic of Barcelona (Barcelona, Spain) and in the Hospital Vall d'Hebron (Barcelona, Spain). All the patients fulfilled the criteria proposed by the European Neuromuscular Centre (29,30),

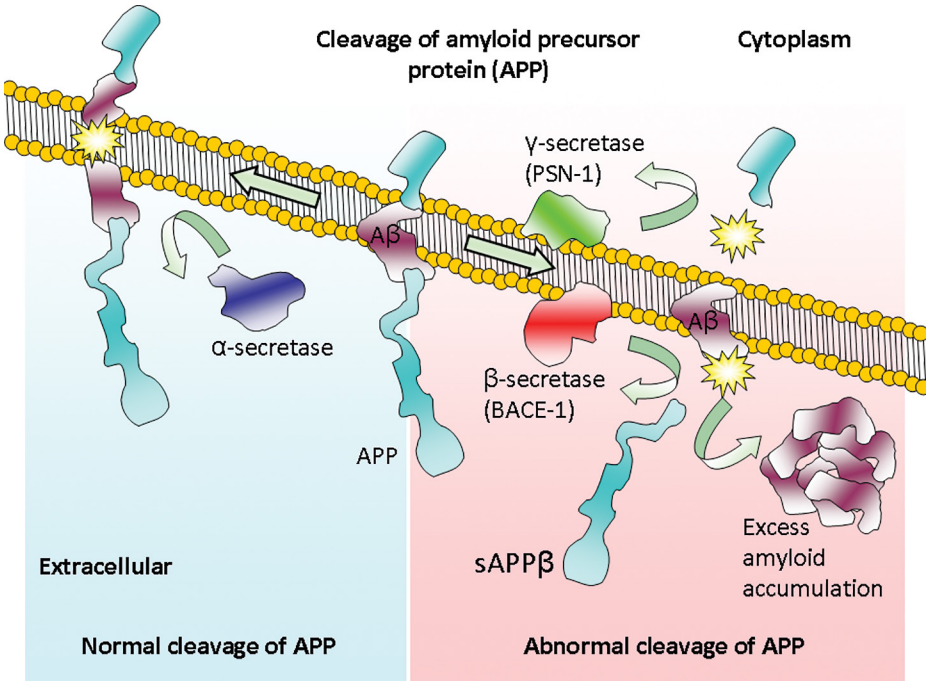

Figure 1. Normal and abnormal cleavage of the amyloid precursor protein. Both pathways are physiologic, but an increase in the abnormal pathway mediated by BACE-1 and PS-1 leads to amyloid-β oligomerization and the accumulation of amyloid-β plaque.

representing definite forms of each disease. Twenty-one patients of sIBM were prospectively and consecutively included in the present study at the time of diagnosis after signing the informed consent previously approved by the ethical committee of the Hospital Clinic of Barcelona. On inclusion, all the sIBM patients completed the inclusion body myositis functional rating scale (IBMFRS), a validated disease-specific functional rating scale (31), scoring 23.6 ± 1.2 out of 40 and presenting clinical features of moderate to advanced sIBM. Parallelly, we included 20 age-gender–paired healthy controls to determine significantly altered biomarkers in sIBM patients. The inclusion criteria for the healthy controls were as follows: age >40 years, absence of family history of mitochondrial disease, absence of muscle disease, viral infection, drug abuse or contact with mitochondrial toxic agents. In addition, patients with inflammatory myopathy different from sIBM (nine patients with DM and five patients with PM) were included to determine the sensitivity and specificity of the biomarkers selected versus sIBM patients.

Sample Collection and Processing

A total of 20 mL peripheral blood were collected from both patients and controls by antecubital vein puncture in EDTA tubes. Plasma was isolated by centrifugation at 1,500*g* during 15 min and stored at –80°C until analysis.

Inflammatory Molecules Analysis

The concentration of soluble inflammatory molecules was determined using a Human Cytokine Plex (Bio-Rad) according to the manufacturer's instructions. The molecules determined were IL-6 and TNF-α. Plates were analyzed on a Luminex 100™ instrument (Luminex) by using Bio-Plex Manager™ Software (Bio-Rad). Concentrations were obtained by standard calibration curves. All measurements were performed in duplicate. Results were expressed in picograms per milliliter (pg/mL).

Mitochondrial-Related Molecules Analysis

Circulating mtDNA was isolated from plasma with a QIAGEN Amp Blood Mini Kit and stored at 4°C for further analysis for a maximum of 24 h after extraction. Free circulating mtDNA was assessed by quantitative real-time polymerase chain reaction in an Applied Biosystems 7500 Real Time PCR System by the amplification of a fragment of the mitochondrial *12SrRNA* gene as reported previously (32). Circulating mtDNA content was expressed as the number of copies of mtDNA per milliliter of plasma. Circulating FGF-21 was measured by an enzyme-linked immunosorbent assay (ELISA) (Biovendor R&D) using an internal curve of standards run in duplicates, and the results were expressed as picograms per milliliter (pg/mL) (33). Plasma levels of CoQ were assessed by high-pressure liquid chromatography (HPLC) in reverse form with electrochemical detection of the reduced and oxidized molecule. Values were expressed as micromoles per liter (μmol/L) (34).

Degenerative Molecules Analysis

Plasma levels of BACE-1, PS-1 (concretely PS-1-NTF) and sAPPβ were analyzed with the ELISA sandwich enzyme immunoassay technique using an internal curve of standards and run in duplicates following the manufacturer's instructions (reference SEA718Hu, SEC200Hu and MBS165363, respectively; USCN-Life-Science), and values were expressed as nanograms per milliliter (ng/mL).

Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM) either as absolute units or as a percentage of increase or decrease between groups. Odds ratio and Fisher test were used to calculate gender distribution and its statistical differences among groups. Nonparametric statistical analysis was performed to select candidate

biomarkers with significantly altered expression in sIBM patients with respect to healthy controls by using the independent sample Mann-Whitney *U* test. In addition, correlations were assessed using Spearman linear regression analysis. Selected biomarkers were further tested among the different groups of patients with inflammatory myopathies using the independent sample Mann-Whitney *U* test. Additionally, for evaluating the predictive capacity of these selected biomarkers to discriminate between sIBM subjects and either healthy controls or the group of patients with inflammatory myopathies other than sIBM, binary logistic regression was performed to assess the sensitivity and specificity of each molecule tested. Furthermore, the Omnibus test, the Hosmer-Lemeshow goodness-of-fit, the ROC curve and the area under the curve (AUC) were also performed to assess the reliability of these molecules for diagnosis. In all cases, a p value <0.05 was considered statistically significant.

RESULTS

There were no differences in terms of age and gender among the groups. Table 1 shows all the clinical and demographical data of the patients included. IBMFRS test confirmed moderate to advanced level of severity for sIBM disease, but did not render statistical significant association to further evaluate molecular biomarkers.

We found remarkable trends toward altered plasma expression of inflammatory and mitochondrial biomarkers in the plasma of sIBM patients. The plasma levels of IL-6 and TNF-α in sIBM patients were higher compared with healthy controls (43.9 ± 29% versus 18.2 ± 23%, respectively). The circulating mtDNA and FGF-21 values were also greater in the plasma of sIBM patients compared with healthy controls (10.2 ± 49% versus 52 ± 40%, respectively), whereas plasma CoQ levels were lower at 3.7 ± 8%. However, none of these alterations was significant (Figure 2).

Table 1. Demographic and clinical data of the three study populations.

	Sporadic inclusion body myositis (n = 21)	Controls (n = 20)	Dermatomyositis and polymyositis (n = 14)	Statistical significance
Demographic data				
Male (n (%))	10 (47.6)	13 (65)	6 (43)	NS
Female (n (%))	11 (52.3)	7 (35)	8 (57)	NS
Age (years) (mean ± SEM)	67.7 ± 2.3	68.5 ± 1.4	59.3 ± 2.8	NS
Disease progression data				
IBMFRS test	23.6 ± 1.66	–	–	–

NS, nonsignificant; SEM, standard error of the mean. The IBMFRS is a disease-specific, 10-point functional rating scale for patients with sIBM. This test classifies the clinical features of sIBM (with a maximum score of 40) according to the impossibility to perform daily activities such as dressing, personal hygiene and swallowing (0 score).

Interestingly, all the plasma biomarkers of amyloidogenic degeneration were significantly increased in the plasma of sIBM patients compared with healthy controls. BACE-1 was significantly increased (102 ± 29.6%) in plasma of sIBM patients compared with healthy controls (38,409 ± 5,629 versus 18,999 ± 2,487; $p = 0.003$). PS-1 was also significantly increased in these patients (31.58 ± 26.9%) compared with the healthy control cohort (1.82 ± 0.18 versus 1.33 ± 0.10; $p = 0.003$), and sAPPβ levels also showed a strong trend to an increase in sIBM patients compared with healthy controls; however, they were not statistically significant (19.8 ± 4.1 versus 15.2 ± 2.5; $p = 0.054$). In addition, a positive correlation was found between BACE-1 and PS-1 plasma levels ($R^2 = 0.087$; $p < 0.05$). The biomarkers showing significant differences between sIBM patients and healthy controls (amyloidogenic molecules) were further evaluated in a third study population composed of patients with DM and PM, the non-sIBM inflammatory myopathy group. This third study population showed similar results compared with the healthy control group, with only 1.2 ± 13.1% in the case of BACE-1 (19,244 ± 2,494 versus 18,999 ± 2,487, respectively), increasing to 1.5 ± 6% for PS-1 values (1.35 ± 0.08 versus 1.33 ± 0.1, respectively) and decreasing by 12.7 ± 6.5% in the case of sAPPβ (13.33 ± 1 versus 15.26 ± 2.53, respectively). None of these differences were statistically significant

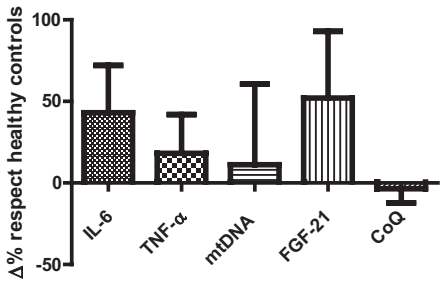

Figure 2. Plasma levels of potential inflammatory (IL-6 and TNF-α) and mitochondrial (free mtDNA, FGF-21 and CoQ) biomarkers in plasma of sIBM patients. Values are expressed as percentage of increase or decrease with respect to healthy controls. No statistically significant differences were found between sIBM patients and controls. However, increased levels of these molecules in sIBM (especially IL-6, TNF-α and FGF-21) suggest evidence of their implication in the pathogenesis of sIBM.

(p = NS) (Figure 3). Specificity of the ELISA kits for the amyloidogenic molecules were confirmed by Western blot in both BACE-1 and PS-1. Similar patterns of expression were found in those experiments regarding differences between sIBM patients and controls (data not shown).

Consequently, when sIBM patients were compared with the non-sIBM group (healthy controls + DM + PM), BACE-1, PS-1 and sAPPβ showed a significant increase of 99.5 ± 29.2%, 34.8 ± 13.3% and 36.5 ± 28.2%, respectively (38,409 ± 5,629 versus 19,244 ± 2,494, $p = 0.001$; 1.82 ± 0.18

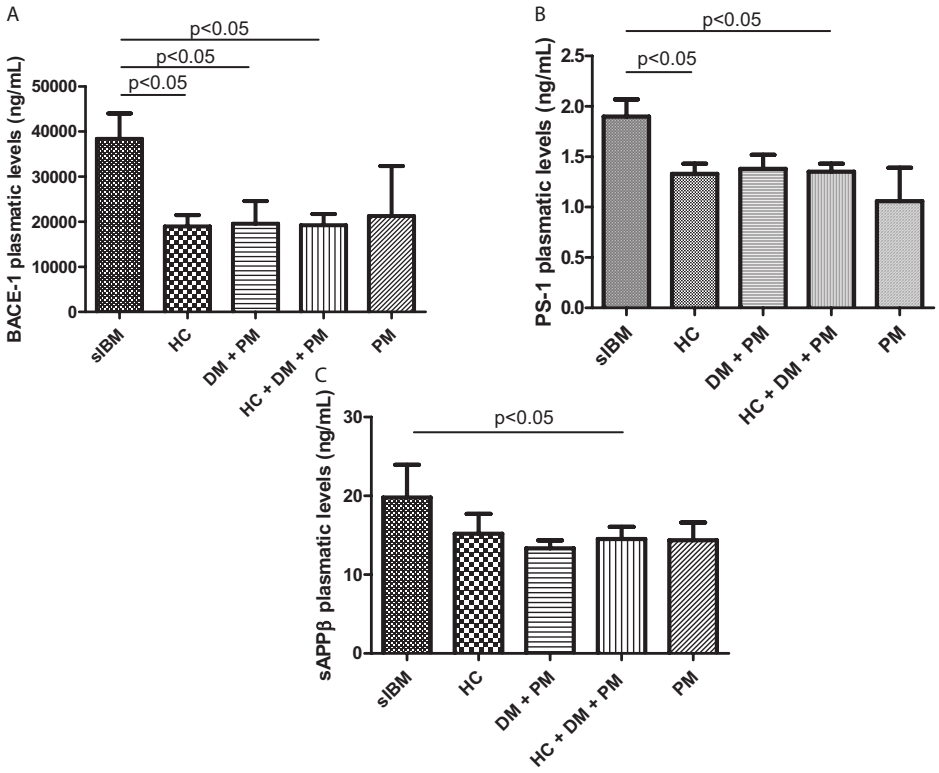

Figure 3. BACE-1 (A), PS-1 (B) and sAPPβ (C) plasma levels in sIBM patients compared with healthy controls (HC), non-sIBM inflammatory myopathy patients (DM + PM) and non-sIBM patients (HC + DM + PM). HC, healthy controls; DM, dermatomyositis; PM, polymyositis. Increased levels of these amyloidogenic molecules in plasma from sIBM patients compared with healthy controls, DM and PM demonstrate their implication in sIBM disease and also strengthen their possible use for diagnostic purposes.

versus 1.35 ± 0.08, $p = 0.024$, and 19.8 ± 4.1 versus 14.5 ± 1, $p = 0.03$, respectively) (Figure 3).

Finally, sIBM patients were compared with PM patients alone, with BACE-1, PS-1 and sAPPβ levels showing the same increased pattern, although these differences did not reach statistical significance, probably because of small sample size of the PM group (38,409 ± 5,629 versus 21,269.6 ± 11,068, $p = 0.103$; 1.82 ± 0.18 versus 1.06 ± 0.33, $p = 0.085$; 19.8 ± 4.1 versus 14.3 ± 2.2, $p = 0.173$, respectively) (Figure 3).

The Omnibus test and the Hosmer-Lemeshow goodness-of-fit test revealed that BACE-1, PS-1 and sAPPβ were suitable as a predictive tool to discriminate sIBM from the other study cohorts. Overall, the sensitivity and specificity were 74.5, 65.5 and 66.7%, respectively, with AUC of 0.77, 0.62 and 0.68, respectively. Combined analysis of these molecules did not show better sensitivity and specificity with respect to the overall values (Table 2).

DISCUSSION

At present, sIBM disease entails two main problems: difficulty for achieving early diagnosis and the lack of effective treatment (7,12,35). The aim of this study was to perform the first screening of circulating molecules potentially involved in the etiopathology of sIBM to promote both advances in understanding the etiology of this disease as well as the development of diagnostic tools. The understanding of the etiopathology of sIBM is crucial to find effective treatment, and improvements in the diagnosis of this disease are essential to reduce the invasiveness of the current approaches, the need for a second or third biopsy to ensure diagnosis and the potential confusion with similar diseases and to facilitate early detection and follow-up. The data provided by the present study demonstrate evidence of plasma biomarkers in a peripheral tissue that is by far more accessible than the target tissue of sIBM; also, these data prevent the need to perform a second muscle biopsy to confirm the diagnosis.

Despite being reported as key molecules in inflammatory processes and mitochondrial bioenergetics, the plasma levels of IL-6, TNF-α, free mtDNA, FGF-21 and CoQ were not significantly altered at plasma level. However, most of these molecules showed strong trends to being altered in the plasma of these patients, for example, IL-6 and TNF-α,

Table 2. Logistic binary regression results in patients with sporadic inclusion body myositis (sIBM) compared with the remaining cohorts (healthy controls + DM + PM).

Molecule	Sensitivity (%)	Specificity (%)	Overall	AUC
Separately				
BACE1	45.0	91.2	74.1	0.77 ± 0.07
PS1	38.1	82.4	65.5	0.62 ± 0.08
sAPPβ	11.1	97.0	66.7	0.68 ± 0.7
Combined				
BACE1 + PS1 + sAPPβ	50.0	87.9	74.1	
BACE1 + PS1	55.0	85.3	74.1	
BACE1 + sAPPβ	44.4	87.9	72.5	

Logistic binary regression results in patients with sporadic inclusion body myositis (sIBM) compared with the remaining cohorts (healthy controls + DM + PM). The sensitivity and specificity of each molecule are shown separately and combined in addition to the AUC of each molecule.

which are also reportedly altered in muscle (16,17). There may be a parallelism between the muscle and plasma levels of these molecules, and since these molecules are not useful to discriminate among other inflammatory myopathies, they may provide information as to the inflammatory status of the patients. Additionally, this inflammation does not seem to be triggered, at least significantly, by circulating mtDNA released from chronically injured muscle cells.

On considering the implication of mitochondria in sIBM, it was also suggested that FGF-21 is altered in these patients. Although the liver is the main producer of FGF-21 (36), muscle was also described to secrete this endocrine factor, and its production is known to be increased as a consequence of primary but not secondary mitochondrial disorders (37). In this regard, we found circulating FGF-21 levels to be increased, albeit not significantly, in these patients. Because all the subjects studied were free of metabolic disorders involving hepatic lesions, muscle may be directly involved in this trend to an increase in sIBM patients. Although it was not possible to evaluate liver and muscle biopsies from the subjects of this study for ethical reasons, this increase in FGF-21 levels seems to reinforce the assumption of mitochondrial implication in the etiopathogenesis of sIBM.

Coenzyme Q was evaluated because of its important involvement in mitochondrial respiratory chain function, as well as its implication in the formation of Tau aggregates (22). However, this molecule was not found to be altered in plasma. Nonetheless, further studies should evaluate the levels of this coenzyme in muscle biopsy where tau aggregation occurs.

On analyzing the molecules related to muscle degeneration and the formation of β-amyloid depositions, we found a significant increase in these biomarkers in plasma of sIBM patients. PS-1 and especially BACE-1 were dramatically increased, suggesting that the increase in these levels is responsible for the formation of β-amyloid depositions in sIBM. In physiological conditions, the processing of APP is carried out by the two pathways: the non-amyloidogenic pathway with α-secretase and the amyloidogenic pathway with BACE-1 and PS-1. However, in nonpathologic conditions, the amyloidogenic pathway is so diminished that there is no relevant formation of β-amyloid. In sIBM, we propose that the amyloidogenic pathway is altered and the levels of the molecules involved in this process (BACE-1 and PS-1) are dramatically increased, pathologically accelerating the formation of β-amyloid depositions. Additionally, the increase of these molecules in muscle (27), where they exert their function-causing protein depots, is also transferred to an increase in plasma levels of these molecules. Likewise, overexpression of sAPPβ, the resulting fragment of APP processed by BACE1, also demonstrated increased BACE1 activity, thereby reinforcing this theory.

The clinical onset of sIBM is similar to that of other inflammatory myopathies. Years of disease evolution are often required, and second biopsies are needed to confirm the diagnosis of sIBM. Consequently, in clinical practice, patients diagnosed with DM and especially PM are the best subjects to test the sensitivity and specificity of the biomarkers selected for sIBM diagnosis.

In the present study, the plasma expression of amyloidogenic markers (BACE-1, PS-1 and sAPPβ) in patients with inflammatory myopathies including DM and PM were similar to those of healthy controls, thereby strengthening the amyloid theory, since, despite sharing some clinical and pathologic features similar to sIBM, these other inflammatory myopathies do not present inclusion bodies. Based on these findings, we can conclude that increased expression of amyloid-related molecules in plasma is specific of sIBM. These findings strengthen the possibility of using those selected molecules as appropriate candidates for the diagnosis of sIBM and as potential biomarkers for discriminating between sIBM and other inflammatory myopathies. Regarding the sensitivity and specificity, we can conclude that, among the biomarkers selected, BACE1 levels are the best parameter for discriminating between sIBM patients and controls or other inflammatory myopathies, since the specificity and sensitivity achieved with the addition of PS-1 or sAPPβ did not increase the diagnostic precision compared with the use of BACE-1 quantification alone. sAPPβ and especially PS-1 levels are also altered in sIBM patients, confirming their involvement in the etiology of this disease.

These findings also strengthen the idea that sIBM is related to AD, at least with respect to its pathogenic mechanisms, showing the same kind of lesion in different tissues (muscle fiber and neurons, respectively). That is why sIBM is also known as muscle AD (23,24,38,39).

CONCLUSION

Considering the difficulties in diagnosing sIBM on the basis of clinical and anatomo-pathological findings, we propose that plasma BACE-1 levels may be a potential circulating biomarker for helping to achieve the diagnosis of sIBM. Because the clinical onset of sIBM and other inflammatory myopathies may be similar, and PM is often misdiagnosed as sIBM, further research should be done to validate in bigger sample size cohorts if those biomarkers could be suitable to ensure sIBM diagnosis in case of ambiguity for differential diagnosis.

The limitation of this study is the sample size, especially of the DM and PM groups. Further studies including more patients are needed to evaluate the usefulness of amyloidogenic biomarkers to establish the severity and evolution of sIBM through the follow-up of a cohort over time.

ACKNOWLEDGMENTS

This study has been funded by Fondo de Investigación Sanitaria (FIS 0229/08, 00462/11 and 01199/12) granted by ISCIII and Fondo Europeo de Desarrollo Regional (FEDER), Fundació Cellex, Fundación para la Investigación y la Prevención del SIDA en España

(FIPSE 360745/09 and 360982/10), Suports a Grups de Recerca de la Generalitat de Catalunya (SGR 09/1158 and 09/1385) and CIBER de Enfermedades Raras (CIBERER, an initiative of ISCIII).

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Cite this article as: Catalán-García M, *et al.* (2015) BACE-1, PS-1 and sAPPβ levels are increased in plasma from sporadic inclusion body myositis patients: surrogate biomarkers among inflammatory myopathies. *Mol. Med.* 21:817–23.

5. GENERAL DISCUSSION

The understanding of mechanisms underlying pathological processes in diseases is key for the development of new diagnostic/prognostic biomarkers and novel therapeutical approaches, either pharmacological or non-pharmacological. This is especially challenging in the field of rare diseases, where information and research is even scarcer than in other more prevalent diseases. In a rare disease scenario, the patient usually counts with fewer resources to deal with the disease, and frequently therapeutical strategies the patient could follow after the diagnosis are quite limited. Curing or attenuating the disease symptoms in these cases are often out of our possibilities, and many times clinical community do not have a solid plan for treating and diagnosing such rare diseases.

This is the case of sIBM, and although many therapeutic approaches have been tested and administrated to sIBM patients through the years, there is no evidence of any of them to be useful for the treatment of sIBM symptoms or, even more ambitious, curing the disease. The mechanism that triggers sIBM pathological processes needs to be elucidated to develop a better diagnostic and therapeutic algorithm. Ideally, early diagnostic and therapeutic strategies should be established to be able to stop the disease in its early stages because preventing the disease is easier than curing it. Until then, every step in the research of sIBM pathogenesis is crucial for scientific community to generate a more exhaustive overview of all the elements that participate in this disease, and that could be directly or indirectly determine its aetiology.

In the present work, we aimed to elucidate how mitochondrion is participating in the sIBM pathogenesis and also understand how deregulated plasmatic molecules related to inflammation, mitochondrial abnormalities or amyloidogenesis could be implicated in disease aetiology and used as accessory diagnostic/prognostic biomarkers.

One of the main complaints in the study of sIBM is that, even from the first sIBM case ever reported (4) until now, despite diagnostic criteria and clinical manifestations have been more studied and accurately described, there are still inherent difficulties in the diagnosis of the disease, especially in its early stages.

One of the main difficulties is that being a rare disease limits the number of patients susceptible of study. For this reason, the possibility to have access to a cohort of patients like the one we have herein studied has infinite value.

The data provided in this thesis is presented in two parts. The first is related to the characterization of the abnormal mitochondrial fingerprint in sIBM patients and the second, a plasmatic screening in search for inflammatory, mitochondrial or amyloidogenic biomarkers. Both studies conclude with interesting results which hopefully will extend the knowledge on sIBM.

Evidence of mitochondrial abnormalities in sIBM have been demonstrated by many authors at histological level, but only some authors have studied the mitochondrial lesion in sIBM at molecular level, and there is only little information about mitochondrial dysfunction in the disease (39). In the first part of this thesis, we conducted an exhaustive analysis of the mitochondria at genetic, structural, functional and dynamic level in a cohort of sIBM patients. Most of the parameters have been parallelally measured in the target tissue of the

disease (muscle) and in peripheral and less invasive tissues (as peripheral blood mononuclear cells, PBMCs) in order to, first, determine specific molecular mitochondrial impairment in the disease and, second, its potential extension to other tissues that allow the establishment of diagnostic or prognostic tools for longitudinal follow-up.

Both tissues showed altered mitochondrial profile with common molecular alterations (including CIV/CS activity) but also specific tissue-depending fingerprint (as mitochondrial dynamics in muscle or mitochondrial protein expression in PBMC).

General mitochondrial overview in muscle tissue of sIBM patients can be summarized with the following altered parameters regarding mitochondrial genetics, mitochondrial function and mitochondrial dynamics: 57% of mtDNA deletions, 36% mtDNA depletion, a 30.31 % decreased CIV activity normalized to mitochondrial mass and a 37% reduction of mitochondrial fusion protein OPA1 transcript. These findings definitively demonstrate mitochondrial impairment, this time from a molecular point of view, in sIBM patients. We additionally propose, for the first time, a mechanistic explanation for this impairment. Mitochondrial dynamics through OPA1 transcript levels have been found to be impaired, but also OPA1 and MFN2 protein content were decreased in muscle from sIBM, although not significantly. These findings indicate an unbalanced fusion and fission in mitochondria and, as a consequence of this impairment, mitochondria's renewal may be affected and genetic errors may be accumulated due to lack of mitochondrial exchange of genetic material and abnormal mitochondrial damaged clearance. The optimal interaction between mitochondria in terms of fusion and fission helps to concentrate the genetic and protein alterations in a small group of mitochondria that would be destroyed through mitophagy. If this process is not occurring, mitochondria will accumulate defects, and that is how we could explain the mtDNA alterations (either deletions or depletion) that present mitochondria from sIBM patients. Muscle is one of the most energetic demanding tissues of the organism. Consequently, mitochondrial dynamics and proper renewal are essential processes to maintain the standard physiology of muscle (103-105) that may take highlighted relevance in disease. Interestingly, mtDNA multiple deletions are also a well-known phenomenon occurring in sIBM patients (34-37), and it has not only been confirmed in our population, but also in correlation with OPA1 levels, being the sIBM patients with mtDNA deletions the cohort with worst mitochondrial dynamics state. This strengthens the hypothesis that alterations of mitochondrial genome could be caused by a poor mitochondrial dynamics. Downstream, this mtDNA instability could be key for the development of other mitochondrial alterations, including the functional impairment, present in sIBM patients.

Mitochondrial DNA codifies for up to 13 proteins, and some of them belong to subunits of MRC complex IV. One of the consequences of this depletion and deletions, regarding our results, could be the decreased activity of CIV that was observed in sIBM patients. In addition, the unbalanced mitochondrial dynamics could also contribute to the CIV malfunction, as spontaneous defects that could occur in the MRC will accumulate and they will not be eliminated properly. Complex IV dysfunction was first detected, in sIBM patients, at histological level, as COX- fibbers. Muscle cells, in order to compensate this CIV malfunction, seem to raise the number of mitochondria (measured as citrate synthase content), although not significantly. Increased mitochondrial biogenesis was first detected

by the presence of RRF in the histological staining. This mitochondrial mass increase may explain why trends to decreased CIV activity become significant when normalizing to CS activity. With the unbalance of mitochondrial dynamics that may probably lead to impaired mitochondrial genetics and further mitochondrial dysfunction, cells may be facing a vicious circle of lesion that could explain the disarrangements that have been observed in histologically in the tissue from sIBM patients. However, this is just a mechanistic surmise and further investigations in this direction should be performed to definitively demonstrate the real causality of this process. In any case, mitochondrial dynamics unbalance and genetic and functional problems seem to go hand in hand in sIBM development and confirm the most relevant histological features previously described in sIBM patients.

The mitochondrial profile in PBMC from sIBM patients was slightly different from that of muscle, but it also reveals mitochondrial disarrangements. As a common feature between muscle and PBMC, we found the same alteration in CIV activity, this time both in absolute and relative values, showing, for the first time, that sIBM may be characterized by a systemic compromise clinically manifested in muscle tissue. However, differential features are observed between muscle and PBMC. In PBMCs, mtDNA levels would not present the depletion observed in muscle. Our explanation for that differences would be based on the own nature of both tissues. PBMC, unlike muscle, have a greater capacity for renewal due to its shorter mean life expectancy. These cells are created and destroyed with a higher ratio than muscle cells, which could remain with the organism for its whole life aging with the organism. For this, mitochondrial errors, despite being also present in PBMC (and probably in other peripheral tissues), would preferentially accumulate in post mitotic tissues as muscle, where they may represent relevant molecular alterations leading to clinical manifestations.

PBMCs, interestingly, would also develop mechanisms to try to compensate the molecular disarrangements. Muscle cells may increase mitochondrial biogenesis to compensate mitochondrial dysfunction. PBMCs may specifically deregulate the synthesis of mitochondrial proteins to reach the same aim. Concretely they seemed to increase the nuclear-encoded *COXIV* subunit*,* respect the mitochondrial-encoded COXII. The mechanistic explanation for this homeostatic intent would be that, as PBMCs mitochondria would be altered and CIV function would not be working properly, nuclear genome will try to generate more CIV subunits in front of a mitochondrial genome unable to produce proteins at the same rhythm, leading to an unbalanced expression of mitochondrial vs. nuclear proteins related to CIV structure.

However, we cannot ignore that in sIBM an inflammatory process is occurring, and muscle, but especially PBMC, could be influenced by this chronic inflammatory process. The reason why PBMC are altered in sIBM needs further investigation, but we think these results demonstrate that mitochondrial alteration in those patients is not confined to the muscle tissue, but also extends to, at least , PBMC.

The alteration profile in both tissues, muscle and PBMC, reveals first, that sIBM is without any doubt a multi-factorial disease, but mitochondrial impairment is likely to be an important factor in sIBM development, and second, the fact that a systemic mild mitochondrial alteration might be occurring in sIBM, but muscle seems to be the most

affected tissue of the disease, for some reason, probably due to its dependence on energy supply and post mitotic nature that makes muscle prone to aging and defect storage.

Finally, these findings also provide evidence for the potential usage of this and other peripheral tissues as models of the disease and diagnostic or prognostic biomarkers to follow-up patients in a less invasive way than performing the usual muscle biopsy.

Our investigations in the search for non invasive ways to monitorize sIBM did not end here. Recently, the relationship of sIBM with AD and amyloidogenesis has become more and more evident. *Askanas et al.* have developed a strong investigation in this field, supported by a large number of publications that deepen in the β-amyloid accumulation in sIBM disease and its processing and consequences (94, 95, 106, 107). On the other hand, the absence of a solid diagnostic biomarker in sIBM is something that difficult its diagnosis, one of the main problems with the disease. In this sense we aimed to evaluate different molecules related to inflammation, to mitochondria and, specially, to amyloidogenesis in the plasma from sIBM patients compared to controls and also compared to a cohort of patients with other inflammatory myositis (with DM and PM), to try to find altered and specific unbalance of plasmatic levels of these molecules that may be used as non-invasive, sensible and specific diagnostic or prognostic tools.

The inflammatory and mitochondrial related molecules that were analyzed in plasma of our cohort of sIBM patients (IL-6, TNF-α, circulating immunogenic mtDNA, FGF-21 and CoQ) showed non-significant differences between cases and controls. However, amyloidogenic molecules in plasma from sIBM patients showed more interesting tendencies. In physiological conditions, APP can be processed by two pathways, the non-amyloidogenic and the amyloidogenic one. BACE-1, PS-1 and sAPPβ are involved in specific steps from the amyloidogenic processing of the APP, and its activity favour the formation of Aβ deposits. In non-pathologic conditions, the amyloidogenic pathway is so diminished that there is no relevant formation of Aβ deposits. In diseases like AD or sIBM, the amyloidogenic processing of APP seems to be increased over the non-amyloidogenic processing of APP, executed by other enzymes (like α-secretase). Due to the increase in Aβ formation and also to errors in the clearance of the Aβ, the oligomerization and subsequent accumulation of the Aβ occurs. We found that plasmatic levels of this three molecules involved in Aβ formation (BACE-1, PS-1 and sAPPβ) were increased in plasma from sIBM patients respect to controls, but also respect to a group of DM and PM patients, other inflammatory myopathies distinct from sIBM. Our hypothesis is that the increase of these molecules in sIBM would increase the amyloidogenic processing of APP that would lead to an increased Aβ production in this disease. This process, prolonged in time and aggravated by an alleged imbalance in the UPR, would lead to protein accumulation and finally to the apparition of the rimmed vacuoles typical from sIBM.

BACE-1 was previously found increased in muscle from sIBM patients (94), at transcript level and, based in our findings, this increase seems to be also reflected in the plasmatic content of this molecule. However, we do not only found an increase of BACE-1, but also an increase of PS-1, another enzyme involved in the same reaction. In addition, over expression of sAPPβ, the resulting fragment of APP processed by BACE-1 reinforces our theory that the rest of enzymes involved in the amyloidogenic processing of APP are increased in sIBM

patients. Why these molecules are present in plasma is something that has to be further studied, but we speculate that, due to muscle degeneration, muscle tissue is being slowly destroyed and muscle cell content could be released to plasma, increasing the levels of these molecules in plasma of affected patients.

The clinical onset of sIBM is very similar to the onset of other inflammatory myopathies and, in absence of specific biomarkers of sIBM, years of evolution are needed to diagnose the disease with solid evidences. Thus, for putative use in clinical settings, the potential utility of any biomarker should be tested in other inflammatory myopathies like DM and PM, to asses sIBM specificity. Our results indicated that the levels of the amyloidogenic molecules in DM and PM were very similar to healthy controls, demonstrating that the amyloidogenic process is only triggered in sIBM and not in other inflammatory myopathies. On the basis of such results we can conclude that the increased expression of amyloidogenic molecules in plasma is specific of sIBM and, thus, we may consider these molecules as useful potential biomarkers for discriminating between sIBM and other inflammatory myopathies.

Among the three amyloidogenic molecules tested and differentially expressed in sIBM (BACE-1, PS-1 and sAPPβ), the quantification of BACE-1 levels is the best parameter for discriminating between sIBM and the other cohort groups, since the sensitivity and specificity values reflected that we did not obtain a better diagnostic precision when adding PS-1 and sAPPβ biomarkers into the diagnostic algorithm. However, since all three molecules showed significant differences between groups, we can also conclude that all of them are involved in the disease aetiology.

One of our major concerns is that sIBM seems to begin with the inflammatory process in the early steps of the disease, while the degenerative process seems to appear in later stages (108-110). This fact is crucial, since all the patients that were studied presented moderate to severe sIBM progression Thus, we cannot ensure that these biomarkers will also be altered in early stages of the disease. Next step should be the screening of these molecules in a cohort of patients presenting potential signs of sIBM at prodromic stage, and then follow-up this cohort to explore how many of them ended with a solid diagnose of sIBM. With this longitudinal study, we could elucidate if these proposed biomarkers would be specific of late stages or if they are also present in earlier stages of the disease, which would increase its value for diagnostic purposes. Unfortunately, the longitudinal study entails a strong difficulty in the identification of the study cohort, first because sIBM is very rare and infrequent and, second, because its development is very progressive and patients use to wait until the signs of muscle atrophy are evident to visit the doctor, and normally when this happens the disease stage is clearly advanced.

Another limitation of these biomarkers is that the increase of these molecules is specific in sIBM with respect to other inflammatory myopathies but they can also be altered in other diseases coursing with β-amyloid accumulation, like AD. In patients with AD, sIBM may be suspected on the base of altered BACE-1, PS-1 and sAPPβ levels, because these biomarkers. Despite there are no studies of sIBM and AD co-morbidity with a large cohort of patients it is foreseeable that, an AD patient in a late stage of the disease, may present sIBM symptoms. However muscle tissue of AD-patients has never been studied, because probably we assign its limited movement is due to the neurologic degeneration. Studies in AD patients in search

for sIBM symptoms, as well as search of symptoms of AD in sIBM patients should be performed to understand the real connection between these two entities, and if the triggering factor is common, or we only appreciate pathologic features in common, as a consequence of different mechanistic processes. In case of common mechanistic link between both pathologies, it would be interesting to understand why clinical and molecular disarrangements are ascribed to distinct tissues, both of postmitotic nature and highly energy dependent.

sIBM is a rare disease with no effective treatment. Although only a reduced part of the population will suffer it, their quality of life will be drastically reduced. For this reason, new information about the pathological features of the disease is of high relevance and essential to provide supportive assistance for affected patients. The understanding of altered mechanisms of diseases and the detection of which parameters could be altered are the basis for a proper development of new diagnostic tools, potential therapies and patient guides. With the present work we deeply explore mitochondria in sIBM, highlighting which aspects of mitochondrial genetics, structure, function and dynamics are altered in this disease. As mitochondria is key for muscle energy supply, and also exerts several other important functions including the regulation of cell death, this organelle is without any doubt playing a role in sIBM development, either primary or secondary.

In addition, the relationship between sIBM and AD has been gaining strength in the last years and the present thesis supports this hypothesis. Both diseases seem to have common pathological processes in their development, especially in protein accumulation, but in different tissues, muscle and brain respectively. To also explore amyloidogenic feature in sIBM, and considering that early diagnosis is one of the major problems of sIBM, we aimed to describe altered plasmatic molecules that could help us both to understand pathological processes but also the diagnosis of the disease. The substitution of muscle biopsy for one of the proposed biomarkers is not feasible at the moment, until further validation is developed, but the analysis of these molecules could provide additional and valuable information to avoid performing additional muscle biopsies, with the relevant benefit it may provide to the patient.

Mitochondrial impairment is involved and detectable in sIBM etiopathogenesis not only at histological level, but also at molecular level, in the target tissue of the disease, as well as in more peripheral tissues. The screening of plasmatic inflammatory, mitochondrial and amyloidogenic biomarkers of the disease may help in the achievement of less invasive diagnosis, prognosis or follow-up.

With this thesis, we were willing to make a step forward in the understanding of sIBM widening the scope of knowledge available for the scientific and clinical community but we would specially expect to benefit sIBM patients in the future. In addition, our laboratory has developed a new research area that, taking advantage of the expertise of the clinical staff of our research unit in inflammatory myopathies, will not end with this thesis and will continue unveiling pathogenic features of this and other rare diseases with muscular affection to be nearer of the awaited effective therapy, in this case, for sIBM patients.

6. CONCLUSIONS

- Mitochondrial molecular profile in muscle from sIBM patients is altered in accordance with the mitochondrial histological features previously described. These abnormalities consisted in mtDNA disturbances, decreased complex IV/citrate synthase activity and mitochondrial dynamics deregulation.

- In addition, the presence of mtDNA deletions in muscle from these patients correlates with a more severe impairment in mitochondrial dynamics and with lower mtDNA content, demonstrating that proper mitochondrial dynamics is essential for mitochondrial homoeostasis and muscle function in these patients.

