# EFECTES DE LA INFECCIÓ PEL VIH I DELS FÀRMACS ANTIRETROVIRALS ENVERS EL MITOCONDRI: LES CÈL·LULES MONONUCLEARS DE SANG PERIFÈRICA COM A MODEL D'ESTUDI

**SÒNIA LÓPEZ MORENO**

**Tesi Doctoral**

# 9. ANNEX

La doctoranda ha participat activament en l'elaboració de diversos estudis, alguns dels quals estan estretament relacionats amb el tema de la seva Tesi Doctoral, i d'altres, tot i que es basen en l'anàlisi del funcionalisme mitocondrial, es distancien dels objectius concrets de la present tesi. A continuació, es detallen en ordre cronològic les publicacions científiques que d'aquests treballs se'n deriven.

Addicionalment, s'inclouen les Jornades i els Congressos relacionats amb el tema de la Tesi en els que la doctoranda ha participat amb la presentació de pòsters i/o comunicacions orals.

# REVERSIBLE MITOCHONDRIAL RESPIRATORY CHAIN IMPAIRMENT DURING SYMPTOMATIC HYPERLACTATEMIA ASSOCIATED WITH ANTIRETROVIRAL THERAPY

*AIDS RESEARCH AND HUMAN RETROVIRUSES*

2003 Nov;19(11):1027-1032, ORIG

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 19, Number 11, 2003, pp. 1027–1032


# Short Communication

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

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## 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.**

HYPERLACTATEMIA 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 myopathy[3] and lipodystrophy syndrome[4]), few works have directly studied mitochondrial respiratory chain (MRC) function in patients with hyperlactatemia.[5–8] Although a recent study[9] 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

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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 lipoatrophy, 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 academia (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/$mm^3$ 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 suspension[13] 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′-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 $MgCl_2$, 0.25 pmol/$\mu$l of each primer, and 10 ng of DNA in 20 $\mu$l of final volume. The PCR reactions for nuclear gene amplification contained 2 mM $MgCl_2$, 0.3 pmol/$\mu$l of each primer, and 10 ng of DNA in 20 $\mu$l 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 quocient 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-

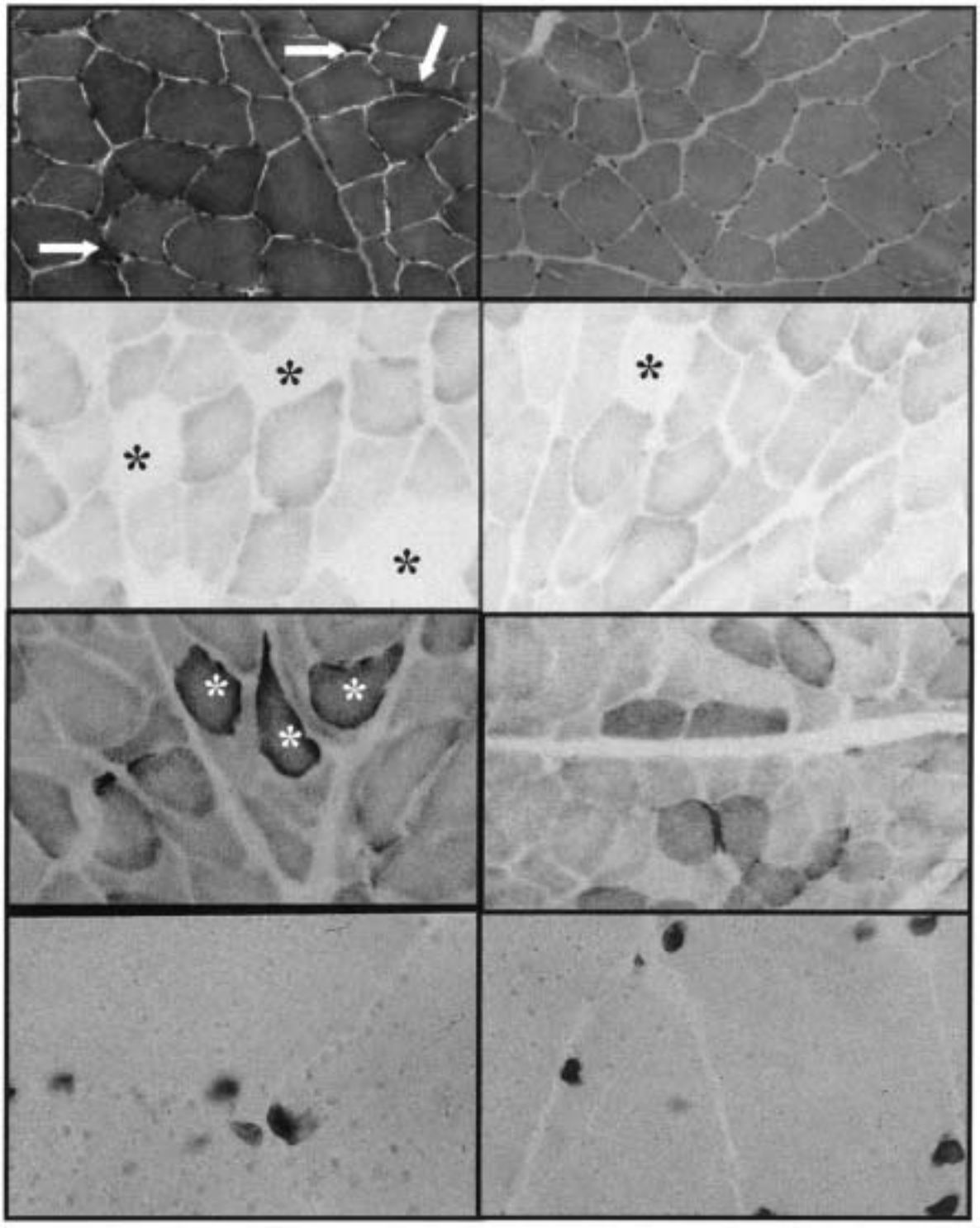

**FIG. 1.** Histological study of skeletal muscle during symptomatic hyperlactatemia (left) and after clinical and analytical recovery (right). First line: Gomori's trichrome staining demonstrating ragged-red fibers (RRF, arrows). Second line: Cytochrome *c* oxidase reaction demonstrating negative fibers (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).

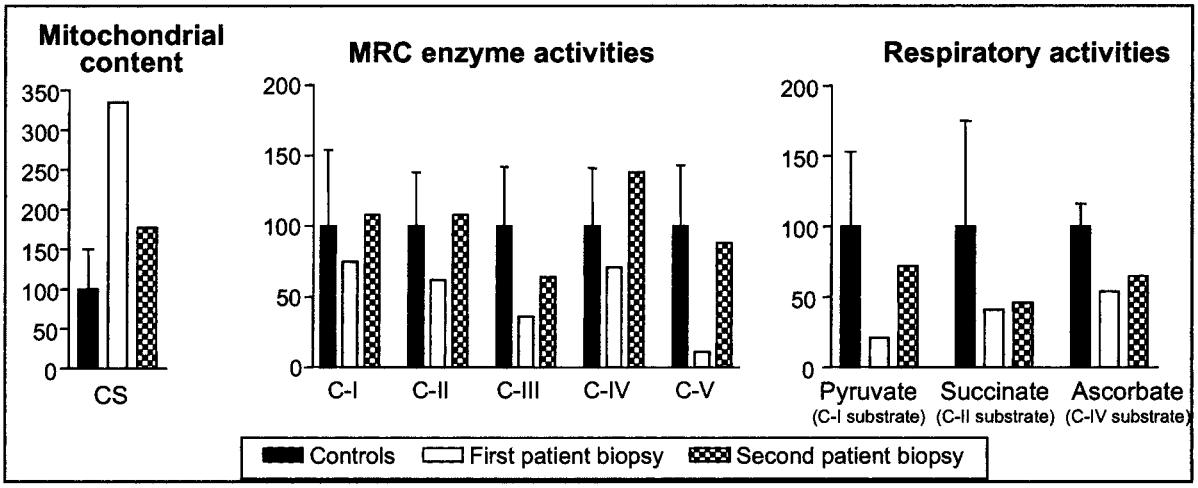


**FIG. 2.** Results of biochemical studies of mitochondrial respiratory chain function. *y*-axis are arbitrary values, where 100 is assigned to the mean of control group for each variable. Bars denote SD, which was obtained from six healthy men. CS, citrate synthase; MRC, mitochondrial respiratory chain;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

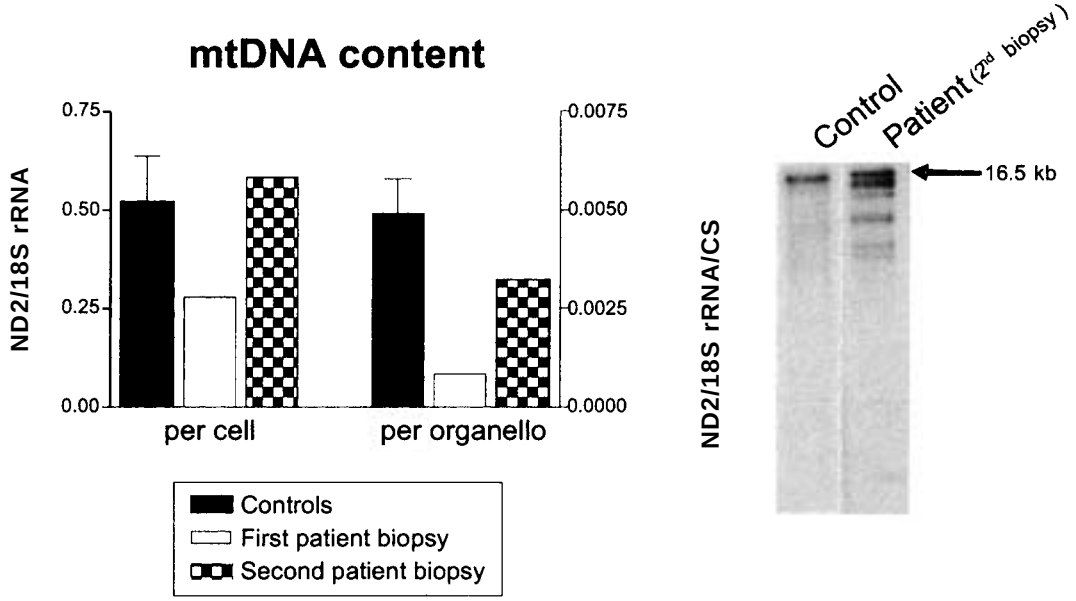


**FIG. 3.** Results of genetic analysis. Left: Results of mtDNA quantification (by cell and by organello) using real time PCR methodology (bars denote SD, which was obtained from six healthy men). CS, citrate synthase activity. Right: Radioactive Southern blot analysis of muscle mtDNA from the patient (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 hyperlactatemic 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 hyperlactatemic 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 $\gamma$-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.

## ACKNOWLEDGMENTS

We thank Mrs. Anna Beato for her expertise and technical support and M. Gómez (Ph.D.) for critical comments. This study has been partially supported by grants from the Fundación para la Investigación y la Prevención del Sida en España (FIPSE 3102-00), Fundació La Marató de TV3 020210, and Suport a Grups de Recerca de la Generalitat de Catalunya (2001/SGR/00379).

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EFFECT OF SMOKING CESSATION ON MITOCHONDRIAL RESPIRATORY CHAIN FUNCTION

*JOURNALOF TOXICOLOGY CLINICAL TOXICOLOGY*

2003; 41(3): 223-8, ORIG

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Journal of Toxicology
CLINICAL TOXICOLOGY
Vol. 41, No. 3, pp. 223–228, 2003

ARTICLE

# Effect of Smoking Cessation on Mitochondrial Respiratory Chain Function

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## ABSTRACT

*Objective*. Chronic smoking has been associated with diverse mitochondrial respiratory chain (MRC) dysfunction in lymphocytes, although inhibition of complex IV activity is the most consistent and relevant finding. These mitochondrial abnormalities have been proposed to contribute to pathogenesis of diseases associated with tobacco consumption. We assessed MRC function in peripheral lymphocytes from heavy smokers after cessation in smoking habit. *Patients and Methods*. We studied MRC function from peripheral lymphocytes of 10 healthy chronic smoker individuals (age $43 \pm 6$ years; 50% women) before cessation of tobacco consumption ($t_0$), and 7 ($t_1$) and 28 ($t_2$) days after cessation. Smoking abstinence was ascertained by measuring carboxyhemoglobin levels and carbon monoxide (CO) concentration in exhaled breath. Ten healthy nonsmoker individuals matched by age and gender were used as controls. Lymphocytes were isolated by Ficoll's gradient, and protein content was determined by Bradford's technique. MRC function was studied through double means: 1) individual enzyme activities of complex II, III, and IV were analyzed by means of spectrophotometry; 2) oxygen consumption was measured polarographically using pyruvate, succinate, and glycerol-3-phosphate (complex I, II, and III substrates, respectively) after lymphocyte permeabilization. Enzyme and oxidative activities were corrected by citrate synthase activity. *Results*. Smokers showed a significant decrease in complex IV activity ($p = 0.05$) and also in respiration of intact lymphocytes ($p = 0.05$) compared to controls. Eight chronic smokers remained abstinent during the study. Smoking cessation was associated with a significant recovery of complex IV ($p = 0.01$) and complex III ($p = 0.05$) activities. Oxidative activities did not show any change during the study. *Conclusion*. Chronic smoking is associated with a decrease of complex IV and III activities of MRC, which return to normal values after cessation of tobacco smoking.

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DOI: 10.1081/CLT-120021102
0731-3810 (Print); 1097-9875 (Online)

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## INTRODUCTION

Smoking habit is directly connected with a vast number of human diseases involving most organs and tissues. Mechanisms by which smoking causes its deleterious effects are varied and not completely elucidated. Mitochondria have been proposed as one of the targets of tobacco smoke compounds (1). These organella are present in nearly all cellular lines and play an important role in two critical cell functions: they are the common pathway for a number of initiators of apoptosis (a physiological process by which cells are removed when their DNA is damaged by noxious agents), and their respiratory chain is the main source of cellular energy liberated as ATP molecules.

Cigarette smoke contains short and long-lived free radicals and also can stimulate endogenous cellular production of other highly reactive oxygen species (ROS), both inducing protein oxidation, lipid peroxidation (2), and mitochondrial DNA damage (3). This oxidative damage further results in mitochondrial depolarization and final cellular apoptosis (4,5). On the other hand, components of enzyme mitochondrial machinery, especially those integrating MRC, can be directly disturbed by tobacco smoke at different steps (6–8), which could result in a decrease in ATP synthesis (8). Although mitochondrial respiratory chain (MRC) abnormalities have been described in chronic smokers, the reversibility of these effects after smoking cessation has not been previously ascertained. Therefore, we assessed MRC function in peripheral lymphocytes from chronic smokers and assessed changes associated with smoking cessation.

## PATIENTS AND METHODS

We included 10 chronic smokers who entered the "Tobacco Deshabituation Unit" of our hospital. Their cigarette consumption was between one and three packs a day, at least during the last five years before this study. All of them were otherwise healthy people with no previous history of metabolic or neuromuscular diseases. In all cases, a complete blood count and chemistry screen (glucose, creatinine, sodium, potasium, calcium, and creatinphosphokinase) were performed and normality of all tests was required to be included in the protocol.

To perform mitochondrial studies, 30 mL of venous blood were obtained in active smokers, and also 7 and 28 days after smoking cessation. Lymphocytes were isolated through a Ficoll's gradient and the protein content of the final lymphocyte suspension was measured using the Bradford's protein-dye binding principle (9).

Smoking status was ascertained at the three times of the study by measuring carbon monoxide content in exhaled air (COEA) by means of an electrochemical transducer (Dräger Pac III, Dräger Safety Inc, Pittsburgh, PA), and blood levels of carboxyhemoglobin (COHb) through CO-oximetry (Blood gas Analyzer CIBA-CORNING 800 System, Switzerland). COHb levels less than 2% and COEA levels less than 10 ppm were respectively required to consider the individual as abstinent. As control values, we used those obtained from 10 healthy nonsmoker individuals matched by age, gender, and physical activity with respect to the smokers group. All individuals gave their informed consent and the Ethic Committee of our hospital revised and accepted the present protocol.

MRC function from lymphocytes was assessed using a double approach. First, we determined the individual enzyme activity for complex II (succinate–ubiquinone reductase, EC 1.3.99.1), complex III (ubiquinol–cytocrome *c* reductase, EC 1.10.2.2), and complex IV (cytochrome *c* oxidase, EC 1.9.3.1) spectrophotometrically (UVIKON 920, Kontron®, Switzerland). Second, oxygen consumption was polarographically determined in intact lymphocytes (Hansatech Instruments Limited®, Norfolk, England). We studied spontaneous respiration, as well as respiratory rates using pyruvate, succinate, and glycerol-3-phosphate as substrates for complex I, II, and III, respectively. The concentration of substrates, complete methodology, and experimental conditions used in all these assays have been described previously (10–13).

In order to account for any eventual difference in the mitochondrial content of peripheral lymphocytes, results were expressed as absolute and relative activities as well. Relative activities were obtained in relation of citrate synthase (EC 4.1.3.7) activity, an enzyme of Krebs' cycle which is considered as a good marker of mitochondrial content. The measurement of citrate synthase activity was carried out spectrophotometrically under the conditions described elsewhere (14).

Results are expressed as percentages, mean ± standard deviation, and 95% confidence intervals using SPSS 10.0 statistical package. Comparisons between the three determinations performed in smokers and the control values of nonsmokers were performed by unpaired t-test. No corrections were made for multiple comparisons. The changes of enzyme activities and oxygen consumption of smoker individuals at the different times of the study (immediately before and 7 and 28 days after smoking cessation) were assessed by

a repeated measures ANOVA using the general lineal model of SPSS. Differences were considered significant when p value was less than 0.05.

## RESULTS

We initially included 10 smoker individuals, although only 8 subjects (43 ± 6 years; 50% women) remained abstinent until the end of the study and yielded all necessary data to be finally included in the study. COHb and COEA in active smokers were 5.1 ± 2.3% and 28.1 ± 7.6 ppm, respectively, which were significantly higher than those observed in controls (0.5 ± 0.3% and 3.6 ± 2.0 ppm, respectively; $p < 0.001$ for both). After smoking cessation, COHb and COEA values decreased to 0.6 ± 0.3% and 3.1 ± 2.0 ppm respectively, at day 7, and 0.8% ± 0.3 and 3.1 ± 2.2 ppm respectively, at day 28, which fitted into normal ranges.

Mitochondrial content of lymphocytes at the beginning of the study did not differ between both groups, as judged by citrate synthase activities (smokers: 127 ± 60 nmol/min/mg protein; controls: 136 ± 24 nmol/min/mg protein; p = NS). Cessation of the smoking habit was not associated with any significant change in citrate synthase activity in the smokers group (127 ± 40 nmol/min/mg protein on day 7 and 110 ± 23 nmol/min/mg protein on day 28).

Results were similar irrespective of whether they were expressed as absolute or relative activities. Relative enzyme activities are presented in Fig. 1. In active smokers, only complex IV activity was significantly lower than that of controls (33% inhibition; CI: 5% to 61%; $p < 0.05$). After smoking cessation, complex IV activity showed a progressive and significant recovery up to normal values after 28 days ($p = 0.002$). At that time, a 56% (CI: 10% to 102%) of complex IV recovery respect to initial values was observed (Fig. 2). Complex III activity showed a slight decrease (21% inhibition; CI: −9% to 51%; p = NS) in active smokers compared to controls, and cessation of smoking was associated with a statistically significant increase in its activity over time ($p = 0.04$).

Polarographic studies in lymphocytes from active smokers showed a significant decrease in spontaneous oxygen consumption compared to controls (26% inhibition; CI: 6% to 46%; $p < 0.05$); however, no differences were observed when respiratory state 3 rate was measured for each particular substrate (Fig. 3).

The behavior of oxidative activities after smoking cessation remained relatively stable with all substrates, and we did not observe any significant change during the study.

## DISCUSSION

The present study confirms that complex IV enzyme activity is inhibited in smokers (7), and that this inhibition progressively disappears shortly after smoking habit cessation. Additionally, our data indicate that complex III activity is also slightly inhibited by chronic

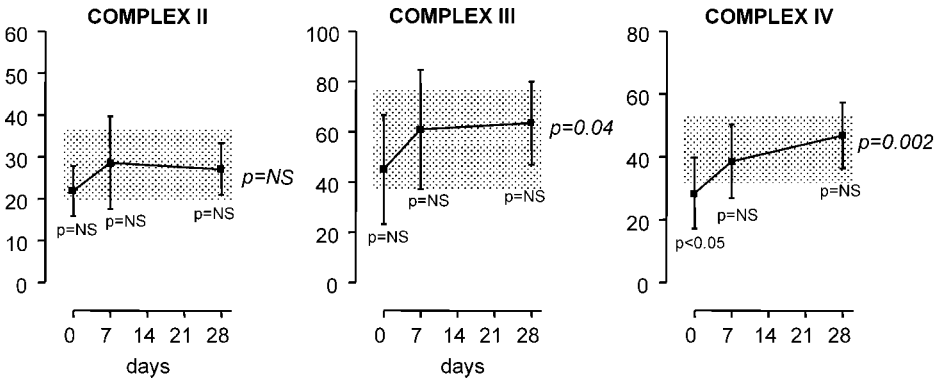


***Figure 1.*** Relative enzyme activities (means with 95% CI) of smokers before (day 0) and after stopping (days 7 and 28) to smoke. There are no units for the Y axis because they represent the ratio between the absolute complex activity ( × 100) and citrate synthase activity. Shadowed squares denote 95% CI for the mean of control values (nonsmoker individuals). P values under the bars refer to the comparisons between each measure in smoker individuals and the control values (unpaired t test), while p values at the right of each graphic refer to the comparisons of the three measures performed in the smoker individuals along the time (general lineal model for repeated measures). NS: not significant.

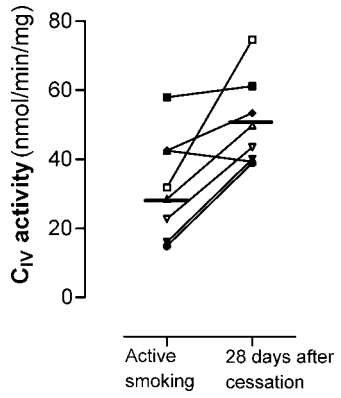


*Figure 2.* Initial and final complex IV absolute activities for each individual patient. Bars denote the means.

smoking and exhibits a progressive, statistically significant increase coinciding with smoking cessation. These changes in MRC complexes seem to be accompanied of a decrease in spontaneous oxidative cellular capacity, while oxidation of selected substrates remains unaltered. In whole, we consider that these findings add further evidence that MRC is an additional target of cigarette smoke.

Our data agree with most of the previously reported investigations in animal models. Some in vivo and in vitro studies on animal mitochondria have demonstrated that complex IV is inhibited by inhaling cigarette smoke, and that there is a close relationship with the length of smoke exposure (8,15–17). In these animal models, oxidative capacity of mitochondria resulted a decrease in substrates that entered complex I (17–19), II (17,18) or IV (19) level. Gairola et al. (20), based on the spectroscopic measurement of the cytochromes in their reduced state, proposed that multiple cytochrome constituents should be involved in the interaction of whole cigarette smoke and intact mitochondria.

However, Örlander et al. (21,22) and ourselves (7) in humans have consistently demonstrated that complex IV inhibition is the main alteration in chronic smokers. Similarly, Smith et al. (6) studied the activity of complexes of MRC in mitochondria obtained from platelets of chronic smokers and found 24% and 8% decrease in complex I and IV activities, respectively, although the inhibition was statistically significant only for the former. Therefore, different complexes of MRC seem to be inhibited in chronic smokers, and we believe that the differences observed could depend on the specific tissue involved. Similar findings among tissues have been described in some mitochondria-related neurodegenerative diseases (23,24).

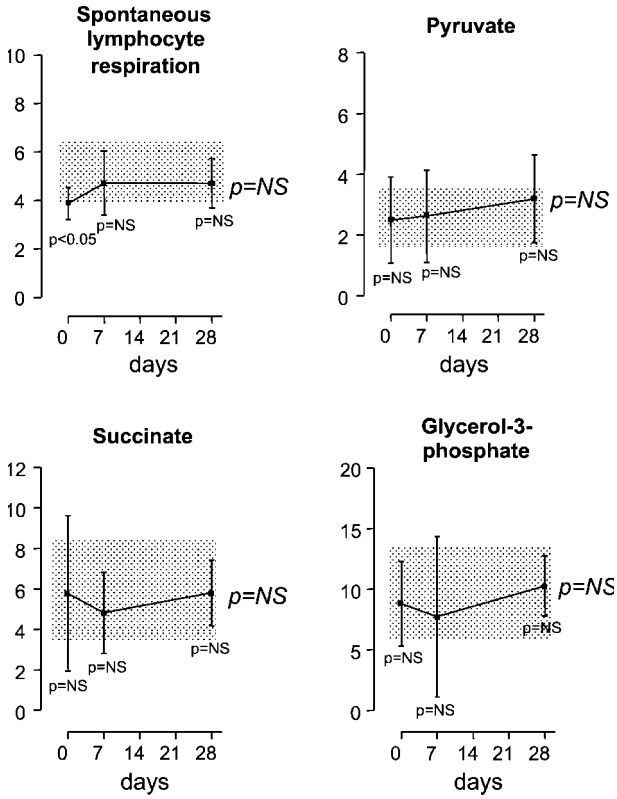


*Figure 3.* Relative oxidative activities (means with 95% CI) of smokers before (day 0) and after stopping (days 7 and 28) to smoke. There are no units for the Y axis because they represent the ratio between the absolute oxidative activity ( × 100) and citrate synthase activity. Shadowed squares denote 95% CI for the mean of control values. P values under the bars refer to the comparisons between each measure in smoker individuals and the control values (unpaired t test), while p values at the right of each graphic refer to the comparisons of the three measures performed in the smoker individuals along the time (general lineal model for repeated measures). NS: not significant.

The most interesting finding in our study is the reversibility of complex IV inhibition after smoking cessation. However, the ultimate significance of this complex IV inhibition induced by smoking, although reversible, has not yet been determined. In fact, long-term inhibition of complex IV activity could lead to a MRC dysfunction, decreased ATP generation, enhanced ROS production, DNA damage (25), and eventually enhanced apoptosis (26) or abnormal cell proliferation (27). Therefore, reversibility of the MRC dysfunction after smoking cessation, even after many years of heavy smoking habit, does not necessarily imply that long-term damage can be avoided and all those persistent changes could play a role in some smoking-related diseases, as

can be seen in clinical practice when patients develop cancer or progressive lung diseases even many years after stopping the smoking habit. To support this hypothesis, we have also demonstrated an increase in oxidative damage of lymphocyte membranes of chronic smokers (7), which additionally could potentiate MRC dysfunction at different levels leading to greater cellular damage as part of a vicious circle.

The exact mechanism for complex IV inhibition, the most consistent finding, is a matter of debate. It has been suggested that CO or other of the thousands of molecules contained in cigarette smoke could contribute to such an inhibition. In experimental studies on animals CO has been largely recognized as a competitive inhibitor of complex IV (7,28,19). Although direct toxicity on complex IV has not been definitively confirmed in humans, this hypothesis seems very likely since we have repeatedly observed that the activity of complex IV is severely depressed in patients acutely poisoned by CO, and that this activity slowly recuperates after removing the patient from the CO exposure (30,31). In addition, in an in vitro model we have recently found that complex IV of human muscle mitochondria is inhibited by progressive concentrations of CO (32).

In conclusion, although the inhibition of mitochondrial complex IV activity in chronic smokers is reversible, long-term inhibition of MRC function could be involved in the development of multiorganic alterations even after smoking cessation.

## ACKNOWLEDGMENTS

We thank Anna Beato for her valuable technical support to carry out experimental studies, and Dolors Marin (Tobacco Deshabituation Unit) for providing us the individuals of the study. This study has been supported in part by grants PGC PM99-0038 of Ministerio de Educación y Cultura, FIPSE 3102-01 and 2001/SGR/00379. Jose R. Alonso was a recipient of a grant from Hospital Clínic of Barcelona (Premi Fi de Residència 1999).

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*PHARMACOLOGY AND TOXICOLOGY*

2003 Sep;93:142-146, ORIG

Josep R Alonso, Francesc Cardellach, **SÒNIA LÓPEZ**, Jordi Casademont, Òscar Miró.

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**Pharmacology & Toxicology**
*ISSN 0901-9928*

# Carbon Monoxide Specifically Inhibits Cytochrome C Oxidase of Human Mitochondrial Respiratory Chain

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(Received December 18, 2002; Accepted Jun 16, 2003)

*Abstract:* Carbon monoxide (CO) toxicity is the result of a combination of tissue hypoxia and direct CO-mediated damage at a cellular level, since not all the signs and symptoms presented can be explained only by the formation of carboxyhaemoglobin. Mitochondria, specially the electron transport chain, seem to be the target for CO at a subcellular level. However, the direct effect of CO in individual complexes of the human mitochondrial respiratory chain has not been completely elucidated. We here studied the *in vitro* effect of CO on individual complexes of the mitochondrial respiratory chain of human mitochondria. We obtained muscle tissue from 10 healthy people who underwent orthopaedic surgery for hip replacement. Isolated mitochondria were incubated for 5 min. under CO concentrations of 50, 100 and 500 ppm. Afterwards, enzymatic activities of individual complexes of the mitochondrial respiratory chain were assessed *in vitro* and compared with those obtained in basal (synthetic air without CO) conditions. Cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain) activity showed a decrease from 836±439 nmol/min./mg of mitochondrial protein after air incubation to 670±401, 483±182, and 379±131 nmol/min./mg after 50, 100 and 500 ppm of CO incubation, respectively (20%, 42% and 55% decrease in cytochrome *c* oxidase activity). This gradual decrease in cytochrome *c* oxidase was found to be statistically significant ($P<0.001$). Other complex activities showed no any significant variation. Carbon monoxide is toxic for mitochondria in man, altering the mitochondrial respiratory chain at the cytochrome *c* oxidase level. This inhibition in cytochrome *c* oxidase may play a role in the development of the symptoms observed in acute CO poisoning, and in some diseases related to smoking.

Carbon monoxide (CO) is a colourless, odourless and non-irritant toxic gas produced by the incomplete combustion of hydrocarbons. Although a very modest amount of CO is normaly produced in man by the catabolism of haemoglobin, larger amounts absorbed from exogenous sources can lead to poisoning and eventually death (Myers 1990; Horner 2000). Clinical signs and symptoms of acute CO poisoning are not specific, but include tachycardia, tachypnoea, headache, nausea, vomiting, dizziness, weakness, difficulty in concentration or confusion, visual changes, syncope, seizures, abdominal pain and muscle cramping (Ernst & Zibrak 1998).

The pathophysiology of CO toxicity appears to be the result of a combination of tissue hypoxia and direct CO-mediated damage at a cellular level. Haemoglobin has a high affinity for CO, leading to formation of carboxyhaemoglobin (Rodkey *et al.* 1974). Hypoxia is due to the formation of carboxyhaemoglobin that reduces the transport of oxygen and impairs the release of oxygen from oxyhaemoglobin in tissues (Roughton & Darling 1944). Since there is no precise relationship between carboxyhaemoglobin values and the symptoms presented (Coburn 1979; Hardy & Thom 1994), other pathophysiological mechanisms for CO toxicity have been suggested, such as reoxygenation injury, lipid peroxidation and oxidative stress (Thom 1990; Zhang & Piantadosi 1992), as well as binding to cellular proteins including myoglobin and cytochromes (Coburn 1979; Zhang & Piantadosi 1992). In this sense, we have reported a marked and sustained inhibition of cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain) activity in human lymphocytes after acute CO poisoning (Miró *et al.* 1998a).

