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

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Tesi Doctoral

5. RESULTATS

RESULTATS EN PACIENTS INFECTATS PEL VIH AFECTES DE LIPODISTRÒFIA

5.1. RESULTATS EN PACIENTS INFECTATS PEL VIH AFECTES DE LIPODISTRÒFIA

5.1.1. Participació mitocondrial en el desenvolupament de la síndrome de lipodistròfia com a efecte secundari associat al tractament antiretroviral en pacients infectats pel VIH.

Aquest apartat fa referència als resultats que es deriven dels estudis realitzats en pacients infectats pel VIH que estan rebent TARGA i que no presenten cap evidència clínica de sida, però que han desenvolupat la síndrome de lipodistròfia (LD) durant l'administració del tractament ARV. Així, els treballs d'investigació que es presenten han estat específicament dirigits a determinar la implicació mitocondrial en la etiopatogènia de la LD en aquests pacients.

PARTICIPACIÓN MITOCONDRIAL EN LA LIPODISTROFIA ASOCIADA AL TRATAMIENTO ANTIRRETROVIRAL DE GRAN ACTIVIDAD DE PACIENTES INFECTADOS POR EL VIRUS DE LA INMUNODEFICIENCIA HUMANA

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Participación mitocondrial en la lipodistrofia asociada al tratamiento antirretroviral de gran actividad de pacientes infectados por el virus de la inmunodeficiencia humana

Sr. Editor: Hemos leído con interés la excelente revisión de Paredes et al[1] sobre el al abordaje terapéutico de la lipodistrofia en pacientes infectados por el virus de la inmunodeficiencia humana (VIH) en tratamiento con antirretrovirales de gran actividad (TARGA) publicada recientemente en su Revista. Sin duda, el tratamiento correcto del síndrome pasa por un mejor conocimiento de los mecanismos fisiopatológicos subyacentes al mismo. En este sentido, entre los mecanismos propuestos, los autores comentan la participación de una hipotética disfunción mitocondrial, la cual ha sido invocada repetidamente como una teórica causa de la lipodistrofia[2,3]. Esto ha sido así de acuerdo con diferentes hechos. Por una parte, por la bien conocida capacidad de la zidovudina para inhibir la actividad de la gammapolimerasa encargada de la replicación del ADN mitocondrial. Por otra parte, el desarrollo en algunos pacientes con lipodistrofia asociada a TARGA de acidosis láctica e insuficiencia hepática aguda parecidas a las que se observan en las encefalopatías mitocondriales también apuntaría hacia una disfunción mitocondrial. Finalmente, la semejanza fenotípica que presenta la lipodistrofia asociada a TARGA con la lipomatosis simétrica múltiple o enfermedad de Madelung, trastorno este último frecuentemente asociado a deleciones únicas o múltiples del ADN mitocondrial y actividad disminuida de la citocromo-c-oxidasa o complejo IV de la cadena respiratoria mitocondrial (CRM), también apoyaría la hipótesis de la participación mitocondrial en la primera. Sin embargo, recientemente se ha puesto en duda esta disfunción[4]. Con todo, aún son muy escasos los estudios que abordan directamente esta hipótesis en pacientes afectados por la lipodistrofia asociada al TARGA. En este sentido, nuestro grupo presentó un caso de lipodistrofia asociada a TARGA en el que la paciente presentaba alteraciones en la funcionalidad de la CRM del músculo esquelético, a la vez que múltiples deleciones en el ADN mitocondrial en dicho tejido y adipocitos de tejido graso subcutáneo[5]. Esta observación inicial motivó la extensión del estudio a 7 pacientes afectados del síndrome, y en todos ellos la disfunción mitocondrial y las deleciones múltiples estaban presentes[6]. Hemos realizado una revisión de los estudios llevados a cabo hasta la fecha en los que se contrastaba esta hipótesis y que han sido comunicados en diversos congresos durante el último año; estos estudios se resumen en la tabla 1[7-10]. Como puede verse, los resultados son todavía discrepantes, y si bien unos autores confirman nuestros hallazgos, en otros estudios no se han encontrado alteraciones en los tejidos analizados. En definitiva, coincidimos con Paredes et al en señalar la posible existencia de una disfunción mitocondrial en la lipodistrofia asociada a TARGA, aunque su confirmación, así como la de los mecanismos por los cuales se produce, todavía no está definitivamente establecida.

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TABLA 1

Resumen de los estudios mitocondriales de pacientes positivos para el VIH afectados de lipodistrofia asociada a tratamiento antirretroviral de gran actividad publicados hasta la fecha

Autores	N.º de casos	Tejido estudiado	Histología	Bioquímica	ADN mitocondrial	Se indica participación mitocondrial en la lipodistrofia
Gómez et al[6]	7	Linfocitos	NP	NR	NR	Sí
	7	Músculo esquelético	Indicativas de alteración mitocondrial	Alteraciones oxidativas, déficit de actividad de los complejos III y IV	Deleciones múltiples (sin mutaciones puntuales ni depleción)	
	2	Grasa subcutánea	NR	NR	Deleciones múltiples (sin mutaciones puntuales ni depleción)	
Walker et al[7]	11	Grasa subcutánea	NR	NR	Depleción	Sí
Shikuma et al[8]	8	Grasa subcutánea	NR	NR	Depleción (sin deleciones ni inserciones)	Sí
Negredo et al[9]	14	Músculo esquelético	Similares al grupo control	Sin deficiencias	NR	No
Vigano et al[10]	6	Linfocitos	NP	Sin incremento de apoptosis ni despolarización de la membrana mitocondrial	NR	No

NP: no procede; NR: no realizado.

- SÍNTESI DE L'ESTUDI

El present treball consisteix en una carta dirigida a l'editor de la revista Medicina Clínica, a través de la qual vàrem voler transmetre la idea de que en l'etiopatogènia de la síndrome de LD com a efecte secundari associat al TARGA pot veure-s'hi implicada una disfunció mitocondrial. Aquest primer treball de la Tesi aporta informació addicional a la revisió presentada per R. Paredes i col·laboradors, en la que es tracten en profunditat les opcions terapèutiques disponibles en aquell moment contra la LD en relació al TARGA en pacients infectats pel VIH (Paredes R, Med Clin (Barc) 2001).

Cal situar el present treball en una època en què la participació mitocondrial en el desenvolupament de la LD era una mera hipòtesi, i els treballs publicats al respecte eren escasos i molt pobres en quant a anàlisi de la morfologia i funció mitocondrial.