- Mitochondrial imbalance is not only confined to the target tissue of the disease but it is also present in PBMC. Such mitochondrial alterations showed common (complex IV dysfunction/citrate synthase) and differential (COXII/COXIV protein synthesis) abnormal pattern compared to muscle and constitute a less invasive approach.

- These results reinforce the theory that mitochondrial imbalance in sIBM is systemic, but probably additional abnormal processes occurring in muscle (such as inflammation and degeneration) and its highly energy dependant nature are crucial for its selective involvement.

- Amyloidogenic molecules are increased in plasma from sIBM patients (BACE-1, PS-1 and sAPPβ), and trends towards deregulation are observed in the mitochondrial and inflammatory markers tested, confirming their etiopathological implication in the disease.

- Sensitivity and specificity analysis show that PS-1, sAPPβ and mostly BACE-1, represent a good predictive non-invasive tool for the diagnosis of sIBM, especially in distinguishing this disease from other inflammatory myopathy (DM&PM).

- The abnormal molecular phenotype of sIBM patients not only contribute to clarify the pathogenesis of the disease, but may also help in the development of new diagnostic tools or therapeutic targets for this disease.

7. REFERENCES

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8. AKNOWLEDGEMENTS

Abans que res, voldria agraïr als membres que formen part del tribunal d'aquesta tesi pel seu temps i la seva dedicació, i especialment al Dr. **Josep Maria Grau**, co-director d'aquesta tesi. Ha estat per mi una mà amiga en tot moment i en definitiva, un exemple del que algú espera d'un tutor de tesi, i molt més. Totes les qualitats professionals, científiques i humanes que convergeixen sempre en unes paraules amables i uns consells de valor incalculable. Gràcies per donar-me la oportunitat d'aprendre treballant al teu costat, i per la direcció d'aquesta tesi, que ha estat fluïda i fàcil crec que per ambdues bandes. No vull doncs desaprofitar aquesta oportunitat per posar de manifest la profunda admiració que sento per vostè. Gràcies Josep Maria.

Qui hauria dit que el dia que vaig travessar la porta d'aquell petit laboratori, tan col·lapsat de material per tot arreu i sense espai pràcticament per treballar, la meva vida canviaria per complert. I es que aquell 302 estava també col·lapsat de criatures increïbles. Han sigut 5 anys i durant aquest temps ha passat moltíssima gent pel laboratori, ja sigui pel 302 o després pel Cellex 4B, però intentaré no deixar-me a ningú.

First of all, I want to thank all the people from the University College of London, but especially to **Mhoriam** and **Charlotte** for helping me and being wonderful people. Also I want to thank **Linda, Bernadett, Emma, Ben** and all the other people! You all make me feel like home, and this is very important when living abroad.

Agraïr per començar a tots els estudiants de Màster i Grau que han anat passant pel grup, i que han omplert el laboratori amb la seva energia. A la **Jennifer** pel seu humor peculiar, al **Sergio** pel seu caos ordenat i passió per la ciència, al **Vicente** per ser una persona que reuneix moltíssimes qualitats tant a nivell professional com personal (y por escuchar mis teorias sobre GoT!), a l'**Adriana** per ser natural, divertida i tranquil·la i per fer-me passar molt bones estones, i a L'**Àngela**, que ha hagut de sofrir, igual que l'**Adriana**, l'odissea fer unes pràctiques sota la meva tutela**. Àngela**, gràcies per la teva paciència, la teva entrega i el teu sentit de l'humor. Arribaràs allà on vulguis. Al **Àlex mito-vàscul**, el teu nom ho diu tot, tots ens barallem per tu. Segueix sent com ets perquè es l'únic que necessites.

De tots els estudiants de màster m'agradaria destacar-ne a dos, i són el **Francesc** i la **Sarai**, que van entrar amb mi a fer el màster al laboratori. Companys de primeres batalles, em fa molt feliç veure com ambdós heu tirat endavant la carrera investigadora que de forma tímida i discreta vam començar junts. I de quina manera. **Sarai**, la teva personalitat trenca totes les barreres que el món et pugui posar. Ets una tia increïble que aconseguirà sempre el que vol, i que a més ho faràs envoltada de gent espectacular com tu, perquè és el teu poder. Gràcies per haver-me deixat formar part d'aquesta gran aventura que ha sigut Londres. Vols saber com et veuen els meus ulls? Com una estrella del rock. **Francesc**, admiro de tu la teva senzillesa i practicitat, el teu humor àcid i que per molt que facis la teva vida a Girona, tinc la sensació que sempre estàs a prop, apunt per apuntar-te a tots els plans.

Merche, abans que res vull agrair-te la teva dedicació quan es tracta de preparar coses i activitats perquè el nostre laboratori segueixi sent-ho. Però es que a més a més he descobert en tu una persona increïble. Gràcies per ser una apassionada de l'art en totes les seves disciplines, perquè amb tu sempre es pot tenir una conversa interessant, perquè barreges els zasques més divertits amb una sensibilitat que no en sap de barreres i per tot el

temps que inverteixes en els que t'envolten. La família 302 és com és per culpa de gent com tu. **Siscu,** darrere de totes les bromes i aquest posat de "paso de tot" que et representa, s'hi troba una persona sensible i dedicada als seus amics. A tu t'he d'agrair el teu bon humor, els teus consells, els teus "tours" i sobretot els piropos que em fots. A més de tot això, tinc la sensació que ets una d'aquelles persones que esta a un truc de distancia i que sempre estarà disposat a dedicar-me una estona.

Del grup Predimed, a la **Rosa** per la certesa de saber que sempre he necessitat alguna cosa, has estat disposada a ajudar-me. A les infermeres que han anat passant pel grup: la **Laia,** l'**Anna** i sobretot a la **Paula**, amb les seves rialles i la seva perenne actitud amable i positiva. A l'**Àlex,** per la teva transparència i per la forma desenfadada que tens de veure la vida. A la **Mireira Urpí** i a la **Sara**. A la **Gemma Sasot** per la seva actitud divertida, pels seus comentaris picants, per les seves rialles i sobretot perquè darrere de tot això hi ha una grandíssima personeta amb la que sempre pots comptar, i que s'ha anat tornat més i més important a la meva vida. I per ser una fantàstica amiga invisible! A la **Palmi** por ser esa persona tan especial, sumamente observadora y atenta, que además siempre se muestra tal y como es, y eso nos encanta. De ti me quedo con tus momentos divertidos, con la pasión y dedicación que profesas y con esa capacidad luchadora que he visto en ti, siempre dispuesta a sonreir y seguir batallando aun cuando la situación no es favorable. A la **Gemma Chiva** perquè he trobat en tu una persona molt afí a mí, amb qui puc compartir apart de moltes bromes i tonteries, moltíssimes aficions. Gràcies per tots els concerts, per no deixar-me marxar mai, per una birra de cirera més, per convidar-me al teu país de pas i per les balenes i les aurores. Gràcies per la teva passió i per la forma com mires al món. És una de les coses que més admiro de tu, i es que la passió per les coses es quelcom que cada cop escasseja més. També vull destacar dues qualitats imprescindibles en una amistat, la confiança i la sinceritat. A tu te'n sobren de les dues. A més, vull que quedi per escrit: t'admiro.

El vascul-team es un petit oasis de gent increïble enmig d'un gran desert de persones que van i venen. **Roser**, intercanviant les dues primeres paraules amb tu ja et fas una idea de quin tipus de persona tens al davant. Natural, senzilla, agradable i a la vegada extremadament intel·ligent, sense por a dir la teva opinió quan saps que pots aportar llum a les coses. Amb el poc temps que fa que ens coneixem ja sé que vals moltíssim la pena. Ets el tipus de gent que mola tenir al costat. **Nekane,** la nostra relació va ser bastant neutre al principi, però poc a poc vam començar a teixir connexions amb coses que ens eren afins als dos fins al punt que t'has tornat imprescindible per a mi. La teva part extremadament detallista contrasta amb els teus moments de caos i entropía, creant una barreja que et fa sumament especial. De tu admiro la teva capacitat crítica envers el món, i l'habilitat multi-tasking que et caracteritza. Gràcies pels "corre cinc minuts", per fer els millors regals d'AI, per ser sempre una orella que et pot escoltar i per qüestionar-t'ho tot. Tu ets, com el teu nom, única. **Ester,** no sé ni per on començar. Ha sigut una gran sort haver-me creuat amb algu com tu en aquesta vida. D'entre les moltissímes qualitats que professes sense adonarte'n, i que donarien per una altra tesi, a mi n'hi ha una que m'agrada en particular, i es que fas que la gent del teu voltant se senti especial. Admiro de tu la teva naturalitat i el teu esperit, màgic i únic en el mon. A tu t'he d'agrair la teva confiança, la teva alegria natural i espontània i que t'hagis volgut involucrar en una de les meves bogeries i ho hagis fet tan fàcil. Gràcies pels "vomito" i els "no puc", perquè a vegades no ens cal ni una paraula per saber exactament que estem pensant i per totes les teves excentricitats, però sobretot

per les petites coses del dia a dia, les quals sento infinitament afortunat d'haver pogut compartir amb tu. Sé que el "no canviïs mai" es molt típic, però no canviïs mai Ester, ja que el món seria una miqueta pitjor. **Marc,** ja vaig tenir oportunitat d'escriure't uns agraïments per la teva tesi, però mai esta de més tornar-ho a fer i menys tenint en compte la magnitud de la situació. Deixant de banda les moltíssimes coses que tenim en comú i que sens dubte han propiciat que la nostra relació sigui més especial, més enllà de tot això trobo en tu un esser humà reservat però amb infinites capes que sempre vols seguir destapant i descobrint. Ets tot un món, millor dit, tot un univers confinat en un cos d'esser humà. Quin millor resum que gràcies per tots els nostres moments, que en són moltíssims i per tenir sempre els braços oberts disposats a encaixar-ne uns altres. I que per molt de temps puguin ser els meus.

Mito-team! Les meves Mito-girls!!! **Ester Tobi**, gràcies per haver-me ensenyat tantíssimes coses a nivell laboral però també personal. Perquè amb tu es pot parlar de qualsevol cosa i sempre estàs disposada a donar un cop de mà. Per la teva actitud lluitadora i la capacitat de saber sobreposar-te a l'adversitat amb un gran somriure. I perquè encara que diguis que no, ets una mitogirl de los pies a la cabeza! A **Diana,** por todas tus palabras mejicanas que me hacen partir de risa, por tu imborrable sonrisa que siempre, siempre puedes encontrar aunque no la busques, por tu actitud alegre y desenfadada, por dejarme abusar de la confianza y hacerte consultas médicas y en definitiva por ser una compañera genial que no todos tienen la suerte de tener. **Ingrid,** quina grandíssima sort tenir-te com a companya de feina. Mica en mica he anat descobrint en tu diferents capes, i cada cop que conec una part de tu que no coneixia, me n'adono de la increïble persona que tinc al costat. Tens una actitud envejable per afrontar les adversitats, sempre positiva però alhora racional. Tens un gust exquisit per la música, i una forma especial d'escoltar-la. I tot i que a vegades els cartellets i detallets et semblen un gra massa, no pots amagar que ets una persona dedicada 100% a la gent que t'envolta, i fas el que calgui perquè tothom estigui a gust. Ets, el que en el meu univers es considera un 10. **Mariona,** per mi ets la companya infatigable de mil batalles. Amb tu tinc la sensació que sempre estaràs allà, per si necessito algo. Gràcies per tantes i tantes coses, algunes banals com l'aquari de pops bebés o altres importants com el tros d'amiga que m'emporto d'aquesta etapa. Gràcies per preguntar-me coses sobre Batman, per un brindis amb Blue Moon al Howl at the Moon o per demostrar-me que ets igual de genial com a companya de feina, de viatge o com a amiga. Gràcies per posar seny al caos i raó al deliri. Gràcies per ser sempre una orella que escolta les meves preocupacions, una boca que riu les meves tonteries, unes mans que SEMPRE estan disposades a ajudar-me i en definitiva, gràcies per ser la Mariona. Perdut en algun lloc, en una altre realitat paral·lela, hi ha un Marc que no et coneix i és una mica menys feliç.

Si he de parlar d'agraïments d'aquest doctorat, aquestes pàgines es poden resumir en una sola frase: **Gloria**, **Cons**, gràcies per CREURE EN MI. Gràcies per donar-me la oportunitat de treballar i aprendre al vostre costat. Encara no sé què us devia passar pel cap, però en aquell moment li vau donar sentit a tot. Si penso en la infinitat de coses bones que m'han passat a arrel de començar el meu camí amb vosaltres, me n'adono que no tinc dies ni anys per agrair-vos-ho tot.

Cons, si t'hagués de definir d'alguna manera, ho tinc clarissim: mai he conegut ni mai coneixeré algú com tu. La teva bondat infinita i el teu altruisme m'ha deixat més i més perplex cada dia que passava al teu costat. Gràcies per tenir sempre una paraula amable,

per no voler mai generar conflictes, per entregar-te i desviure't pels demés i per ser una companya de feina i amiga de valor incalculable. La innocència es una qualitat que m'agrada molt en les persones, i a tu te'n sobra pels quatre costats. Gràcies per les teves bromes lingüístiques, i per estar sempre de bon humor. Per la teva sensibilitat i pel teu "good mood". Ets una d'aquelles persones que tothom vol tenir a prop. Jo també. **Glòria,** simplement gràcies. Si has sigut peça clau i fonamental d'aquest doctorat a nivell professional, qualsevol paraula se'm queda curta per descriure el que has suposat per mi a nivell personal. N'estic segur que el Marc que va començar a fer el doctorat i el Marc que l'abandona es molt diferent, i si es millor es en gran part gràcies a tu. Gràcies per la teva entrega als altres i la teva generositat. Gràcies per dedicar sempre temps que no tens als conflictes i preocupacions alienes. Gràcies per enaltir les meves virtuts i obviar els meus moltíssims defectes, i perquè amb tu, la paraula bondat pren un nou significat. Gràcies perquè, amb el teu exemple i sense adonar-te'n, m'has descobert una manera diferent d'enfocar la vida. Gràcies per ser jefa (per molt que no t'agradi!), companya i amiga i compaginar-les de forma magistral. Gràcies perquè a més de tot això, que no es poc, ens hem divertit moltíssim, i per procurar sempre que el bon ambient domini sobre la resta. Gràcies per tots els bons moments de congrés, i per les nostres reunions discutint resultats. Tot i que a vegades tenim punts de vista i maneres de fer diferents, sempre hem coincidit en el que realment es important, i el millor resum es que sempre he cregut que fèiem un gran equip. Gràcies Glòria.

Per últim volia agraïr al nostre cap de grup, el Dr. **Francesc Cardellach**, per reunir tantíssimes qualitats tan professionals com humanes, i per ser sempre una referència per a mi. Gràcies per la teva practicitat i per dir les coses sempre tal i com son sense perdre mai el tracte amable.

Fora del laboratori també hi ha hagut molta gent que m'ha ajudat, potser sense adonar-se'n, a seguir lluitant en aquesta etapa. Enumerar a tothom és impossible, però intentaré citar almenys a alguns de vosaltres.

Voldria agrair especialment al "Consejo de sabios", la increïble gent que vaig conèixer durant la carrera i amb la que hi segueixo tenint molt bona relació, cosa que per mi es molt preuat: **Marc,** m'has demostrat que tot i el que tots dos ja sabem (jaja!) ets un amic de veritat, algú en qui puc confiar i que sempre hi serà per si necessito alguna cosa. Gràcies per escoltar-me sempre i per ser el millor company batalles (màgiques). **Marcos,** quizá nuestra relación ha sido mas intensa en los últimos años que en los años de carrera, pero la verdad es que para mi ya eres imprescindible. Gracias por nuestras coñas, por la paciencia y en definitva por ser un colega de los buenos. **Samir,** estic molt feliç de tenir-te com amic, i t'agraeixo que sempre estiguis pendent de la gent que t'envolta. A tu, un amant de quantificar, vull que sàpigues que la teva amistat per mi es inquantificable. **Juanmi,** gracias por ser el Juanmi. Ese tio que es distinto a cualquier otra persona que puedas conocer. Gracias porque conversar contigo es siempre estimulante, porque me encanta nuestro pique marvel-DC, y porque contigo puedo hablar de cosas con las que con muy poca gente puedo. No cambies nunca, aunque estoy seguro que no tenías pensado hacerlo. **Chefo,** gracias por tantos metalzones, conciertos, ensayos y muchísimas cosas mas que hemos hecho juntos. Por ser un tio que siempre esta dispuesto a dar todo lo que tiene por sus colegas. Y por traerme las cosas a casa. **Jofre,** ets un tio de puta mare. Gràcies per les

aficions que compartim, pel teu sentit de l'humor i sobretot, per la nostra nit especial fa uns anys a la Salamandra :P. Gracias al resto del consejo también, **Pablo, Chus, Javi, Sergio, Tomás**, por leernos y tenernos al dia, y ser en el fondo un buen lugar donde ir a quejarse de vez en cuando o a reir de la misma forma.

Gracias a mis antiguos compañeros de banda. **Yael,** han sido muchísimos los ensayos, conciertos, festivales, tallers, cenas, idas y venidas con "La Bestia", tantos que son incontables. Muchas gracias por esos años, que fueron de los mejores de mi vida. Juntos teníamos un sueño, y a base de mucho trabajo, hicimos algo maravilloso. Brindo por ello. **Samu,** nuestro encuentro fue casual, pero todo lo que vino después estuvo muy lejos de serlo. Gracias por hacerme siempre partir el culo, por tu dedicación en la banda, por las maratones de cine gore en tu casa y por estar ahí siempre. Joder, lo hemos pasado de puta madre tio. Devouring Doom siempre significara mucho para mi, y no por el grupo, sino por vosotros y nuestra amistad, y por los años dorados.

Álex, entraste en la banda cuando esta ya anunciaba signos de marchitarse, pero ese tiempo fue crucial para convertirnos en lo que somos ahora. Fue una época de aprendizaje y crecimiento continuo, pero lo más importante es que te conservo como amigo, y es lo que me hace mas feliz. Siento que siempre vamos a estar conectados por nuestra pasion con la música. Alex, eres único, gracias por ser mi amigo.

Fa una miqueta més de tres anys vaig aventurar-me en un projecte que m'ha portat moltes coses bones, les millors de les quals son les que venen a continuació: **Arnau**, gràcies per la teva entrega i perquè ho fas tot fàcil. Perquè més que un cop de mà, ens estàs regalant quelcom molt més valuós, el teu temps i la teva dedicació. **Martí,** gràcies per la teva alegria perenne i pel bon rotllo que desprens. Ha sigut una incorporació molt clau, tant al grup com a la meva vida. **Joan,** collons m'encantes tio. Sento que ens entenem moltíssim a tots els nivells i m'agrada tocar al costat d'un tio tan de puta mare com tu. Ets de lo milloret que un es pot trobar. **Xavi,** quina sort que tinc d'haver-te conegut. M'encanta la capacitat que tens de fer sentir a tothom a gust i encara que no te'n recordis de les notes, ja saps que t'estimo molt i a més m'agrada que sigui així! Ets un tio únic! **Ferran,** deixarem de banda el rollo que et portes quan vas taja, ets un tio fácil, agradable, optimista i a més, sempre ens fotem uns riures amb qualsevol parida. A més, se que dins teu hi ha una persona amb la que sempre hi pots comptar. I òbviament, fer unes partidetes a algun joc!

Per últim vull agrair aquesta tesi a la meva família. Als meus avis, tiets, cosins. Tinc una família molt nombrosa però tots ells saben que han sigut un gran suport per a mi.

Quería dedicar especialmente a **Marta y Josep Lluís** su cariño incondicional desde el primer momento, su sencillez y el haberme tratado siempre de la mejor manera que uno podría esperar. Gràcies, moltes gràcies **Lluís i Aura**, heu estat i sou encara un mirall on mirar-se i un exemple d'on aprendre. Sempre m'heu ajudat moltíssim i heu estat a sobre meu, aconsellant-me si necessitava res i estimant-me i fer-me sentir de la família des del primer moment. De fet, la paraula família pren sentit per mi amb persones com vosaltres. Així mateix, les vostres proeses, l' **Aina** i la **Tanit**, irradien amb la seva llum i energia els dies més grisos. Són un miracle, i em sento afortunat de tenir-les tan a prop.

Finalment, volia agrair especialment al meu cercle més proper. Sense vosaltres res d'això existiria.

Albert, jo que sempre havia estat fill únic i amb 12 anys vas arribar tu i ens vas trastocar a tots! Amb la teva energia, la teva vitalitat. Quan eres petit, els ulls et brillaven amb l'ànsia que tenies per descobrir el món. Tot ho volies tocar, tot ho havies d'explorar. I a mesura que et vas fer gran, ens vas deixar perplexos amb la teva inventiva, la teva imaginació i la teva creativitat. Com a germà gran, reconec que alguna vegada t'he fet alguna que altra putada, però espero que no em tinguis rencor, ja que tu també me n'havies fet alguna, però si vols ens quedem en pau! Sé que com a germà gran, he sigut un mirall on mirar-te, però vull que sàpigues que jo de la mateixa manera, sempre he admirat moltíssimes qualitats que tu tens i que a mi em falten. Espero que no perdis mai la il·lusió que sempre has tingut per descobrir, per créixer i per ser feliç. El que si que et deixo que perdis, són les emprenyades que pillaves de petit jaja! T'estimo Albert.

Al **Ricard** gràcies pel teu optimisme, la teva bondat i per haver pensat en mi sempre com en un fill, treballant incansablement perquè mai em faltés de res. Perquè amb tu sempre he pogut tenir una conversa sobre literatura o qualsevol altre inquietud, quan jo era àvid de coneixement i tu m'il·lustraves amb el teu. Sempre has procurat afegir seny a les situacions i guiar-me si algun cop m'he deixat ajudar, que no és gaire sovint. Moltíssimes gràcies per tot. Sé que sempre puc comptar amb tu pel que calgui.

A la **Dolors**, per ser el contrapunt perfecte per la tempesta que és el meu pare, per cuidar-me sempre i tractar-me com si fos un fill. Per saber redreçar amb maduresa els conflictes que sorgien quan jo era més petit, i que no entenia. Perquè en un principi em vas acceptar com una conseqüència d'estar amb el papa, i vas aprendre ràpidament a estimar-me amb tendresa fins a ser part imprescindible de la meva vida. Gràcies Loli, t'estimo.

Papa, no puc imaginar-me una vida en la que tu no siguis el meu pare. Gràcies a tu sóc qui sóc i com sóc. De tu he agafat la teva bondat, el teu optimisme i la capacitat de forjar grans amistats. Mentiria si digués que també el teu esperit aventurer, ja que aquí, com a tantes altres coses, no t'arribo ni a la sola de la sabata. Lo millor és que per ensenyar-me coses, només has hagut de ser tu mateix. Tot el que has aconseguit és perquè t'ho has treballat tu i fixa't, ni jo mateix puc fer una llista de tota la gent que sé que t'estima i t'aprecia. M'agrada pensar que una de les bones coses que has aconseguit en aquesta vida sóc jo mateix i aquesta tesi es una forma d'agrair-tho. Saps què penso molt sovint? Que quina sort he tingut de que siguis el meu pare. T'estimo fins a l'infinit papa.

Mama, eterna lluitadora. Des de sempre t'has desviscut per donar-me tot el que necessitava, tot el que em fes falta i més. Has anteposat les necessitats dels teus fills a les teves pròpies, i tot i així a vegades has rebut a canvi una mala paraula. Mai, mai a la vida et podré agraïr tot el que has fet per mi, tot el suport que m'has donat i tota la dedicació que has professat per fer-me tirar endavant. Jo encara no tinc fills i no sé com deu ser, però el que si que sé es que mares com tu n'hi ha poques. Així que aquesta tesi, és una petita mostra, tot i que insuficient, per donar-te les gràcies. Aquesta tesi és culpa teva, així que vull que la sentis teva, perquè sense tu, tot això no hagués passat. T'estimo mama. Gràcies per tot, i per tantes coses que no caben en aquesta llista.

Cris, l'escriptura d'aquests agraïments pren tot el seu sentit quan es tracta de tu. Senzillament gràcies, per ser i per estar. Gràcies per la teva alegria, la teva senzillesa i la teva naturalitat. Gràcies perquè amb tu al costat, tot es fa de sobte més fàcil i tinc la sensació que no hi ha obstacle que no es pugui superar. Gràcies per aquests deu anys, que espero que es tornin inquantificables. Trobar-nos va ser una sort, però tot el que va venir a continuació es molt més que això, es viure en un somni, un que hem construït a la nostra manera. Gràcies per estimar-me amb tots els meus defectes i fer-ho amb aquesta naturalitat. Cada any que passa descobreixo una nova part de tu que fa que no em cansi mai de voler viure aventures al teu costat. Gràcies per la forma amb la que mires el món, per la dedicació que professes envers la gent que t'estimes, per tenir sempre ganes de passar-t'ho bé. Per acompanyar-me a concerts i a les pelis de superherois, perquè tot el que et proposo et sembla sempre bé, per la teva capacitat d'adaptar-te i d'estar a gust allà on sigui. Gràcies per aquest univers que hem construït junts, i on s'hi està tant i tant bé. Gràcies per ser tu i jo contra el món. Gràcies per sorprendre'm quan menys m'ho espero i més ho necessito. I gràcies per tot el que ens queda per viure. Cris, que la última cançó no acabi mai.

Per acabar, gràcies a la MÚSICA, per fer-me tenir els peus a terra i l'ànima a l'univers.

9. ANNEXES

J. Cell. Mol. Med. Vol XX, No X, 2016 pp. 1-8

HIV-1 promonocytic and lymphoid cell lines: an *in vitro* model of *in vivo* mitochondrial and apoptotic lesion

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Received: February 3, 2016; Accepted: July 21, 2016

Abstract

To characterize mitochondrial/apoptotic parameters in chronically human immunodeficiency virus (HIV-1)-infected promonocytic and lymphoid cells which could be further used as therapeutic targets to test pro-mitochondrial or anti-apoptotic strategies as *in vitro* cell platforms to deal with HIV-infection. Mitochondrial/apoptotic parameters of U1 promonocytic and ACH2 lymphoid cell lines were compared to those of their uninfected U937 and CEM counterparts. Mitochondrial DNA (mtDNA) was quantified by rt-PCR while mitochondrial complex IV (CIV) function was measured by spectrophotometry. Mitochondrial-nuclear encoded subunits II–IV of cytochrome-c-oxidase (COXII-COXIV), respectively, as well as mitochondrial apoptotic events [voltage-dependent-anion-channel-1(VDAC-1)-content and caspase-9 levels] were quantified by western blot, with mitochondrial mass being assessed by spectrophotometry (citrate synthase) and flow cytometry (mitotracker green assay). Mitochondrial membrane potential (JC1-assay) and advanced apoptotic/necrotic events (AnexinV/propidium iodide) were measured by flow cytometry. Significant mtDNA depletion spanning 57.67% ($P < 0.01$) was found in the U1 promonocytic cells further reflected by a significant 77.43% decrease of mitochondrial CIV activity ($P < 0.01$). These changes were not significant for the ACH2 lymphoid cell line. COXII and COXIV subunits as well as VDAC-1 and caspase-9 content were sharply decreased in both chronic HIV-1-infected promonocytic and lymphoid cell lines (<0.005 in most cases). In addition, U1 and ACH2 cells showed a trend (moderate in case of ACH2), albeit not significant, to lower levels of depolarized mitochondrial membranes. The present *in vitro* lymphoid and especially promonocytic HIV model show marked mitochondrial lesion but apoptotic resistance phenotype that has been only partially demonstrated in patients. This model may provide a platform for the characterization of HIV-chronicity, to test novel therapeutic options or to study HIV reservoirs.

Keywords: apoptosis • cell models • HIV-infection • HIV progression • *in vitro* modelling • mitochondria

Introduction

Data regarding the potential role of mitochondria in the cytopathogenicity of human immunodeficiency virus (HIV) have led to great interest in the study of the relationships between these two entities [1]. Mitochondria have been closely linked to HIV infection, as a target of the deleterious effects of both HIV [2] and antiretroviral therapy (ART) [3] in relation to their involvement in the development of apoptosis. Mitochondrial and apoptotic alterations have been widely described in both naïve and treated patients *in vivo* [4, 5].

Human immunodeficiency virus has tropism for hematopoietic cells [6]. This lentivirus is able to infect either lymphocytes or monocytes and macrophages, but the latter cells do not seem to undergo apoptosis following infection and represent a potential viral reservoir [7]. Mitochondrial and apoptotic lesions in both lymphocytes and monocytes have been associated with accelerated HIV progression in naïve patients [8, 9] and in peripheral tissues from treated patients as a result of secondary effects of medication [10]. A positive correlation

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doi: 10.1111/jcmm.12985

has been described between apoptosis in peripheral blood lymphocytes and monocytes and the severity of HIV infection [11].

Whether mitochondrial and apoptotic malfunctions are causal factors rather than a consequence of differential HIV progression patterns remains to be elucidated. It is still unknown whether adequate mitochondrial function contributes to preventing mitochondrial-driven apoptosis of the defence cells, increasing the organism defences against a specific viral antigen. It would be conceivable that correct mitochondrial function protects defence cells to undergo apoptosis, which, in turn, would slow down the infective capacity of HIV progression.

The interaction between mitochondria, apoptosis and HIV has been well-described in HIV-infected patients [12, 13] and several groups including ours, have assessed the association between mitochondrial and apoptosis alterations and the severity of HIV infection [8, 9, 14]. Nevertheless, there is very little information on adequate mitochondrial characterization of *in vitro* models of HIV infection. Different animal models such as primate models have been used to further explore the disease and/or its treatment [15]. However, high ratios of cost/effectiveness requiring complex facilities have been associated with these models. Cell models could contribute to solve these disadvantages. In this study, we used two different types of cell models which represent the main target cells for HIV (monocytes and lymphocytes). Human immunodeficiency virus-1-infected U1 and ACH2 cell lines are, respectively, promonocytic and lymphoid cell lines derived from uninfected U937 (same U1 cell line but uninfected) and CEM (same ACH2 cell line, but uninfected) precursor cells. U1 and ACH2 lines are characterized by harbouring one and two stable integrated copies of the HIV-1 genome which replicates at a low rate, comparable to slow progression of the infection in patients. In these cell lines, HIV-1 latently auto-replicates itself, constituting a worldwide model to study chronic HIV infection [16, 17]. One previous study has shown apoptotic resistance involving modulation of the apoptotic mitochondrial pathway in persistently infected HIV-1 cells [18], however, accurate characterization of mitochondrial mechanisms and mitochondrial-derived apoptosis has yet to be performed in these models of chronically infected cells.

The hypothesis of this study was that the mitochondrial and apoptotic damage initiated by HIV infection may contribute to the persistence of infection chronicity and progression. As a proof-of-concept, we expected mitochondrial genetics, function, expression and apoptotic levels to be altered in both chronically HIV-1-infected promonocytic and lymphoid cell lines. These parameters were initially determined in the chronically HIV-1-infected promonocytic and lymphoid cell lines U1 and ACH2, being U937 and CEM non-HIV-infected cells used as *in vitro* cellular controls respectively. Mitochondrial and apoptotic involvement in the progression of HIV infection could lead to the use of putative mitochondrial or apoptotic therapeutic strategies to deal with the chronicity of HIV patients.

In depth characterization of the mitochondrial and apoptotic pathways in these cell models may lead to elucidate whether these *in vitro* models resemble *in vivo* alterations observed in patients, providing a platform to test potential targets (such as mitochondrial or apoptotic therapeutic targets) to fight HIV infection.

Materials and methods

Cell lines

Chronically infected HIV-1 promonocytic (U1) and lymphoid (ACH2) are cloned cell lines derived by limiting dilution cloning of U937 or CEM cells surviving an acute infection with HIV-1 (LAV-1 strain) first generated by Folks *et al.* [19, 20].

U1 and ACH2 cell lines, as well as their non-HIV-infected counterparts U937 and CEM, respectively, were cultured at 37°C in a fully humidified atmosphere with 5% CO_2 in RPMI-1640 medium (BioWhittaker, LONZA Portsmouth, NH, USA) supplemented with 10% foetal calf serum, and 1% penicillin-streptomycin, by trained personnel in P3 facility cores devoted to viral replication.

Mitochondrial and apoptotic parameters and experimental setups were simultaneously evaluated in these HIV-1-infected cell lines and their corresponding uninfected controls, and were run in parallel with both infected and control cell lines. Cell cultures were grown into 12 experiments (including four lines in parallel). Experimental measurements were run, at least, in triplicates.

Mitochondrial DNA depletion through multiplex real time PCR

A mitochondrial DNA (mtDNA) depletion study was performed as described. Total DNA was phenol-chlorophorm-extracted, spectrophotometrically quantified and diluted at 5 ng/μl. Multiplex real-time PCR (PCR Applied Biosystems (Foster City, CA, USA) 7500 Real Time PCR System) was performed with 96 round bottom well plates with the simultaneous determination of the mitochondrial 12S ribosomal RNA (mt12SrRNA) gene and the constitutive nuclear RNAseP gene (nRNAseP). The former used mtF805 (5′-CCACGGGAAACAGCAGTGAT-3′) and mtR927 (5′-CTATTGACTTGGGTTAATCGTGTGA-3′) with the TaqMan Probe 6FAM-5′-TGCCAGCCACCGCG-3′-MGB (Sigma-Aldrich, St. Louis, MO, USA). The latter used a commercial kit (4304437; Applied Biosystems). Each well included 25 ng of total genomic DNA diluted in 20 μl total reaction mixture containing: 1× TaqMan Universal PCR Master Mix (ABI P/N 4304437), 1 μl RNAseP commercial kit and 125 nM of each mtDNA primer and 125 nM of mtDNA probe.

The PCR was set at 2 min. at 50°C, 10 min. at 95°C, followed by 40 cycles each of 15 sec. of denaturalization at 95°C and 60 sec. of annealing/extension at 60°C. The mt12SrRNA gene was normalized by determining the nRNAseP nuclear gene and expressed as mt12SrRNA/nRNAseP ratio.

Mitochondrial function by enzymatic activities

Complex IV (CIV) enzymatic activity was measured as an experimental parameter [21] representative of mitochondrial function. The enzymatic activity of CIV was measured following national standardization rules of the Spanish network for the study of mitochondrial respiratory chain (MRC) enzyme activities. This enzymatic assay was spectrophotometrically measured at 37°C at a wavelength of 550 nm including an internal control of pig muscle sample with known reference values and expressed as nmols/min.mg protein.

Protein subunits content and mitochondrial apoptotic events by western blot

Mitochondrial and nuclear DNA encoded subunits (COXII and COXIV, respectively) of CIV, in addition to voltage-dependent anion chanel-1 (VDAC-1) and caspase-9 content were analysed by western blot. In brief, 20–30 μg crude cell lysates were mixed 1:5 with a solution containing 50% glycerol, 10% SDS, 10% β-mercapto-ethanol, 0.5% bromophenol blue and 0.5 M Tris (pH 6.8), incubated at 99°C for 5 min. for protein denaturalization and electrophoresed on 0.1% SDS ranging from 7% to 13% of polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes for 7 min. using an automatic system. Blots were probed with (*i*) a monoclonal antibody (moAb) recognizing the mtDNA-encoded human COXII subunit as a marker of the mitochondrial protein synthesis rate (A6404; Molecular Probes, Eugene, OR, USA), (*ii*) a moAb elicited against the nuclear DNA encoded human COXIV subunit as a marker of the nuclear protein synthesis rate (A21347; Molecular Probes) and (*iii*) an anti-β-actin moAb (A5441; Sigma-Aldrich) as a loading control of overall cell protein content. The protein subunits content was normalized by the content of β-actin signal to establish the relative abundance per overall cell protein. Antimouse and anti-rabbit secondary antibodies were used depending on the primary antibody [22]. Mitochondrial and nuclear protein synthesis ratios were expressed, respectively, as COXII/β-actin and COXIV/β-actin.

Apoptosis approaches were measured by western blot (as aforementioned) using the moAb raised against VDAC-1 (529536, antiporin 31HL; Calbiochem, Darmstadt, Alemania) as a marker of early apoptotic mitochondrial events and moAB against caspase-9 (ab2324; Abcam, Cambridge, UK) as marker of advanced apoptotic mitochondrial events, normalized by β-actin content and expressed as the VDAC-1/β-actin and caspase-9/β-actin ratios.

Mitochondrial content quantified by citrate synthase enzymatic activity

Citrate synthase (CS) enzymatic activity was measured as a reliable marker of mitochondrial content [21]. Enzymatic activity was spectrophotometrically measured following national standardization rules of the Spanish network for the study of MRC enzyme activities. This enzymatic assay was performed at 37°C including an internal control of pig muscle sample with known reference values and assessed at a wavelength of 412 nm and expressed as nmols/min.mg of protein.

Mitochondrial content, mitochondrial depolarization and advanced apoptosis/necrosis by flow cytometry

Mitochondrial content was determined by mitotracker green (MTG), and mitochondrial membrane potential (MMP) was estimated by JC-1 staining as reported elsewhere [23, 24] in cell lines either in the presence or absence of the mitochondrial pro-apoptotic and depolarizing stimuli valinomycin. Advanced apoptosis and necrosis phenomena were assessed using the annexin V and propidium iodide (PI) ratio by means of flow cytometric analysis. Briefly, a total of 1 ml of complete culture media containing roughly 2×10^5 cells was prepared for different reaction procedures and subjected to incubation: (*i*) in the absence of any dye used for the autofluorescence calculation, (*ii*) with 200 nM MTG fluorophore (M-7514; Molecular Probes) for 30 min. for mitochondrial content quantification, (*iii*) with 0.02% JC1 dyer (T-3168; Molecular Probes) for 10 min. for MMP assessment, (*iv*) with 0.02% JC1 fluorophore plus 0.05% valinomycin pro-apoptotic and depolarizing stimuli reagent (60403; Sigma-Aldrich) for 10 min. and (*v*) with 0.05% annexin V plus PI (556463; BD Biosciences, East Rutherford, NJ, USA) for 10 min. to assess advanced apoptotic and necrotic events. All cytometric analyses were performed in a FACScalibur cytometer with an argon ion laser tuned at 488 nm and a diode laser tuned at 635 nm (Becton Dickinson, San José, CA, USA). Results were expressed as median or percentage of cells with specific fluorescence.

Statistics

Results were expressed as mean ± S.E.M. Descriptive statistics were performed using the Statistical Package for the Social Sciences (SPSS) software (IBM inc. SPSS Statistics Chicago, IL, USA). A single filter was applied to discard extreme values. Statistical analysis was performed with non-parametric Kruskal–Wallis H and Mann–Whitney *U*-tests, and the level of significance was considered at $P < 0.05$ (for a confidence interval of $\alpha = 95\%$).

Results

Mitochondrial DNA content showed a significant decrease in the promonocytic latently infected HIV-1 cell line U1 compared to its respective uninfected U937 control cell line (204.64 ± 28.07 *versus* 483.34 ± 97.61; $P < 0.01$). However, the lymphoid latently infected HIV-1 cell line ACH2 showed non-significant variations of mtDNA content compared to the uninfected CEM control cells (227.32 ± 42.99 *versus* 162.59 ± 22.35, P = NS; Fig. 1).

Mitochondrial CIV enzymatic activity was then quantified. In agreement with the above results, CIV enzymatic activity significantly decreased in infected promonocytic U1 cells with respect to uninfected U937 control cells (10.57 ± 2.46 *versus* 46.84 ± 7.30, $P < 0.01$). On the other hand, this value remained non-significantly altered, although showing a trend to decrease in the infected ACH2 lymphoid cell line compared to uninfected CEM control cells (17.86 ± 6.12 *versus* 24.16 ± 3.45, P = NS; Fig. 2).

