

ANEXO

12. Anexo

Este apartado recopila las colaboraciones del doctorando en otros trabajos distintos a los objetivos de la tesis. En cada uno de los artículos presentados se detalla la contribución realizada.

Absence of mitochondrial dysfunction in polymyalgia rheumatica. Evidence based on a simultaneous molecular and biochemical approach.

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Òscar Miró , Diana Jarreta, Jordi Casademont, Antoni Barrientos, Benjamín Rodríguez, Montse Gómez, Virginia Nunes, Álvaro Urbano-Márquez, Francesc Cardellach.

Contribución: estudio molecular del mtDNA mediante la técnica de Southern para detectar posibles reordenamientos.

Absence of mitochondrial dysfunction in polymyalgia rheumatica

Evidence based on a simultaneous molecular and biochemical approach

Òscar Miró¹, Diana Jarreta¹, Jordi Casademont¹, Antoni Barrientos¹, Benjamín Rodríguez², Montserrat Gómez², Virginia Nunes², Álvaro Urbano-Márquez¹, and Francesc Cardellach¹

¹Muscle Research Unit, Department of Internal Medicine, Hospital Clínic, "August Pi i Sunyer" Biomedical Research Institute (IDIBAPS), Medical School, University of Barcelona, ²Department of Molecular Genetics, Oncology Research Institute (IRO), L'Hospitalet de Llobregat, Barcelona, Spain

Objective: To investigate the molecular and biochemical profile of skeletal muscle mitochondria of patients with isolated polymyalgia rheumatica (PMR).

Patients and Methods: We included patients with a recent diagnosis of PMR and as control healthy individuals submitted to orthopedic surgery. Skeletal muscle was obtained from quadriceps, thus was mitochondria immediately isolated. Long polymerase chain reaction and Southern blot transference were performed to detect deleted mtDNA molecules. Mitochondrial oxidative activity using different substrates and individual enzyme activity of respiratory chain complexes were assessed to search for any biochemical dysfunction.

Results: Fifty-one individuals (PMR=25, controls=26) were included. Mean age was 72 (11) years; 45% were females. We found no significant increase of deleted mtDNA molecules in PMR patients compared to controls. Both groups differed neither on oxygen consumption ($p=NS$ for all substrates) nor enzymatic activity ($p=NS$ for all complexes).

Conclusions: Skeletal muscle mitochondria are molecularly and biochemically unaffected in PMR.

Key words: polymyalgia rheumatica, mitochondria, mitochondrial DNA, electron transport chain, enzyme activity, oxidative activity, mitochondrial DNA deletions

The etiopathogenesis of polymyalgia rheumatica (PMR) remains essentially unknown. The proposed autoimmune phenomenon, vasculitic mechanism, synovial inflammation, genetic factors or previous viral infections do not, on their own, satisfactorily explain the syndrome (1, 2). During the last years, some molecular and biochemical findings have suggested that mitochondria from skeletal muscle of patients affected by PMR could be involved in the pathogenesis of the disease (1, 3, 4). However, since accumulation of multiple and varied mtDNA deletions takes place during normal aging in the postmitotic tissues such as skeletal muscle (5), in the absence of biochemical dysfunction of the mitochondrial respiratory chain (MRC), the pathophysiological relevance of these molecular abnormalities is uncertain. In order to answer this question, we designed a prospective, case-control study to investigate skeletal muscle mitochondrial changes in PMR by means of a simultaneous molecular and biochemical approach.

Material and methods

1 Selection of patients and controls

We prospectively included patients with untreated, newly diagnosed PMR (6) without symptoms or signs suggesting a concomitant GCA (cephalea, visual loss, jaw claudication, abnormal temporal pulses) seen at an urban, university and tertiary care hospital from January 1996 to March 1998. As controls, we included otherwise healthy individuals submitted to orthopedic surgery because of accidental fracture of femur or hip prosthesis placement. Since general anesthesia *per se* may decrease the rates of mitochondrial oxidation (7, 8), we only included patients who underwent regional anesthesia through subarachnoidal blockade. They were carefully matched with PMR patients considering those factors that have been found to be confounding variables in previous studies of the mitochondrial function: age (9), gender (10), smoke habit (10, 11), and physical activity (11, 12). Physical activity during the month previous to muscle biopsy was evaluated using the Steinbrocker et al.'s classification for rheumatoid arthritis (13) slightly modified (8). We excluded those patients with a family history of neuromuscular disorder and those individuals in which histological muscular findings of a specific neuromuscular disorder were found (i.e. neurogenic atrophy). All individuals or their families were previously informed of the protocol study and gave

Francesc Cardellach, Muscle Research Unit, Department of Internal Medicine, Hospital Clínic, Villarroel 170, SP-08036, Barcelona, Spain

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written permission. The study was approved by the Ethical Committee of our Institution.

2 Muscle biopsy

The muscular samples were obtained by open biopsy from the vastus lateralis of the quadriceps in all cases and processed routinely as explained elsewhere (14).

3 Molecular studies

Total DNA was extracted from muscle following standard procedures. Long polymerase chain reaction (PCR) was performed using the Long Expand™ kit (Boehringer-Mannheim, Indianapolis, IN, USA) with primers XL-1 (CCC ACA GTT TAT GTA GCT TAC CTC CTCA; 571–598 position) and XL-2 (TTG ATT GCT GTA CTT GCT TGT AAG CATG; 16220–16193 position) following the manufacturer's recommendations. The PCR product was run on an 0.8 agarose gel and visualized under UV light after staining with ethidium bromide. When a supplementary band was obtained by long PCR, an additional Southern blot analysis was performed, digesting with Pvu II and hybridizing with a whole mtDNA probe labeled with [α -³²P] dCTP.

4 Biochemical studies

4.1 Isolation of mitochondria. Fragments of muscle for biochemical studies were immediately placed into an adequate medium and processed for pure mitochondrial obtaining in less than 5 minutes, as previously explained (15). All steps were carried out at 4 °C. The final protein concentration of pure mitochondrial suspension was determined utilizing the principle of protein-dye binding (16) and expressed as mitochondrial yield, calculated from the rate between final mitochondrial protein content (μ g) and initial muscle weight (mg).

4.2 Polarographic studies. Oxygen utilization was measured polarographically in a volume of 0.25 mL with a Clark oxygen electrode in a micro water-jacketed cell at 37 °C (Hansatech Instruments Limited, Norfolk, England). Rate of oxygen consumption stimulated by ADP (state 3 rate) was assessed using pyruvate (complex I substrate), succinate (complex II substrate), glycerol-3-phosphate (glycerol-3-phosphate dehydrogenase substrate, which feeds the MRC with one electron entering at complex III level), and ascorbate (complex IV substrate) under previously explained conditions (8, 15, 17).

4.3 Spectrophotometric studies. Specific activity of the complexes I, II, III, and IV of the mitochondrial respiratory chain (MRC), as well as of glycerol-3-phosphate dehydrogenase was measured spectrophotometrically (UVIKON 922, Kontron AS, Zurich, Switzerland) at 37 °C in a total volume of 1 mL as explained elsewhere (8, 15, 17, 18).

5 Statistical analysis

Results from the quantitative variables were expressed as the mean (1 SD); percentages were used for the qualitative variables. The normality of the distribution of quantitative variables was assessed by means of Kolmogorov-Smirnov goodness-of-fit test. Comparisons between groups were carried out using the bilateral unpaired t-test (Mann-Whitney U test if variables were not distributed normally or had different variances) for quantitative variables and chi square test with Yate's correction for qualitative variables. Values of $p < 0.05$ were considered statistically significant.

Results

We included 51 individuals. Mean age was 72 (11) years, 45% were females, 9% active smokers and 22% had an impaired physical activity. Twenty-five individuals were affected by PMR and the remainder constituted the control group. The main clinical and analytical data of patients suffering from PMR are presented in table I. Titers for antinuclear antibodies ranged from 1/80 to 1/320; in these patients, other criteria of systemic lupus erithematous were searched for, but always unsuccessfully.

In 12 (48%) of the PMR patients and 12 (46%) controls at least one supplementary band by PCR could be demonstrated, suggesting the existence of mtDNA deleted molecules ($p = \text{NS}$). Nonetheless, when those samples were analyzed by Southern blot, only a 16.5 kb band corresponding the wild type mtDNA was seen, which means that the amount of deleted molecules was insignificant.

Mitochondrial yield was similar for both groups: 0.257 (0.081) and 0.305 (0.180) μ g mitochondrial protein/mg of skeletal muscle for PMR patients and controls, respectively ($p = \text{NS}$). When the biochemical function of skeletal muscle mitochondria were evaluated, no statistically significant differences were found between groups, neither in mitochondrial oxidative capacity (Figure 1), nor in individual enzymatic activities (Figure 2).

Table I. Clinical and analytical data of patients suffering from polymyalgia rheumatica.

	PMR patients (N=25)
Clinical symptoms	
Myalgia	25 (100%)
Asthenia	11 (48%)
Weakness	8 (32%)
Arthralgia	8 (32%)
Weight loss (>10%)	6 (24%)
Fever (>38°C)	3 (12%)
Muscle atrophy	3 (12%)
Analytical data	
Hemoglobin (N.V.: 120–150 g . L ⁻¹)	117 (22)
Leukocyte count (N.V.: 4–11 cells . 10 ⁹ . L ⁻¹)	8.4 (2.2)
Platelets (N.V.: 150–400 cells . 10 ⁹ . L ⁻¹)	318 (104)
Erythrocyte rate sedimentation (N.V.: <20 mm . h ⁻¹)	62 (35)
Reactive C protein (N.V.: <0.008 g . L ⁻¹)	0.042 (0.034)
Alpha-2-globuline (N.V.: 3.6–8.0 g . L ⁻¹)	9.1 (1.0)
Haptoglobin (N.V.: 0.5–2.8 g . L ⁻¹)	4.53 (1.43)
Fibrinogen (N.V.: 1.5–4.5 g . L ⁻¹)	5.1 (1.3)
Nutritional parameters	
Albumin (N.V.: 37–53 g . L ⁻¹)	37 (4)
Pre-albumin (N.V.: 0.25–0.45 g . L ⁻¹)	0.21 (0.07)
Transferrin (N.V.: 2.2–3.5 g . L ⁻¹)	2.33 (0.43)
Retinol binding protein (N.V.: 0.02–0.06 g . L ⁻¹)	0.036 (0.016)
Zinc (N.V.: 0.8–1.2 mg . L ⁻¹)	0.69 (0.27)
Immunological tests	
Positive rheumatoid factor	3 (12%)
Positive antinuclear antibodies	7 (28%)
Positive anti-mitochondrial antibodies	0 (0%)

N.V.: normal values.

Discussion

In the present study, we have failed to detect any data of mitochondrial dysfunction in patients

suffering from PMR when these organelle were studied in a simultaneous molecular and biochemical approach. Our results add further evidence to some morphological studies (19, 20) reporting mitochondrial indennity in PMR.

Since PCR is a very sensitive method and can detect minimal amounts of mutated mtDNA molecules, it is not surprising to find amplification of deleted molecules both in PMR patients and controls, particularly if it is taken into account that the majority of healthy people with age over 70 exhibits a little amount of deleted mtDNA molecules when several cycles of PCR are performed (5). In classical mitochondrial disorders only deleted mtDNA proportions over 60% result in a biochemical respiratory chain dysfunction (21). Therefore, our findings of low percentage of deleted mtDNA molecules by PCR (but not in Southern blot) in approximately 50% of patients and controls should probably be considered as functionally irrelevant.