MITOCHONDRIAL DNA DEPLETION AND RESPIRATORY CHAIN ENZYME DEFICIENCIES ARE PRESENT IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF HIV-INFECTED PATIENTS WITH HAART-RELATED LIPODYSTROPHY

ANTIVIRAL THERAPY

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

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The main objective of the present study was to ascertain if mitochondrial DNA (mtDNA) depletion as reported in HIV-infected patients with highly active antiretroviral therapy (HAART)-related lipodystrophy (LD) implies any degree of mitochondrial respiratory chain (MRC) dysfunction. For this purpose, we evaluated HIV patients on different HAART schedules with LD (group A; *n*=12) and on HAART but without LD (group B; *n*=12), and untreated HIV-infected patients as controls (group C; *n*=24). mtDNA content was determined on peripheral blood mononuclear cells (PBMCs) with a real-time PCR method. Complex II, III and IV activities of the MRC were simultaneously measured spectrophotometrically, as were spontaneous and stimulated oxygen consumption by PBMCs. Compared to controls (group C, 100%), patients with LD (group A) showed a decreased mtDNA content (54%, *P*<0.001), which was associated with a decline in complex III (62%, *P*<0.05) and IV activity (69%, *P*<0.05) (both complexes partially encoded by mtDNA), but not in complex II activity (exclusively encoded by nuclear DNA). Patients in group B showed a similar pattern of mitochondrial dysfunction but to a lesser extent and without statistical significance. Respiratory activities in both treated groups (A and B) did not differ in comparison with controls. We conclude that mtDNA depletion occurring during HAART is associated with deficiencies in MRC complexes partially encoded by mtDNA, which are detectable by PBMCs. Presented in 'Late Breakers and Hot Topics' session at *6th International Congress on Drug Therapy in HIV Infection*, Glasgow, UK, 17–21 November 2002.

Introduction

Toxic effects on mitochondria were first reported [1] with the antiretroviral zidovudine, but several *in vitro* and clinical studies have subsequently demonstrated that all nucleoside analogue reverse transcriptase inhibitors (NRTIs) are associated with some degree of mitochondrial dysfunction [1–4], even in asymptomatic patients [5]. The effects of NRTIs on mitochondria are mainly due to their capacity to inhibit gamma-polymerase (γ-pol), the enzyme responsible for mitochondrial DNA (mtDNA) replication. Recent studies have demonstrated that mtDNA depletion or deletions are present in fat [6,7], skeletal muscle [8–10] and liver [8] of patients with lipodystrophy (LD), and some authors have, accordingly, suggested that such a decrease in mtDNA could be related to the development of lipodystrophic changes. Since mtDNA only encodes for two ribosomal RNA, 22 transfer RNA and 13 messenger RNA, all exclusively involved in the translation of subunits of complexes I, III, IV and V of the mitochondrial respiratory chain (MRC), the only possible phenotypic expression of mtDNA defects is MRC dysfunction [11]. This dysfunction is demonstrable by measuring both enzyme activity of MRC complexes and whole mitochondrial capacity to oxidize substrates coupled with oxygen consumption. Therefore, simultaneous analysis of MRC function along with the determination of mtDNA content are the clues to linking genetic findings in HIV patients on highly active antiretroviral therapy (HAART) with the development of LD [12].

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

Patients and methods

Patients

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

Methods

Obtaining PBMCs

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

mtDNA studies

Total DNA was extracted by standard phenol-chloroform procedures. Rearrangements in mtDNA were also examined by Southern blot hybridization using a mitochondrial ND4 gene as the probe [10]. For mitochondrial DNA quantification, nuclear 18s rRNA housekeeping gene and the highly conserved mitochondrial ND2 gene were quantified separately by real-time quantitative PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals, Germany). The PCR amplification of a 500 bp fragment length of the 18S rRNA gene was performed by using the forward 5′-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/µl of each primer, 10 ng of DNA in 20 µl of final volume. The PCR reactions for nuclear gene amplification contained 2 mM $MgCl_2$, 0.3 pmol/µl of each primer, and 10 ng of DNA in 20 µl of final volume. The PCR amplification programme consisted of a single denaturation-enzyme-activation step of 10 min at 95°C, followed by 35 cycles (for 18S rRNA gene) and 29 cycles (for the ND2 gene). Each cycle consist of a denaturation step (2 s at 95°C, for r18S gene and 0 s at 94°C, for the ND2 gene) an annealing step (10 s at 66°C, for18S rRNA gene and 10 s at 53°C, for the ND2 gene), and an extension step (20 s at 72°C, for 18S rRNA gene and 10 s at 72°C, for the ND2 gene), with a temperature-transition rate of 20°C/s. The fluorescent product was detected at the last step of each cycle by single acquisition. The method used a double-stranded DNA dye (SYBR Green I) to continuously monitor product formation. The sensitivity of SYBR Green I detection is limited by non-specific product formation, which is monitored by fluorescence acquisition at temperatures at which only specific products are double-stranded [15]. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C, cooling at 20°C/s to 72°C for the ND2 gene and 76°C for the 18S rRNA, and slowly heating it at 0.2°C/s to 94°C with continuous fluorescence collection. Melting curves were used to determine the specificity of the PCR products. The results were expressed as the ratio of the mean mtDNA value of duplicate measurements to the mean nuclear DNA value of duplicate measurements (mtDNA/nDNA) [16].

MRC enzyme activities

Measurement of enzyme activity (in nmol/min/mg of protein) of individual complexes of the MRC was performed spectrophotometrically (UVIKON 920, Kontron®, Switzerland) [17]. Since isolated complex I and V activities cannot be measured in whole cells (due to the absence of activation of the former with decylubiquinone and due to a strong oligomycin-insensitive ATPase activity of the latter) [18], we only determined complex II, complex III and complex IV activities

(being the first exclusively encoded by nuclear DNA and the two latter partially encoded by mtDNA) following Rustin *et al.* [19], but slightly modified for complex IV measurement [20].

PBMC respiratory activity

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

Statistical analysis

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

Results

We included 12 HIV-infected patients on HAART with LD and 12 HIV-infected individuals on HAART without LD. As controls, 24 naive HIV-infected patients with respect to treatment were used. Clinical data are shown in Table 1. All patients receiving treatment demonstrated a significantly lower viral load than untreated controls. Patterns of LD were peripheral lipoatrophy in 75% of the cases and mixed in 25%. No case of isolated fat accumulation was observed. Six patients in group A had hypertriglyceridaemia, four had hypercholesterolaemia and two hyperglycaemia. Only one patient in group B had hyperglycaemia and hypertriglyceridaemia.