Thereafter, the content of mitochondrial protein subunits was assessed by the expression of both the mtDNA-encoded COXII (Fig. 3A) and the nuclear encoded COXIV (Fig. 3B), components of the MRC CIV. The expression of these two subunits was reduced in both chronically HIV-1-infected cell lines studied compared to their respective uninfected control cell lines (U1 *versus* U937 and ACH2 *versus* CEM, $P < 0.001$ in all cases).

Moreover, early and advanced apoptotic mitochondrial events estimated as VDAC-1 and caspase-9 content, respectively, were significantly reduced in infected U1 and ACH2 infected cell lines *versus* their respective uninfected counterparts (U937 and CEM, respectively, $P < 0.05$ in all cases, except for caspase-9 in lymphoid cell lines: $P = 0.079$; Fig. 4A and B).

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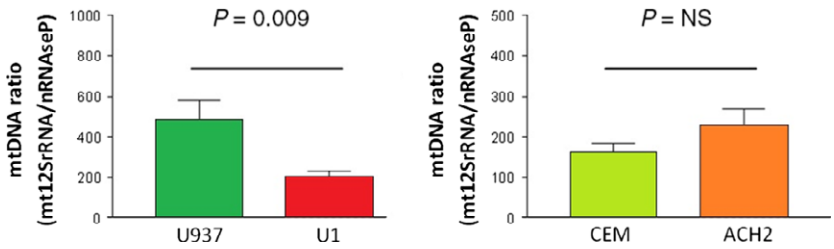

Fig. 1 mtDNA content. This figure shows the mtDNA *versus* nuclear DNA ratio (expressed in arbitrary units) in the infected promonocytic U1 cell line with respect to its uninfected U937 control (left), $P < 0.01$, as well as in the HIV-1-infected lymphoid ACH2 cell line with respect to its CEM control (right), P = NS. MtDNA: mitochondrial DNA, Mt12SrRNA: mitochondrial 12SrRNA ribosomal gene, nRNAseP: nuclear RNAseP gene, NS: not significant.

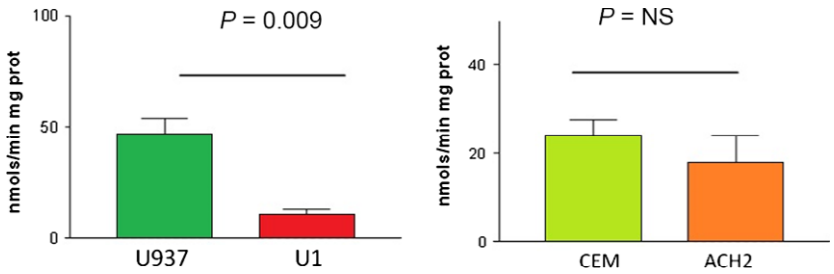

Fig. 2 CIV enzymatic activity. This figure shows the enzymatic activity of complex IV of the mitochondrial respiratory chain of promonocytic (left) and lymphoid (right) cell lines expressed in nmols/min mg protein. A significant decrease in complex IV activity was observed in the infected promonocytic cell line U1 with respect to its uninfected U937 control, $P < 0.01$. NS: not significant.

The mitochondrial content did not show any statistically significant differences, quantified by either CS activity (Fig. 5) or by the MTG assay (data not shown).

Finally, the comparisons with mitochondrial membrane depolarization in addition to advanced apoptosis and the necrosis markers assessed by flow cytometry did not reach statistical significance, although some parameters showed a bias. Thereby, the U1 promonocytic infected cell line showed a trend to lower levels of depolarized mitochondria than their uninfected U937 control cells, although these differences were not statistically significant (0.22 ± 0.16 *versus* 2.38 ± 2.38 respectively). This trend was slight in the HIV-1-infected ACH2 lymphoid cell line *versus* uninfected CEM cells (9.99 ± 9.74 *versus* 12.44 ± 10.85, P = NS). The formerly described increment pattern was also exhibited in promonocytic U1 infected cells *versus* U937 uninfected control when cells were exposed to a pro-apoptotic reagent (valinomycin) (1.54 ± 1.54 *versus* 10.22 ± 10.22, respectively), but this exacerbation pattern was not observed in ACH2 lymphoid cells *versus* uninfected control CEM cells (13.72 ± 11.51 *versus* 12.91 ± 10.88 respectively).

The rate of cells of either promonocytic or lymphoid origin undergoing apoptosis or necrosis measured by annexin V plus PI did not render significant differences. A trend towards a decrease was observed in HIV-1-infected U1 promonocytic cells with respect to their U937 controls (9.92 *versus* 27.63 respectively), whereas an increase was observed in HIV-1-infected ACH2 lymphoid cells in comparison with their CEM control cells (23.40 *versus* 4.32).

Discussion

In this study, we compared mitochondrial and apoptotic parameters in chronically HIV-1-infected promonocytic and lymphoid cell lines with their uninfected counterparts to evaluate whether they could be used as HIV infection models of the *in vivo* mitochondrial and apoptotic lesion characteristic of HIV-infected patients. Most of the mitochondrial and apoptotic parameters were altered in the HIV-infected cell lines, especially in the promonocytic lineage, resembling *in vivo* alterations in case of mitochondrial findings. However, contrarily to what is observed *in vivo*, both promonocytic and lymphoid HIV-infected models showed resistance to undergo apoptosis. In the case of the promonocytic infected cell line U1, this may lead to a useful model to study HIV reservoirs, frequently established in these cell lineages. However, *in vivo* apoptosis of lymphocytes followed by infection was not observed in the ACH2 model, except for the trends of annexin V and PI measured by flow cytometry.

Mitochondrial and apoptotic abnormalities have been postulated to be the basis of collateral effects such as the development of hyperlactatemia, lipodystrophy and neuropathy associated with both HIV infection and its treatment [25–28]. Although most mitochondrial changes are considered to be the result of HIV apoptotic capacity and the secondary effects derived from ART [12], there is a growing evidence of the crucial role of mitochondria in the dynamics of HIV infection [29]. Despite the interest in several areas of the HIV infection process such as HLA, polymorphisms in viral co-receptors,

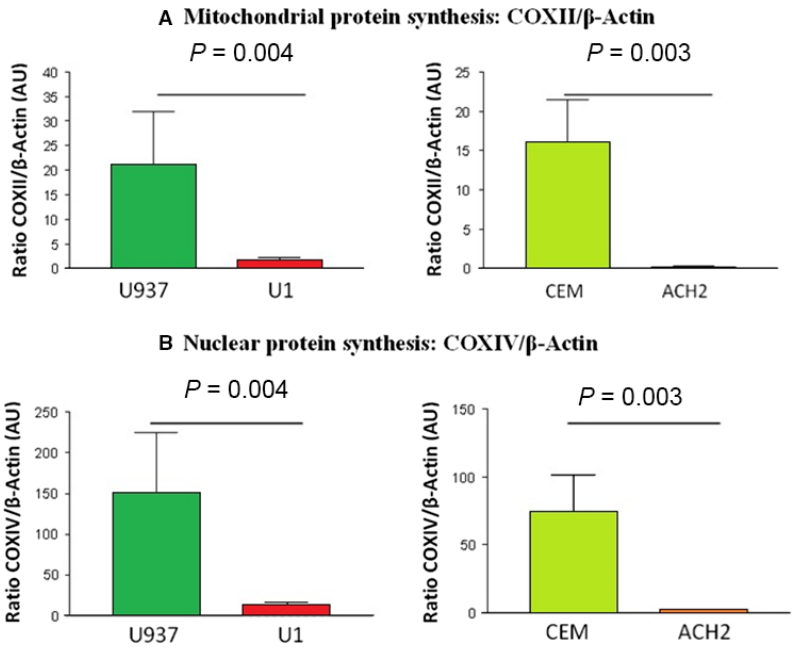

Fig. 3 (**A**) COXII content. This figure shows COXII protein subunit content in arbitrary units with expression of the levels of mitochondrial DNA encoded subunit II of complex IV of promonocytic (left) and lymphoid (right) cell lines normalized by β-actin content. A significant decrease in mitochondrial protein synthesis was observed in promonocytic and lymphoid U1 and ACH2 infected cell lines with respect to their uninfected U937 and CEM controls. COX: cytochrome c oxidase, AU: arbitrary units. (**B**) COXIV content. This figure shows COXIV protein subunit content in arbitrary units with expression of the levels of nuclear DNA encoded subunit IV of complex IV of promonocytic (left) and lymphoid (right) cell lines normalized by β-actin content. A significant decrease in nuclear protein synthesis was observed in promonocytic and lymphoid U1 and ACH2 infected cell lines with respect to their uninfected U937 and CEM controls. COX: cytochrome c oxidase, AU: arbitrary units.

antibodies, chemokines and defensins, among others [30, 31], the role of mitochondria has become outstanding in this process [29, 32].

The mitochondrial and apoptotic damage induced by HIV infection may exacerbate these deleterious effects in a kind of vicious cycle, terminating in the loss of cell defence capability of the host and the progression of infection. Studies performed to date have highlighted the need to establish an *in vitro* model to further investigate possible therapeutic approaches to revert mitochondrial or apoptotic damage and determine novel tools to fight HIV progression.

Mitochondrial parameters

In our cell models, most of the mitochondrial parameters were affected in both cell lines. Mitochondrial genome content and functional enzymatic activity were sharply depleted in U1 promonocytic cells, without being significantly modified in the ACH2 lymphoid cells. Both infected cell lines exhibited a marked decrease in MRC subunit expression which, in case of the promonocytic cells, goes in agreement with the outstanding dysfunction of the MRC. The expression of mitochondrial protein subunits of MRC was above 90% decreased in most cases compared to their uninfected counterparts. These mitochondrial and nuclear encoded subunits were diminished in both HIV-1-infected cell lines, suggesting both mitochondrial and nuclear gene malfunctions in all cases.

Apoptotic parameters

A link between HIV infection and apoptosis has been widely reported [33]. Some viral proteins interact with mitochondrial targets, leading to apoptosis through different pathways [34, 35]. This is the case of the viral protein R (Vpr), the HIV-1-trans-activating protein (Tat) and the viral protease (Pr), which can directly interact with components of the mitochondrial permeability transition pore (Vpr interacts with the adenine nucleotide translocator ANT attached to VDAC, thus prompting mitochondrial depolarization) [32], translocate proapoptotic proteins into the mitochondria (Tat mediates Bim translocation) [33], activate caspases (Vpr activates caspases 3 and 9) [34] or inactivate anti-apoptotic proteins (Vpr inactivates HAX-1 and Pr inactivates Bcl-2) [34, 35], ultimately causing cell death. Consequently, the marked significant decrease in the early and advanced apoptotic markers (VDAC-1 and caspase-9 proteins) in both infected cell lines studied was unexpected. These results are in agreement with the lower levels of early apoptotic events of depolarized mitochondrial membranes observed with JC1 by flow cytometry in both infected U1 and ACH2 cell lines, although such trends did not achieve statistical significance.

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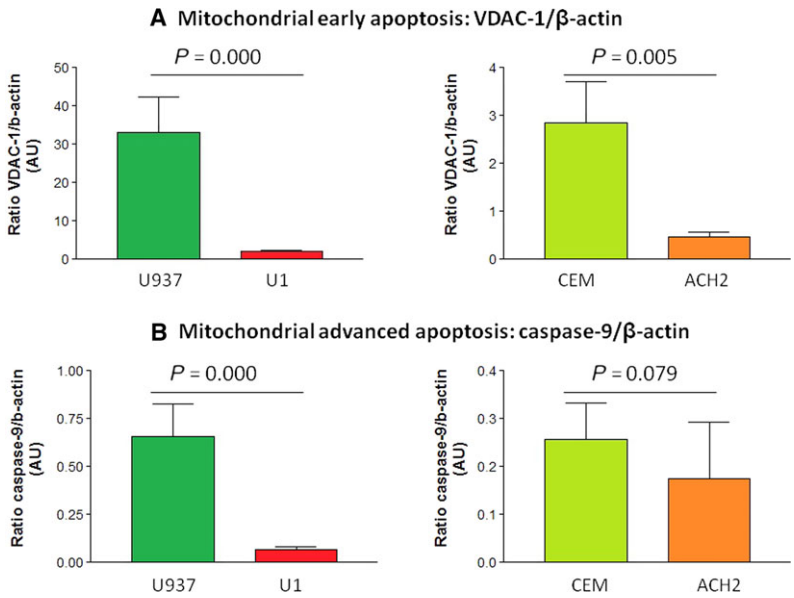

Fig. 4 (**A**) VDAC-1 content. This figure shows the VDAC-1 content expressed in arbitrary units. The expression of the levels of VDAC-1 content normalized by β-actin of promonocytic (left) and lymphoid (right) cells. A significant decrease in early mitochondrial apoptosis was observed in promonocytic and lymphoid U1 and ACH2 infected cell lines with respect to their uninfected U937 and CEM controls. VDAC-1: voltage-dependent anion channel-1, AU: arbitrary units. (**B**) Caspase-9 content. This figure shows the caspase-9 content expressed in arbitrary units. The expression of the levels of caspase-9 content normalized by β-actin of promonocytic (left) and lymphoid (right) cells. A decrease in advanced mitochondrial apoptosis was observed in promonocytic (significant) and lymphoid (non-significant) U1 and ACH2 infected cell lines with respect to their uninfected U937 and CEM controls. AU: arbitrary units.

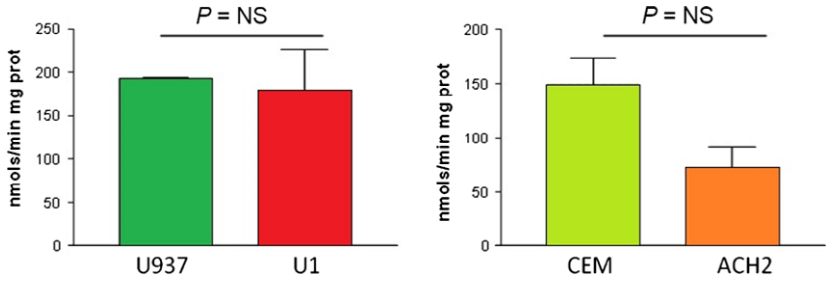

Fig. 5 Mitochondrial content. This figure shows the mitochondrial content measured by citrate synthase enzymatic activity expressed in nmols/min mg protein of promonocytic (left) and lymphoid (right) cells. No significant changes were observed in infected promonocytic and lymphoid U1 and ACH2 cell lines with respect to their uninfected U937 and CEM controls. NS: not significant.

Our data strongly indicate that latently infected cells are less susceptible to undergo apoptosis compared to uninfected cells. These results support a previous study [18] which was mainly focused on the reaction of specific cell lines against different exogenous pro-apoptotic and stress-induced stimuli. Although HIV infection most frequently leads to cell death, the latently HIV-1-infected cell lines studied only showed a reduced apoptotic behaviour which may be indicative of a viral strategy to survive [36]. In this sense, both promonocytic and lymphoid HIV-1-infected cell lines showed different patterns in advanced apoptosis and necrosis phenomena tested by flow cytometry through annexin V plus PI staining. A trend to decrease in advanced apoptotic events was observed in the infected U1 promonocytic cell lines compared to their controls, whereas a tendency to increase was observed in infected ACH2 lymphoid cell lines. These data are also in agreement with the lower sensitivity to apoptosis previously observed in promonocytic *versus* lymphoid cells [37].

Validation of both *in vitro* models

Considering all these results, our findings suggest that these cell models may at least partially reproduce *in vitro* the alterations observed *in vivo* in HIV-infected patients [4, 8, 9, 12, 13] that should be further characterized in depth.

From a mitochondrial point of view, both promonocytic and lymphoid cell lines showed pronounced abnormalities with slightly different patterns. Previous studies reported different behaviour for promonocytic and as well as lymphoid cell lines [37] or monocytes/lymphocytes [36] suggesting that different molecular mechanisms may depend on cell types considered. In our study, although both promonocytic and lymphoid cell lines showed changes in MRC expression, only the promonocytic cells (not the lymphoid lineage) showed significant alterations at the mitochondrial genetic and functional levels. In this context, it was surprising to notice the significant reduced content of subunits COXII and COXIV in the lymphoid infected lines despite the trends towards increased levels of mtDNA and only slightly reduced enzymatic activity of the complex IV. It would be conceivable that, despite the decrease in the subunit protein levels is observed, the threshold to observe a defective functionality of the enzyme had not been yet reached, probably due to the homeostatic up-regulation of mtDNA content in these cells. All these mechanisms were not observed in promonocytic infected cell lines, which showed decreased ratios in all genetic, functional and expression mitochondrial parameters fully resembling *in vivo* conditions.

From the apoptotic point of view, both infected cell lines showed a trend to a resistance to apoptotic events.

A previous study reported lower MMP in peripheral blood mononuclear cells from HIV patients, as well as higher apoptotic/necrotic events in these cells [35]. In this study non-significant trends were found in MMP. In addition, most of apoptotic/necrotic parameters measured (mitochondrial pore and caspase-9 activation or phosphatidylserine expression and nuclear DNA fragmentation) yielded to similar results suggesting the resistance to undergo apoptosis of HIV-infected cell models [18, 33] and, as opposite as *in vivo* conditions of blood cells not becoming reservoirs [4, 5].

Our findings point out the use of the present *in vitro* models as largely useful for mitochondrial monitoring. We encourage other researchers to explore in depth apoptotic events in this and other *in vitro* models. Importantly, our results clearly mimic the *in vivo* mitochondrial features of HIV infection, (especially in the promonocytic cell line), rather than the *in vivo* apoptotic conditions. Apoptotic resistance in infected lines might be derived from the genetic modification of the cancerous lineages that do not undergo apoptosis following viral genome insertion. Promonocytic apoptotic resistance may reflect the *in vivo* percentage of monocytes capable to constitute reservoirs and, subsequently, become more resistant to apoptosis. Resistance to apoptosis of ACH2 lymphoid cell line should be taken with caution when willing to reproduce *in vivo* pathophysiology of HIV infection. The observed findings, suggest that such alterations might have HIV clinical consequences as previously described *in vivo* [11] and point out to the involvement of mitochondrial status in HIV progression and chronicity. In addition, this study provides complementary data to the apoptosis approaches previously considered [15].

In spite of strained HIV-1 replication in the infected cell lines used in this study, the assessment of the mitochondrial and apoptotic parameters investigated led to the description of significant changes compared to the respective uninfected control cell lines. It remains unknown whether these disturbances would be increased in activated HIV-1-infected cell lines (with the addition of HIV replication inducer factors).

A limitation of this study was the resistance to apoptosis of the cancerous infected cell lines, which remain viable regardless of HIV infection and would have died in natural conditions. Nonetheless, despite these limitations, due to the same cancerous origin of both control and infected lines, it is presumable that the significant changes found between cell lines are comparable and attributable to the infection itself. Thereby, we consider that the models studied may constitute adequate *in vitro* models to monitorize the patient dynamics at apoptotic and especially at mitochondrial level.

In summary, this study provides evidence that the mitochondrial and, in a lesser extent, apoptotic pathways are seriously affected in latently infected HIV-1 promonocytic and lymphoid cells. The availability of an *in vitro* HIV model is essential for translational research. Studies using these cell models of HIV infection may lead to the development of novel therapeutic tools [38], including apoptotic and mitochondrial targets [39], as strategies to reverse the lesions exerted by the virus.

Acknowledgements

Both U1 and ACH2 cell lines were first created by Dr. Thomas Folks through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, which we would like to thank. Foremost, we would like to express our gratitude to Florencio Varas for his critical review of the manuscript and for providing writing assistance. This work was supported by Fundación para la Investigación y la Prevención del SIDA en España (grant number FIPSE 360982/10); Fundació Cellex, Suports a Grups de Recerca de la Generalitat de Catalunya 2014-2016 (grant number SGR 2014/376) and Fondo de Investigación Sanitaria (grant numbers FIS 00462/11, FIS 01199/12, FIS 01455/13, FIS 01738/13, FIS 00903/15 and FIS 00817/15), InterCIBER (PIE1400061) and CIBER de Enfermedades Raras (CIBERER), initiatives of ISCIII and FEDER.

Conflicts of interest

None of the 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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J. Cell. Mol. Med. Vol XX, No X, 2016 pp. 1-9

Mitochondrial toxicity and caspase activation in HIV pregnant women

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Received: November 10, 2015; Accepted: June 12, 2016

Abstract

To assess the impact of HIV-infection and highly active anti-retroviral treatment in mitochondria and apoptotic activation of caspases during pregnancy and their association with adverse perinatal outcome. Changes of mitochondrial parameters and apoptotic caspase activation in maternal peripheral blood mononuclear cells were compared at first trimester of pregnancy and delivery in 27 HIV-infected and -treated pregnant women *versus* 24 uninfected pregnant controls. We correlated immunovirological, therapeutic and perinatal outcome with experimental findings: mitochondrial DNA (mtDNA) content, mitochondrial protein synthesis, mitochondrial function and apoptotic caspase activation. The HIV pregnancies showed increased adverse perinatal outcome (OR: 4.81 [1.14–20.16]; $P < 0.05$) and decreased mtDNA content ($42.66 \pm 5.94\%$, $P < 0.01$) compared to controls, even higher in naïve participants. This depletion caused a correlated decrease in mitochondrial protein synthesis ($12.82 \pm 5.73\%$, $P < 0.01$) and function ($20.50 \pm 10.14\%$, $P < 0.001$), not observed in controls. Along pregnancy, apoptotic caspase-3 activation increased $63.64 \pm 45.45\%$ in controls ($P < 0.001$) and $100.00 \pm 47.37\%$ in HIV-pregnancies ($P < 0.001$), in correlation with longer exposure to nucleoside analogues. HIV-infected women showed increased obstetric problems and declined genetic and functional mitochondrial parameters during pregnancy, especially those firstly exposed to anti-retrovirals. The apoptotic activation of caspases along pregnancy is emphasized in HIV pregnancies promoted by nucleoside analogues. However, we could not demonstrate direct mitochondrial or apoptotic implication in adverse obstetric outcome probably because of the reduced sample size.

Keywords: HIV • pregnancy • HAART • mitochondrial toxicity • perinatal outcome

Introduction

The effectiveness of highly active antiretroviral treatment (HAART) regimens in reducing mother-to-child vertical transmission (MTCT) of HIV-infection and in delaying disease progression has been demonstrated and should therefore be offered to all pregnancies [1–3]. Anti-retroviral (ARV) treatment comprised of two nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI) is recommended in pregnancy by the United States, World Health Organization and European guidelines [4, 5]. Additional measures including selective caesarean or avoidance of breastfeeding are strongly recommended. However, adverse pregnancy outcome have been increasingly reported by several observational studies in HIV-infected women exposed to HAART [6–11]. Anti-retrovirals and HIV-infection have been associated with pre-eclampsia, stillbirth, pre-term labour, low birth weight and intrauterine growth restriction (IUGR) [12–17].

One of the most serious complications associated with ARVs is mitochondrial toxicity. Mitochondrial-derived clinical effects of NRTI

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doi: 10.1111/jcmm.12935

have been firmly established in HIV-infected non-pregnant adults [18–20]. These negative effects depend on the capacity of NRTIs to inhibit DNA polymerase gamma, the enzyme devoted to mitochondrial DNA (mtDNA) replication, leading to a decrease in mtDNA copy number and quality, which may, finally, cause mitochondrial dysfunction [18]. Depletion of mtDNA has been extensively described in different tissues of human and animal models (placenta, foetal cord blood, heart, adipose tissue, skeletal muscle, brain and kidney, among others) leading to mitochondrial morphologic, metabolic and energetic abnormalities [21–25]. Such mitochondrial disturbances are enhanced by HIV, which has been additionally blamed for triggering apoptosis [26]. Associated clinical syndromes have been expanded to include lipoatrophy, peripheral neuropathy, cardiomyopathy, lactic acidosis and hepatic steatosis [27, 28]. Moreover, accelerated 'mitochondrial aging' associated with ARV may contribute to cardiovascular disease, malignancies and frailty [29].

Maternal death has been described as a result of lactic acidosis in women receiving long-term treatment with a combination of NRTI [30, 31], especially in the third trimester of pregnancy and in ARV schedules including two NRTIs. Fortunately, severe maternal mitochondrial toxicity associated with NRTI during pregnancy appears to be rare and is reversible on treatment discontinuation. However, milder forms of mitochondrial toxicity are commonly reported and may have future long-term effects.

Although ARV use during pregnancy is considered safe, data on ARV and pregnancy, especially in HAART, are insufficient, and safety and long-term health consequences are currently unknown. The few studies on long-term HAART exposure have focused on foetal and perinatal ARV effects [32–37], but rarely on maternal-related problems which may, in turn, affect children.

We recently described that HAART toxicity may cause subclinical mitochondrial damage in pregnant women and their newborn [38] by reducing mtDNA levels, mitochondrial protein synthesis and mitochondrial function. Additionally, increased apoptosis through caspase-3 activation was observed in HIV-pregnant women, but not in their children, cross-sectionally, at delivery.

The aim of this study was to investigate the impact of HIV-infection and HAART on mitochondria and apoptotic caspase activation during pregnancy to assess their implication in the increase of adverse perinatal outcome characteristic of HIV-pregnancies to establish potential prenatal prognosis markers.

Materials and methods

Design

We performed a single-site, controlled observational study without intervention to determine longitudinal mitochondrial toxicity and apoptotic caspase activation (from the first trimester of gestation to delivery) in maternal peripheral blood mononuclear cells (PBMC) of HIV-infected pregnancies compared to uninfected controls.

Study population

Twenty-seven asymptomatic HIV-1-infected and twenty-four uninfected women were consecutively included during routine prenatal care at first trimester of gestation in the Hospital Clinic of Barcelona (Barcelona, Spain).

Controls and cases were age and parity matched. The inclusion criteria for pregnant women were: >18 years of age, single pregnancy, delivery >22 weeks of gestation and, for HIV-patients, previous diagnosis of HIV-infection.

Patients taking potentially toxic drugs for mitochondria and with familial history of mitochondrial disease were excluded.

The Ethical Committee of our hospital approved the study and it was performed following the Declaration of Helsinki. All participants provided written consent.

Clinical results

A database was created to collect epidemiological, immunovirological, therapeutic, obstetric, perinatal and experimental data.

Maternal epidemiological parameters included information on maternal age, race and illegal substance abuse.

Immunovirological parameters for HIV-infected women consisted in quantifying comorbidity with HCV infection, plasmatic HIV-viral load (by rtPCR), $CD4^+$ T-cell count (by flow cytometry) and time from HIV infection to delivery.

Therapy was administered to all HIV-pregnant women following international guidelines. HIV-infected women were stratified according to ARV use during pregnancy. Women naïve for ARV before pregnancy started HAART (double-NRTI schedule and either one PI or NNRTI drug) during the second trimester of gestation to prevent MTCT.

Information regarding obstetric and perinatal outcome included: parity, mode of delivery, gestational diabetes mellitus, pre-eclampsia (new onset of hypertension of >140 mmHg systolic or >90 mmHg diastolic pressure and >300 mg proteins/24 hrs urine after 20 weeks of gestation), foetal death (>22 weeks of pregnancy), gestational age at delivery, pre-term birth (<37 weeks of gestation), birth weight, small newborn for gestational age (<10th percentile), 5-min Apgar score <7, neonatal admission to intensive care unit and global adverse perinatal outcome.

Finally, experimental data included maternal measures of mitochondrial and apoptotic caspase activation at first trimester of gestation and delivery.

Sample collection and processing

At the first trimester of pregnancy and immediately after delivery, 20 ml of peripheral blood was collected in EDTA tubes to isolate PBMC by Ficoll gradient and stored at −80°C until analysis. Protein content was measured by Bradford protein dye binding-based method [39].

Mitochondrial studies in maternal PBMC

Mitochondrial DNA quantification

Total DNA was extracted by phenol-chloroform procedure. A fragment of mitochondrial DNA-encoded ND2 and nuclear DNA-encoded 18SrRNA

genes were amplified separately in triplicate by quantitative rtPCR using the Roche Lightcycler-thermocycler [20]. Mitochondrial DNA content was expressed as the ratio between mitochondrial and nuclear DNA amount (ND2mtDNA/18SrRNA nDNA content).

Mitochondrial protein synthesis

We performed Western blotting of 20 μg total cell protein through 7/13% SDS-PAGE and posterior immunoquantification of the mitochondrial DNA-encoded and located COXII subunit (25.6 kD) with respect to the nuclear DNA-encoded and mitochondrially located COXIV subunit (15 kD) to compare relative mitochondrial to nuclear protein synthesis (COXII/COXIV) [20].

Mitochondrial enzymatic function

Mitochondrial respiratory chain complex II+III (CII+III) enzymatic activities were measured by spectrophotometry according to Rustin *et al.* [37, 40] by following the increase in absorbance at 550 nm of reduced cytochrome c generation (complex III product) after succinate addition (complex II substrate). Specific enzymatic activities were expressed as nanomols of product per minute and milligram of protein (nmols/min.mg prot).

Apoptotic caspase activation studies

We performed Western blotting of 20 μg total cell protein by 7/13% SDS-PAGE and posterior immunoanalysis of active (cleaved) caspase-3 pro-apoptotic protein expression (17–19 kD) normalized by the content of β-actin (47 kD) as a cell loading control. Results were expressed as caspase-3/β-actin relative content and were interpreted as a marker of advanced apoptotic events [41].

Additionally, caspase-9 enzymatic activity was measured by means of the luminescent assay Caspase-Glo® Assay (Ref: G8210, Promega Corporation, Madison, WI 53711 USA) using 20 μg of PBMCs' protein. Briefly, 50 μl of diluted sample and 50 μl of kit Caspase-Glo® reagent were mixed in an opaque reading multiwell plate following manufacturer's instructions. After 36 min. of incubation, the plate was read in a Modulus™ II Microplate Multimode Reader and caspase- 9 activity was calculated based on relative luminescent units normalized to sample protein amount.

Statistical analysis

Clinical and epidemiological parameters were expressed as means and range interval and experimental results as means and S.E.M. or as a percentage of increase/decrease at delivery compared to first trimester of pregnancy.

Adverse perinatal outcome, mitochondrial and apoptotic caspase activation results of HIV-infected women were compared to those of uninfected controls to assess the impact of HIV and/or ARV. Additionally, different correlations were sought between: molecular and functional mitochondrial parameters (to ascertain dependence of mitochondrial function on mitochondrial genome) and clinical and experimental data (to assess mitochondrial or apoptotic basis of obstetric and perinatal outcome).

Nonparametric tests were used to determine: case–control differences (Mann–Whitney independent sample analysis), odds ratio (chi-squared test) and parameter correlation (Spearman's rank coefficient). Significance was set at 0.05.

Results

Clinical data

Table 1 shows epidemiological characteristics of the participants and immunovirological and therapeutic data of HIV mothers. All pregnant women were Caucasian, ranging 25–42 years. Non-significant differences were observed in maternal clinical data between HIV-positive and HIV-negative women.

Most HIV-infected women were under HAART before pregnancy (85%) and only four cases (15%) were ARV-naïve and started HAART at the second trimester of gestation. Highly active ARV treatment was given to all patients at delivery to avoid MTCT, consisting of two NRTI and either PI (55.5%) or NNRTI (29.5%). The mean time of HIV-infection and HAART treatment prior to delivery were 84 and 48 months respectively. At delivery, all women had undetectable viral load and received at least 6 months of double-NRTI treatment. Neither patients nor controls presented clinical manifestations of mitochondrial toxicity.

Perinatal outcome

Table 2 shows obstetric and neonatal outcome of the study cohort. All infants were HIV-uninfected with no clinical symptoms of mitochondrial toxicity, and all received 6-week zidovudine chemoprophylaxis to prevent MTCT. These pregnancies showed a trend to increased gestational diabetes (7.4% *versus* 4.0%), decreased gestational age at delivery (37.5 *versus* 38.6), pre-term birth (25.9% *versus* 8.0%), reduced birth weight (2879 g *versus* 3170 g), small newborn for gestational age (22.2% *versus* 4.0%) and intensive care unit admission (11.1% *versus* 4.0%). However, only global adverse perinatal outcome (pre-term birth and small for gestational age events) were significantly increased among HIV-positive pregnancies [40.7% *versus* 12.0%, OR: 4.81 (1.14–20.6); $P < 0.05$].

Mitochondrial and apoptotic maternal PBMC analysis

Table S1 shows raw data and statistics of all tested experimental parameters.

MtDNA content

A highly significant progressive reduction of PBMC mtDNA content was observed in HIV pregnancies (42.66 ± 5.94%, $P < 0.01$), not observed in uninfected pregnant controls (18.34 ± 11.73%, P = NS), being even greater in naïve HIV-infected pregnant women (50%, P = NS; data not shown).

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Table 1 Epidemiologic, immunovirologic and therapeutic characteristics of HIV-infected and uninfected pregnant women

	HIV-positive ($n = 27$)	HIV-negative ($n = 24$)	*P*
Maternal age at delivery*	34.7 (27–42)	33.6 (25–41)	NS
Illegal drug use, *N* (%)	0	0	–
Alcohol use, *N* (%)	0	0	–
HCV infection, *N* (%)	3 (11.1)	1 (4)	NS
HIV RNA copies per ml at delivery*	62.3 (49–250)	–	–
CD4 T-cell count per ml at delivery*	560.2 (97–1242)	–	–
Time from diagnosis of HIV infection to delivery (months)*	84 (4–228)	–	–
NRTI before pregnancy (months)*	48 (0–106)	–	–
NNRTI before pregnancy (months)*	3 (0–86)	–	–
PI before pregnancy (months)*	12 (0–97)	–	–
NAÏVE (HAART 2nd–3rdtrimesters), *N* (%)	4 (15)	–	–
HAART all trimesters, *N* (%)	23 (85)	–	–
2 NRTI+1 PI, *N* (%)	15 (55.5)		
2 NRTI+1 NNRTI, *N* (%)	8 (29.5)		

*Data are presented as means and range interval.
HCV: hepatitis C virus; HIV: human immunodeficiency virus; HAART: highly active antiretroviral treatment; NRTI: nucleoside-analogue reverse transcriptase inhibitor; NNRTI: non-nucleoside analogue reverse transcriptase inhibitor; NS: not significant; PI: protease inhibitors; RNA: ribonucleic acid; *N*: number.

Mitochondrial protein synthesis and function

Along pregnancy, HIV women also showed a significant decrease in the mitochondrial protein synthesis rate and function, not observed in controls (Fig. 1).

The protein synthesis (COXII/IV expression ratio; Fig. S1) dropped 12.82 ± 5.73% in HIV mothers ($P < 0.01$) but only 6.25 ± 11.25% in controls (P = NS), and mitochondrial respiratory chain activity of complex II+III significantly decreased in HIV-infected mothers (20.50 ± 10.14%, $P < 0.001$), but not significantly in controls (6.64 ± 10.39%, P = NS).

Apoptotic caspase activation

Along pregnancy, HIV-infected pregnant women and healthy controls presented a marked and significant increase in apoptotic caspase-3 activation of 100.00 ± 47.37% and 63.64 ± 45.45%, respectively, with respect to baseline ($P < 0.001$ in both cases) (Fig. 1 and Fig. S1). This difference in the apoptotic caspase-3 activation between cases and controls was significant ($P < 0.05$). Similar findings were observed in the measurement of Caspase-9 enzymatic activity by means of apoptotic increase along pregnancy in both cohorts of HIV-infected women and uninfected controls (88.38 ± 41.88 *versus* 15.76 ± 36.83, respectively, P = NS), higher for HIV patients (data not shown).

Associations between molecular and clinical parameters

Genetic and functional mitochondrial parameters

In treated HIV-infected pregnant women, the mitochondrial genome content was positively and significantly correlated with mitochondrial function measured as CII+CIII enzymatic activity in the first trimester of gestation ($P < 0.05$, $R^2 = 0.16$; Fig. 2A).

The mitochondrial genome level was also positively and significantly correlated with mitochondrial protein synthesis, by COXII/IV measurement, in HIV-positive pregnancies at delivery ($P < 0.05$; $R^2 = 0.19$; Fig. 2B).

Mitochondrial and apoptotic parameters and immunovirological and therapeutic features

A significant, positive correlation was found between increased caspase-3 activation and longer exposure to NRTI prior to pregnancy, in the first trimester of gestation ($P < 0.05$; $R^2 = 0.16$; Fig. 3A) and at delivery (data not shown).

Table 2 Obstetric and neonatal outcome of the study cohorts

	HIV positive (n = 27)	HIV negative (n = 24)	OR (95% CI)
Gestational diabetes mellitus, *N* (%)	2 (7.4)	1 (4)	1.84 (0.15–21.7)
Pre-eclampsia, *N* (%)	0	0	–
Foetal death, *N* (%)	0	0	–
Gestational age at delivery (weeks)*	37.5 (32.2–41.2)	38.6 (38.3–40.3)	P = NS
Preterm birth (<37 weeks of gestation), *N* (%)	7 (25.9)	2 (8)	3.85 (0.71–20.7)
Birth weight (g)*	2879 (1940–4040)	3170 (3130–3320)	P = NS
Small newborn for gestational age (<10th percentile), *N* (%)	6 (22.2)	1 (4)	6.51 (0.72–59.19)
5-min Apgar score <7, *N* (%)	0	0	–
Neonatal intensive care unit admission, *N* (%)	3 (11.1)	1 (4)	2.87 (0.28–29.67)
Global adverse perinatal outcome, *N* (%)	11 (40.7)	3 (12)	4.81 (1.14–20.16) $P < 0.05$

*Data are presented as means and range interval.
95% CI: 95% confidence interval of the mean; *N*: number; NS: not significant; OR: odds ratio.

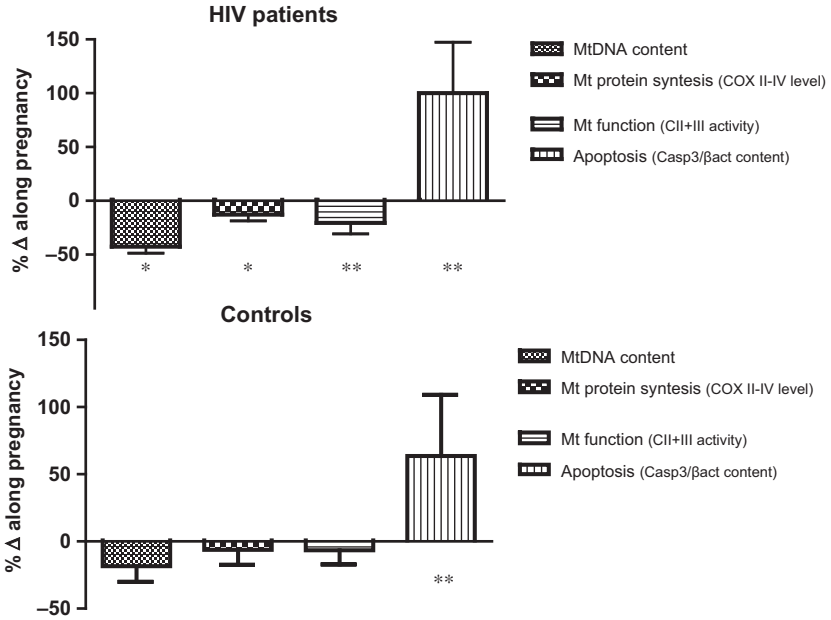

Fig. 1 Mitochondrial parameters and apoptotic caspase-3 activation. Percentage of increase/decrease from first trimester to delivery of mitochondrial parameters or apoptotic caspase-3 activation in HIV participants and uninfected controls along pregnancy. MtDNA: mitochondrial DNA; COX-II/IV: Mitochondrial protein synthesis; CII+III: mitochondrial complex II+ complex III enzymatic activity; caspase-3/β-Actin: apoptotic caspase-3 activation. *$P < 0.01$/**$P < 0.001$.

A significant, positive correlation was also found between increased caspase-3 activation in the first trimester and longer HIV infection ($P < 0.05$; $R^2 = 0.20$; Fig. 3B).