Even accepting that a slight increase of mtDNA deletions could exist in PMR, it could hardly produce significant biochemical impairment, since mitochondrial oxidative phosphorylation processes are highly efficient and have a great degree of functional reserve (22). In this sense, PMR patients showed no impairment for oxidizing substrates entering at different levels of the MRC nor in the enzymatic activity of individual complexes. The normality of the simultaneous studies also support the structural integrity of the mitochondrial membranes and the correct assembly of the complexes which are contained in them. In addition, the ATP production seems to be unaffected in PMR as judged

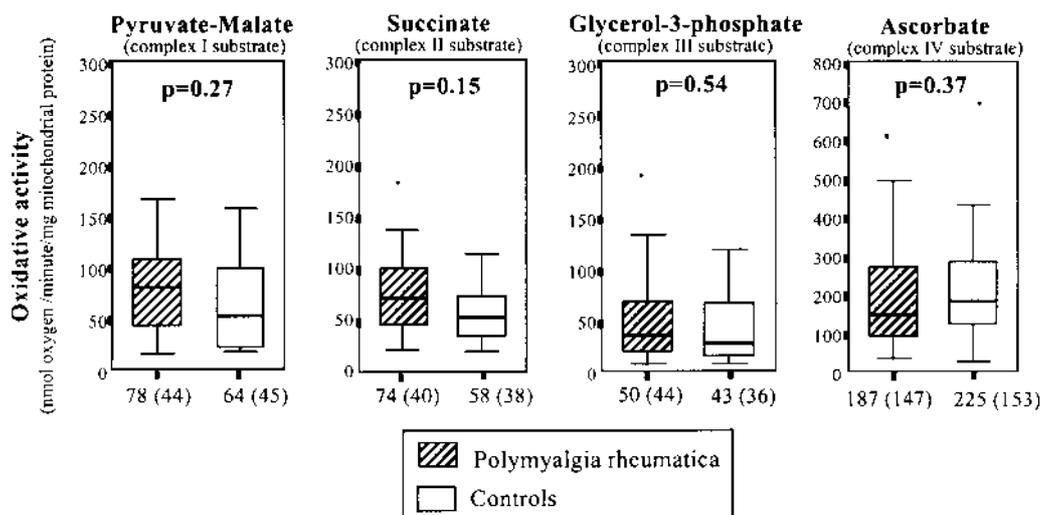


Fig. 1. Box-plots corresponding to oxidative activity of mitochondria with different substrates. Boxes includes 50% of values (between percentiles 25% and 75%) and whiskes includes 100%. Dots are extreme values and outliers. Solid bars correspond to the medians, and values under X-axis to the means (SD).

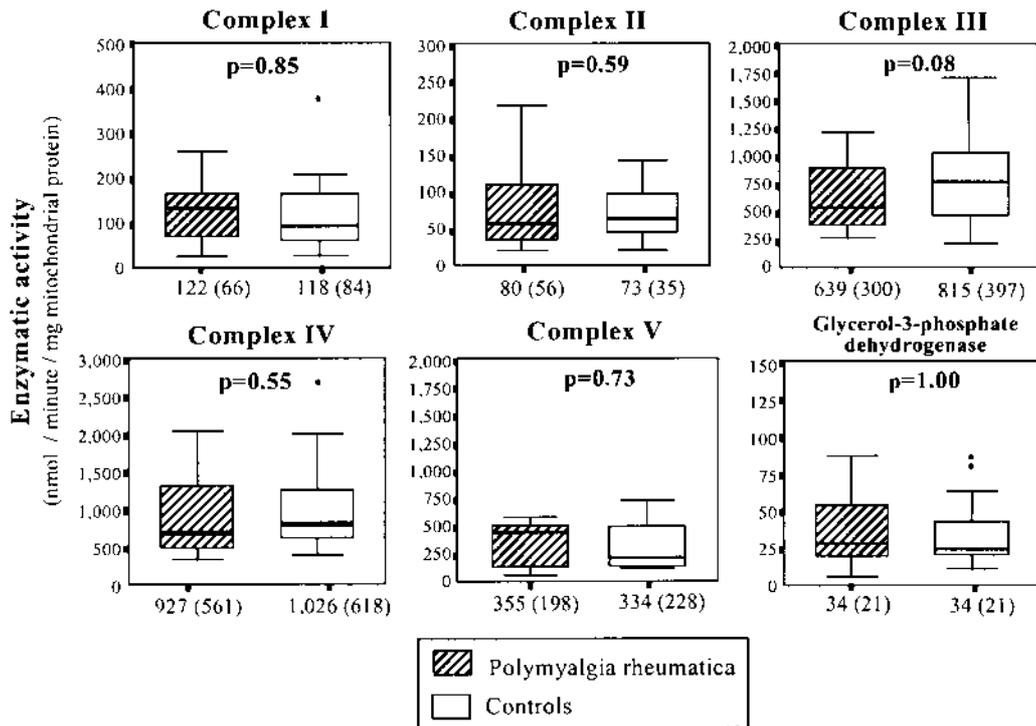


Fig. 2. Box-plots corresponding to enzymatic activity of the complexes of mitochondrial respiratory chain. Dots are extreme values and outliers. Solid bars correspond to the medians, and values under X-axis to the means (SD).

by the normality of the activity of ATPase (complex V), the main responsible in rendering the energy stored as electrochemical gradient across the inner mitochondrial membrane during the electron transient through MRC (22).

Our observations contrast with previous reports that suggested that mitochondria could play a role in the pathogenesis of PMR based on the findings of an increased number of histochemical and ultrastructural abnormalities (1, 3), decreased enzymatic activity in some complexes of the MRC (1, 3), and multiple mtDNA deletions (4). However, the high level of fluctuations of the biochemical results, as well as the lack of consideration of some confounding factors (8–12) in the analysis of the mitochondrial function could have influenced their findings. In fact, our biochemical results are in agreement with those reported in a recent study performed by Mattei et al. (23), who demonstrated a normal energy utilization by muscle in patients with PMR using P-31 nuclear magnetic resonance spectroscopy. Assuming that the mitochondria supplies more than 90% of the cellular energy requirements, they deduced that a disturbance of MRC is very unlikely in PMR. We hence conclude that the results of our simultaneous molecular and biochemical study provide strong evidence to support that mitochondria remain essentially unaffected in PMR.

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Presence of a Major WFS1 Mutation in Spanish Wolfram Syndrome Pedigrees

Molecular Genetics and Metabolism 72, 72-81 (2001)

Montse Gómez-Zaera, Tim M. Strom, Benjamín Rodríguez, Xavier Estivill, Thomas Meitinger, and Virginia Nunes.

Contribución: búsqueda de mutaciones LHON en los individuos control mediante PCR-RFLP.

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http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WNG-45V294RC&_coverDate=01%2F31%2F2001&_alid=250443892&_rdoc=1&_fmt=&_orig=search&_qd=1&_cdi=6962&_sort=d&view=c&_acct=C000053451&_version=1&_urlVersion=0&_userid=1517318&md5=aa97e8b2a5a54ab502357b2ba96a165e

Mitochondrial DNA depletion and respiratory chain enzyme deficiencies are present in peripheral blood mononuclear cells of HIV-infected patients with HAART-related lipodystrophy

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Òscar Miró , Sònia López, Enric Pedrol, Benjamín Rodríguez-Santiago, Esteban Martínez, Anna Soler, Ana Milinkovic, Jordi Casademont, Virginia Nunes, Josep M. Gatell, Francesc Cardellach.

Contribución: extracción de DNA, cuantificación del mtDNA y del nDNA mediante la PCR en tiempo real conjuntamente con Sònia López. Cálculo del ratio mtDNA/nDNA mediante las rectas de regresión determinadas con los estándares. Escritura del apartado “*mtDNA studies*” del manuscrito.

Mitochondrial DNA depletion and respiratory chain enzyme deficiencies are present in peripheral blood mononuclear cells of HIV-infected patients with HAART-related lipodystrophy

Òscar Miró^{1*}, Sònia López¹, Enric Pedrol², Benjamín Rodríguez-Santiago³, Esteban Martínez⁴, Anna Soler², Ana Milinkovic⁴, Jordi Casademont¹, Virginia Nunes³, Josep M Gatell⁴ and Francesc Cardellach¹

¹Mitochondrial Research Laboratory, Muscle Research Unit, Department of Internal Medicine, Hospital Clínic, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), School of Medicine, University of Barcelona, Barcelona, Catalonia, Spain

²HIV Unit, Department of Internal Medicine, Hospital Fundació-Asil de Granollers, Granollers, Barcelona, Catalonia, Spain

³Medical and Molecular Genetics Centre-IRO, Hospital Duran i Reynals, L'Hospitalet de Llobregat, Barcelona, Catalonia, Spain

⁴Department of Infectious Diseases, Hospital Clínic, August Pi i Sunyer Biomedical Research Institute (IDIBAPS), School of Medicine, University of Barcelona, Barcelona, Catalonia, Spain

*Corresponding author: Tel: +34 93 227 55 39; Fax: +34 93 227 55 39; E-mail: omiro@clinic.ub.es

The main objective of present study was to ascertain if mitochondrial DNA (mtDNA) depletion as reported in HIV-infected patients with highly active antiretroviral therapy (HAART)-related lipodystrophy (LD) implies any degree of mitochondrial respiratory chain (MRC) dysfunction. For this purpose, we evaluated HIV patients on different HAART schedules with LD (group A; n=12) and on HAART but without LD (group B; n=12), and untreated HIV-infected patients as controls (group C; n=24). mtDNA content was determined on peripheral blood mononuclear cells (PBMCs) with a real-time PCR method. Complex II, III and IV activities of the MRC were simultaneously measured spectrophotometrically, as were spontaneous and stimulated oxygen consumption by PBMCs. Compared to controls (group C, 100%), patients with LD (group A) showed a decreased mtDNA content

(54%, P<0.001), which was associated with a decline in complex III (62%, P<0.05) and IV activity (69%, P<0.05) (both complexes partially encoded by mtDNA), but not in complex II activity (exclusively encoded by nuclear DNA). Patients in group B showed a similar pattern of mitochondrial dysfunction but to a lesser extent and without statistical significance. Respiratory activities in both treated groups (A and B) did not differ in comparison with controls. We conclude that mtDNA depletion occurring during HAART is associated with deficiencies in MRC complexes partially encoded by mtDNA, which are detectable by PBMCs. Presented in 'Late Breakers and Hot Topics' session at 6th International Congress on Drug Therapy in HIV Infection, Glasgow, UK, 17–21 November 2002.

Introduction

Toxic effects on mitochondria were first reported [1] with the antiretroviral zidovudine, but several *in vitro* and clinical studies have subsequently demonstrated that all nucleoside analogue reverse transcriptase inhibitors (NRTIs) are associated with some degree of mitochondrial dysfunction [1–4], even in asymptomatic patients [5]. The effects of NRTIs on mitochondria are mainly due to their capacity to inhibit gamma-polymerase (γ -pol), the enzyme responsible for mitochondrial DNA (mtDNA) replication. Recent studies have demonstrated that mtDNA depletion or deletions are present in fat [6,7], skeletal muscle [8–10] and liver [8] of patients with lipodystrophy (LD) and some authors have, accordingly, suggested that such a decrease in mtDNA could be related to the

development of lipodystrophic changes. Since mtDNA only encodes for two ribosomal RNA, 22 transfer RNA and 13 messenger RNA, all exclusively involved in the translation of subunits of complexes I, III, IV and V of the mitochondrial respiratory chain (MRC), the only possible phenotypic expression of mtDNA defects is MRC dysfunction [11]. This dysfunction is demonstrable by measuring both enzyme activity of MRC complexes and whole mitochondrial capacity to oxidize substrates coupled with oxygen consumption. Therefore, simultaneous analysis of MRC function along with the determination of mtDNA content are the clues to linking genetic findings in HIV patients on highly active antiretroviral therapy (HAART) with the development of LD [12].

However, the use of the classical target tissues of primary mitochondrial disorders (skeletal muscle or liver) is difficult to conceive for routine clinical evaluation of patients on HAART who develop LD. In this setting, peripheral blood mononuclear cells (PBMCs) are easier to obtain than such tissues and, very recently, Côte *et al.* [13] have used venous buffy coats to demonstrate a depletion of mtDNA in HIV-infected patients developing lactic acidosis, another side effect linked to mitochondrial toxic effects of HAART. Accordingly, in the present study, we have comprehensively analysed mtDNA and MRC function on PBMCs from patients who developed LD during HAART in order to detect the simultaneous existence of both genetic and biochemical deficiencies.

Patients and methods

Patients

HIV patients on diverse HAART schedules without personal or familial history suggestive of mitochondrial disease or neuromuscular disorders with relevant LD as the only clinical abnormality at the time of initiating the study were included (group A). LD was defined as self-reported by patients and confirmed by the investigator, who classified LD as isolated peripheral lipoatrophy, isolated central fat accumulation or mixed syndrome. We also studied patients on HAART but without LD (group B). As controls, we included naive HIV patients with respect to treatment (group C). The three groups were matched for age (± 10 years) and gender. Time on HAART was recorded. Basal levels of triglycerides, cholesterol and glycaemia, as well plasma HIV viral load and CD4 lymphocyte count were determined. The Ethical Committee of our institution approved the protocol and informed consent was obtained from all the patients.