Tobacco smoke is an important source of CO. Accordingly, the more a person smokes, the higher the carboxyhaemoglobin levels (Puente-Maestu *et al.* 1998), with known adverse effects on oxygen transportation by haemoglobin (McDonough & Moffatt 1999). However, the direct effects of smoke CO at a cellular level and its implication in diseases to related smoking remain to be completely elucidated. Similar to other investigators we have found an inhibition in mitochondrial respiratory chain function, mainly on mitochondrial cytochrome *c* oxidase, due to tobacco smoke (Örlander *et al.* 1979; Gvozdjáková *et al.* 1984; Larsson & Örlander 1984; Gvozdjak *et al.* 1987; Pryor *et al.* 1992; Miró *et al.* 1999). These findings suggest that the CO of tobacco smoke itself might play a role in this mitochondrial dysfunction.

Although CO is known to be toxic to mitochondria (Coburn 1979), only a few studies have evaluated its pathogenic mechanism at a subcellular level in animal models (Piantadosi *et al.* 1985; Brown & Piantadosi 1990; Zhang & Pianta-

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dosi 1992), and confirmation in man has not been completely achieved. In addition, only a few studies based on CO poisoning have ruled out the effect of the coexisting hypoxia (Haab 1990), and studies with smokers have not excluded the participation of other substances in the observed mitochondrial effect.

Our aim was to investigate the specific effect of CO itself on the mitochondrial respiratory chain of human mitochondria in the absence of any confounding factor.

## Material and Methods

*Patients*. We obtained muscle samples from the quadriceps muscle (vastus lateralis) from patients submitted to orthopaedic surgery (hip prothesis placement) under regional anaesthesia (Miró *et al.* 1999). We excluded patients with a previous diagnosis of neuromuscular disease and/or familial history of neuromuscular disorder. All the individuals were similar in age and physical activity and all were non-smokers in order to avoid factors that have been considered to be confounding variables in studies on mitochondrial function (Cardellach *et al.* 1989; Smith *et al.* 1993; Boffoli *et al.* 1994; Barrientos *et al*. 1996). Non-smokers were considered to be individuals that had never smoked or had abstained from smoking during the previous year. We included only persons who walked on the street every day, following the Steinbrocker classification (Steinbrocker *et al.* 1949). The subjects were informed about the study protocol and all provided written informed consent to participate. The study was approved by the Ethics Committee of our hospital. A total of 10 persons were included in the study.

*Obtaining mitochondria*. The muscle samples were obtained at the begining of the orthopaedic procedure and placed into media with 20 mM Tris (pH 7.2), 250 mM sucrose, 40 mM KCl, 2 mM EGTA and 1 mg/ml of bovine serum albumin (BSA) at 4°, and processed in less than 5 min.

Muscle mitochondria were isolated using a standard methodology employed in our laboratory (Cardellach *et al.* 1991 & 1992), slightly modified according to Rustin *et al.* (1994) for minute amounts of human skeletal muscle, as previously reported (Miró *et al*. 1998a, b & c). The pellet of crude mitochondria was resuspended in media with 20 mM Tris (pH 7.2), 250 mM sucrose, 40 mM KCl and 2 mM EGTA. All the steps were carried out at 4°. Protein concentration was determined by the Bradford method (Bradford 1976). The approximate time between skeletal muscle biopsy and mitochondrial isolation (ready to start biochemical assays) was about 45 min. Spectrophotometric studies were carried out immediately afterwards.

*Biochemical studies*. According to different conversion charts of CO in exhaled air (COEA) to carboxyhaemoglobin and data from smokers, allowed professional exposures to CO, and patients with CO poisoning, synthetic air ($N_2$ 79% $O_2$ 21% CO 0 ppm) was used as the gas control, and concentrations of 50 ppm, 100 ppm and 500 ppm CO (Linde Abelló®, Barcelona, Spain) for the determinations.

Samples were incubated for 5 min. at 37° under these different gas mixtures of CO-enriched air. Measurement of the specific activity of the following complexes of the respiratory chain was performed by spectrophotometry (UVIKON 922, Kontron, Zurich, Switzerland): NADH-ubiquinone reductase (complex I), succinate-ubiquinone reductase (complex II), ubiquinol-cytochrome *c* reductase (complex III), and cytochrome *c* oxidase (complex IV). A

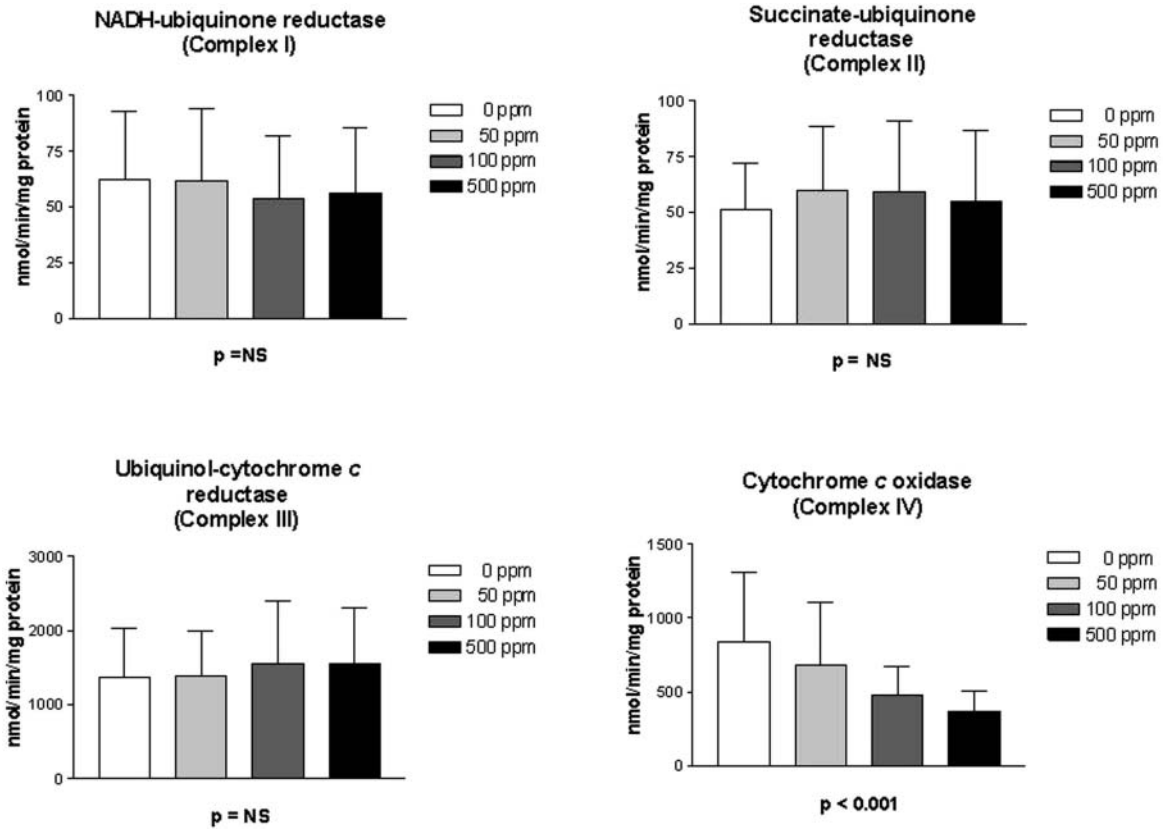


Fig. 1. Absolute enzyme activities.

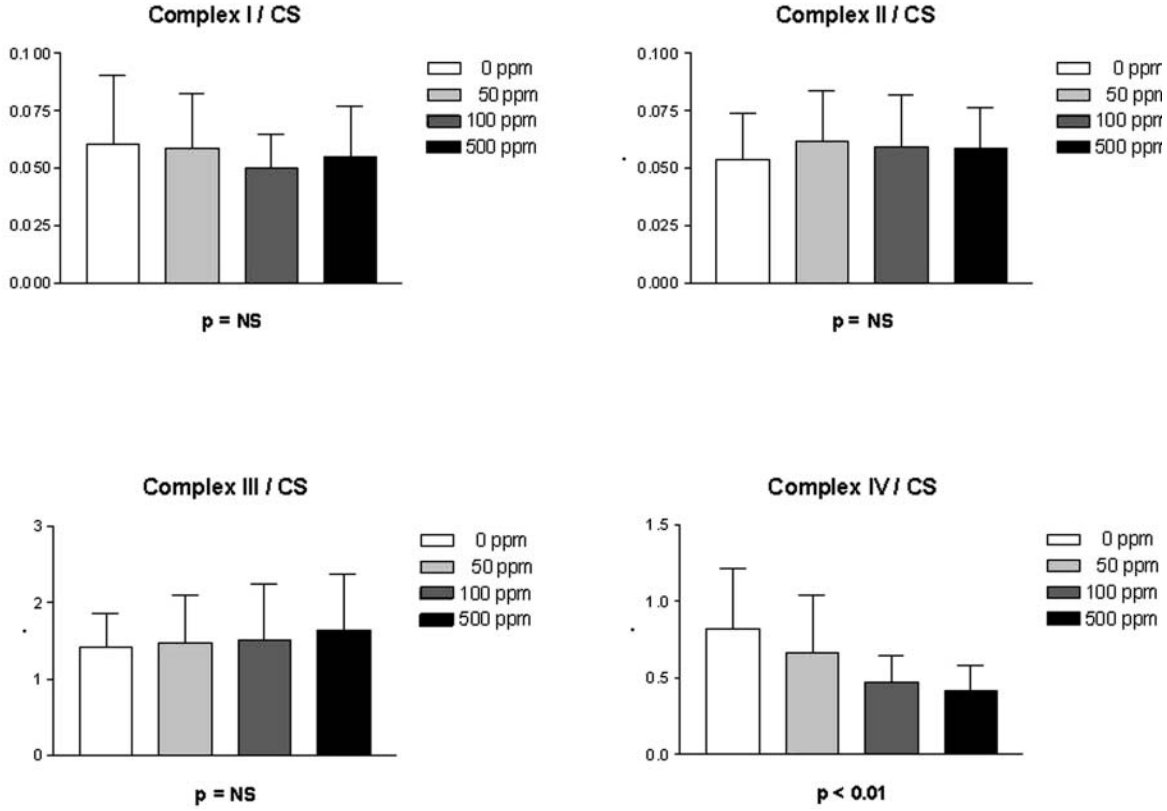


Fig. 2. Relative enzyme activities.

total of 10–40 µg of mitochondrial protein was required to determine the activity of each complex, except for complex IV, for which 2–4 µg was used (Miró *et al*. 1998a, b & c). Assays were performed at 37° in a total volume of 1 ml according to Chretien *et al.* (1994) and Rustin *et al.* (1994). Absolute enzyme activities were expressed as nmols of reduced or oxidized substate/min./mg of mitochondrial protein. Relative enzyme activities were presented as ratios related to cytrate synthase activity (Rustin *et al.* 1994).

*Statistical analyses*. Data were processed using a SPSS software. Results from quantitative variables were expressed as mean± standard deviation. We used one-way ANOVA for repeated measures to determine differences among them, and checked for a linear trend using a polynomial contrast of first degree. Values of $P<0.05$ were considered as statistically significant.

## Results

Biopsies were obtained from 6 men and 4 women. Similar to previous studies (Miró *et al.* 1999) we did not find gender differences in enzyme activities (results not shown). Fig. 1 shows the activity of the mitochondrial respiratory chain enzymes after exposure to the different CO concentrations. Increased concentrations of CO showed a progressive and significant decrease in cytochrome *c* oxidase activity ($P<0.001$) with no changes being observed in other complex enzyme activities. Basal activity was 836±439 nmol/min./mg of mitochondrial protein, which decreased to 670±401 nmol/min./mg of mitochondrial protein after CO incubation at 50 ppm, to 483±182 nmol/min./mg of mitochondrial protein at 100 ppm, and lastly to 379±131 nmol/min./mg of mitochondrial protein at 500 ppm, representing a decrease of 20%, 42% and 55% respectively in cytochrome *c* oxidase activity.

After correcting absolute enzyme rates by citrate synthase activity in order to obtain their relative activities, again only cytochrome *c* oxidase (complex IV) activity showed the same pattern of inhibition associated with increasing exposures of CO concentrations ($P<0.05$) (fig. 2).

## Discussion

The present study reports the effects of exposures to increased CO concentrations on mitochondrial respiratory chain components of human mitochondria. We incubated aliquots of different concentrations of CO following standard procedures for gas exposure. According to Henry's law, incubation media were saturated with each gas mixture. Thus, it was assumed that the samples had absorbed sufficient CO to detect any acute potential effect arising from CO exposure in the studied parameters. On the other hand, muscle samples were obtained from persons during similar physical activity, and illnesses that could interfere with mitochondria activity were excluded by evaluation of medical history, physical examination and routine blood analyses.

We observed a significant, progressive inhibition in cytochrome *c* oxidase activity parallel to increased concen-

trations of CO in each medium while other enzyme activities did not show any change in relation to the presence of CO. Cytochrome *c* oxidase inhibition by CO could explain previously reported findings on mitochondrial function in animals inhaling tobacco smoke (Gvozdjáková *et al.* 1984), smokers (Örlander *et al.* 1979; Larsson & Örlander 1984; Miró *et al.* 1999) and patients with acute CO poisoning (Miró *et al.* 1998a, b & c). Since oxygen was always present and no other toxic substance was added in the media, this inhibition may be explained by a reduction of prosthetic group cytochrome $a_3$ of cytochrome *c* oxidase by CO (Ellis *et al.* 1986).

The progressive inhibition of cytochrome *c* oxidase activity by increased exposures of CO concentrations may produce an overall decrease in mitochondrial respiratory chain function. Such a disturbance of mitochondrial respiratory chain function could lead to a decay in mitochondria energy production and, eventually, to cellular injury or dysfunction, especially in "high metabolic rate" tissues exposed to CO such as the central nervous system, heart muscle or skeletal muscle. Indeed, muscular weakness and impaired heart function have been reported and demonstrated in CO poisoning (Tritapepe *et al.* 1998; Raub *et al.* 2000; Yanir *et al.* 2002) and in heavy smokers (Örlander *et al.* 1979; Gvozdjakova *et al.* 1984). On the other hand, the disturbance in mitochondrial respiratory chain function may also lead to a major production of reactive oxygen species (Cadenas & Davis 2000). Our results support previously reported data that suggest a higher oxidative stress on cells after CO exposure which may explain some of the clinical features of CO poisoning not only due to increased carboxyhaemoglobin levels, i.e. delayed neuropsychiatric syndrome (Thom 1993). In fact, repeated sessions of hyperbaric oxygenotherapy in patients with nearly normal carboxyhaemoglobin seem to reduce the incidence of the syndrome, indicating that subcellular effects of CO should be taken into account in the pathophysiology of this syndrome (Weaver *et al.* 2002; Cardellach *et al.* 2003).

There are some limitations in establishing an exact correlation between CO levels used in *in vitro* studies and the clinical symptoms of acute CO poisoning *in vivo*. Carbon monoxide absortion depends on minute ventilation, relative $CO/O_2$ concentrations in the environment and the length of exposure (Forbes *et al.* 1945). On the other hand, after being absorbed, CO distribution depends on tissue perfusion, as any substance in the blood-stream. These two factors make it impossible to correlate *in vitro* CO concentrations with similar concentrations in blood. Moreover, tissue sensitivity differs according to its metabolic rate (Ray 1997), and there is always a certain degree of hypoxia because of carboxyhaemoglobin which may magnify the effects of CO. Finally, CO and hypoxia can lead to cell death, and thus, irreversible tissue damage.

In conclusion, we demonstrate that CO by itself is toxic to human mitochondria and alters the mitochondrial respiratory chain at the cytochrome *c* oxidase level. This inhibition may play a role in some CO-related alterations explained not only by hypoxia, such as acute CO poisoning, and diseases to related smoking. Our data on mitochondrial respiratory chain dysfunction by CO agree with previous findings by our group and those of others, but no quantitative correlations may be made because of the design of the study. In spite of these limitations, we believe that our findings contribute to the understanding of the pathogenic mechanisms for the clinical features involved in these situations. However, the severity of the clinical manifestations depends not only on the degree of poisoning but also on the presence of other factors such as tissue hypoxia and previous tissue function, i.e. underlying illness.

Acknowledgements

We thank Anna Beato for her valuable technical support in experimental studies, and the Traumatology Department of our hospital for providing us with the participants in the study. This study has been supported in part by grant PGC PM99-0038 of Ministerio de Educación y Cultura. Jose-R. Alonso was a recipient of a grant from the Hospital Clínic of Barcelona (Premi Fi de Residència 1999).

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ANÁLISIS *EX VIVO* DE LA FUNCIÓN MITOCONDRIAL EN PACIENTES INTOXICADOS POR MONÓXIDO DE CARBONO ATENDIDOS EN URGENCIAS

*MEDICINA CLÍNICA (BARCELONA)*

2004 Mar 27;122(11):401-6, ORIG

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# Análisis *ex vivo* de la función mitocondrial en pacientes intoxicados por monóxido de carbono atendidos en urgencias

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Artículo 92.648

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FUNDAMENTO Y OBJETIVO: **Diversos estudios experimentales en animales han demostrado que el monóxido de carbono (CO) tiene capacidad para unirse al complejo IV de la cadena respiratoria mitocondrial (CRM) y distorsionar el funcionalismo mitocondrial. Sin embargo, se desconoce si esta circunstancia se produce en la práctica clínica diaria de los pacientes que acuden a urgencias tras haber sufrido una intoxicación aguda por CO. El objetivo de este trabajo fue evaluar desde diferentes perspectivas si existe algún tipo de disfunción mitocondrial en los pacientes que han sufrido una intoxicación aguda por CO.**
PACIENTES Y MÉTODO: **Se incluyeron 10 pacientes que acudieron a urgencias tras sufrir una intoxicación aguda por CO (carboxihemoglobina inicial, 20,4 [6]%). Siete de ellos recibieron tratamiento con oxigenoterapia hiperbárica. A todos ellos se les extrajo 20 ml de sangre durante la fase aguda de la intoxicación, a los 3-5 días y 10-14 días del episodio agudo. A partir de estas muestras se aislaron linfocitos para realizar los estudios mitocondriales, que consistieron en la determinación del contenido mitocondrial a través de la actividad de la citrato sintasa (nmol/min/mg proteína), la actividad enzimática (nmol/min/mg proteína) de los complejos III y IV de la CRM (ambos contienen citocromos), la actividad oxidativa (nmol oxígeno consumido/min/mg proteína) espontánea y estimulada mediante la administración de glutamato y succinato, y la cuantificación de la peroxidación lipídica utilizando ácido cis-parinárico. Estos parámetros se cuantificaron tanto por célula (valores absolutos) como por mitocondria (valores relativos). Los resultados se compararon con los valores control históricos de nuestro laboratorio procedentes de 130 individuos.**
RESULTADOS: **Durante la fase aguda de la intoxicación no se observaron cambios en el contenido mitocondrial de los pacientes con respecto al grupo control, pero sí una inhibición significativa de la actividad enzimática de los complejos III y IV de la CRM asociada a un descenso de todas las actividades oxidativas, tanto si la estimación se hacía por célula como si se hacía por organela (mitocondria). Aunque todas estas actividades se recuperaron a lo largo del tiempo ($t_1$ y $t_2$), sólo en el caso del complejo IV y de la actividad oxidativa estimulada por glutamato dicha recuperación resultó estadísticamente significativa. No obstante, ni siquiera en estos casos las actividades finales alcanzaron los valores control. Aunque se observó una tendencia al incremento de la peroxidación lipídica, este aumento no alcanzó significación estadística.**
CONCLUSIONES: **En el presente estudio confirmamos *ex vivo* la existencia de una inhibición de la actividad de la CRM en pacientes intoxicados por CO que consultan a un servicio de urgencias. Dicha disfunción continúa siendo detectable transcurridos 14 días del episodio agudo. Esta inhibición del funcionalismo mitocondrial podría desempeñar algún papel patogénico en los síntomas y signos tardíos que en ocasiones presentan estos enfermos.**

*Palabras clave:* Monóxido de carbono. Intoxicación. Mitocondria. Cadena respiratoria. Urgencias.

## Analysis *ex vivo* of mitochondrial function in patients attended in an emergency department due to carbon monoxide poisoning

BACKGROUND AND OBJECTIVE: **Many experimental studies in animals have demonstrated that carbon monoxide (CO) has the ability to bind to complex IV of the mitochondrial respiratory chain (MRC) inhibiting its function. It is unknown, however, if this situation is also present in patients who are admitted to an emergency department because of acute CO poisoning. The objective of this study was to evaluate from different points of view whether or not mitochondrial function is abnormal in patients admitted because of an acute CO poisoning.**
PATIENTS AND METHOD: **Ten patients with an acute CO poisoning admitted in an emergency department were included in the study. Initial carboxyhemoglobin was 20.4 (6)%. Seven of these patients received hyperbaric-oxygen therapy. In all the patients, lymphocytes from 20 mL of blood were obtained at admission ($t_0$), and at days 3-5 ($t_1$), and 10-14 ($t_2$). Mitochondrial content was estimated through citrate synthase activity (nmol/min/mg protein). Enzymatic activity of complexes III and IV (both containing cytochromes) as well as oxidative activities were measured. Lipid peroxidation was ascertained by means of cis-parinaric acid fluorescence. All the values were given as absolute values, and were corrected according to the mitochondrial content (relative values). The results were compared with the control values obtained from 130 historical normal individuals.**
RESULTS: **During acute poisoning ($t_0$), there were no changes in mitochondrial content. On the other hand, there was a significant inhibition of the enzymatic activity of complexes III and IV, and a decrease in all oxidative activities, considering both absolute and relative values. Although all the activities showed a trend to recuperation with time ($t_1$ y $t_2$), statistical significance was only observed for complex IV and for the oxydative activity stimulated with glutamate.**
CONCLUSIONS: **In the present study we confirm that an inhibition of the MRC can be demonstrated *ex vivo* in patients attended in an emergency department due to acute CO poisoning. The inhibition is still present 14 days after the acute event. This mitochondrial dysfunction may play a pathogenic role in the persisting or delayed sings and symptoms that these patients occasionally refer.**

*Key words:* Carbon monoxide. Poisoning. Mitochondrial. Respiratory chain. Emergency.

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El presente trabajo ha sido galardonado durante el XV Congreso Nacional de Medicina de Urgencias y Emergencias (Valencia, 11-14 de junio de 2003) con el Primer Premio de Investigación Clínica en Toxicología Clínica patrocinado por Laboratorios Lainco S.A.

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Recibido el 26-6-2003; aceptado para su publicación el 19-9-2003.

Se calcula que la intoxicación aguda por monóxido de carbono (CO) tiene una incidencia en los países desarrollados de 17,5 casos por cada 100.000 habitantes y es aún hoy un diagnóstico poco reconocido e infradiagnosticado en los servicios de urgencias[1]. Esto es así a pesar de que la intoxicación por CO continúa siendo en muchos de estos países, incluyendo España, la primera causa de muerte por intoxicación[2-4]. En los casos fatales, la muerte sobreviene por la hipoxia tisular que produce la unión reversible y competitiva del CO al grupo hemo de la hemoglobina, de donde desplaza al oxígeno. Así, cuando lo habitual es que los valores de carboxihemoglobina (COHb) se sitúen por debajo del 3% en personas no fumadoras, cuando éstos alcanzan el 50% se considera que la muerte se produce de manera prácticamente indefectible[5]. En cambio, cuando el grado de intoxicación por CO no es de una magnitud suficiente para causar el fallecimiento del individuo, la unión del CO a la hemoglobina es revertida con el paso de las horas (el tiempo de vida media de dicha unión se sitúa entre 5 y 6 h). La rapidez de este proceso de reversibilidad puede verse acelerado por la administración de oxígeno a una concentración del 100% y/o a una presión superior a la atmosférica, las cuales son las principales medidas terapéuticas a administrar en los casos atendidos en urgencias[6].

Sin embargo, a pesar de que a la mayoría de los pacientes que han sufrido una intoxicación aguda no mortal por CO se les puede dar de alta de los servicios de urgencias a las pocas horas, una proporción de ellos puede desarrollar secuelas neurológicas, especialmente cognitivas, que bien están presentes ya en ese momento, bien aparecen o se intensifican durante los días o semanas posteriores a la intoxicación. Aunque con menor frecuencia, esto se observa incluso en pacientes que han sufrido una intoxicación moderada por CO (valores de COHb entre el 20 y el 30%) y es por ello que recientemente se ha defendido la utilización de la oxigenoterapia hiperbárica en ellos con objeto de prevenir el desarrollo de este síndrome neurológico tardío[7].

La explicación fisiopatológica de este síndrome aún no se conoce bien. Entre los mecanismos que se cree podrían desem-

TABLA 1

**Principales características clínicas de los pacientes incluidos en el estudio**

| | Intoxicados por CO (n = 10) | Controles (n = 130) |
|---|---|---|
| Edad (años)* | 39 (19) | 37 (12) |
| Sexo (mujer) | 40% | 35% |
| Fumador habitual | 40% | 44% |
| Valores de carboxihemoglobina* | | |
| $t_0$ | 20,4 (6,0)% | 2,2 (2,5%)** |
| $t_1$ | 1,7 (0,9)% | NP |
| $t_2$ | 1,6 (1,2)% | NP |
| Síntomas agudos | | |
| Disnea | 40% | NP |
| Náuseas | 60% | NP |
| Cefalea | 100% | NP |
| Torpor mental | 80% | NP |
| Debilidad | 70% | NP |
| Inestabilidad | 30% | NP |
| Ataxia | 20% | NP |
| Síntomas crónicos | | |
| Disnea | 0% | NP |
| Náuseas | 30% | NP |
| Cefalea | 30% | NP |
| Torpor mental | 10% | NP |
| Debilidad | 10% | NP |
| Inestabilidad | 0% | NP |
| Ataxia | 0% | NP |
| Tratamiento hiperbárico | 70% | NP |

CO: monóxido de carbono; $t_0$: intoxicación aguda; $t_1$: 3-5 días tras la intoxicación aguda; $t_2$: 10-14 días tras la intoxicación aguda; NP: no procede. *Se indica la media (error estándar); **p < 0,001.

peñar algún papel se encuentra la citotoxicidad causada por la unión del CO a otras moléculas del organismo que, aparte de la hemoglobina, contienen el grupo hemo. Entre ellas, se encuentran la mioglobina y los citocromos y, entre estos últimos, los citocromos $bc_1$ y $aa_3$, que forman parte de algunos de los complejos proteínicos que integran la cadena respiratoria mitocondrial (CRM) (fig. 1). Esta CRM es la encargada del transporte de electrones, generados en diferentes vías catabólicas, con la finalidad última de aprovechar la energía de este transporte altamente exergónico para la síntesis de adenosín trifosfato. Es fácil intuir que la interrupción del normal funcionamiento de esta CRM por la acción del CO pudiera ocasionar un déficit energético celular y un incremento de las reacciones oxidativas intracelulares y contribuir, en última instancia, a la disfunción tisular y a la aparición de síntomas y signos. No obstante, debido a la inaccesibilidad del tejido diana (sistema nervioso central) para realizar estudios mitocondriales en los individuos intoxicados por CO, esta hipótesis sólo se ha podido contrastar en modelos experimentales animales. De los resultados de estos estudios se desprende que la hipótesis del daño mitocondrial como mecanismo efector para la aparición de signos y síntomas tardíos tras la intoxicación por CO es muy plausible[8-12]. Durante los últimos años nuestro grupo ha iniciado una serie de aproximaciones para intentar contrastar esta teoría en humanos. Para ello, se ha estudiado el funcionalismo mitocondrial de linfocitos de sangre periférica de pacientes intoxicados de forma aguda por CO que han acudido al servicio de urgencias. Dado que los resultados preliminares han mostrado que los linfocitos de sangre periférica pueden constituir un buen modelo para el análisis de los efectos toxicológicos del CO[13,14], se diseñó el presente estudio con la finalidad de realizar una valoración integral de los efectos que produce el CO sobre la CRM en la práctica clínica no experimental. Se parte de la hipótesis de que en pacientes intoxicados de forma aguda por CO es posible detectar una inhibición de la actividad de los complejos de la CRM que contienen citocromos, y que esta inhibición tiene repercusión en el funcionalismo global de dicha CRM e incluso puede llegar a causar un incremento del daño oxidativo.

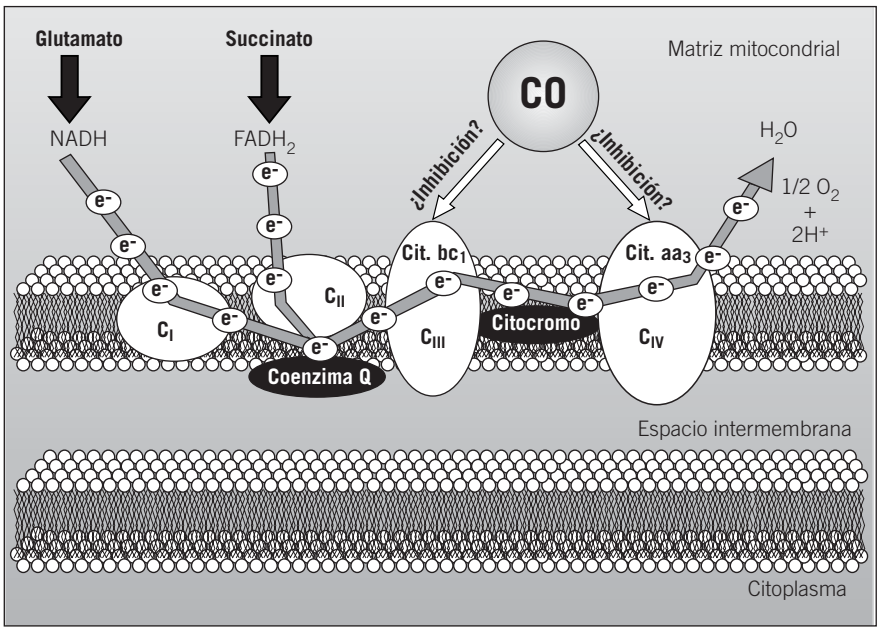


*Fig. 1. Esquema de la cadena respiratoria mitocondrial, la cual se encuentra integrada por 4 complejos proteicos multienzimáticos ($C_I$: complejo I; $C_{II}$: complejo II; $C_{III}$: complejo III; $C_{IV}$: complejo IV) y 2 transportadores móviles de electrones (coenzima Q y citocromo c). Glutamato y succinato son 2 sustratos que se utilizan en el estudio de la actividad oxidativa mitocondrial y que actúan como dadores de electrones en los puntos que muestra el esquema. El resultado final que se registra en los estudios polarográficos es el consumo de oxígeno asociado a la administración de los mencionados sustratos. Las abreviaturas cit. $bc_1$ y cit. $aa_3$ corresponden a los citocromos $bc_1$ y $aa_3$, respectivamente, y son los lugares donde podría unirse el monóxido de carbono (CO).*

## Pacientes y método

Se incluyeron en el estudio 10 pacientes que acudieron al servicio de urgencias con síntomas derivados de la exposición aguda al CO y valores de COHb superiores al 10%. Se excluyó a los que padecían otras enfermedades o que estuviesen recibiendo fármacos. Las características clínicas de los pacientes se recogen en la tabla 1. En todos los casos la exposición a CO podía considerarse «pura», ya que la fuente de exposición consistía en sistemas de calefacción o calentadores con combustión incompleta por una ventilación insuficiente. En 7 de estos pacientes había criterios suficientes para indicar oxigenoterapia hiperbárica para completar el tratamiento, y así se hizo. A todos estos pacientes se les extrajo, antes de recibir tratamiento, 20 ml de sangre venosa para la realización de los estudios mitocondriales que se detallan más adelante. El Comité Ético de Investigación de nuestro centro aprobó el protocolo y se obtuvo el consentimiento informado de los pacientes incluidos.