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

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

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

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

Discussion

Lipodystrophy is one of the most prevalent side effects of HAART [21,22]. Clinical similarities with symmetric multiple lipomatosis, a syndrome in which mitochondrial dysfunction has been proven [23], led to the

Table 1. Clinical data

	Controls (untreated) (n=24)	Patients (on HAART without LD) (n=12)	Patients (on HAART with LD) (n=12)
Age (years) (mean ±SEM)	40 ±3	40 ±4	44 ±4
Gender female (%)	25	25	25
Viral load (log_{10} copies) (mean ±SEM)	5.2 ±0.3	1.8 ±0.3*	2.8 ±0,4*
Lymphocyte CD4 count (per mm^3) (mean ±SEM)	221 ±58	455 ±55	351 ±49
Time on HAART (months) (mean ±SEM)		21 ±3	31 ±5
HAART containing d4T, but not PI (%)		42	42
HAART containing PI, but not d4T (%)		25	25
HAART containing d4T and PI (%)		33	33

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

Figure 1. Mitochondrial DNA content [*P<0.001; respect to control (untreated) group]

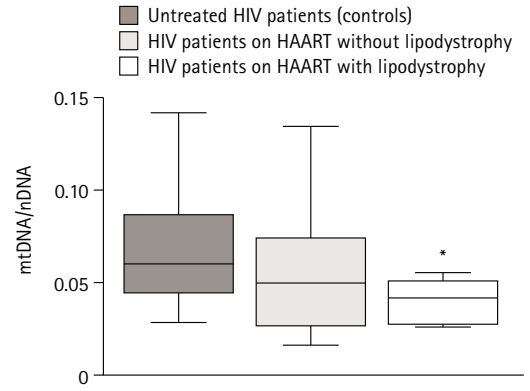
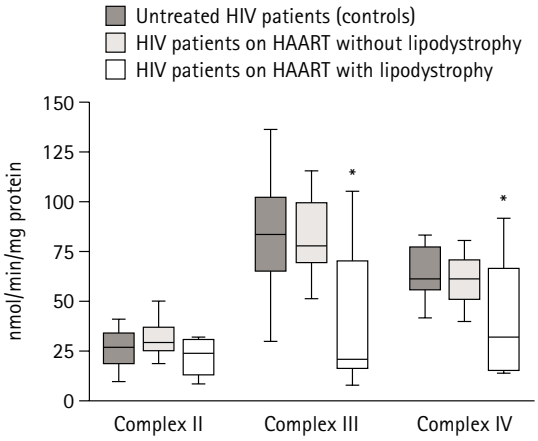


Figure 2. Enzyme activity of mitochondrial respiratory chain complexes [*P<0.05; respect to control (untreated) group]



mitochondrial hypothesis for HAART-related LD. Since then, data suggesting the participation of mitochondria in the pathogenesis of LD have steadily increased [7,8]. Although our study does not address experimentally the responsibility of mtDNA depletion in adipose tissue to cause LD, it adds new data possibly pointing to that direction. Our results regarding mtDNA depletion in PBMCs from patients developing LD are consistent with data from subcutaneous adipose tissue of patients with this syndrome. Shikuma *et al.* [6] reported a significant, but not quantified, decrease in mtDNA content irrespective of the sample site of the subcutaneous fat, while Walker *et al.* [7] found that the mtDNA content in subcutaneous adipose tissue of patients on HAART with LD was 61% of that from patients on HAART without LD. Although it has been reported that the toxic effects are not equally evident in different tissues, as demonstrated by quite different results in mtDNA quantification of paired samples of fat and PBMCs [24], in our experience mtDNA contained in PBMCs seems to be also sensitive to the toxic effects of antiretroviral drugs and may, therefore, potentially be used to investigate this side effect in HIV patients on HAART.

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

The decreased complex III and IV activities (both enzymes partially encoded by mtDNA) along with mtDNA depletion strengthens the pathogenic link between these two findings. However, at least in

Figure 3. Oxygen consumption

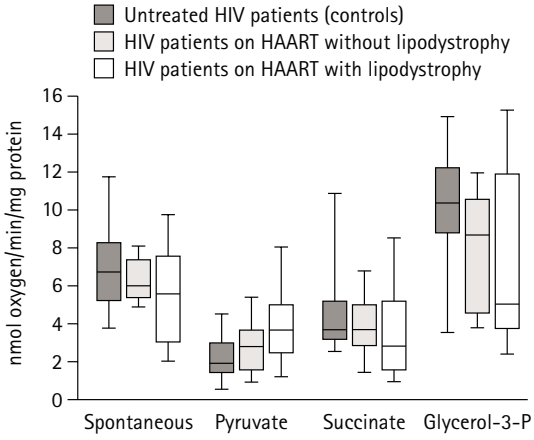
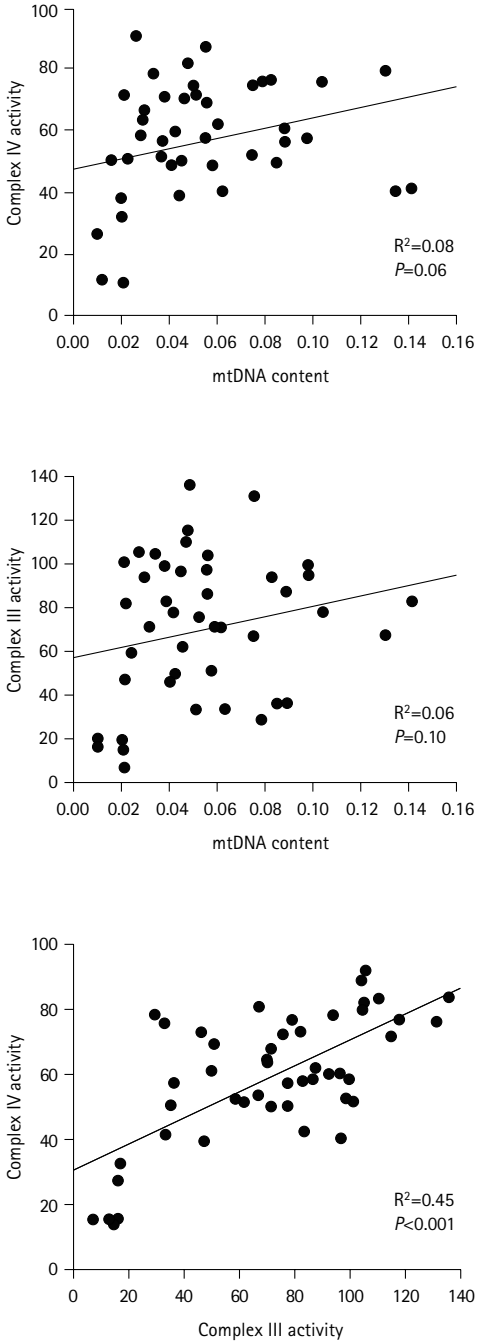