Methods

Obtaining PBMCs

20 ml of peripheral blood was used to perform all the studies. PBMCs were isolated by centrifugation using a Ficoll's gradient. Protein content was measured by means of Bradford's protein-dye binding principle [14]. Less than 200 platelets were present in each final sample.

mtDNA studies

Total DNA was extracted by standard phenol-chloroform procedures. Rearrangements in mtDNA were also examined by Southern blot hybridization using a mitochondrial ND4 gene as the probe [10]. For mitochondrial DNA quantification, nuclear 18s rRNA housekeeping gene and the highly conserved mitochon-

drial ND2 gene were quantified separately by real-time quantitative PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals, Germany). The PCR amplification of a 500 bp fragment length of the 18S rRNA gene was performed by using the forward 5'-ACGGACCAGAGC-GAAAGCAT-3' and the reverse 5'-GGACATCTAAGGGCATCACAGAC-3' primers. For the mitochondrial ND2 gene, the forward 5'-GCCCTAGAAATAAACATGCTA-3' and the reverse 5'-GGGCTATTCCTAGTTTTATT-3' primers were used for the amplification of a 200 bp fragment length. The PCR reactions for mitochondrial gene amplification contained 3 mM MgCl₂, 0.25 pmol/ μ l of each primer, 10 ng of DNA in 20 μ l of final volume. The PCR reactions for nuclear gene amplification contained 2 mM MgCl₂, 0.3 pmol/ μ l of each primer, and 10 ng of DNA in 20 μ l of final volume. The PCR amplification programme consisted of a single denaturation-enzyme-activation step of 10 min at 95°C, followed by 35 cycles (for 18S rRNA gene) and 29 cycles (for the ND2 gene). Each cycle consist of a denaturation step (2 sec at 95°C, for r18S gene and 0 sec at 94°C, for the ND2 gene) an annealing step (10 sec at 66°C, for 18S rRNA gene and 10 sec at 53°C, for the ND2 gene), and an extension step (20 sec at 72°C, for 18S rRNA gene and 10 sec at 72°C, for the ND2 gene), with a temperature-transition rate of 20°C/sec. The fluorescent product was detected at the last step of each cycle by single acquisition. The method used a double-stranded DNA dye (SYBR Green I) to continuously monitor product formation. The sensitivity of SYBR Green I detection is limited by non-specific product formation, which is monitored by fluorescence acquisition at temperatures at which only specific products are double-stranded [15]. After amplification, a melting curve was acquired by heating the product at 20°C/sec to 95°C, cooling at 20°C/sec to 72°C for the ND2 gene and 76°C for the 18S rRNA, and slowly heating it at 0.2°C/sec to 94°C with continuous fluorescence collection. Melting curves were used to determine the specificity of the PCR products. The results were expressed as the ratio of the mean mtDNA value of duplicate measurements to the mean nuclear DNA value of duplicate measurements (mtDNA/nDNA) [16].

MRC enzyme activities

Measurement of enzyme activity (in nmol/min/mg of protein) of individual complexes of the MRC was performed spectrophotometrically (UVIKON 920, Kontron®, Switzerland) [17]. Since isolated complex I and V activities cannot be measured in whole cells (due to the absence of activation of the former with decylubiquinone and due to a strong oligomycin-insensitive

ATPase activity of the latter) [18], we only determined complex II, complex III and complex IV activities (being the first exclusively encoded by nuclear DNA and the two latter partially encoded by mtDNA) following Rustin et al. [19], but slightly modified for complex IV measurement [20].

PBMC respiratory activity

Oxygen usage was measured polarographically using a Clark electrode in a water-jacketed cell at 37°C (Hansatech Instruments Limited®, Norfolk, UK). We determined spontaneous PBMC oxygen consumption rates (in nmol oxygen/min/mg of protein). Afterwards, we permeabilized PBMC membranes with 1% digitonin and added malate (2 mM) plus pyruvate (5 mM) (complex I substrate, electrons flow through complexes I, III and IV), succinate (20 mM) (complex II substrate, electrons flow through complexes II, III and IV) and glycerol-3-phosphate (10 mM) (complex III substrate, the electrons flow through complexes III and IV) in the presence of adequate inhibitors. Complete methodology is reported elsewhere [17,18].

Statistical analysis

Results are expressed as mean \pm SEM. Normality of the variables distribution was ascertained by the Kolmogorov-Smirnov test. Comparisons between patients (groups A and B) with respect to the control group (group C), as well as between groups A and B, were performed using the unrelated Student's t-test. Linear regression analysis was employed to uncover any relationship between quantitative variables. Statistical significance was accepted when a *P*-value was less than 0.05.

Results

We included 12 HIV-infected patients on HAART with LD and 12 HIV-infected individuals on HAART without LD. As controls, 24 naive HIV-infected patients with respect to treatment were used. Clinical

data are shown in Table 1. All patients receiving treatment demonstrated a significantly lower viral load than untreated controls. Patterns of LD were peripheral lipoatrophy in 75% of the cases and mixed in 25%. No case of isolated fat accumulation was observed. Six patients in group A had hypertriglyceridaemia, four had hypercholesterolaemia and two hyperglycaemia. Only one patient in group B had hyperglycaemia and hypertriglyceridaemia.

Molecular analysis of mtDNA of patients on HAART (irrespective of the presence of LD) did not uncover any rearrangement. However, the mtDNA content of patients with LD was only 54% of that found in controls ($P<0.001$), while patients on HAART but without LD had 76% of that found in controls ($P=NS$) (Figure 1).

Significant decreases in complexes III and IV (both partially encoded by mtDNA), but not in complex II, were found in patients on HAART with LD, but not in those on HAART without LD. Specifically, complex III activity of patients with LD was 62% ($P<0.05$) of that in untreated controls, while for complex IV the remaining activity was 69% ($P<0.05$) (Figure 2). There were no statistically differences between patients on HAART with and without LD.

Respiratory activity, either spontaneous or stimulated with pyruvate, succinate or glycerol-3-phosphate, did not differ between either group of treated patients and the control group (Figure 3).

Figure 4 shows the relationship between mtDNA content, complex III activity and complex IV activity, all of them found to be decreased in patients with LD. As can be seen, a significant direct relationship was only present for complex III and IV activities, while only a trend of relationship was observed for such activities and mtDNA content.

Discussion

Lipodystrophy is one of the most prevalent side effects of HAART [21,22]. Clinical similarities with

Table 1. Clinical data

	Controls (untreated) (n=24)	Patients (on HAART without LD) (n=12)	Patients (on HAART with LD) (n=12)
Age (years) (mean \pm SEM)	40 \pm 3	40 \pm 4	44 \pm 4
Gender female (%)	25	25	25
Viral load (log ₁₀ copies) (mean \pm SEM)	5.2 \pm 0.3	1.8 \pm 0.3*	2.8 \pm 0.4*
Lymphocyte CD4 count (per mm ³) (mean \pm SEM)	221 \pm 58	455 \pm 55	351 \pm 49
Time on HAART (months) (mean \pm SEM)		21 \pm 3	31 \pm 5
HAART containing d4T, but not PI (%)		42	42
HAART containing PI, but not d4T (%)		25	25
HAART containing d4T and PI (%)		33	33

* $P<0.001$ respect to untreated control group.

Figure 1. Mitochondrial DNA content (**P*<0.001)

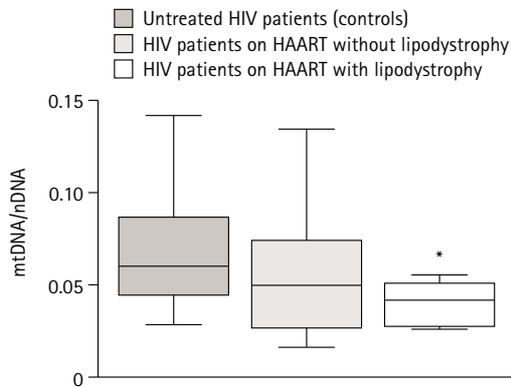
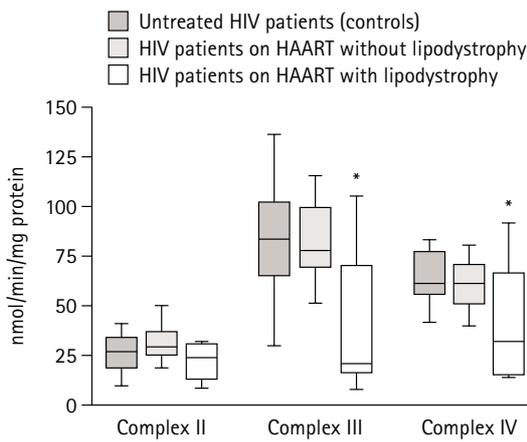


Figure 2. Enzyme activity of mitochondrial respiratory chain complexes (**P*<0.05)



symmetric multiple lipomatosis, a syndrome in which mitochondrial dysfunction has been proven [23], led to the mitochondrial hypothesis for HAART-related LD. Since then, data suggesting the participation of mitochondria in the pathogenesis of LD have steadily increased [7,8]. Although our study does not address experimentally the responsibility of mtDNA depletion in adipose tissue to cause LD, it adds new data possibly pointing to that direction. Our results regarding mtDNA depletion in PBMCs from patients developing LD are consistent with data from subcutaneous adipose tissue of patients with this syndrome. Shikuma *et al.* [6] reported a significant, but not quantified, decrease in mtDNA content irrespective of the sample site of the subcutaneous fat, while Walker *et al.* [7] found that the mtDNA content in subcutaneous adipose tissue of patients on HAART with LD was 61% of that from patients on HAART without LD. Although it has been reported that the toxic effects are

not equally evident in different tissues, as demonstrated by quite different results in mtDNA quantification of paired samples of fat and PBMCs [24], in our experience mtDNA contained in PBMCs seems to be also sensitive to the toxic effects of anti-retroviral drugs and may, therefore, potentially be used to investigate this side effect in HIV patients on HAART.

Previous studies evaluating the mitochondrial effects of HAART on peripheral blood cells [25,26] had failed to demonstrate any significant decline in mtDNA abundance. Methodological aspects could explain the discrepancy with our data. On one hand, one of the greatest difficulties that investigators have is to analyse homogeneous groups of patients with respect to the type and time on antiretroviral treatment. Undoubtedly, length of time on HAART is a crucial factor in the development of mtDNA depletion (and MRC dysfunction) and LD. In our series, the anti-retroviral schedules of the patients in groups A and B (treated patients) were similar and, although not identical, the length of time on these HAART schedules did not differ statistically. Furthermore, we believe that the real-time PCR methodology currently employed provides a better approach to quantify mtDNA, because it is more sensitive in the clinically relevant low range of mtDNA:nDNA values. This advantage could be even more evident when the biological samples analysed are not those typically affected by mitochondrial disorders, such as PBMCs. In this sense, Côté *et al.* [13] have satisfactorily used real-time PCR in buffy coats of patients developing symptomatic lactic acid elevation, another complication associated with mitochondrial toxicity of HAART, and have demonstrated that the mtDNA content in such patients is only 39% of that from untreated patients.

The decreased complex III and IV activities (both

Figure 3. Oxygen consumption

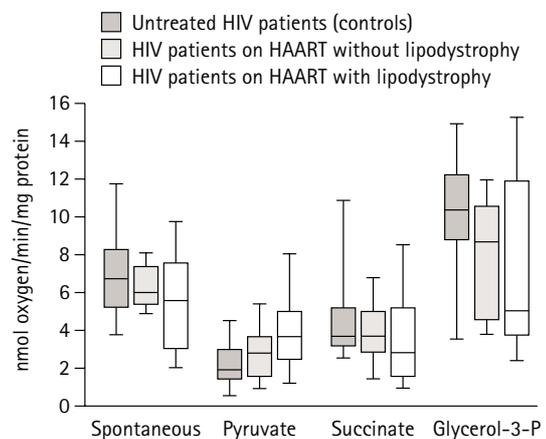
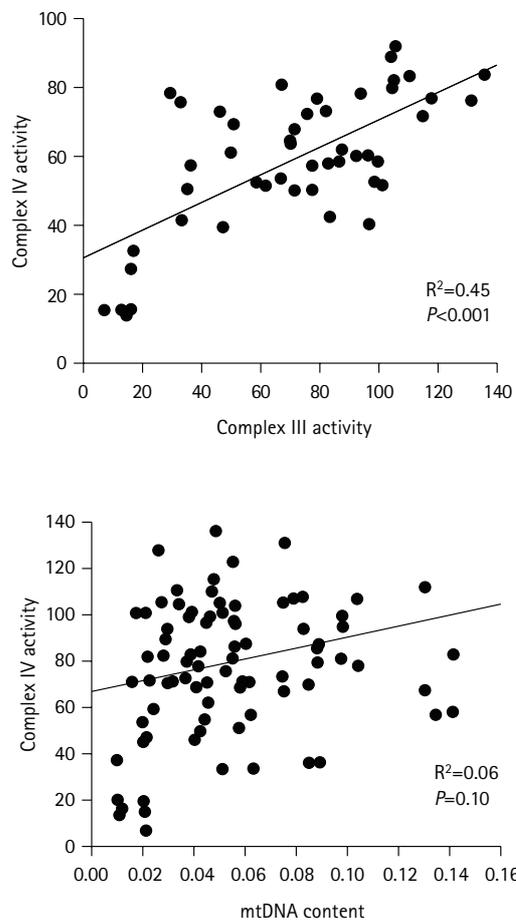


Figure 4. Relationship between the diverse mitochondrial parameters



enzymes partially encoded by mtDNA) along with mtDNA depletion strengthens the pathogenic link between these two findings. However, at least in PBMCs, these disturbances were not enough to inhibit the whole respiratory capacity, either spontaneous or stimulated with substrates of complexes I, II and III. This does not rule out the fact that MRC abnormalities found in PBMC can be more pronounced in target tissues leading to an overt MRC dysfunction. For example, we demonstrated 14% and 22% of residual activities for complexes III and IV, respectively, in skeletal muscle of patients with LD combined with a marked decline in whole respiratory capacity [9,10]. This lack of concordance among tissues is explained by the well-known tissue specificity of inherited mitochondrial diseases, which is attributed to heteroplasmy among mitochondria within a given cell and among cells in a particular tissue, as well as the dependence of oxidative phosphorylation of each tissue [27]. In addition, tissue specificity is also present for NRTI-induced mitochondrial dysfunction, with a differentiated tissue involvement depending on the kind of NRTI used

[4,27] and even for the same patient for a specific NRTI drug [28]. On the other hand, mechanisms by which HAART causes mitochondrial dysfunction may not solely be a consequence of NRTI effects on mitochondrial DNA synthesis via γ -pol inhibition. Our finding that relatively moderate mtDNA depletion caused a detectable decrease of complex III and IV enzyme activity also suggests the existence of alternative mechanisms for HAART toxicity.