El diseño del presente estudio contemplaba 2 fases diferenciadas. En la primera, transversal, los resultados obtenidos en la fase aguda de la intoxicación por CO ($t_0$) se compararon con los de un grupo control formado por 130 individuos sin enfermedad conocida que habían realizado una donación voluntaria de sangre, los cuales constituyen los valores de referencia históricos de nuestro laboratorio. La segunda fase del diseño, longitudinal, consistió en la repetición de los estudios mitocondriales a los pacientes intoxicados por CO al cabo de 3 a 5 días ($t_1$) y de 10 a 14 días ($t_2$) de haber sido tratados y dados de alta.

El aislamiento de linfocitos se realizó mediante centrifugación en un gradiente de densidad de Ficoll (Histopaque®-1077, Sigma Diagnostics, St. Louis MO63178,

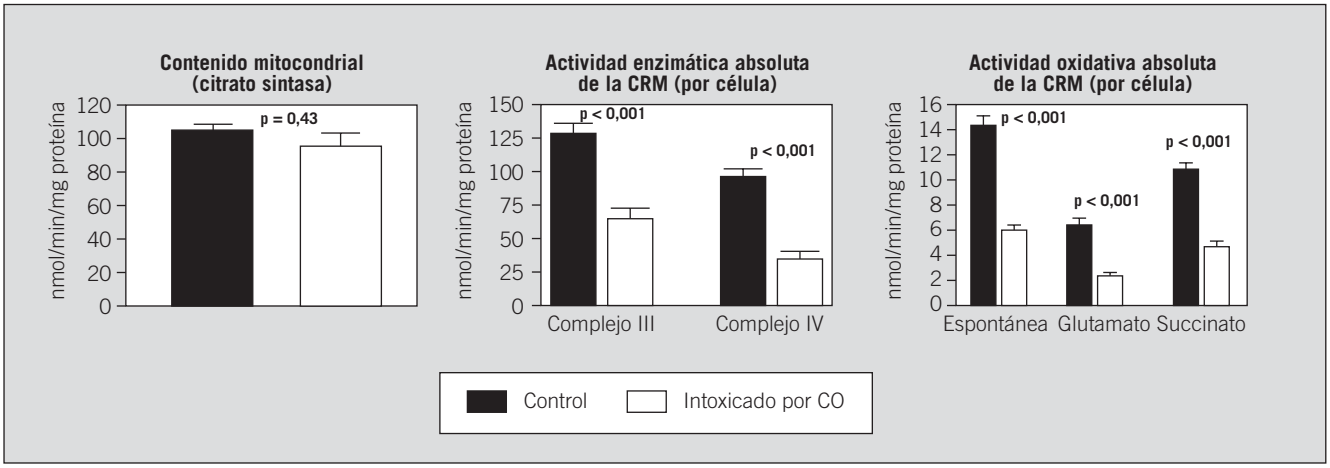


*Fig. 2. Resultados –media (error estándar)– obtenidos en los controles y en los pacientes en el momento agudo de intoxicación por monóxido de carbono (CO) por lo que respecta a contenido mitocondrial (izquierda), actividad enzimática (centro) y actividad oxidativa (derecha). Los resultados se expresan como actividades absolutas (por célula). CRM: cadena respiratoria mitocondrial.*

EE.UU.). Los linfocitos así obtenidos se resuspendieron en 100-150 µl de solución salina tamponada con fosfato y la concentración de proteína celular se determinó siguiendo el principio de unión colorante-proteína propuesto por Bradford[15].

El cálculo del contenido mitocondrial se realizó mediante la determinación espectrofotométrica (UVIKON 922, Kontron®, Suiza) a 412 nm de la actividad de la citrato sintasa (Enzyme-Code [EC]: 4.1.3.7), que es una enzima del ciclo de Krebs que se encuentra en la matriz mitocondrial y cuya concentración permanece altamente constante en el interior de la mitocondria. Por dicho motivo, diversos autores consideran que su actividad es un buen marcador del contenido mitocondrial de una célula o tejido[16,17].

Mediante espectrofotometría se determinó la actividad de los complejos III (EC 1.10.2.2, el cual contiene citocromo $bc_1$) y IV (EC 1.9.3.1, el cual contiene citocromo $aa_3$) de la CRM. Los experimentos se realizaron a 37 ºC siguiendo el método propuesto por Rustin et al[17], ligeramente modificado para el complejo IV[18]. Los resultados obtenidos en cuanto a actividad absoluta (determinada en nmol/min/mg proteína) reflejan la actividad enzimática por célula. También se calcularon las actividades relativas de estos complejos dividiendo por la actividad de la citrato sintasa, las cuales son un reflejo de las actividades enzimáticas por organela (mitocondria).

La actividad oxidativa se determinó mediante polarografía (medida en nmol oxígeno consumido/min/mg proteína) con un electrodo de Clark a 37 ºC (Hansatech Instruments Limited®, Norfolk, Reino Unido). En condiciones experimentales previamente definidas[16,19] se midió el consumo de oxígeno (respiración espontánea) de los linfocitos intactos, y posteriormente el consumo de oxígeno obtenido tras permeabilizar la membrana celular con digitonina al 1% y añadir glutamato-malato (sustrato que cede electrones al complejo I) y succinato (sustrato que cede electrones al complejo II). Mediante la utilización de glutamato-malato se valora el correcto transporte de electrones del complejo I a la coenzima Q, al complejo III, al citocromo c, al complejo IV y al agua, mientras que mediante el succinato se valora el correcto transporte de electrones del complejo II a la coenzima Q, al complejo III, al citocromo c, al complejo IV y al agua (fig. 1). Al igual que para las actividades enzimáticas, los valores absolutos de actividad oxidativa se dividieron también por la citrato sintasa para obtener los valores relativos, ya que mientras los primeros expresan la actividad oxidativa por célula, los segundos la expresan por organela.

El daño oxidativo se valoró mediante la determinación del grado de peroxidación lipídica de las membranas linfocitarias. Para ello se utilizó el ácido cis-parinárico, el cual se une a los ácidos grasos haciéndose fluorescente. Esta fluorescencia se pierde cuando existe peroxidación de los ácidos grasos, de manera que una mayor pérdida de fluorescencia es sinónimo de una mayor peroxidación lipídica. Para la realización de los experimentos, se colocaron 100 µg de proteína linfocitaria en una cubeta con 3 ml de PBS, previamente bombeado con nitrógeno, que contenía 5 µmol de ácido cis-parinárico (Molecular Probes®, Eugene, OR, EE.UU.), se incubó a 37 ºC en oscuridad y posteriormente se determinó cada 3 min la fluorescencia de la muestra durante un total de 15 min con longitudes de onda de 318 nm de excitación y 410 nm de emisión[20,21].

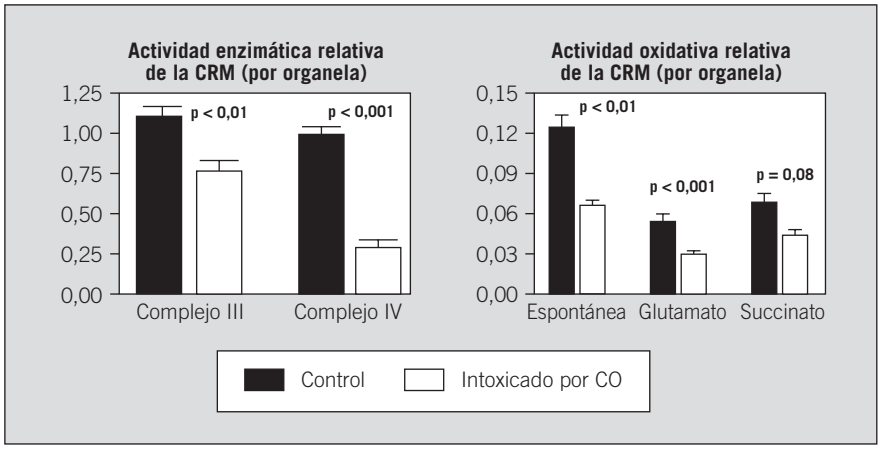


*Fig. 3. Resultados –media (error estándar)– obtenidos en los controles y en los pacientes en el momento agudo de la intoxicación por monóxido de carbono (CO) por lo que respecta a actividad enzimática (izquierda) y actividad oxidativa (derecha). Los resultados se expresan como actividades relativas (por organela), las cuales se obtienen a partir de dividir la actividad absoluta por la actividad de la citrato sintasa (marcador de contenido mitocondrial). CRM: cadena respiratoria mitocondrial.*

### *Análisis estadístico*

Los datos cualitativos se expresan como porcentajes y los cuantitativos como media (error estándar). Para la comparación de las variables cualitativas se utilizó el modelo lineal general para muestras independientes (estudio transversal) o muestras repetidas (estudio longitudinal y estudio de la curvas de cis-parinárico). En todos los casos, se consideró que existían diferencias estadísticamente significativas cuando el valor de p fue inferior a 0,05.

## Resultados

Durante la fase de intoxicación aguda por CO ($t_0$), la estimación del contenido mitocondrial a través de la determinación de la actividad de la citrato sintasa no mostró cambios respecto al contenido mitocondrial del grupo control (fig. 2, izquierda). Sin embargo, la cuantificación de las actividades enzimáticas de los complejos III y IV de la CRM (los 2 que contienen citocromos) evidenció descensos significativos en ambos casos, que fueron del 50% el grado de inhibición para el primero y del 65% para el segundo (fig. 2, centro). Finalmente, respecto a la capacidad oxidativa mitocondrial medida en forma de consumo de oxígeno en condiciones óptimas, se pudo observar un descenso significativo de aquélla, con porcentajes de inhibición que oscilaban entre el 58 y el 64%, según las condiciones del experimento (esto es, si se valoraba el consumo de oxígeno de manera espontánea o estimulado con la administración de glutamato o de succinato) (fig. 2, derecha). Cuando estos resultados de actividades enzimáticas y oxidativas se expresaron no por célula sino por organela (dividiendo los va-

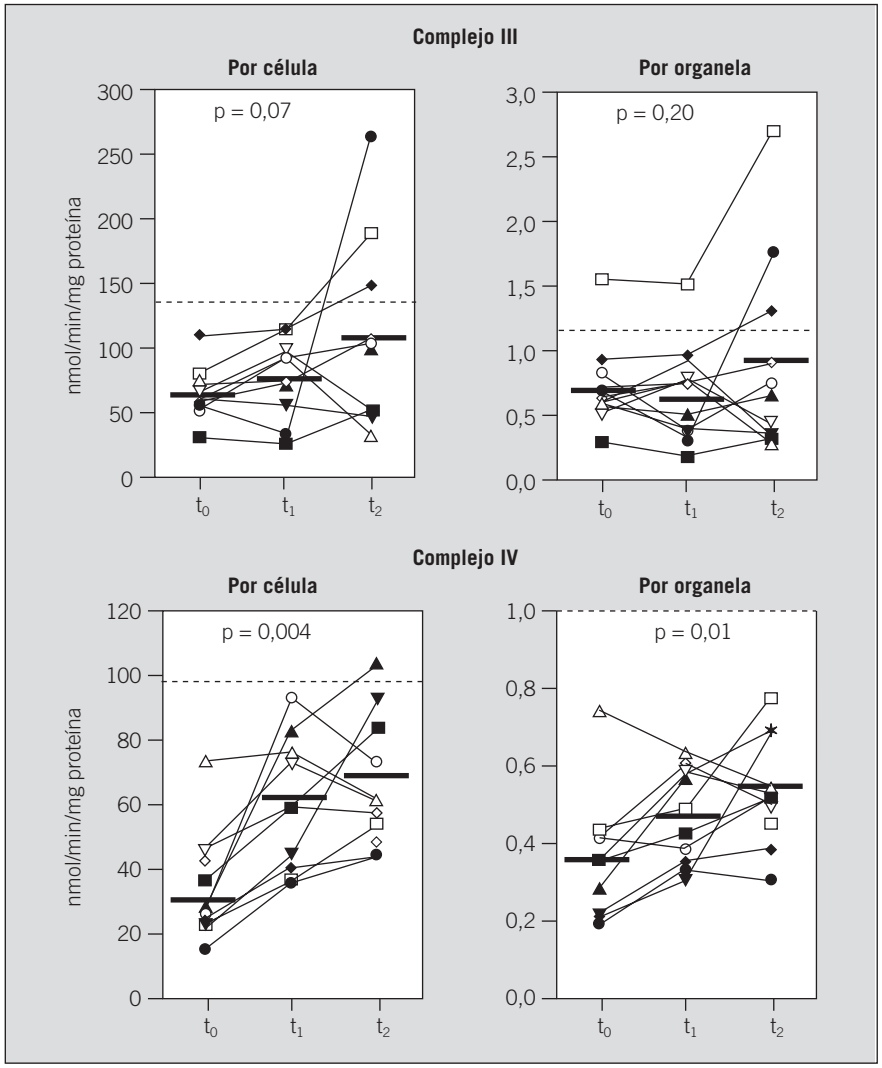


*Fig. 4. Evolución a lo largo del tiempo ($t_0$: intoxicación aguda; $t_1$: 3-5 días tras la intoxicación aguda; $t_2$: 10-14 días tras la intoxicación aguda) de las actividades enzimáticas de los pacientes intoxicados de forma aguda por monóxido de carbono (CO), expresadas tanto por célula (valores absolutos) como por organela (valores relativos). Las líneas continuas indican las medias de los pacientes en los diferentes momentos del estudio, y la línea discontinua, la media del grupo control.*

lores absolutos por la actividad de la citrato sintasa), se observó igualmente un descenso en todos los parámetros mitocondriales evaluados con respecto al grupo control (fig. 3). No obstante, los porcentajes de inhibición resultaron inferiores (oscilaron entre el 30 y el 65%) y, para el caso particular de la actividad oxidativa estimulada con succinato, dicha inhibición no alcanzó la significación estadística.

En las figuras 4 y 5 se presenta la evolución de las actividades enzimáticas y oxidativas, respectivamente, de los pacientes intoxicados por CO durante los días posteriores a la intoxicación ($t_1$ y $t_2$) y cuando los valores de COHb se habían normalizado. Aunque en todos los casos se observó una recuperación de la actividad, ésta sólo resultó significativa para el complejo IV y para la actividad oxidativa estimulada con glutamato, lo cual sucedía tanto si la valoración era celular como organular. A pesar de ello, prácticamente en ningún caso las actividades finales llegaron a alcanzar los valores del grupo control.

Respecto a la valoración del daño oxidativo, las curvas de fluorescencia del ácido cis-parinárico (fig. 6) mostraron un incremento de la peroxidación de los lípidos de membrana (mayor pérdida de fluorescencia) tanto durante la fase aguda de la intoxicación por CO ($t_0$) como durante los días posteriores ($t_1$ y $t_2$), si bien ninguno de estos incrementos de peroxidación alcanzó una significación estadística.

## Discusión

Los principales hallazgos del presente estudio de funcionalismo mitocondrial *ex vivo* en pacientes intoxicados de forma aguda por CO han sido la demostración de que el CO causa una inhibición profunda y sostenida de los complejos III y IV de la CRM, y que dicha inhibición repercute en la capacidad oxidativa mitocondrial. Estas inhibiciones presentan una lenta recuperación durante los días posteriores a la intoxicación aguda, de manera que al cabo de 14 días del episodio aún se detectan actividades disminuidas respecto al grupo control. En conjunto, esta disfunción mitocondrial podría ayudar a explicar la persistencia de síntomas y signos tardíos tras una intoxicación aguda por CO a pesar de que los valores de COHb se hayan normalizado por completo.

Si bien Haldana[22] ya sospechó la alteración mitocondrial del complejo IV (citocromo c oxidasa) de la CRM, no fue hasta 1939 cuando se comprobó por primera vez *in vitro* la unión del CO al citocromo $a_3$ de dicho complejo IV[23]. Sin embargo, hubo que esperar hasta la década de los ochenta para que una serie de experimentos llevados a cabo por el grupo de Piantadosi y Brown[8-12] constataran *in vivo* en modelos animales esta unión del CO al complejo IV. Además, estos autores encontraron que el CO es capaz de inhibir el metabolismo cerebral aun después de haberse normalizado los valores de COHb, e incluso aunque se administrase de forma concomitante oxigenoterapia hiperbárica[10]. En 1998 nuestro grupo presentó los primeros estudios *ex vivo* en humanos, realizados en 3 pacientes que habían presentado una intoxicación por CO, y cuyos resultados indicaban que esta inhibición del complejo IV de la CRM tenía relevancia clínica[13]. Los datos que aquí se presentan, obtenidos a partir de estudios en linfocitos de sangre periférica, confirman definitivamente esta hipótesis. Por otra parte, aunque es bien conocido que el complejo III de la CRM contiene citocromo $bc_1$, no hemos encontrado referencias en la bibliografía en las que se objetive que dicho complejo también resulte inhibido de forma significativa por el CO, por lo que éste es un aspecto en el que deberá profundizarse en un futuro.

Una cuestión interesante de nuestros resultados es por qué, a pesar de la utilización de tratamiento hiperbárico en la mayoría de casos (7 de 10), éste no consigue normalizar de forma absoluta las alteraciones mitocondriales detectadas. Una explicación podría ser que, a pesar de que la afinidad del CO y del oxígeno *in vitro* es menor para los citocromos que para la hemoglobina, existe una diferente capacidad *in vivo* del oxígeno para alcanzar estas moléculas. Además, el tratamiento hiperbárico persigue como objetivo normalizar las cifras de COHb (que además se utiliza como marcador del tratamiento) y es posible que, cuando este objetivo se

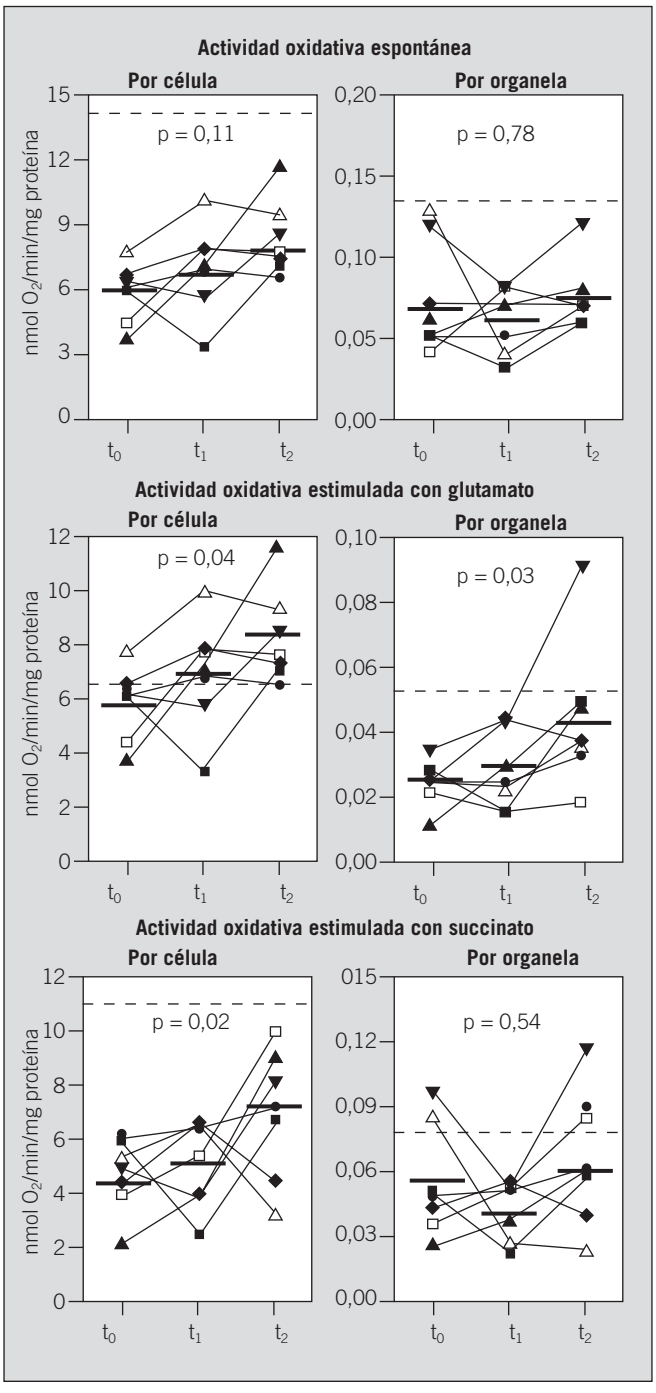


*Fig. 5. Evolución a lo largo del tiempo ($t_0$: intoxicación aguda; $t_1$: 3-5 días tras la intoxicación aguda; $t_2$: 10-14 días tras la intoxicación aguda) de las actividades oxidativas de los pacientes intoxicados de forma aguda por monóxido de carbono (CO), expresadas tanto por célula (valores absolutos) como por organela (valores relativos). Las líneas continuas indican las medias de los pacientes en los diferentes momentos del estudio, y la línea discontinua, la media del grupo control.*

hibición enzimática de los complejos III y IV. Cuando existe una inhibición de algún complejo de la CRM, el flujo de electrones a través de ella se ve reducido y decrece el consumo de oxígeno. Cuando el complejo afectado es el III o el IV (como sucede en nuestro caso), este descenso del consumo de oxígeno se hace evidente tanto si se mide la respiración celular espontánea como la estimulada mediante sustratos que ceden electrones al complejo I (glutamato) o al complejo II (succinato) (fig. 1). El resultado esperable ante un descenso de la actividad oxidativa es que parte del potencial reductor contenido en estos electrones se derive hacia la reducción incompleta del oxígeno, con la consiguiente formación de radicales libres que lesionarán moléculas biológicas. De hecho, ya en condiciones normales hasta un 5% del oxígeno consumido por la mitocondria se convierte en radicales oxidativos[24]. Sin embargo, este último extremo (el del incremento del daño oxidativo) no ha podido demostrarse en el presente estudio, al menos por lo que se refiere a la peroxidación lipídica, ya que, si bien se encontró una tendencia a que estuviera incrementada, las diferencias con el grupo control no resultaron estadísticamente significativas. Es posible que el corto tiempo de exposición de los pacientes intoxicados por CO no sea suficiente para que se produzca el daño oxidativo y, en este sentido, hemos podido comprobar cómo en personas que presentan de forma

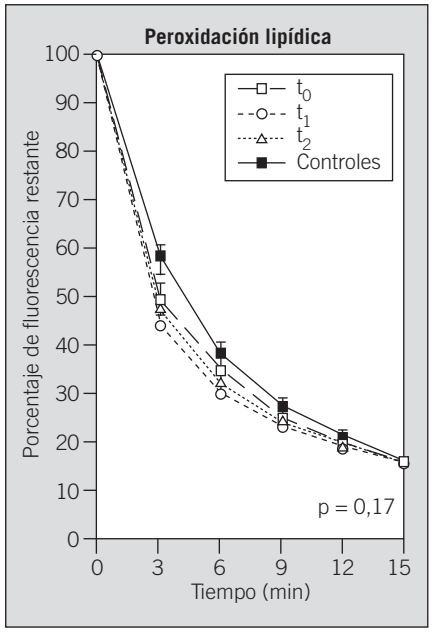


*Fig. 6. Curvas de fluorescencia –media (error estándar)– del ácido cis-parinárico obtenidas en el grupo control y en el grupo de pacientes intoxicados por monóxido de carbono (CO) en los diferentes momentos del estudio ($t_0$: intoxicación aguda; $t_1$: 3-5 días tras la intoxicación aguda; $t_2$: 10-14 días tras la intoxicación aguda). A mayor pérdida de fluorescencia, mayor peroxidación lipídica de las membranas.*

alcanza, la unión del CO a moléculas tisulares (y entre ellas los citocromos) aún no haya sido completamente revertida. Los datos experimentales anteriormente comentados de la ineficacia del tratamiento hiperbárico para revertir el descenso del metabolismo cerebral causado por el CO[10] apoyarían esta hipótesis. Dado que el presente estudio tiene un número de casos limitado, no es posible establecer comparaciones entre los grupos que recibieron tratamiento hiperbárico y los que no lo hicieron, y serán necesarios estudios posteriores que valoren si tratamientos hiperbáricos más prolongados podrían ser capaces de modificar nuestros hallazgos. De hecho, recientemente se ha demostrado que las sesiones repetidas con oxigenoterapia hiperbárica reducen el riesgo de padecer secuelas cognitivas durante las semanas siguientes a una intoxicación aguda por CO[7].

En cualquier caso, lo que parece evidente a partir de nuestros experimentos es que la capacidad oxidativa mitocondrial resulta marcadamente afectada por la in-

ma crónica valores elevados de COHb (fumadores crónicos) este incremento de la peroxidación lipídica sí que se produce[20].

Una hipótesis alternativa para explicar nuestros resultados es que la existencia de estas inhibiciones no fuese debida a la unión del CO con los citocromos, sino a la hipoxia tisular sufrida durante el episodio agudo secundario a la unión del CO con la hemoglobina. Sin embargo, existen estudios experimentales que demuestran que la actividad del complejo IV de la CRM no se encuentra inhibida tras 72 h de hipoxia tisular (conseguida a través de mecanismos distintos de la intoxicación por CO)[25]. Dado que todas las alteraciones encontradas en el presente estudio persistían en mayor o menor grado 14 días después de la intoxicación, creemos más plausible que sea la unión del CO a los grupos prostéticos de los citocromos contenidos en los complejos III y IV la responsable última de la disfunción de la CRM detectada en el presente estudio[26].

Quisiéramos resaltar el hecho de que los resultados que aquí se presentan se han obtenido a partir del estudio de linfocitos extraídos de sangre periférica. La mayoría de estos linfocitos circulan en sangre en su forma inactivada, por lo que su actividad metabólica es baja y las mitocondrias no están sometidas a una demanda de actividad. Además, su contenido en mitocondrias es reducido, pues se estima en 15-20 organelas por célula[27]. Por todo ello, es posible que nuestros resultados subestimen lo que en realidad acontece en los tejidos diana, como es el sistema nervioso central, con un elevado nivel metabólico. En este sentido, dado que las neuronas son altamente dependientes del metabolismo celular aerobio y que son células posmitóticas en las que el daño mitocondrial resulta más difícil de eliminar, parece lógico pensar que la inhibición enzimática y oxidativa en estas células sea mayor y/o más mantenida que la que hemos observado en los linfocitos, y que incluso en este tejido dicha inhibición pueda conducir a un incremento de la lesión oxidativa, hecho este último que no hemos podido constatar en los linfocitos.

Aunque el número de pacientes estudiados pueda parecer bajo, su homogeneidad en cuanto a la fuente de exposición al CO, la extensa valoración mitocondrial que se ha realizado y la consistencia interna de los resultados obtenidos creemos que otorgan fiabilidad a nuestros resultados. Además, el hecho de que con este número limitado ya se obtengan diferencias significativas habla en favor de la existencia de todas estas disfunciones que el presente estudio describe. Ante estos resultados, cabría plantearse la conveniencia de, además de la oxigenoterapia hiperbárica, administrar de forma concomitante cofactores y vitaminas que se han mostrado eficientes en algunos casos de enfermedades mitocondriales primarias[28] con la finalidad de minimizar, tanto cuantitativa como cualitativamente, el desarrollo de un síndrome neurológico tardío[14]. Sin duda, éste será un campo de investigación a tener en cuenta durante los próximos años, y futuros trabajos con modelos *in vitro* profundizarán a buen seguro en los mecanismos patogénicos de la intoxicación por CO. En cualquier caso, y como conclusión, creemos que nuestros datos demuestran claramente que la CRM es una diana relevante en pacientes intoxicados de forma aguda con CO y que, a través de este mecanismo, podrían explicarse algunos síntomas tardíos observados en estos pacientes.

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# ENZYMATIC DIAGNOSIS OF OXIDATIVE PHOSPHORILATION DEFECTS ON MUSCLE BIOPSY: BETTER ON TISSUE HOMOGENATE OR ON A MITOCHONDRIA-ENRICHED SUSPENSION?

*MEDICAL SCIENCE MONITOR*

2004 Sep;10(9):CS49-53

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© Med Sci Monit, 2004; 10(9): CS49-53
**PMID:** 15328490

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Case Study

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**Received:** 2003.10.16
**Accepted:** 2003.12.30
**Published:** 2004.09.01

# Enzymatic diagnosis of oxidative phosphorylation defects on muscle biopsy: Better on tissue homogenate or on a mitochondria-enriched suspension?

**Authors' Contribution:**
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

**Jordi Casademont[ADEF], Milagrosa Perea[B], Sonia López[C], Anna Beato[C], Òscar Miró[DE], Francesc Cardellach[FG]**

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**Source of support:** This study was funded in part by Fundació La Marató de TV3 1710/01, SGR 00399 and Red-G011/03.

## Summary

**Background:** The enzymatic analysis of mitochondrial respiratory chain (MRC) complexes of skeletal muscle is an important step in the diagnosis of mitochondrial disorders. Because of its lesser turbidity and increased sensitivity, mitochondrial fractionation has been increasingly considered the diagnostic method of choice compared with the more classical analysis of muscle homogenate. In circumstances in which mitochondria become abnormal in number, size or shape, the process of mitochondrial enrichment made by sequential centrifugation and washing may favor the selection of the most normal mitochondria, eliminating the most abnormal ones. In this situation, the study of muscle homogenate, paradoxically, may better reflect what happens *in vivo*.

**Case Report:** To exemplify this situation we present a 60-year-old woman with a complete mitochondrial phenotype and a 70% heteroplasmic presence of the mtDNA A3243G mutation in muscle tissue. The respiratory and enzymatic activities from mitochondria-enriched muscle suspension were within normal control limits. In contrast, when muscle homogenate was studied, enzyme activities of complexes I, III, and V were found to be decreased.

**Conclusions:** Although mitochondria-enriched muscle suspensions are usually more informative than muscle homogenates for studies of MRC, in some situations it may be necessary to study both to uncover the biochemical defect.

**key words:** **mitochondria • respiratory chain • heteroplasmy • MELAS • enzymatic diagnosis**

**Full-text PDF:** http://www.MedSciMonit.com/pub/vol_10/no_9/4260.pdf

**Word count:** 2435
**Tables:** 2
**Figures:** 3
**References:** 22

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## BACKGROUND

Mitochondrial respiratory chain (MRC) dysfunctions are increasingly recognized as a cause of human disease. Their diagnosis relies on clinical suspicion plus a combination of imaging, histopathological, genetic, and biochemical studies of affected tissues [1,2]. The biochemical analysis of MRC oxidative phosphorylation (OXPHOS) has the advantage of providing a "functional" view of the respiratory chain. This can be very important in diseases in which clinical manifestations are very heterogeneous, histopathological studies of available tissues are not always informative, and there is no screening test for the genetic defects underlying most of the MRC deficiencies. Furthermore, many suspected nuclear mutations remain to be described, and even the presence of a genetic defect is not easy to interpret. The coexistence in different proportions of abnormal and wild-type mitochondria – phenomena known as heteroplasmy – and the appearance of somatic mutations in the mitochondrial genome with increasing age [1–4] may account for these difficulties.

OXPHOS biochemical studies include the determination of individual enzyme activities from complexes I to V, which constitute the MRC. They can be performed in tissue homogenates, in permeabilized whole cells, or in mitochondria isolated from tissue or cultured cells [5–9]. For diagnostic purposes, skeletal muscle has been the preferred tissue due to its accessibility and frequent involvement in disease, and enzymatic analyses performed in muscle homogenates are the most widely used methods. In recent years, however, because of its lesser turbidity and increased sensitivity, mitochondrial fraction, freshly isolated from biopsied muscle, has been increasingly considered the diagnostic method of choice [6,10] and adopted as the standard enzymatic diagnostic procedure by many laboratories.