Figure 4. Relationship between the diverse mitochondrial parameters



PBMCs, these disturbances were not enough to inhibit the whole respiratory capacity, either spontaneous or stimulated with substrates of complexes I, II and III. This does not rule out the fact that MRC abnormalities found in PBMC can be more pronounced in target tissues leading to an overt MRC dysfunction. For example, we demonstrated 14% and 22% of residual activities for complexes III and IV, respectively, in skeletal muscle of patients with LD combined with a marked decline in whole respiratory capacity [9,10]. This lack of concordance among tissues is explained by the well-known tissue specificity of inherited mitochondrial diseases, which is attributed to heteroplasmy among mitochondria within a given cell and among cells in a particular tissue, as well as the dependence of oxidative phosphorylation of each tissue [27]. In addition, tissue specificity is also present for NRTI-induced mitochondrial dysfunction, with a differentiated tissue involvement depending on the kind of NRTI used [4,27] and even for the same patient for a specific NRTI drug [28]. On the other hand, mechanisms by which HAART causes mitochondrial dysfunction may not solely be a consequence of NRTI effects on mitochondrial DNA synthesis via γ-pol inhibition. Our finding that relatively moderate mtDNA depletion caused a detectable decrease of complex III and IV enzyme activity also suggests the existence of alternative mechanisms for HAART toxicity.

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

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- SÍNTESI DE L'ESTUDI

En aquest estudi hem volgut determinar si la depleció d'ADNmt que presenten els pacients que han desenvolupat la síndrome de LD com a efecte secundari associat al TARGA és detectable en les CMSP i resulta suficientment important com per a què la funció normal mitocondrial es vegi alterada.

L'estudi l'hem realitzat en pacients infectats pel VIH amb LD que reben diferents combinacions TARGA. Hem comparat els resultats amb un grup control de pacients VIH positus en TARGA però sense cap efecte secundari que pugui estar associat al tractament ARV, i amb un segon grup control format per pacients infectats pel VIH que mai han rebut TARGA.

- CONCLUSIONS

Els pacients amb LD tenen el contingut d'ADNmt de les CMSP disminuït respecte al grup de pacients infectats que no reben TARGA.

La depleció de l'ADNmt que presenten els pacients amb LD s'associa amb deficiències en l'activitat enzimàtica dels complexos del sistema OXPHOS que estàn parcialment codificats pel genoma mitocondrial.

La respiració mitocondrial de les CMSP dels pacients amb LD no es veu críticament afectada per les alteracions mitocondrials trobades en l'estudi, tot i que manifesta una tendència a disminuir la seva capacitat oxidativa respecte als dos grups control.

MITOCHONDRIAL STUDIES IN HAART-RELATED LIPODYSTROPHY: FROM EXPERIMENTAL HYPOTHESIS TO CLINICAL FINDINGS

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Mitochondrial studies in HAART-related lipodystrophy: from experimental hypothesis to clinical findings

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Chronic use of antiretrovirals (ARVs) to treat HIV infection, along with more prolonged patient survival, has been associated with an increase in adverse drug effects in HIV-infected patients on treatment. It has been proposed that some of these adverse effects (including myopathy, cardiomyopathy, anaemia, hyperlactataemia/lactic acidosis, pancreatitis, polyneuritis and lipodystrophy) could be mediated by mitochondrial (mt) toxicity. From the experimental data, it has been proposed that nucleoside analogue reverse transcriptase inhibitors (NRTIs) also inhibit γ-polymerase, the enzyme devoted to replicate (and, to a lesser extent, repair) mtDNA. It is now widely accepted that the use of most NRTIs in HIV-infected patients is associated with mtDNA depletion. Although cross-sectional studies suggest that certain ARVs, especially stavudine, are more toxic to mitochondria, the differences among different highly active ARV therapy (HAART) schedules detected in the analysis of longitudinal studies are not so clear. These types of study in previously untreated individuals suggest that the greatest mtDNA loss appears at the beginning of the treatment. Conversely, in ARV-experienced patients, the potential beneficial effects of HAART changes in terms of mtDNA content remain controversial and must be further investigated. Functional studies accompanying genetic investigations are needed for the correct pathogenic interpretation of the mtDNA abnormalities.

Introduction

The clinical use of antiretrovirals (ARVs) to treat HIV infection began in 1986 with the introduction of zidovudine (AZT), an analogue of thymidine nucleoside with proven capacity to inhibit HIV reverse transcriptase (RT). Since then, more than 20 ARVs that act at different steps of the HIV life cycle have been approved. With this wide therapeutic arsenal, the current standard treatment for HIV infection consists of a combination of several ARVs in so-called highly active ARV therapy (HAART). As a consequence, HAART use has achieved a marked decrease in patient mortality and has shifted the concept of HIV infection from a highly mortal disease to a chronic illness.

However, chronic use of ARVs together with prolonged patient survival has also been associated with an increase in adverse drug effects. It has been proposed that some of these adverse effects (including myopathy, cardiomyopathy, anaemia, hyperlactataemia/lactic acidosis, pancreatitis, polyneuritis and lipodystrophy) could be mediated by mitochondrial (mt) toxicity. Nonetheless, despite the large number of basic and clinical studies published on this hypothesis during the last 10 years, a unifying theory that explains the clinical manifestations of ARV mt damage is still lacking. One of the main reasons for this is that most of these studies have been performed *in vitro* and were either non-reproducible or have not been examined in the setting of human studies. Consequently, it is currently unknown why certain patients develop adverse effects in a particular tissue or why patients with similar accumulated doses of ARVs express different patterns of adverse effects. It is possible that ARV-related factors (different rates of tissue incorporation, transportation into mt compartment or intra-mt phosphorylation for each ARV), patient-related factors (mtDNA polymorphisms) or tissue-related factors [oxidative phosphorylation (OXPHOS) dependence] play a significant role in this heterogeneity. Therefore, while for some of these adverse effects (such as toxic myopathy or lactic acidosis) a strong relationship with mt dysfunction has been proven [1–6], in others (especially lipodystrophy) controversies remain. In this review, we cover the field from experimental hypothesis to clinical studies and analyse the level of evidence with respect to the relationship between ARV adverse effects and mt toxicity, with particular attention to mt participation in lipodystrophy.

Molecular basis of ARV toxicity on mitochondria

In addition to inhibiting HIV-RT, *in vitro* studies suggest that nucleoside analogue RT inhibitors (NRTIs) are also able to inhibit β and γ human DNA polymerases, although a current demonstration of such direct inhibition by NRTIs in humans is lacking. From a pathogenic point of view, whereas the inhibition of β-polymerase (devoted to the repair nuclear of DNA) seems not to be of clinical relevance, the inhibition of γ-polymerase (γ-pol, devoted to the replication and, to a lesser extent, the repair of mtDNA) has been proposed to participate in most of the secondary effects associated with the clinical use of NRTIs [7]. Although γ-pol inhibition is considered a specific class effect of these drugs, the magnitude is not the same for all NRTIs, at least under experimental conditions. Based on experimental laboratory data, different NRTIs have been classified from a higher potency to impair γ-pol activity to a lower potency as follows: zalcitabine (ddC) > didanosine (ddI) > stavudine (d4T) >>> AZT > lamivudine (3TC) > abacavir (ABV) = tenofovir (TDF) [8–10].