No correlations were found between mitochondrial maternal experimental parameters and immunovirological status.

Mitochondrial and apoptotic parameters and obstetrics results

The global adverse perinatal outcome were significantly increased in HIV pregnancies, but did not correlate with mitochondrial or apoptotic findings.

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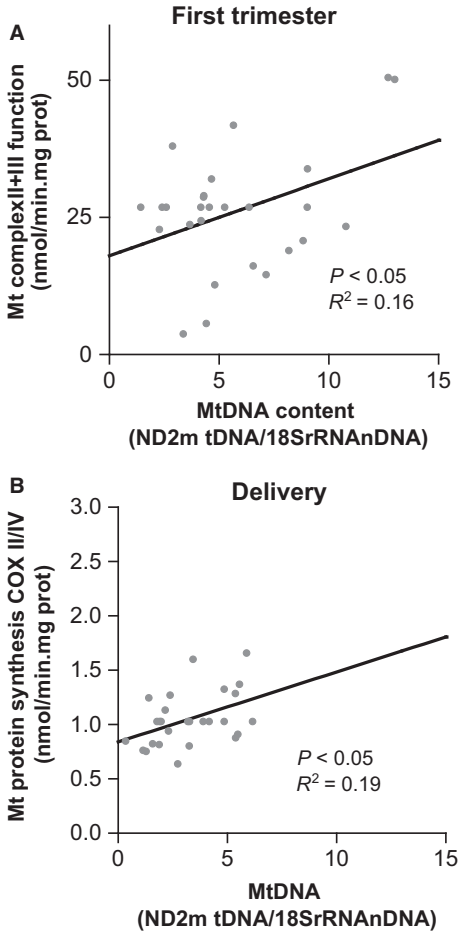

Fig. 2 Associations between genetic and functional mitochondrial parameters in HIV women on HAART. (**A**) Association between mitochondrial complex II+III function and mitochondrial DNA content at first trimester. (**B**) Association between mitochondrial protein synthesis COXII/IV and mitochondrial DNA at time of delivery.

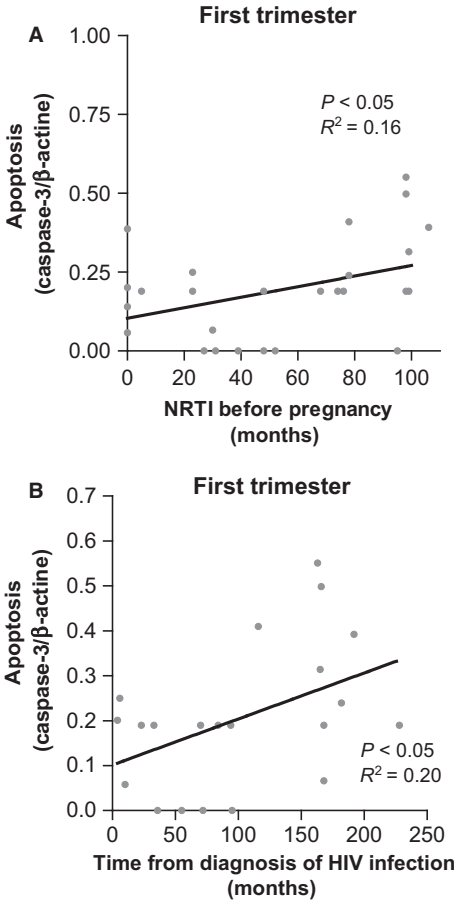

Fig. 3 Association between mitochondrial parameters or apoptotic caspase-3 activation and inmunovirologic and therapeutic characteristics of HIV women on HAART. (**A**) Association between apoptotic caspase-3 activation and months using NRTI prior to pregnancy. (**B**) Association between apoptotic caspase-3 activation and months of HIV-infection.

Discussion

Anti-retrovirals are indispensable in the treatment and prevention of HIV infection. Although their use during pregnancy is considered safe, there are still lingering concerns about long-term health consequences.

Several studies have demonstrated mitochondrial toxicity in animal models, HIV-infected infants and adults on NRTI therapy and newborns exposed *in utero* to ARVs [22, 24, 37, 38, 47], but it is currently unknown whether HIV-pregnancy may be an additional risk for the onset of mitochondrial toxicity.

Several physiopathological mechanisms have been proposed to explain the adverse clinical effects in HIV pregnancies. Mitochondrial bioenergetics conditions foetal growth and early postnatal adaptation. Given that mitochondria are exclusively inherited from the maternal ovum, early exposure of the ova and the mitochondria to ARV affects foetal development. We have demonstrated that oocytes from infertile HIV-infected HAART-treated women show a decreased mtDNA content that could explain their poor reproductive outcome [19]. Several mitochondrial alterations are associated with initiation of mitochondrial biogenesis and activation during early states of embryo development. The mtDNA content and expression levels of genes involved in the maintenance and regulation of biogenesis change during human foetal development. The foetus is exposed to the potentially stressful *in utero* environment created by maternal ARV-associated metabolic toxicities and placental transferred ARVs at critical points in its development.

Colleoni *et al.* have recently described significantly decreased mtDNA in non-infected women carrying IUGR fetuses compared to control pregnancies. The authors concluded that in HIV-uninfected pregnancies mtDNA content and mitochondrial function may help recognizing adverse perinatal outcome [42]. It is unknown whether

mitochondrial depletion is a predictor factor for poor pregnancy outcome in HIV patients. Indeed, only two studies have evaluated the association between mitochondrial toxicity and poor pregnancy outcome in HIV-infected pregnant women on HAART. Both analyse mitochondrial status in the third trimester of gestation. Nasi *et al.* described a decrease in mtDNA content in subcutaneous fat of HIV-pregnant women taking ARV compared to uninfected women without ARV [43]. The second report, published by our group, described that HAART toxicity may cause subclinical mitochondrial damage in blood of HIV-pregnant women and their newborn compared to uninfected pregnancies [27]. To our knowledge, the present work is the first longitudinal study investigating the evolution of mtDNA content, mitochondrial protein synthesis, mitochondrial function and apoptotic caspase activation in HIV-infected women during pregnancy or the potential use of these parameters as pronostics factors to predict adverse perinatal outcome in HIV pregnancies.

In this study we found a higher prevalence of adverse perinatal outcome in the HIV cohort compared with controls, thereby confirming the deleterious effect of both the virus and the treatment on foetal development as well as validating our sample.

During pregnancy, we observed a progressive decrease in mtDNA content which was significantly higher in HIV-infected pregnant women. Our results agree with two previous studies performed in pregnant women which evaluated blood mtDNA content along pregnancy [38–42]. Colleoni *et al.* reported a significant decrease in mtDNA content in the blood of uninfected pregnant women in the first, second and third trimesters compared to non-pregnant women [42]. This decrease was higher in HIV-pregnancies [38] and, according to the present findings, would be partially developed along pregnancy.

The mtDNA depletion observed in our HIV cohort was even greater in naïve pregnant women who started HAART during the second trimester of gestation compared with pregnancies under HAART prior to conception. This finding could be explained by the initiation of ARV, abruptly increasing mitochondrial toxicity and resulting in a dramatic decrease in mtDNA content which is maintained over time. This theory is in agreement with a previously published study by our group [44].

In our study we have demonstrated that a decrease in mtDNA leads to a significant reduction in downstream mitochondrial protein synthesis and mitochondrial function in pregnancies complicated with HIV infection and ARV. Correlations were found between genetic and functional mitochondrial parameters demonstrating (*i*) that proper mitochondrial functionalism relies on proper levels of mtDNA copies and (*ii*) the etiopathogenic cause of the dysfunction observed is because of NRTI toxicity and interference in mtDNA replication.

Under pathological conditions, mitochondrion triggers apoptosis. Consequently, any mitochondrial disarrangement may have fatal cell consequences. An important pre-requisite for a successful pregnancy is that the maternal immune system does not reject the foetus and thus, cellular immune response could be essential. Apoptosis has also been shown to play an important role in promoting maternal immune tolerance to paternal antigens expressed by trophoblastic cells [45], which is a physiological process during pregnancy. While apoptosis is thought to be important as a normal physiological feature for foetal or placental development, enhanced levels may also be involved in the pathological conditions. Higher apoptosis levels may have implications in adverse perinatal outcome. A greater incidence of apoptosis has been observed in conditions such as pre-eclampsia and IUGR, suggesting that appropriate regulation of apoptosis is important for normal pregnancy [46].

Blood cells apoptotic activation of caspase-3 increased along pregnancy in both our cohort of HIV-patients under treatment and in controls. In uninfected pregnancies the apoptotic rate of caspase-3 activation probably increases as a physiological mechanism to delete newborn cells from the maternal blood [45]. However, this increase was significantly enhanced in HIV pregnancies in concordance with accumulated, previous NRTI exposure. Apoptosis of uninfected cells is a key element of HIV pathogenesis and is believed to be the driving force behind the selective depletion of $CD4^+$ T cells leading to immunodeficiency. We found that HIV infection and ARV have a significant impact on apoptotic activation of PBMC's caspases. In the first trimester of gestation HIV-positive women showed higher levels of PBMC apoptotic caspase-3 activation specially in those with longer exposure to HIV or NRTI. Additionally, at delivery, HIV-infected women longer exposed to NRTI showed higher levels of PBMC caspase-3 activation. These results are in agreement with previously published work [38].

However, we did not find any association between mtDNA content, mitochondrial protein synthesis, mitochondrial function or apoptotic caspase activation and adverse perinatal outcome in HIV patients. We found an increased prevalence of adverse pregnancy events in HIV pregnancies and enhanced trends towards mitochondrial impairment and apoptotic caspase activation along pregnancy in accordance to HIV or NRTI exposure, but did not find a significant association between these molecular findings and poor obstetric outcome. We did not find the presence of different patterns of mitochondrial toxicity and apoptotic caspase activation in HIV pregnancies correlating with a distinct pattern of clinical expression, probably because of the small sample size, which made patient stratification and statistical findings difficult. Other constraints of this study may be the presence of different types of HAART and time-exposure to HIV or ARVs, characteristic of observational studies and personalized treatment interventions, which on the other hand may enclose findings to reality. The inclusion of a control group of non-pregnant HIV-infected and treated women may have been useful to assess longitudinal mitochondrial and apoptotic toxicity of HIV and HAART without potential gestation interference. However, such studies are extensively documented in the bibliography [47] and, additionally, the interest of the present work was focused on obstetric problems and, thus, in pregnant women. Additionally, to overcome methodological pitfalls and strength, the significance of reported findings, the deregulation of bioenergetics capacity or apoptotic status in studied patients was assessed, in parallel, by Western Blotting and functional measures, which rendered similar results. However, detailed mechanistic pathways underlying HIV- and HAART-associated toxicity in HIV pregnancies should be further elucidated.

In conclusion, although pathogenetically plausible with these findings, it is not yet possible to prove a cause–effect association of adverse perinatal outcome with mitochondrial and apoptotic toxicity of HIV and NRTIs exposure during pregnancy. As a consequence, we

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did not succeed in the secondary objective of this study; identify epidemiological risk factors and prognostic markers of mitochondrial toxicity or apoptotic caspase activation for potentially associated poor clinical results in HIV-infected mothers.

Several groups have proposed that monitoring possible markers of mitochondrial dysfunction in peripheral blood of pregnant women may be useful for detecting preclinical NRTI toxicity. Furthermore, prospective studies in HIV pregnancies under HAART are needed, to determine whether the incidence of mitochondrial disorders differs according to the regimen used and to develop predictive models to identify mothers–infants at highest risk. In an era of expanding treatment options, minimizing toxicities is possible and necessary. The role of mitochondria and apoptosis in physiological conditions must also be clarified to define the relevance of mitochondrial or apoptotic alterations in HIV pregnancies.

As a proof-of-concept, the present study has been conducted in a single-site centre and in a limited population. The short- and long-term health consequences of mitochondrial toxicity and apoptosis in HIV pregnancies should be further investigated in larger cohorts. There is a crucial need to fully understand the scope and depth of this problem through continued basic and clinical research evaluating the effects of foetal and maternal ARV exposure to better understand the morbidity associated with mitochondrial toxicity or apoptosis in pregnant women exposed to HIV and HAART. The current challenge is to design new ARV schedules with reduced harmful mitochondrial and apoptotic effects.

Acknowledgements

This study has been funded by Fundación para la Investigación y la Prevención del SIDA en España (FIPSE 360745/09 and 360982/10), Suports a Grups de Recerca de la Generalitat de Catalunya (2014/SGR/376), Fundació Cellex, Fondo de Investigación sanitaria (FIS PI14/00005, PI15/00903 and PI15/00817) and InterCIBER PIE1400061, granted by ISCIII and Fondo Europeo de Desarrollo Regional (FEDER), and CIBER de Enfermedades Raras (CIBERER, an initiative of ISCIII). We thank the collaboration and the valuable help of Ester Tobias and Mireia Nicolàs as laboratory technicians, Donna Pringle for language assistance and we are especially in debit to all the participants.

Conflict of interest

None of the above mentioned authors have any financial, consultant, institutional and other relationship that might lead to bias or a conflict of interest for the information contained on the present manuscript.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Western Blotting results of COXII, COXIV, Caspase3 and β-actin in HIV-infected and treated or uninfected pregnant women (patients and controls, respectively) at first trimester of gestation (1T) and at delivery (D).

Table S1 Raw data of mitochondrial DNA, protein synthesis, mitochondrial respiratory chain activity of complex II+III and apoptotic rate of caspase-3 activation in HIV-infected and treated or uninfected pregnant women.

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Journal of Cellular and Molecular Medicine published by John Wiley & Sons Ltd and Foundation for Cellular and Molecular Medicine.

Decreased Mitochondrial Function Among Healthy Infants Exposed to Antiretrovirals During Gestation, Delivery and the Neonatal Period

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Background: Antiretroviral (ARV)-associated mitochondrial toxicity in HIV/ARV-exposed healthy infants is a concern. Clinically relevant toxicity is rare. Hyperlactatemia is common but nonspecific, both increased and decreased mitochondrial DNA (mtDNA) level has been reported. Mitochondrial function has scarcely been investigated.

Methods: In a prospective observational study of 133 HIV/ARV-exposed infants, mtDNA content was measured with quantitative real-time polymerase chain reaction, and mitochondrial respiratory chain enzymatic activity of complex IV (CIV) and mitochondrial mass (MM) were assessed spectrophotometrically from cryopreserved peripheral blood mononuclear cells obtained at 6 weeks and 3, 6 and 12 months of age and compared with a control group.

Results: Most mothers (88%) received combined ARV therapy during pregnancy, and 92% of infants received zidovudine monotherapy. No infant had clinical evidence of mitochondrial disease during follow-up. Nonsignificant higher MM and lower mtDNA levels (normalized by MM) were observed over time in HIV/ARV-exposed infants. MM-normalized CIV activity was consistently lower in HIV/ARV-exposed children than in controls over time (0.09 vs. 0.35, 0.12 vs. 0.38, 0.13 vs. 0.24 and 0.14 vs. 0.24 nmol/min/mg at 6 weeks and 3, 6 and 12 months; $P = 0.014$, $P < 0.0001$, $P = 0.065$ and $P = 0.011$, respectively) and showed a linear trend toward normalization with age ($P < 0.01$). In HIV/ARV-exposed infants, an inverse correlation between CIV activity and mtDNA levels was observed until 6 months of age ($r = -0.327$, $P = 0.016$; $r = -0.311$, $P = 0.040$ and $r = -0.275$, $P = 0.046$).

Conclusions: Mitochondrial-encoded CIV activity was consistently lower among HIV/ARV-exposed healthy infants and inversely correlated with mtDNA levels, suggesting upregulation of the latter.

Accepted for publication March 18, 2015.

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Presented at the 18th Conference on Retroviruses and Opportunistic Infections (CROI), February 2011, Boston, MA (poster board 750).

Supported by the Fundación para la Investigación y la Prevención del SIDA en España (grant numbers FIPSE 36612/06 and FIPSE 360982/10); Fundació Cellex, Fondo de Investigación Sanitaria (grant numbers FIS 00462/11, FIS 01199/12, FIS01738/13 and FIS 01455/13); Suports a Grups de Recerca de la Generalitat de Catalunya (grant numbers SGR 14/376 and 14/505) and CIBER de Enfermedades Raras (CIBERER, an initiative of ISCIII).

The authors have no conflicts of interest to disclose.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (www.pidj.com).

ISSN: 0891-3668/15/3412-1349

DOI: 10.1097/INF.0000000000000894

Key Words: antiretrovirals, HIV-exposed healthy infants, mitochondrial DNA, mitochondrial function

(*Pediatr Infect Dis J* 2015;34:1349–1354)

The routine use of highly active antiretroviral (ARV) therapy (HAART) in HIV-infected pregnant women has led to a dramatic decrease in mother-to-child HIV transmission rates to below 1%–2%. Recommended HAART regimens during pregnancy include a dual nucleoside reverse transcriptase inhibitor (NRTI) backbone together with a third drug, usually a protease inhibitor or a non-NRTI. HIV-exposed infants routinely receive zidovudine (ZDV) prophylaxis for the first 4–6 weeks of life.[1–3] NRTIs are known to inhibit both HIV reverse transcriptase and human DNA-γ-polymerase, an endogenous enzyme dedicated to the replication and repair of the mitochondrial DNA (mtDNA) genome.[4–6]

Several adverse effects have been attributed to NRTI-related mitochondrial toxicity in adult and pediatric HIV-infected patients chronically receiving these drugs.[7–11] Genetic and biochemical findings consistent with mitochondrial injury have also been described in HIV/ARV-exposed healthy children in European and American cohorts, ultimately leading to infrequent clinical syndromes similar to those of inherited mitochondrial diseases.[12–14] Smaller studies have reported hyperlactatemia rates ranging from 48% to 92%, with infants generally symptom free and showing a trend toward normalization during the first year of life.[15,16]

With regard to mtDNA content in the peripheral blood mononuclear cells (PBMCs; lymphocytes and monocytes), some studies have shown mtDNA depletion in these patients,[17,18] whereas others have reported increased mtDNA levels when compared with ARV-unexposed controls.[19–21] No differences were observed between HIV/ARV-exposed patients and healthy controls in mtDNA deletions or mitochondrial haplotypes,[22] mitochondrial RNA content[20] or telomere length.[17,23] Conversely, AC/TG mtDNA mutations were more common in both HIV-infected mothers and their uninfected infants, raising concern about long-term outcomes, because these mutations have been associated with aging and age-associated diseases.[24]

We recently demonstrated significant decreases in the mitochondrial protein synthesis rate and mitochondrial respiratory chain (MRC) enzyme activity in maternal and umbilical cord blood PBMCs from mother–child pairs exposed to ARV compared with healthy controls.[25] Interestingly, only MRC complex II function was preserved, because this is independent of mtDNA depletion. These results are in line with previous studies showing mitochondrial dysmorphology, mtDNA depletion and altered MRC function in placenta and umbilical cord PBMCs from animal models[26] exposed to NRTIs and also in ARV-treated HIV-infected pregnant women.[21,27,28] Two recent studies failed to demonstrate differences in MRC function between HIV/ARV-exposed and

unexposed neonates, assessed by means of the complex II:IV ratio of cytochrome c oxidase in frozen neonatal PBMCs obtained within the first 48 hours of life.[19,21] However, no data are available on MRC function over the first months of life, a critical period of time in terms of energy requirements, when an MRC dysfunction would represent an intermediate step in the pathogenic pathway between NRTI-associated mtDNA damage and the potential development of hyperlactatemia and clinical disease in HIV/ARV-exposed healthy infants. We hypothesized that NRTI-related mtDNA depletion would lead to mitochondrial dysfunction in the first year of life in HIV-uninfected infants who were exposed to ARV during gestation, birth and the neonatal period.

MATERIALS AND METHODS

We conducted a prospective observational study of children born to HIV-infected mothers who were followed up in the outpatient clinic of a single tertiary care pediatric center in Barcelona, Spain. From January 2000 to December 2012, 296 mother–child pairs were enrolled in the cohort, and the HIV mother-to-child HIV transmission rate was 2.03% (95% confidence interval: 0.97–3.09); all infants born from January 2000 to May 2005 were eligible for this particular study. As per protocol, informed consent was obtained, and demographic, clinical and laboratory data were routinely collected on all mother–child pairs at enrollment. A clinical interview and a complete physical examination were performed at every visit (at birth, at 2–3 and 6 weeks, at 3, 6 and 12 months of age and yearly thereafter until 18 years of age). Complete blood count and serum biochemistry were obtained together with whole blood proviral HIV-DNA (Amplicor HIV, Roche, Basel, Switzerland) until 2004 and HIV-RNA load quantification (CA HIV-1 Monitor, Roche; limit of <50 copies/mL) thereafter at every visit during the first year of life. Uninfected HIV status beyond the age of 12 months was always confirmed with an HIV antibody test.

Infants were eligible for the study when vertically transmitted HIV infection was ruled out; they had been exposed to ARV during gestation, labor and/or the neonatal period and they did not present any other medical condition that might lead to mitochondrial dysfunction (eg, congenital heart defects or inborn metabolism errors). A control group of age- and gender-matched healthy infants referred to our laboratory for routine blood analysis before elective minor pediatric surgery was used. For ethical reasons, control infants provided a single blood sample each. Study-specific informed consent was obtained from parents or legal guardians, and local ethical committee approval was given.

For this particular study, PBMCs were obtained from 3–5 mL of venous blood at 6 weeks and at 3, 6 and 12 months of age, together with results of routine clinical laboratory tests. PBMCs were isolated on Percoll discontinuous gradients after blood sedimentation in dextran.[29] This methodology allows platelet decontamination. Isolated PBMCs were cryopreserved at −80°C and were used to assess the following mitochondrial parameters. Mitochondrial mass (MM) was estimated by citrate synthase (CS) activity (in nmol/min/mg of protein), an enzyme located in the mitochondrial matrix and which acts in Krebs cycle and which is considered a reliable marker of mitochondrial content.[30,31] As a representative of replication efficiency, mtDNA content was quantified with quantitative real-time polymerase chain reaction (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals®, Germany), separately analyzing a fragment of the highly conserved ND2 mitochondrial-encoded (*mtND2*) gene and a sequence of the housekeeping nuclear-encoded RNA polymerase II gene (*nRPII*).[32] The results were expressed as the ratio between *mtND2* and *nRPII* genes. As an indicator of functional efficiency, MRC enzymatic activity of complex IV (CIV) was measured spectrophotometrically (in nmol/min/mg of protein).[33] All absolute measurements were corrected with MM to exclude any influence of mitochondrial proliferation or reduction in such values. This correction was performed by dividing mtDNA content and CIV activity by CS activity. In case of insufficient PBMC sample, the determination of CIV enzymatic activity was prioritized. The evolution over time of venous blood lactate levels obtained at the same time points in this population had already been published and was also used as a marker of mitochondrial function.[15]

Other variables that were relevant to this study included mother's age and ethnicity, history of AIDS, hepatitis C virus coinfection, third-trimester HIV RNA viral load and CD4 T-lymphocyte cell count, type and timing of ARV therapy, a history of substance abuse during pregnancy, mode of delivery, intrapartum use of ZDV, infant's sex, Apgar scores, gestational age at birth and birth weight, type and timing of exposure to ARV during the neonatal period and infant's hepatitis C virus status.

Statistical Analysis

Qualitative variables were expressed as percentages, whereas quantitative variables were expressed as mean/median values and standard deviation/range. Normality of data was ascertained with Kolmogorov–Smirnov test. Comparisons between groups were carried out using the Student *t* test, and the association between continuous variables was assessed by means of Pearson correlation test. Nonparametric tests were used as appropriate. All tests were 2 tailed, and a *P* value lower than 0.05 was considered significant. Statistical analysis was performed with the Statistical Package for the Social Sciences version 18.0 (SPSS, Chicago, IL).

RESULTS

The final study group consisted of 133 consecutive HIV-uninfected ARV-exposed infants born to 126 HIV-infected mothers (including 7 sets of twins) from January 2000 to May 2005; samples from 73 healthy control infants were available for the 4 different timepoints. Previously 5 HIV-exposed children had been excluded because of HIV infection (n = 1), congenital heart defects (n = 2) and extreme prematurity (n = 2). Data regarding gestation, birth and neonatal clinical variables of the HIV/ARV-exposed group are summarized in the Table, Supplemental Digital Content 1, http://links.lww.com/INF/C251. In HAART-treated mothers, the mean/median duration of HAART during pregnancy was 29.6/36 weeks (range: 2–40 weeks). Oral ZDV was implemented in most of the neonates at birth; 11 infants (8.3%) received combination ARV prophylaxis because of late diagnosis of maternal HIV infection or uncontrolled viral replication at delivery. Individual and cumulative gestation and total exposure to NRTIs of infants included in the study are summarized in Table 1. None of the ARV-exposed infants developed clinical signs or symptoms consistent with mitochondrial disease during follow-up.

HIV/ARV-exposed infants showed higher nonsignificant mean values in MM (estimated by CS) when compared with controls: 101.9 versus 66.7, 85.1 versus 65.8, 94.7 versus 67.3 and 100.3 versus 93.3 nmol/min/mg of protein, at 6 weeks and 3, 6 and 12 months, respectively (*P* = NS in all cases). Mean mtDNA content (mtND2:RPII ratios normalized by CS) was nonsignificantly lower in HIV/ARV-exposed infants when compared with controls over time: 0.009 versus 0.011, 0.010 versus 0.016, 0.008 versus 0.020 and 0.010 versus 0.014 at 6 weeks and 3, 6 and 12 months, respectively (*P* = NS in all cases). Because of the low number of samples available for mtDNA determination in the control group (6, 8, 12 and 8 samples at 6 weeks and 3, 6, and 12 months of age, respectively), these results should be approached with caution.

TABLE 1. Individual and Cumulative Gestational Exposure to Different Nucleoside Reverse Transcriptase Inhibitors and Tenofovir (in Weeks)

Drug	n	Median	Range
Zidovudine (ZDV)	96	26	2–40
Lamivudine (3TC)	125	35	2–40
Stavudine (d4T)	35	37	2–39
Didanosine (ddI)	18	37	11–40
Abacavir (ABC)	4	27	17–37
Tenofovir (TDF)	2	38.5	37–40
Gestational cumulative exposure to different NRTIs	127	66	4–111
Prenatal and postnatal cumulative exposure to different NRTIs	133	54	6–117

Note that some children were exposed to >2 drugs because of changes in maternal HAART regimens during pregnancy.

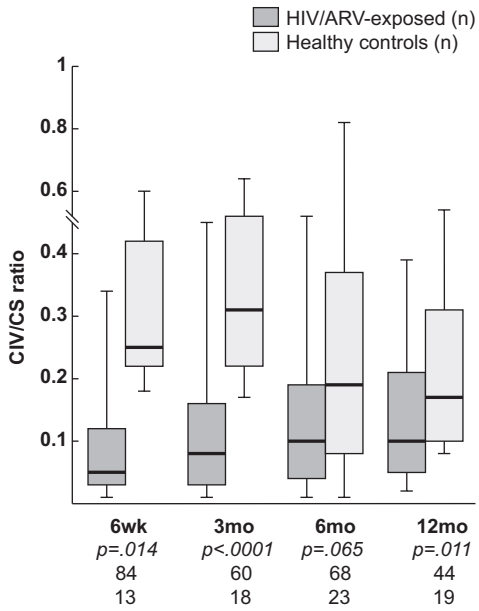

FIGURE 1. Evolution over time of mitochondrial respiratory CIV enzymatic activity expressed as nmol/min/mg of protein normalized by mitochondrial mass estimated by CS activity in HIV/ARV-exposed infants and healthy controls. Solid line represents the median, the box represents the interquartile range and the range is indicated by a vertical bar.

Conversely, MM-normalized mean CIV enzymatic activities were consistently lower in HIV/ARV-exposed infants at all time points: 0.09 versus 0.35 ($P = 0.014$), 0.12 versus 0.38 ($P < 0.0001$), 0.13 versus 0.24 ($P = 0.065$) and 0.14 versus 0.24 ($P = 0.011$) nmol/min/mg of protein per mitochondrion at 6 weeks and 3, 6 and 12 months, respectively (Fig. 1). Among HIV/ARV-exposed patients, CIV enzymatic activity showed a linear trend toward normalization with age ($P < 0.01$) and an inverse correlation with mtDNA levels up to the age of 6 months (r = −0.327, $P = 0.016$ at 6 weeks; r = −0.311, $P = 0.040$ at 3 months and r = −0.275, $P = 0.046$ at 6 months), but not at 1 year of age (r = −0.271, $P = 0.211$; Fig. 2). CIV enzymatic activity and mtDNA levels did not correlate with mean lactate levels (2.88, 2.78, 1.89 and 1.71 mmol/L at 6 weeks and 3, 6 and 12 months, respectively; normal range: 0.77–2.44 mmol/L) at any of the timepoints.

Neither CIV activity nor mtDNA levels were associated with the rest of the baseline maternal and infant variables, including maternal age, AIDS, ethnicity, hepatitis C virus coinfection, exposure to other drugs during gestation, antepartum CD4 T-lymphocyte cell count and HIV viral load, gestational age at delivery, infant gender, birth weight and Apgar score. When exposure to ARV during gestation was taken into account, neither the type (individual drugs or regimens; Fig. 3) nor the timing of exposure (to an individual drug or the accumulated exposure to different NRTIs) was associated with differences in mtDNA levels or CIV activity, except for the maternal use of ZDV, which was associated with lower mean mtDNA levels at different timepoints: 0.96 versus 1.28 ($P = 0.027$), 0.91 versus 1.15 ($P = 0.071$), 0.77 versus 1.09 ($P = 0.002$) and 0.91 versus 1.28 ($P = 0.037$) mmol/L at 6 weeks and 3, 6 and 12 months, respectively.

DISCUSSION

This is the first study to longitudinally assess mitochondrial function in a cohort of HIV/ARV-exposed healthy infants. Mitochondrial function has been very scarcely investigated in this population. To date, only 2 cross-sectional studies have analyzed the COX II (encoded by mtDNA):COX IV (encoded by nuclear DNA) ratio, 2 subunits of MRC cytochrome c oxidase, in PBMCs obtained within 48 hours of delivery. The authors of both studies[19,21] reported nonsignificant lower mitochondrial function in infants exposed to ARV (81 and 20 HIV/ARV-exposed infants, respectively) when compared with controls (48 and 26 control infants). Interestingly, both studies observed significantly higher mtDNA content in these neonates as well.[19,21]

Like the other authors,[19,21] we used the partially mitochondrially encoded CIV to assess mitochondrial function in frozen PBMCs and then adjusted the enzymatic activity results with MM to correct for possible differences in the absolute number of mitochondria per cell. As hypothesized, we observed lower CIV values in HIV/ARV-exposed children at all timepoints when compared with healthy controls. Our data nicely depict a reversible mitochondrial dysfunction that tends to gradually normalize over the first year of life, and they are in agreement with the previously reported inverse evolution in the plasma lactate levels (from higher to normal values) in the same cohort of patients,[15] although lactate levels and CIV activity values did not correlate in this study. In spite of these findings, none of the patients in our study developed signs or symptoms consistent with mitochondrial dysfunction. Why only a very small percentage of these children develop clinical symptoms may be at least partially related to the mitochondrial threshold effect.[4] Of note, CIV activity values were still significantly lower in ARV-exposed infants at the age of 12 months, underscoring the fact that early mitochondrial toxicity may persist until later in life and should always be kept in mind by physicians caring for these children.[34,35] In fact, median age at clinical onset in the 2 largest case series of mitochondrial disease in HIV/ARV-exposed healthy children was 7[12] and 16 months.[14]

We observed nonsignificant lower mtDNA levels in HIV/ARV-exposed infants than in controls. In previous studies, conflicting results were reported, with some studies showing mtDNA depletion[17,18] and others reporting increased mtDNA content when compared with ARV-unexposed controls.[19–21] Methodological differences regarding how mtDNA results were reported, methods for PBMC isolation, platelet contamination, timing of blood sampling and type and duration of ARV exposure may explain these contradictory results. In our study, we were able to assess mtDNA content longitudinally in a large cohort of HIV/ARV-exposed infants, PBMCs were free of platelets and mtDNA levels were normalized to MM; unfortunately, the number of mtDNA determinations

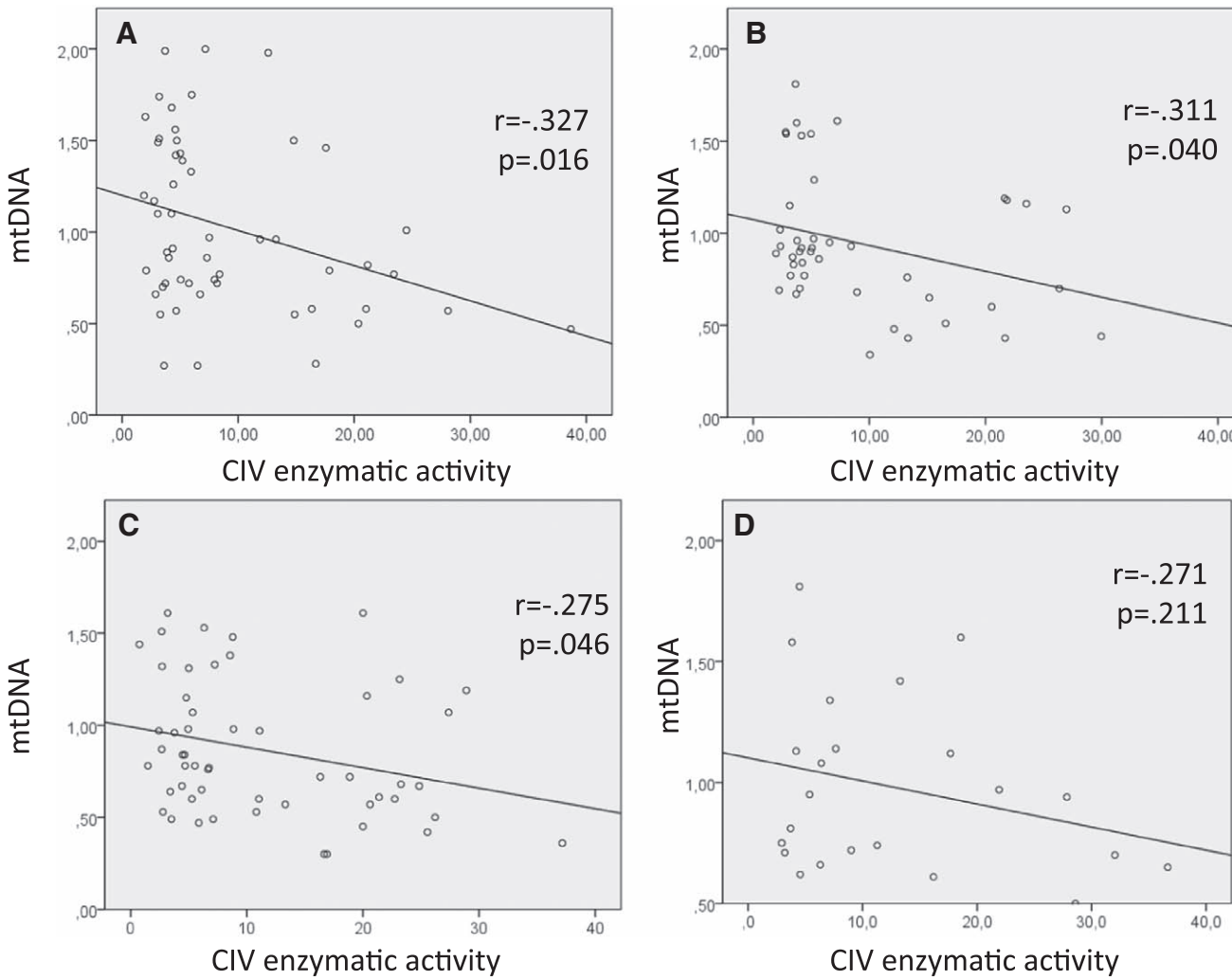

FIGURE 2. Charts showing inverse correlation between mtDNA (y-axis; mtND2:nRPII ratio, absolute values) and CIV enzymatic activity (x-axis; nmol/min/mg of protein, absolute values) in HIV/ARV-exposed children at (A) 6 weeks and (B) 3, (C) 6 and (D) 12 months of age.

in the control group was very low, and this may have hampered our results. In any case, mtDNA levels remained fairly stable (0.009, 0.010, 0.008 and 0.010 mtND2:RPII ratio normalized by CS at 6 weeks and 3, 6 and 12 months of age, respectively) in the HIV/ARV-exposed population and did not show an increasing or decreasing trend over time, as opposed to CIV enzymatic activity. These results are consistent with those reported by Aldrovandi et al[18] in a cohort of 411 healthy HIV-unexposed pediatric patients aged 0–18 years, in whom PBMC mtDNA levels did not show age-related differences.

The mitochondrial toxic effect derived from HIV itself has been well demonstrated in ARV-naïve HIV-infected patients but cannot be directly invoked in the ARV-exposed HIV-uninfected child. Therefore, in this population, other explanations for mitochondrial dysfunction are needed, including NRTI-related toxicity but also possible NRTI-unrelated mechanisms, especially mitochondrial toxicity affecting the maternal tissues associated with fetal development, such as placenta and cord blood.[19,26,28,36,37] It is unlikely that this phenomenon can be explained solely by a single mechanism; rather it is the end result of several causes and pathogenic pathways.

Leaving aside the controversial results in mtDNA content when compared with controls, several recent studies suggest that an increase in mtDNA content initially counteracts mitochondrial toxicity in the HIV/ARV-exposed infant. Higher mtDNA content in PBMCs has been reported in this population,[19–21] and, importantly, Aldrovandi et al[18] reported lower mtDNA content in ARV-unexposed infants born to HIV-infected mothers when compared with patients exposed to ZDV monotherapy during gestation, as well as a further increase in those exposed to HAART; moreover, the longer the exposure, the greater the effect on mtDNA.[18,20] Albeit weak, the inverse correlation between CIV enzymatic activity and mtDNA we observed up to the age of 6 months supports this hypothesis and is consistent with very similar findings reported by Brogly et al[22] in a group of 18-month-old HIV/ARV-exposed children who later developed clinical signs of mitochondrial dysfunction. Instead, in the absence of ARV exposure, mtDNA content and mitochondrial function would directly correlate;[19] unfortunately, we could not verify this because of the low numbers.