Enzymatic activities refer to mitochondrial protein content and are greatly dependent on the quality of preparation. For this reason, they are usually corrected for variations in purity by standardizing to a mitochondrial marker such as citrate synthase, a mitochondrial enzyme not related to the respiratory chain, belonging to the tricarboxylic acids cycle [6,11]. It is also interesting to perform a simultaneous control study to exclude any possible abnormality due to technical problems [12,13]. Even with such precautions it is not infrequent to find a patient with a clinical picture of a full-blown mitochondrial disease, even with the identified genetic defect, but with normal enzymatic studies on enriched mitochondrial suspensions. This poses questions about the ability of a given laboratory to detect OXPHOS defects and is one of the possible reasons why many laboratories do not routinely perform such studies for diagnostic purposes, in spite of their potential information. During enrichment by differential centrifugation and washing there may be a purification and a selective loss of the most abnormal mitochondria, i.e. significant variations in size or density due to paracrystalline or amorphous inclusions. When abnormal mitochondria are very abundant, mitochondrial fractionation may be less informative than simple tissue homogenates to uncover MRC dysfunctions.

## CASE REPORT

We have reviewed the enzymatic studies of suspected mitochondrial disorders from the last five years in our laboratory. Five patients (two with Kearns-Sayre and large mtDNA mutations, two with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) and the 3243 tRNA Leu mutation, and one patient with mitochondrial encephalopathy and ragged-red fibers (MERRF) with the 8363 tRNA Lys mutation) rendered normal results on mitochondrial extracts, while the respiratory complexes were abnormal on muscle homogenate.

To exemplify this situation, we present a 58-year-old woman with a prolonged history of fatigue, diffuse muscle weakness, behavioral disorders, hypertension, and neurosensorial hearing loss. In the last two years she developed myoclonus, episodic vomiting, fluctuating level of consciousness without neurological focality, and symptoms of progressive dementia. Laboratory studies revealed increased serum creatinine levels (3.4 mg/dl), a renal clearance of 7%, lactate dehydrogenase of 503 U/l, and creatine kinase of 166 U/l. Serum glucose was slightly elevated (177 mg/dl) and serum lactate was 49 mg/dl (normal: 5 to 22). The electroencephalogram showed diffuse slowing with bilateral epileptiform discharges, and photomyoclonic activity. Renal ultrasound studies showed atrophic kidneys, suggestive of chronic renal failure.

Family history indicated neurosensorial deafness and headaches in her mother, intellectual impairment and renal insufficiency of unknown etiology in one of her two sisters, and deafness in the other sister and two nieces. Her brother was 71 years old and asymptomatic.

While under study, she presented a sudden decrease in the level of consciousness and left hemiplegia. A CT scan showed an occipital hemorrhage, but also a diffuse cerebral atrophy with enlargement of the ventricles, hypodensity of the white matter, and basal ganglia calcifications (Figure 1). A muscle biopsy of the deltoid muscle showed the typical ragged-red fibers (RRF) in modified Gomori's trichrome, negative on cytochrome oxidase (COX), and hyperactive on oxidative histochemical reactions (Figure 2). Electron microscopy disclosed the presence of abundant subsarcolemmal mitochondria, abnormal in size and shape, some of which had paracrystalline inclusions (Figure 3). Genetic studies disclosed the heteroplasmic A to G mutation at 3243 tRNALeu$^{(UUR)}$ characteristic of MELAS [14] in muscle from the patient and in lymphocytes of her asymptomatic brother and two nieces, in proportions ranging from 35 to 70% of mtDNA.

### Biochemical analyses

200 mg of muscle tissue were cut with scissors and homogenized (5% w/v) in a medium containing 0.25 M sucrose, 40 mM KCl, 2 mM EGTA, 20 mM Tris-HCl (pH 7.2), and 1 mg/ml BSA, in a 2 ml Potter-Elvehjem glass homogenizer equipped with a motor-driven Teflon pestle. The homogenized tissue was filtered through a piece of nylon cloth and the homogenate obtained divided into two parts: one kept frozen at –80°C and the other used for mitochondrial extraction.

1. *Isolation of mitochondria*: The mitochondrial fraction was obtained through differential centrifugation at 4°C. The homogenate was centrifuged at 2000 rpm for 8 min. The supernatant was collected and kept on ice. The remaining

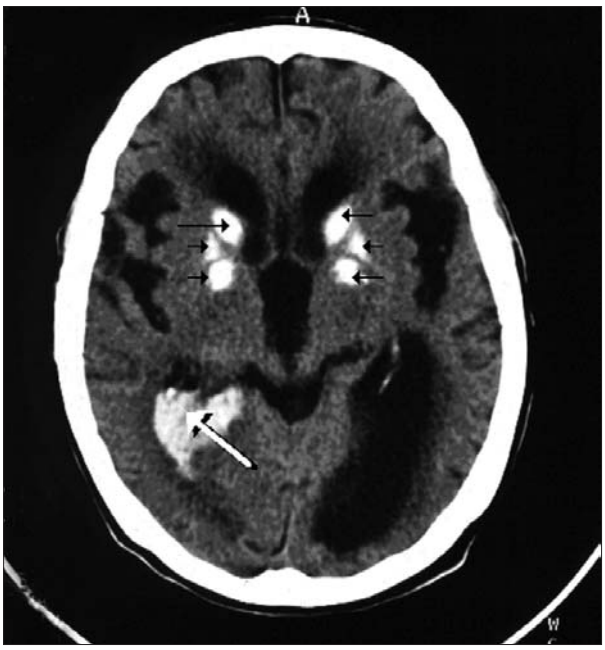

**Figure 1.** CT scan showed an occipital hemorrhage with ventricular contamination (large white arrow), but also a diffuse cerebral atrophy with enlargement of the ventricles, and basal ganglia calcifications (small black arrows).

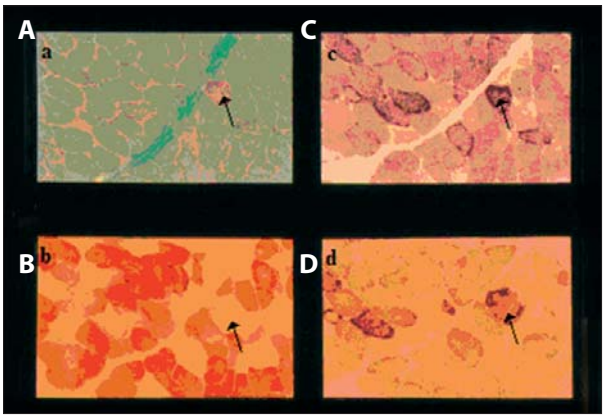


**Figure 2.** Sequential sections of deltoid muscle biopsy. Note a typical ragged-red fiber (arrow) in modified Gomori's trichrome (**A**), negative on COX (**B**) and hyperactive on NADH (**C**) and SDH (**D**).

pellet was resuspended in a small volume of the initial medium and the process repeated for enrichment of the mitochondrial suspension. The supernatant obtained in this step was mixed with the first and centrifuged at 10,000 rpm for 8 min. The resulting pellet was resuspended in homogenizing medium plus 5% (v/v) Percoll. Finally, another centrifugation was done at 10,000 rpm for 8 min. The pellet corresponded to the mitochondrial fraction and was resuspended in the initial solution.

2. *Protein quantification*: Protein content was measured according to Bradford's technique[15] with deduction of BSA content from the blank.

3. *Polarographic studies*: Oxygen utilization was measured polarographically in 0.25 ml of standard medium (pH 7.40)

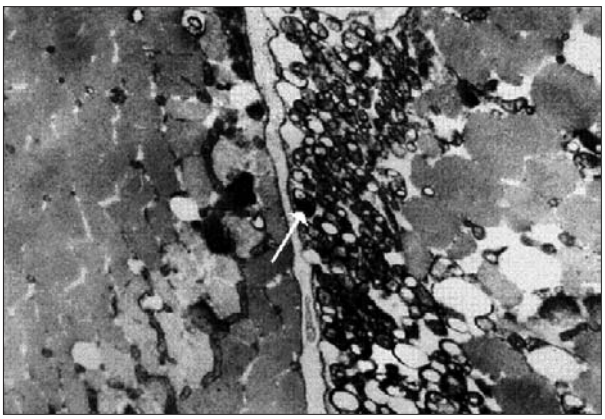

**Figure 3.** Abnormal mitochondria in number, shape, and size are present in subsarcolemmal areas. Note the presence of some paracrystalline inclusions (white arrow).

containing 0.3 M mannitol, 10 mM KCl, 5 mM MgCl2, 10 mM KH2PO4, and 0.25 mg of BSA with a Clark oxygen electrode in a micro water-jacketed cell at 37°C (Hansatech Instruments, Norfolk, UK). The following assays were performed in intact mitochondria: oxidation of glutamate (20 mM) plus malate (10 mM); oxidation of succinate (12 mM); and oxidation of ascorbate (4 mM) plus TMPD (120 µM) according to Rustin et al. [6]. The appropriate inhibitors were added in each experiment. The rate of oxygen consumption stimulated by adenosine 5'-diphosphate (ADP, 0.4 mM, state 3 rate) was assessed for each substrate, and expressed as nmols oxygen/min/mg of mitochondrial protein.

4. *Spectrophotometric studies*: Absolute enzyme activities for each MRC complex were spectrophotometrically quantified (UVIKON 922, Kontron AS, Switzerland). 10–40 µg of protein were used to determine the activity of complexes, except for complex IV, for which 2–4 µg were utilized [16]. Assays were performed at 37°C in a total volume of 1 ml.

Measurement of Complex I (rotenone-sensitive NADH-ubiquinone oxidoreductase, EC 1.6.99.3), complex II (succinate-ubiquinone reductase, EC 1.3.99.1), complex III (ubiquinol-cytochrome c reductase, EC 1.10.2.2), complex IV (cytochrome c oxidase, EC 1.9.3.1), and complex V (oligomycin-sensitive ATPase, EC 3.6.1.34) were performed as previously described [6,17].

Measurement of Citrate Synthase (CS, EC 4.1.3.7): The assay was performed at 412 nm in a medium containing 10 mM $KH_2PO_4$ (pH 7.8, 37°C), 2 mM EDTA, 1 mg/ml BSA, 0.1% Triton X-100, 0.1 mM Acetyl-CoA, and 2 mM 5,5'-Dithiobis(2-nitrobenzoic acid) as acceptor. The reaction started with the addition of 12 mM oxalacetic acid. Enzyme activities were expressed as nmol of reduced or oxidized substrate/min/mg of mitochondrial protein.

5. *Tissue homogenate enzymatic studies*: Once aware of the studies performed on mitochondrial extractions, spectrophotometric enzymatic analyses were repeated in the homogenate kept frozen, without methodological variations with respect to mitochondrial fractionation. The results were expressed per mg of homogenate protein.

**Table 1.** Enzymatic activities and oxidation rates.

| Mitochondria | | | | |
|---|---|---|---|---|
| **Spectrophotometric analyses** | | | | |
| | **Absolute values** | | **Relative to CS values** | |
| CI | 191 | (30–276) | 0.11 | (0.09–0.15) |
| CII | 367 | (75–445) | 0.21 | (0.12–0.36) |
| CIII | 1024 | (513–2338) | 0.85 | (0.75–1.29) |
| CIV | 838 | (197–1213) | 0.47 | (0.32–0.98) |
| CV | 951 | (101–1217) | 0.53 | (0.15–0.52) |
| CS | 1775 | (316–2490) | – | |
| **Polarographic studies** | | | | |
| Piruvate-Malate (substrate CI) | | | 20 | (18–119) |
| Succinate-Malate (substrate CII) | | | 21 | (20–70) |
| Glycerol-3-P (substrate CIII) | | | 98 | (20–133) |
| Ascorbate (substrate CIV) | | | 6 | (5–43) |

Results of spectrophotometric enzyme activities are expressed as nmols of reduced or oxidized substrate/min/mg of mitochondrial protein.
Rate of oxygen consumption measured by polarography is expressed as nmols oxygen/min/mg of mitochondrial protein.
In parenthesis are the ranges of normal values in our laboratory.

**Table 2.** Enzymatic activities.

| Homogenate | | | | |
|---|---|---|---|---|
| **Spectrophotometric analyses** | | | | |
| | **Absolute values** | | **Relative to CS values** | |
| CI | 34 | (8–35) | 0.05 | (0.06–0.33) |
| CII | 158 | (23–105) | 0.24 | (0.09–0.57) |
| CIII | 302 | (140–373) | 0.45 | (0.85–3.31) |
| CIV | 261 | (50–279) | 0.39 | (0.36–1.22) |
| CV | 146 | (81–188) | 0.22 | (0.37–2.19) |
| CS | 667 | (51–439) | – | |

Results of spectrophotometric enzyme activities are expressed as nmols of reduced or oxidized substrate/min/mg of homogenate protein. Abnormal results are expressed in bold.
In parenthesis are the ranges of normal values in our laboratory.

## Results

Enzymatic activities of complexes I to V were normal on fresh mitochondrial suspensions either uncorrected or corrected by citrate synthase activity (Table 1). Oxygen consumption analyses were also normal, independently of the substrate used (Table 1).

In spite of such normal results, and in view of the strong suspicion of a mitochondrial disease, we also measured enzymatic activities on muscle homogenate based on the hypothesis that the most morphologically abnormal mitochondria had been eliminated in the process of mitochondrial recovery. When the results of OXPHOS enzymes were standardized for CS, a decrease in the activity of complexes I, III, and V was clearly demonstrated (Table 2).

## Discussion

The patient under discussion presented a phenotype strongly suggestive of a mitochondrial disease. The muscle biopsy disclosed abundant RRF, the histologic hallmark of mitochondrial disorders [2], and genetic analyses confirmed a significant proportion of a heteroplasmic A to G mutation at 3243 tRNALeu$^{(UUR)}$ in the muscle of the patient and in the lymphocytes of some relatives. The diagnosis of MELAS was established. Surprisingly, however, the enzymatic analyses of MRC on mitochondria-enriched muscle suspensions rendered normal results.

Diseases due to mtDNA mutations usually present as heterogenous clinical syndromes. In many instances the genetic defect is known, but the pathogenesis and the relation between the molecular target of the mutation and the clinical phenotype remains obscure. Moreover, a simple direct biochemical consequence of the protein or RNA alteration due to the mutation often cannot be found. The case herein reported is not exceptional.

Many explanations have been provided. The most obvious is that due to the presence of multiple mtDNA copies in the same cell, and the possibility for a mutation to have different degrees of heteroplasmy, phenotypic expression varies from tissue to tissue depending on the threshold effect [18–20]. Partial defects can therefore be overlooked in analyzed tissues because normal mitochondria may supplement and balance abnormal mitochondria due to the intermitochondrial complementation phenomena. This, in theory, may apply to all heteroplasmic mitochondrial diseases. The problem is that when abundant RRF are found in histochemical muscle preparations it is difficult to conclude that the structure can be affected without any dysfunction.

In homoplasmic mtDNA diseases the situation is different. The best studied examples relate to homoplasmic mutations leading to nonsyndromic neurosensorial hearing loss and to Leber's hereditary optic neuroretinopathy (LHON) [21]. Both diseases are exquisitely tissue specific from a clinical point of view and normal enzymatic analyses of MRC in usually available tissues such as lymphocytes, fibroblasts, or muscle may be difficult to extrapolate.

Another possible explanation is that MRC enzyme activities have a very high scattering of control data, frequently of over one order of magnitude. Such results tend to overlap with those of patients, especially in cases of partial defects.

Possible solutions to overcome such diagnostic difficulties include a double approach to analyze MRC dysfunction: on the one hand, enzymatic activities by spectrophotometry, and on the other, oxygen consumption through polarography. Unfortunately, polarographic oxygen consumption can only be analyzed on freshly extracted tissues and

not on frozen material, which limits its generalized utilization. Furthermore, in situations such as that discussed herein in which we hypothesize that there is a bias in mitochondrial extraction, oxygen consumption analysis may render normal results for the same reason why enzymatic studies are normal.

Due to the tight balance between respiratory chain activities required for an optimal functioning of MRC and to avoid significant leakage of free radicals, some authors have stressed the importance of using relative activities between complexes: if the activity of one complex is within normal values, but relatively low in relation to other complexes in the same sample, relative activities will uncover slight decreases in the activity of one given complex [6,11]. Moreover, ratios between complex activities tend to follow a normal distribution with narrow ranges, thus avoiding the overlap between controls and patients. This approach has the inconvenience that it renders a lot of results (as many as 20 ratios can be calculated for every study on isolated mitochondria) and consequently increases the difficulty in interpreting the results, as well as the possibility of a finding a spuriously significant result. It also fails to recognize generalized OXPHOS defects.

In normal conditions, mitochondria show a great variation in the average sedimentation coefficient [22]. Based on this coefficient and known centrifugation parameters, a specific procedure has been set up to extract mitochondria from each tissue. Mitochondrial diseases usually disclose abnormal mitochondria either in number, size, or shape. When mitochondria are abnormal, the process of mitochondrial extraction made through sequential centrifugation may favor the selection of the most normal mitochondria while the most abnormal ones are eliminated. As mitochondria proliferate, the protein content in mg of mitochondria per mg of tissue (used as an estimate of proper mitochondrial extraction procedure) may be normal. In this situation, respiratory and enzymatic analyses of mitochondrial suspensions may render false normal results. In contrast, when muscle homogenates are studied, no mitochondrial selection is performed, and when enzymatic activities are normalized by CS activity, the results may better reflect what happens *in vivo*.

## CONCLUSIONS

We still have insufficient knowledge of some basic aspects of mitochondrial structure and function, and it is possible that in certain cases the MRC function remains normal in clear-cut mitochondrial diseases for the above-mentioned, or other, circumstances. But simply refining the usual methods of analysis, as the herein proposed simultaneous study of mitochondrial extractions and tissue homogenates, may increase the profitability of enzymatic studies. We conclude that although mitochondria-enriched muscle suspensions are usually more informative than muscle homogenates for studies of MRC, in some clinical situations in which abundant abnormal mitochondria exist it may be necessary to study both to uncover the biochemical defect.

**Acknowledgements**

Due to the characteristics of the article, we may have biased our citations toward reviews. We apologize to the many authors whose efforts may have been overlooked as a result.

CS

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# MITOCHONDRIAL EFFECTS OF A 24-WEEK COURSE OF PEGYLATED-INTERFERON PLUS RIBAVIRIN IN ASYMPTOMATIC HCV/HIV CO-INFECTED PATIENTS ON LONG-TERM TREATMENT WITH DIDANOSINE, STAVUDINE OR BOTH

*ANTIVIRAL THERAPY*

2004 Dec;9(6):969-77

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# Mitochondrial effects of a 24-week course of pegylated-interferon plus ribavirin in asymptomatic HCV/HIV co-infected patients on long-term treatment with didanosine, stavudine or both

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**Background: It has been suggested that the addition of ribavirin (RBV) as a part of the treatment for chronic hepatitis C virus (HCV) in HIV co-infected patients on didanosine (ddI) or stavudine (d4T) might increase the nucleoside-induced impairment of mitochondrial function.**
**Design: Comparative study to investigate the impact on mitochondrial function of adding RBV to a long-term treatment with ddI, d4T or both in HCV/HIV non-cirrhotic, asymptomatic patients. We included 26 patients: 16 continued with their current antiretroviral therapy (control group) and 10 patients received a concomitant 24-week course of RBV plus pegylated interferon (PEG-IFN) $\alpha$-2b therapy (HCV-treated group).**
**Methods: We assessed peripheral blood mononuclear cells mitochondrial DNA (mtDNA) content and mitochondrial respiratory chain (MRC) function at baseline and at 24 weeks of follow-up. In the HCV-treated group we performed additional determinations at 12 weeks during anti-HCV therapy and 24 weeks after finishing anti-HCV therapy.**
**Results: Times on ddI or d4T exposure were 194 ±54.9 and 131 ±66.5 weeks in the HCV-treated and control groups, respectively. There were no differences either in mtDNA content, the enzyme activity of MRC complexes or clinical parameters at baseline. Throughout the study, mitochondrial measurements remained stable within groups and without differences when we compared HCV-treated and control groups.**
**Conclusions: In our study, the addition of RBV and PEG-IFN during a 24-week period in HCV/HIV non-cirrhotic, asymptomatic patients on long-term ddI, d4T or both had no impact on mitochondrial function. These findings could suggest that additional triggers are required to achieve a critical threshold in the degree of mitochondrial damage needed for symptoms to develop.**

## Introduction

The efficacy of hepatitis C virus (HCV) treatment has dramatically increased with the combination strategy of pegylated interferon (PEG-IFN) plus ribavirin (RBV) [1,2], leading physicians to consider this therapeutic approach in patients co-infected with HIV and HCV, due to the faster progression of liver damage in such patients [3,4]. However, there are several reports of a worse response to HCV therapy in co-infected patients than in HCV mono-infected populations [5–8]. Drug-related toxicity is an important issue in HIV patients treated for chronic hepatitis C. Major concerns have arisen after recently reported cases of lactic acidosis, in particular in patients taking didanosine (ddI), stavudine (d4T) or both [6,9]. The aetiopathogenic mechanism may be enhanced mitochondrial toxicity of nucleoside reverse transcriptase inhibitors (NRTIs) [10,11] due to the interaction with RBV. The increased exposure to the active triphosphorylated anabolite of ddI during RBV treatment would support this hypothesis [12]. A recent United States Food and Drug Administration (USFDA) warning therefore recommends using RBV with caution in

combination with ddI, based on cumulative case reports [13].

The toxic effect of NRTI therapy is usually investigated by studying mitochondrial DNA (mtDNA) depletion [14], but that marker does not seem to be a reliable indicator of mitochondrial dysfunction [15]. The complementary direct determination of oxidative activity offers further evaluation, as has been shown in HIV patients on highly active antiretroviral therapy (HAART) [16]. Even though a possible decrease in mtDNA levels might occur after the addition of RBV to a current NRTI regimen, its real impact on mitochondrial function would also need to be demonstrated [17].

Given that there is no information available to date that allows us to assess the targeted impact on mitochondria of adding RBV to NRTI, we designed the present study to evaluate the evolution of both mtDNA content and mitochondrial respiratory chain (MRC) function in that setting, along with clinical (anthropometric changes) and analytical determinations (lactate and pyruvate).

## Materials and methods

### Study design

We carried out a comparative, retrospective study of 26 HCV/HIV co-infected patients under a stable HAART regimen containing ddI, d4T or both. Ten patients received a 24-week course of RBV and PEG-IFN α-2b therapy (HCV-treated group), and the remaining 16 patients continued their HAART regimen and did not receive any anti-HCV therapy (control group). The primary objective was to assess the impact on mitochondrial function of adding RBV and PEG-IFN to the current HAART. Secondarily, we aimed to investigate whether or not those findings correlated with anthropometric evaluations (lipodystrophy).

### Study population

All patients attended the HIV outpatient clinic at the Hospital Universitari Germans Trias i Pujol, which takes care of 2300 patients, 43% of whom are HCV co-infected. From August 2001 to May 2002, we included in the HCV-treated and in the control group HCV/HIV co-infected patients of either gender aged 18 years or older, who were taking a stable HAART including ddI, d4T or both for more than 1 year. We considered cumulative time of NRTI exposure as the sum of consecutive antiretroviral strategies including ddI, d4T or both, as shown in Figure 1, for HCV-treated patients. HCV infection was confirmed in both groups by a second generation enzyme linked immunoabsorbent assay. We excluded patients with cirrhosis suggested by clinical or echographic criteria or confirmed by liver biopsy. We also excluded patients with positive circulating surface hepatitis B virus antigen and those who self-reported daily intake of alcohol greater than 40 g. Women of childbearing age were also excluded if they were, or might become, pregnant or were lactating.

### Treatment groups

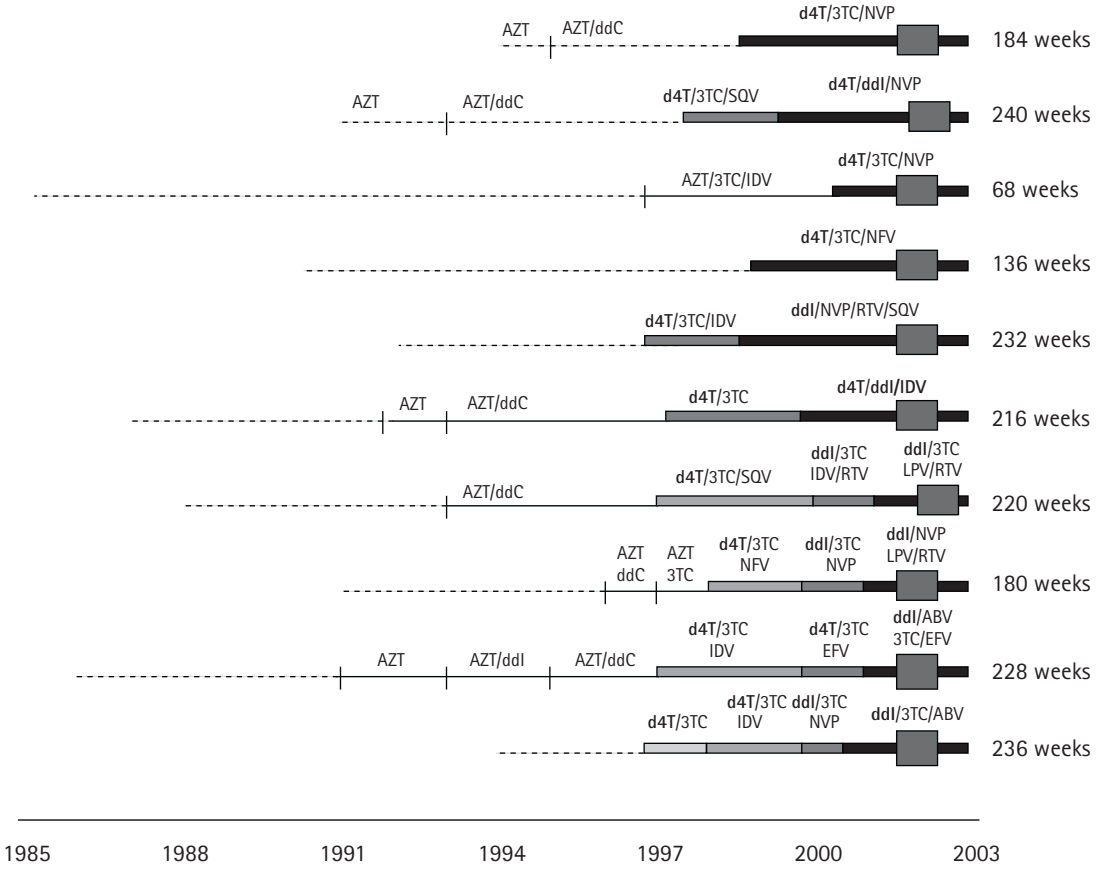
The HCV-treated patients were recruited and screened from a group of 27 patients enrolled in a trial of directly observed treatment against HCV with PEG-IFN α-2b (1.5 μg/kg/weekly) and a fixed dose of RBV (800 mg/daily) [18]. For the present study, patients were selected if they fulfilled the entry criteria and once they had completed 24 weeks of HCV therapy without dosage modifications. HCV therapy was stopped if the patient harboured a HCV genotype 3 or if a patient harbouring HCV genotype 1 or 4 lacked virological response at 24 weeks. Eleven patients (two patients with genotype 3, six with genotype 1 and three with genotype 4) fulfilled the inclusion criteria, but one patient harbouring genotype 3 was not enrolled because blood samples were not available. The reasons for excluding 16 other candidate patients from the study were that the HAART strategy did not include ddI or d4T in six cases, ddI had been taken for less than 1 year in one case, four cases were naive for antiretroviral therapy, RBV was temporarily interrupted because of dyspepsia with normal lactate levels in one case and two cases were under ddI or d4T use but discontinued HCV therapy before 6 months. Another two patients harbouring HCV genotypes 1 and 4 with virological response at week 24 were excluded from the present study as they had to fulfil a 48-week course of PEG-IFN plus RBV. We did not detect any symptomatic hyperlactataemia or lactic acidosis in patients who were excluded.

The other 16 patients in the control group were naive for HCV therapy. These patients did not receive anti-HCV therapy because of patient refusal (five patients), delayed treatment (seven patients) or psychiatric conditions other than drug consumption (four patients). The HCV genotype distribution was similar between groups (eight patients with genotype 1, three with genotype 3, three with genotype 4; genotype unavailable for two patients). They were consecutively selected from our database if they had been receiving the same NRTI combination as the included HCV-treated patients for more than 1 year and the therapy was maintained during the study period (from August 2001 to May 2002).

### Mitochondrial function evaluation

Peripheral blood mononuclear cells (PBMCs) were used to perform all studies. In the HCV-treated group, cellular samples were obtained prior to initiating HCV

**Figure 1.** NRTI exposure and time on hepatitis C virus therapy in the HCV-treated group




Cumulative time on NRTI, including d4T, ddI or both is expressed in weeks. Squares represent period of HCV therapy. AZT, zidovudine; ddC, zalcitabine; 3TC, lamivudine; ABV, abacavir; ddI, didanosine; d4T, stavudine; EFV, efavirenz; SQV, saquinavir; NVP, nevirapine; NFV, nelfinavir; LPV, lopinavir; IDV, indinavir; RTV, ritonavir.

therapy, at 12 weeks, 24 weeks (when RBV and PEG-IFN were stopped) and 48 weeks (6 months after completion of HCV treatment). In the control group, two cellular samples were obtained for comparison at baseline and 24 weeks. PBMCs were isolated according to a standard method of centrifugation using a Ficoll gradient (Sigma Chemical Co., St Louis, Miss., USA).

*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 polymerase chain reaction (LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany). The results were expressed as the ratio of the mean of duplicate measurements of mtDNA to the mean of duplicate measurements of nuclear DNA (mtDNA/nDNA).

*Enzyme activities of MRC complexes.* We determined the individual enzyme activity for complex II (succinate-ubiquinone reductase, EC 1.3.99.1), complex III (ubiquinol-cytochrome c reductase, EC 1.10.2.2) and complex IV (cytochrome c oxidase, EC 1.9.3.1) by spectrophotometry (Uvikon 920; Kontron, Switzerland). All experiments were performed in duplicate at 37ºC.

The complete methodology of all mitochondrial studies described above has been reported more extensively elsewhere [19–21].

## Clinical and anthropometric evaluation

In the HCV-treated group, data of medical visits consisted of complete clinical and anthropometric examinations including body mass index (BMI), and hip and waist measurements, which were recorded at baseline, 12 weeks, 24 weeks and 48 weeks of follow-up. In the control group, the same parameters at baseline

and at 24 weeks were available for comparison. The presence of lipodystrophy was also evaluated in HCV-treated patients using an objective measure, the validated lipodystrophy case definition score (LCDS) [22]. This score allowed us to sum up all the clinical, anthropometric and biochemical data and to measure changes during the study period. A score greater than zero defined the presence of lipodystrophy. The reported sensitivity and specificity of the model without body-imaging data is 73% and 71%, respectively [22]. In control patients, BMI was also available for comparison at baseline and at 24 weeks.

### Biochemical analyses

Fasting plasma samples were obtained weekly from HCV-treated patients during the first month and every month afterwards until week 24. A minimum rest of 10 min before phlebotomy was required. Extraction was performed without a tourniquet or fist clenching and the samples were processed within 20 min. Lactate, pyruvate, ratio of lactate to pyruvate and bicarbonate levels were determined an average of 10 different times during the study period for every patient to optimize the consistency of data.

### Statistical analyses

Results were expressed as percentages for qualitative variables and as means and standard deviations for quantitative variables. For comparisons between cases and controls, we used the Fisher exact test and the unpaired *t*-test (or the alternate Welch test when variances were unequal), respectively. We used the two-way analysis of variance for variables with repeated measures to search for significant differences within subjects or between groups. Relationships between quantitative variables were assessed by linear regression. For all tests, *P* values less than 0.05 were considered statistically significant. Statistical analyses were performed with SPSS v10.0 for Windows (SPSS, Inc., Chicago, Ill., USA). All data were recorded on a database program (Microsoft Access 97 for Windows; Microsoft Corp., Redmont, Wash., USA).