NRTIs inhibit γ-pol through four different mechanisms encompassing their effects as: i) mtDNA chain terminators (once incorporated into a growing strand, DNA replication is abruptly halted), ii) competitive inhibitors (competing with natural nucleotides to be incorporated into growing DNA chains by γ-pol), iii) inductors of errors in the fidelity of mtDNA replication (inhibiting the exonucleolytic proofreading function of γ-pol) and iv) contributors to the decrease of mtDNA reparatory exonuclease activity (resisting exonucleolytic removal by exonuclease activity of γ-pol because of the lack of the group 3′OH in NRTIs) [11]. As a final consequence, the efficiency of γ-pol is decreased and the whole cellular content of mtDNA is reduced, while the percentage of point mutations [12] and deletions [13] in such genomes increases. Since mtDNA only codifies for functional proteins corresponding to mt respiratory chain complexes I, III, IV [cytochrome C oxidase (COX)] and V, the final and only possible consequence of mtDNA damage (if the magnitude is great enough to surpass compensatory mechanisms) is to cause respiratory chain dysfunction and low ATP synthesis. Other studies carried out on different cell lines have also demonstrated decreased synthesis of some of these mt-encoded proteins [14,15]. Furthermore, respiratory chain dysfunction can cause a loss of mt membrane potential and an increase in mt-driven apoptosis, a cascade of events that has been demonstrated for AZT and d4T [16].

Experimental studies have also invoked other additional mechanisms aside from γ-pol inhibition to completely explain NRTI-associated mt toxicity. For example, AZT is able to i) inhibit the ATP/ADP translocator from rat liver and heart *in vitro*, thereby limiting the OXPHOS mt capacity [17,18], ii) reduce protein glycosylation [19] and iii) to decrease protein synthesis [20].

The effects of ARV drug classes other than NRTIs against mitochondria have been less frequently studied and evaluated. Although it seems that non-NRTIs (NNRTIs) have no toxic effects on mtDNA, they could interfere with apoptotic pathways and eventually lead to some secondary harmful effects against mitochondria. Recent data indicate that in laboratory assays, efavirenz (EFV) acts as an inductor of the caspasa-dependent apoptotic mt pathway [21]. Also, few and controversial data have been reported regarding mt effects of protease inhibitors (PIs). While some authors have found that PIs can induce loss of mt membrane potential [22], others have suggested that PIs could exert a beneficial role due to their anti-apoptotic properties [23]. In a very recent review, Badley identified at least five distinct mechanisms for the anti-apoptotic effects of PIs: i) decreasing expression of apoptosis regulatory molecules, ii) caspase inhibition, iii) altering proliferation, iv) inhibiting calpain and v) avoiding loss of mt transmembrane potential [24]. However, under certain circumstances (particularly high doses of PIs) a paradoxical pro-apoptotic effect can be observed in transformed cell lines *in vitro* and implanted mouse models [24]. Clearly, further studies are required with this drug class to define the exact interactions with mitochondria, especially with apoptotic pathways. Finally, there are no current reports evaluating the effects of fusion inhibitors on mitochondria.

The bulk of data generated under experimental laboratory conditions, briefly mentioned above, have been of crucial relevance in order to propose and, in some cases define, the exact mechanisms by which ARV cause mt side effects. However, results from *in vitro* experimental studies on cultured cells or animals cannot always be directly extrapolated to what occurs *in vivo* in HIV-infected individuals. Laboratory models usually consist of non-HIV infected cells or animals and, nowadays, there are data consistently indicating that HIV itself causes some mt disarrangements, including mtDNA depletion [4,25–28] and respiratory chain dysfunction [25–29]. Thus, experimental studies do not take into consideration the role of HIV in facilitating or magnifying ARV-related mt toxic effects and do not include the beneficial effects of ARV in limiting HIV-related mt damage by controlling HIV infection. Additionally, ARV pharmacokinetics are not accounted for in models based on cell cultures, and the effects of the long-term use of ARVs are probably not accurately evaluated either. Moreover, experimental studies are

mainly limited to ascertaining the effects of an isolated ARV and, in clinical practice, ARVs are managed as combinations of three or four drugs that are concomitantly administered to patients. Finally, the exact pathogenic significance of experimental findings to explain certain adverse effects (such as lipodystrophy) which appear during HAART, is not established by cellular models. Therefore, this review encompasses mt data from studies performed in HIV-infected patients receiving ARV who have developed lipodystrophy.

Cross-sectional studies in HIV-infected individuals developing lipodystrophy

The first direct data of mt involvement in lipodystrophy was described in a case report of an HIV-infected woman suffering from lipodystrophy. The patient exhibited multiple mtDNA deletions in skeletal muscle and subcutaneous fat along with a marked respiratory chain dysfunction in skeletal muscle and peripheral blood mononuclear cells (PBMCs) [30]. Soon after this, mtDNA depletion was found in subcutaneous fat of eight patients developing lipodystrophy [31]. Since then, case series and cross-sectional studies have both progressively appeared. Currently, mt abnormalities (and especially mtDNA depletion) have been identified in several tissues of HIV-infected patients with lipodystrophy on HAART, including skeletal muscle [13,32,33], liver [32], adipose tissue [13,31,34,35], sperm [36] and PBMCs [12,37]. However, some other authors have not found mt abnormalities [38–41]. Table 1 summarizes the published data from transversal studies of HIV patients developing lipodystrophy. As one can see, the evaluated tissues are diverse and there is great heterogeneity of the methodologies for studying mitochondria. For example, Southern blot methodology to measure mtDNA content has been replaced by real-time PCR technology, which renders greater sensitivity, accuracy and reliability than the former. Moreover, it is highly unusual for a single work to provide morphological, genetic and functional data on mitochondria. These facts can explain, at least in part, the lack of current consensus regarding the exact role of mt dysfunction in lipodystrophy. Additionally, since the majority of data comes from HIV lipodystrophic patients who have been receiving ARV for many years, there are important limitations in interpreting the results. For example, the prior drug exposure is different in practically every patient and other uncontrolled patient-related factors [such as age, smoking habit, intake of drugs (other than ARV) which can interfere with mt function] can act as confounding factors, which may severely limit the power of the cross-sectional studies.