Although mtDNA content remained stable over time, MM was higher at all timepoints in the HIV/ARV-exposed group, although differences were not statistically significant. This supports

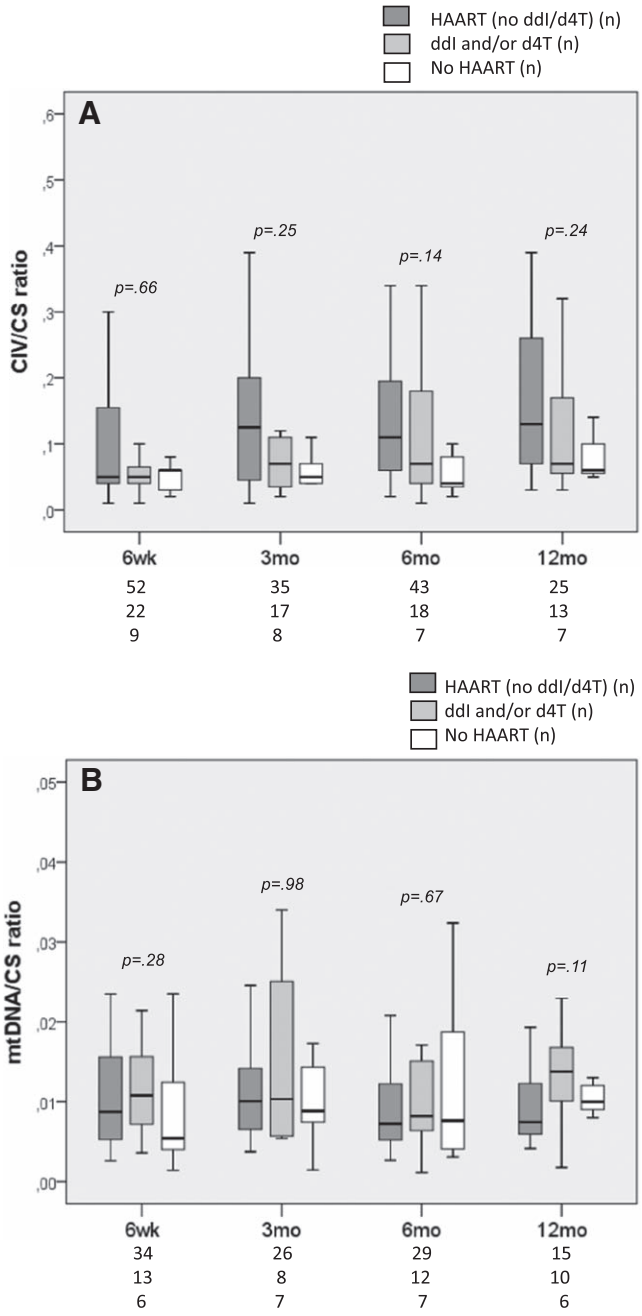

FIGURE 3. CIV enzymatic activities (A; expressed as nmol/min/mg of protein normalized by CS, median values) and mtDNA values (B; expressed as mtND2:nRPII ratios normalized by CS, median values) at all timepoints according to the maternal antiretroviral regimen during gestation [no HAART, no antiretrovirals or zidovudine monotherapy; didanosine (ddI) and/or stavudine(d4T)-based HAART regimens and other HAART regimens]. Solid line represents the median; the box represents the interquartile range and the range is indicated by a vertical bar.

the hypothesis that the increase in mtDNA content reported by others is driven by an increase in the absolute number of mitochondria in PBMCs, rather than by an increase in mtDNA replication, as previously suggested.[21] Actually, this phenomenon is also observed in congenital respiratory chain disorders, and subsarcolemmal mitochondrial accumulations remain a minor diagnostic criteria for these disorders in patients younger than 16 years.[38]

Our observational study has several limitations. First, the number and volume of cryopreserved samples was insufficient to complete planned analysis, especially among controls, and samples from birth were not available. Second, for ethical reasons, a group of ARV-unexposed infants born to HIV-infected mothers was not available, while control healthy patients provided a single sample, potentially leading to higher interindividual variability. Some of the ARVs that the mothers in the study received are no longer recommended, such as didanosine, stavudine and nelfinavir. Current HAART regimens include many new ARVs that were not being used at the time the samples were collected. Newer NRTIs show a much lower propensity to cause mitochondrial toxicity.[39,40]

In summary, our results show for the first time the decreased mitochondrial function that tends to normalize over the first year of life in HIV/ARV-exposed healthy children. The inverse correlation between mtDNA and CIV activity values suggests an upregulation of the former, although the pathogenic pathway for this remains unclear. Further studies are needed to better characterize mitochondrial toxicity in HIV/ARV-exposed healthy children, with special attention paid to the long-term follow-up of this population into adulthood.

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J Antimicrob Chemother 2015; **70**: 2330–2336
doi:10.1093/jac/dkv101 Advance Access publication 28 April 2015

Journal of Antimicrobial Chemotherapy

Mitochondrial and apoptotic *in vitro* modelling of differential HIV-1 progression and antiretroviral toxicity

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Received 12 November 2014; returned 25 January 2015; revised 5 March 2015; accepted 22 March 2015

Objectives: *Ex vivo* analysis of mitochondrial function may reveal HIV progression and the impact of ART. We propose a mitochondrial and apoptotic *in vitro* model using Jurkat T cells incubated with plasma. The objectives of this study were to evaluate mitochondrial and apoptotic lesions in this model in relation to HIV progression, and to assess the effect of >1 year of standard non-thymidine-containing therapy.

Methods: This was a cross-sectional comparison among three age- and gender-matched groups ($n=19\times3$): healthy non-HIV-infected participants, HIV-infected long-term non-progressors (LTNPs) and standard antiretroviral-naive chronically infected patients [standard progressors (Sps)], longitudinally evaluated before (Sp1) and after (Sp2) >1 year of efavirenz+tenofovir+emtricitabine therapy. We analysed mitochondrial DNA content by RT–PCR, mitochondrial function by spectrophotometry, mitochondrial protein synthesis by western blot analysis, mitochondrial dynamics by western blot analysis (MFN2), apoptotic transition pore formation by western blot analysis (VDAC-1) and mitochondrial membrane potential and annexin V/propidium iodide fluorescence by flow cytometry.

Results: There was a decreasing non-significant trend towards lower mitochondrial parameters for HIV-infected values with respect to uninfected control reference values. HIV progression (LTNP versus Sp1) was associated with decreased mitochondrial genetic, functional and translational parameters, which partially recovered after treatment intervention (Sp2). Mitochondrial fusion showed a trend to decrease non-significantly in Sp patients compared with LTNP patients, especially after therapy. All apoptotic parameters showed a trend to increase in Sp1 with respect to LTNP, followed by recovery in Sp2.

Conclusions: We proposed an *in vitro* model for mitochondrial and apoptotic assessment to test the effects of HIV infection and its therapy, resembling *in vivo* conditions. This model could be useful for clinical research purposes.

Keywords: *in vitro* model, HIV progressors, mitochondrial function, apoptosis

Introduction

Under no treatment, most HIV-infected patients show a progressive decrease in CD4+ T cell counts over time that is inversely related to plasma HIV RNA. ART suppresses HIV replication and prevents CD4+ T cell loss. A small subset of HIV-infected patients (1%–5%), named long-term non-progressors (LTNPs), spontaneously maintain undetectable HIV RNA in plasma and their CD4 cell counts may be preserved over time without antiretroviral (ARV) intervention.[1]

HIV may cause mitochondrial damage and promote apoptosis. Different degrees of evidence have been found in *in vitro*, cell and

animal models but also in the *ex vivo* analysis of different tissues of infected patients, including PBMCs.[2,3]

Given that (i) T lymphocytes are responsible for the immune defence capacity of the organism and that these cells constitute the main target cells for viral replication, consequently acting as both rescuers and prey for HIV infection, and (ii) mitochondria are the main organelles responsible for enhancing or triggering cell apoptosis, the hypothesis that mitochondrial status within T lymphocytes may determine differential HIV progression seems plausible. Some studies demonstrate that mitochondrial disturbances induced by HIV may lead to the death of T cells and promote faster progression of HIV infection. One of these studies demonstrated that the mitochondrial and apoptotic lesions in PBMCs of LTNPs were reduced with respect to naive standard progressor (Sp) subjects,[4] demonstrating variability of both phenomena depending on the course of infection: LTNPs presented intermediate mitochondrial and apoptotic alterations with respect to uninfected healthy volunteers (lacking any lesion) and naive Sp subjects (presenting the highest level of mitochondrial and apoptotic alterations).[4]

Several later studies indicated that mitochondria are important agents of differential progression of HIV infection. For instance, while some studies have failed to demonstrate that genetic variants in nuclear-encoded mitochondrial genes influence AIDS progression,[5] others have reported that certain mitochondrial DNA (mtDNA) haplogroups (specifically J and U5A) influence AIDS progression[6] and specific disorders related to HIV infection.[7]

ART may at least partially restore mitochondrial function by controlling HIV replication. However, some ARV agents may have mitochondrial toxicity. Although the capacity of the formerly used thymidine NRTIs to cause mitochondrial lesions is well established[8–11] and has been postulated to be responsible for several adverse effects,[12–14] it remains unknown whether therapies that do not include non-thymidine-NRTIs are safer. Even though the evolution of less-toxic drugs is the principal aim of the scientific, clinical and pharmaceutical communities and current ARVs seem to be safer for the patient in most recent schedules, further information regarding the details of the mitochondrial toxicity associated with such combination treatments is needed. It is still unknown whether the current therapy combinations, such as ARV regimens including the two NRTIs tenofovir and emtricitabine and the NNRTI efavirenz are safer than those administered in the past.[15] It is not only NRTIs that have been associated with mitochondrial toxicity and secondary effects of medication, since controversial results have also been observed regarding the NNRTI efavirenz, which has been associated with cell apoptosis,[15–18] although to a lesser extent than NRTI-derived agents.

Currently, there is no gold standard test to assess mitochondrial function in HIV-infected patients. Mitochondrial DNA (mtDNA) has been widely used, but the method requires cells and provides limited information on mitochondrial function.

We propose an *in vitro* model of mitochondrial analysis using Jurkat T cells incubated with plasma. Plasma samples can be easily obtained and preserved until assessment. We evaluated the results obtained in groups of different subjects according to whether they were HIV-infected, and, if HIV-infected, according to whether HIV replication was controlled either spontaneously or by a standard ARV regimen.

The present study aimed to: (i) establish an *in vitro* culture model for study based on the capacity of the plasma of different types of progressor patients to induce mitochondrial and apoptotic lesions; (ii) investigate the HIV-derived molecular mechanisms of mitochondrial and apoptotic lesions in the model in relation to the differential progression of HIV infection (LTNP versus Sp); and (iii) determine the mitochondrial and apoptotic effects of current therapeutic schedules (tenofovir + emtricitabine + efavirenz), which are presumably less toxic than the thymidine NRTI-containing ones in this model.

Methods

Design

This was a multicentric, controlled, cross-sectional and longitudinal study.

Patients

The present work consisted of: (i) a cross-sectional study including plasma samples from non-HIV-infected controls ($n=19$), LTNP patients ($n=19$) and standard ARV-naive progressors (Sp1 group); and (ii) a longitudinal study including plasma samples of the same Sp1 individuals ($n=19$) after >12 months of ART, consisting of two NRTIs (tenofovir + emtricitabine) and one NNRTI (efavirenz) (Sp2 group).

All patients signed an informed consent form previously approved by the ethics committee of each participating centre. Sample recruitment was also approved at each centre and was performed with the collaboration of the infectious diseases departments of four tertiary care hospitals: Hospital General of Granollers (Granollers, Spain), Hospital Universitari Joan XXIII (Tarragona, Spain), Hospital de la Santa Creu i Sant Pau (Barcelona, Spain) and Hospital Clinic of Barcelona (Barcelona, Spain).

Inclusion criteria

A control group, matched for age and gender with the study groups, of healthy non-infected volunteers was included to obtain normal reference values within the general population.

(i) LTNP group: patients presenting asymptomatic HIV infection, lacking opportunistic infections, not undergoing ARV therapy and with long-term non-progression of the disease were considered.[4] Specifically, subjects with >15 years of infection, stable CD4+ T cell counts persistently >500 cells/mm^3 and plasma viral load <5000 copies/mL, in the absence of ARV.
(ii) Sp1 group: HIV-infected patients with standard progression before initiation of ARV (naive) and presenting with CD4+ T cell counts <350 cells/mm^3 and viral load up to 35000 copies/mL.
(iii) Sp2 group: Sp1 subjects after receiving >12 months of tenofovir + emtricitabine + efavirenz ARV.

Exclusion criteria were a personal or family history of mitochondrial or neuromuscular disease or contact with drugs having known or potential toxicity for mitochondria (e.g. aminoglycosides, linezolid or antipsychotics).

Clinical data

Clinical and epidemiological variables of the patients and controls are shown in Table 1.

Model for study

After an overnight fast, 20 mL of whole blood from all patients and controls was collected in EDTA tubes and the plasma was obtained by 15 min of centrifugation at 1500 **g**.

Table 1. Clinical and epidemiological data for patients and controls providing plasma

	Controls	LTNP	Sp1	Sp2	*P*
Sample size (*n*)	19	19	19	19	—
Age, years (mean ± SEM)	41.06 ± 3.98	44.89 ± 3.06	37.58 ± 2.53	38.58 ± 2.53	NS
Male (%)	46.15	47.36	78.94	78.94	NS
ARV schedule	—	—	—	tenofovir + emtricitabine + efavirenz	—
Log_{10} viral RNA, copies/mL (mean ± SEM)	—	2.83 ± 0.25	4.44 ± 0.23	1.62 ± 0.017	<0.05[a]
CD4+ T cell count, cells/mm^3 (mean ± SEM)	—	732.47 ± 55.94	478.00 ± 75.82	628.78 ± 35.22	<0.05[b]

[a]Significantly higher in Sp1 than in LTNP and Sp2.
[b]Significantly higher in LTNP and Sp2 than in Sp1.

Jurkat cells (of an immortalized T lymphocyte cell line from a patient with acute lymphocytic leukaemia) were cultured, grown to 250 000–500 000 cells/mL and incubated for two complete viral replication cycles (16 h), at identical passage numbers, in complete medium [RPMI + 1% (v/v) penicillin/streptomycin], containing 10% (v/v) plasma from the patients and controls. The experimental procedures always included a sample from each study group to be analysed in parallel. Immediately after this incubation, an aliquot of each sample was analysed by flow cytometry, while the remaining material was cryopreserved at −80°C until molecular and biochemical analysis.

Experimental assays

Protein quantification

The BCA assay was used to calculate the total protein cell content (Pierce BCA Protein Assay Kit #23225; Thermo Scientific). Mitochondrial functional parameters were expressed in relation to the total amount of cell protein.

mtDNA levels

To quantify mtDNA content, total DNA was isolated by the phenol–chloroform method and RT–PCR was performed (Applied Biosystems) as reported elsewhere.[19]

Mitochondrial function

Spectrophotometry was performed to assess complex IV (CIV) and cytochrome *c* oxidase (COX) enzyme activity of the mitochondrial respiratory chain (MRC) and mitochondrial content by measuring citrate synthase (CS) enzyme activity.[20–22] MRC enzyme activities were expressed as relative units, normalized for mitochondrial content, estimated by CS activity[23] (COX/CS).

Mitochondrial protein synthesis

Western blot analysis was performed to quantify the mitochondrial-encoded subunit II of COX (COXII) and nuclear-encoded subunit IV of COX (COXIV) of the MRC by using 20 μg of total cell protein with 7%/13% SDS–PAGE and immunodetection with the specific corresponding antibodies.[24]

Mitochondrial dynamics

Western blot analysis was performed to quantify mitofusin-2 (MFN2) relative to β-actin by using 20 μg of total cell protein with 7%/13% SDS–PAGE and immunodetection using the mouse monoclonal anti-MFN2 antibody ab56889 (Abcam®, UK) diluted 1/2000 in 1% milk.

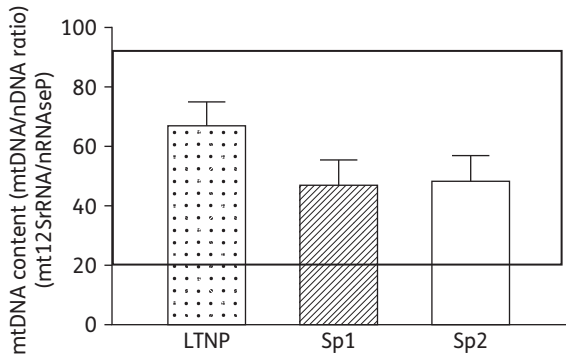

Figure 1. mtDNA content, expressed as the ratio of the mitochondrial 12SrRNA gene with respect to the constitutive nuclear RNAseP gene (mtDNA12SrRNA/nDNARNAseP). The normal range of non-infected controls is shown as a box (mean ± SD).

Apoptotic rate

Quantification of early and late apoptotic rates was performed by using three different measures. First, voltage-dependent anion channel-1 (VDAC-1) protein content relative to β-actin content was assessed by western blot analysis, as previously described.[24] Second, flow cytometry was used to assess mitochondrial membrane potential (MMP) using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethyl benzimidazolo carbocyanine iodide (JC1), by quantifying cells with depolarized mitochondria as a percentage of the total number of cells.[25] Finally, cells double-stained for annexin V and propidium iodide were quantified by flow cytometric analysis as a percentage of the total number of cells, as reported elswhere.[26]

Statistical analysis

Statistical analysis was performed using the Mann–Whitney test for non-parametric variables and statistical significance was set at $P < 0.05$.

Results

Clinical data and participant characteristics

We performed 76 *in vitro* assays to test 19 plasma samples from each study group (healthy controls, LTNP, Sp1 and Sp2) with identical Jurkat T cells.

Men predominated in the Sp1 and Sp2 groups, while in the LTNP and control groups the gender distribution tended to be balanced. As expected, CD4 counts and viral load in HIV-infected patients were different according to case definition (LTNP, Sp1 and Sp2).

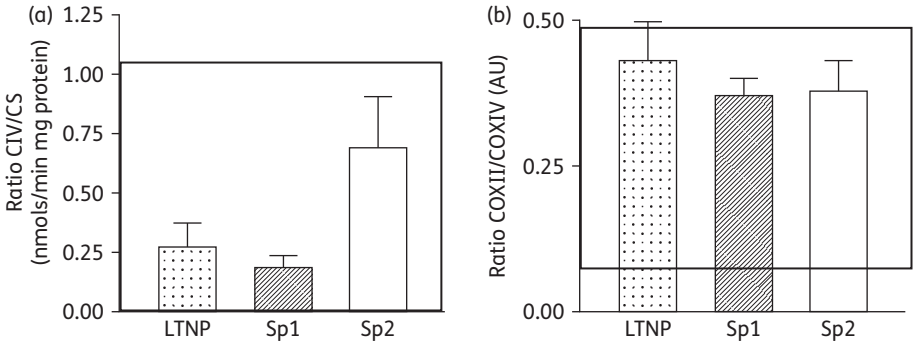

Figure 2. Mitochondrial function and mitochondrial protein synthesis. (a) Mitochondrial function: CIV/CS activity ratio. Relative activity of COX or CIV per mitochondrion (CS). (b) Mitochondrial protein synthesis: COXII/COXIV ratio. Protein synthesis expressed as mitochondrial COXII/COXIV ratio. AU, arbitrary units. The normal range of non-infected controls is shown as a box (mean ± SD).

Mitochondrial parameters

Most of the parameters tended to be consistently lower in HIV-infected patients than the reference values derived from the non-infected control group, although this was not statistically significant.

The mtDNA content was 30% lower in the Sp1 group than in the LTNP group and was 2% higher in the Sp2 group than in the Sp1 group (Figure 1).

Regarding mitochondrial function, the relative value of COX or CIV enzyme activities normalized per mitochondrion (CIV/CS ratio) in the Sp1 group was 58% of the value in the LTNP group and was 146% higher in the Sp2 group than in the LTNP group (Figure 2a).

Mitochondrial protein synthesis, expressed as the COXII/COXIV ratio, was 14% lower in Sp1 than in LTNP and was maintained in Sp2 with respect to the Sp1 level (Figure 2b).

Intergenomic coordination between mitochondrial and nuclear genomes, measured as the correlation between the levels of expression of the mitochondrial-encoded COXII and the nuclear-encoded COXIV, was positive, which was statistically significant in all groups (Figure 3).

Mitochondrial dynamics, measured as MFN2/β-actin expression, gradually decreased, being 24% lower in Sp1 and 44% lower in Sp2 than in LTNP (Figure 4).

Apoptotic parameters

The apoptotic parameter VDAC-1/β-actin content, an indicator of transition pore formation and apoptosis initiation, was 22% higher in Sp1 and 50% lower in Sp2 with respect to the LTNP group. LTNP and Sp2 values were within control reference values (Figure 5). Accordingly, the percentage of cells with depolarized mitochondria with respect to total number of cells, a marker of MMP loss and early apoptosis, was 10% higher in Sp1 and 14% lower in Sp2 with respect to LTNP. Sp2 values were within control reference values (Figure 6).

Finally, the percentage of cells with double staining for annexin V and propidium iodide with respect to the total number of cells, an indicator of advanced apoptotic status, was 8% higher in Sp1 and 1% lower in Sp2 with respect to the LTNP group. Values for all groups were within the control reference values (Figure 7).

In summary, there were no statistically significant differences in most of the mitochondrial and apoptotic parameters depending on HIV progression (LTNP versus Sp1) or treatment intervention (Sp1 versus Sp2). However, there was a trend towards differential levels of mitochondrial and apoptotic lesions, with an increase in the Sp1 group with respect to the LTNP group, and a partial recovery in the Sp2 group. This pattern was consistent for all parameters.

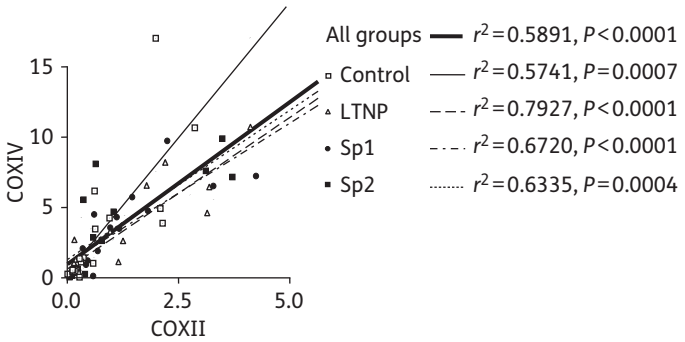

Figure 3. Intergenomic cross-talk. Intergenomic coordination between nuclear and mitochondrial genomes, analysed as the correlation of the mitochondrion-encoded COXII subunit and the nuclear-encoded COXIV subunit. Statistical significance was accepted at $P<0.05$.

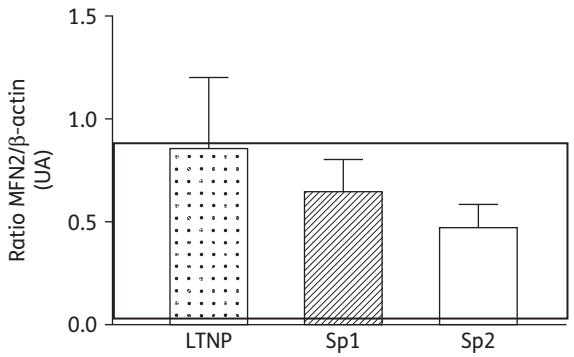

Figure 4. Mitochondrial dynamics. MFN2 content expressed as the MFN2/β-actin ratio. The normal range of non-infected controls is shown as a box (mean ± SD).

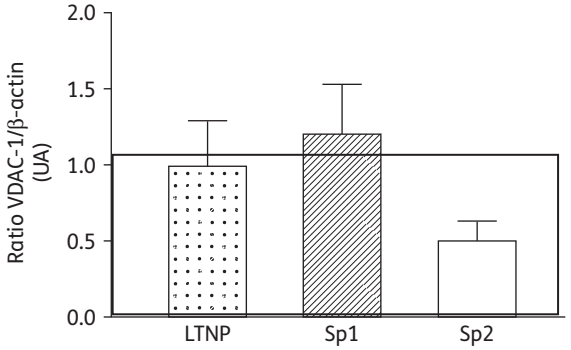

Figure 5. Apoptosis initiation. VDAC-1 content expressed as the VDAC-1/β-actin ratio, as an indicator of mitochondrial transition pore formation for apoptosis initiation. The normal range of non-infected controls is shown as a box (mean ± SD).

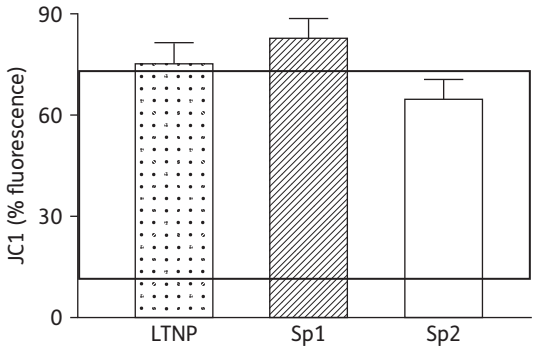

Figure 6. Early apoptosis. Mitochondrial depolarization analysis using the MMP sensor JC1, expressed as the percentage of cells with depolarized mitochondria with respect to the total number of cells, an indicator of early apoptosis. The normal range of non-infected controls is shown as a box (mean ± SD).

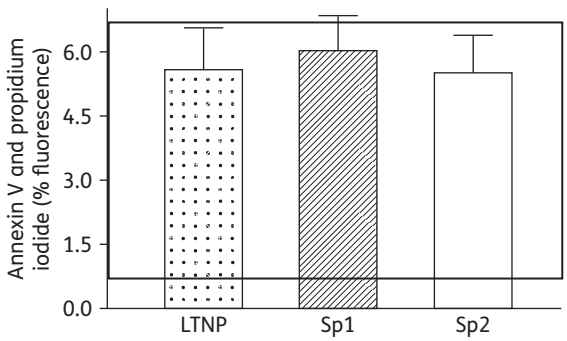

Figure 7. Advanced apoptosis. Apoptosis and cell viability measurement using the markers annexin V and propidium iodide, respectively, expressed as the percentage of cells with double fluorescence for both markers with respect to the total number of cells, an indicator of late apoptosis. The normal range of non-infected controls is shown as a box (mean ± SD).

Discussion

Since the explosion of interest in HIV and ARV toxicity, there has been increasing demand for the establishment of animal or cell models to determine the underlying mechanisms of HIV progression, as well as to test the adverse effects of NRTI and toxic mechanisms.[27] Distinct experimental models have been developed, most of them based on animal experimentation.[28] Consequently, determining the pathophysiological effects of ARV on animals has become important in understanding the risks associated with current therapeutic approaches, in which mitochondrial toxicity is important.[29] However, animal models present serious disadvantages, such as high costs, extensive animal facilities and differential viral progression, immunological and drug toxicity mechanisms, with respect to human models.

Within this framework, we aimed to create an *in vitro* culture model using a Jurkat T cell line incubated with plasma of different HIV progressors, in order to: (i) reproduce differential mitochondrial and apoptotic status in distinct progressions of the infection, according to the findings observed in a previously described *ex vivo* study;[4] (ii) demonstrate whether mitochondria are involved in differential HIV progression; and (iii) establish an *in vitro* platform to test the mitochondrial and apoptotic toxicities of ARV schedules containing non-thymidine NRTIs. The establishment of an *in vitro* model of differential HIV progression would allow the development of strategies for the prevention of cell impairment and for the use of therapeutic assays in a standardized way.

Even though most of the differences in the mitochondrial and apoptotic parameters characterized in the cell model used in the present study did not reach the level of significance, we found a consistent trend towards a common pattern of gradual increase in mitochondrial lesions and apoptotic rates related to faster progression of HIV infection. The proposed culture model using Jurkat cells with plasma from patients partially reproduces the findings observed in *ex vivo* studies,[4] as it consistently shows intermediate mitochondrial lesion and apoptosis rates that are higher in naive Sp patients than in LTNP patients.

The present work advocates a feasible and simple cell culture model in which it is possible to evaluate the mitochondrial and apoptotic toxicity and/or safety of one of the most frequent current ARV combinations used as an initial treatment, consisting of tenofovir + emtricitabine + efavirenz. This model made it possible to detect differential trends of mitochondrial function in the populations studied. In the model, mitochondrial lesions and apoptotic injury promoted by the virus and enhanced in typical progressors versus LTNPs, occurred in Sp2 patients after >12 months of receiving an ARV schedule including tenofovir + emtricitabine + efavirenz. A recent study described depletion in mtDNA and mitochondrial dysfunction in CI and CIV enzyme activities in adipose tissue of HIV-infected patients receiving tenofovir + emtricitabine plus efavirenz or atazanavir/ritonavir,[30] although there was no evidence of any mitochondrial and apoptotic T cell effect associated with >12 months of treatment with tenofovir + emtricitabine + efavirenz. In the proposed *in vitro* study model, the fact that the Sp2 group tended to partially recover its mitochondrial parameters and show a decrease in all apoptotic events supports the concept that this specific ARV schedule seems to be safe with respect to cell viability. A common pattern such as this (observed in mitochondrial genetic and functional parameters as well as in apoptotic events) may point out the association between mitochondrial status and HIV progression, placing mitochondria as a key orchestrator, and the main source of cell apoptotic events, within the T cell population (Jurkat cells in our model).

Secondary conclusions can be extracted from the present study. Interestingly, we noticed in this *in vitro* model that coordination between the nuclear and mitochondrial genomes remained unaltered in all groups despite differential HIV progression or treatment intervention, as our findings showed a significant and positive correlation among the contents of structural protein subunits of the COX enzymes of the MRC, encoded by the different genomes. Thus, this intergenomic interaction is not compromised within different types of HIV progression or an ARV consisting of tenofovir+emtricitabine+efavirenz. Additionally, mitochondrial dynamics, which encompass the processes of mitochondrial fusion and fission for organelle renewal and recycling, recently associated with diverse pathologies,[31] have also been studied in the proposed model of HIV infection by MFN2 measurement (mitochondrial fusion). In our study model, MFN2 gradually decreased in faster HIV progressors with respect to the LTNP group. Although these findings are consistent with previously reported associations between Vpr viral protein and decreased MFN2,[32] the lower levels of MFN2 found in treated Sp2 patients require an alternative hypothesis. Notably, experimental data are lacking in the field of mitochondrial dynamics in HIV infection.

Even though the findings derived from the proposed model led to a homogeneous pattern for all the mitochondrial and apoptotic parameters, comparable to *in vivo* data, this *in vitro* model presents some limitations. First, the fact that most of the parameter differences did not achieve statistical significance could be due to the small sample size. However, some patients, such as those in the LTNP group, are uncommon due to the very low prevalence of the LTNP status. Second, despite their analogy to T lymphocytes, Jurkat cells belong to a cancerous cell line and present a high glycolytic metabolism rather than an oxidative and mitochondrial metabolism. These cell features, associated with increased proliferative ratios, confer on these cells a reduced mitochondrial involvement as well as high resistance to the development of apoptosis. However, there have been a remarkably wide range of scientific studies using Jurkat cells as a model to investigate the roles of HIV and ARV molecular mechanisms.[33] Third, differential HIV progression is a multifactorial trait comprising at least two variables: the virus and its host. The proposed study model discards the endogenous cells of the host, and thus their mitochondria, replacing them with Jurkat cells, in order to elucidate whether the influence of HIV progression and ARV toxicity rely on a factor in the plasma. The replacement of endogenous T cell lymphocytes with a Jurkat cell line avoids the requirement of a huge amount of blood from patients. Furthermore, the use of Jurkat cells makes it possible to keep the cells alive in a cell culture system and to avoid dealing with activation of the coagulation cascade. Their use reduces host differences, but discards one of the main factors promoting slow HIV progression or ARV toxicity. Finally, other factors have been described as contributing resistance to HIV- or ARV- secondary effects, in addition to mitochondria. The hypothesis to explain differential HIV progression in the present model considering the mitochondrion as a target organelle within a target cell is not detrimental to other reportedly protective factors, such as HLA, co-receptor polymorphisms, antibodies, cytokines and antimicrobial peptides ($\alpha/\beta/\theta$ defensins).[34] When considering ARV toxicity, pharmacogenetic variability among individuals in drug processing should also be considered. Lastly, another potential limitation of this study is the possibility that the experimental conditions, such as temperature and concentrations, were not optimal for the reproduction of *in vivo* conditions.

From a translational point of view, if mitochondrial and apoptotic cell lesions are the basis of future clinical onset, the improvement of such lesions would minimize the adverse effects of medication and the sociosanitary costs associated with the management of infected individuals, potentially improving the quality of life and lifespan of the patient.

Our *in vitro* model partially reproduced the differential mitochondrial and apoptotic lesions characteristic of HIV progression previously described in patients and indicated potential pathways for slowing viral progression by reducing mitochondrial impairment and apoptotic events. Moreover, in this cell model the therapeutic tenofovir+emtricitabine+efavirenz combination was confirmed as a first-line therapy that is safe with respect to mitochondrial function.

In summary, we have proposed a simple and feasible study model that makes it possible to evaluate the effects of HIV infection and its therapy on mitochondrial function. This model could be useful in clinical research. Further studies will be needed to confirm our observations, to determine their clinical relevance and to test the potential use of serum and/or fresh plasma in the incubation process. The main limitation of this study is that the method did not reveal any significant differences among different groups, and thus it needs further validation, including a proof of concept with statistically significant results.

Funding

This work was supported by: Fundació Privada Cellex, *Fundación para la Investigación y la Prevención del SIDA en España* (grant number FIPSE 360982/10); Fundació Cellex, *Fondo de Investigación Sanitaria* (grant numbers FIS 00462/11, FIS 01199/12, FIS 01455/13 and FIS 01738/13); *Suports a Grups de Recerca de la Generalitat de Catalunya* 2014-2016 (grant number SGR 2014/376); and CIBER de Enfermedades Raras (CIBERER, an initiative of ISCIII).

Transparency declarations

None to declare.

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Mitochondrial disturbances in HIV pregnancies

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Background: Mitochondrial consequences from foetal exposure to HIV infection and antiretrovirals could be further investigated.

Objective: The main objective of this study was to evaluate maternofoetal mitochondrial disturbances in HIV infection and antiretroviral administration in human pregnancies as the aetiopathogenic basis of suboptimal perinatal-clinical features.

Design: Cross-sectional, prospective, observational, exploratory and controlled study.

Methods: Clinical/epidemiological data of 35 HIV-infected pregnant women and 17 controls were collected. Mitochondrial DNA (mtDNA) and RNA (mtRNA) content (real time-PCR), enzymatic activities and content (spectrophotometry) were measured in leucocytes. Genetic-functional, maternofoetal and molecular-clinical correlations were assessed.

Results: Birth weight was lower in infants from HIV-infected mothers compared with controls. MtDNA values were slightly decreased in HIV cases, although not reaching statistical significance. MtRNA values were lower in HIV-infected mothers. Similarly, binary complex II+III enzymatic activity decreased to 50% in both HIV-infected mothers ($44.45 \pm 3.77\%$) and their infants ($48.79 \pm 3.41\%$) ($P = 0.001$ and $P < 0.001$). Global CI+III+IV enzymatic activity was lower in HIV-infected mothers and infants ($90.43 \pm 2.39\%$ and $51.16 \pm 9.30\%$) ($P < 0.005$ and $P < 0.05$). MtDNA content correlated with function in mothers and infants. Maternofoetal parameters correlated at genetic and functional levels.

Conclusion: HAART toxicity caused mitochondrial damage in HIV-infected pregnant women and their newborns, being present at a genetic and functional level with a maternofoetal correlation.

AIDS 2015, **29**:5–12

Keywords: antiretrovirals, HAART, HIV infection, HIV pregnancies, in-utero exposure, mitochondrial dysfunction and perinatal outcomes

Introduction

The current implementation of recommendations for universal prenatal HIV counselling and testing, the gestational use of antiretroviral therapy (HAART), scheduled caesarean section delivery and avoidance of breastfeeding, has led to a reduction in HIV mother-to-child transmission (MTCT) rates from around 20–25% to 1–2% in developed countries [1,2]. Widespread use of antiretroviral drugs has been accepted for the prevention

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Received: 9 December 2013; revised: 4 September 2014; accepted: 9 September 2014.

DOI:10.1097/QAD.0000000000000486

ISSN 0269-9370 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins

of MTCT despite the lack of data related to safety in human pregnancies [3,4]. The potential clinical risks associated with antiretroviral exposure in HIV-infected pregnant women and foetuses have been described by controversial observational studies [5–8] and potential mitochondrial implication has seldom been taken into account. There are limited data on possible toxicities in this population, and the large number of confounding factors limits any conclusions [9]. Antiretrovirals have been associated with adverse pregnancy outcomes such as preeclampsia, foetal death, preterm birth and low birth weight [10]. There are some studies reporting these negative effects of in-utero antiretroviral exposure in animal models [11].

Although antiretroviral therapy is required to suppress viral replication, leading to a decrease in MTCT rates and avoidance of disease progression, its derived mitochondrial toxicity has been widely described in adults, especially concerning the use of nucleoside reverse transcriptase inhibitors (NRTI), which are known to inhibit mitochondrial DNA (mtDNA) polymerase γ [12] and may therefore lead to mitochondrial dysfunction [13]. Other antiretroviral groups included in the backbone of therapeutic regimens such as protease inhibitors and non-NRTI are also known to cause mitochondrial deficiencies mainly through the development of apoptosis [14]. Subclinical mitochondrial molecular consequences from in-utero exposure of foetuses to HIV infection and antiretroviral drugs have not been completely elucidated as well as their association with the perinatal clinical outcomes in human pregnancies. Mitochondrial alterations may entail many important and heterogeneous secondary adverse events such as neuropathies, lactic acidosis, hyperlactataemia, lipodystrophy or myopathies, common to primary mitochondrial disease [15], but they have also been suggested to play a role in fertility [16] and foetal development [10]. Recently, adverse neurochemical and behavioural effects derived from transplacental exposure to zidovudine have been described in a mouse model together with a potential protector role of L-acetylcarnitine on mitochondrial function [17].

The mitochondrial genome encodes for proteins of the mitochondrial respiratory chain. The relationship between mitochondrial genetic and functional parameters has already been demonstrated, both in antiretroviral-exposed animals and newborn [18]. Our group carried out a previous study to investigate the role of in-utero antiretroviral exposure in mitochondrial function in mononuclear cells isolated from chord blood from the newborn [10]. In the present study, mitochondrial parameters were measured directly from peripheral blood mononuclear cells of the infant for two purposes: to further confirm our previous results from chord blood, comparing the findings from chord blood with those of peripheral blood and to further investigate the transcriptional level and the general assessment of the global mitochondrial respiratory chain, as these parameters were not considered in our last work [10].

Our previous findings showed a decrease in global mitochondrial function of the complete respiratory chain in a perinatally HIV-infected paediatric population [19] leading to analysis of this combined general mitochondrial enzymatic activity in infants exposed to antiretroviral drugs during gestation in the present study.

We hypothesized that antiretroviral-derived mitochondrial toxicity is present in HIV-infected and treated mothers and their foetuses exposed *in utero,* that the type and severity of the maternal involvement may be similarly reflected in the newborns and that this mitochondrial damage may underlie the clinical perinatal outcomes in HIV-infected human pregnancies.

The main objective of the present study was to evaluate the subclinical mitochondrial implication within the context of HIV infection and antiretroviral exposure in human pregnancies. We therefore aimed to (i) assess mitochondrial parameters in HIV-infected pregnant women and their infants exposed to antiretroviral *in utero*, (ii) correlate the genetic and functional levels of mitochondrial parameters and (iii) correlate the maternofoetal relationship of the mitochondrial parameters.

Methods

Design

We performed a single-site, cross-sectional, prospective, case-controlled observational and exploratory study with an inclusion period from 2007 to 2012.

Patients

Fifty-two mother–infant couples were recruited in this study. Thirty-five were classified as HIV-infected mothers (with their noninfected infants) and the control group included 17 HIV-uninfected mother–child pairs in follow-up because of other infections susceptible to be vertically transmitted, such as hepatitis C or B virus, syphilis or Chagas. All cases and controls were consecutively included during their routine prenatal care at the last trimester of gestation in the tertiary care Hospital St Joan de Déu of Barcelona (Barcelona, Spain), while the experimental procedures were performed in the Faculty of Medicine, Hospital Clínic of Barcelona (Barcelona, Spain).

Sample collection and processing

Twenty millilitres of peripheral blood were collected from the mothers in EDTA-tubes, with 2–5 ml being collected from their infants at the age of 6 weeks. Peripheral blood mononuclear cells were obtained by a Ficoll density gradient centrifugation procedure [20] divided into aliquots and stored at −80°C until analysis. Samples

from mother–child pairs in the control group were eligible only when maternal infection had definitely been ruled out in the infant, usually some months after collection.

Clinical analysis

Clinical data were collected through detailed questionnaires at inclusion and at delivery. Anthropometric data of the infant were also collected at delivery.