## Results

We analysed results for 26 patients, 10 in the HCV-treated group and 16 in the control group. Baseline individual clinical characteristics are summarized in Table 1. It is remarkable that individuals from the HCV-treated group were receiving NRTI for a significantly longer period of time than individuals in the control group. Current NRTI combinations (HCV-treated/controls) during the time of the study were distributed in the patient and control groups as follows: ddI plus d4T (2/4), d4T plus lamivudine (3/7) and ddI plus abacavir or ddI as the only NRTI (5/5). Figure 1 represents the complete pharmacological histories of patients from the HCV-treated group.

The content of mtDNA and the enzyme activity of MRC complexes II, III and IV did not differ between groups at baseline or at 24 weeks (Table 2). The follow-up until 48 weeks of HCV-treated patients did not demonstrate any significant modification for any mitochondrial parameters (Figure 2). A trend was seen for patients who were receiving a HAART regimen containing ddI plus d4T or ddI to exhibit lower mtDNA content and MRC function than those receiving a HAART regimen containing d4T plus 3TC (inter-group *P* value=0.06) (Figure 3), but no significant changes were found within any group over the course of the study. Moreover, no cases of metabolic acidosis or symptomatic hyperlactataemia were observed during HCV therapy, as shown by no significant within-subject changes in repeated measures of serum lactate, pyruvate or bicarbonate levels for those receiving treatment for HCV.

Anthropometric parameters were similar for both groups at baseline, but BMI was significantly lower in the HCV-treated group than in the control group at 24 weeks (20.5 ±1.8 and 22.5 ±3.0, respectively; *P*=0.02). Follow-up of HCV-treated cases showed that BMI returned to baseline values after stopping HCV therapy (BMI 21.1 ±1.3 at 48 weeks). In the HCV-treated group, five patients presented with lipodystrophy at baseline (LDCS ≥0), and the remaining five individuals

**Table 1.** Comparison of baseline characteristics between the HCV-treated group and the control group

| | HCV-treated group (*n*=10) (PEG-IFN+RBV+HAART) | Control group (*n*=16) (only HAART) | *P* value |
|---|---|---|---|
| Male/female | 6/4 | 10/6 | 1.0 |
| Age, years | 35.5 ±3.5 | 38.6 ±6.8 | 0.19 |
| Elapsed time on NRTI, weeks | 194 ±54.9 | 131 ±66.5 | 0.02 |
| CD4+, cells/mm$^3$ | 559.9 ±219.7 | 670.6 ±251.2 | 0.26 |
| HIV load (<200 copies/mm$^3$) | 100% | 100% | – |
| Weight, kg | 62.1 ±7.7 | 65 ±13.6 | 0.49 |
| Body mass index, kg/m$^2$ | 21.3 ±1.7 | 22.4 ±2.7 | 0.36 |

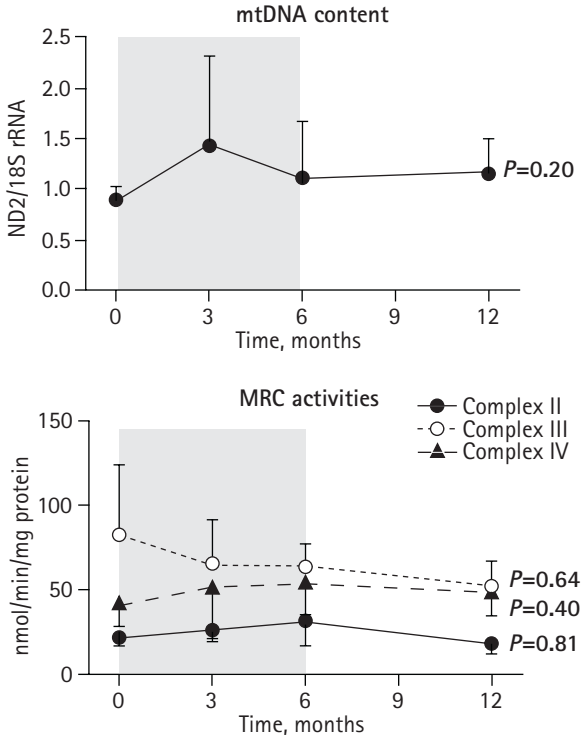
**Table 2.** Mitochondrial data at baseline and after 6 months in the HCV-treated group and the control group

| | HCV-treated group (*n*=10) (PEG-IFN+RBV+HAART) | Control group (*n*=16) (only HAART) | *P* value |
|---|---|---|---|
| DNA content (ND2/18S rRNA) | | | |
| At baseline | 0.9 ±0.1 | 1.2 ±0.6 | 0.07 |
| After 6 months | 1.1 ±0.6 | 1.0 ±0.6 | 0.68 |
| Complex II activity, nmol/min/mg protein | | | |
| At baseline | 22 ±2 | 24 ±9 | 0.86 |
| After 6 months | 31 ±14 | 25 ±11 | 0.23 |
| Complex III activity, nmol/min/mg protein | | | |
| At baseline | 82 ±41 | 55 ±24 | 0.08 |
| After 6 months | 63 ±14 | 69 ±34 | 0.54 |
| Complex IV activity, nmol/min/mg protein | | | |
| At baseline | 41 ±13 | 41 ±10 | 1.00 |
| After 6 months | 54 ±20 | 44 ±19 | 0.21 |

did not (LDCS <0). After initiating HCV therapy, there were no significant changes in LDCS from baseline to 24 weeks (–1.71 ±8.47 and –0.57 ±9.13; *P*=0.5) and none of the patients with an LDCS <0 developed lipodystrophy during the 48 weeks of follow-up. When we analysed this HCV-treated group according to whether patients presented lipodystrophy or not, we also failed to detect a significant difference for any of the mitochondrial parameters evaluated. Figure 4 shows the results of changes in mtDNA content related to lipodystrophy.

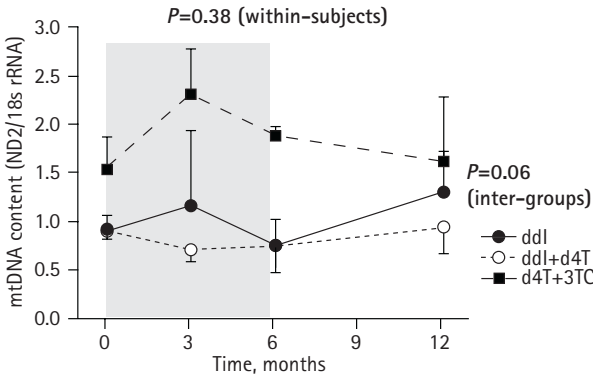
Although all HCV-treated patients received 800 mg of RBV daily, exposure adjusted by weight was different in every patient. Moreover, the mean daily RBV dosage by weight increased significantly between the determinations at baseline and 24 weeks (12.3 ±1.5 to 13.1 ±1.4 mg/kg; *P*=0.002) of HCV therapy. However, we did not find a significant correlation between the baseline dose of RBV adjusted by weight and the decrease in BMI (r= –0.09; *P*=0.79) or with the changes of LDCS (r=0.28; *P*=0.42)

**Figure 2.** Evolution of the mitochondrial parameters in patients from the HCV-treated group (treated with PEG-IFN plus RBV in addition to HAART)



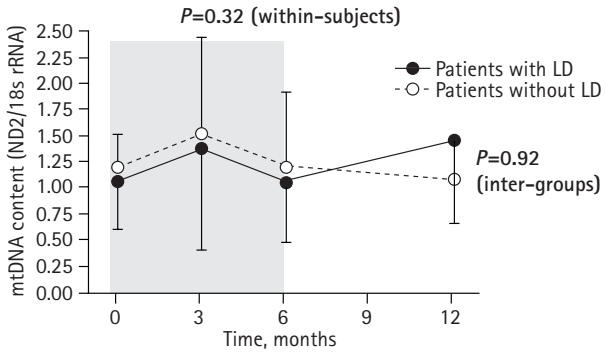

*P* values were calculated using the general linear model for repeated measures. Points denote the means and bars denote the SDs. Shadowed areas represent the time on treatment for HCV infection.

**Figure 3.** Evolution of mtDNA content in patients from the HCV-treated group (treated with PEG-IFN plus RBV in addition to HAART) according to the type of NRTI included in the HAART




*P* values were calculated using the general linear model for repeated measures. Points denote the means and bars denote the SDs. Shadowed area represents the time on treatment for HCV infection.

**Figure 4.** Evolution of mtDNA content in patients from the HCV-treated group (treated with PEG-IFN plus RBV in addition to HAART) according to whether they were suffering from lipodystrophy or not




*P* values were calculated using the general linear model for repeated measures. Points denote the means and bars denote the SDs. Shadowed area represents the time on treatment for HCV infection. LD, lipodystrophy.

## Conclusions

The main objective of our study was to assess the mitochondrial impact of a 24-week course of RBV plus PEG-IFN anti-HCV therapy in asymptomatic, non-cirrhotic HIV co-infected patients on long-term HAART with ddI, d4T or both. We failed to demonstrate significant changes in any of the mitochondrial parameters evaluated in PBMCs through three repeated measures over a 24-week period of HCV therapy. In addition, we observed no differences in mitochondrial parameters between those patients receiving RBV plus PEG-IFN and ddI, d4T or both and a control group receiving only a similar NRTI combination. Likewise, we found no association between mitochondrial parameters and lipodystrophy during HCV therapy. In our study population, the lack of differences between groups in the clinical and analytical parameters evaluated at baseline and the assessment of mitochondrial function in a parallel period of time strengthen the validity of the study design.

Several *in vitro* and clinical studies have demonstrated that NRTIs are associated with a decrease of mtDNA content and mitochondrial function [23–26], ddI and d4T being the most frequent NRTIs associated with mtDNA depletion [23,27]. An increased risk of mitochondrial toxicity attributed to the addition of RBV to a NRTI combination including ddI, d4T or ddI plus d4T has been found [6,9,28]. The mechanism suggested for the development of symptoms is the impairment of mitochondrial function due to a pharmacological synergistic effect between NRTIs and RBV. *In vitro* studies indicate that RBV may increase the intracellular metabolism of ddI into its active phosphorylated anabolite [2′,3′dideoxy-adenosine 5′-triphosphate (ddATP)], possibly leading to major inhibition of the DNA polymerase-γ activity [9,29] that is responsible for mitochondrial replication. The actual mechanism leading to an increase in mitochondrial dysfunction due to d4T and RBV interaction remains to be clarified, given the antagonist effect of RBV on the phosphorylation of d4T *in vitro*.

The life-threatening consequences of some adverse events reported in HCV/HIV co-infected patients on anti-HCV therapy including RBV and ddI, d4T or both, has prompted the USFDA to recommend avoiding ddI when treating HCV infection. The USFDA's Adverse Event Reporting System estimates an increased risk of mitochondrial toxicity in patients receiving RBV and ddI, or RBV with ddI and d4T [13]. However, the USFDA's assessments were based on reports of identified adverse events suggestive of mitochondrial toxicity rather than on knowledge of the real incidence of such toxicity in this context. Reports from large trials probably come closer to the true incidence. Among the 412 patients in the RIBAVIC study [6], six patients developed symptomatic hyperlactataemia and five developed pancreatitis, representing an overall incidence of mitochondrial toxicity of 2.6% during the study period. In the APRICOT study, the largest one performed to date in co-infected patients, the frequency of events related to mitochondrial toxicity was 2.3%, with similar distribution in arms with and without RBV [8]. Indeed, this incidence does not appear to be very different from the estimated rate of lactic acidosis (four to five cases per 1000 person-years) [30,31] or symptomatic hyperlactataemia (13–15 cases per 1000 person-years) [32] in HIV patients receiving a regimen including NRTIs, particularly when both ddI and d4T are used [31,33].

As mentioned, in asymptomatic HCV/HIV co-infected patients on long-term treatment with ddI, d4T or both (194 ±54.9 weeks), we did not find evidence of reduction in either mtDNA content or in MRC enzyme activity in PBMCs at any time point after the addition of RBV for 24 weeks, even in the two patients on ddI plus d4T-containing regimens for whom a tendency toward lower mtDNA content was observed. These results are different from those from a cross-sectional study in which mtDNA content fell in asymptomatic HIV patients who had received more than 24 weeks of ddI plus d4T therapy [21]. However, according to other authors, the decline in the mtDNA content occurs over the first 6 months of NRTI therapy with no evidence of decline beyond that time [34]. This finding may explain the stability of mitochondrial evaluation in two separate samples in our non-HCV-treated patient population. However, the low number of

patients on ddI plus d4T in our study must be taken into account when interpreting our results.

None of the patients in our study presented symptomatic hyperlactataemia or hyperamylasaemia during the follow-up period. We can hypothesize that in asymptomatic HCV/HIV co-infected patients with a supposed impairment in mitochondrial function at the time of initiating anti-HCV therapy, the addition of RBV needs additional triggers to produce symptomatic hyperlactataemia. Another explanation could be that in co-infected patients on anti-HCV therapy, symptoms associated with hyperlactataemia do not develop unless a threshold in the mitochondrial function has been achieved before the addition of RBV. Finally, it could be that including subjects who had tolerated 1 year or more of a HAART regimen containing d4T, ddI or both contributed to selecting patients with a greater chance of tolerating the addition of HCV therapy.

One of the factors acting as a trigger may be the inability of the liver to clear lactate. Interestingly, in the recent report by Moreno *et al.* [28], 49% of the patients were cirrhotic, which could partially explain their high rate of mitochondrial toxicity with ddI and RBV combination. However, in the Cox multivariate analyses performed by Moreno *et al.*, baseline amylase levels and a HAART regimen including three or more NRTIs but not liver histology at baseline, were predictors of mitochondrial toxicity-related adverse events. We excluded cirrhotic patients, so there might be a preserved compensatory clearance of lactate in our patients, which could underestimate the effects of RBV in cirrhotic patients suitable to receive anti-HCV therapy. Nevertheless, recent data from cirrhotic patients in the APRICOT study have stated ddI, but not RBV, to be associated with hepatic decompensation in the multivariate analysis [35]. Finally, other important triggers for toxicity have been described such as alcohol consumption [36], liver steatosis [37,38] and individual susceptibility [16].

Clinically, the proportion of dysfunctional mitochondria inside a cell, and therefore the proportion of cells with dysfunctional mitochondria in each organ system, may be relevant for the development of symptomatic complications such as lipodystrophy [16,39,40]. We did not detect a worsening of lipodystrophy in our patients. Moreover, the reversibility of the observed loss of BMI argues against a significantly increased effect of RBV on lipodystrophy, at least for a short period of time, although a cumulative toxic effect of RBV over longer periods cannot be firmly ruled out. We did not find a significant correlation between RBV exposure and anthropometric evaluations. Nevertheless, further studies in that setting are needed, especially when higher doses of RBV would be used to diminish the rate of RNA-HCV relapses in HCV/HIV co-infected patients [41].

We obtained our results by analysing PBMCs. It is theoretically possible that PBMC parameters underestimate the effects of drugs on mitochondria from other tissues and that decreased mtDNA content and MRC function or both – in tissues such as the liver or skeletal muscle – are in fact present during RBV and PEG-IFN therapy. However, we recently demonstrated that PBMCs provide a valid model with which to investigate the effects of HAART in HIV-infected asymptomatic individuals, even though those cells are not the target of the adverse mitochondrial effects of HAART [21]. Furthermore, the findings obtained from PBMCs are consistent with those obtained by Walker *et al.* [34] in hepatocytes and those obtained by Shikuma *et al.* [39] in adipocytes, in which a depletion in mtDNA content is mainly produced in NRTI-treated patients in comparison with naive patients. We suggest, therefore, that PBMCs constitute a good and easy model for studying precocious NRTI toxicity.

In summary, in this study population, mitochondrial parameters remained unaltered when RBV plus PEG-IFN were added for 24 weeks to HAART, even in patients taking the more harmful NRTIs for mitochondrial damage (ddI, d4T or both), suggesting that mitochondrial toxicity needs additional triggers to produce clinical manifestations of lactic acidosis. However, given the lack of reliable markers that allow lactic acidosis to be predicted before symptoms develop, as well as the seriousness of such a development in the absence of an effective treatment for it, the clinical implications of our findings should be interpreted with caution.

## Acknowledgements

Financial support was received from Red Temática Cooperativa de Investigación en SIDA (grant number RIS 173), Fundación para la Investigación y la Prevención del Sida en España (grant FIPSE 3102-00), Fundació La Marató de TV3 (grant number 020210) and the Clinical Research Support Foundations for AIDS Research (grant number 173) and for Mitochondrial Research (grant number V-2003-REDG011-O).

We thank ME Kerans for revising the language in the manuscript.

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Received 12 May 2004, accepted 30 September 2004

LOPINAVIR/RITONAVIR PLUS NEVIRAPINE AS A NUCLEOSIDE-SPARING APPROACH IN ANTIRETROVIRAL-EXPERIENCED PATIENTS (NEKA STUDY)

*JOURNAL OF ACQUIRED IMMUNE DEFICIENCY SYNDROME*

2005 Jan 1;38(1):47-52

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# Lopinavir/Ritonavir Plus Nevirapine as a Nucleoside-Sparing Approach in Antiretroviral-Experienced Patients (NEKA Study)

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**Objectives:** To compare the efficacy and safety of a nucleoside-sparing approach with a conventional highly active antiretroviral therapy (HAART) regimen in antiretroviral-experienced patients with prolonged viral suppression.

**Methods:** Pilot study including 31 antiretroviral-experienced patients with HIV RNA <80 copies/mL. Subjects were randomly assigned to lopinavir/ritonavir (LPV/rtv) 400/100 mg BID plus nevirapine (NVP) 200 mg BID (NVP group, n = 16) or LPV/rtv plus the 2 previous NRTIs (NRTI group, n = 15). The primary endpoint was the percentage of subjects who maintained viral suppression at week 48. Changes in lipid metabolism, mitochondrial parameters, and LPV trough levels were also assessed.

**Results:** All patients maintained viral suppression after 48 weeks. No subject discontinued therapy because of adverse events. HDL cholesterol increased by 28% at week 24 ($P < 0.0001$) and 10% after 48 weeks of follow-up ($P = 0.319$) in the NVP group. In the NRTI group, LDL cholesterol increased by 14% at week 48 ($P = 0.076$). Mitochondrial DNA/nuclear DNA ratio and mitochondrial respiratory chain complex IV activity showed a trend toward increasing in the NVP group. Mean (SD) LPV trough levels were 6340 (2129) ng/mL in the NRTI group and 5161 (2703) ng/mL in the NVP group ($P = 0.140$).

**Conclusions:** In antiretroviral-experienced subjects with sustained viral suppression, dual therapy with NVP plus LPV/rtv at standard dosage was as potent and safe as standard-of-care HAART at 48 weeks of follow-up. This approach may reduce mitochondrial toxicity and improve LPV/rtv-associated lipid abnormalities. The results of this pilot study support the study of this approach in a larger, randomized trial.

**Key Words:** NRTI-sparing regimen, nevirapine, lopinavir, mitochondrial toxicity, lipid profile, tolerability, pharmacokinetic study

(*J Acquir Immune Defic Syndr* 2005;38:47–52)

## INTRODUCTION

Limitations on achieving complete HIV eradication render it necessary to maintain highly-active antiretroviral treatment (HAART) over long periods,[1] which may lead to the development of antiretroviral-associated toxicities.[2–4] The current standard-of-care HAART regimens include a backbone of 2 nucleoside reverse transcriptase inhibitors (NRTIs). This antiretroviral family inhibits the mitochondrial gamma-DNA polymerase, and previous studies have shown decreases in mitochondrial DNA content to appear soon after starting NRTI-based treatments.[5] In addition, mitochondrial dysfunction has been recognized as one of the pathogenic mechanisms of NRTI-related side effects such as peripheral neuropathy, pancreatitis, liver dysfunction, or even lipoatrophy, which is one of the most stigmatizing features related to antiretroviral therapy.[6–10] Conversely, a relationship has been reported between protease inhibitor (PI)-based antiretroviral regimens and metabolic disorders such as hyperlipidemia, glucose intolerance, and changes in body shape, mainly fat accumulation.[2,3]

All these antiretroviral drug-derived toxicities may compromise treatment compliance and may represent a major obstacle for achieving durable control of viral replication.[4,11–13] Thus, different strategies aimed at avoiding or delaying these toxicities are currently under evaluation. Although NRTI-sparing

Received for publication March 10, 2004; accepted August 11, 2004.
José Moltó was supported by contract SCO/1620/2003 from the Fundació per a la Recerca Biomédica Germans Trias i Pujol in collaboration with the Spanish Health Department. Oscar Miró and Sònia López were supported by Fundación para la Investigación y la Prevención del Sida en España (FIPSE 3102-00), Fundació La Marató de TV3 (020210), Suport als Grups de Recerca (2001/00379), and Redes de Investigación en Mitocondrias (V-2003-REDG011-O) y en Sida (Redg173) del Fondo de Investigaciones Sanitarias.
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regimens may be an adequate approach for this purpose, data currently available on this strategy are rather scarce.[14–16]

The objective of this study was to assess the efficacy and safety of a standard dosage of lopinavir/ritonavir (LPV/rtv) in combination with nevirapine (NVP), as an NRTI-sparing simplification approach in antiretroviral-experienced subjects with long-term viral suppression.

## PATIENTS AND METHODS

### Design and Study Population

The Nevirapine-Kaletra Study (NEKA) study is a pilot, prospective, randomized, and open-label study conducted in antiretroviral-experienced patients with long-lasting viral suppression.

Patients included in this study had been on a PI- or a nonnucleoside reverse transcriptase inhibitor (NNRTI)-based HAART regimen for at least 9 months and had plasma HIV-1 RNA <80 copies/mL in at least 2 determinations within the previous 6 months. Subjects with previous experience with LPV/rtv, suspected or documented NNRTI resistance, or intolerance to NVP were excluded from the study. Transaminase levels >5 times above the upper normal limit, active opportunistic infection in the previous 6 months, poor treatment compliance, and pregnant or breast-feeding women were other exclusion criteria. The ethical committee of our hospital approved the trial and all subjects gave written consent before inclusion in the study.

### Study Medication

Subjects were randomly assigned to replace the current PI or NNRTI with LPV/rtv (Kaletra, Abbott Lab, Abbott Park, IL, USA, 400/100 mg BID), maintain the 2 NRTIs (NRTI group), or switch therapy to NVP (Viramune, Boehringer, Ingelheim Gmbh, Ingelheim am Rheim, Germany, 200 mg BID) plus LPV/rtv (400/100 mg BID) (NVP group). Patients without previous exposure to NVP received a daily dose of 200 mg for the first 2 weeks, increasing to 200 mg of NVP twice daily thereafter.

### Endpoints

The primary endpoint of the study was the percentage of subjects from each group who maintained viral suppression at week 48. Secondary endpoints included changes in CD4 and CD8 cell counts as well as in liver enzymes, lipid metabolism, and mitochondrial parameters during follow-up. Plasma LPV and NVP trough levels were also assessed at steady-state conditions.

### Follow-up and Assessments

Adverse events and physical examination were assessed at baseline, at weeks 4 and 12, and every 12 weeks thereafter. Blood samples for HIV-1 RNA quantification, $CD4^+$ and $CD8^+$ T-cell count, hematology, clinical chemistry, and lipid profile evaluations (including total cholesterol, high- and low-density lipoprotein [HDL and LDL] cholesterol, triglycerides, and apopoproteins A and B) were taken after at least 8 hours' fasting, at baseline and every 12 weeks. Self-perceived and dual-energy x-ray absorptiometry (DEXA)-assessed lipodystrophy was evaluated at baseline and every 24 weeks during the follow-up, as well as anthropometric measurements (weight, body mass index, waist-hip ratio, and circumferences by Harpenden anthropometric tape). Lipodystrophy was defined by patients' report of peripheral fat wasting (upper and lower extremities, face, or buttocks) or central fat accumulation (abdominal, dorsocervical, or breast) and confirmed by physician.

HIV-1 RNA was quantified by NASBA assay (Organon, Teknika, Barcelona, Spain; limit of detection of 80 copies/mL). Flow cytometry was used to perform $CD4^+$ and $CD8^+$ T-lymphocyte counts.

Peripheral blood mononuclear cells (PBMCs) were obtained at baseline and at week 48 in the first 11 recruited patients. The ratio between mitochondrial (mt) and nuclear (n) DNA was quantified using real-time polymerase chain reaction with fluorescent detection[17] (LightCycler, Fact Sheet DNA Meter, SYBR Green I, Roche Molecular Biochemicals®, Mannheim, Germany). Additionally, changes from baseline in respiratory mitochondrial chain (RMC) complex III and IV activities were spectrophotometrically assessed at week 48. mtDNA content and RMC enzyme activities were expressed both by cell (normalizing by protein content measured through Bradford's methodology) and by mitochondria (normalizing by citrate synthase). In all cases, results were expressed as percentages of activity with regard to baseline (100%).

LPV and NVP plasma trough levels were determined in steady-state conditions by high-performance liquid chromatography and were considered to be suboptimal if they were <5000 and 3400 ng/mL, respectively.[18–21]

### Statistical Analysis

Comparisons between groups were performed using SPSS Version 10.0 statistical software (Cary, NC). Variable distribution was explored by the Kolmogorov-Smirnov test. Variables with a normal distribution were described as mean (SD) and compared with the Student *t* test. Median and interquartile range (IQR) were employed to describe variables that did not follow a normal distribution, which were compared by using Mann-Whitney nonparametric test. Proportions were compared by a $\chi^2$ test or Fisher exact test, where appropriate. Differences were considered statistically significant at $P < 0.05$.

Only patients whose outcomes were measured at specific time points were included in the on-treatment analysis. The intention-to-treat analysis involved all patients enrolled in the study, and those who discontinued treatment were considered as failures. Missing plasma viral load observations were considered as failures unless they had been <80 copies/mL immediately before and after the missing value, in which case it was assumed to be undetectable.

## RESULTS

### Study Population

A total of 31 patients were included into the study between May and December 2001. Sixteen subjects were randomly assigned to receive NVP plus LPV/rtv (NVP group), and 15 maintained their current 2-NRTI backbone and only replaced the PI or NNRTI by LPV/rtv (NRTI group). Baseline characteristics were similar between the 2 groups (Table 1).

**TABLE 1.** Baseline Characteristics of Patients Enrolled

| | Total n = 31 | NVP Group n = 16 | NRTI Group n = 15 | *P* |
|---|---|---|---|---|
| Gender | | | | |
| Male | 24 (77) | 13 (81) | 11 (73) | |
| Female | 7 (23) | 3 (19) | 4 (27) | NS |
| Age, y, mean (SD) | 37.6 (11.2) | 41.2 (8.1) | 32.6 (14.2) | NS |
| Risk group | | | | |
| IVDU | 8 (26) | 4 (25) | 4 (27) | |
| Homosexual | 13 (42) | 7 (44) | 6 (40) | |
| Heterosexual | 10 (32) | 5 (31) | 5 (33) | NS |
| AIDS | 7 (22) | 3 (19) | 4 (27) | NS |
| Clinical lipodystrophy | 8 (26) | 4 (25) | 4 (27) | |
| Baseline ARV therapy | | | | |
| NRTIs + PI | 18 (58) | 9 (56) | 9 (60) | |
| NRTIs + NNRTI | 13 (42) | 7 (44) | 6 (40) | |
| NRTIs | | | | |
| AZT+3TC | 8 (26) | 5 (31) | 3 (20) | NS |
| ddI+d4T | 7 (23) | 3 (19) | 3 (20) | |
| ABV+ddI | 6 (19) | 3 (19) | 3 (20) | |
| 3TC+d4T | 3 (10) | 2 (13) | 1 (7) | |
| Other (ddI+3TC, ddI+TDF…) | 7 (23) | 3 (19) | 5 (33) | |
| Years since HIV diagnosis, mean (SD) | 7.3 (4.9) | 6.2 (4.6) | 9.6 (4.6) | NS |
| Months since last detectable HIV-1 RNA, mean (SD) | 19.8 (10.6) | 18.6 (4.4) | 21.7 (16.6) | NS |
| $CD4^+$ T-cell count (cells/$mm^3$), mean (SD) | 547 (238) | 555 (195) | 645 (348) | NS |

Data are n(%) except where noted.

ABV, abacavir; ARV, antiretroviral; AZT, zidovudine; ddI, didanosine; d4T, stavudine; IVDu, intravenous drug user; 3TC, lamivudine; TDF, tenofovir disoprokil fumarate.

Two subjects, both assigned to the NVP group, were lost to follow-up.

## Virologic and Immunologic Assessments

At week 48 of follow-up, all patients maintained viral suppression in the on-treatment analysis. Likewise, in the intention-to-treat analysis, plasma viral load was <80 copies/mL in 87.5% of the subjects in the NVP group and in 100% of the patients in the NRTI group ($P = 0.484$).

Absolute $CD4^+$ T-cell count increased in both study groups during follow-up while $CD8^+$ T-cell count remained stable (Fig. 1). At week 48, mean (SD) increase from baseline in CD4 cell count was 300 (124) cells/$mm^3$ in the NVP group (95% CI 37–563; $P = 0.028$), and 155 (197) cells/$mm^3$ in the NRTI group (95% CI −328 to +639; $P = 0.462$). There were no significant differences between the 2 arms in absolute $CD4^+$ T-cell count at week 48 ($P = 0.813$).

## Adverse Events

The proportion of patients who developed adverse events during follow-up was similar in both study groups (37.5% in the NVP group and 33.3% in the NRTI group; $P = 0.894$). Gastrointestinal complaints (diarrhea, vomiting, and abdominal disturbances) were the most frequently observed adverse events. They mainly appeared during the first 12 weeks of therapy, were usually mild (grade I or II) and transient in all the subjects, and no patient discontinued the study medication because of them.

Mean transaminase levels did not show any significant change from baseline, and no patient developed acute clinical hepatitis during follow-up. Likewise, mean GGT values did not significantly vary in patients in the NVP group (from 100 U/L at baseline to 72 U/L at week 48; $P = 0.365$) or in the NRTI group (from 76 U/L at baseline to 35 U/L at week 48; $P = 0.540$), without statistically significant differences between the 2 arms after 48 weeks of follow-up ($P = 0.082$).

## Lipid Profile and Lipoprotein Assessment

Total cholesterol levels increased in both arms during follow-up (Fig. 2). At week 48, percentage augmentation in total cholesterol levels was 14% in the NVP group ($P = 0.039$) and 13% in the NRTI group ($P = 0.036$), without significant differences between the groups ($P = 0.400$). HDL cholesterol increased by 28% at week 24 ($P < 0.0001$) and by 10% at week 48 ($P = 0.319$) in the NVP group but decreased by 13% at week 48 in the NRTI group ($P = 0.138$). The same was observed for apoprotein AI levels, with a mean increase of 12.6% at week 24 ($P < 0.0001$) and 6.8% at week 48 ($P = 0.452$) in the NVP group, while it did not vary in the NRTI group (mean increase of 0.8% at week 48, $P = 0.843$). As for the LDL cholesterol and apoprotein B levels, there were no significant changes among the patients in the NVP group during the study. On the contrary, subjects assigned to the NRTI group presented a percentage increase of 14% in LDL cholesterol ($P = 0.076$) and of 37% in apoprotein B levels ($P = 0.004$) at week 48 of follow-up (Fig. 2).