In addition, mtDNA depletion has also been described in patients not showing clinically relevant ARV adverse effects [42–44]. Some cross-sectional studies have reported that, in asymptomatic individuals, mtDNA depletion is greater in patients receiving d4T as the backbone of HAART in comparison with other HAART regimens [43–45]. This is consistent with the greater *in vitro* affinity of this drug for γ–pol than other currently used NRTIs. However, the finding of mtDNA depletion in asymptomatic individuals limits the pathogenic conclusions from the aforementioned transversal studies of patients with lipodystrophy, since mtDNA depletion could be interpreted as a class drug effect of NRTI that, if investigated, may be demonstrable in most patients receiving such drugs, irrespective of the presence or absence of adverse effects. In fact, it has been reported very recently that mtDNA depletion is even present in asymptomatic HIV-uninfected infants born to HIV-infected women receiving ARV during pregnancy [46]. There are, therefore, reasonable doubts regarding the real value of abnormal findings in HIV-infected individuals with lipodystrophy. In this setting, data from longitudinal studies evaluating the same mt parameters using the same tissues from the same individuals should help to better understand the findings reported by cross-sectional studies.

Longitudinal studies in previously untreated HIV-infected individuals

Sequential studies performed in naive patients starting ARV are of special interest because they eliminate the hypothetical effects of ARV taken by patients prior to the current ARV or HAART regimen that is being evaluated. The main endpoint evaluated in such studies is usually the mtDNA content, since this is the direct effect of NRTIs. In a pioneering study, Reiss' group [47] reported the effects on the mtDNA content of PBMCs of starting ARV therapy with different ARV strategies, prior to the HAART era. They observed that, after 1 year of treatment, patients receiving AZT in monotherapy exhibited a 27% decrease of mtDNA with respect to baseline and that, when AZT was associated with ddC or ddI, the decrease was 44% and 49%, respectively. It is remarkable that the greatest part of this molecular side effect was already present as early as 4 weeks after starting the NRTIs. More recently, these investigators have also studied HIV-naive patients starting HAART and have found that, after 4 years on treatment, both d4T- and AZT-based HAART are associated with a profound mtDNA depletion in PBMCs, with this decrease being greater in the former HAART schedules (75% of decrease) than in the latter (63% of decrease) [48]. Our laboratory has

Table 1. Results of cross-sectional studies published until December 2004 in HIV-patients receiving ARV with lipodystrophy

Reference	Year	Tissue	*n*	Morphology	mtDNA	MRC function
Miro *et al.* [30]	2000	Skeletal muscle	1	Lipid+ COX–/SDH+	M. deletions	Abnormal (C3 def., C4 def., O_2 consumption def.)
		Fat	1	?	M. deletions	
		Blood cells	1	–	Normal	Abnormal (C3 def., C4 def., O_2 consumption def.)
Shikuma *et al.* [31]	2001	Fat	8	?	Depletion	?
Zaera *et al.* [13]	2001	Skeletal muscle	7	Lipid+	M. deletions	Abnormal (C3 def., C4 def., O_2 consumption def.)
		Fat	2	?	M. deletions	?
White *et al.* [36]	2001	Sperm	3	?	M. deletions	?
Walker *et al.* [34]	2002	Fat	11	Lipid+ mt. inclusions	Depletion	?
Cossarizza *et al.* [40]	2002	Blood cells	6	Normal	Normal	Normal
McComsey *et al.* [39]	2002	Blood cells	10	?	Normal	?
Roge *et al.* [41]	2002	Skeletal muscle	7	?	?	Normal
Vittecoq *et al.* [32]	2002	Skeletal muscle	19	COX-/SDH+/ COX-II def.	M. deletions, depletion	Abnormal (C1 def., C3 def., C4 def.)
		Liver	5	?	Depletion (one out of three cases)	Abnormal (C4 def.)
		Blood cells	12	?	Normal	Normal
Miro *et al.* [37]	2003	Blood cells	12	?	Depletion	Abnormal (C3 def., C4 def.)
Cossarizza *et al.* [38]	2003	Blood cells	23	?	Increase	?
Martin *et al.* [12]	2003	Blood cells	5	?	Point mutations	?
Chapplain *et al.* [35]	2004	Skeletal muscle	10	COX–	?	Abnormal (C3 def., C4 def.)
Christensen *et al.* [33]	2004	Fat	13	?	Depletion	?
		Blood cells	13	?	Normal	?

M, multiple; C1, C3 and C4, complexes I, III and IV of the mt respiratory chain, respectively; *n*, number of patients studied; ?, not performed; lipid+, lipid deposition; COX–, cytochrome c oxidase negative fibres; mt, mitochondrial; SDH+, succinate dehydrogenase hyper-reactive fibres; def., deficiency.

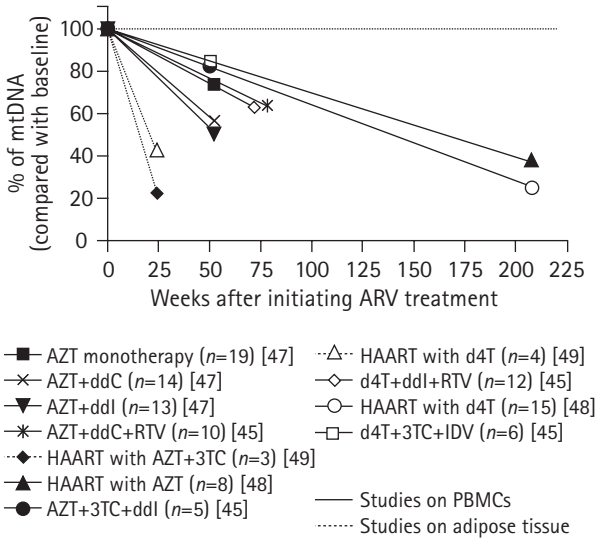
studied 11 patients naive for ARV, four of them starting with d4T+ddI-based HAART and seven of them starting with AZT+3TC-based HAART. As with previously discussed studies, we have also found greater harmful effects of d4T on PBMCs' mtDNA abundance, with a 28% decline in mtDNA content 6 months after starting HAART containing d4T+ddI, a situation that it is not observed in those who started HAART containing AZT+3TC.

However, not all longitudinal studies have shown greater mtDNA depletion in HAART schemes containing d4T. Petit *et al.* [45] also evaluated the effect of starting four different HAART regimens on mtDNA content of PBMCs after a variable time on treatment (from 50–80 weeks). They found more modest negative effects on mtDNA abundance (ranging from 15–38% of reduction) than those found by Reiss *et al.* and they did not find clear differences between schemes containing d4T and those containing AZT. Interestingly, the sequential analysis of mtDNA content evolution demonstrates again that the greatest decay is achieved during the first weeks of treatment. Nolan's group has been the only team to study the effects of the introduction of HAART on mtDNA content in the subcutaneous

fat of naive patients [49] – the target tissue of the lipodystrophy syndrome. They found that when HAART included AZT+3TC, patients developed a 78% decrease in mtDNA after 24 weeks of treatment, whereas when HAART included d4T+3TC, the magnitude of the decrease was 58%. They have further reported that, in such patients, the decrease in mtDNA fat content is accompanied by COX deficiencies demonstrated through immunohistochemical reactions [50].