As per protocol, informed consent was obtained and epidemiological and obstetric parameters included information on maternal age, race, parity, and mode of delivery. Information regarding perinatal outcomes for both HIV-infected women and controls included the following data: preeclampsia (new onset of hypertension of >140 mmHg or >90 mmHg of SBP and DBP, respectively, and >300 mg proteins/24 h of urine), gestational age at delivery, preterm birth (<37 weeks of gestation), birth weight, infant small for gestational age (<10th percentile), 5-min Apgar score below 7, time and type of antiretroviral exposure, neonatal admission to ICU and global adverse perinatal outcome.

Molecular analysis of mitochondrial parameters

Protein content was measured according to the Bradford protein–dye binding-based method [21].

Total DNA was extracted by standard phenol–chloroform procedures. We analysed mtDNA content by the amplification of the mitochondrial gene mt12SrRNA and the nuclear constitutive gene nRNAseP using Applied Biosystems real-time quantitative PCR (Foster City, California, USA) in a 96-well plate and expressed in relative units as the ratio between mtDNA and nuclear DNA (mt12SrRNA/nRNaseP). The amplification procedure was performed as follows:

To determine mitochondrial mtDNA: MtF805 (5′-CCA CGGGAAACAGCAGTGAT-3′) was used as the 12SrRNA forward primer and MtR927 (5′-CTAT TGACTTGGGTTAATCGTGTGA-3′) was used as the 12SrRNA reverse primer, using a TaqMan sonda 6FAM-5′-TGCCAGCCACCGCG-3′-MGB, of Applied Biosystems.

To determine nuclear DNA a commercial kit was used (RNase P Control Reagent VIC, part no 4316844; Applied Biosystems).

The conditions for the amplification cycles for both genes were: 2 min at 50°C, 10 min at 95°C, 40 denaturalisation cycles of 15 s at 95°C and 60 s of annealing step at 60°C.

Total RNA was extracted by affinity microcolumns of Nucleospin (Düren, Germany), following the instructions of the commercial kit. Reverse transcription was performed by using random hexamer primers before the RT-PCR experiment.

mtRNA was quantified amplifying a fragment of the conserved mitochondrial gene ND2 (using the forward 5′-GCCCTAGAAATAAACATGCTA-3′ primer and the reverse 5′-GGGCTATTCCTAGTTTTATT-3′ primer) and the constitutive nuclear gene 18SrRNA (using the forward primer 5′-ACGGACCAGAGCGAAAGCAT-3′ and the reverse primer 5′-GGACATCTAAGGGCA TCACAGAC-3′ primer). Both genes were quantified separately by real-time quantitative PCR (LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany) and the results were finally expressed by the ratio between mtRNA and nuclear RNA (mtND2/n18SrRNA). The conditions for the amplification cycles were single denaturization–enzyme-activation step of 10 min at 95°C followed by 29 cycles (for the ND2 gene) and 35 cycles (for the 18SrRNA gene). Each cycle consisted in a denaturation step (0 s at 94°C for the ND2 gene and 2 s at 95°C for the 18SrRNA gene), an annealing step (10 s at 53°C for the ND2 gene and 10 s at 66°C for the 18SrRNA gene), and an extension step (10 s at 72°C for the ND2 gene and 20 s at 72°C for the 18SrRNA gene).

Mitochondrial function was measured spectrophotometrically according to the Rustin *et al.* [22] and Miró *et al.* [23] methodologies. We assessed the enzymatic activities of the isolated complexes: complex II (CII), complex IV (CIV) and binary combination enzymatic activities: complex II+III (CII+III), glycerol-3-phosphate dehydrogenase+complex III (G3PDH+CIII) and complete mitochondrial respiratory chain activity: complex I+III+IV (CI+III+IV) of the mitochondrial respiratory chain.

We measured mitochondrial content by citrate synthase activity (EC 4.1.3.7) with spectrophotometric measurements of the absorbance at 412 nm. Citrate synthase is a mitochondrial enzyme of the Krebs cycle which is widely considered as a reliable marker of mitochondrial content [22].

All the enzymatic activities were obtained as absolute values in nanomoles of synthesized product or consumed substrate per minute and milligram of protein (nmoles/min/mg protein) units and afterwards as relative values normalized by citrate synthase activity to relativize the enzymatic activity by mitochondrial content. The remaining genetic or transcriptional analyses were also normalized to citrate synthase activity to relativize parameters to mitochondrial mass.

Statistical analysis

Epidemiologic, clinical and mitochondrial data of HIV-infected women and their infants were compared with those of uninfected mother–child pairs to assess the

presence of obstetric/perinatal problems and mitochondrial damage due to in-utero exposure to HAART. Additionally, different correlations were sought between: (i) genetic and functional mitochondrial parameters (to ascertain dependence of mitochondrial function on mitochondrial genome content), (ii) mother-to-child mitochondrial parameters (to determine maternal influence on infant cellular condition) and (iii) clinical and experimental data (to assess mitochondrial implication in obstetric problems and perinatal outcomes).

Statistical analyses were performed by means of the SPSS 15.0 (Chicago, Illinois, USA) program using Mann–Whitney nonparametric tests to search for independent sample differences, chi-square tests were used to calculate odds ratio values (OR; 95% confidence interval [CI]; significance) and the Spearman's rank correlation coefficient was used to correlate parameters (R^2 and significance). Clinical parameters were expressed as mean ± SD and experimental results were expressed as mean ± SEM or percentages with respect to the means of controls, and the level of significance was set at 0.05 for all the statistical tests.

Results

Clinical data

The clinical and epidemiologic characteristics of the HIV-infected mothers and their infants and the control group have been summarized in Table 1.

The maternal, labour, neonatal prophylactic treatments are shown in Table 2. The incidence of preeclampsia in the HIV pregnancies was not higher compared with the controls. The incidence of preterm birth was higher in infants from HIV-infected mothers with respect to those from control pregnant women (36.36 vs. 21.42%), although this was not statistically significant. Additionally, the birth weight was significantly lower in HIV-exposed and antiretroviral-exposed newborn compared with controls (2689.35 ± 615.92 vs. 3292.65 ± 540.45, $P = 0.001$).

Molecular data of mitochondrial parameters

The mitochondrial mass amount was not compromised in HIV-infected mothers or their infants as shown in Fig. 1. All the absolute mitochondrial parameters were relativized per mitochondrial mass by normalizing per citrate synthase enzymatic activity.

The mitochondrial genome (mtDNA) showed a trend towards depletion in both HIV-infected mothers and their infants with respect to controls (1.06 ± 0.21 and 0.97 ± 0.20 vs. 5.05 ± 2.58 and. 2.05 ± 0.95 mtDNA 12SrRNA/nDNA RNAseP arbitrary ratio units, $P < 0.1$ for both). At the transcriptional level, mitochondrial RNA showed a decrease in HIV-infected mothers with respect to the control group (0.65 ± 0.16 vs. 5.50 ± 2.63 mtRNA ND2/nRNA 18SrRNA arbitrary ratio units, P = NS), attaining statistical significance in their infants (0.29 ± 0.46 vs. 4.78 ± 2.65 mtRNA ND2/nRNA 18SrRNA arbitrary ratio units, $P < 0.01$) (Fig. 2a and b).

Mitochondrial function, assessed in isolated complex II and complex IV enzymatic activities, was not compromised in HIV-infected mothers and their infants with respect to controls; CII: (0.22 ± 0.01 and 0.21 ± 0.01 vs. 0.25 ± 0.03 and 0.29 ± 0.04 nmole/min mg protein, P = NS in all cases); CIV: (0.34 ± 0.029 and 0.34 ± 0.024 vs. 0.87 ± 0.24 and 0.39 ± 0.055 nmole/min mg protein, P = NS in all cases). The isolated enzymatic activity of glycerol-3-phosphate dehydrogenase (G3PDH) was not compromised in any case. G3PDH enzymatic activity combined to CIII was not significantly reduced in cases with respect to the controls. The measurement of binary enzymatic activity CII+CIII decreased to 50% both in HIV-infected mothers and their infants compared with controls (0.25 ± 0.01 vs. 0.45 ± 0.07, $P = 0.001$), (0.21 ± 0.01 vs. 0.41 ± 0.059 nmole/min mg protein, $P < 0.001$) (Fig. 2a and b).

Table 1. Clinical and epidemiologic characteristics of the HIV-infected mothers and their infants included in the study.

	Control mothers	Control infants	HIV-infected mothers	HIV-exposed infants
Sample size (*n*)	17	17	35	35
Age at blood draw (mean ± SD)	34.3 years ± 5.7	1.3 months ± 0.7	31.4 years ± 7.3	2.0 months ± 0.7
Female gender (%)	100	58.82	100	41.93
Mean $CD4^+$ lymphocyte count (cells/μl^3 ± SD)	NA	NA	624.8 ± 316.6	641.75 ± 304.17
Mean viral load (Log10 viral load copies/ml ± SD)	NA	NA	0.36 ± 1.0	NA
Antiretroviral therapy				
3 NRTI (*n*)	NA	NA	2	0
2 NRTI + 1 or 2 PI, or 1NNRTI (*n*)	NA	NA	22	0
1 NRTI + 1 tNRTI or 1 PI (*n*)	NA	NA	5	1
ZDV monotherapy (*n*)	NA	NA	0	30
Obstetric parameters				
Preterm birth (%)	NA	21.4	NA	36.4
Birth weight (mean gr ± SD)	NA	3292 ± 540	NA	2689 ± 615*

NNRTI, nonnucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; PI, protease inhibitor; tNRTI, nucleotide reverse transcriptase inhibitor; ZDV, zidovudine. *$P = 0.001$.

Table 2. Maternal, labour and neonatal prophylactic treatments.

Maternal pregnancy treatments	Maternal labour treatments	Neonatal treatments
Protease inhibitor-based egimens, $n=20$	Intravenous ZDV, $n=32$	ZDV monotherapy, $n=33$
ZDV/3TC+LPV/r, $n=7$	Untreated, $n=3$	ZDV+NVP, $n=1$
ZDV/3TC+SQV/r, $n=3$		ZDV+3TC+NVP, $n=1$
TDF/FTC+LPV/r, $n=2$		
ZDV/3TC+FPV/r, $n=2$		
ABC/3TC+LPV/r, $n=1$		
ABC+TDF+LPV/r, $n=1$		
3TC+d4T+LPV/r, $n=1$		
ZDV/3TC+NFV, n=1		
TDF/FTC+ATV/r, $n=1$		
TDF/FTC+SQV/r, $n=1$		
NNRTI-based regimens, $n=12$		
ZDV/3TC+NVP, $n=6$		
ZDV+FTC+EFV, $n=2$		
ABC/3TC+NVP, $n=1$		
ABC+ddI+NVP, $n=1$		
ABC+FTC+NVP, $n=1$		
ABC/3TC/ZDV, $n=1$		
ZDV monotherapy, $n=1$		
Untreated, $n=2$		

1st trimester exposure to antiretrovirals, 21/33 mothers (63.6%), median (range) duration of antiretroviral treatment during pregnancy: 33.5 (4–40) weeks. 3TC, lamivudine; ABC, abacavir; ATV/r, atazanavir/ritonavir; d4T, stavudine; ddI, didanosine; EFV, efavirenz; FPV/r, fosamprenavir/ritonavir; FTC, emtricitabine; LPV/r, lopinavir/ritonavir; NFV, nelfinavir; NNRTI, nonnucleoside reverse trancriptase inhibitors; NVP, nevirapine; SQV/r, saquinavir/ritonavir; TDF, tenofovir; ZDV, zidovudine.

General assessment of the whole mitochondrial respiratory chain enzymatic activity through analysis of CI+III+IV showed a significant decrease in both HIV-infected mothers and their infants compared with the control groups (0.20 ± 0.05 and 0.21 ± 0.03 vs. 2.09 ± 0.82 and 0.43 ± 0.11; $P < 0.01$ and $P = 0.05$) (Fig. 2a and b).

Mitochondrial genetic and functional associations

Mitochondrial genome content correlated with mitochondrial function measured as CII+CIII/citrate synthase enzymatic activity (Fig. 3a) in both groups of mother–child pairs, whether HIV-infected or not.

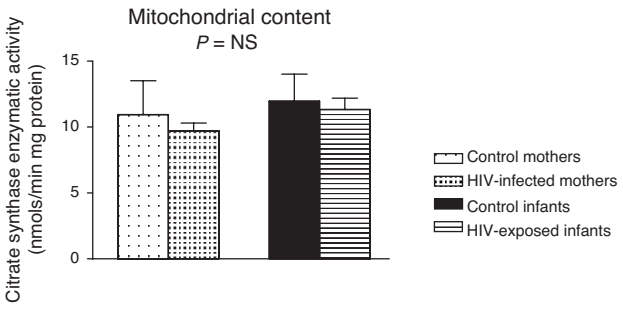

Fig. 1. Mitochondrial content measured by citrate synthase enzymatic activity of mothers and infants in both the control and case groups. Mann–Whitney nonparametric test.

Maternofoetal associations

The mitochondrial parameters of the mothers and their infants positively correlated at a genetic level, measured as mtDNA content, as well as at the functional level, with CII+CIII/citrate synthase measurement (Fig. 3b and c) in both cohorts. No consistent molecular and clinical associations were found in the present study (Fig. 3d and e).

Discussion

Although mitochondrial toxicity has been demonstrated in in-utero exposure to antiretroviral drugs in animal models, specifically secondary to NRTIs [24], more information on this subject in human HIV pregnancies could be addressed.

Previous studies have demonstrated HAART-induced mtDNA depletion in oocytes from HIV-infected women that may impair their reproductive capacity [16].

The present study does not show a higher incidence of preeclampsia in HIV pregnancies. There were no cases of foetal death in any of our cohorts. However, the incidence of preterm birth tended to be higher and birth weight was significantly lower in infants born to HIV-infected mothers with antiretroviral treatment. Previous studies have demonstrated the implication of mitochondria in abnormal perinatal foetal weight in non-HIV or HAART-exposed mothers [25]. The clinical data in our study suggest an association of HAART toxicity in the context of HIV infection and the presence of perinatal outcomes in human pregnancies. We therefore wished to determine whether the mitochondrion remains behind these clinical manifestations, as the aetiopathogenic basis.

As the viral load of the HIV-infected mothers was undetectable and the infants were confirmed to be HIV-negative, the mitochondrial toxicity observed in our HAART-exposed cohorts was attributed to antiretroviral exposure but not to HIV infection itself.

Mitochondrial amount was not affected in HIV-infected mothers and their in-utero antiretroviral-exposed newborns. However, the mitochondrial genome showed a trend towards depletion in both HIV-infected mothers and their infants with respect to controls. These findings are in accordance with previous studies reporting mtDNA depletion in newborn from HIV-infected and treated mothers [26–29]. The trend to mtDNA depletion observed in the present study was reflected downstream at the transcriptional and functional levels, displaying an organelle dysfunction with significantly lower mtRNA levels in HIV-exposed infants and a decrease in the combined binary and global enzymatic activities of the mitochondrial respiratory chain, respectively. Despite

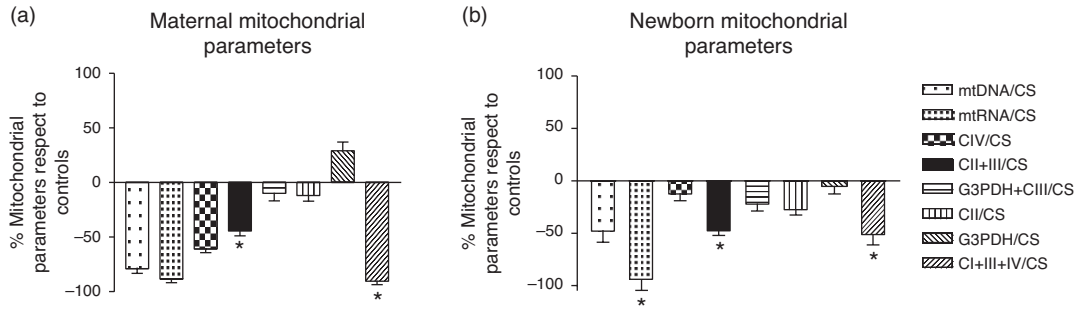

Fig. 2. (a) Maternal mitochondrial parameters and (b) Mitochondrial parameters of the infants. Percentage of increase/decrease of the mitochondrial parameters in HIV-infected mothers with respect to the mean values of uninfected controls. CIII, complex III; CII+III, complex II+III; CI+III+IV, complex I+III+IV; CIV, complex IV; G3PDH, glycerol-3-phosphate dehydrogenase; MtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA. $*P = 0.05$. Mann–Whitney nonparametric test.

the general involvement of these global enzymatic activities, isolated complexes were not compromised. These findings are consistent with our previous studies in perinatally HIV-infected paediatric patients in which we found significant alterations of the global enzymatic activity of mitochondria in the absence of any suboptimal function of single complexes [19]. Our results suggest the presence of mild alterations in individual complexes of the mitochondrial respiratory chain which are only noticeable through the measurement of binary or global enzymatic activities, thus, reaching a detectable threshold as a summatory effect of these mild alterations.

The positive and significant correlation found between mitochondrial genetics and functional binary enzymatic activity strengthens the idea of a genetic defect affecting the general function of the mitochondria and, subsequently, cell viability. This fact demonstrates the dependence of mitochondrial function on mitochondrial genetics.

The significant positive correlation between the maternofoetal mitochondrial parameters suggests that the toxicity caused by HAART in the context of HIV infection in human pregnancies has a similar impact on both the mother and fetus. Consequently, our findings show that the mtDNA depletion, the decrease in mtRNA content and the general dysfunction of the mitochondrial respiratory chain observed in HIV-infected mothers also occurred in their newborn.

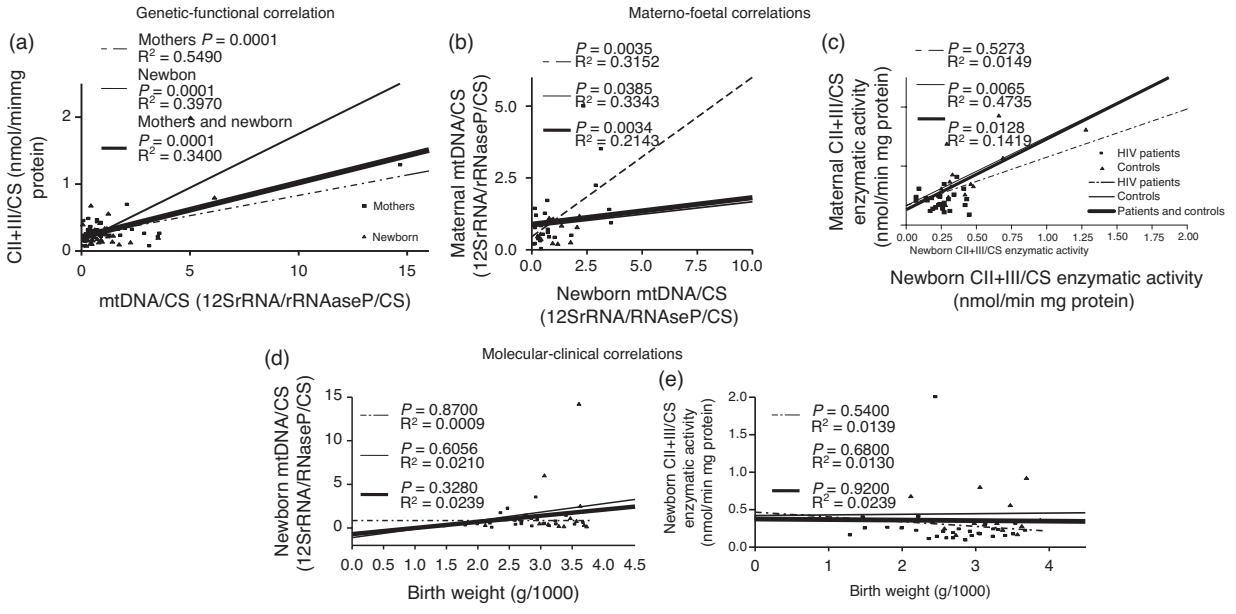

Fig. 3. Mitochondrial genetic-functional, maternofoetal and molecular-clinical correlations. (a) Mitochondrial genome and function in mothers and newborn; (b) Mitochondrial genome; (c) Mitochondrial function; (d)Mitochondrial genome and birth weight; (e) Mitochondrial function and birth weight. CII+III, complex II+III enzymatic activity; CS, citrate synthase; MtDNA, mitochondrial DNA.

As the use of chord blood may be questioned as a proper model of study due to a possible contamination with maternal cells, we assessed all the molecular parameters in samples from the peripheral blood (specifically monocytes and lymphocytes) of infants obtained at the age of 6 weeks to further confirm the results of our previous study performed in chord blood cells from mother–newborn couples [10]. The findings derived from the present work confirm the presence of a mitochondrial lesion following the same general pattern of maternofoetal correlation.

Although abnormal perinatal outcomes and mitochondrial alterations were more prevalent in HIV-infected mothers and their infants with respect to uninfected controls, the results obtained from this study do not confirm an association between the mitochondrial defects and the clinical manifestations observed (preterm birth and low birth weight). Further studies in larger cohorts are necessary to confirm a potential relationship between antiretroviral exposure and clinical morbidities in HIV-infected mothers and newborn.

Finally, the presence of a mitochondrial lesion derived from antiretroviral intake both in HIV-infected pregnant women and in their newborns exposed *in utero* indicates that the toxic effects associated with HAART cross the placenta and affect the HIV-negative, but antiretroviral in-utero exposed newborn in a similar manner. As the antiretroviral intake ensures that viral loads are decreased to undetectable levels, it is expected that the damage in mitochondrial function in the HIV-infected mothers should be rather attributed to antiretroviral drugs. However, it is generally considered that the infant may sustain some mitochondrial dysfunction caused by the maternal virus infection, in the absence of any drug therapy, [28]. The HAART-derived toxicity in fertility or pregnancy context confirms previously documented results in chord blood cells from HIV-uninfected but HAART-exposed newborn [9].

Some limitations of this study are worthy to be mentioned. In spite of the previously mentioned fact that the mitochondrial toxicity in our cohort has been attributed to antiretroviral exposure rather than to HIV infection itself, it is not possible to completely dissect the role of mother's HIV infection and HAART because all HIV-infected-pregnant women are currently treated. Furthermore, the heterogeneity of therapeutic schedules and the clinical history of each patient, with differential cumulative antiretroviral drug intake, hampers the possibility of further specific descriptions of the molecular mechanisms together with more focalized antiretroviral-specific conclusions.

In summary, the findings of the present study demonstrate a significantly lower birth weight and genetic and functional maternofoetal mitochondrial toxicity in HIV-infected mothers and their infants, although no relationship was found among these clinical and molecular parameters.

Acknowledgements

The authors are indebted to Donna Pringle for the English correction. The authors are indebted to Fundación para la Investigación y la Prevención del SIDA en España (FIPSE) and CIBER de Enfermedades Raras (CIBERER, an initiative of ISCIII).

Source of funding: This work was supported by Fundación para la Investigación y la Prevención del SIDA en España [grant numbers FIPSE 36612/06, FIPSE 360982/10, FIPSE 360745/09]; Fundació Cellex, *Fondo de Investigación Sanitaria* [grant numbers FIS 00462/11, FIS 01199/12, FIS PI13/01738]; *Suports a Grups de Recerca de la Generalitat de Catalunya* [grant number SGR 14/376] and CIBER de Enfermedades Raras (CIBERER, an initiative of ISCIII).

Role of each of the authors in the study reported: C.M. is the main author of the manuscript as she was in charge of most of the experimental procedures; A.N.J. is the coordinator of the Infectious Disease Unit of the paediatric hospital Sant Joan de Déu. He organises the inclusion of the patients; G.G. is the postdoctoral research who coordinates the experimental research procedures; N.R. is the person in charge of the inclusion of the controls; M.C. is responsible for the enzymatic activities measurement; M.B. is responsible for the mitochondrial DNA content quantification; M.G.M. is responsible for the analysis of the total cell protein content; E.T. is the laboratory technician in charge of the preparation and cryopreservation of the samples; S.H. is the gynaecologist in charge of the management of the obstetric data; F.C. is the head of the Internal Medicine Department who is in charge of the final review of the study; O.M. is the clinician in charge of the management and analysis of the clinical data; C.F. is the head of the Infectious Disease Unit of the paediatric hospital who coordinates the inclusion of the samples and the management of the project.

Conflicts of interest

There are no conflicts of interest.

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Addiction Biology

HUMAN STUDY

doi:10.1111/adb.12183

Molecular basis of reduced birth weight in smoking pregnant women: mitochondrial dysfunction and apoptosis

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ABSTRACT

In utero exposure of fetuses to tobacco is associated with reduced birth weight. We hypothesized that this may be due to the toxic effect of carbon monoxide (CO) from tobacco, which has previously been described to damage mitochondria in non-pregnant adult smokers. Maternal peripheral blood mononuclear cells (PBMCs), newborn cord blood mononuclear cells (CBMCs) and placenta were collected from 30 smoking pregnant women and their newborns and classified as moderate and severe smoking groups, and compared to a cohort of 21 non-smoking controls. A biomarker for tobacco consumption (cotinine) was assessed by ELISA (enzyme-linked immunosorbent assay). The following parameters were measured in all tissues: mitochondrial chain complex IV [cytochrome *c* oxidase (COX)] activity by spectrophotometry, mitochondrial DNA levels by reverse transcription polymerase chain reaction, oxidative stress by spectrophotometric lipid peroxide quantification, mitochondrial mass through citrate synthase spectrophotometric activity and apoptosis by Western blot parallelly confirmed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay in placenta. Newborns from smoking pregnant women presented reduced birth weight by 10.75 percent. Materno-fetal mitochondrial and apoptotic PBMC and CBMC parameters showed altered and correlated values regarding COX activity, mitochondrial DNA, oxidative stress and apoptosis. Placenta partially compensated this dysfunction by increasing mitochondrial number; even so ratios of oxidative stress and apoptosis were increased. A CO-induced mitotoxic and apoptotic fingerprint is present in smoking pregnant women and their newborn, with a lack of filtering effect from the placenta. Tobacco consumption correlated with a reduction in birth weight and mitochondrial and apoptotic impairment, suggesting that both could be the cause of the reduced birth weight in smoking pregnant women.

Keywords Carbon monoxide (CO), intrauterine growth restriction (IGR), pregnancy, tobacco.

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INTRODUCTION

Tobacco is one of the most common toxic drugs in developed countries, being associated with high mortality and morbidity rates. Smoking is a major health burden and is a huge determinant of socio-economic policies and budgets. In fact, smoking is a risk factor for six out of the eight leading causes of death worldwide. Although most pregnant women reduce or give up tobacco consumption during pregnancy, it is estimated that about 20–50 percent of pregnant women continue to smoke during pregnancy in developed countries (Bouhours-Nouet *et al.* 2005). The toxic effects of tobacco are especially dangerous during pregnancy, presenting a negative impact on gestation, the fetus and even on the fertility of women (Sepaniak, Forges & Monnier-Barbarino 2006). Smoking

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pregnant women show a higher rate of premature delivery, intrauterine fetal death, smallness for gestational age, sudden infant death and respiratory problems during childhood (Mitchell *et al.* 1993; Pattenden *et al.* 2006). Intrauterine growth restriction (IUGR) is among the most frequent toxic effects in these children and entails a reduction in birth weight between 90 and 200 g (Pringle *et al.* 2005; Alberry & Soothill 2007). IUGR is associated with several adverse perinatal outcomes such as cardiac dysfunction (Crispi *et al.* 2008; Eixarch *et al.* 2011) and delayed metabolic effects in adulthood. The mechanism of this toxicity remains largely unknown.

Tobacco contains more than 4000 toxic compounds, with carbon monoxide (CO) being one of the most important among them. This gas has a great affinity to the biological molecules that bind to oxygen by porphyrin rings containing iron, such as haemoglobin, myoglobin, cytochrome P450 and cytochrome *c* oxidase (COX), or mitochondrial respiratory chain (MRC) complex IV. Carbon monoxide binds very tightly to these structures, preventing the union binding of oxygen. When CO binds to haemoglobin, it prevents oxygen delivery to the cells, causing tissue hypoxia. At a cellular level, the binding of CO to cytochrome aa3 from mitochondrial COX inhibits the enzymatic activity of this complex, which is one of the most important elements in the MRC responsible for cell respiration, energy supply and cell survival (Cardellach *et al.* 2003a; Piantadosi, Carraway & Suliman 2006; Queiroga, Almeida & Vieira 2012). Inhibition of MRC by CO has been reported *in vitro* through experimental models (Alonso *et al.* 2003; Piantadosi *et al.* 2006) and *in vivo* in acute CO-poisoned patients (Cardellach, Miro & Casademont 2003b; Miro *et al.* 2004; Garrabou *et al.* 2011a,b) as well as in chronic or acute smoking subjects (Cardellach *et al.* 2003a; Garrabou *et al.* 2011b). Mitochondria, the energy power unit of the cell, produce reactive oxygen species (ROS) as by-products of respiration. In stress conditions, this basal production of ROS could be enhanced, leading to a state of oxidative stress (Piantadosi *et al.* 2006). Inhibition of COX by CO can lead to mitochondrial hypoxia and a decrease in the phosphorylation system, leading cells to energy failure and, at the same time, an increment of ROS production (Tibbles & Edelsberg 1996; Miro *et al.* 1998b, 1999, 2000; Gorman *et al.* 2003). These ROS may damage proteins, lipids, and glucidic and nucleotidic structures especially in mitochondria, where they originate. Consequently, as the mitochondrial genome encodes for 13 proteins of the MRC, ROS-derived mitochondrial DNA (mtDNA) damage may lead to mitochondrial dysfunction, in a kind of vicious circle that increases the production of new ROS.

Mitochondria are also the organelle that triggers and amplifies the signs of programmed cell death or apoptosis. Oxidative stress can lead to serious downstream cell consequences such as apoptosis. Recent studies have suggested that increased levels of voltage-dependent anion channel protein (VDAC, especially the isoform VDAC1) are responsible for both mitochondrial ROS production and induction of apoptosis (Abu-Hamad, Sivan & Shoshan-Barmatz 2006; Sasaki *et al.* 2012). VDAC is a mitochondrial protein located at the outer mitochondrial membrane and is known to be a key regulator protein of cell life and death (Zaid *et al.* 2005; Baines *et al.* 2007; Shoshan-Barmatz & Golan 2012). Its function relies upon allowing the transit of metabolic molecules along the mitochondrial outer membrane such as ATP, NADH and the proapoptotic cytochrome *c*, and it is highly involved in calcium signalling and metabolic crosstalk.

Some studies have analysed the implications of mtDNA levels in fetal growth in non-smoking women. Gemma *et al.* reported that low weight newborns present less mtDNA content in the umbilical cord (Gemma *et al.* 2006) and Pejznochova and colleagues described the same in mononuclear cells [cord blood mononuclear cells (CBMC)] (Gemma *et al.* 2006; Pejznochova *et al.* 2008). However, none of these studies assessed the molecular upstream aetiology or downstream consequences for cell of mtDNA decay and none have analysed the involvement of tobacco consumption in birth weight reduction.

Smoking has been associated with both the increase of IUGR and the mitochondrial toxicity. As mitochondria are responsible for the supply of energy and are essential for cell survival, we hypothesized that dysfunction in this organelle during fetal development may lead to an adverse perinatal outcome manifested as a reduction in birth weight.

Since the specific toxic mechanism leading to a reduction in newborn weight in fetuses exposed *in utero* to tobacco remains unknown, we aimed to assess whether mitochondrial dysfunction produced by CO may play a role. Thus, we performed a wide mitochondrial study in maternal peripheral blood mononuclear cells (PBMCs), fetal CBMCs and placenta of smoking pregnant women and their newborn at delivery, correlating the clinical data with the experimental results to assess the potential mitochondrial and/or apoptotic aetiopathogenesis of this obstetric problem.

MATERIALS AND METHODS

Study design

We performed a single-site, cross-sectional, case–control observational study.

Study population

Fifty pregnant women were prospectively and consecutively included in the present study during their routine

prenatal care at the first trimester of gestation in the Materno-Foetal Medicine Department of the Hospital Clinic of Barcelona (Barcelona, Spain).

Mitochondrial studies of 30 smoking pregnant women, 21 non-smoking pregnant controls and their newborns were performed in maternal PBMC, fetal CBMC and placenta at delivery. Controls and cases were matched for age. The smoking pregnant women were divided into two groups depending upon the amount of self-reported cigarettes consumed at inclusion: moderate smoking [less than 10 cigarettes per day ($n = 16$)] and severe smoking [more than 10 cigarettes per day ($n = 14$)].

The inclusion criteria were as follows: maternal age ≥18 years, singleton pregnancy and gestational age at delivery after 22.0 weeks (dated by first trimester crown rump length) and absence of family history of mitochondrial disease, viral infection or drug abuse.

All individuals were informed and signed written consent to be included in this protocol, which was approved by the Ethical Committee of our hospital following the Declaration of Helsinki.

A database was created to collect epidemiological, obstetric data, perinatal outcome and experimental results.

Clinical data

Extensive clinical data were collected through detailed questionnaires at each visit and at delivery. The anthropometric data of the newborns were also collected at delivery.

The epidemiological and obstetric parameters included information on maternal age, ethnicity, parity and mode of delivery. Information regarding perinatal complications for both smoking pregnant women and controls included gestational age at delivery, preterm birth (<37 weeks of gestation), gestational diabetes mellitus, low weight for gestational age (<10th percentile; Slancheva & Mumdzhiev 2013), IUGR, birth weight and growth percentile.

Sample collection and processing

Immediately after delivery, 20 ml of peripheral blood was obtained from women by antecubital vein puncture and 20 ml of umbilical vein blood was collected from their newborns in EDTA tubes. This method of newborn blood extraction was designed to prevent invasive sample collection and increase study participation. In both types of samples, the plasma was collected and stored at −80°C after centrifugation at 1500 *g* for 15 minutes, and mononuclear cells were isolated by Ficoll density gradient centrifugation, and divided into aliquots and stored at −80°C until analysis. In addition, a placenta biopsy was obtained at delivery and stored at −80°C until homogenization (5 percent w/v) to perform experimental studies. A sample of maternal urine was also collected for analytical purposes.

Tobacco consumption and quantification of cotinine levels

A complete questionnaire (Fagerström test) to assess smoking dependence was performed at inclusion in all the mothers studied. To assess the reliability of self-reported tobacco consumption and the capacity of the toxic compounds of tobacco to cross the placental barrier to reach the fetus, we quantified the cotinine levels (a biomarker of the amount of tobacco consumed) both in the plasma of pregnant women and the newborns as well as in maternal urine (Haddow *et al*. 1987; Jauniaux *et al*. 1999). Plasma samples were evaluated with a Calbiotech (Spring Valley, CA, USA) ELISA (enzyme-linked immunosorbent assay) kit (reference CO096D). Urine samples were analysed with a Gernon kit (reference GN 020600) and a standard curve was made with the calibrating 'Multi-droga' (reference GN 020999) by Gernon. Samples were processed with the auto-analyser Les Metrolab2300 following kit specifications. Cotinine concentrations were expressed in nanograms per milliliter (ng/ml).

Mitochondrial analysis

Information concerning experimental data included the collection of maternal, fetal and placental mitochondrial parameters.

To assess mitochondrial status, we measured COX activity, oxidative stress levels (lipid peroxidation), mtDNA content and mitochondrial mass in maternal PBMC, fetal CBMC as well as in placenta homogenate.

Mitochondrial complex IV (COX) enzyme activity

Mitochondrial complex IV (COX; E.C. 1.9.3.1) enzyme activity was measured spectrophotometrically according to the Rustin and Miro methodologies (Rustin *et al*. 1994; Miro *et al*. 1998a). Specific enzymatic activities are expressed in absolute values as nanomoles of substrate consumed per minute and milligram of cell protein (nmol/minute·mg protein).

Mitochondrial complex IV (COX) protein content

Mitochondrial complex IV protein expression was measured by Western blot [7–13 percent sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunodetection] and expressed as the ratio between COX-II subunit (encoded by the mtDNA) and COX-IV subunit (encoded by the nuclear genome) (Garrabou *et al*. 2009).

Oxidative stress

Lipid peroxidation levels are an indicator of oxidative damage of ROS in cellular lipid compounds. Lipid peroxidation was quantified using the Oxys Research kit of DeltaClone (Deltaclon, Madrid, Spain) by the spectrophotometric measurement of malondialdehyde (MDA) and 4-hydroxyalkenal (HAE), both products of fatty acid peroxide decomposition, normalized by protein content (μM MDA + HAE/mg protein) (Cardellach *et al.* 2003b; Garrabou *et al.* 2012).

Mitochondrial DNA quantification

To evaluate mtDNA depletion, total DNA was obtained by the standard phenol–chloroform extraction procedure. Fragments of the mitochondrial-encoded *ND2* gene and the nuclear-encoded *RNApol-II* gene were amplified in triplicate and separately by quantitative reverse transcription polymerase chain reaction using the Roche Lightcycler thermocycler (Moren *et al.* 2012). The relative content of mtDNA was expressed as the ratio between mitochondrial to nuclear DNA amount (mtDNA ND2/nDNA RNApol-II).

Mitochondrial mass

To assess mitochondrial number, we performed the spectrophotometric measurement of citrate synthase activity (CS; E.C. 4.1.3.7), a mitochondrial enzyme of the Krebs cycle widely considered as a reliable marker of mitochondrial content. Citrate synthase activity was expressed as nanomoles of product per minute and milligram of protein (nmol/minute·mg protein).

Apoptotic analysis

Mitochondrial VDAC levels

To assess cell apoptosis in maternal PBMC, newborn CBMC and placental homogenate, we measured the ratio of VDAC1/β-actin protein expression by Western blot by 7–13 percent SDS-PAGE and immunodetection. As β-actin is a cell protein with highly conserved expression, we used it to normalize VDAC1 content to total cell mass (Moren *et al.* 2012).

TUNEL assay

In parallel, we performed the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) (Gavrieli, Sherman & Ben-Sasson 1992; Negoescu *et al.* 1996) the incidence of apoptosis in placenta. This is a histological staining technique used to detect nuclear DNA fragmentation that results from caspase signalling cascades. Briefly, frozen placentas were cut in a slicing microtome at 7–8 μm and immunoreacted with the 'In Situ Cell Death detection' (reference 11 684 817 910) of Roche to detect apoptotic nucleus following the manufacturer's instructions. Three different observers counted the apoptotic nucleus, and values were expressed as the ratio of positive (red) nucleus per a total (red and blue) 1000 nuclei.

Protein content

The protein content was measured using the Bradford protein-dye binding-based method (Bradford 1976) to relativize mitochondrial and apoptotic parameters.

Statistical analysis

We assessed the normal distribution of the parameters using the Kolmogorov–Smirnov test. Statistical analysis was performed to determine differences between both the moderate and the severe groups of tobacco consumption and the control group using the independent sample *t*-test. In addition, correlations between clinical and/or experimental parameters were assessed using Pearson's linear regression analysis.

Results were expressed as means and standard error means (SEM). In all cases, a P value less than 0.05 was considered statistically significant.

RESULTS

Clinical data

The clinical, epidemiological and obstetric data of smoking pregnant and control women are summarized in Table 1, according to the amount of tobacco consumed (more or less than 10 cigarettes/day) together with the perinatal outcomes of the study cohorts.

There were no differences in the epidemiologic characteristics between smoking pregnant women and controls with a mean age of 31.29 years.

Obstetric data and perinatal results showed trends towards an increased frequency of adverse outcomes in smoking pregnancies regarding the incidence of preterm delivery, gestational diabetes, IUGR or growth percentile, except for gestational age at delivery, which was similar among the cohorts. Remarkably, the only significant difference between the smoking pregnant women and the controls was the newborn birth weight at delivery.

The birth weight was reduced in newborns of smoking pregnant women. In comparing moderate consumers with controls, the newborn weight was decreased by 9.3 percent [3133 ± 100.93 g versus 3451.66 ± 93.85 g, P = not significant (NS)]. Newborns from the severe smoking group showed a statistically significant 12.2 percent decrease in birth weight with respect to controls (3030.42 ± 163.67 g versus 3451.66 ± 93.85 g, $P < 0.05$). No differences in fetal sex

Table 1 Clinical, epidemiological and perinatal data of the patients and controls studied at delivery.