Although mean LDL/HDL cholesterol ratio increased in the NRTI group during follow-up (from 2.07 at baseline to 2.5

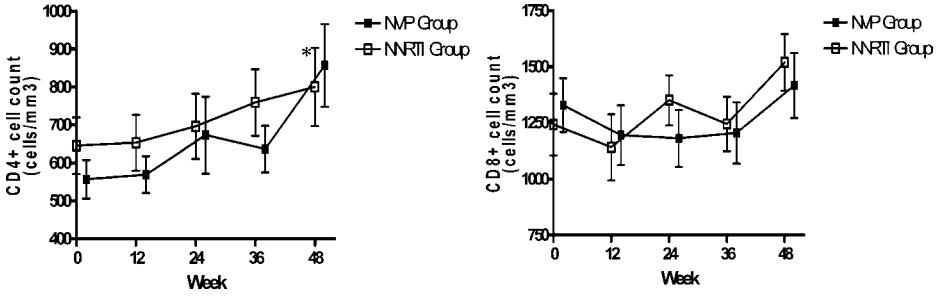


**FIGURE 1.** Changes in absolute $CD4^+$ and $CD8^+$ T-cell count during follow-up in the NVP and NRTI groups. *$P < 0.05$ for comparisons from baseline.

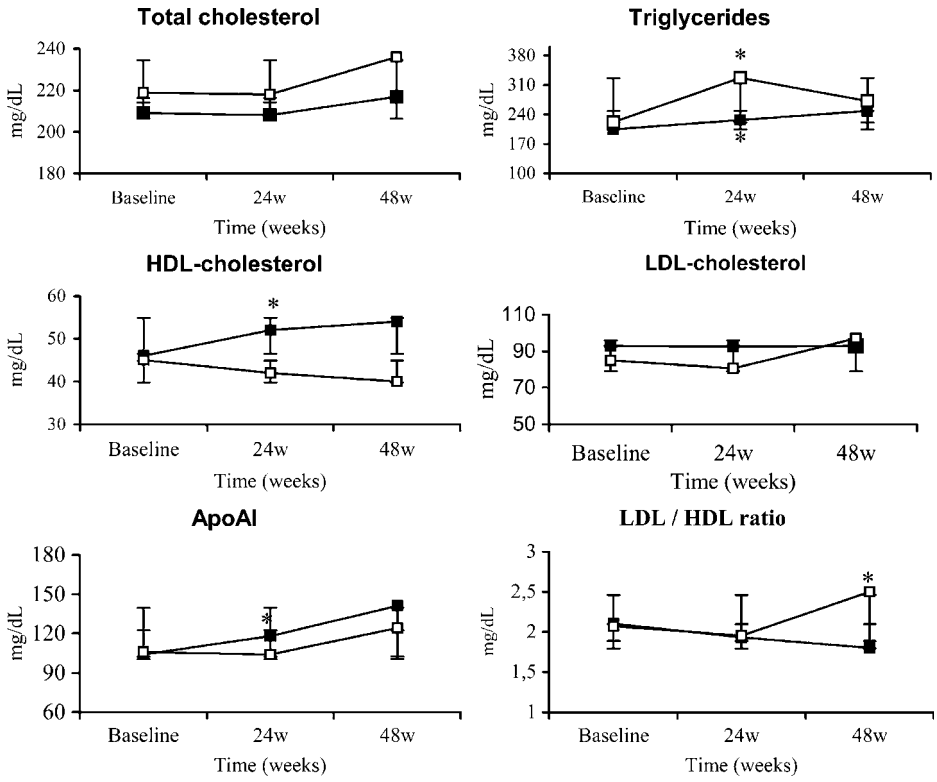


**FIGURE 2.** Changes in lipid profile during follow-up in the NVP group (*filled squares*) and the NRTI group (*open squares*). *$P < 0.05$ for comparisons from baseline.

at week 48; $P = 0.02$), it decreased slightly in the NVP group (from 2.1 at baseline to 1.8 at week 48; $P = 0.10$) (Fig. 2).

Fasting triglyceride levels augmented from baseline in both study groups. In the NRTI group the percentage increase from baseline was 103.8% ($P = 0.017$) and 18% ($P = 0.120$) at weeks 24 and 48, respectively. Likewise, patients in the NVP group experienced a mean increase in triglyceride levels of 25.8% ($P = 0.048$) and 56% ($P = 0.056$) at weeks 24 and 48, respectively. There were no significant differences regarding the fasting triglyceride levels at week 48 between groups (Fig. 2).

Two patients in the NVP group and 1 in the NRTI group were receiving lipid-lowering agents at baseline. However, 1 subject in the NVP group was able to stop pravastatin during follow-up. On the contrary, 2 patients in each group had to start statins during the study, following the National Cholesterol Education Program criteria.

## Mitochondrial Toxicity and Lipodystrophy Assessments

After 48 weeks of NRTI discontinuation, mtDNA/nDNA ratio reached a median increment of 36 and 117% according to whether the results were considered by cell or by mitochondria, respectively, although such increases did not reach statistical significance ($P = 0.22$ and $P = 0.17$, respectively). On the contrary, no substantial changes in the mtDNA/nDNA ratio were observed among subjects who continued NRTI therapy by cell ($P = 0.68$) or by mitochondria ($P = 0.66$). Regarding the mitochondrial respiratory chain, complex III and IV activity remained quite stable throughout the study in both groups of patients, but a trend toward increased complex IV activity expressed by mitochondria was observed for patients who discontinued NRTIs ($P = 0.05$).

A new case of self-perceived lipoatrophy was reported during follow-up in the NRTI group. A loss of fat in lower extremities and buttocks was evidenced through the study in this subject, who was receiving didanosine, lamivudine, and LPV/rtv. On the contrary, 2 subjects assigned to the NVP group had a marked improvement in peripheral lipoatrophy after the cessation of NRTIs, zidovudine and lamivudine in both cases, which was confirmed by anthropometric measurements. However, DEXA scans did not show any significant change in fat in any of the study groups.

## Pharmacokinetics

Mean NVP trough level was 6611 (2207) ng/mL in patients assigned to the NVP group, and no subject presented suboptimal NVP trough levels.

Mean (SD) lopinavir trough levels were 6340 (2129) and 5161 (2703) ng/mL in the NRTI and in the NVP group, respectively ($P = 0.140$). Although 44% of the patients in the NRTI group and 58% of the subjects in the NVP group presented LPV trough levels $<5000$ ng/mL ($P = 0.369$), all patients maintained LPV trough levels above the previously reported protein-binding corrected 95% CI for wild-type HIV-1.[19,20]

# DISCUSSION

NVP plus standard doses of LPV/rtv showed similar antiviral potency to that of conventional HAART regimens in HIV-infected patients with prolonged viral suppression. Additionally, this dual NRTI-sparing approach was safe and well

tolerated, improved lipid profile, and showed a trend toward increasing mtDNA content and RMC enzyme activities.

Despite 48 weeks of dual antiretroviral therapy, the maintenance of viral suppression in all our patients may be accounted for by the high antiviral activity of this drug combination. Previous studies with LPV/rtv monotherapy have reported a similar antiretroviral potency to that of conventional 3-drug antiretroviral regimens, as well as a high proportion of patients maintaining viral suppression.[22,23] In addition, lopinavir reaches high enough plasma levels to provide an elevated inhibitory quotient, precluding the development of viral resistance.[24] These issues may have contributed to the virologic success of this strategy.

Although it is currently recommended to increase LPV/rtv dosage when it is combined with NNRTIs,[25] our patients received a standard dosage of LPV/rtv to preserve comparability between the treatment groups regarding LPV/rtv-associated toxicity. The proportion of our patients with suboptimal LPV plasma trough levels was similar between the 2 study groups. In addition, all patients showed LPV levels above the protein-binding corrected 95% CI described for the wild-type HIV-1 strains.[19,20] These data agree with a previous study showing successful antiviral efficacy in patients receiving standard dosage of LPV/rtv plus NVP and 2 NRTIs.[26] The high LPV inhibitory quotient may help to maintain viral suppression in patients with limited PI exposure and undetectable viral load despite the drugs being given at standard doses. Nevertheless, until more experience in this regard is available, monitoring lopinavir plasma levels should be recommended in all patients receiving NVP plus LPV/rtv at standard dosage.

In our study no differences were found regarding body shape changes between groups. Interestingly, only patients who discontinued the NRTIs tended to improve mitochondrial parameters. These data agree with other data reported[5] and encourage considering NRTI-sparing approaches to limit NRTI-induced mitochondrial damage and the consequent development of lipoatrophy.

In our study, the heterogeneity of NRTI combinations received at enrollment should be taken into account due to the different impact of each nucleoside analogue on mitochondrial toxicity. The continuation of stavudine in almost one-third of patients in the NRTI group may have had a strong influence in the lack of improvement of mitochondrial parameters in this group. However, none of the 3 patients who reported changes in fat redistribution during the study were receiving stavudine at baseline. This supports the significant impact on mitochondrial function of other nucleosides beside stavudine. Although this remains to be confirmed, our results suggest an association between the improvement in mitochondrial parameters in PBMCs and the recovery of fat loss. The easiness and reproducibility of the PBMC mitochondrial study should prompt further studies to confirm its clinical usefulness.

In addition, increased adipocyte apoptosis in subcutaneous fat, which has been proposed as the final pathogenic mechanism of lipoatrophy, could render the morphologic changes in subcutaneous fat irreversible.[27] As a consequence, NRTI-sparing regimens might be offered to HIV-infected patients ideally before or at the very beginning of lipoatrophy development.

Lipid disorders such as hypercholesterolemia or hypertriglyceridemia have been related to the use of LPV/rtv and some NRTIs.[22,28,29] We observed an increment in fasting total cholesterol and triglyceride levels in both study groups after enrollment. Nonetheless, only the subjects in the NVP group experienced an increase in HDL cholesterol and apoprotein AI levels, both related to a lower vascular risk. In contrast, LDL cholesterol increased in subjects assigned to the NRTI group, which may favor the development of atherosclerosis. Previous studies, including either antiretroviral-naive patients starting an NVP-based HAART regimen[30] or those switching from PIs to NVP,[31–34] sustain the beneficial effect of this NNRTI on lipid profile. Our study revealed a similar benefit on lipid metabolism even when NVP is combined with a PI. This better atherogenic profile in patients receiving NVP plus LPV/rtv may imply a lower risk of developing cardiovascular events in this population.

In conclusion, dual antiretroviral therapy with NVP plus LPV/rtv at standard dosage was as potent and safe as standard HAART care regimens after 48 weeks in antiretroviral-experienced subjects with prolonged viral suppression. Our results support the suitability of this NRTI-sparing approach to prevent mitochondrial damage and NRTI-related adverse events, as well as to ameliorate PI-associated lipid abnormalities. Nevertheless, due to the small size of the study, the benefits of this approach should prompt a larger, randomized trial.

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MITOCHONDRIAL RESPIRATORY CHAIN IN BRAIN HOMOGENATES: ACTIVITIES IN DIFFERENT BRAIN AREAS IN PATIENTS WITH ALZHEIMER'S DISEASE

*AGING CLINICAL AND EXPERIMENTAL RESEARCH*

2005;17(1):1-7

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ORIGINAL ARTICLES

# Mitochondrial respiratory chain in brain homogenates: activities in different brain areas in patients with Alzheimer's disease

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*ABSTRACT.* ***Background and aims:*** *The potential influence of impaired oxidative metabolism in the modulation of manifestations in sporadic Alzheimer's disease (AD) has attracted much attention in the last 50 years. Unfortunately, many clinical and experimental results aiming at proving this hypothesis are still controversial. The aim was to study the enzymatic activities of respiratory chain (RC) complexes I through V in three brain areas of a group of patients with definite AD, and to compare the results with a group of normal brains. We simultaneously assessed the lipid peroxidation of the samples as a measure of free radical damage.* ***Methods:*** *The specific activity of the individual complexes of the RC was measured spectrophotometrically, and the loss of cis-parinaric acid fluorescence was used to determine the chemical process of lipid peroxidation.* ***Results:*** *We were not able to detect differences in any of the analyzed RC enzymatic activities, or in the level of lipid peroxidation between patients with AD and controls. Instead, differences were found in the number of mitochondria and in the intrinsic enzymatic activities of complexes III and IV in various brain areas.* ***Conclusions:*** *Spectrophotometric enzymatic analyses of respiratory complexes in brain homogenates do not support the primary contribution of mitochondrial RC dysfunction in the pathogenesis of AD.*

*(Aging Clin Exp Res 2005; 17: 1-7)*



## INTRODUCTION

Sporadic Alzheimer's disease (AD) is the most common neurodegenerative disease. It is clinically characterized by early impairment of recent memory, followed by progressive intellectual dysfunction (1). Definite diagnosis relies on pathological studies, which disclose selective pyramidal neuronal death, accumulation of intraneuronal neurofibrillary tangles and extracellular fibrillar senile plaques (2).

Extensive data accumulated since the late 1940s have documented that the metabolic rate for oxygen is decreased in AD brains, supporting the hypothesis that impaired oxidative metabolism plays a role in the development of clinical disability (for a recent review, see ref. 3). On the other hand, epidemiological data show a female-to-male ratio of 3.6-3.8:1 in the parental generation of probands, suggesting that a maternally inherited genetic factor may be involved (4, 5).

As mitochondria are the main source of energy in the cell and as mitochondrial DNA (mtDNA) is inherited *via* the maternal line, many studies have been directed toward their study. Findings have been abundant but very variable (6-8). Initial work suggested that point mutations in the mitochondrial-encoded cytochrome *c* oxidase subunit genes segregated with AD patients (9), but more recent evidence shows that the presumed mutations were polymorphisms in nuclear pseudogenes (10). Other mutations in mitochondrially encoded Complex I or tRNA genes (11, 12) have not been confirmed by other studies (13, 14).

In the absence of uniform mtDNA findings, and as this genome only codes for proteins of the mitochondrial respiratory chain (RC), a mitochondrial bioenergetic defect – and specifically a RC defect – may be the clue to impute a pathogenic role to mitochondria in AD. Again, several kinds of dysfunction have been described,

*Key words:* Alzheimer's disease, dementia, free radicals, mitochondria, neurodegenerative disorders, respiratory chain.

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Received July 23, 2003; accepted in revised form March 3, 2004.

a reduction in complex IV activity being the most frequent finding. This reduction has been described in platelets and fibroblasts (15, 16), although it has been neither confirmed by other investigators (17), nor further reported. Other extraneurological tissues such as lymphocytes disclose normal mitochondrial function (18). In postmortem brain tissue, although the most frequent finding is reduced complex IV enzyme activity (19-22), other authors have found normal complex IV activity, whereas activities of complexes II and III (23), or complex V (24), are decreased.

The reasons for these discrepancies are unclear, although they are probably explained by methodological factors such as variations in AD diagnostic criteria, methods in enzymatic studies, the influence of the age of patients, or delay in processing samples after death. In 1997, a cytochrome *c* oxidase defect was transferred from AD platelets to cybrids, and the ensuing cybrid cell lines showed increased free radical production (25). This finding suggested that the variability in mitochondrial genetic and enzymatic findings may reflect secondary changes as a result of the progressive accumulation of free radicals in mitochondria (26, 27). In normal conditions, about 5% of the oxygen consumed by mitochondria is converted into free radicals, as by-products of the RC (28). As free radicals are highly reactive species, it is conceivable that they cause damage near the site of their formation, i.e., mtDNA and RC itself. This would further impair the function of the respiratory machinery which, in turn, would promote the generation of more free radicals, oxidative damage of membrane phospholipids and proteins, and more mtDNA mutations, in a sort of catastrophic vicious cycle (29). Similar mechanisms based on a free radical hypothesis have been proposed to modulate the expression of other neurodegenerative diseases, atherosclerosis, and even aging itself (30-32). However, until now, this admittedly very attractive possibility remains speculative.

In an attempt to gain additional insights into this issue, we took advantage of the brain material stored in the Brain Bank, University of Barcelona-Hospital Clínic. In all cases, the material was carefully classified from a neuropathological point of view, and hypothetical influencing and confounding variables were recorded. Complete RC analysis and appraisal of brain lipid peroxidation as a marker of free radical damage was undertaken.

## METHODS

The Brain Bank of the University of Barcelona-Hospital Clínic was created in 1990. It stores brains obtained from voluntary donations belonging either to patients with neurological illnesses, or to "normal" individuals – from a neurological point of view – who died in diverse circumstances. In all cases, a standard data collecting protocol is followed, including thorough neuropathological examinations to establish the final neuropathological diagnosis (33, 34).

Twelve brains that fulfilled the pathological criteria of definite AD, with Braak's staging V or VI, were included in the present study. Eight normal brains were added as a control group. From each brain, samples of approximately 250 mg were obtained from the frontal cortex, cerebellar cortex and hippocampus. All biochemical and enzymatic studies were performed consecutively over a 6-week period of time.

### *Preparation of homogenates*

Homogenates from frozen brain areas 3.5% (w/v) were prepared in 50 mM of Tris-buffer (pH 7.5), 100 mM of potassium chloride, 5 mM of magnesium sulfate, and 1 mM of ethylene diaminetetraacetic acid. Tissues were disrupted by 7 strokes at 800 rpm in a glass-Teflon homogenizer and filtered through two layers of cheesecloth. The homogenates were centrifuged at 2000 rpm for 3 min. Pellets were discarded and the supernatant used for biochemical studies. Protein content in the supernatant was measured according to Bradford's protein-dye binding principle.

### *Mitochondrial respiratory chain enzyme assays*

Measurement of the specific activity of the individual complexes of the respiratory chain was performed spectrophotometrically (UVIKON Spectrophotometer 922, KONTRON Instruments, Zurich, Switzerland). A total of 40-70 µg of homogenate protein was used to determine the activity of each complex, except for complex IV, for which 8-15 µg of protein were used (35). Measurements of complex I (Rotenone-sensitive NADH-Decylubiquinone Oxidoreductase), complex II (Succinate Decylubiquinone DCPIP Reductase), complex III (Ubiquinol Cytochrome *c* Reductase), complex IV (Cytochrome *c* Oxidase), and complex V (Oligomycin-sensitive ATP-synthase) were performed at 37ºC in 1 mL of medium (36, 37). Measurement of citrate synthase (CS) was performed at 412 nm following the reduction of 2 mM of 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of 0.1 mM acetyl-CoA and 12 mM oxalacetic acid in 1 mL of medium containing 10 mM $KH_2PO_4$ (pH 7.8, 37ºC), 2 mM EDTA, 1 mg/ml BSA and 0.1% Triton X-100. Enzyme activities were expressed as nmol of reduced or oxidized substrate $\cdot min^{-1} \cdot$mg of protein$^{-1}$.

### *Relative enzymatic activities*

CS is a Krebs-cycle enzyme outside the respiratory chain which may be used as a marker of the number of mitochondria present in homogenates. When the activities of RC complexes are normalized for CS, 'relative' activities are obtained, which better reflect the intrinsic activity of each enzyme in a given tissue.

Table 1 - *Main clinical characteristics of patients and controls.*

| | Age | Sex (F/M) | Duration of disease | Smoking habit | Drugs | Time after death |
|---|---|---|---|---|---|---|
| AD patients | | | | | | |
| n=12 | 76.2±6.9 | 6/6 | 4.8±3.7 | 5 | Neuroleptics, 4<br>Benzodiazepines, 3 | 13.2±6.2 |
| Controls | | | | | | |
| n=8 | 70.4±6.0 | 3/5 | -- | 4 | Benzodiazepines, 1 | 14.1±5.8 |
| | *p*=NS | *p*=NS | -- | *p*=NS | *p*=NS | *p*=NS |

Age and duration of disease expressed in years ± SD.
Time from death refers to delay in processing samples after death, expressed in hours ± SD.

### *Lipid membrane peroxidation*

Brain homogenates (100 µg protein) were labeled with 5 µM *cis*-parinaric acid (Molecular Probes, Eugene, OR) in a cuvette containing 3 mL of nitrogenized PBS. They were then incubated in the dark at 37ºC, and fluorescence at 318-nm excitation and 410-nm emission was measured at 3-min intervals over 30 min, as described (38). Measures were related to the first determination in each case, and the loss of *cis*-parinaric acid fluorescence was used to measure the chemical process of peroxidation (the greater the loss, the higher the peroxidation).

### *Statistical analysis*

Results are given as means ±standard deviations (SD). Fisher's exact test was used to compare qualitative variables. For quantitative variables, a Kolgomorov-Smirnov goodness-of-fit test was used to ascertain if samples were normally distributed. A two-way ANOVA for repeated measures was used to test differences in enzymatic activities or peroxidation in different brain areas between AD patients and controls. This approach means that, if there are no one-way differences (for example, between groups), from a statistical point of view the groups are considered together to test the other way (for example, between areas) and *vice versa*, increasing the overall power of the analysis. A p-value of less than 0.05 was considered statistically significant. If the ANOVA was significant, a Tukey *post-hoc* test was applied to determine where the difference lay.

## RESULTS

There were no differences when comparing the groups of AD patients and controls as regards age, sex, duration of disease, smoking habit, psychoactive drugs taken at the time of death, or mean time elapsing from death to freezing of brain material (Table 1). The time elapsing after death to collecting and storing brain material ranged from 4.5 to 21.5 hours. During this period, there was a slight decrease in all enzymatic activities analyzed, but in any case it did not reach statistical significance, either considering AD patients and controls as independent groups or taking them together (Fig. 1).

Analysis of enzymatic activities revealed that the samples were normally distributed.

CS activity did not differ between AD patients and controls. Instead, there were significant differences among brain areas for CS activity, indicating that the number of mitochondria was higher in the cerebellum, followed by frontal cortex and hippocampus (Fig. 2). When the activities of the respiratory chain complexes were normalized for CS, no differences were found between AD patients and controls (Fig. 3). On the contrary, there were differences regarding the activities for complexes III and IV in different brain areas. In both cases, maximum activity occurred in the frontal cortex, followed by hippocampus and cerebellum (Fig. 3).

As regards membrane lipid peroxidation studies, we did not find any differences between AD patients and controls, or among the three areas of the brain analyzed (Fig. 4). The degree of lipid peroxidation did not correlate with the time elapsing after death (data not shown).

## DISCUSSION

In the present study, we found that both RC enzymatic activities and the number of mitochondria vary among the three brain areas examined. Once the activities of RC enzymes were corrected for the number of mitochondria present in homogenates, the intrinsic enzyme activities were different for complexes III and IV, maximal activity occurring in the frontal cortex, followed by hippocampus and cerebellum. Apart from the biological interest of this observation, which confirms that the central nervous system is very heterogeneous in terms of mitochondrial bioenergetics (39), these variations are useful as an internal check, to ensure that the sensitivity of enzymatic measurements undertaken in the present study were sufficient to detect subtle variations in activity.

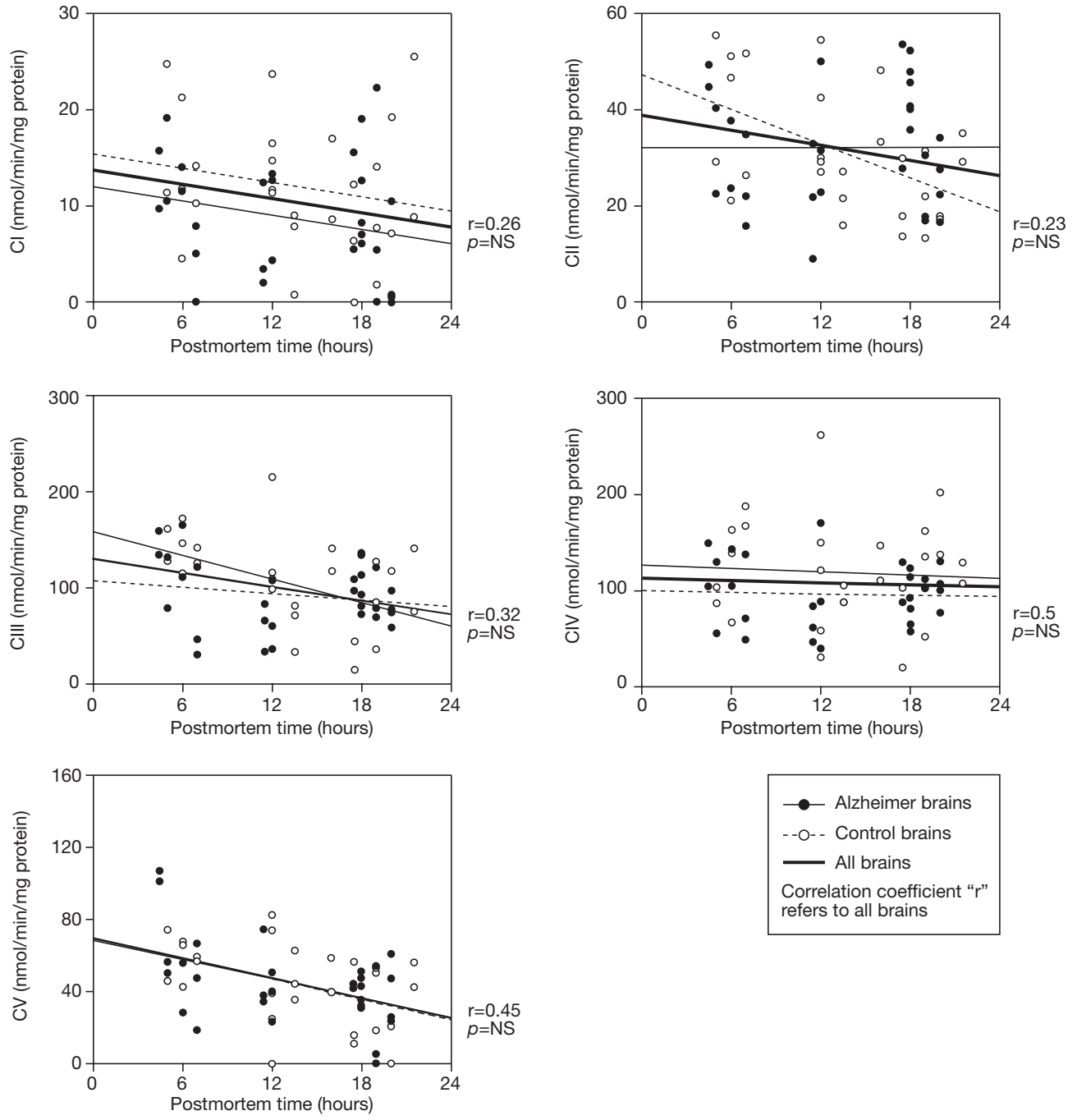


Figure 1 - *Activities of respiratory chain complexes related to time after death. In no case did tendency to decrease reach statistical significance in this period of time. Note that for complex V, three regression lines overlap, so that there appears to be only one line.*

Instead, we were not able to detect differences in any of the analyzed RC enzymatic activities between AD patients and controls. As our AD patients were in an advanced stage of disease at the time of death, and as the control patients did not present any pathological criteria of AD, the lack of significant differences between the two groups argues against inherent impairment in the ability of the brain to oxidize substrates *via* the various respiratory chain complexes. Similarly, we did not find any differences in the level of membrane lipid peroxidation, as an indirect

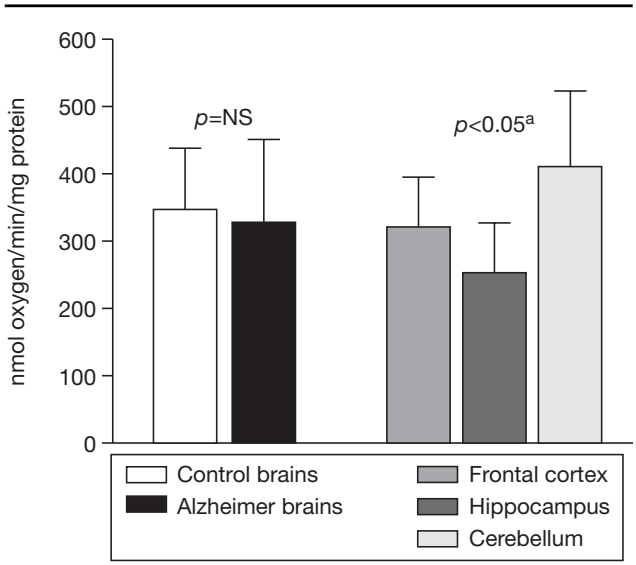


Figure 2 - *Citrate synthase activity as indicator of number of mitochondria in brain homogenates. No differences were found between AD patients and controls, but differences were statistically significant when different brain areas were compared. Tukey* post-hoc *test indicated that differences lay between cerebellum and either frontal cortex or hippocampus ([a]). Bars: means; error bars: SD.*

method of ascertaining free radical damage. This leads to the conclusion that free radical damage plays no particularly important role in the modulation of clinical manifestations of AD, at least through damage to RC complexes.

One limitation of the present study is that enzymatic measurements were made in autopsy brain material. Nonetheless, we demonstrate that brains obtained at autopsy can be reliably used to study the activity of mitochondrial RC enzymatic activities, provided that samples are correctly frozen and stored within an interval of 4 to 21.5 hours after death. We base this conclusion on the fact that, although during this period there was a decrease in the activity of the individual complexes, including complex I, classically considered the most prone to lose activity (40), this decrease was not statistically significant (Fig. 1).

The reliability of enzymatic studies in biological material obtained postmortem has generated many debates. This is probably one of the reasons for the relatively low number of reports using this approach to evaluate mitochondria, despite the fact that abnormal function is essential to impute a pathogenic role to genetic, structural or other defects. A conclusion similar to ours was previously obtained using mouse brains as an experimental model (41). The preservation of brain material for enzymatic analyses also seems to apply to lipid peroxidation studies, which may be performed in the above-mentioned period of time without any significant decrease in fluorimetry related to time postmortem. Experimental procedures support the suggestion that oxidative damage does not play a role in these ranges of postmortem time (42).