Taken as a whole (Figure 1), the aforementioned results indicate that commencing HAART is uniformly associated with mtDNA depletion. As previously discussed, the vast majority of cross-sectional studies have found greater mt toxicity for d4T-based HAART than other HAART schedules. However, no clear conclusions regarding a greater capacity to induce mtDNA depletion by a particular ARV or HAART scheme can be depicted from the data reported in longitudinal analysis of previously untreated patients. Additionally, such a decline seems to mainly occur early after starting ARV. This observation is consistent with the cross-sectional study performed in liver by Walker *et al.* [51]. They found that mtDNA content declines during the initial 6 months of treatment with dideoxynucleotides, with no further decline beyond this time. One possible explanation is that while HAART adverse effects on mtDNA kinetics due to γ-pol inhibition are clearly manifest at initial stages of treatment, they are further counteracted by the reduction of HIV viraemia achieved by HAART. Since it has been demonstrated that HIV infection *per se* is associated with a decrease in mtDNA content [4,25,28], the control of HIV infection may limit the mt side effects of HIV itself. As a result, the initial exponential decline in mtDNA content may be followed by a steady-step phase (Figure 2).

Figure 1. Changes in mtDNA content in HIV-naive patients starting antiretrovirals

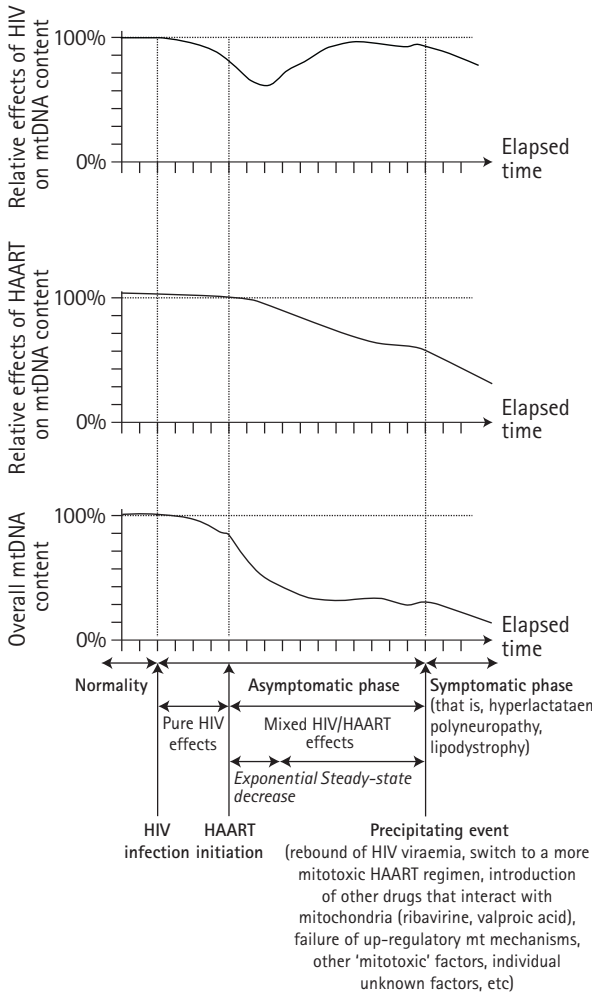


Longitudinal studies published up to December 2004. *n*, number of patients studied; ARV, antiretroviral; HAART, highly active antiretroviral therapy; mt, mitochondrial; PBMCs, peripheral bloodmononuclear cells; 3TC, lamivudine; ABV, abacavir; AZT, zidovudine; ddC, zalcitabine; ddI, didanosine; d4t, stavudine; IDV, indinavir; RTV, ritonavir.

Longitudinal studies of the effects of HAART switching in HIV-infected individuals

From a clinical point of view, it seems that once lipodystrophy appears, changes in HAART schedules do not lead to a rapid improvement of the syndrome. One possible explanation could be that adipocytes involved

Figure 2. Proposed model of action of HIV and ARVs on mtDNA content



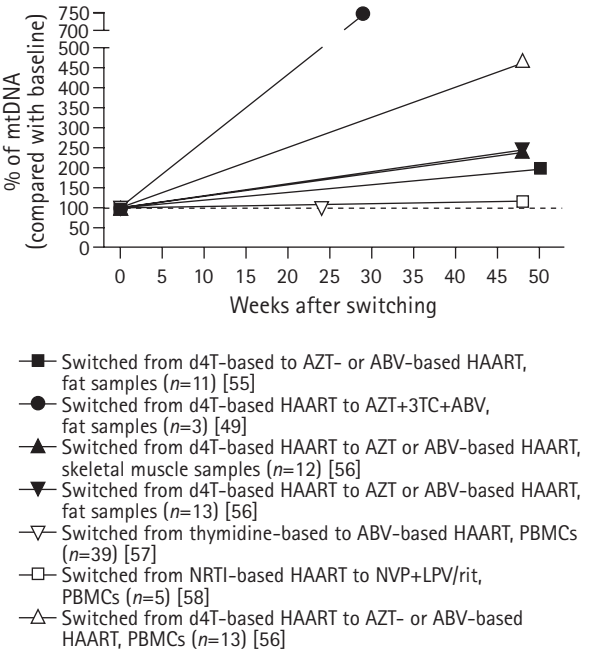
ARV, antiretroviral; mt, mitochondrial; HAART, highly active antiretroviral therapy.

in lipodystrophic changes are severely and, for the most part, inevitably damaged. Since patients with lipodystrophy usually exhibit a mixed pattern of peripheral lipoatrophy and central adiposity, it has been postulated that apoptotic activation could be responsible for lipoatrophy while decreased mt metabolism could result in lipid accumulation in visceral and central adipocytes [49].

Based on experimental data regarding the affinity of NRTIs for γ-pol, it has been proposed that switching to a HAART scheme that does not contain dideoxynucleotides could eventually improve lipodystrophy. Very recently, data from the MITOX study [52] have shown that, in patients receiving HAART who have developed lipodystrophy, the substitution of d4T or AZT by ABV (one of the NRTIs with the weakest affinity for γ-pol), is associated with a significant increase in body fat content 2 years after ABV introduction. Unfortunately, no molecular studies of mtDNA content at 2 years accompanied this report. The demonstration of the reversibility of mtDNA depletion associated with HAART changes occurring concurrently with the regression of lipodystrophy would strengthen the existence of an mt role in the development of this syndrome. This close relationship between clinical manifestations and laboratory findings has been better approached for HAART-related hyperlactataemia. For example, Montaner *et al.* [53] studied eight patients receiving d4T-based HAART who developed hyperlactataemia, and demonstrated that, after discontinuation or substitution of d4T, the disappearance of the clinical signs and symptoms correlated with an increase of over 200% in the mtDNA content in PBMCs. Similarly, we have reported that reversibility of mt abnormalities with HAART interruption is not limited to mtDNA depletion in patients developing hyperlactataemia, but also implicates the restoration of previously altered respiratory chain function [54].