As commented above, despite the lack of significance, patients with LD received HAART for a longer period than patients without LD. Therefore, the duration of HAART itself may be the only explanation for our findings. In fact, it is conceivable that the mitochondrial toxic effects of antiretrovirals begin as soon as treatment is introduced, and it is only when mtDNA depletion achieves a certain magnitude and leads to biochemical dysfunction that LD becomes clinically evident. Consistent with this idea, recent data from Henry *et al.* [29] have also shown that mtDNA does not decrease in lymphocytes of HIV patients on HAART while they remain asymptomatic. Furthermore, although our study has the limitations of a relatively few number of subjects in each treated group as well as the absence of an objective definition of LD, it seems to confirm the presence of a MRC dysfunction together with mtDNA depletion in PBMC of patients with LD. The exact mechanism by which such mitochondrial abnormalities finally lead to LD remains, however, elusive. Since skeletal muscle biopsies, classically used to evaluate mitochondrial disorders, are not practical for routine monitoring of HAART toxicity, the present study is also remarkable because it suggests that PBMCs may be an easily obtainable sample to further investigate and uncover precocious changes in MRC function, perhaps even before LD becomes clinically evident.

Acknowledgements

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Reversible mitochondrial respiratory chain impairment during symptomatic hyperlactatemia associated with antiretroviral therapy.

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Òscar Miró , Sònia López, Esteban Martínez, Benjamín Rodríguez-Santiago, José L. Blanco, Ana Milinkovic, José M. Miró, Virginia Nunes, Jordi Casademont, Josep M. Gatell, Francesc Cardellach.

Contribución: extracción de DNA, cuantificación del mtDNA y del nDNA mediante la PCR en tiempo real y cálculo del ratio mtDNA/nDNA mediante las rectas de regresión determinadas con los estándares. Búsqueda de reordenamientos del mtDNA mediante *Southern blot*. Escritura del párrafo del manuscritodedicado a las técnicas de análisis del mtDNA . Figura 3 del artículo correspondiente a la imagen del *Southern blot*.

Short Communication

Reversible Mitochondrial Respiratory Chain Impairment During Symptomatic Hyperlactatemia Associated with Antiretroviral Therapy

ÒSCAR MIRÓ,¹ SÒNIA LÓPEZ,¹ ESTEBAN MARTÍNEZ,² BENJAMÍN RODRÍGUEZ-SANTIAGO,³ JOSÉ L. BLANCO,² ANA MILINKOVIC,² JOSE M. MIRÓ,² VIRGINIA NUNES,³ JORDI CASADEMONT,¹ JOSEP M. GATELL,² and FRANCESC CARDELLACH¹

ABSTRACT

Direct evidence confirming the hypothesis that a dysfunction of the mitochondrial respiratory chain (MRC) underlies the pathogenesis of hyperlactatemia associated with highly active antiretroviral therapy (HAART) is scarce. We studied mitochondrial DNA (mtDNA) content and MRC function in the skeletal muscle of an HIV-infected patient during an episode of symptomatic hyperlactatemia. Skeletal muscle biopsy was performed during the episode when the patient was symptomatic and 3 months later when the patient was clinically recovered. Assessment of mitochondria was performed using histological, polarographic, spectrophotometrical, and Southern blot and real time PCR DNA quantification methods. The histological study disclosed extensive mitochondrial impairment in the form of ragged-red fibers or equivalents on oxidative reactions. These findings were associated with an increase in mitochondrial content and a decrease in both mitochondrial respiratory capacity and MRC enzyme activities. Mitochondrial DNA content declined to 53% of control values. Mitochondrial abnormalities had almost disappeared later when the patient became asymptomatic. Our findings support the hypothesis that MRC dysfunction stands at the basis of HAART-related hyperlactatemia.

HPERLACTATEMIA is observed in up to 10% of HIV-infected patients on antiretroviral therapy. In most cases, blood lactate elevation is usually mild (less than 2-fold) and patients remain asymptomatic. With higher plasma lactate concentration, patients may develop fatigue, weakness, abdominal pain, weight loss, tachycardia, and/or exertional dyspnea. Occasionally, hyperlactatemia may lead to severe lactic acidosis and death.^{1,2}

Due to their central role in intermediary metabolism, mitochondria could participate in such a metabolic imbalance. Despite the fact that mitochondrial dysfunction has been widely investigated in other complications of antiretroviral therapy (as zidovudine-related myopathy³ and lipodystrophy syndrome⁴),

few works have directly studied mitochondrial respiratory chain (MRC) function in patients with hyperlactatemia.⁵⁻⁸ Although a recent study⁹ has demonstrated mtDNA depletion in peripheral blood mononuclear cells (PBMCs) obtained from patients with hyperlactatemia, the clinical repercussion of such a depletion is uncertain because any abnormality in mitochondrial DNA (mtDNA) in a pathogenic role must necessarily cause overt MRC dysfunction.¹⁰

A 50-year-old HIV-1-infected man was admitted in May 2001 because of malaise, fatigue, weight loss, increasing abdominal discomfort, and dyspnea for the previous 6 weeks. He had been diagnosed with HIV infection in 1988 and treated with

¹Mitochondrial Research Laboratory, Muscle Research Unit, Department of Internal Medicine, and ²Infectious Disease Unit, Hospital Clínic, "August Pi i Sunyer" Biomedical Research Institute (IDIBAPS), School of Medicine, University of Barcelona, Barcelona, Catalonia, Spain.

³Medical and Molecular Genetics Centre-IRO, Hospital Duran i Reynals, L'Hospitalet de Llobregat, Barcelona, Catalonia, Spain.

antiretroviral drugs for the past 8 years (including zidovudine for 55 months, zalcitabine for 12 months, didanosine for 55 months, stavudine for 55 months, saquinavir for 20 months, lamivudine for 12 months, ritonavir for 19 months, nelfinavir for 28 months, and nevirapine for 12 months). At the time of the study, he had been treated with stavudine 40 mg/12 hr, didanosine 400 mg/24 hr, indinavir 800 mg/12 hr, ritonavir 100 mg/12 hr, gemfibrozil 600 mg/12 hr, and allopurinol 300 mg/24 hr for at least the previous 6 months. Body fat changes consisting of abdominal accumulation, gynecomastia, and lipodystrophy, as well as hypertriglyceridemia and hyperuricemia, had been noticed in February 1999. On admission, the patient was cachectic. Serum lactate was 49 mg/dl (normal: 5–22), but there was no acidemia (pH 7.39, bicarbonate 21 mmol/liter). He had mild abnormalities in liver and pancreas function tests. Serological markers for hepatitis C and B viruses were negative. CD4 cell count was 450/mm³ and HIV-1 RNA was <20 copies/ml. The patient was considered to have symptomatic hyperlactatemia. A first muscle biopsy was indicated, and all antiretroviral drugs were immediately withdrawn. The condition of the patient progressively improved and serum lactate and liver and pancreas tests returned to normal concentrations 3 months after discontinuation of antiretroviral therapy. At this time, when the patient was asymptomatic and was not receiving antiretroviral drugs, a second muscle biopsy was performed.

Four samples of quadriceps were obtained in each biopsy. One was used for histological studies as explained elsewhere.¹¹ A second sample was used to spectrophotometrically measure enzyme activities of complexes I to V of MRC on muscle homogenate.¹² Results were corrected by citrate synthase activity in order to normalize for mitochondrial content.¹² The third sample was used to polarographically determine oxygen consumption on fresh mitochondrial suspension¹³ using pyruvate-malate, succinate, and ascorbate as substrates of complexes I, II, and IV of MRC, respectively.¹⁴ Total DNA was extracted by standard phenol-chloroform procedures from the fourth sample.

Rearrangements in mtDNA were examined by Southern blot hybridization using a mitochondrial ND4 gene as the probe.¹⁵ For mitochondrial DNA quantification, the nuclear 18S rRNA housekeeping gene and the highly conserved mitochondrial ND2 gene were quantified separately by real time quantitative polymerase chain reaction (PCR) (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals, Mannheim, Germany). The PCR amplification of a 500-bp fragment length of the 18S rRNA gene was performed by using the forward 5'-ACGGACCAGAGCGAAAGCAT-3' and the reverse 5'-GGACATCTAAGGGCATCACAGAC-3' primers. For the mitochondrial ND2 gene, the forward 5'-GCCCTAGA-AATAAACATGCTA-3' and the reverse 5'-GGGCTATTCC-TAGTTTTATT-3' primers were used for the amplification of a 200-bp fragment length. The PCR reactions for mitochondrial gene amplification contained 3 mM MgCl₂, 0.25 pmol/μl of each primer, and 10 ng of DNA in 20 μl of final volume. The PCR reactions for nuclear gene amplification contained 2 mM MgCl₂, 0.3 pmol/μl of each primer, and 10 ng of DNA in 20 μl of final volume. The PCR amplification program consisted of a single denaturation-enzyme-activation step of 10 min at 95°C, followed by 35 cycles (for 18S rRNA gene) and 29 cycles (for the ND2 gene). Each cycle consists of a denaturation

step (2 sec at 95°C for the 18S gene and 0 sec at 94°C for the ND2 gene), an annealing step (10 sec at 66°C for the 18S rRNA gene and 10 sec at 53°C for the ND2 gene), and an extension step (20 sec at 72°C for the 18S rRNA gene and 10 sec at 72°C for the ND2 gene), with a temperature-transition rate of 20°C/sec. The fluorescent product was detected at the last step of each cycle by single acquisition. The method used a double-stranded DNA dye (SYBR Green I) to continuously monitor product formation. The sensitivity of SYBR Green I detection is limited by nonspecific product formation, which is monitored by fluorescence acquisition at temperatures at which only specific products are double stranded. After amplification, a melting curve was acquired by heating the product at 20°C/sec to 95°C, cooling at 20°C/sec to 72°C for the ND2 gene and 76°C for the 18S rRNA, and slowly heating it at 0.2°C/sec to 94°C with continuous fluorescence collection. Melting curves were used to determine the specificity of the PCR products. The results were expressed as the ratio of the mean mtDNA value of duplicate measurements to the mean nuclear DNA value of duplicate measurements (ND2/18S rRNA).¹⁷ To also express mtDNA content by organello, we divided the ND2/18S rRNA quotient by citrate synthase activity. The second muscle biopsy from the patient rendered enough biological material to perform a Southern blot hybridization using a mitochondrial ND4 gene probe in order to detect mtDNA deletions.⁴

All the results were expressed as the percentage with respect to the mean (\pm SD) of control values (100%) obtained from six healthy men.

The first histological study showed 5–10% of ragged-red fibers (RRF) or RRF equivalents and the presence of abundant lipid droplets in 50% of myocytes. In the second biopsy, RRF or equivalents had disappeared while only 5% of myocytes still showed an abnormal amount of lipid droplets (Fig. 1).

A marked increase (335%) in mitochondrial content during the symptomatic episode of hyperlactatemia was demonstrated. After treatment withdrawal and disappearance of clinical symptoms, mitochondrial content decreased but still remained above control values (177%, Fig. 2).