A second limitation is the number of patients (12) and controls (8) analyzed, due to the difficulty in finding appropriate material for our study. It may be argued that the absence of enzymatic decreases in AD is due to a statistical error type 2, i.e., low power to detect significant differences due to a small sample. To overcome the difficulty in increasing the number of samples, we studied three brain areas for every individual, using a two-way ANOVA for comparisons. In fact, it is as if we had stud-

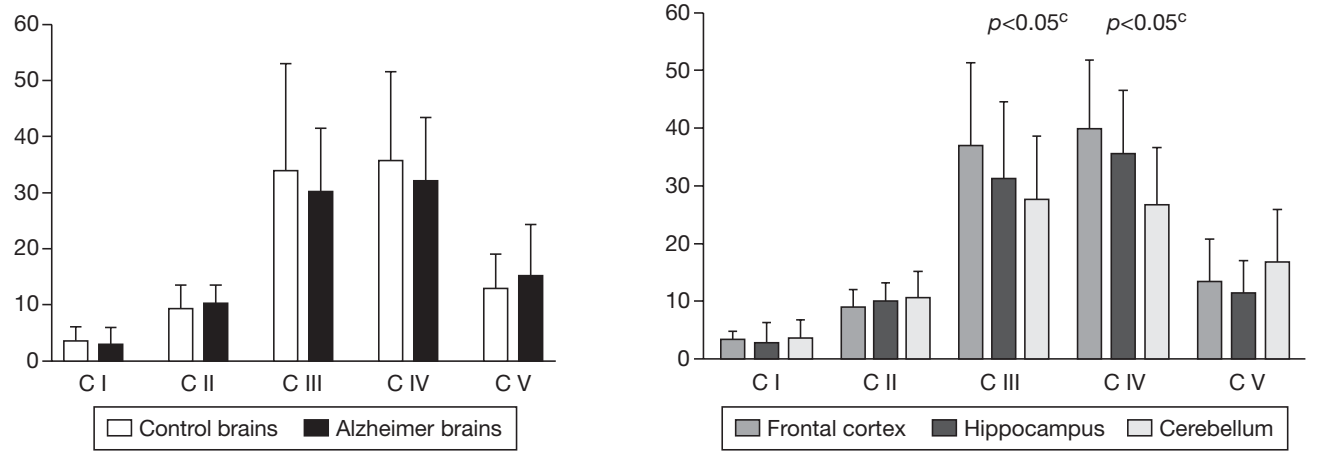


Figure 3 - *When enzymatic activities of RC complexes were corrected by CS to normalize for mitochondrial content, no differences were found between AD and control brains, for all areas taken together (left). Instead, differences between areas were found for complexes III and IV (right). p: ANOVA test. Tukey* post-hoc *test showed that differences lay between frontal cortex and cerebellum ([c]). Bars: means; error bars: SD.*

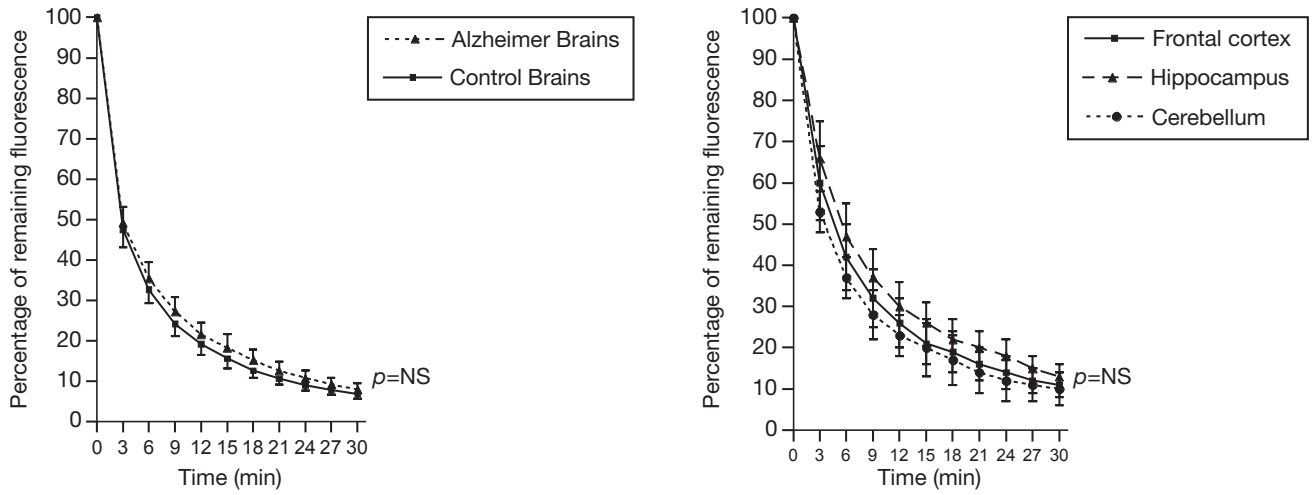


Figure 4 - *Membrane lipid peroxidation assessed as loss of* cis*-parinaric acid fluorescence (the greater the loss, the higher the peroxidation). No differences were found either between AD patients and controls (left) or different brain areas (right).*

ied 20 individuals (12 patients + 8 controls) to compare the differences between brain areas, and 60 individuals (20 frontal cortex + 20 cerebellar cortex + 20 hippocampus) to compare AD patients *vs* controls, which, in terms of statistical power, are relatively large numbers.

Our findings are in apparent contradiction with previous reports demonstrating a reduction in oxidative/energy metabolism in AD dementia by means of various biochemical and imaging techniques (5). It should be noted that enzyme activities were measured here in ideal *in vitro* situations, which may not reflect what actually happens *in vivo*. There is always the question of causation: is the reduction of brain metabolism a cause or an effect of brain dysfunction? The activity of complexes may be down-regulated due to reduced neuronal activity. In this case, the reduced activity would probably not be detected by analyzing the intrinsic activities of individual RC complexes. Furthermore, oxidative capacity may be negatively influenced at levels other than respiratory complexes proper, i.e., pyruvate dehydrogenase or α-ketoglutarate complexes, ADP/ATP transporters, and others. Polarographic studies of the oxidative capacity of intact mitochondria would be ideal in analyzing this issue, because they reflect not only the activity of RC complexes but also analyze the integrity of diverse intermediary steps, from the addition of a substrate to oxygen uptake. Unfortunately, polarography cannot be performed on frozen material. Lastly, when studying tissue homogenates, the heterogeneous behavior of mitochondrial enzymes between neurons (43) is not taken into account and may contribute to these contradictory conclusions. Single neuron studies aimed at determining RC performance within individual neurons may be a possible future approach to test if a defect in a small proportion of key neurons plays any role in the pathogenesis of AD.

## CONCLUSIONS

AD probably results from an interplay of various genetic, environmental and aging influences. From the present data, obtained from enzymatic analyses of RC complexes on brain homogenates, the putative role of a primary mitochondrial RC defect in AD cannot be confirmed.

## ACKNOWLEDGEMENTS

We thank the Brain Bank, University of Barcelona-Hospital Clínic for help in providing biological material and relevant information from patients included in this study. Funding came from the Fundació La Marató de TV3 (2002-2102/97 and 1710/01), and SGR 00279 and 00379.

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NON-INVASIVE DIAGNOSIS OF MITOCHONDRIAL DYSFUNCTION IN HAART-RELATED HYPERLACTATEMIA

*CLINICAL OF INFECTIOUS DISEASES*

2005;IN PRESS

Glòria Garrabou, Eduard Sanjurjo, Òscar Miró, Esteban Martínez, Ana B. Infante, **SÒNIA LÓPEZ**, Francesc Cardellach, Josep M Gatell and Jordi Casademont.

October 18, 2005

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## NON-INVASIVE DIAGNOSIS OF MITOCHONDRIAL DYSFUNCTION IN HAART-RELATED HYPERLACTATEMIA

To the editor:

Hyperlactatemia developed by patients under highly active antiretroviral therapy (HAART) has been suggested to be caused by mitochondrial dysfunction [1-6]. This hypothesis is based on the presence of mitochondrial DNA (mtDNA) depletion and/or mitochondrial respiratory chain (MRC) dysfunction in liver [1,2], skeletal muscle [3,4] and/or peripheral blood mononuclear cells (PBMC) [5,6]. However, these techniques are laborious, time consuming and some of them require invasive biopsies.

Non-invasive methods have been developed for the screening of mitochondrial function in primary myopathies [7-9], but have not been applied to the study of HAART-mediated mitochondrial damage. We have tested the utility of the forearm aerobic exercise test (FAET) [8] in two HIV-infected patients during an episode of HAART-related hyperlactatemia and after resolution. To check mitochondrial function, we have simultaneously measured mtDNA content by quantitative real-time PCR and complex IV MRC enzymatic activity by spectrophotometry, in biopsed skeletal deltoid-muscle homogenate and PBMC, as previously described [10,11]. Results were compared to 10 healthy non-HIV-infected controls.

The FAET measures venous oxygen saturation (VOS) in forearm's blood before (time 0), during aerobic exercise (minute 1, 2 and 3) and after 1 minute of resting (minute 4), to monitor oxygen saturation and to detect cellular oxygen uptake. The aerobic effort consists on intermittent static handgrip exercise at 33% of the intended Maximal Voluntary Contraction force set up for each subject. In primary mitochondrial disorders there is less oxygen desaturation on venous blood during aerobic exercise, indicating an oxidative phosphorilation impairment [8].

Both HIV-patients, infected before 1997, were on treatment with didanosine and stavudine (plus tenofovir -patient A- or efavirenz -patient B-). They were admitted because of fatigue, plus anorexia and weight loss in case of patient A. Serum lactate levels were 3'26 and 2'69 mmol/L (normal: 0'4-2), CD4 cell count 624 and 439/mm3 and HIV-1 RNA 198 copies/mL or undetectable (patient A and B, respectively). After 6 weeks of HAART disruption (patient A) or a ten-week-switching to lamivudine, tenofovir and efavirenz (patient B), both patients were assymptomatic.

The mitochondrial analysis performed on PBMCs and muscle homogenate during the hyperlactatemic phase confirmed the existence of a mitochondrial dysfunction, with a 20-80% complex IV activity reduction and a 65-80% of mitochondrial DNA depletion, depending on tissues. These deficiencies returned to normality after clinical resolution of hyperlactatemia (Figure 1). At the same time, both patients exhibited a marked decrease of oxygen utilisation during the symptomatic phase using the FAET, since venous oxygen saturation (VOS) only diminished 30% respect to baseline (100%) instead of the 50%-decrease found in controls, indicating a reduced blood oxygen uptake [8]. After clinical recovery the results of both patients matched the values of healthy persons (Figure 1).

Mitochondrial studies and FAET matched clinical manifestations in these two HIV-patients during symptomatic HAART-related hyperlactatemia and after clinical recovery. The possibility that antiretroviral drugs (i.e. ddI or d4T) were the direct cause of abnormal FAET results instead of hyperlactatemia seems improbable, in view of previous metabolic studies (12). The FAET, accordingly, seems to be a useful non-invasive tool for the detection of mitochondrial dysfunction developed during HAART-induced hyperlactatmia.

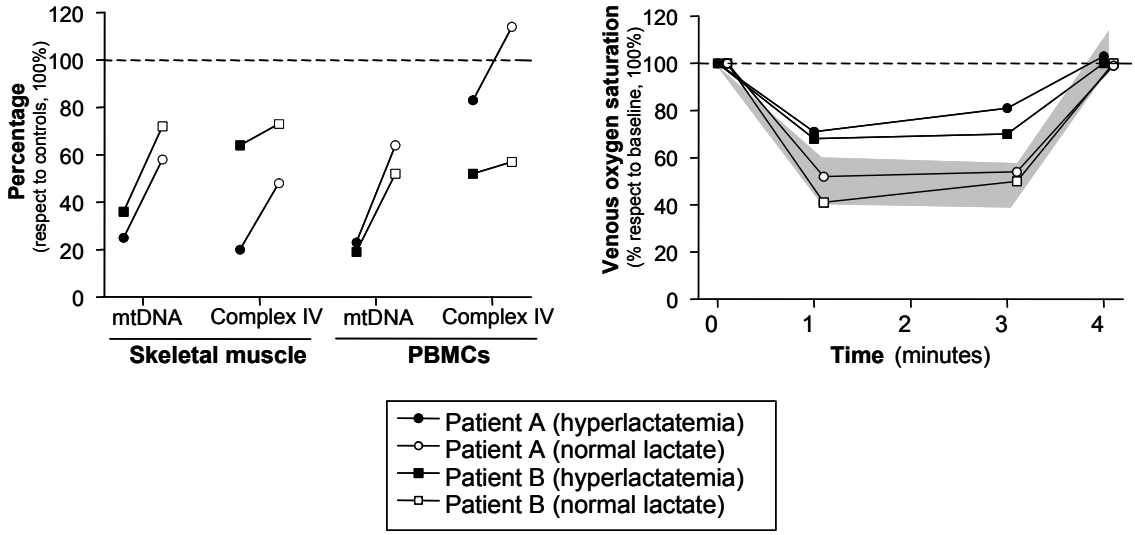


**Figure 1:** MRC complex IV enzymatic activity and mtDNA content in muscle homogenate and PBMC, in percentage compared to controls (left). FAET in percentage respect to baseline (right). Black marks represent the two HIV-patients during symptomatic hyperlactatemia and white marks 6 months after recovery. Shaded grey area represents the normal control ranges (MRC: mitochondrial respiratory chain, mtDNA: mitochondrial DNA, PBMC: peripheral blood mononuclear cells, FAET: Forearm Aerobic Exercise Test).

## Acknowledgments

This study has been supported by *Fundació la Marató de TV3 (02/0210* and *01/0710), Redes de Investigación en Mitocondrias (V2003-REDC06E-0) y SIDA (Redg 173)* and *Suports a Grups de Recerca de la Generalitat de Catalunya (2001/SGR/00379).*
No conflicts of interest exist.

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IN VIVO EFFECTS OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPIES CONTAINING THE PROTEASE INHIBITOR NELFINAVIR ON MITOCHONDRIALLY-DRIVEN APOPTOSIS

*ANTIVIRAL THERAPY*

2005;IN PRESS

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Para: MIRO, OSCAR (URGENCIES)

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# In-vivo effects of highly active antiretroviral therapies containing the protease inhibitor nelfinavir on mitochondrially-driven apoptosis.

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**Background: *In vitro* studies have reported controversial effects of protease inhibitors (PIs) on mitochondrially-driven apoptosis. Additionally, since PIs in the clinical setting are almost always given in combination with nucleoside analogues, which may have negative effects on mitochondrial DNA (mtDNA), the impact of PI-containing HAART on apoptosis and mtDNA content is unclear.**
**Methods: A cross sectional study was performed including 20 HIV negative patients (HIV-), 16 HIV positive antiretroviral-naïve patients (HIV+) and 17 HIV positive patients receiving the PI nelfinavir (NFV) plus AZT+3TC or ddI+d4T (HIV+PI) as first line antiretroviral treatment for at least 12 months. Peripheral blood mononuclear cells (PBMCs) were isolated. Bcl-2 expression (anti-apoptotic) and the levels of cleaved, active form, of caspase-9 (pro-apoptotic) were determined by Western blot. An index of mitochondrially-driven apoptotic activation was estimated calculating the ratio caspase-9/Bcl-2. mtDNA content measured by real-time PCR.**
**results: Bcl-2 expression was lower in HIV+ than in HIV- patients ($p<0.01$), while levels of caspase-9 were higher ($p=0.001$). The caspase-9/Bcl-2 ratio was significantly increased in HIV+ respect to HIV- individuals ($p<0.001$). mtDNA content was also decreased in HIV+ compared to HIV- patients ($p<0.001$). The HIV+PI group exhibited a trend to normalization for Bcl-2 expression and caspase-9 compared to HIV+ group, while the caspase-9/Bcl-2 ratio significantly improved (decreased, $p<0.05$ compared to HIV+). The mtDNA content in the HIV+PI group was similar to that of the HIV+ group, although the results of mtDNA content differed depending on whether NFV was combined with AZT+3TC (preserved) or with ddI+d4T (depleted). Conversely, no differences were found in apoptotic markers between the two subgroups of HIV+PI.**
**Conclusions: NFV-based PI-containing HAART regimens may exert some beneficial effects counteracting the increased mitochondrially-driven apoptosis present in HIV-infected people.**

## Introduction

The negative mitochondrial effects of nucleoside analogues used in current HAART strategies have been firmly established. These negative effects depend on the capacity of these analogues to inhibit DNA γ-polymerase, the only enzyme devoted to replicating (and to a lesser extend, to repairing) mtDNA, thus, leading to a decrease in mtDNA which, in turn, may finally cause mitochondrial dysfunction (1-3). Such mitochondrial abnormalities may be magnified by the effects of HIV itself, since it has been demonstrated that this virus causes diffuse mitochondrial alterations, probably through the activation of apoptotic mechanisms triggered by HIV proteins (4-6).

On the other hand, the effects of protease inhibitors (PI) on mitochondria continue to be controversial. It has been reported that these drugs can either inhibit or induce mitochondrially-driven apoptosis in leukocytes and endothelial cells (7-13). Additionally, a switch from apoptosis inhibition to induction is observed when high concentrations of PIs are used (8,12). The PI nelfinavir (NFV) constitutes an example of these controversies. While some *in vitro* studies showed that NFV prevents mitochondrially-driven apoptosis by blocking transmembrane potential loss (11) and/or by inhibiting adenine nucleotide translocator pore function in mitochondria (14), others demonstrated that NFV induces apoptosis and, accordingly, it could be hypothetically used as antitumorals on the basis of this pro-apoptotic capacity (9,13).

Studies are scarce in the clinical setting, where PIs are given in combination with other antiretrovirals and especially with nucleoside analogues. It is unknown whether such combinations of antiretrovirals increase or decrease mitochondrial damage and/or mitochondrially-driven apoptosis. Additionally, most *in vitro* investigations have been performed using the low concentration range of PIs required for viral inhibition (7-9). We therefore evaluated the effects of HAART regimens containing the PI NFV on peripheral blood mononuclear cell (PBMC) levels of Bcl-2, a main anti-apoptotic mitochondrial protein, and of the active cleaved-form of caspase-9, a major effector protein of mitochondrially-driven apoptosis. We have also measured PBMC mtDNA content.

## Materials and Methods

We designed a cross-sectional study including HIV-uninfected individuals (HIV-), HIV-infected antiretroviral-naïve patients (HIV+) and HIV-infected patients receiving a first-line NFV-based PI-containing HAART regimen (HIV+PI). These latter group included HIV+PI individuals consecutively visited as outpatients, who were asymptomatic regarding HIV infection and antiretroviral adverse effects, with an uninterrupted duration of at least 12 months of first line HAART. Concerning the nucleoside backbone, HIV+PI patients were receiving either d4t+ddI or AZT+3TC. On the other hand, HIV- patients were recruited from voluntary blood donors and HIV+ patients from asymptomatic ambulatory patients. All patients were attended at the Hospital Clinic and Hospital de Granollers and gave informed consent. The Ethics Committees of both hospitals approved the protocol.

All patients were assessed in order to exclude any clinical evidence of lipodystrophy, and to confirm normal cholesterol, triglycerides, glucose and lactate plasma levels. We recorded clinical and demographic data. In all cases, 20 mL of venous blood were obtained to perform mitochondrial studies on PBMCs, which were isolated following standard procedures (15).

An aliquot of PBMC was used to assess parameters indicative of mitochondrially-driven apoptosis. Crude protein extracts containing 20 µg protein were mixed with 1/5 vol of a solution containing 50% glycerol, 10% SDS, 10% 2-mercaptoethanol, 0.5% bromophenol blue and 0.5 M Tris (pH 6.8), incubated at 90ºC for 5 min and electrophoresed on 0.1% SDS/13% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, USA). Blots were probed with a mouse polyclonal antibody for human Bcl-2 (Santa Cruz Biotechnology sc-599, Santa Cruz, CA, USA)(1:500) and with a rabbit polyclonal antibody specific for the cleaved, active form, of human caspase-9 (Cell Signaling Technology 9505, Beverly, MA, USA)(1:1000). The monoclonal mouse antibody against β-actin (Sigma A5441, St Louis, MI, USA) (1:10.000) was used to normalize caspase-9 immunoreactive signals for equal cell protein loading. The mitochondrial protein voltage-dependent anion carrer (VDAC) or porin was determined using a monoclonal mouse antibody (Calbiochem Anti-Porin 31HL, Darmstadt, Germany) (1:1000) to refer changes in the mitochondrial protein Bcl-2 to the overall content of mitochondrial protein present in cell extracts. Immunoreactive material was detected with a horseradish peroxidase-coupled anti-mouse (Bio-Rad 170-6516, Hercules, CA, USA) (1:3000) or anti-rabbit (Santa Cruz Biotechnology sc-2004, Santa Cruz, CA, USA) (1:3000) secondary antibody and the enhanced chemiluminiscence (ECL) detection system (Amersham, Buckinghamshire, UK). Immunoblot analysis resulted in a 26 kD band for Bcl-2, a 35 kD band for the cleaved, active form, of caspase-9, a 47 kD band for β-actin, and a 31 kD band for VDAC, as expected (Figure 1). The intensity of the signals was quantified by densitometric analysis (Phoretics 1D Softward, Phoretic International Ltd, Newcastle,UK). The quotient Caspase-9 / Bcl-2 expression was arbitrarily used as an "apoptotic index" to quantify the extent of activation of PBMC mitochondrially-driven apoptosis.

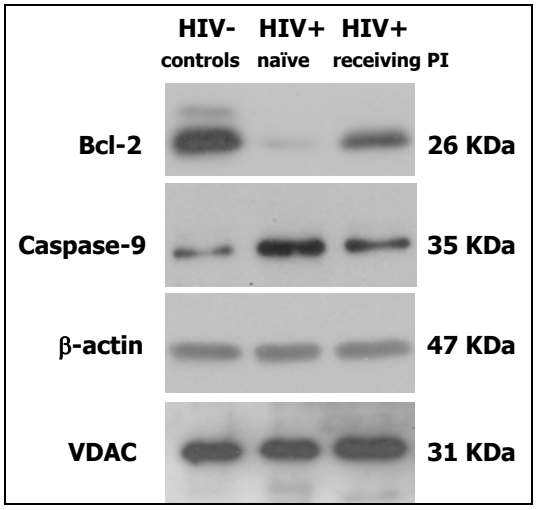


**Figure 1:** Example of immunoblot analysis of Bcl-2 and of active, truncated form, of caspase-9. Each lane corresponds to 20 µg of protein extracts from peripheral blood mononuclear cells. Panels at the bottom correspond to immunoblot with markers of the overall cell protein loading (β-actin) and the mitochondrial protein content of samples (VDAC). The size of the specific immunoreactive signals is indicated on the right.

A second aliquot was used for total DNA extraction by means of a standard phenol-chloroform procedure. For mtDNA quantification, the nuclear housekeeping 18S rRNA gene and the highly conserved mitochondrial ND2 gene were amplified separately in duplicate by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals®, Germany). The mtDNA content was expressed as the ratio of mtDNA to nuclear DNA, as previously reported (15).

Results were expressed as percentages and mean±SD, and compared by means of chi square test and Mann-Whitney U test, respectively. Linear regression analysis was employed to assess any relationship between quantitative variables. Values of p less than 0.05 were considered statistically significant.

## Results

We included 53 individuals: 16 HIV-, 20 HIV+ and 17 HIV+PI. In the latter group, 8 patients were receiving NFV combined with AZT+3TC and 9 combined with ddI+d4T. The clinical characteristics of the patients are summarized in table 1. The only difference observed concerned HIV viral load which was greater in HIV+ than HIV+PI, as expected. There were no differences between the HIV+PI subgroups of patients receiving ddI+d4T or AZT+3TC.

**Table 1:** Baseline clinical characteristics of the individuals included in the study.

| | **HIV–** (n=16) | **HIV+ naïve** (n=20) | **HIV+ receiving PI** (n=17) | **p value** |
|---|---|---|---|---|
| **Age** (years) | 40±9 | 38±8 | 41±9 | 0.62 |
| **Gender male** (%) | 69 | 72 | 82 | 0.64 |
| **HIV viral load** (log HIV RNA copies/ µL) | - | 4.9±0.8 | 2.1±0.9 | <0.001 |
| **CD4+ lymphocyte count** (cells/µL) | - | 303±185 | 395±195 | 0.40 |
| **Time receiving antiretrovirals** (months) | - | - | 18±9 | - |

As shown in figure 2, Bcl-2 expression was lower in the HIV+ than in the HIV- group (p<0.01), while caspase-9 was higher (p=0.001). The levels of processed caspase-9 at a given amount of Bcl-2 (caspase-9/Bcl-2 ratio) were very significantly increased in HIV+ compared to HIV- individuals (p<0.001). HIV+PI patients exhibited a trend to normalize Bcl-2 expression as well as caspase-9 levels, while the caspase-9/Bcl-2 ratio was significantly improved (decreased, p<0.05 respect to HIV+).

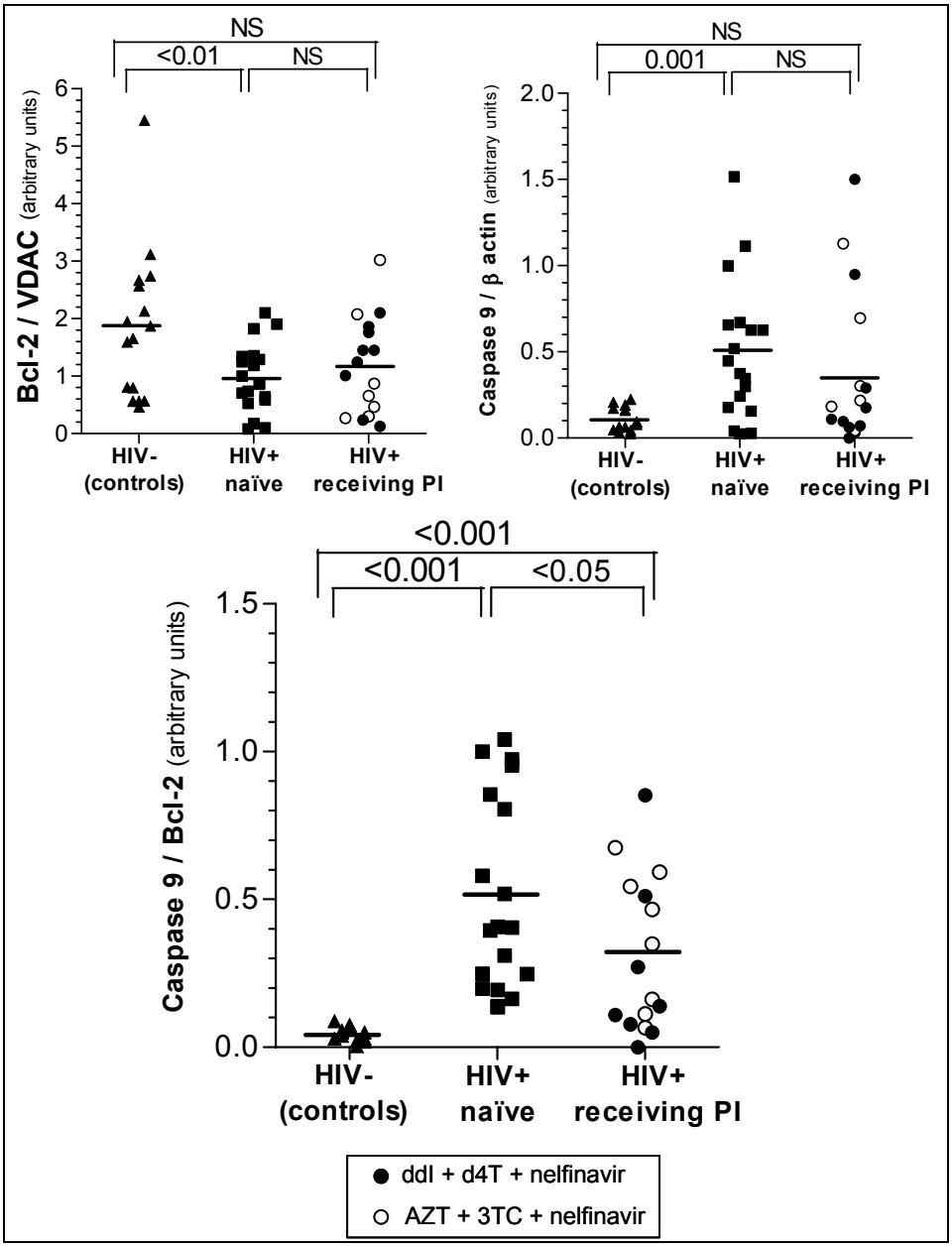


**Figure 2:** Bcl-2 and caspase-9 protein expression determined by Western-blot (up) and the apoptotic index of mitochondrially-driven apoptosis activation (down).

With respect to mtDNA content, both HIV+ and HIV+PI showed mtDNA depletion (52% and 50% of the remaining content, respectively) compared with HIV- (figure 3).

The changes in the apoptosis parameters were comparable in both subgroups of HIV+PI patients irrespective of the nucleosides used in combination with NFV (figure 4). Conversely, we observed a different behavior in the mtDNA content of HIV+PI patients depending on the nucleoside combination used, since patients with ddI+d4T showed a greater mtDNA depletion than with AZT+3TC ($p<0.001$, figure 4).

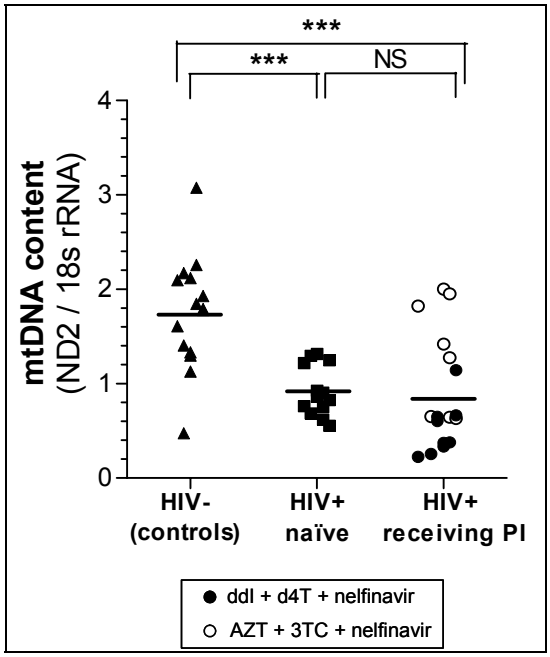


Figure 3: **Mitochondrial DNA content**

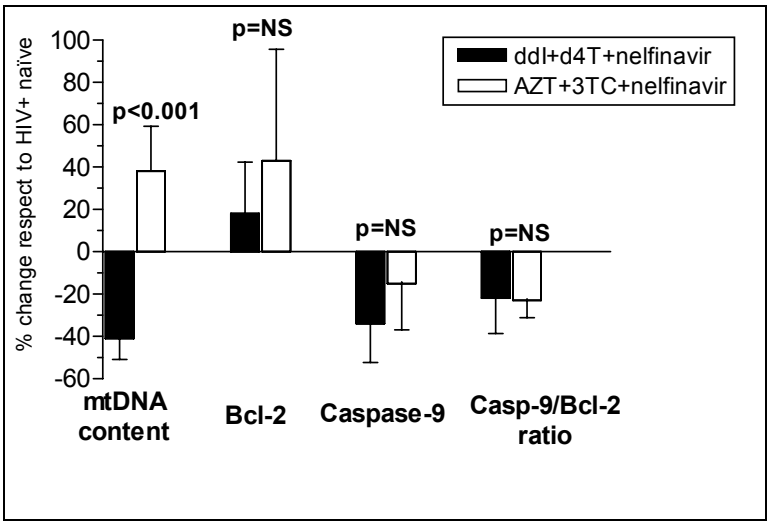


**Figure 4:** Comparison of the mitochondrial and apoptotic changes (respect to HIV+ group) observed in the two subgroups of HIV+PI (ddI+d4T+nelfinavir and AZT+3TC+nelfinavir).

We did not find any relationship between mtDNA content, complex IV activity and the mitochondrially-driven apoptotic parameters measured in present study (data not shown).

## Conclusions

The present study confirms the association of HIV infection with enhancement of mitochondrially-driven apoptotic pathways as well as mtDNA depletion in PBMCs. However, while the treatment of HIV infection with a NFV-based PI-containing HAART did not normalize the mtDNA content (in fact, the mtDNA content worsened in the subgroup receiving ddI+d4T), it was associated with an improvement in the apoptotic parameters involving mitochondrial pathways. Additionally, this positive effect on mitochondrially-driven apoptosis was

observed irrespective of the backbone of nucleoside analogues used in combination with NFV. These findings indicate that, at least in response to PI-containing HAART, the two relevant mitochondrial disturbances described in HIV-infected patients (i.e. enhancement of mitochondrially-driven apoptosis and reduction in mtDNA levels) can be independently influenced by the clinical use of anti-retroviral therapy.

Previous *in vitro* experiments have indicated that the anti-apoptotic action of PIs do not involve down-regulation of apoptosis regulatory molecules (11,16). The present *in vivo* study in PBMCs from HIV-infected patients confirms that NFV-based PI-containing HAART reduces apoptotic activation through mechanisms that do not involve an improvement in the HIV infection-mediated reduction in Bcl-2 levels. The fact that, at similar levels of Bcl-2, caspase-9 levels were lowered in treated patients (reduced caspase-9/Bcl-2 ratio in treated versus untreated HIV+ patients) points to caspase-9 processing itself or other events upstream from this process as the potential targets of the anti-apoptotic action. Although a direct effect of PIs on caspase-9 processing can be hypothesized (caspase-9 activation is the result of pro-caspase-9 proteolisis), research in recent years has not demonstrated any direct inhibitory effects of PIs on proteolitic activation of cellular caspases (11,17), including caspase-9 (18). Alternatively, other experimental studies have proposed that the antiapoptotic action of NFV occurs at the level of mitochondria, downstream from the activation of pro-apoptotic members of the Bcl-2 family, and involving, as a primary event, the opening of the mitochondrial permeability transition pore and subsequent activation of caspase-9 (14). The results of our study on PBMCs from treated patients, in which caspase-9 reduction by NFV-containing HAART occurs in the absence of Bcl-2 changes, are in accordance with this model of NFV action.