Epidemiologic data	*Non-smoking pregnant women* (n = *21*)	*Moderate smoking pregnant women* (n = *14*)	*Severe smoking pregnant women* (n = *16*)
Maternal age at delivery (mean ± SEM)	33.00 ± 1.09	30.94 ± 1.50	29.93 ± 1.60
No. of cigarettes/day (mean ± SEM)	0	7.31 ± 0.65**	18.35 ± 1.17**
Cotinine levels in maternal plasma (mean ± SEM)	1.75 ± 0.06	53.43 ± 14,80**	88.8 ± 11.30**
Cotinine levels in newborn's plasma (mean ± SEM)	1.66 ± 0.07	45.16 ± 12.65*	114.28 ± 14.27**
Cotinine levels in maternal urine (mean ± SEM)	181.52 ± 80.40	548.03 ± 212.28	1195.57 ± 300.01*
Obstetric data	*Non-smoking pregnant women* (n = *21*)	*Moderate smoking pregnant women* (n = *14*)	*Severe smoking pregnant women* (n = *16*)
Gestational age at delivery (mean ± SEM)	39.77 ± 0.26	39.58 ± 0.38	38.59 ± 0.71
Preterm delivery [*n* (percent)]	0 (0)	0 (0)	2 (12.5)
Gestational diabetes mellitus [*n* (percent)]	1 (4.8)	3 (21.4)	1 (6.3)
Low weight for gestational age [*n* (percent)]	1 (4.8)	2 (14.3)	3 (18.8)
Intrauterine growth restriction [*n* (percent)]	1 (4.8)	2 (14.3)	3 (18.8)
Birth weight (mean ± SEM)	3451.66 ± 93.85	3133.75 ± 100.93	3030.42 ± 163.67*
Growth percentile (mean ± SEM)	52.81 ± 5.70	37.75 ± 7.68	37.14 ± 9.23

Differences compared with controls: *$P < 0.05$; **$P < 0.001$.

distribution were observed among groups that might bias differences in birth weight due to higher proportion of men with increased weight in any group.

Cotinine levels

Cotinine levels in the plasma of pregnant women were found to be altered in comparing moderate and severe smokers with the control group [53.43 ± 14.80 and 88.8 ± 11.30 versus 1.75 ± 0.06 (ng/ml), respectively, $P < 0.0001$ in both cases] and urine [548.03 ± 212.28 and 1195.57 ± 300.01 versus 181.52 ± 80.49 (ng/ml), respectively, P = NS and $P < 0.0001$, respectively]. Differences in cotinine content also significantly differed in the plasma of newborn between both moderate and severe smokers with respect to controls [45.16 ± 12.65 and 114.28 ± 4.65 versus 1.66 ± 0.07 (ng/ml), respectively, $P < 0.0001$ in both cases].

Mitochondrial parameters

Mitochondrial COX enzyme activity

Mitochondrial COX activity was decreased by 24.9 percent in the PBMC of pregnant women with moderate tobacco consumption compared with the non-smoking group (41.77 ± 3.87 nmol/minute·mg protein versus 55.61 ± 6.41 nmol/minute·mg protein, P = NS). Maternal COX activity of the severe smoker group was decreased by 23.91 percent compared with the control group (42.31 ± 4.65 nmol/minute·mg protein versus 55.61 ± 6.41 nmol/minute·mg protein, P = NS). Newborn CBMC COX activity of moderate and severe smokers was also decreased by 11.2 percent and 24.2 percent, respectively, compared with the control group (41.19 ± 5.59 nmol/minute·mg protein and 35.16 ± 5.42 versus 46.38 ± 6.89 nmol/minute mg protein, P = NS in both cases). However, these differences were not statistically significant. No alterations in COX enzymatic activity were found in the placenta (Figs 1–3).

Mitochondrial COX protein content

No statistically significant differences were found in COX II/COX IV relative protein content between moderate smokers, severe smokers and controls in none of the three analysed tissues. However, COX protein expressed showed a small trend to be increased in newborn CBMC from moderate and severe smoking group with respect to controls (0.53 ± 0.06 versus 0.50 ± 0.05 versus 0.40 ± 0.03, respectively; P = NS).

Oxidative stress

Lipid peroxidation was significantly increased by 52 percent in PBMC of severe smoking women with respect to the control group (0.38 ± 0.05 μM MDA + HAE/mg protein versus 0.25 ± 0.03 μM MDA + HAE/mg protein, respectively, $P < 0.05$). Similarly, in the CBMC of the severe smoking group, lipid peroxidation was also increased by 20.75 percent compared with the control group (0.64 ± 0.09 μM MDA + HAE/mg protein versus 0.53 ± 0.04 μM MDA + HAE/mg protein, P = NS). In placenta, this parameter was also significantly increased by 41.6 percent in the severe smoking group with respect to the control group (19.42 ± 2.16 μM MDA + HAE/mg protein versus 13.7 ± 0.75 μM MDA + HAE/mg protein, respectively, $P < 0.05$). The moderate smoking group did

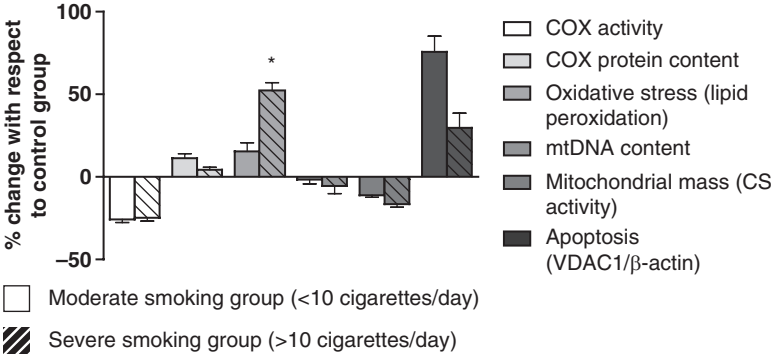

Figure 1 Mitochondrial and apoptotic parameters in maternal PBMC. PBMC = peripheral blood mononuclear cells; COX = cytochrome *c* oxidase; mtDNA = mitochondrial DNA; CS = citrate synthase; VDAC1 = voltage-dependent anion channel protein

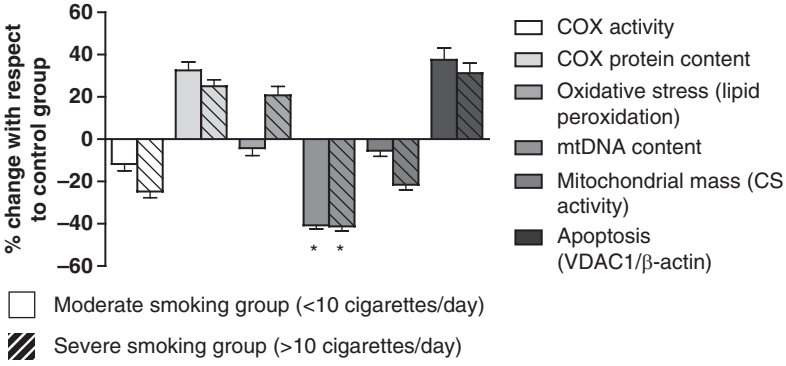

Figure 2 Mitochondrial and apoptotic parameters in newborn CBMC. CBMC = cord blood mononuclear cells; COX = cytochrome *c* oxidase; mtDNA = mitochondrial DNA; CS = citrate synthase; VDAC1 = voltage-dependent anion channel protein

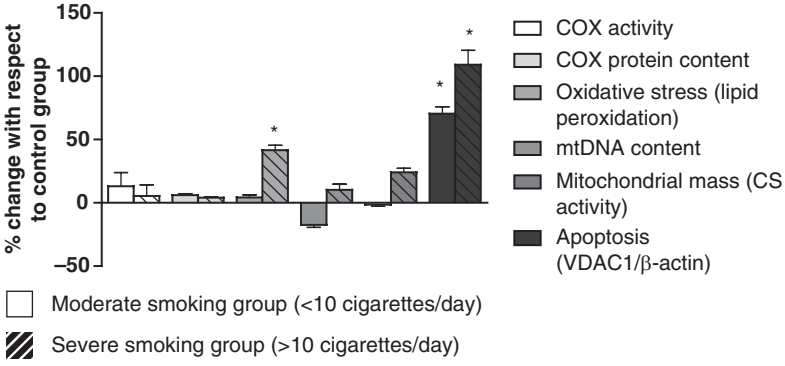

Figure 3 Mitochondrial and apoptotic parameters in placenta
COX = cytochrome *c* oxidase; mtDNA = mitochondrial DNA; CS = citrate synthase; VDAC1 = voltage-dependent anion channel protein

not show increased oxidative stress compared with control subjects in PBMC, CBMC or placenta (Figs 1–3).

Mitochondrial DNA content

Regarding the quantity of mtDNA, a trend to depletion was observed in PBMC from smoking pregnant women. This trend became statistically significant in comparing the fetuses exposed to moderate tobacco consumption with respect to controls, being 40.20 percent (1.01 ± 0.11 mtDNA ND2/nDNA RNApol-II versus 1.69 ± 0.22 mtDNA ND2/nDNA RNApol-II, respectively, $P < 0.05$). In the case of the fetuses exposed to severe tobacco consumption, mtDNA levels were also decreased by 40.83 percent with respect to the control group (1.00 ± 0.14 mtDNA ND2/ nDNA RNApol-II versus 1.69 ± 0.22 mtDNA ND2/ nDNA RNApol-II, respectively, $P < 0.05$). No depletion was found in the placenta (Figs 1–3).

Mitochondrial content

Mitochondrial content measured by citrate synthase activity (nmol/minute·mg protein) in PBMC and CBMC of smoking pregnant women and their newborns showed a trend to decrease in both the moderate and the severe smoking groups with respect to the controls. However, in placenta there was a trend to increase by 24.15 percent in the severe smoking group compared with controls (32.97 ± 3.29 nmol/minute·mg protein versus 26.43 ± 1.37 nmol/minute·mg protein, respectively, P = NS) (Figs 1–3).

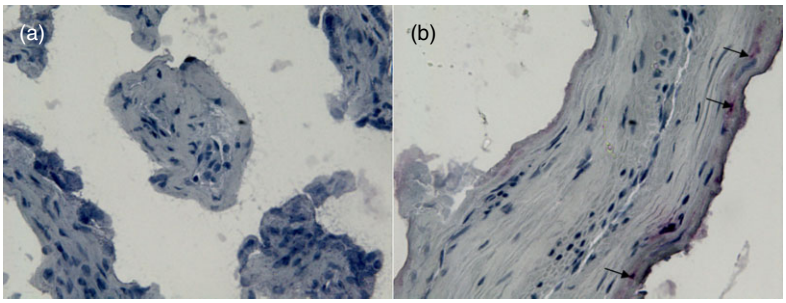

Figure 4 TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay in placenta. (a) Non-apoptotic control placenta. (b) Apoptotic placenta (arrows indicate specifically stained fragmented nucleus of apoptotic cells) from severe smoking pregnant women

Apoptotic studies

Apoptotic changes measured with the ratio of VDAC1/β-actin content were increased in all the subjects and tissues of both smoking groups studied. These differences were not statistically significant for the PBMC and CBMC of smoking pregnant women and newborns. However, the VDAC1/β-actin ratio was significantly increased by 70.5 percent in the placenta of moderate consumers compared with the control group (0.75 ± 0.09 versus 0.44 ± 0.07; respectively, $P < 0.05$). The VDAC1/β-actin ratio was also significantly increased by 109.09 percent in the placenta of the severe consumption group with respect to the control women (0.92 ± 0.2 versus 0.44 ± 0.07; respectively, $P < 0.05$). The apoptotic TUNEL assay showed statistically significant increased apoptosis in the placenta between severe smokers and the control group (1.10 ± 0.37 positive nucleus/1000 total nuclei versus 0.06 ± 0.06 positive nucleus/1000 total nuclei, respectively, $P < 0.01$). All these results are represented in Figs 1–4.

Clinical, molecular and materno-filial correlations

Cotinine levels in plasma were positively correlated with the number of cigarettes smoked per day both in mothers ($R^2 = 0.428$; $P < 0.001$) and in newborns ($R^2 = 0.602$; $P < 0.001$). A significant, positive correlation was also found between plasma materno-fetal cotinine levels ($R^2 = 0.764$; $P < 0.001$). A significant negative correlation was found between the newborn weight and cotinine levels in urine from mothers ($R^2 = 0.160$; $P < 0.01$). Newborn weight was also negatively correlated with the number of cigarettes consumed per day ($R^2 = 0.146$; $P < 0.01$).

Cotinine levels in plasma of the newborns were positively correlated with lipid peroxidation of placenta ($R^2 = 0.130$; $P < 0.05$) and was negatively correlated with mtDNA levels in CBMC ($R^2 = 0.133$; $P = 0.05$). The number of cigarettes smoked per day was positively correlated with lipid peroxidation in placenta ($R^2 = 0.125$; $P < 0.05$) and was negatively correlated with COX activity in maternal PBMC ($R^2 = 0.086$; $P < 0.05$). The VDAC/β-actin levels from maternal PBMC negatively correlated with COX activity from maternal PBMC ($R^2 = 0.150$; $P < 0.01$) and with COX activity from newborn CBMC ($R^2 = 0.100$; $P < 0.05$). In addition, the VDAC/β-actin levels of the CBMC of newborns was positively correlated with lipid peroxidation from the placenta ($R^2 = 0.150$; $P < 0.01$) and was negatively correlated with COX activity from maternal PBMC ($R^2 = 0.140$; $P < 0.01$).

In comparing the PBMC from mothers and the CBMC from newborns, we found positive correlations in lipid peroxidation ($R^2 = 0.188$; $P < 0.05$) and COX activity ($R^2 = 0.522$; $P < 0.001$). In addition, lipid peroxidation from the mothers was negatively correlated with COX activity in newborns ($R^2 = 0.094$; $P < 0.05$). These materno-filial correlations were also observed between VDAC1/β-actin levels of the mothers and their newborns, being positively correlated ($R^2 = 0.270$; $P < 0.001$) with similar correlations in VDAC/β-actin levels from mothers and placenta ($R^2 = 0.110$; $P < 0.05$). The most representative of these correlations is represented in Fig. 5.

DISCUSSION

The pregnancies of smoking women present an increased incidence of adverse perinatal outcomes, especially reduced newborn weight. Previous findings have associated mitochondrial alterations with abnormal birth weight in non-smoking women (Gemma *et al.* 2006). As mitochondria are key organelles in providing energy supply and triggering apoptosis and tobacco-derived CO has been demonstrated to be a mitochondrial toxin, we hypothesized that mitochondrial toxicity and CO could both be key factors leading to reduced birth weight in smoking pregnant women.

The mitochondrial toxicity of tobacco smoke has previously been demonstrated in chronic or acute smoking non-pregnant individuals (Miro *et al.* 1999; Cardellach *et al.* 2003a) but not in the fetuses exposed *in utero* to tobacco. Acute exposure to tobacco in non-pregnant individuals is directly associated with immediate inhibition of COX (Garrabou *et al.* 2011a) and chronic tobacco consumption promotes both COX inhibition and an increase in oxidative stress levels (Alonso *et al.* 2003; Garrabou

et al. 2011b). This is the first description of a chronic tobacco lesion fingerprint present not only in the PBMC of smoking women but also in the CBMC of their newborns and placenta.

The correlation between cotinine levels and the number of self-reported cigarettes consumed per day demonstrated the reliability of the Fagerström test and the accuracy of self-reporting. Cotinine levels and the number of cigarettes smoked per day had also a negative correlation with newborn weight, thereby demonstrating, in this dose–response manner, that tobacco consumption is the main cause of low weight in newborns.

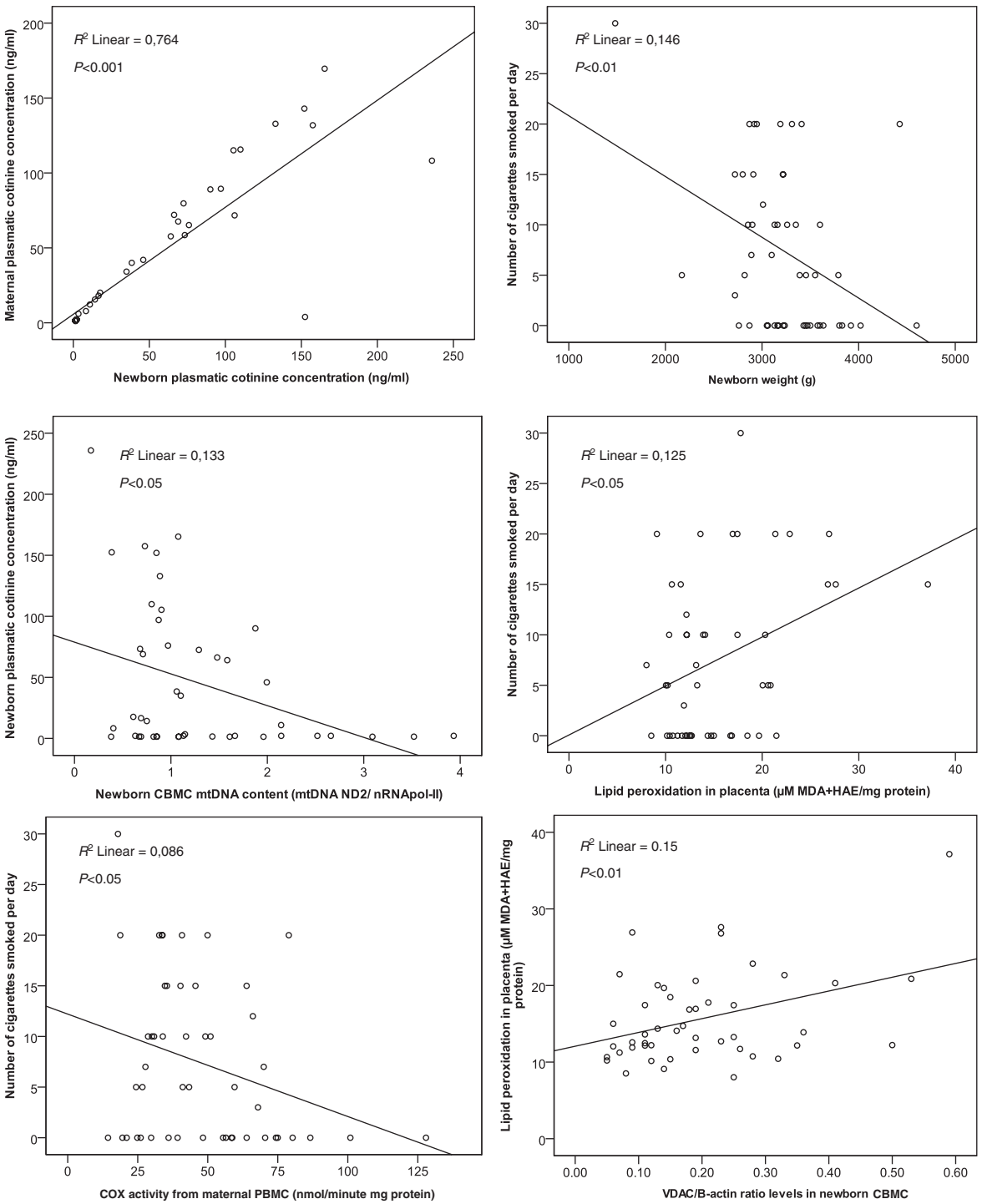

Figure 5 Molecular, clinical and materno-fetal correlations showing a relationship between tobacco consumption, mitochondrial toxicity and reduced newborn weight

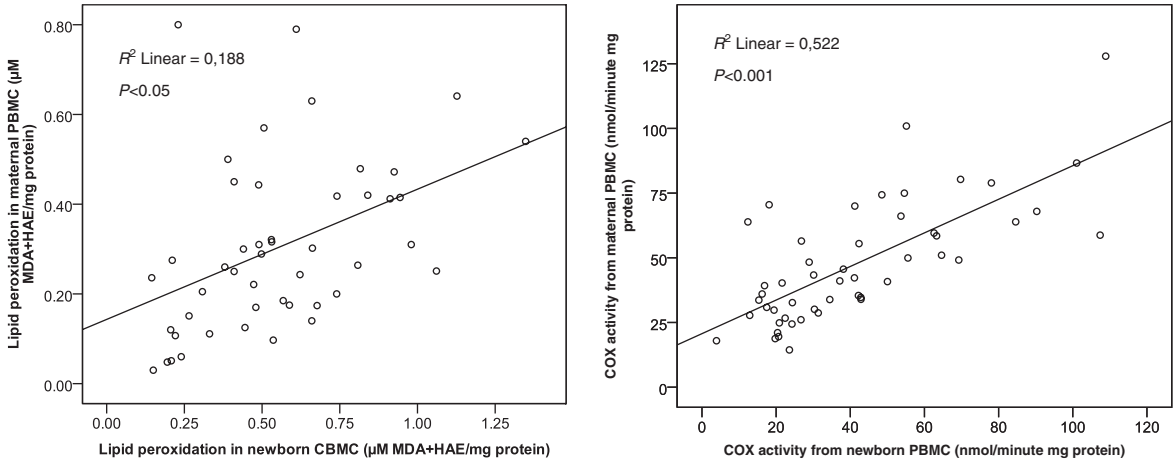

Figure 5 *Cont.*

We found a materno-fetal correlation in cotinine levels in maternal and fetal plasma, suggesting that some toxic compounds of tobacco cross the placental barrier and reach the fetus practically unhindered. Newborns exposed to tobacco during fetal development would therefore receive compounds of tobacco through maternal blood in this crucial stage of development with little filtering activity by the placenta. Lack of placental protection against the toxic effects of tobacco would explain the correlation in mitochondrial and apoptotic status between smoking mothers and exposed newborns.

Although some of the findings reported are not statistically significant, probably due to the small size of the sample, the aetiological mechanism that leads maternal PBMC and fetal CBMC with tobacco exposure to cell death is, probably among others, the CO-mediated COX inhibition.

In these pregnancies, there is a strong trend towards COX inhibition that impairs MRC function, leading to an increase in ROS production and consequent mtDNA depletion. Conserved COX protein content reinforces the hypothesis that COX dysfunction is caused by a CO-mediated inhibition and not by a decrease of COX expression due to mtDNA depletion. Interestingly, this seems to be a dose-dependent response to oxidative stress as only severe smoking pregnancies showed significantly increased oxidative stress in PBMC, CBMC and placenta. Moderate consumers seemed to detoxify lower levels of ROS. These ROS compounds react with cell structures such as sugars, proteins, lipids and also nucleic acids, especially in mitochondria, where they originate. These ROS could lead to mtDNA damage that could cause the mtDNA depletion observed, being especially relevant in the CBMC of newborns and secondarily in placenta. In this case, as in COX, the presence of the toxic compound seems to be more important than the amount of it, as both severely and moderately exposed fetuses showed mtDNA depletion in a kind of qualitative, rather than quantitative response. Depletion and additional destruction of protein and lipid structures such as MRC complexes or mitochondrial inner membrane may lead to a mitochondrial function failure and the production of new increase in ROS. Mitochondria may be involved in a vicious circle of ROS production that may end with programmed cell death or apoptosis, as we observed with the increased levels of VDAC1/β-actin expression in all the samples/tissues of the smoking pregnancies and correlations among mitochondrial and apoptotic parameters. We observed increased levels of the VDAC1/β-actin protein, as a trend in PBMC and CBMC but being statistically significant in placenta. As VDAC1/β-actin levels are highly involved in mitochondrial-dependent apoptosis (Abu-Hamad *et al.* 2006), we performed the TUNEL assay in placenta in order to confirm placental apoptosis. Our hypothesis was clearly confirmed and we can conclude that placental apoptosis is involved in the pathophysiology of adverse perinatal outcomes, especially in the low birth weight observed and documented in smoke-exposed newborns. Placental apoptosis has been previously associated with a higher incidence of adverse obstetric outcomes in non-smoking women, especially with pre-eclampsia (Kim *et al.* 2007; Cali *et al.* 2013). This increase in apoptosis is probably caused in part by the mitochondrial dysfunction due to CO-mediated COX inhibition and the consequent oxidative stress and genetic lesions. However, there could be other toxic compounds in tobacco, solid or volatile, that may lead to ROS enhanced production and mitochondrial dysfunction through an independent mechanism from CO-mediated inhibition of COX, as well as the hypoxia caused by CO binding to haemoglobin, which could affect these parameters. Nicotine alterations of haemodynamic and uterine

blood flow (Krishna 1978) may also be playing a role in observed molecular and obstetric results.

The toxic effects of tobacco seem to be different in each tissue, and this could be due to the nature of the tissue. PBMC and CBMC have a short lifetime and are continuously being replaced by the spleen, presenting fewer trends towards accumulation of toxic effects with respect to placenta, a tissue created to protect and feed the fetus during the 9 months of gestation. We hypothesized that in order to counter this toxic effect of CO, the placenta raises the number of mitochondria to increase mitochondrial function as a mechanism of compensation. We observed this finding as a trend in the measurement of citrate synthase activity. This homeostatic up-regulation of mitochondrial number would explain why the placenta does not present decreased COX activity or mtDNA depletion. Similar homeostatic mechanisms have been described in primary mitochondrial diseases and in *in vitro* models of oxidative stress response (Lee *et al*. 2000). However, and as tobacco consumption is known to produce alterations in the Krebs cycle (Vulimiri *et al*. 2009), results on mitochondrial mass based upon citrate synthase activity should be explored deeper in further investigations.

The small size of the study cohorts may have limited some statistical findings. This may be the biggest limitation of the present study. Probably observed trends in experimental results and obstetric differences may become significant in bigger sample sizes. For instance, significant differences in newborn birth weight between smoking and non-smoking pregnancies were reflected as strong trends in rates of low weight for gestational age and IUGR, but were not significant. On the contrary, most of these statistical trends became significant when we analysed both moderate and severe smoking pregnancies together against controls, but we thought that dose-dependent analysis of tobacco toxicity would be of higher interest for the present study, despite loosing statistical power.

In this article, we describe the toxic relationship among smoking, COX inhibition, derived mitochondrial oxidative and genetic lesion, and birth weight reduction. However, our data suggest that probably the relationship among these parameters is more complex than this simple linear association, so it would be of great interest to investigate further in this topic.

There is a correlation both between the number of cigarettes consumed per day or cotinine levels with mitochondrial or apoptotic disarrangements and between tobacco consumption and birth weight. Similar weight reduction have been reported in newborn with inherited primary mitochondrial diseases (Munnich *et al*. 1992), suggesting that mitochondrial disarrangements may stand at the basis of toxic birth weight reduction in pregnancies of smoking women. In addition, experimental materno-fetal correlations have demonstrated that mitochondrial toxicity affects both the women and their newborn in the same manner. Mitochondrial dysfunction and apoptosis may play a key role in the reduced birth weight of newborns of smoking pregnant women.

Acknowledgements

This study has been funded by *Fondo de Investigación Sanitaria (FIS)* (0229/08, 00462/11 and 01199/12) granted by ISCIII and *Fondo Europeo de Desarrollo Regional (FEDER)*, *Fundació Cellex*, *Fundación para la Investigación y la Prevención del SIDA en España (FIPSE)* (360745/09 and 360982/10), *Suports a Grups de Recerca de la Generalitat de Catalunya* (SGR 14/376 and 09/1385) and CIBER de Enfermedades Raras (CIBERER), (an initiative of ISCIII).

Disclosure/Conflict of Interests

None of the authors of the present article have any conflict of interest regarding the information contained in the article submitted. Additionally, the present article has not been submitted elsewhere.

Authors Contributions

GG conceived the study, together with FC, and also supervised all the data collection and analysis. ASH was responsible for the inclusion of patients and collected the samples at delivery for laboratory processing. MCG carried out the experimental analysis of mitochondrial function and genetics and also drafted the article and performed the statistical analysis of the molecular parameters with GG. CM participated in sample collection and processing and molecular apoptotic quantification. ET was responsible for reactive and material acquisition and preparation and provided technical support in all the experimental assays. SC performed the analysis of oxidative stress and the statistical analysis of these results. ML, FF and ASH collected the clinical, epidemiological and perinatal data, and participated in the revision of the manuscript adding new concepts of high clinical relevance. As an expert in histological measurements, JMG supervised the sample preparation and staining for the TUNEL assay. FC participated in the design of the study together with GG and provided support and supervision to both the clinical and molecular aspects of the study. All authors critically reviewed content and approved final version for publication.

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Autoimmunity Reviews 13 (2014) 363–366

Contents lists available at ScienceDirect

Autoimmunity Reviews

journal homepage: www.elsevier.com/locate/autrev

Review

Diagnosis and classification of sporadic inclusion body myositis (sIBM)

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ARTICLE INFO

Article history:
Accepted 13 November 2013
Available online 12 January 2014

Keywords:
Inclusion body
Myositis
Myopathy
Inflammation

ABSTRACT

Sporadic inclusion body myositis (sIBM) is the most common acquired muscle disease in elderly individuals, particularly men. Its prevalence varies among ethnic groups but is estimated at 35 per one million people over 50. Genetic as well as environmental factors and autoimmune processes might both have a role in its pathogenesis. Unlike other inflammatory myopathies, sIBM causes very slowly progressive muscular weakness and atrophy, having a distinctive pattern of muscle involvement and different forms of clinical presentation. In some cases a primary autoimmune disease coexists. Diagnosis is suspected on clinical grounds and is established by typical muscle pathology. As a rule sIBM is refractory to conventional forms of immunotherapy.

Contents

1. Introduction

Sporadic inclusion body myositis (sIBM) is a progressive degenerative inflammatory disorder in skeletal muscle of unknown etiology. In addition, is one of the three main subsets of inflammatory myopathies, the other two being polymyositis and dermatomyositis. Although all of these conditions include inflammation in the endomysium, muscle fiber necrosis, elevation of serum muscle enzymes and varying degrees of muscle weakness, sIBM is often misdiagnosed as polymyositis. sIBM should be distinguished from hereditary inclusion body myopathies (hIBM) in which histologic and ultrastructural findings resemble those of sIBM with one clear exception: the absence of inflammation.

2. Epidemiology

The prevalence of sIBM is estimated at between 4.5 to 9.5 per one million rising to 35 per million for people over 50 years old. Recent studies have reported differences in prevalence with respect to geographical location, being 1.0 in Turkey [1], 4.7 in Netherlands [2], 9.8 in Japan [3] or 50 in Western Australia [4]. A number of discrepancies

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http://dx.doi.org/10.1016/j.autrev.2014.01.016

suggest that these numbers underestimate the true prevalence of this myopathy. On the basis of clinical reports from reference centers worldwide, it seems that sIBM is the most common acquired myopathy in patients above 50 years, affecting men slightly more frequently than women [5,6].

3. History

In 1971 Yunis and Samaha coined the term IBM for the definition of a myopathy that clinically resembled a chronic polymyositis but was pathologically characterized by the presence of vacuoles containing cytoplasmic degradation products with fibrillary nuclear and cytoplasmic inclusions [7]. A few years previously some authors had reported clinical cases suggestive of IBM. Since then, large series of patients have been described.

4. Pathogenesis

Although the underlying cause of sIBM is unclear, it seems that at least three processes might occur in parallel: a primary immune process due to T-cell mediated cytotoxicity, a non-immune process characterized by vacuolization and intracellular accumulation of amyloid-related molecules probably due to MHC-class I-induced stress [6,8], and mitochondrial dysfunction.

Choi et al. [9] demonstrated the elevated expression of transglutaminases 1 and 2 in the vacuoles of sIBM, co-localizing with amyloid-related proteins. They suggest that these enzymes participate in the formation of insoluble amyloid deposits and may thereby contribute to progressive debilitating muscle disease. This topic has been explored by Selva-O'Callaghan et al. [10] with interesting results. Genetic factors are presumed to play a role in sIBM based on an association between sIBM and certain HLA genes, in particular HLA — DR3. This association is present in nearly 75% of the cases, but this figure may vary in different ethnic groups [6,11,12]. Many recent studies have shown parallelism between sIBM and Alzheimer's disease, focusing on similarities between brain and muscle cells of Alzheimer and sIBM, respectively. These similarities include cellular aging, oxidative and endoplasmic reticulum stresses, mitochondrial abnormalities, proteasome inhibition and multiprotein aggregates [13–15].

5. Clinical manifestations

sIBM causes weakness and atrophy of the distal and proximal muscles and involvement of the quadriceps and deep finger flexors are clues to early diagnosis. The pattern is sometimes asymmetric resembling a motor neuron disease. Neck flexors and extensors are frequently affected. Heat drop and camptocormia (selective atrophy and weakness of paraspinal muscles) may occur, even as a form of clinical presentation. Facial involvement is rare but can be observed in HIV-related cases. Dysphagia occurs in up to 60% of patients with sIBM and again may be the form of presentation in rare cases. Sensory function is normal as well are tendon reflexes, but they become diminished or absent as the atrophy of major muscles occurs. The clinical course is always chronic or very chronic, lasting for years after the onset of symptoms and the diagnosis of the disease. Disease progression is slow but steady resembling that of a muscular dystrophy.

6. Differential diagnosis

sIBM is often misdiagnosed as polymyositis or other diseases and is frequently only suspected retrospectively when a patient with presumed polymyositis does not respond to therapy. In a patient complaining of falls due to weakness at the knees and feet with atrophic thighs and without paresthesias or cramps the most plausible diagnosis is sIBM. Useful data regarding differential diagnoses are shown in Table 1.

Table 1
Differential diagnoses (prominent data for each condition).

Motor neuron disease:	Hyperreflexia, cramps, fasciculations Typical EMG
Polymyositis:	Subacute (weeks to months) Proximal and symmetrical muscle weakness High CK levels
Vacuolar myopathies: (myofibrillar myopathies, hIBM)	Lack of inflammation, negative MHC HLA-class I

7. Pathological features

The common findings in muscle biopsy are perivascular and endomisial inflammatory infiltrates of varying degrees, rimmed vacuoles in atrophic fibers (Fig. 1), the presence of partial cellular invasion by CD8 cells, frequent cytochrome oxidase (COX)-negative cellules, β-amyloid and tau deposits and the upregulation of MHC class I antigens in healthy muscle cells. In addition abnormal mitochondrial changes such as ragged-red fibers are frequently observed. Some of theses features can be observed in Fig. 1. Nuclear and/or cytoplasmic filamentous inclusions of 16–20 nm are seen in electron microscopy examination [16]. Recent studies suggest that abnormal accumulation of extranuclear TDP-43, a nucleic acid-binding protein, in sarcoplasm of IBM muscle cells may be toxic through its binding to RNA [17,18]. On some occasions an additional muscle biopsy must be performed if pathological changes are suggestive but not consistent.

8. Biochemical features

Creatine kinase (CK) serum levels are moderately elevated but can be normal. Unlike other inflammatory conditions acute phase reactants are normal in sIBM.

9. Serological features

Different autoantibodies can be detected in a percentage of sIBM patients. Antinuclear antibodies (20%), rheumatoid factor (13%), anticardiolipin antibodies (10%), antiRo antibodies (10%) are the most frequently reported. In 2011, an autoantibody to an approximately 43 kDa human muscle protein was identified in 52% of IBM samples, 0% of other autoimmune myopathy samples and 0% of normal samples [19,20]. In about 10% of the cases dysproteinemia can also be detected [21].

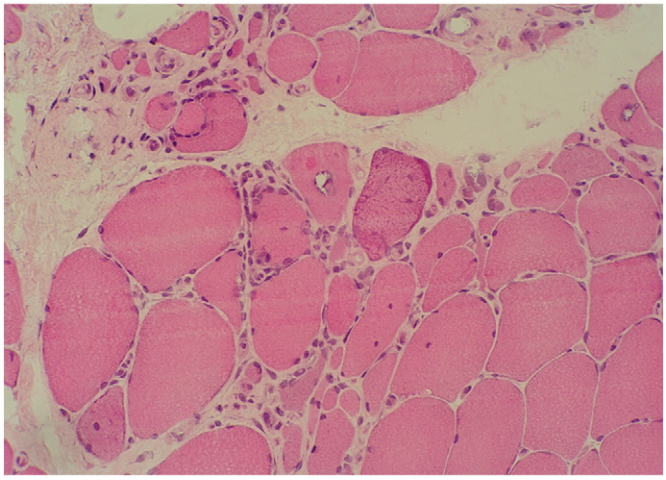

Fig. 1. Variability in fiber size with prominent connective tissue. Mononuclear cells in the endomysium as well as invading a healthy muscle cell can be observed. Rimmed vacuoles are present on at least two fibers. A typical ragged-red fiber is also observed in the center of the picture. HE on frozen muscle biopsy from an sIBM patient.

Table 2
Diagnostic criteria proposed for sIBM.

Clinical features:
- Duration of illness >6 months
- Age at onset > 30 years
- Slowly progressive muscle weakness and atrophy: selective pattern with early involvement of quadriceps femoris and finger flexors (frequently not symmetric)
- Dysphagia
Laboratory features:
- Serum CK levels might be high but can be normal
- EMG: myopathic or mixed patterns, with both short and long duration motor unit potentials and spontaneous activity
Muscle biopsy:
- Myofiber necrosis and regeneration
- Endomysial mononuclear cell infiltrate (in variable degree)
- Mononuclear cell-invasion of non-necrotic fibers (mainly CD8)
- MHC class I expression in otherwise morphologically healthy muscle fibers
- Vacuolated muscle fibers (rimmed vacuoles)
- Ubiquitin-positive inclusions and amyloid deposits in muscle fibers
- Nuclear and/or cytoplasmic filamentous inclusions of 16–20 nm on electron microscopy
- COX-negative fibers

10. Associated disorders

Several autoimmune disorders have been reported in association with sIBM [6,21–23], including pernicious anemia, dermatitis herpetiformis, psoriasis, Sjögren syndrome, SLE, rheumatoid arthritis, common variable immunodeficiency, idiopathic thrombocytopenic purpura, Hashimoto's thyroiditis, dermatomyositis and gluten sensitivity enteropathy. In a recent study Ray et al. demonstrated the evidence of humoral autoimmunity in sIBM [24]. Unlike dermatomyositis, sIBM should not be considered as a paraneoplastic condition. In addition HIV infection can be associated with sIBM with progressive muscular disease despite the good control of the infection (normal CD4 values as well as viral load).

11. Diagnostic criteria

The definite diagnostic procedure is a biopsy of the muscle. Although individual pathological features are all non specific and can also be seen in other myopathies and neurogenic disorders, their co-occurrence in the same biopsy allows the diagnosis of sIBM. Table 2 shows the diagnostic criteria for sIBM. The criteria for diagnosis of sIBM were first proposed by Griggs et al. in 1995, with minor modifications in 2002 and were finally reviewed by Dalakas in 2007. Table 3 presents the diagnostic categories (definite, probable and possible sIBM) [5,6,25].

12. Prognosis

The severity of the disease is poorly associated with the degree of inflammatory changes found in muscle biopsies and although treatment with corticosteroids might reduce the inflammation, it does not stop the degenerative changes and has little or no effect on the degree of weakness. sIBM is a relentlessly progressive disorder: most patients requiring a walking aid after 5 years and the use of wheelchair after about 10 years. sIBM patients often die due to a complication of their debilitating progressive disease (aspirative pneumonia) or because an unrelated condition.

Table 3
Diagnostic categories.