Enzyme activity decreased for all complexes of MRC during the acute phase of hyperlactatemia. The decrease was specially marked for complexes III and V, which showed only 36% and 11% of residual activity, respectively. Three months later, a general recovery of activities was observed, returning all of them into the control range (Fig. 2).

Regarding respiratory activity (Fig. 2), oxidation of all substrates tested in the symptomatic phase was found to be decreased (21% for pyruvate, 41% for succinate, 54% for ascorbate). However, the second study of the patient was not associated with a full recovery of respiratory activity.

Analysis of mtDNA relative abundance disclosed 47% of depletion with respect to control values in the first study when expressed by cell, which reached 83% when expressed by organello. Complete restoration of mtDNA content was ascertained in the second biopsy, 3 months after highly active antiretroviral therapy (HAART) discontinuation (Fig. 3). The Southern blot study of the second skeletal muscle specimen disclosed the presence of several mtDNA deletions of different size (Fig. 3).

We have demonstrated mitochondrial proliferation, deficient MRC complexes III and V activities, a general decay of mito-

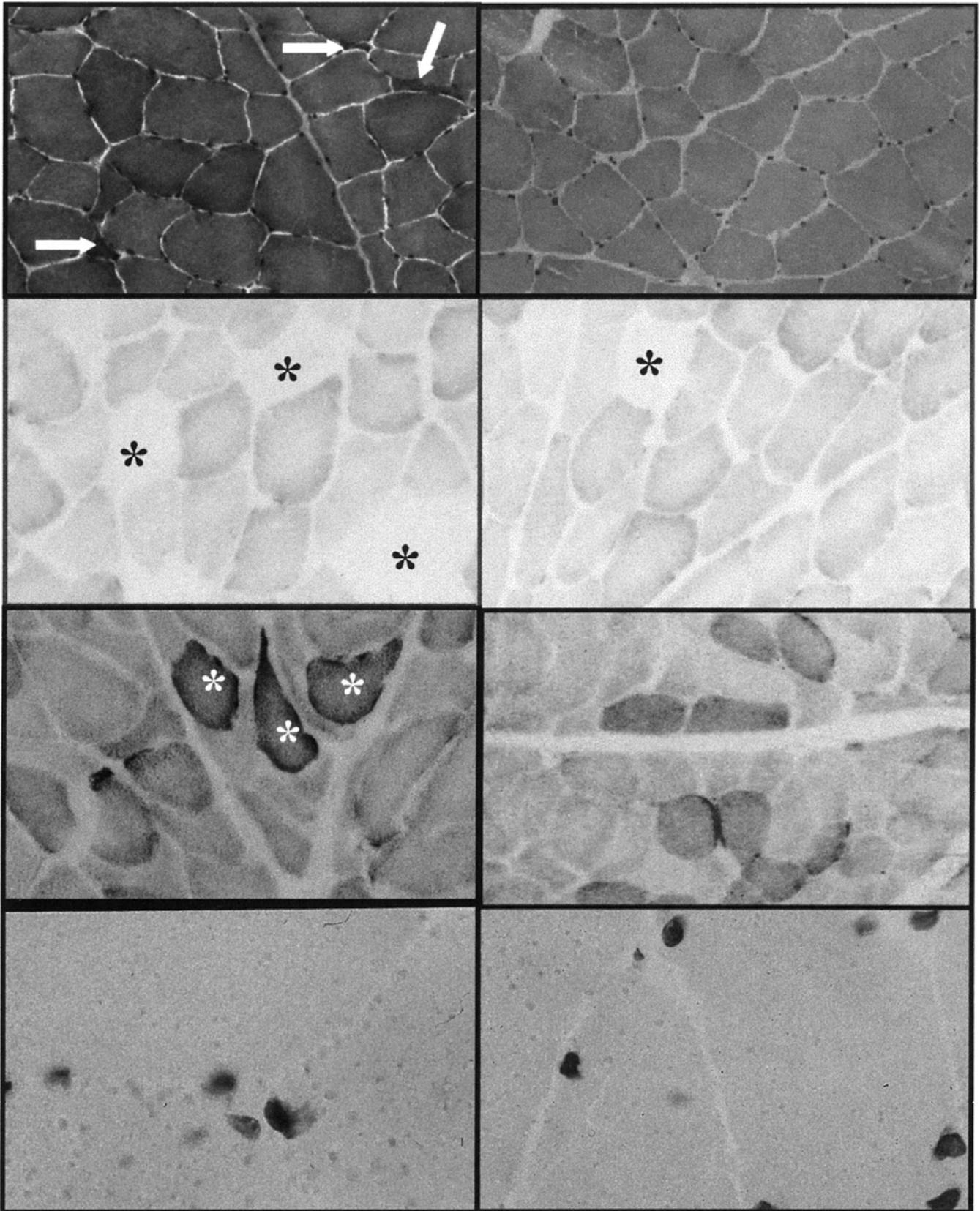


FIG. 1. Histological study of skeletal muscle during symptomatic hyperlactatemia (left) and after clinical and analytical recovery (right). First line: Gomori's trichrome staining demonstrating ragged-red fibers (RRF, arrows). Second line: Cytochrome *c* oxidase reaction demonstrating negative fibers (RRF equivalents, asterisks). Third line: Succinate dehydrogenase reaction demonstrating hyperactive fibers (RRF equivalents, asterisks). Forth line: Oil red O staining demonstrating deposition of neutral lipid droplets, which are more prominent in the first skeletal muscle biopsy (left).

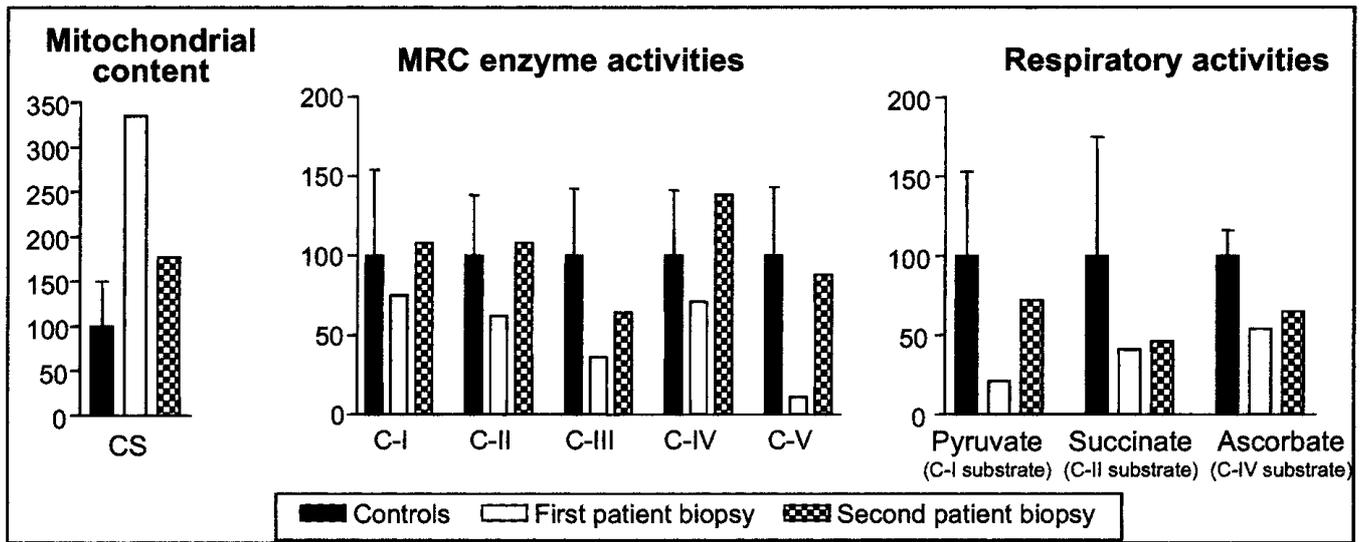


FIG. 2. Results of biochemical studies of mitochondrial respiratory chain function. y-axis are arbitrary values, where 100 is assigned to the mean of control group for each variable. Bars denote SD, which was obtained from six healthy men. CS, citrate synthase; MRC, mitochondrial respiratory chain; C-I, complex I; C-II, complex II; C-III, complex III; C-IV, complex VI; C-V, complex V.

chondrial respiratory capacity, and mtDNA depletion during a symptomatic episode of hyperlactatemia in an HIV-infected patient. Mitochondrial DNA depletion was even more evident when expressed by organello than by cell. A similar study performed 3 months later, when the patient became asymptomatic, showed a marked improvement in most of these mitochondrial parameters. At that time, the patient was receiving no anti-

retroviral drugs, while the rest of the treatments were maintained. This fact argues against the hypothetical role of gemfibrozil in our pathological findings because although gemfibrozil use has been described as causing decreased state 3 respiration stimulated by malate-pyruvate,¹⁸ the drug was maintained during the time that most of the abnormal mitochondrial parameters returned to normal.

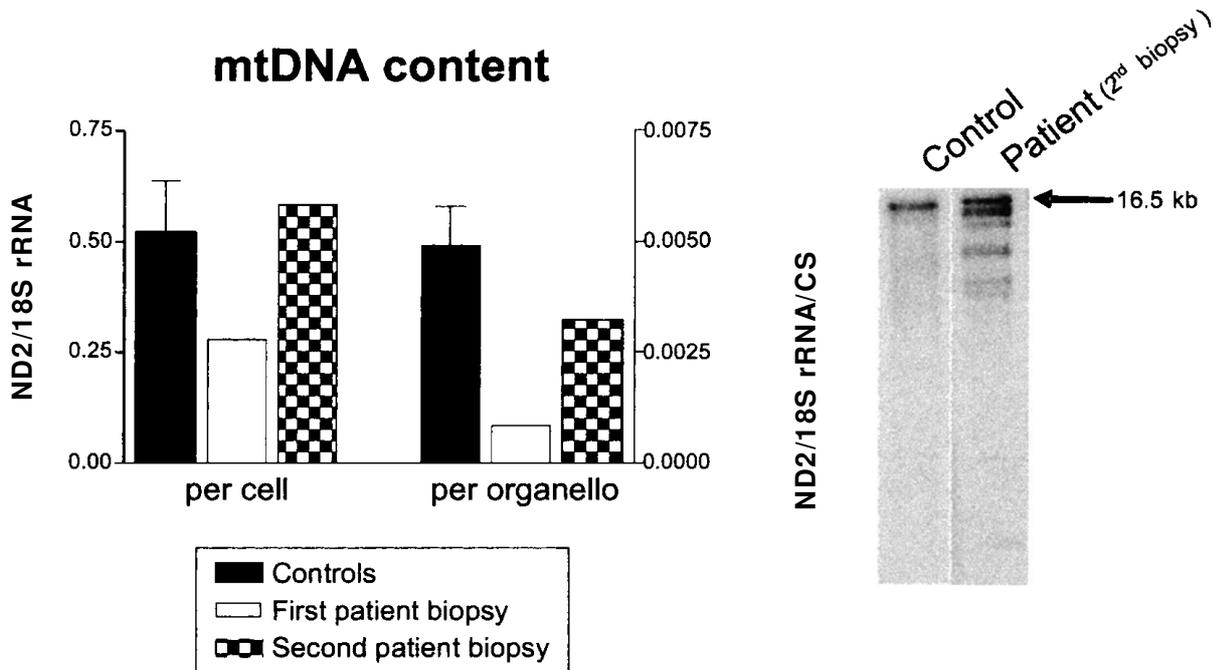


FIG. 3. Results of genetic analysis. Left: Results of mtDNA quantification (by cell and by organello) using real time PCR methodology (bars denote SD, which was obtained from six healthy men). CS, citrate synthase activity. Right: Radioactive Southern blot analysis of muscle mtDNA from the patient (second biopsy) and a healthy control. DNA samples were *Pvu*II digested, electrophoretically separated, and probed with the mitochondrial ND4 gene. Arrow indicates the wild-type mtDNA molecule (right).