On the other hand, the concomitant occurrence of apoptotic inhibition by PIs and mtDNA depletion by NRTIs would not be contradictory in this scenario and seems to occur in an independent manner. In fact, we observed that the apoptotic benefits of a NFV-based PI-containing HAART had been achieved to a very similar extent in both, patients with depleted mtDNA and patients with preserved mtDNA. Additionally, *in vitro* studies support the concept that apoptotic modulation can occur in the cell regardless of changes in mtDNA levels. In fact, experimental models of mtDNA depletion in cells have indicated maintenance of mitochondrial membrane potential (19) and cells lacking mtDNA can maintain their responsiveness to activate apoptosis via disruption of mitochondrial membrane potential (20).

We believe that our results agree with the hypothesis of a double positive effect of NFV when used in the treatment of HIV infection: one depending on their antiviral capacity by means of HIV protease activity inhibition and another depending on their immunomodulatory properties through the apoptosis modulation via mitochondrial pathways. The latter effect now seems to be firmly established, since in uninfected people taking NFV+AZT+3TC (one of those used in a subgroup of our HIV+PI patients) for post-exposition prophylaxis, surface expression of apoptosis-related ligands and receptors was unaltered, but mitochondrially-driven apoptosis susceptibility was significantly inhibited (21). This anti-apoptotic activity could explain why in some patients CD4+ cells increase despite a non-observable antiviral effect (22), or why in some cases in which PIs are discontinued a deterioration of CD4+ count has been observed despite maintaining full viral suppression (23). We are unaware whether the present results are limited to NFV or could be extended to other PIs. This probably depends on the main specific mechanism by which NFV exerts its beneficial anti-apoptotic effects: directly acting as a booster of mitochondrial defense mechanisms or indirectly reducing the HIV viral load and the pro-apoptotic processes caused by the virus. If the latter is the relevant mechanism, at least some of the beneficial effects found in the present study would be present in other PI-containing HAART, since reduction of viremia is a common characteristic of all PIs. On the other hand, since our study design did not include a HAART combination using a non-PI third drug (for example, non-nucleoside analogue reverse transcriptase inhibitor), we can not definitively rule out that the observed antiapoptotic effects are the result of the non-specific effects of NRTIs themselves. However, since the beneficial effects on apoptotic were observed in both NRTI

combination subgroups, we believe that NFV have played a significant role in such effects.

In summary, HIV-infected patients without evident metabolic or body fat abnormalities receiving a NFV-based PI-containing HAART show anti-apoptotic effects on PBMCs and these effects are independent of the effects on mtDNA content. This supports the concept of some beneficial activity linked to PIs in addition to their benefits through direct antiviral therapy.

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A continuació es citen les Jornades i Congressos Internacionals en els que la doctoranda ha presentat, com a pòster o com a comunicació oral, diversos treballs d'investigació realitzats durant els seus anys de formació, i que han donat lloc a l'elaboració dels articles que conformen aquesta Tesi.

Autors: F Cardellach, J Alonso, Ò Miró, **S López**, A Beato, J Casademont.
Títol: *'Effect of carbon monoxide on cytochrome c oxidase of skeletal muscle mitochondria'*.
Tipus de participació: Late breaker (LB91), Poster.
Congrés: *'Society of Toxicology 41st Annual Meeting'*.
Publicació: Official Journal of the Society of Toxicology, Vol. 66, Number 1-S, March 2002; Oxford University Press.
Lloc de celebració: Nashville, TS (Tenessi), Març 17-21, 2002.

Autors: F Cardellach, JM Grau, **S López**, A Beato, A Urbano-Márquez, J Casademont.
Títol: *'Primary biliary cirrhosis and mitochondrial myopathy. A rare association'*.
Tipus de participació: Poster P417.
Congrés: *'Xth International Congress on Neuromuscular Diseases'*.
Publicació: Journal of the Neurological Sciences. Supplement to volume 199(2002), Elsevier.
Lloc de celebració: Vancouver, Canada, 7-12 Juliol, 2002.

Autors: J Casademont, **S López**, A Beato, Ò Miró, F Cardellach.
Títol: *'The importance of simultaneous study of muscle homogenate and mitochondrial enriched suspensions in the diagnosis of mitochondrial respiratory Chain disorders'.*
Tipus de participació: Poster P419.
Congrés: *'Xth International Congress on Neuromuscular Diseases'*.
Publicació: Journal of the Neurological Sciences. Supplement to volume 199(2002), Elsevier.
Lloc de celebració: Vancouver, Canada, 7-12 Juliol, 2002.

Autors: JM Grau, M Picón, **S López**, A Beato, J Casademont, Ò Miró, F Cardellach.
Títol: *'Toxic mitochondrial myopathy related to alpha-1-antitrypsin augmentation therapy'*.
Tipus de participació: Poster P422.
Congrés: *'Xth International Congress on Neuromuscular Diseases'*.
Publicació: Journal of the Neurological Sciences. Supplement to volume 199(2002), Elsevier.
Lloc de celebració: Vancouver, Canada, 7-12 Juliol, 2002.

Autors: Cardellach F, Miró Ò, Godayol M, Fabregat ME, **López S**, Casademont J, Gomis R.
Títol: *'Mitochondrial respiratory chain (MRC) activity of pancreatic islets from mice fed with a high fat diet'.*
Tipus de participació: Pòster (P29)
Congrés: *'5th European Meeting on Mitochondrial Pathology (EUROMIT5)'*.

Publicació: Mitochondrion 2001, Vol. 1, Suppl. 1, pp: S34. Elsevier.
Lloc de celebració: Venècia (ITÀLIA) Any: 19-23 Setembre, 2001

Autors: Casademont J, **López S**, Miró Ò, Gómez-Zaera M, Pedrol E, Nunes V, Cardellach F.
Títol: *'Mitochondrial respiratory chain involvement in antiretroviral therapy- realted lipodystrophy'*.
Tipus de participació: Pòster (P34)
Congrés: *'5th European Meeting on Mitochondrial Pathology (EUROMIT5)'*.
Publicació: Mitochondrion 2001, Vol. 1, Suppl. 1, pp: S36. Elsevier.
Lloc de celebració: Venècia (ITÀLIA) Any: 19-23 Setembre, 2001

Autors: Miró Ó, Martínez E, Soler A, **López S**, Milinkovic A, Pedrol E, Casademont J, Gatell JM, Cardellach F.
Títol: *'Mitochondrial function in lymphocytes from non-lipodystrophic HIV-patients on different HAART schedules'*.
Tipus de participació: Pòster (P4)
Congrés: *'3th European Workshop on lipodystrophy and metabolic disorders'*
Publicació: pp: 12-12
Lloc de celebració: Marbella (ESPANYA) Any: Abril 25-27, 2002

Autors: **López S**, Miró O, Martínez E, Rodríguez B, Milinkovic A, Pedrol E, Casademont J, Nunes V, Gatell JM, Cardellach F.
Títol: *'Effects of different first-line HAART regimens on the content and function of lymphocyte mitochondria of HIV-infected patients withouth lipodystrophy'*.
Tipus de participació: Late-breaker (LBPeB9027), Pòster
Congrés: *'XIV International AIDS Conference'*
Lloc de celebració: Barcelona (ESPANYA) Any: Juliol 7-12, 2002

Autors: Miró Ò, Casademont J, **López S**, Pedrol E, Soler A, Beato A, Cardellach F.
Títol: *'Mitochondrial respiratory deficiencies in antiretroviral therapy-related lipodystrophy'*.
Tipus de participació: Pòster (TuPeB4506)
Congrés: *'XIV International AIDS Conference'*
Lloc de celebració: Barcelona (ESPANYA) Any: Juliol 7-12, 2002

Autors: **López S**, Miró Ò, García M, Martínez E, Milinkovic A, Blanco J.L, Pedrol E, Beato A, Casademont J, Cardellach F.
Títol: *'Sequencial analysis of mitochondrial respiratory chain function of HIV-infected patients on highly active antiretroviral therapy'*.
Tipus de participació: CD Abstract publication.
Congrés: *'XIV International AIDS Conference'*
Lloc de celebració: Barcelona (ESPANYA) Any: Juliol 7-12, 2002

Autors: **López S**, Miró Ò, García M, Martínez E, Milinkovic A, Soler A, Blanco JL, Pedrol E, Beato A, Casademont J, Cardellach F.
Títol: *'Assessment of mitochondrial toxicity of diverse highly active antiretroviral therapy regimens by a simultaneous genetic and biochemical approach'*.
Tipus de participació: **Presentació com Comunicació oral** (Abstract 24).
Congrés: *'4th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2002; 7:L15. International Medical Press. University of California (San Diego).
Lloc de celebració: San Diego, Califòrnia (ESTATS UNITS D'AMÈRICA) Any: Setembre 22-25, 2002

Autors: Miró Ò, **López S**, Pedrol E, Rodríguez-Santiago B, Martínez E, Soler A, Casademont J, Nunes V, Gatell JM, Cardellach F.
Títol: *'Mitochondrial DNA depletion is associated with biochemical dysfunction in lymphocytes from HIV-infected patients with lipodystrophy'*.
Tipus de participació: Pòster (Abstract 68)
Congrés: *'4th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2002; 7:L45. International Medical Press. University of California (San Diego).
Lloc de celebració: San Diego, Califòrnia (ESTATS UNITS D'AMÈRICA) Any: Setembre 22-25, 2002

Autors: **López S**, Miró Ò, García M, Martínez E, Milinkovic A, Blanco JL, Rodríguez-Santiago B, Beato A, Casademont J, Cardellach F.
Títol: *'Reversible Mitochondrial DNA depletion and mitochondrial respiratory chain dysfunction in symptomatic hyperlactataemia'*.
Tipus de participació: Pòster (Abstract 69)
Congrés: *'4th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2002; 7:L46. International Medical Press. University of California (San Diego).
Lloc de celebració: San Diego, Califòrnia (ESTATS UNITS D'AMÈRICA) Any: Setembre 22-25, 2002

Autors: **López S**, Miró Ò, García M, Martínez E, Milinkovic A, Soler A, Blanco JL, Pedrol E, Beato A, Casademont J, Cardellach F.
Títol: *'Assessment of mitochondrial toxicity of diverse HAART regimens by a simultaneous genetic and biochemical approach'*.
Tipus de participació: **Presentació com a Comunicació oral en 'Late breakers and Hot topics session'** (PL14.1 (1), Pòster).
Congrés: *'Sixth International Congress on Drug Therapy in HIV Infection'*
Publicació: eJournal of the International AIDS Society (eJIAS) 2002.
Lloc de celebració: Glasgow (ANGLATERRA) Any: Novembre 17-21, 2002

Autors: Miró Ò, **López S**, Pedrol E, Rodríguez-Santiago B, Martínez E, Soler A, Casademont J, Nunes V, Gatell J.M, Cardellach F.
Títol: *'Mitochondrial DNA depletion is associated with biochemical dysfunction in lymphocytes from HIV infected patients with lipodystrophy'*.
Tipus de participació: **Presentació com a Comunicació oral en 'Late breakers and Hot topics session'** (PL14.1 (2), Pòster).
Congrés: *'Sixth International Congress on Drug Therapy in HIV Infection'*.
Publicació: eJournal of the International AIDS Society (eJIAS) 2002.
Lloc de celebració: Glasgow (ANGLATERRA) Any: Novembre 17-21, 2002

Autors: **López S**, Miró Ò, García M, Martínez E, Milinkovic A, Blanco J.L, Rodríguez B, Beato A, Casademont J, Cardellach F.
Títol: *'Reversible mitochondrial DNA depletion and mitochondrial respiratory chain dysfunction in symptomatic hyperlactatemia'*.
Tipus de participació: Pòster (P119).
Congrés: *'Sixth International Congress on Drug Therapy in HIV Infection'*.
Publicació: eJournal of the International AIDS Society (eJIAS) 2002.
Lloc de celebració: Glasgow (ANGLATERRA) Any: Novembre 17-21, 2002

Autors: **López S**, Miró Ò, Martínez E, Pedrol E, Beato A, Deig E, Gatell JM, Casademont J, Cardellach F.
Títol: *'Does HIV infection by itself have any effect on mitochondrial DNA content?'*.
Tipus de participació: Pòster (Abstract 54)
Congrés: *'5th International workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2003; 8:L40. International Medical Press.
Lloc de celebració: París (FRANÇA) Any: Juliol 8-11, 2003

Autors: **López S**, Negredo E, Miró Ò, Ruíz L, Clotet B, Casademont J, Cardellach F.
Títol: *'Effects of switching to a highly active antiretroviral therapy regimen containing tenofovir on diverse mitochondrial parameters'*.
Tipus de participació: Pòster (Abstract 55)
Congrés: *'5th International workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2003; 8:L41. International Medical Press.
Lloc de celebració: París (FRANÇA) Any: Juliol 8-11, 2003

Autors: Miró O, **López S**, Fernández-Solá J, Martínez E, Pedrol E, Milinkovic A, Casademont J, Gatell JM.
Títol: *'Increased apoptosis in skeletal muscle, highly active antiretroviral therapy and lipodystrophy'*.
Tipus de participació: Pòster (Abstract 57)
Congrés: *'5th International workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2003; 8:L42. International Medical Press.

Lloc de celebració: París (FRANÇA) Any: Juliol 8-11, 2003

Autors: Miró O, **López S**, Martínez E, Pedrol E, Casademont J, Cardellach F.
Títol: *'Time on antiretroviral therapy and Mitochondrial DNA content and cytochrome c oxidase activity'*.
Tipus de participació: Pòster (Abstract 58)
Congrés: *'5th International workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2003; 8:L43. International Medical Press.
Lloc de celebració: París (FRANÇA) Any: Juliol 8-11, 2003

Autors: **López S**, Miró O, Martínez E, Pedrol E, Beato A, Deig E, Gatell JM, Casademont J, Cardellach F.
Títol: *'Has HIV infection by itself any effect on Mitochondrial (mt) DNA content?'*.
Tipus de participació: Pòster (Abstract 441)
Congrés: *'The 2nd IAS Conference on HIV Pathogenesis and Treatment'*
Publicació: Antiviral Therapy 2003; 8 (Suppl. 1): S300.
Lloc de celebració: París (FRANÇA) Any: Juliol 13-16, 2003

Autors: **López S**, Negredo E, Miró Ò, Ruiz L, Clotet B, Casademont J, Cardellach F.
Títol: *'Effects of switching to a HAART regimen containing tenofovir on diverse Mitochondrial parameters'*.
Tipus de participació: Pòster (Abstract 739)
Congrés: *'The 2nd IAS Conference on HIV Pathogenesis and Treatment'*
Publicació: Antiviral Therapy 2003; 8 (Suppl. 1): S389.
Lloc de celebració: París (FRANÇA) Any: Juliol 13-16, 2003

Autors: Garrabou G, **López S**, Negredo E, Puig J, Ruiz L, Sanjurjo E, Casademont J, Cardellach F, Clotet B, Miró Ò.
Títol: *'Addition of tenofovir to a didanosine-based highly active antiretroviral therapy increases mitochondrial toxicity'*.
Tipus de participació: Póster P1.
Congrés: *'6th European Meeting on Mitochondrial Pathology (EUROMIT6)'*.
Publicació: Biochim Biophys Acta 2004; 1657:24. Bioenergetics. Elsevier.
Lloc de celebració: Nijmegen (HOLANDA) Any: Juliol 1-4, 2004

Autors: Casademont J, **López S**, Picón M, Garrabou G, Infante A, Miró Ò, Cardellach F.
Títol: *'The effect of 'atypical' antipsychotics on the OXPHOS system in peripheral blood mononuclear cells'*.
Tipus de participació: Pòster P75.
Congrés: *'6th European Meeting on Mitochondrial Pathology (EUROMIT6)'*.
Publicació: Biochim Biophys Acta 2004; 1657:50. Bioenergetics. Elsevier.
Lloc de celebració: Nijmegen (HOLANDA) Any: Juliol 1-4, 2004

Autors: Cardellach F, Miró Ò, **López S**, Martínez E, Pedrol E, Milinkovic A, Deig E, Garrabou G, Casademont J, Gatell JM.
Títol: *'Mitochondrial Abnormalities on peripheral blood mononuclear cells of HIV-infected patients'*.
Tipus de participació: Pòster P102.
Congrés: *'6th European Meeting on Mitochondrial Pathology (EUROMIT6)'*.
Publicació: Biochim Biophys Acta 2004; 1657:61. Bioenergetics. Elsevier.
Lloc de celebració: Nijmegen (HOLANDA) Any: Juliol 1-4, 2004

Autors: **López S**, Garrabou G, Rodríguez de la Concepción M, Martínez E, Pedrol E, Giralt M, Cardellach F, Gatell JM, Vilarroya F, Casademont J, Miró Ò.
Títol: *'Mitochondrial DNA depletion in asymptomatic HIV-infected patients receiving didanosine plus stavudine-based antiretroviral regimen seems to be compensated by up-regulatory mechanisms'*.
Tipus de participació: Pòster P186.
Congrés: *'6th European Meeting on Mitochondrial Pathology (EUROMIT6)'*.
Publicació: Biochim Biophys Acta 2004; 1657:92. Bioenergetics. Elsevier.
Lloc de celebració: Nijmegen (HOLANDA) Any: Juliol 1-4, 2004

Autors: Miró Ò, Negredo E, **López S**, Ruíz L, Garrabou G, Sanjurjo E, Casademont J, Cardellach F, Clotet B.
Títol: *'Mitochondrial effects of adding tenofovir to a HAART regimen containing didanosine'*.
Tipus de participació: Pòster WePeB5896.
Congrés: *'XV International AIDS Conference'*.
Publicació: MedGenMed. 2004 Jul 11;6(3):WePeB5896 [eJIAS. 2004 Jul 11;1(1):WePeB5896].
Lloc de celebració: Bangkok (TAHILÀNDIA) Any: Juliol 11-16, 2004

Autors: O Miro, **S López**, M Rodríguez, E Martínez, E Pedrol, G Garrabou, J Casademont, F Vilarroya, F Cardellach, JM Gatell.
Títol: *'Up-regulatory mechanisms compensate mitochondrial DNA depletion in asymptomatic individuals receiving didanosine plus stavudine (ddI+d4T)'*.
Tipus de participació: Pòster.
Congrés: *'XV International AIDS Conference'*.
Publicació: MedGenMed. 2004 Jul 11;6(3): [eJIAS. 2004 Jul 11;1(1):].
Lloc de celebració: Bangkok (TAHILÀNDIA) Any: Juliol 11-16, 2004

Autors: Garrabou G, **López S**, Infante AB, Negredo E, Puig J, Ruiz L, Sanjurjo E, Casademont J, Cardellach F, Clotet B, Miró Ò.
Títol: *'Addition of tenofovir to a didanosine-based HAART does not increase mitochondrial DNA depletion but decreases cytochrome c oxidase function and mitochondrial mass'*.
Tipus de participació: Pòster (Abstract 26)
Congrés: *'6th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*

Publicació: Antiviral Therapy 2004; 9:L19. International Medical Press.
Lloc de celebració: Washington, DC (ESTATS UNITS D'AMÈRICA) Any: Octubre 25-28, 2004

Autors: **López S**, Garrabou G, Martínez E, Domingo P, Fontdevila J, Gatell JM, Infante AB, Gallart X, Milinkovic A, Cardellach F, Casademont J, Miró Ò.
Títol: *'Mitochondrial studies in adipose tissue of HIV-infected patients without fat redistribution'*.
Tipus de participació: Pòster (Abstract 27)
Congrés: *'6th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2004; 9:L20. International Medical Press.
Lloc de celebració: Washington, DC (ESTATS UNITS D'AMÈRICA) Any: Octubre 25-28, 2004

Autors: **López S**, Garrabou G, Rodríguez de la Concepción M, Martínez E, Pedrol E, Giralt M, Cardellach F, Gatell JM, Villarroya F, Casademont J, Miró Ò.
Títol: *'Mitochondrial DNA depletion in asymptomatic HIV-infected patients receiving didanosine plus stavudine-based HAART regimen seems to be compensated by up-regulatory mechanisms'*.
Tipus de participació: Pòster (Abstract 28)
Congrés: *'6th Internacional Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2004; 9:L20. International Medical Press.
Lloc de celebració: Washington, DC (ESTATS UNITS D'AMÈRICA) Any: Octubre 25-28, 2004

Autors: **López S**, Garrabou G, Fernández-Solà J, Pedrol E, Badía E, Infante AB, Martínez E, Cardellach F, Gatell JM, Casademont J, Miró Ò.
Títol: *'HIV infection is associated with increased skeletal apoptosis assessed by TUNEL'*.
Tipus de participació: Pòster (Abstract 29)
Congrés: *'6th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2004; 9:L21. International Medical Press.
Lloc de celebració: Washington, DC (ESTATS UNITS D'AMÈRICA) Any: Octubre 25-28, 2004

Autors: Garrabou G, **López S**, Sanjurjo E, Infante AB, Riba J, Casademont J, Cardellach F, Miró Ò.
Títol: *'Mitochondrial dysfunction of HAART-related hyperlactatemia is demonstrable by non-invasive studies'*.
Tipus de participació: Pòster (Abstract 35)
Congrés: *'6th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'*
Publicació: Antiviral Therapy 2004; 9:L24. International Medical Press.
Lloc de celebració: Washington, DC (ESTATS UNITS D'AMÈRICA) Any: Octubre 25-28, 2004

Autors: **López S**, Garrabou G, Martínez E, Domingo P, Fontdevila J, Gatell JM, Infante AB, Gallart X, Milinkovic A, Cardellach F, Casademont J, Miró Ò.
Títol: *'Mitochondrial studies in adipose tissue of HIV-infected patients without fat redistribution'*.

Tipus de participació: **Presentació com a Comunicació oral en 'Late breakers and Hot topics session'** (PL7.3).
Congrés: *'7th International Congress on Drug Therapy in HIV infection'*
Publicació: eJournal of the International AIDS Society (eJIAS) 1 (2), 2004.
Lloc de celebració: Glasgow, UK (ESCÒCIA) Any: Novembre 14-18, 2004

Autors: **López S**, Garrabou G, Rodríguez de la Concepción M, Martínez E, Pedrol E, Giralt M, Cardellach F, Gatell J, Villarroya F, Casademont J, Miró Ò.
Títol: *'Compensatory mechanisms preserve mitochondrial function in HIV patients on didanosine plus stavudine exhibiting mitochondrial DNA depletion'*.
Tipus de participació: Pòster (P183)
Congrés: *'7th Internacional Congress on Drug Therapy in HIV Infection'*
Publicació: eJournal of the International AIDS Society (eJIAS) 1 (2), 2004.
Lloc de celebració: Glasgow, UK (ESCÒCIA) Any: Novembre 14-18, 2004

Autors: Garrabou G, **López S**, Fernández-Solà J, Pedrol E, Badía E, Infante AB, Martínez E, Cardellach F, Gatell JM, Casademont J, Miró Ò.
Títol: *'Addition of tenofovir to a didanosine-based HAART does not increase mitochondrial DNA depletion but decreases citochrome c oxidase function and mitochondrial mass*.
Tipus de participació: Pòster (P184)
Congrés: *'7th International Congress on Drug Therapy in HIV infection'*
Publicació: eJournal of the International AIDS Society (eJIAS) 1 (2), 2004.
Lloc de celebració: Glasgow, UK (ESCÒCIA) Any: Novembre 14-18, 2004

Autors: **López S**, Garrabou G, Fernández-Solà J, Pedrol E, Badía E, Infante AB, Martínez E, Cardellach F, Gatell JM, Casademont J, Miró Ò.
Títol: *'HIV infection is associated with increased skeletal apoptosis assessed by TUNEL'*.
Tipus de participació: Pòster (P188)
Congrés: *'7th International Congress on Drug Therapy in HIV infection'*
Publicació: eJournal of the International AIDS Society (eJIAS) 1 (2), 2004.
Lloc de celebració: Glasgow, UK (ESCÒCIA) Any: Novembre 14-18, 2004

Autors: Milinkovic A, **López S**, Miró Ò, Vidal S, Fernández X, Arnaiz JA, Blanco JL, Leon A, Larrousse M, Lonca M, Laguno M, Gatell JM, Martínez E.
Títol: *'A randomized open study comparing the impact of reducing stavudine dose versus switching to tenofovir on mitochondrial function, metabolic parameters, and subcutaneous fat in HIV-infected patients receiving antiretroviral therapy containing stavudine'*.
Tipus de participació: Pòster (Abstract 857)
Congrés: *'12th Conference on Retroviruses and Opportunistic Infections' (CROI)*
Lloc de celebració: Boston, Massachusetts, (ESTATS UNITS D'AMÈRICA) Any: Febrer 22-25, 2004

Autors: Garrabou G, Sanjurjo E, **López S**, Infante AB, Ramos J, Cardellach F, Miró Ò, Casademont J.
Títol: 'Estudis no invasius i invasius en el diagnòstic de la hiperlactatèmia pel tractament antiretroviral de gran activitat (TARGA)'.
Tipus de participació: Pòster A-16.
Congrés: 'Xiè Congrés Català-Balear de Medicina Interna'
Lloc de celebració: Barcelona, Catalunya, (Espanya) Any: Maig 18-20, 2005

Autors: **López S**, Garrabou G, Miró Ò, Villarroya J, Rodríguez de la Concepción M, Escayola R, Giralt M, Gatell JM, Cardellach F, Casademont J, Villarroya F.
Títol: *'Effects of highly active antiretroviral therapy (HAART) regimens containing the protease inhibitor (PI) nelfinavir on apoptosis of peripheral blood mononuclear cells'*.
Tipus de participació: Pòster (Abstract P-136)
Congrés: *'13th Euroconference on Apoptosis'*
Lloc de celebració: Budapest, Hungría Any: Octubre 1-4, 2005

Autors: Garrabou G, **López S**, Fernández-Solà J, Pedrol E, Badia E, Infante AB, Martínez E, Cardellach F, Gatell JM, Casademont J, Miró Ò.
Títol: *'HIV infection is associated with increased skeletal apoptosis assessed by TUNEL'*.
Tipus de participació: Pòster (Abstract P-253)
Congrés: *'13th Euroconference on Apoptosis'*
Lloc de celebració: Budapest, Hungría Any: Octubre 1-4, 2005

Autors: Garrabou G, **López S**, Sanjurjo E, Infante AB, Larrouse M, Milinkovic A, Martínez E, Riba J, Casademont J, Cardellach F, Miró Ò.
Títol: *'Mitochondrial function in HAART-related hyperlactatemia'*.
Tipus de participació: Pòster (P-70)
Congrés: *'7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'*
Lloc de celebració: Dublín, Irlanda Any: Novembre 13-16, 2005

Autors: Garrabou G, **López S**, Vidal F, Miró Ò, Domingo P, Pedrol E, Villarroya F, Saumoy M, Infante AB, Martínez E, López-Dupla M, Sambeat MA, Deig E, Villarroya J, Rodríguez-Chacón M, Fontanet A, Richart C, Giralt M, Gatell JM.
Títol: *'Peripheral blood mononuclear cells of HIV-1-infected patients long-term non-progressors show mild mitochondrial impairment and low mitochondrially-driven apoptosis'*.
Tipus de participació: Pòster (P-71)
Congrés: *'7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'*
Lloc de celebració: Dublín, Irlanda Any: Novembre 13-16, 2005

Autors: **López S**, Garrabou G, Negredo E, Infante AB, Puig J, Grau E, Gatell JM, Casademont J, Cardellach F, Clotet B, Miró Ò.

Títol: *'Effects of different dosis of didanosine combined with tenofovir on peripheral blood mononuclear cell (PBMC) mitochondrial parameters'*.
Tipus de participació: Pòster (P-75)
Congrés: *'7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'*
Lloc de celebració: Dublín, Irlanda Any: Novembre 13-16, 2005

Autors: **López S**, Garrabou G, Villaroya J, Infante AB, Rodríguez de la Concepción M, Escayola R, Giralt M, Gatell JM, Cardellach F, Casademont J, Villaroya F, Miró Ò.
Títol: *'Protease inhibitors (PI) and apoptosis: studies on peripheral blood mononuclear cells (PBMCs)'*.
Tipus de participació: Pòster (P-68)
Congrés: *'7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'*
Lloc de celebració: Dublín, Irlanda Any: Novembre 13-16, 2005

Autors: Miró Ò, Garrabou G, **López S**, Deig E, Vidal I, Infante AB, Cardellach F, Casademont J, Pedrol E.
Títol: *'Metabolic and mitochondrial changes after 6 months of switching of antiretroviral-experienced patients to enfuvirtide, tenofovir and saquinavir/ritonavir'*.
Tipus de participació: Pòster (P-81)
Congrés: *'7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'*
Lloc de celebració: Dublín, Irlanda Any: Novembre 13-16, 2005

Autors: **López S**.
Títol: 'Posibilidades de estudios en sangre periférica de la toxicidad mitocondrial de los antirretrovirales'.
Tipus de participació: **Ponència**.
Jornada: '1as Jornadas de VIH y Mitocondria' patrocinades pel Grup Bristol-Myers Squibb i organitzades pel Dr. Enric Pedrol Clotet (Unitat d'Infeccions-VIH de l'Hospital General de Granollers) i pel Dr. Òscar Miró Andreu (Grup d'Investigació Muscular, Servei de Medicina Interna, Hospital Clínic i Provincial de Barcelona).
Lloc de celebració: Hotel Plaza, Plaza España 6-8, Barcelona 23 Octubre de 2002.

Autors: **López S**.
Títol: *'Mitochondria and antiretroviral treatment'*.
Tipus de participació: **Ponència**.
Jornada: *'The International Meeting on Mitochondrial Biogenesis and Disease'* organitzat per MitEURO. 'Xarxa temàtica: Biogènesi i Patologia Mitocondrial' (II Plà de Recerca de Catalunya 1997/2000).
Lloc de celebració: Facultat de Biologia. Universitat de Barcelona (UB), Barcelona, Espanya 19 Setembre, 2003.

Autors: **López S**.
Títol: 'El DNA mitocondrial: herramientas diagnósticas y su interpretación en la práctica clínica'.
Tipus de participació: **Ponència**.
Jornada: '2as Jornadas de VIH y Mitocondria' patrocinades pel Grup Bristol-Myers Squibb i organitzades pels Drs. Enric Pedrol Clotet, Òscar Miró Andreu i Pere Domingo Pedrol (Servei de malalties infeccioses, Hospital de la Santa Creu i Sant Pau, Barcelona).
Lloc de celebració: Hotel Plaza, Plaza España 6-8, Barcelona 5 Novembre de 2003.

Autors: **López S**.
Títol: 'Efectos de los tratamientos antirretrovirales de alta potencia sobre la cadena respiratoria mitocondrial'.
Tipus de participació: **Ponència**.
Jornada: '1ª Reunión General de la Red MITOESPAÑA' organitzada pel Dr. Santiago Rodríguez de Córdoba.
Lloc de celebració: Centro de Investigaciones Biomédicas, c/ Ramiro Maetzu, 9, Madrid, Espanya 5 Desembre, 2003.

Autors: **López S**.
Títol: 'Estudios mitocondriales en el laboratorio'.
Tipus de participació: **Ponència**.
Jornada: '3as Jornadas de VIH y Mitocondria' patrocinades pel Grup Bristol-Myers Squibb i organitzades pels Drs. Enric Pedrol Clotet, Òscar Miró Andreu i Pere Domingo Pedrol. Les Jornades tenen el reconeixement d'Interés Sanitari per part de l'Institut d'Estudis Sanitaris del Departament de Sanitat i Seguretat Social de la Generalitat de Catalunya, de l'Observatori de la Salut Dr. Carles Vallbona i de la 'Red Española de Investigación en SIDA' així com el Patrocini Científic del Grup d'Estudi de ls SIDA (GESIDA) de la SEIMC.
Lloc de celebració: Hotel Plaza, Plaza España 6-8, Barcelona 10 Novembre de 2004.