Definite sporadic inclusion body myositis:
· Characteristic clinical features with biopsy confirmation: inflammatory myopathy with autoaggressive T cells, rimmed vacuoles, COX-negative fibers, amyloid deposits or filamentous inclusions and upregulation of MHC class I expression. With these pathlogical findings the presence of other laboratory features are not mandatory.
· Atypical pattern of weakness and atrophy but with diagnostic biopsy features.
Probable sporadic inclusion body myositis:
· Characteristic clinical and laboratory findings but incomplete biopsy criteria (e.g. features of necrotising inflammatory myopathy with T cell invasion but absence of rimmed vacuoles, amyloid deposits, filamentous inclusions and COX-negative fibers).
Possible sporadic inclusion body myositis:
· Atypical pattern of weakness and incomplete biopsy criteria

13. Therapy

Most patients do not respond to antiinflammatory, immunosuppressant or immunomodulatory drugs currently available. Corticosteroids, cytotoxic drugs, intravenous immunoglobulins, antithymhocyte globulin and cytokine-based therapies have been used with poor results in follow-up [5,6]. Some authors have reported reliable data about inefficacy of immunotherapy in sIBM [26]. A small proportion of patients do respond, at least initially, and this probably represents a subgroup in whom the disease is diagnosed early and/or is associated with a primary autoimmune condition [27]. In some centers an initial 3–6 month trial of prednisone and methotrexate or azathioprine is recommended.

Other empirical therapies such as coenzyme Q10, carnitine, myostatin inhibitors and even statins have been used or are under investigation. Exercise therapy and orthotic appliances have confirmed their efficacy in stabilizing muscle strength and functional ability [28].

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Drug and Chemical Toxicology

ISSN: 0148-0545 (Print) 1525-6014 (Online) Journal homepage: http://www.tandfonline.com/loi/idct20

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To cite this article: Constanza Morén, Glòria Garrabou, Antoni Noguera-Julian, Núria Rovira, Marc Catalán, Sandra Hernández, Ester Tobías, Francesc Cardellach, Clàudia Fortuny & Òscar Miró (2013) Study of oxidative, enzymatic mitochondrial respiratory chain function and apoptosis in perinatally HIV-infected pediatric patients, Drug and Chemical Toxicology, 36:4, 496-500, DOI: 10.3109/01480545.2013.776578

To link to this article: http://dx.doi.org/10.3109/01480545.2013.776578

Published online: 27 Mar 2013.

Drug and Chemical Toxicology

http://informahealthcare.com/dct
ISSN: 0148-0545 (print), 1525-6014 (electronic)

Drug Chem Toxicol, 2013; 36(4): 496–500
 DOI: 10.3109/01480545.2013.776578

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SHORT COMMUNICATION

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

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Abstract

Mitochondrial toxicity in perinatally human immunodeficiency virus (HIV)-infected pediatric patients has been scarcely investigated. Limited data are available about HIV or antiretroviral (ARV)-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 on the pathology of HIV and highly active antiretroviral therapy (HAART) in perinatally HIV-infected pediatric patients. We studied 10 healthy control participants and 20 perinatally HIV-infected pediatric patients (10 under ARV 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. Global oxygen consumption was significantly compromised in HIV-infected untreated patients, compared to the control group (0.76 ± 0.01 versus 1.59 ± 0.15; $P = 0.014$). Apoptosis showed a trend to increase in untreated patients as well. The overall complex (C) CI-III-IV activity of the mitochondrial respiratory chain (MRC) was significantly decreased in HIV-infected treated patients with respect to the control group (1.52 ± 0.38 versus 6.38 ± 1.53; $P = 0.02$). No statistically significant differences were found between untreated and HAART-treated patients. These findings suggest the pathogenic role of both HIV and HAART in mitochondrial dysfunction in vertical infection. The abnormalities in mitochondrial genome may be downstream reflected through a global alteration of the MRC. Mitochondrial impairment associated with HIV and HAART was generalized, rather than localized, in this series of perinatally HIV-infected patients.

Keywords

Antiretrovirals, HIV, mitochondrial toxicity, pediatrics

History

Received 26 June 2012
Revised 8 January 2013
Accepted 27 January 2013
Published online 27 March 2013

Introduction

A dramatic decrease in the rates of human immunodeficiency virus (HIV) mother-to-child transmission has been observed subsequent to the implementation of several prophylactic measures in the past 15 years (Connor et al., 1994; Santos et al., 2012; Senise et al., 2011). These measures include the use of antiretrovirals (ARVs) in HIV-infected women, during pregnancy and delivery, as well as in newborns during the neonatal period, the use of elective caesarean section, 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 situation. According to current guidelines, this type of infant will be exposed lifelong to HAART. First-line recommended treatments in infants include two nucleoside reverse-transcriptase inhibitors [NRTIs; most commonly, abacavir (ABC) plus lamivudine (3TC) or emtricitabine (FTC)] and either one protease inhibitor [PI; ritonavir-boosted lopinavir (LPV/rtv)] or a non-nucleoside reverse-transcriptase inhibitor [NNRTI; nevirapine (NVP)] (NIH, 2011; PENTA, 2011).

HIV and HAART are extensively documented to cause mitochondrial toxicity in adults by different pathogenic ways, and this mitochondrial toxicity has been suggested to underlie several secondary effects, such as hyperlactatemia/lactic acidosis, pancreatitis, myopathies, neuropathies and lipodystrophy (Feeney & Mallon, 2010; Walker & Brinkman, 2001). Mitochondrial DNA (mtDNA) depletion, caused by NRTI, 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 (Miró et al., 2003). Additionally, HIV itself is able to induce apoptosis (Badley et al., 2000) and secondarily affect mitochondrial function in adults (Miró et al., 2004).

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However, the specific role of both HIV and HAART on mitochondrial damage in HIV-infected, perinatally infected pediatric patients remains unknown. Our group previously reported a decrease in mtDNA amount in a series of asymptomatic HIV-infected pediatric patients (Morén et al., 2011a). In the present study, we aimed to elucidate whether this mitochondrial abnormality, at the genetic level, is also present at downstream levels from the study of mitochondrial enzymatic activities to oxygen consumption and apoptosis. Only a few studies, usually focused on one aspect of mitochondrial biogenesis (Vigano et al., 2001), have provided controversial information about mtDNA content in HIV-infected treated children (Cossarizza et al., 2002; Saitoh et al., 2007) and, further, the role of apoptosis has not been taken into account. In addition, there is no information reporting on mitochondrial oxidative parameters through polarographic studies in perinatally HIV-infected pediatric patients. In the present study, we assessed different aspects of mitochondrial function to gain insight on mitochondrial toxicology caused by HAART and HIV in vertically infected pediatric patients on and off HAART.

Methods

We performed a cross-sectional, multicentric, observational study. The study was approved by the ethical committee from both the pediatric Hospital Sant Joan de Déu (Barcelona, Spain) and Hospital Clínic of Barcelona (Barcelona, Spain) sanitary centers, and patients and their parents signed informed consent before inclusion. Patients were classified in two categories: uninfected healthy control participants ($n = 10$) 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 the untreated group, and HAART-treated patients ($n = 10$). These patients belong to a larger cohort of HIV-infected pediatric patients followed in Hospital Sant Joan de Déu, a tertiary-care pediatric center. Both control participants and patients were consecutively and parallely included 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 of them were free from any active HIV-related clinical condition, showed a plasmatic HIV-RNA (CA HIV Monitor; Roche, Basel, Switzerland) below 50 copies/mL, and maintained a cluster of differentiation (CD)4 cell count or percentage (flow cytometry, FACSCalibur; BD Biosciences, San Jose, CA) within Centers for Disease Control and Prevention Immunological Category 1 (>350 cells/mm^3 for patients over 12 years of age or >25% for children 12 years of age or less) for at least the preceding 2 years.

All experiments of the study have been performed in peripheral blood mononuclear cells (PBMCs). All samples recruited from patients and control participants were obtained by means of a peripheral venous blood extraction (20 mL), and the PBMC fraction of lymphocytes and monocytes was isolated through a Ficoll gradient (Prilutskii et al., 1990). Experiments of flow cytometry and polarography were performed in fresh samples, and the remaining amount of sample was cryopreserved at −80 °C for further analysis.

Mitochondrial mass was evaluated by citrate synthase (CS) activity measurement by means of spectrophotometry (Barrientos, 2002).

Mitochondrial respiratory chain (MRC) study

As a representative of translation efficiency, mtDNA-encoded cytochrome-c-oxidase (COX) subunit II and nuclear DNA-encoded subunit IV (COXII and COXIV) content of the MRC complex (C) IV (CIV) were measured by Western blot and expressed as the COXII/COXIV ratio (McComsey et al., 2008). As a representative of functional efficiency, mitochondrial CII, CII-III, glycerol-3-phosphate dehydrogenase (G3PDH), G3PDH-CIII, CIV and global CI-III-IV enzymatic activities were measured spectrophotometrically at 37 °C, as reported elsewere (Garrabou et al., 2012; Miró et al., 1998). As a representative of mitochondrial respiration, oxidative activities of fresh PBMCs were measured polarographically 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 and III through succinate oxidation (Sox), G3P oxidation (G3Pox), pyruvate malate oxidation (PMox), glutamate malate oxidation (GMox), using the corresponding specific substrates and inhibitors, and, finally, the global endogenous oxygen consumption in intact cells (Cellox) in the absence of substrates and inhibitors (Barrientos, 2002; Garrabou et al., 2012).

Apoptosis study

Apoptosis was assessed by two different methods: 1) by measuring loss of mitochondrial membrane potential of fresh PBMCs, indicative of early apoptosis development, by flow cytometry through JC-1 fluorescence quantification (Cossarizza et al., 1993) and 2) by determining the truncate or activated form of caspase-3 (Casp-3) content, suggestive of advanced apoptosis development, through Western blot analysis (McComsey et al., 2008).

Statistical analysis

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

Results

Clinical and epidemiological data of the patients included in the study are summarized in Table 1. 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 PI-based HAART regimen (median time on that regimen: 25.7 months; range, 2.0–64.0), whereas the reminder had interrupted HAART several years ago (range, 3.1–7.1). As expected, HIV plasmatic viral load was significantly higher among untreated patients (median values: 4.5 log versus 1.9 log RNA-HIV copies/mL; $P = 0.008$), whereas CD4 cell counts remained similar between groups and within normal values in

Table 1. Clinical characteristics of patients included in the study.

Clinic data of the included patients	HIV infected ($n=20$)	HIV HAART interrupted ($n=10$)	HIV treated ($n=10$)
Age (years)	14.85 ± 0.73	14.86 ± 1.26	15.15 ± 0.91
Male gender (n)	10	8	2
Viral Load (log10 copies RNA/mm^3)	3.19 ± 0.31	4.54 ± 0.10	1.85 ± 0.13
CD4 count (cells/mm^3)	778.35 ± 66.88	731.00 ± 90.64	825.70 ± 100.87
Lactic acid levels (mmol/L)	1.14 ± 0.13	1.03 ± 0.25	1.26 ± 0.082
Antiretroviral duration (months)			25.70 ± 6.88
Time without treatment (months)		48.90 ± 6.94	
Antiretroviral type[a]			
Triple NRTI regimen (TDF + FTC + ABC + PI) (n)			1
Double NRTI regimen			
TDF + d4T or ABC or FTC + PI (n)			5
3TC + ABC + PI (n)			2
Single NRTI regimen (3TC + NNRTI + PI) (n)			2

[a]Only for HIV-treated patients. Results are expressed as mean ± standard error mean.
TDF, tenofovir; d4T, stavudine.

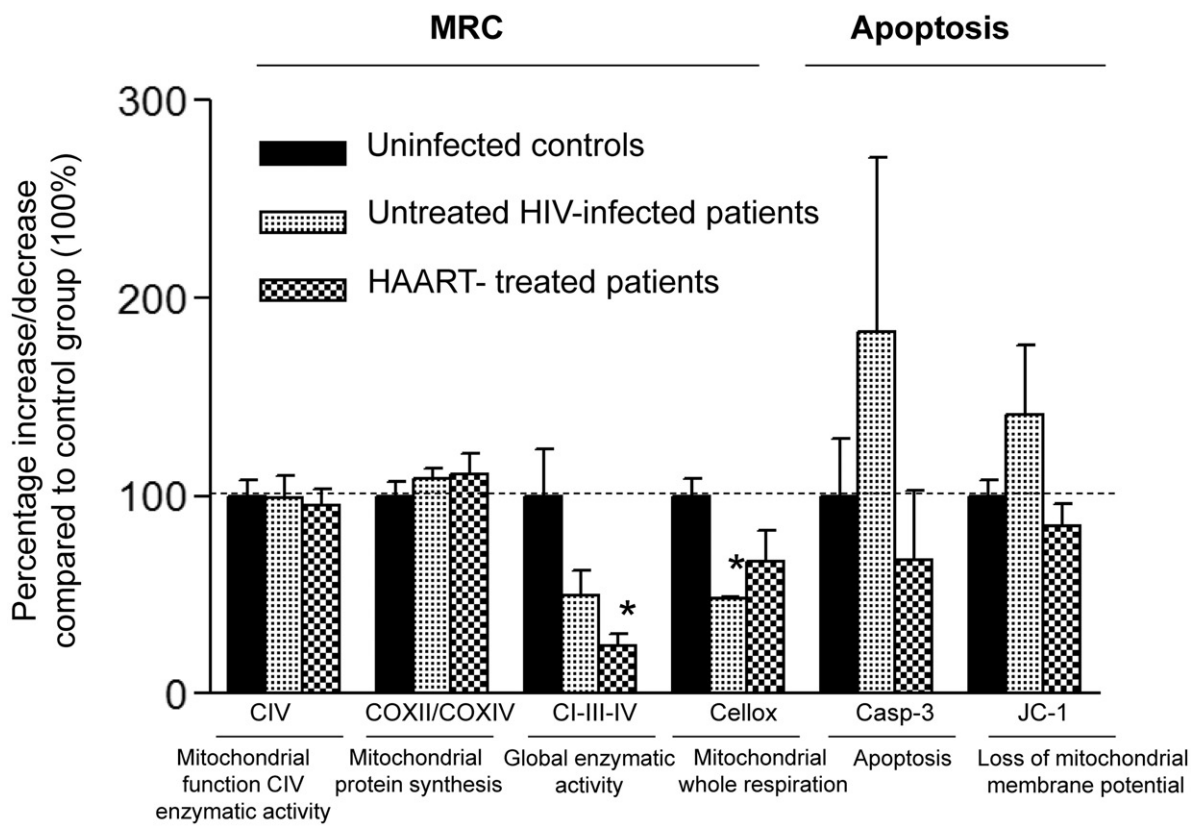

Figure 1. Mitochondrial and apoptotic parameters. Results are expressed as mean percentage of the parameters with respect to controls, represented by the line (100%) (mean ± SEM). CIV: cytochrome-c-oxidase or complex IV; COXII and COXIV: cytochrome-c-oxidase subunits II and IV; CI-III-IV: Complex I-Complex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

all cases [median values: 731 versus 826 cells/mm^3; $P=$ not significant (NS)]. Normal plasmatic lactate levels were also observed in both groups (median value: 1.14 mmol/L; range, 0.74–1.61), and none of the patients presented symptoms consistent with mitochondrial toxicity. A sex- and age-matched control group of 10 healthy volunteers was used.

Mitochondrial content was very similar in both groups with respect to uninfected control participants ($103.0 \pm 8.2\%$ for untreated patients and $91.2 \pm 5.4\%$ for treated patients; $P=$ NS for both).

On analysis of MRC (Figure 1), the translational COXII/COXIV ratio was preserved in HIV-infected patients, irrespectively of whether they were receiving HAART or not. Similarly, functional efficiency of mitochondrial COX (enzymatic activity of CIV), as well as the rest of the complexes of the MRC (CII and CII-III), the G3PDH enzyme, and the combination G3PDH-CIII, were unaffected, although general CI-III-IV activity was significantly reduced (approximately 75%) in HAART-treated HIV-infected patients with respect to the control group (1.52 ± 0.38 versus 6.38 ± 1.53 nmol substrate/min mg of cell protein; $P=0.02$), with this decline not being statistically significant in untreated patients either with respect to the control group or to untreated patients (3.15 ± 0.82 nmol substrate/min mg of

Table 2. Enzymatic and oxidative activities of MRC and G3PDH.

Groups	Control group	Untreated	HAART	*P*-value
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	**

Results are expressed as nmol of oxidized or reduced substrate/min mg of cell protein.

*Significant differences between control group and HAART-treated patients ($P = 0.02$).

**Significant differences between control group and untreated patients ($P = 0.014$).

cell protein; $P =$ NS for both) (Table 2). Respiration capacity by stimulation of specific MRC complexes (I, II and III) was similar in control participants and cases (Table 2). However, global oxygen consumption (of all complexes) in intact nonstimulated cells was significantly reduced (approximately 50%) in HIV-infected untreated patients, compared to the control group (0.76 ± 0.01 versus 1.59 ± 0.15 nmol substrate/min mg of cell protein; $P = 0.014$), with this decline not being statistically significant in HIV-infected patients on HAART, either with respect to control participants or untreated patients (1.06 ± 0.24 nmol substrate/min mg of cell protein; $P =$ NS for both).

Apoptosis, measured by either casp-3 quantification or by mitochondrial membrane potential (MMP) assessment, did not show any significant changes between groups (Figure 1), although a trend to greater activation was found in untreated patients.

Discussion

As we observed in our previous work, a depletion of mtDNA was found, but this was neither reflected in the function or in the subunits content of complex IV of the MRC nor in the enzymatic activities of most complexes of the MRC (Morén et al., 2011a, 2012). In this study, we aimed to further assess a possible alteration in apoptotic pathways or at the level of the oxygen consumption (not previously assessed) in perinatally HIV-infected adolescents. No significant differences in mitochondrial protein synthesis or in the specific function of most MRC complexes between asymptomatic perinatally HIV-infected pediatric patients off or on HAART with respect to the uninfected control group were found, because all groups showed very similar experimental results. Conversely, a significant decrease was observed in the global CI-III-IV enzymatic activity of MRC in HAART-treated patients, as previously reported in a study including symptomatic HIV-infected children (Morén et al., 2011b), as well as in endogenous mitochondrial respiration in intact cells from untreated HIV-infected patients (not previously described). Our findings can be explained through two hypotheses. First, because of technical aspects regarding the stimulation and inhibition of each complex of the MRC; thus, a potential endogenous deficit could be masked by the addition of our exogenous compounds. The second hypothesis considers an additive effect of slight alterations of most of the complexes of the MRC. This means that the sum of many moderate alterations triggers a detectable alteration in global MRC.

To date, there are no data available regarding polarographic studies on oxygen consumption in this population. The observed decrease in mitochondrial respiration does not seem to be related to any intrinsic dysfunction of a specific complex of MRC, because mitochondrial protein synthesis and function in each isolated complex were preserved in our patients. Alternatively, the trend toward an increase in apoptosis observed in the untreated group may indicate that apoptotic activation of untreated HIV patients could rely at the basis of suboptimal cell respiration in this group. Previous studies have reported on the apoptotic and deleterious role of HIV associated with mitochondrial dysfunction in adults (Badley et al., 2000; Miró et al., 2004).

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, generalized effect caused by HAART.

It is remarkable that in all HIV-infected patients, either off or on HAART, there was a decrease of global CI-III-IV activity and of global endogenous oxygen consumption, although statistical significance was not always reached, when compared to the control group.

In our study, we found that mitochondrial abnormalities in PBMCs of vertically HIV-infected young patients were not detected through mitochondrial studies of each individual complex of the MRC, but were revealed with a general MRC assessment. Our findings suggest the presence of a moderate, diffuse mitochondrial impairment resulting from both HIV and HAART, rather than a specific, profound and localized damage in any of the complexes of the MRC. 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 the heterogeneity in the characteristics of the patients, although these are both common methodological limitations in 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 report, for the first time, the 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, through a global alteration of the MRC.

Conclusion

In summary, our results in this case series suggest that mitochondrial impairment associated with HIV and HAART is generalized, rather than localized, in HIV-infected children.

Acknowledgements

The authors thank the collaboration of the Surgical Team of the Hip Unit at the Hospital Clinic, Barcelona. The authors thank Mireia Nicolás for her valuable technical support in carrying out experimental studies.

Declaration of interest

This work has been supported by Fondo para la Investigación y la Prevención del SIDA en España (FIPSE 36612/06, 36572/06, 360745/09 and 360982/10), Fondo de Investigaciones Sanitarias (FIS 0229/08 and 0462/11), Suports a Grups de Recerca de la Generalitat de Catalunya (2009/SGR/1158), Red de Sida (RD 06/006), Fundació Cellex, and CIBER de Enfermedades Raras, an initiative of the ISCIII. Dr. Miró has been a depositary of a Research Intensification grant from ISCIII (Spain) during 2009.

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Mitochondrial Evolution in HIV-Infected Children Receiving First- or Second-Generation Nucleoside Analogues

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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 antiretroviral regimens.

Methods: We followed-up 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) for a period of 2 years for their immunovirological and mitochondrial status, and compared these subjects with a group of untreated HIV-infected patients (n = 10) and uninfected controls (n = 27). We measured T-lymphocyte CD4+ content (flow cytometry), viral load (real-time polymerase chain reaction), and lactate levels (spectrophotometry); we assessed mtDNA content (real-time polymerase chain reaction), mitochondrial protein levels (Western blot), oxidative stress, mitochondrial mass, and electron transport chain function (spectrophotometry) in peripheral 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, respectively; $P = 0.022$). 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, first- and second-generation antiretrovirals, HIV, mitochondrial toxicity, nucleoside reverse transcriptase inhibitors, therapeutic strategies

(*J Acquir Immune Defic Syndr* 2012;60:111–116)

INTRODUCTION

Highly active antiretroviral (ARV) therapy (HAART) reduces the mortality and morbidity of 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 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 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 pediatric patients, for whom this issue has special relevance because they constitute the first generation that will receive ARV treatment throughout their lives.

As in adult patients, HAART-related mitochondrial effects in children have been reported by our group.[7] Rosso et al[8] 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. 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

Received for publication November 15, 2011; accepted February 13, 2012.
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Supported by Fundació Cellex, Fundación para la Investigación y la Prevención del SIDA en España (360745/09, 360982/10, and 36612/06), and Fondo para la Investigación Sanitaria (PI08/00229, PI00462).

The authors have no funding or conflicts of interest to disclose.
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drugs, as follows: zalcitabine > didanosine (ddI) > stavudine (d4T) > zidovudine (ZDV) > lamivudine (3TC)/emtricitabine (FTC) > abacavir (ABC) = tenofovir (TDF).[9,10] The use of zalcitabine (no longer administered) and ddI has been related to pancreatitis[11,12]; d4T has been associated with lipoatrophy,[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-polymerase,[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 pediatric 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 pediatric patients undergoing different ARV regimens 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 ± SEM at baseline, 11.1 ± 0.7 years) on HAART that either included 1gNRTIs (n = 15) or 2gNRTIs (n = 13) was assessed and compared with the status of an untreated group of HIV-infected children (no ARV group; n = 10) and with 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+ content was analysed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA). HIV RNA viral load was quantified by quantitative real-time polymerase chain reaction (CA HIV Monitor; Roche, Basel, Switzerland; limit <50 copies/mL) and plasma lactate levels (a surrogate biomarker of mitochondrial lesion, millimoles per liter) were measured with a spectrophotometric procedure (Cobas Fara II Analyzer; Roche).

Peripheral blood mononuclear cells were isolated from 5–10 mL of venous blood with a Ficoll gradient (Histopaque 1077; Sigma Diagnostics, St Louis, MO)[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), as previously reported.[19] Results were expressed as nanomoles of reduced substrate per minute and per milligram of cell protein (nanomoles per minute per milligram protein).

We assessed mtDNA content by quantitative real-time polymerase chain reaction (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 nDNA content as the ratio of ND2/18SrRNA and normalized by CS.

Mitochondrial protein levels of mtDNA-encoded subunit II (COXII) and nDNA-encoded subunit IV (COXIV) of cytochrome c oxidase or complex IV (CIV) were quantified by Western blot, using the porin voltage-dependent anion carrier protein as a marker of mitochondrial protein loading and β-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 and hydroxyalkenals content at 586 nm,[22] and the results were normalized per total protein content (micromolar malondialdehyde and hydroxyalkenals per milligram protein).

Measurement of the enzymatic activity (also in nanomoles per minute per milligram of protein) of the mitochondrial respiratory chain (MRC) complexes was performed by spectrophotometry (Hitachi 2900; Hitachi Instruments Inc), and the results were referred to overall cell protein and normalized by mitochondrial mass, estimated by CS activity. Because 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: CIV activity at 550 nm according to Rustin et al,[24] slightly modified for minute amounts of biological samples[19,25]; complex II–III at 550 nm; glycerol-3-phosphate dehydrogenase–complex III (G3PDH–CIII) at 550 nm; complex II at 600 nm; and isolated G3PDH at 600 nm.

Statistical analysis was carried out with the SPSS 18.0 program. The results were expressed as 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 plasma viral loads ($P < 0.001$) at both time points when compared with 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 plasma biomarker of mitochondrial dysfunction, were similar in all groups at baseline (Table 1) but were higher in the 1gNRTIs group with respect to HIV-infected untreated controls (1.28 ± 0.08 vs. 0.85 ± 0.08 mmol/L; $P < 0.001$) and also with respect to the

TABLE 1. Clinical, Immunovirological, and Mitochondrial Data of Patients and Controls at Baseline

	1gNRTIs	2gNRTIs	Untreated	Uninfected
Clinical data				
No. subjects (n)	15	13	10	27
Age (yr)	9.53 ± 1.39	13.13 ± 0.91	13.20 ± 1.44	9.96 ± 0.82*
Sex (girls, %)	53	77	30	33
Time on HAART (yr)	9.53 ± 1.39	13.13 ± 0.91	13.20 ± 1.44	—
Immunovirological data				
CD4+ T-cell percentages	32.76 ± 2.51	33.33 ± 2.73	26.00 ± 1.78†	—
Viral load (log HIV RNA, copies/mL)	0.84 ± 0.39	0.81 ± 0.8	4.46 ± 0.24‡	—
Mitochondrial data				
Lactate levels (mmol/L)	1.05 ± 0.13	1.09 ± 0.13	0.93 ± 0.1	—
mtDNA/nDNA (ND2/18SrRNA)	4.25 ± 0.61	3.49 ± 0.61	3.26 ± 0.90	5.82 ± 0.48§
mtDNA/CS (ND2/18SrRNA)	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01§
COXII/β-actin	0.038 ± 0.006	0.065 ± 0.018	0.07 ± 0.029	2.90 ± 0.80‖
COXIV/β-actin	0.14 ± 0.03	0.26 ± 0.08	0.34 ± 0.16	2,29 ± 0.533‖
COXII/CS	0.0003 ± 0.00008	0.0006 ± 0.00018	0.0006 ± 0.00025	0.03 ± 0.012‖
COXIV/CS	0.0013 ± 0.00038	0.0026 ± 0.00084	0.003 ± 0.0014	0.0234 ± 0.006‖
COXII/COXIV subunits	0.36 ± 0.06	0.39 ± 0.08	0.23 ± 0.03	1.08 ± 0.08‖
MDA and HAE (μM/mg protein)	0.57 ± 0.14	0.57 ± 0.05	0.43 ± 0.07	0.59 ± 0.09
CS (nmol/min/mg protein)	127.87 ± 8.43	118.77 ± 8.64	107.33 ± 11.47	118.20 ± 6.10
CII/CS	0.15 ± 0.02	0.15 ± 0.02	0.25 ± 0.07	0.21 ± 0.02
CII–III/CS	0.13 ± 0.02	0.15 ± 0.02	0.15 ± 0.03	0.15 ± 0.02
G3PDH/CS	0.33 ± 0.04	0.32 ± 0.04	0.35 ± 0.46	0.43 ± 0.03
G3PDH–CIII/CS	0.14 ± 0.01	0.16 ± 0.02	0.14 ± 0.019	0.15 ± 0.01
CIV/CS	0.51 ± 0.04	0.51 ± 0.05	0.51 ± 0.06	0.45 ± 0.03

Data are mean ± SEM, except when stated otherwise.

*Significant differences in age of the uninfected controls with respect to the untreated group ($P < 0.05$).

†CD4+ T-cell percentages in untreated group compared with the 2gNRTIs group (26.00 ± 1.78 and 33.33 ± 2.73, respectively; $P < 0.05$).

‡Viral load (HIV RNA copies per milliliter) in the untreated group compared with the 2gNRTIs and 1gNRTIs groups (4.46 ± 0.24, 0.81 ± 0.38, and 0.84 ± 0.39, respectively; $P < 0.001$ for both).

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

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

CII, complex II; CII–III, complex II–III; HAE, hydroxyalkenals; MDA, malondialdehyde.

2gNRTIs group (1.00 ± 0.07 mmol/L; $P = 0.000$) at the second time point, after 2 years (Fig. 2A).

The molecular mitochondrial parameters (mtDNA and the ratio of mitochondrial protein levels) are represented in Figure 2B. MtDNA content was similar in all HIV-infected groups at baseline (Table 1) and 2 years later (0.04 ± 0.008 mmol/L in untreated, 0.04 ± 0.010 mmol/L in 2gNRTIs, 0.04 ± 0.009 mmol/L in 1gNRTIs; *P*, not significant), and remained unchanged over time within the different groups of HIV-infected patients (Fig. 2B). When compared with healthy controls, mtDNA was significantly depleted at baseline in all HIV-infected patients (Table 1) but remained significantly depleted only in the untreated group through the follow-up (0.04 ± 0.008 mmol/L in the untreated group, 0.05 ± 0.006 mmol/L in the healthy controls; $P = 0.048$). These results were consistently similar in relative and absolute values (referring to mitochondrial mass or to nDNA).

Mitochondrial protein levels of subunits COXII and COXIV were decreased in all HIV-infected patients with respect to control reference values at baseline (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 COXII and COXIV remained unaltered along time (Fig. 2B). An example of an immunoblot is provided in 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 (Fig. 2C). Mitochondrial mass was similar at baseline and after 2 years in all groups (Fig. 4).

Enzymatic activities of MRC were similar referred to overall cell protein and to mitochondrial mass. At baseline, MRC enzymatic activities (nanomoles per minute per milligram protein) relativized by CS (nanomoles per minute per milligram 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 ± 0.01, 0.13 ± 0.02, and 0.14 ± 0.02, respectively; $P = 0.012$ and $P = 0.015$; Fig. 4).

Mitochondrial function was preserved over time except for CIV/CS activity, which significantly decreased in the untreated patients and in the 1gNRTIs group (0.256 ± 0.023 and 0.292 ± 0.036, respectively) with respect to baseline (0.519 ± 0.06 and 0.512 ± 0.036; $P = 0.015$ and $P = 0.006$, respectively; Fig. 4). Absolute CIV enzymatic activity per overall cell

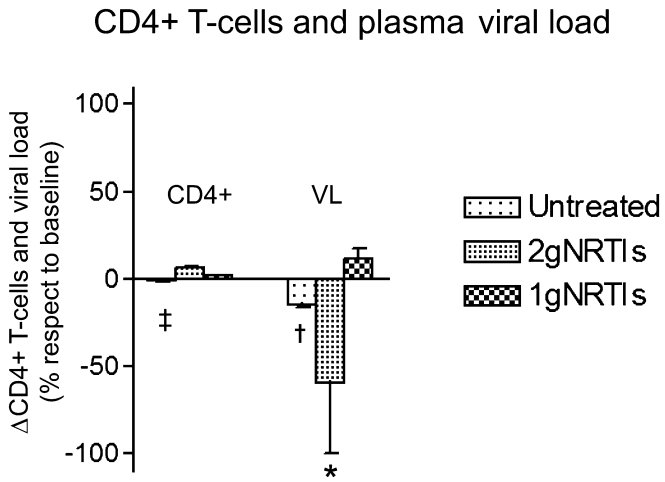

FIGURE 1. Immunovirological parameters. Differences between groups of HIV-infected patients in CD4+ T-cell percentages and HIV plasma viral load (HIV RNA copies per milliliter). The *x* axis represents the baseline of the 3 HIV-infected groups and the columns represent the increase or decrease of the parameters with respect to baseline. Bars represent mean ± SEM. *Viral load (log HIV RNA copies per milliliter) significantly decreased in the 2gNRTIs group compared with baseline (from 0.81 ± 0.38 to 0.33 ± 0.22; $P < 0.05$). †Despite a decrease of almost 1 log at the second time point, the viral load (log HIV RNA copies per milliliter) was still higher in the untreated group when compared with the 2gNRTIs and 1gNRTIs groups (3.81 ± 0.51, 0.33 ± 0.22, and 0.94 ± 0.45, respectively; $P < 0.05$ for both). ‡At the second time point, CD4+ T cells (%) were significantly lower in the untreated group compared with the 2gNRTIs and 1gNRTIs groups (25.70 ± 1.70, 35.53 ± 2.35, and 33.53 ± 1.31, respectively; $P < 0.05$ for both).

protein was significantly decreased in all HIV-infected groups with respect to baseline and with respect to the uninfected controls. At baseline, results on absolute CIV enzymatic activity were as follows: 52.20 ± 5.62 for the untreated patient group, 57.58 ± 5.64 for the 2gNRTIs group, and 65.06 ± 5.84 for the 1gNRTIs group. At the second time point, CIV enzymatic activity was significantly decreased by 26.58% in the untreated patient group (38.33 ± 13.29), by 33.54% in the 2gNRTIs group (38.27 ± 4.93), and by 48.31% in the 1gNRTIs group (33.60 ± 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 are scarce data on mitochondrial toxicity in children. We studied the evolution of mitochondrial parameters in a series of pediatric 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 group is an indicator of a mitochondrial alteration in the former.

MtDNA content was decreased in all HIV-infected groups with respect to the controls at baseline. Thus, HIV-induced and HAART-induced mtDNA depletion was present in the untreated and treated groups, respectively. There was a slight, albeit nonsignificant, 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

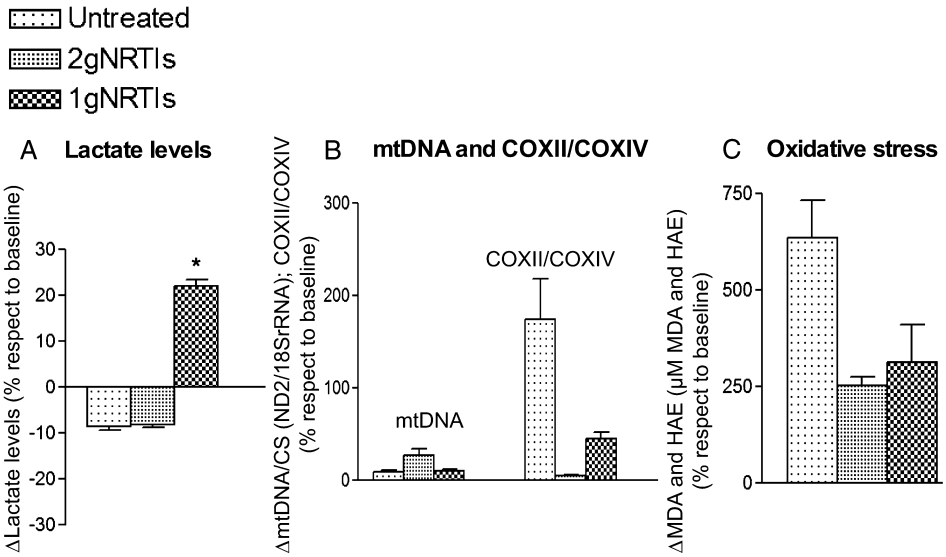

FIGURE 2. A, Lactate levels (millimoles per liter). The *x* axis represents the baseline of the 3 HIV-infected groups and the columns represent the increase or decrease of the parameters with respect to baseline. Bars represent mean ± SEM. *After 2 years, at the second time point, lactate levels were significantly higher in the 1gNRTIs group with respect to the untreated group and the 2gNRTIs group (1.28 ± 0.083, 0.85 ± 0.081, and 1.00 ± 0.071, respectively; $P < 0.05$ for both). B, MtDNA content and mitochondrial protein levels over time in the 3 groups of HIV-infected children. C, Evolution of oxidative stress measurements over time in the 3 groups of HIV-infected children. HAE, hydroxyalkenals; MDA, malondialdehyde.

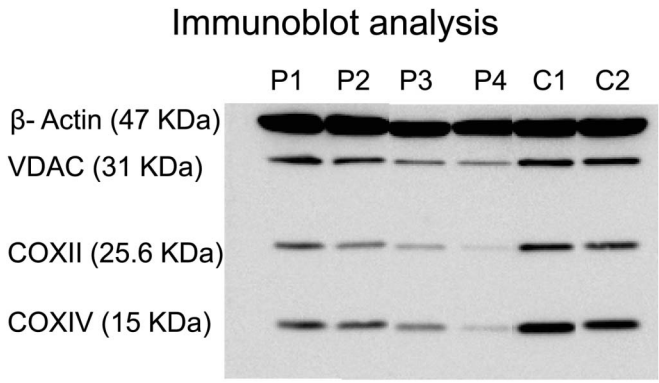

FIGURE 3. An example of an immunoblot analysis of COXII, COXIV, VDAC, and overall cell protein β-actin. C, control; P, patient; VDAC, voltage-dependent anion channel.

potential or the release of material within the mitochondria to the cytosol, such as cytochrome c or the apoptosis-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 plasma mtDNA released from damaged or dead cells, which, in turn, may explain an inflammatory response in the organism.[30]

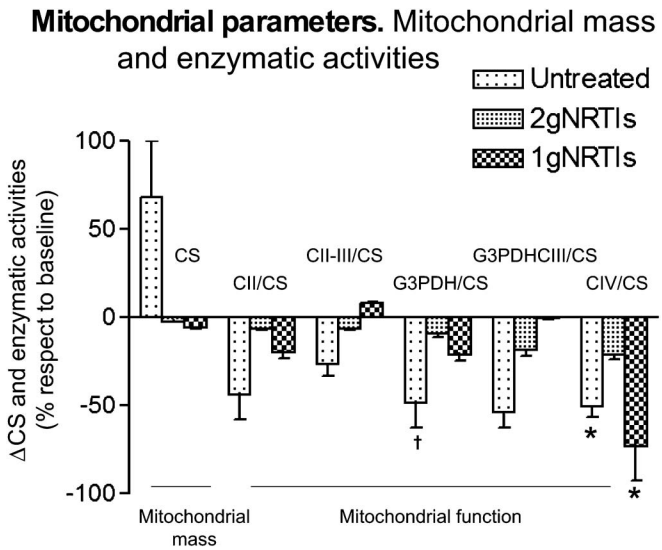

FIGURE 4. Mitochondrial mass and enzymatic activities relativized by CS over time in the 3 groups of HIV-infected children. The *x* axis represents the baseline of the 3 HIV-infected groups and the columns represent the increase or decrease of the parameters with respect to baseline. Bars represent mean ± SEM. *CIV/CS activity significantly decreased in the untreated and 1gNRTIs groups (0.256 ± 0.023 and 0.292 ± 0.036, respectively) with respect to baseline (0.519 ± 0.06 and 0.512 ± 0.036; $P = 0.015$ and $P = 0.006$, respectively). †At the second time point, G3PDH–CIII/CS activity significantly decreased in the untreated group with respect to the 2gNRTIs and 1gNRTIs groups (0.064 ± 0.010, 0.13 ± 0.02, 0.14 ± 0.02; $P = 0.012$ and $P = 0.015$, respectively). CII, complex II; CII-III, complex II–III.

According to the results observed in mtDNA content in all the HIV-infected groups, the results of protein levels of COXII and the ratio of 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] and 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 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 G3PDH–CIII/CS enzymatic activity, which was significantly lower in the untreated group compared with both treated groups. Along this line, in the literature it has been described how HIV triggers mitochondrial impairment, not only at a genetic level 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 percent) in the 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 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 pediatric age. Furthermore, 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 immunologic status. Likewise, HAART changes are often required in pediatric 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.

ACKNOWLEDGMENT

The authors are indebted to Mireia Nicolás for her technical laboratory support.

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