As a whole, our data confirm that multiple mitochondrial dysfunction is involved in the pathogenesis of this syndrome and are in accordance with two previous reports investigating mitochondrial function of skeletal muscle in hyperlactemic HIV-infected patients. Gérard *et al.*⁶ biopsied skeletal muscle in 6 of 14 patients with symptomatic hyperlactatemia and in four of them found complex IV deficiency, with the residual activity ranging from 16% to 36% of control values. Unfortunately, there is a lack of data concerning the evolution after resolution of symptomatic hyperlactatemia in that study. More recently, Church *et al.*⁸ reported abnormal mitochondrial morphology and reduced activities (from 11% to 38%) for all MRC complexes (from I to IV) in skeletal muscle of an HIV-infected child with lactic acidosis and liver failure. Two months after discontinuing treatment with nucleoside analogs, a marked recovery was seen in both clinical status and MRC enzyme activities. Two additional mitochondrial studies^{7,13} also found different MRC complex deficiencies on hepatic biopsies. Consistent with all these facts, our demonstration that not only MRC enzyme activities, but also respiration are concurrently disturbed heightens the functional relevance of such MRC enzyme deficiencies in HAART-related hyperlactatemia. However, our documentation of skeletal muscle mitochondrial dysfunction in the presence of hyperlactatemia does not necessarily mean that this is the only tissue affected, or the main source of increased lactate production in hyperlactemic patients. In fact, Roge *et al.*¹⁹ have shown that skeletal muscle function and response to exercise remained relatively normal in patients with hyperlactatemia.

Côté *et al.*⁹ demonstrated that mtDNA was significantly depleted in PBMCs from HIV-infected patients with symptomatic hyperlactatemia who were receiving HAART. The decrease in mtDNA preceded the rise in venous lactate levels, an observation suggesting that hyperlactatemia is a consequence of mtDNA depletion. However, that study lacks MRC functional studies. Lactate is the product of anaerobic glycolysis. Therefore, hyperlactatemia in normal aerobic conditions may indicate mitochondrial dysfunction. In addition, if mtDNA depletion plays a pathogenic role it must necessarily be via MRC dysfunction, since mtDNA encodes only for certain subunits of certain complexes constituting MRC. Therefore, polarographic and spectrophotometrical studies of MRC must be the clue to link genetic and clinical findings.¹⁰ The case described in the present study, extensively evaluated from an MRC point of view, is consistent with the hypothesis of Côté *et al.*⁹ that mitochondrial toxicity stands at the basis of HAART-related hyperlactatemia.

An interesting aspect of our patient is that in addition to mtDNA depletion, multiple mtDNA deletions were present. One hypothetical mechanism to explain these gene defects, in addition to γ -polymerase inhibition, which causes mtDNA depletion, could be the coexistence of inhibition of mitochondrial processing peptidases by protease inhibitors included in HAART. Although not demonstrated to date, such a hypothetical effect of HAART regimens has also been postulated for mtDNA multiple deletions found in patients with lipodystrophy.^{4,20} Irrespective of all these speculations, all the above-mentioned data seem to confirm that mitochondrial dysfunction is the basis of hyperlactatemia occurring during HAART.

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Address reprint requests to:
Oscar Miró
Muscle Research Unit
Department of Internal Medicine
Hospital Clinic
Villarroel 170
08036 Barcelona
Catalonia, Spain
E-mail: omiro@clinic.ub.es

Mitochondrial effects of antiretroviral therapies in asymptomatic patients.

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Sònia López, Òscar Miró , Esteban Martínez, Enric Pedrol, Benjamín Rodríguez-Santiago, Ana Milinkovic, Anna Soler, Miguel A. García-Viejo, Virginia Nunes, Jordi Casademont, Josep M. Gatell, Francesc Cardellach.

Contribución: conjuntamente con Sònia López: cuantificación del mtDNA y del nDNA mediante la PCR en tiempo real y cuantificación del mtDNA y del nDNA mediante la PCR en tiempo real y cálculo del ratio mtDNA/nDNA mediante las rectas de regresión determinadas con los estándares. Figura 1 del manuscrito conjuntamente con Sònia López y escritura del apartado "*Mitochondrial DNA quantification*" del manuscrito.

Mitochondrial effects of antiretroviral therapies in asymptomatic patients

Sònia López¹, Òscar Miró^{1*}, Esteban Martínez², Enric Pedrol³, Benjamín Rodríguez-Santiago⁴, Ana Milinkovic², Anna Soler³, Miguel A García-Viejo², Virginia Nunes⁴, Jordi Casademont¹, Josep M Gatell² and Francesc Cardellach¹

¹Muscle Research Unit, Department of Internal Medicine, and ²Infectious Disease Unit. Hospital Clínic, 'August Pi i Sunyer' Biomedical Research Institute (IDIBAPS), School of Medicine, University of Barcelona, Barcelona, Catalonia, Spain

³HIV-Unit, Department of Internal Medicine, Fundació Hospital-Asil de Granollers, Granollers, Barcelona, Catalonia, Spain

⁴Medical and Molecular Genetics Center-IRO, Hospitalet de Llobregat, Barcelona, Catalonia, Spain

*Corresponding author: Tel: +34 93 227 93 65; Fax: +34 93 227 93 65; E-mail: omiro@clinic.ub.es

Background: A decrease in the mitochondrial (mt) DNA to nuclear DNA ratio has gained acceptance as a marker of mitochondrial toxicity in treated HIV-infected patients, but the functional meaning of this alteration is unclear. **Methods:** We assessed mtDNA content, mitochondrial content and function in peripheral blood mononuclear cells (PBMCs) of consecutive asymptomatic HIV-infected patients. Patients selected had been receiving a first-line highly active antiretroviral therapy (HAART) regimen for at least 6 months, consisting of zidovudine plus lamivudine or stavudine plus didanosine plus either nelfinavir or nevirapine, or were antiretroviral-naïve. The mtDNA content was assessed by quantitative real-time PCR, mitochondrial content by citrate synthase activity, enzyme activity of complexes III and IV (both partially encoded by mtDNA) of the electron transport chain by spectrophotometry, oxygen consumption by polarography, and oxidative damage in cell membranes by monitoring cis-parinaric acid fluorescence.

Results: Mitochondrial content was significantly lower in all treated groups. Patients receiving stavudine plus didanosine had mtDNA depletion and a decrease in complex IV activity. However, oxygen consumption capacity and lipid peroxidation were unaffected in all groups.

Conclusion: Long-term HAART may induce mitochondrial abnormalities in PBMC mitochondria, which do not necessarily translate into functional abnormalities, at least in asymptomatic patients.

This study was presented in the *4th International Workshop on Adverse Drug Reactions & Lipodystrophy in HIV* (San Diego, Calif., USA, September 2002) and in 'Late Breakers & Hot Topics' session in the *6th International Congress on Drug Therapy in HIV Infection* (Glasgow, UK, November 2002).

Introduction

Antiretroviral therapies containing nucleoside reverse transcriptase inhibitors (NRTIs) may cause toxic effects in mitochondria. NRTIs inhibit HIV replication but can also inhibit human DNA polymerase- γ [1,2] and thus, replication of mitochondrial DNA (mtDNA), leading to depletion of mtDNA and drug toxicity [3,4]. This has justified the investigation of mtDNA to nuclear DNA ratio as a marker of NRTI-related mitochondrial toxicity. The development of clinical manifestations of mitochondrial toxicity in HIV-infected patients receiving NRTI-containing antiretroviral therapy [5] has proven to be consistently associated with a decrease in this ratio. Fortunately, clinical manifestations of mitochondrial toxicity may not develop in a substantial proportion of HIV-infected patients receiving NRTI-containing antiretroviral therapy [6]. In the absence of clinical manifestations of

mitochondrial toxicity, it is not clearly known whether the mtDNA to nuclear DNA ratio may also be decreased [7]. In addition, even in cases where the mtDNA to nuclear DNA ratio has decreased, in asymptomatic HIV-infected patients, the functional meaning of such a disturbance has not been established. Therefore, it is necessary to demonstrate whether patients with mtDNA depletion present any functional disturbance, since the only phenotypic expression of mtDNA deficiency is the dysfunction of the mitochondrial respiratory chain (MRC) [8]. For all these reasons, we simultaneously studied the mtDNA to nuclear DNA ratio along with the mitochondrial content and function in peripheral blood mononuclear cells (PBMCs) from asymptomatic HIV-infected patients receiving different first-line antiretroviral regimens. PBMCs were chosen for this study because of

their accessibility. The main objective of this study was to assess whether mitochondrial abnormalities (genetic or functional) are detected in PBMCs even in the absence of clinically evident adverse effects.

Materials and methods

Patients

We designed a cross-sectional study including HIV-infected patients who fulfilled the following criteria: (a) asymptomatic, without clinically evident body fat changes; (b) normal acid–base equilibrium; (c) on first-line antiretroviral regimen for at least 6 months; (d) greater than 90% compliance with antiretroviral therapy; and (e) lack of personal or familiar history suggestive of mitochondrial disease or neuromuscular disorder. Patients were recruited from two recently reported randomized studies [9,10], and the antiretroviral treatment included four different regimens: one containing stavudine (d4T) plus didanosine (ddI) or zidovudine (AZT) plus lamivudine (3TC) as the nucleoside backbone plus either nelfinavir or nevirapine. A control group consisting of antiretroviral-naïve HIV-infected patients was also studied. Clinical and demographic data for each patient including age, gender, CD4 cells, HIV-1 RNA copies and duration of exposure to HAART were recorded at the time of inclusion. The protocol was approved by the Institutional Ethics Committee of each hospital and all patients provided written informed consent.

Methods

Sample obtaining

A total of 20 ml of venous blood were extracted from each patient and collected in Vacutainer™ EDTA tubes. PBMCs were immediately isolated by Ficoll density gradient centrifugation (Histopaque®-1077, Sigma Diagnostics, St Louis, Mo., USA). Platelet count after PBMC extraction procedure was confirmed to be below 200 per PBMC in each case. Total DNA was obtained by a standard phenol-chloroform extraction procedure from an aliquot of PBMCs and used for genetic studies. The remaining PBMCs were resuspended in 100–150 µl of phosphate buffered saline (PBS) and used directly for polarography, because fresh and intact cells are required for the measurement of the oxidative activity. For the remaining experiments, cells resuspended in PBS were frozen at –80°C until used for the determination of enzymatic activities. Protein content was measured by means of Bradford's protein-dye binding principle [11].

Mitochondrial DNA quantification

For each DNA extract, the housekeeping r18S nuclear gene and the highly conserved mitochondrial ND2

gene were quantified separately by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals®, Germany) [12]. The results were expressed as the ratio of the mean values of duplicate measurements of each gene (mtDNA/nuclear DNA).

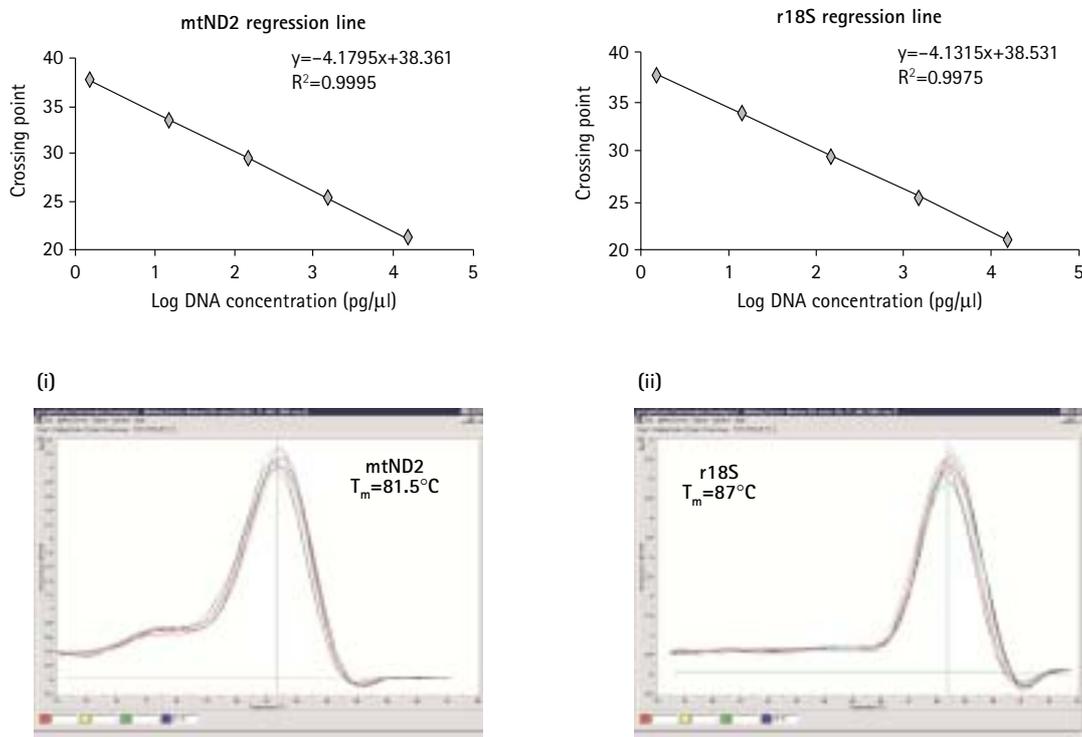
The PCR amplification of a 500 bp fragment length of the nuclear r18S gene was performed by using the forward 5'-ACGGACCAGAGCGAAAGCAT-3' and the reverse 5'-GGACATCTAAGGGCATCACAGAC-3' primers. For the mitochondrial ND2 gene, the forward 5'-GCCCTAGAAATAAACATGCTA-3' and the reverse 5'-GGGCTATTCCTAGTTTTATT-3' primers were used for the amplification of a 200 bp fragment length.

The PCR reactions for the mitochondrial gene amplification contained 3 mM MgCl₂, 0.25 pmols/µl of each primer and 10 ng of the DNA extract in 20 µl of final volume. The PCR reactions for the nuclear gene amplification contained 2 mM MgCl₂, 0.3 pmols/µl of each primer and 10 ng of the DNA extract in 20 µl of final volume. The PCR amplification programme consisted of a single denaturation-enzyme-activation step of 10 min at 95°C, followed by 35 cycles (for the r18S gene) and 29 cycles (for the ND2 gene). Each cycle consists of a denaturation step (2 s at 95°C, for the r18S gene and 0 s at 94°C, for the ND2 gene), an annealing step (10 s at 66°C, for the r18S gene and 10 s at 53°C, for the ND2 gene), and an extension step (20 s at 72°C, for the r18S gene and 10 s at 72°C, for the ND2 gene), with a temperature-transition rate of 20°C/s. The fluorescent product was detected at the last step of each cycle by single acquisition. A double-stranded DNA dye (SYBR Green I) was used to monitor product formation continuously [13]. The sensitivity of SYBR Green I detection is limited by non-specific product formation, which is monitored by fluorescence acquisition at temperatures at which only specific products are double-stranded. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C, cooling at 20°C/s to 72°C for ND2 and 76°C for r18S, and slowly heating it at 0.2°C/s to 94°C with continuous fluorescence collection. Melting curves, as shown in Figure 1, were used to determine the specificity of the PCR products [14].

Biochemical studies

Mitochondrial content: This was estimated by spectrophotometric measurement of citrate synthase activity (EC: 4.1.3.7). Citrate synthase is a mitochondrial matrix enzyme of the Krebs cycle, which remains highly constant in mitochondria and is considered a good marker of mitochondrial content [15,16]. The assay is performed at 412 nm (UVIKON 922, Kontron®, Switzerland) in 1 ml of medium at 37°C,

Figure 1. Real-time PCR



Top: Linear regression lines for mtND2 and r18S sequences. Amplification of mtND2 and r18S derived from amplifying fourfold dilutions of standard DNA provided two log-linear control standard lines. The least squares best fit of the standards is used to calculate the amount of template initially present in the samples. Bottom: melting curves used to determine the specificity of the PCR products. (i) mtND2 and (ii) r18S PCR products after amplification programme containing DNA or without template. The rate of fluorescence change with changing temperature ($-dF/dT$) was plotted as a function of temperature. The mtND2 product T_m was 81.5°C and for r18S was 87°C .

containing 2 mM EDTA, 10 mM KH_2PO_4 , pH 7.8 and 1 mg/ml of BSA, which should be freshly added to the buffered medium. Nearly 30 μg of cell protein is used to determine enzymatic activity. The reaction occurs in the presence of the detergent Triton X-100 [0.1% (v/v)], 0.1 mM acetyl-CoA and 2 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DNTB) as acceptor. The reaction starts with the addition of 12 mM oxalacetic acid. Citrate synthase activity (expressed as nmol of reduced DNTB per min per mg of cell protein) is measured by following, during 4 min, the increase in absorbance resulting from the enzymatic reduction of DNTB.

Oxidative activity by PBMCs (expressed as nmol of oxygen consumed per min per mg protein): measured polarographically with a Clark oxygen electrode in a water-jacketed microcell, magnetically stirred, at 37°C (Hansatech Instruments Ltd, Norfolk, UK) in 250 μl of respiration buffer (RB) consisting of 0.3 M mannitol, 10 mM KCl, 5 mM MgCl_2 , 10 mM KH_2PO_4 , PH 7.4 and 1 mg/ml of bovine serum albumin (BSA), which should be freshly added to RB. Under these conditions, we determined the oxygen consumption rate by intact cells, as well as after permeabilization of the cellular

membrane by incubation with digitonin, followed by the addition of different substrates, thus stimulating electron transfer through the enzymatic complexes that are partially encoded by the mitochondrial genome. The following assays were performed in PBMCs using two polarographic chambers in order to avoid contamination by rotenone:

Chamber A: Ten microlitres of cell suspension (100–200 μg of total protein) are added into the respiration chamber. Then, oxidation of glycerol-3-phosphate 10 mM (complex III substrate) is measured in the presence of rotenone 4 μM , EDTA 3.6 mM, ATP 2 mM, malonate 20 mM and 1 μl of a stock solution of digitonin 1% (w/v) (Sigma Chemical CO, St Louis, Mo., USA), solubilized in distilled water. These conditions allow the electrons to flow from complex III to complex IV to finally reduce an oxygen molecule [15,17].

Chamber B: Ten microlitres of cell suspension (100–200 μg of total protein) are added into the respiration chamber. Then, oxidation of endogenous substrates of intact cells is immediately monitored. Afterwards, oxidation of glutamate 20 mM plus

malate 2 mM (complex I substrate) is determined in the presence of 1 μ l of a stock solution of digitonin 1% (w/v) and ADP 0.4 mM. In this case, electrons flow from complex I to complex III to complex IV to finally reduce an oxygen molecule.

Electron transport chain enzyme activities: Measurement of the specific enzyme activity (expressed as nmol of reduced or oxidized substrate per min per mg of cell protein) of complex III and complex IV of the MRC (both partially encoded by mtDNA) was performed spectrophotometrically (UVIKON 922, Kontron®, Switzerland) in 1 ml of total reaction volume at 37°C according to the methodology of Rustin *et al.* [16], which was slightly modified for complex IV measurement [18].

Measurement of complex III (ubiquinol-cytochrome c reductase, EC 1.10.2.2): The assay is performed at 550 nm using 40 μ M oxidized cytochrome c as acceptor and 80 μ M decylubiquinol as donor in a medium containing 2 mM EDTA, 10 mM KH_2PO_4 , PH 7.8 and 1 mg/ml of BSA, which should be freshly added to the buffered medium. Nearly 40 μ g of cell protein are used to determine the enzymatic activity. The reaction occurs in the presence of 0.24 mM KCN, 4 μ M rotenone and 0.2 mM ATP. The reaction is followed for 3 min. Finally, the addition of 1 μ M antimycin A allows to distinguish between the specific reduction of cytochrome c catalysed by the complex III and the non-enzymatic reduction of cytochrome c directly by both the presence of BSA and by the auto-oxidation of the reduced decylubiquinone. A stock solution of 28 mM decylubiquinol is prepared by dissolving 25 mg decylubiquinone into 2.5 ml dimethyl sulfoxide (DMSO), 250 μ l distilled water and 10 μ l concentrated HCl, with a very large excess of sodium dithionite. After stirring for several minutes, the orange dark solution becomes transparent. Most of the sodium dithionite remains insoluble. The final solution is centrifuged at 9000 rpm for 5 min and the supernatant is transferred to a new vessel and stirred to eliminate the remaining sodium dithionite.

Measurement of complex IV (cytochrome c oxidase, EC 1.9.3.1): The assay is performed at 550 nm using 10 μ M reduced cytochrome c as donor, in an isosmotic medium containing 0.3 M sucrose, 10 mM KH_2PO_4 , PH 6.5 and 1 mg/ml of BSA, which should be freshly added to the buffered medium. Nearly 10 μ g of cell protein is used to determine the enzymatic activity. The detergent laurylmaltoside (2.5 mM) is used to permeabilize the mitochondrial membranes. The initial rate of decrease in absorbance, as a result of the oxidation of reduced cytochrome c, is measured during the first 2 min. Finally, the reaction is inhibited with 0.24 mM KCN in order to obtain specific activity.

Quantification of lipid peroxidation: Oxidative damage of PBMC membranes was measured with assessment of lipid peroxidation using cis-parinaric acid. Cis-parinaric acid is a fatty acid that contains four conjugated double bonds that render it naturally fluorescent, and which is attacked in lipid peroxidation reactions. Accordingly, cis-parinaric acid fluorescence is consumed in lipid peroxidation reactions. Since it readily incorporates into membranes, its loss of fluorescence is used indirectly to monitor the degree of lipid peroxidation. For this purpose, 100 μ g of PBMC protein was placed into 3 ml of nitrogenized PBS containing cis-parinaric acid (5 μ M) (Molecular Probes®, Eugene, Oreg., USA) and incubated in darkness at 37°C. Afterwards, fluorescence was measured at 3 min intervals over 30 min at 318 nm excitation and 410 nm emission [19,20]. The greater the lipid peroxidation, the less fluorescence detected.

Statistical analysis

Qualitative data were expressed as percentages and quantitative data as mean \pm SEM and 95% confidence interval (95% CI). All groups on antiretroviral therapy were compared with the control group by using the χ^2 test and the unpaired Student's t test for qualitative and quantitative variables, respectively. In the Student's t test, normality of the distribution was ascertained using the Kolmogorov-Smirnov test prior to applying parametric tests. For comparison of curves of cis-parinaric experiments, two-way ANOVA was used. In all cases, *P*-values less than 0.05 were considered statistically significant.

Results

Forty-two HIV-infected patients on first-line antiretroviral therapy were included, 22 in the d4T plus ddI group and 20 in the AZT plus 3TC group. There were no differences in age and gender between the groups on antiretroviral therapy and controls. Both groups receiving antiretrovirals had lower plasma HIV-1 RNA values and higher CD4 lymphocyte counts than controls. The duration of exposure to antiretroviral agents was similar for both groups of (Table 1).

Mitochondrial DNA content was significantly decreased only in the group of HIV patients receiving d4T plus ddI, with a remaining mtDNA content of 56% (95% CI: 41–71%; *P*=0.001) when compared with the untreated control group (100%). Conversely, patients receiving AZT and 3TC exhibited preserved amounts of mtDNA in PBMCs (Figure 2).

Mitochondrial content was decreased in both groups on antiretroviral therapy. Compared with the controls (100%), mitochondrial abundance was 68% (95% CI: 50–86%; *P*<0.001) for patients receiving ddI

Table 1. Clinical and demographic data of patients

	Controls; n=25	HAART containing d4T+ddI; n=22	HAART containing AZT+3TC; n=20
Age (years), \pm SEM	37 \pm 1.7	42 \pm 2.6	40 \pm 2.5
Gender (female), %	24	27	25
Lymphocyte CD4 count (per ml), \pm SEM	317 \pm 43.0	407 \pm 42.6	624 \pm 81.4 †
*Log ₁₀ viral load (copies/ml), \pm SEM	5.0 \pm 0.19	1.99 \pm 0.11 †	1.84 \pm 0.16 †
Time on HAART (months), \pm SEM	–	16 \pm 1.6	20 \pm 2.2

*Viral load <50 copies/ml was considered to be 49 copies/ml; † $P \leq 0.001$ with respect to the controls.

plus d4T, and 70% (95% CI: 50–90%; $P=0.001$) for patients receiving AZT plus 3TC (Figure 3).

On spectrophotometry of the specific enzyme activity of complex III of the electron transport chain, no differences were observed on comparison with controls. Conversely, complex IV activity was significantly reduced in HIV-infected patients taking d4T plus ddI (87% of remaining activity, 95% CI: 75–99%; $P=0.04$) (Figure 4).

The results obtained by polarography were particularly noteworthy because mitochondrial oxygen consumption, either by intact cells or stimulated by different substrates, remained within normal values for all treated patients irrespective of the type of antiretroviral regimen (Figure 5).

Similarly, we did not detect an increase in the oxidative damage of PBMC membranes due to lipid peroxidation reactions in treated patients, irrespective of the type of antiretroviral regimen when compared with the control group (Figure 6).

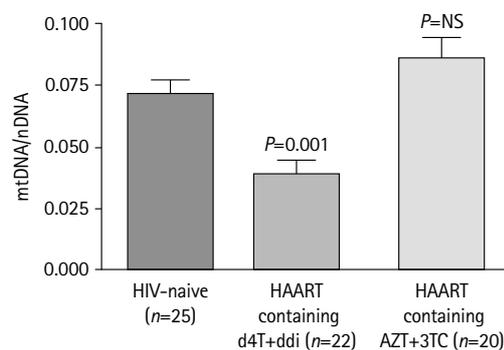
Conclusions

The most important finding in this study was that sub-clinical mitochondrial toxicity was detected in PBMCs from asymptomatic HIV-infected patients receiving antiretroviral therapy. This toxicity was expressed as decreased mitochondrial content in all treatment schedules, while depletion of mtDNA was detected exclusively in patients receiving d4T plus ddI. Interestingly, these declines did not translate into overt abnormalities of mitochondrial function because, aside from a mild decrease in complex IV activity in patients on d4T plus ddI, no differences were present in oxygen consumption by mitochondria and oxidative damage for any HAART schedule.

To our knowledge, these findings have not been previously reported. Different approaches have been used to study mitochondrial function during the clinical use of HAART, with most being addressed to investigate the role of mitochondria in patients with lipodystrophy [21–31] or hyperlactataemia [8,30–35]. In these studies, however, either a group of patients on HAART without symptoms is lacking [8,24–27,30,32–35] or the number of patients included

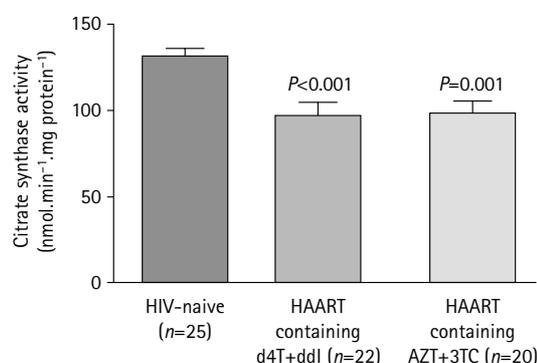
is limited [21–23,28,29,31]. Moreover, they were not specifically designed to investigate the mitochondrial effects of HAART. Only one previous study has specifically addressed this issue and failed to demonstrate mtDNA depletion in 10 asymptomatic patients on different HAART schedules compared with HIV-negative patients. However, the heterogeneity of antiretroviral schedules received by these patients could hypothetically mask the existence of mtDNA depletion for a specific HAART regimen [7]. In contrast with the data reported by Henry *et al.*, our

Figure 2. Mitochondrial DNA (mtDNA) content



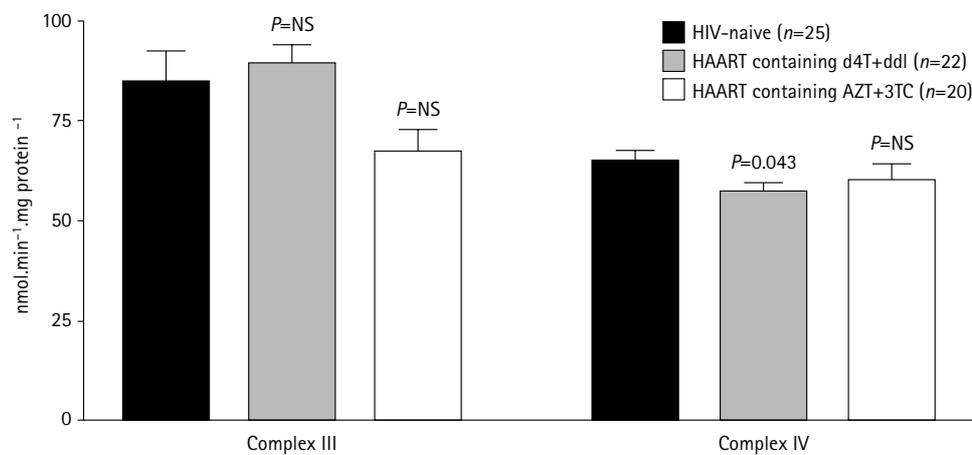
HAART, highly active antiretroviral therapy; d4T, stavudine; ddI, didanosine; AZT, zidovudine; 3TC, lamivudine.

Figure 3. Mitochondrial content



HAART, highly active antiretroviral therapy; d4T, stavudine; ddI, didanosine; AZT, zidovudine; 3TC, lamivudine.

Figure 4. Enzyme activities of complexes III and IV of electron transport chain



HAART, highly active antiretroviral therapy; d4T, stavudine; ddl, didanosine; AZT, zidovudine; 3TC, lamivudine.

cohort of asymptomatic patients on HAART was larger and more homogenous, and undoubtedly showed mitochondrial disturbances.

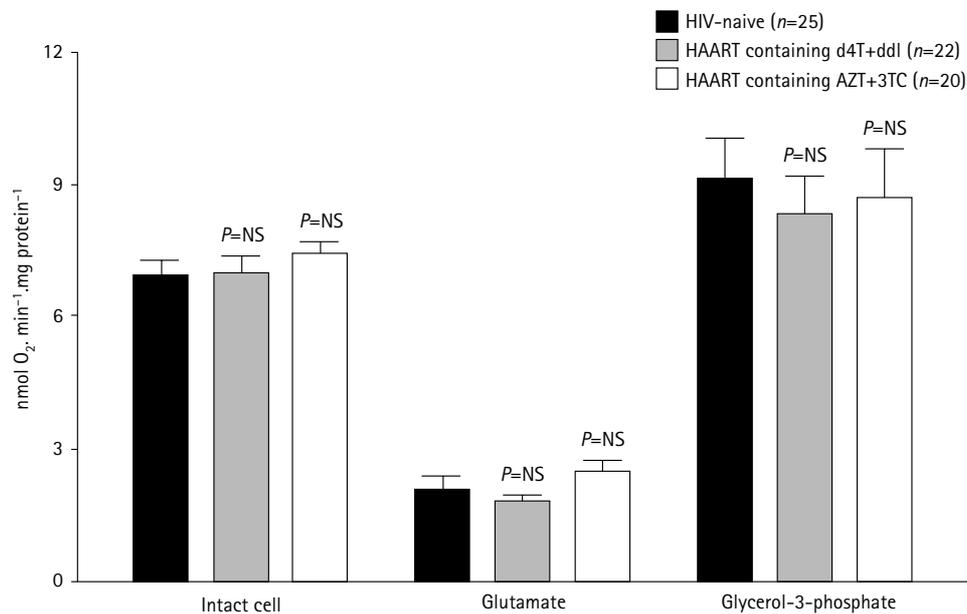
In addition, we also performed an intensive and comprehensive mitochondrial study not limited to only the mtDNA content but also to considering the content and functional capacities. Other authors who have studied the mtDNA to nuclear DNA ratio exclusively in HIV-infected patients receiving antiretroviral therapy found either an increase of this marker compared with the baseline [36] or similar values compared with matched controls [7]. It is biologically plausible that sub-clinical mitochondrial toxicity may be present in asymptomatic HIV-infected patients receiving NRTI-containing antiretroviral therapy. In predisposed patients, this sub-clinical deficit may lead to clinical manifestations in the presence of precipitating factors. There may be an as yet unidentified biological threshold for mitochondrial toxicity, before which there is little evidence of damage at the molecular level. The results of this study confirm that mitochondria are a specific target during the clinical use of HAART.

A decrease in the mtDNA to nuclear DNA ratio in this study was detected exclusively in patients receiving d4T plus ddI. This finding is in accordance with previous studies *in vitro* demonstrating that ddI and d4T have a high potency to inhibit polymerase- γ [37] and, additionally, the triphosphated form of d4T is incorporated into replicating DNA more readily than other currently used NRTI and exerts the greatest inhibition on the human polymerase- γ [2]. This may explain the apparent association between clinical data of mitochondrial toxicity and the concomitant use of ddI and d4T. Our results confirm the relatively higher

mitochondrial toxicity, in terms of a decrease in the mtDNA to nuclear DNA ratio, *in vivo* in patients receiving antiretroviral therapy containing d4T plus ddI. These results are concordant with those of another study in which the combination of nucleosides and a third drug (either a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor) were periodically changed [38]. In that study, there was an asymptomatic depletion of mtDNA in PBMCs in all treatment arms, but the depletion was greater with the regimen containing d4T plus ddI plus efavirenz than with AZT plus 3TC plus nelfinavir or the alternation of the two regimens.

On the other hand, the significance of the decreased mitochondrial content found in association with both HAART regimens analysed in the present study differs from the mitochondrial proliferation observed in the skeletal muscle in primary MRC defects. However, decreases in mitochondrial content have been documented in skeletal muscle and/or PBMCs from patients with type 2 diabetes [39], very premature neonates with immature mitochondrial energy-providing system [40], and obese and normal-weight subjects with age-related mitochondrial changes [41]. In some cases, it has been associated with a deficiency of mitochondrial transcription factor A (mitochondrial destined nuclear protein required for mtDNA replication and expression) [42], exemplifying that nuclear interference leading to mitochondrial disturbances may also be possible in patients on antiretroviral therapy. In fact, Vittecoq *et al.* [27] have recently reported the lack of an increase in mitochondrial content in skeletal muscle and liver of patients on HAART, and even in three out of nine patients in whom mitochondrial content was analysed in PBMCs, the mitochondrial content was

Figure 5. Respiratory activity



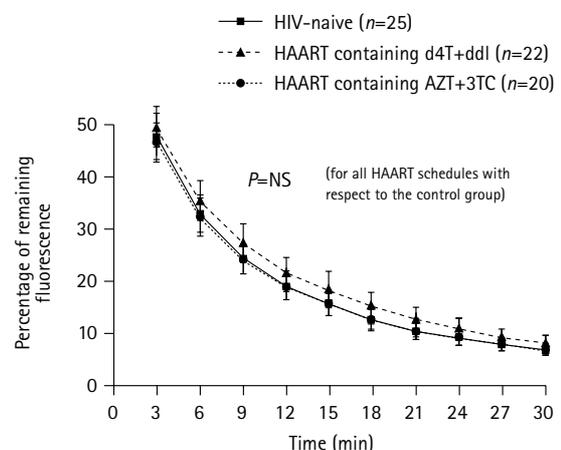
HAART, highly active antiretroviral therapy; d4T, stavudine; ddl, didanosine; AZT, zidovudine; 3TC, lamivudine; Glutamate, glutamate oxidation; Glycerol-3-P, Glycerol-3-phosphate oxidation.

clearly decreased. As HAART up-regulates tumour necrosis factor- α [43], which contributes to increasing the mitochondrial-mediated apoptotic pathway [26], it could be suggested that an enhanced apoptotic process could be present in PBMCs and leads to a generalized decrease in mitochondrial content associated with all the antiretroviral regimens studied.

However, decreases in mitochondrial and mtDNA content do not reach the threshold necessary to cause a significant decay in the overall respiratory capacity of MRC. It is possible that cumulated doses of antiretrovirals may not be sufficient to induce a degree of mtDNA depletion and lead to a decline in respiratory capacity. In fact, all of the patients remained asymptomatic at the time of inclusion in the study and in this setting it is foreseeable that the main MRC function, that is, to oxidize substrates, should remain unaltered. This does not exclude the fact that defects could already be present in other tissues more dependent on oxidative phosphorylation than PBMCs. In this sense, PBMCs are constantly removed from and introduced to peripheral blood. Thus, despite the fact that the HIV-infected patients included had received antiretroviral therapy for a mean of more than 1 year, it is possible that the cells studied were not exposed to antiretroviral therapy for their whole life cycle, and therefore, the drugs were not able to induce significant mtDNA damage to lead to electron transport chain dysfunction. Moreover, since PBMCs do not contain

more than 15–20 mitochondria per cell and their metabolic rate is low in the absence of activation, the incorporation of damaging drugs into mtDNA is slow. It is important to note that at this preclinical stage, an antiretroviral withdrawal should probably be followed by the disappearance of all the reported abnormalities. This potential reversibility is supported by previous experience with AZT myopathy [44] and also with hyperlactataemia [45] caused by antiretrovirals, in

Figure 6. Lipid peroxidation assessed by curves of cis-parinaric acid fluorescence loss over time



HAART, highly active antiretroviral therapy; d4T, stavudine; ddl, didanosine; AZT, zidovudine; 3TC, lamivudine.

which molecular and biochemical disturbances normalized after drugs withdrawal.

Assessment of MRC function is essential prior to making any pathogenic conclusions regarding mtDNA depletion, because mtDNA exclusively contains information related to some MRC proteins, and therefore, the only pathogenic expression of mtDNA lesion is MRC dysfunction. For this reason, although mtDNA depletion has recently been proposed as a marker of clinically evident mitochondrial-related adverse effects [5], this depletion does not necessarily translate into abnormal mitochondrial function. In fact, our data illustrate how, despite the observation of different patterns and degrees of mitochondrial toxic effects by HAART, net oxidative capacity of mitochondria was within the normal range, at least in asymptomatic HIV-infected patients. Therefore, we believe that while a laboratory marker with a clear threshold of clinical relevance has not yet been defined, clinicians should be cautious to take any decision based on the measurement of the mtDNA content.

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