Unfortunately, the data regarding the benefits in terms of mtDNA content of switching a HAART schedule are scarce and have often been obtained in a very limited number of patients with contradictory results [49,55–58] (Figure 3). On one hand, Carr's group [57] reported the largest cohort in which sequential mtDNA content has been determined and did not find evidence of any significant mtDNA change after switching from HAART containing a thymidine analogue to HAART containing ABV, at least after 24 weeks of follow-up. Additionally, the behaviour of these patients was the same as that of a control group in which no HAART changes were introduced. Consistent with Carr *et al.*'s results, we have found that the introduction of a nucleoside-sparing HAART (nevirapine plus lopinavir/ritonavir) to ARV-experienced patients is associated only with a trend toward increasing mtDNA

Figure 3. Effects of HAART changes on mtDNA content

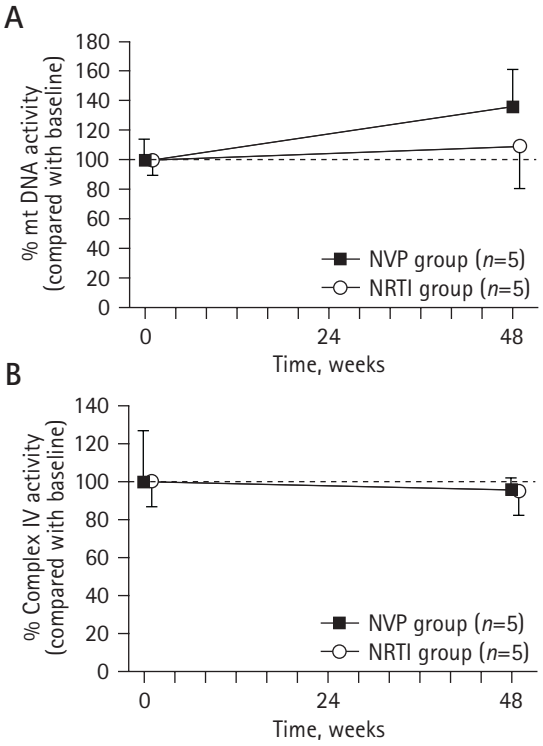


Longitudinal studies published until January 2005. *n*, number of patients studied; 3TC, lamivudine; ABV, abacavir; AZT, zidovudine; LPV/rit, lopinavir/ritonavir; NVP, nevirapine; HAART, highly active antiretroviral therapy; mt, mitochondrial; PBMCs, peripheral bloodmononuclear cells.

without changes in respiratory chain function evaluated through COX activity [58]. Indeed, this group of patients showed very similar changes as those in patients who continued with the same NRTI-containing HAART scheme (Figure 4) [57]. It is noteworthy that these non-relevant effects of switching the HAART regimen obtained by our group and Carr's were achieved using PBMCs of asymptomatic individuals. Although some doubts have been raised regarding the sensitivity of this biological sample to detect mt abnormalities, we have demonstrated that changes in mtDNA are present in asymptomatic patients receiving ARV, thereby demonstrating that PBMCs are reliable in the investigation of mt disorders [44].

In a clash with these results, Nolan's group [49] found a mean increase of 650% of mtDNA in subcutaneous fat from three patients with lipodystrophy who were switched from d4T-based HAART to a HAART combination consisting in AZT+3TC+ABV. Similarly, McComsey's group [55] has reported that mtDNA increased around 100% in the subcutaneous fat of patients with lipodystrophy in whom a d4T-based HAART was replaced by an AZT- or ABV-based HAART scheme. In a more recent and extensive paper, they studied mtDNA content in patients treated with

Figure 4. Longitudinal study comparing the effects of being maintained on a NRTI-containing HAART (NRTI group) or switched to a NRTI-sparing HAART (NVP group) on **(A)** mtDNA content and **(B)** complex IV activity



n, number of patients studied; NVP, nevirapine; HAART, highly active antiretroviral therapy; mt, mitochondrial; NRTI, nucleoside reverse transcriptase inhibitor.

d4T-based HAART and lipodystrophy, and found that the replacement of d4T by AZT or ABV was accompanied by a rebound in mtDNA of 141% in skeletal muscle, 146% in adipose tissue and 369% in PBMCs at week 48 [56]. Additionally, they also observed a restoration of mt respiratory chain complex I activity in skeletal muscle [56], as well as a decrease in apoptosis scores in adipose tissue after the switching. It is noteworthy that this study simultaneously investigates, for the first time, sequential genetic and functional mt parameters along with clinical data from patients with lipodystrophy in whom HAART was switched, and it constitutes the first evidence that the substitution of ABV or AZT for d4T improves the subcutaneous fat content as well as reverting abnormal mt indices and fat apoptosis. The demonstration of deficiencies in the diverse tissues evaluated, as well as a close correlation between mtDNA content in PBMCs and in fat, reinforces the usefulness of PBMCs as subrogates of other more appropriate, but more difficult to obtain, tissues. As the only caveat, the McComsey *et al.* study [55] lacks a control cohort group to assess the exact effects of pharmacological intervention.

Overall, it is important to note that investigations of mtDNA content are not sufficient to correctly ascertain the effects of ARV on mitochondria. The study of functional repercussions of the eventual genetic deficiencies appearing during HAART is probably a more physiopathological approach to knowing the real involvement of mitochondria in the development of lipodystrophy [59]. For example, we have recently reported the presence of up-regulatory mechanisms that compensate for mtDNA depletion developed by patients on treatment with ddI+d4T. Such mechanisms allow maintenance of correct COXII expression (a protein codified by mtDNA, which was depleted in the patients studied), as well as a normal COX activity [60]. Therefore, mtDNA studies only cover one of the multiple aspects of mt function and, consequently, can only offer partial explanations. On the other hand, lipodystrophy probably has a multifactorial mt aetiology where events other than mtDNA depletion play a pivotal role. Among them, ARV modifications of mt-dependent apoptotic pathways, as well as of overall biology of adipose tissue, are being intensively investigated [61]. Regardless of the mechanism, the important question that remains to be answered is how altered mt function leads to the massive modifications observed in adipose tissue of patients developing HAART-related lipodystrophy.

Conclusions

Nowadays, it has been demonstrated that the use of most of NRTIs in HIV-infected patients is associated with mtDNA depletion. Although cross-sectional studies suggest that certain ARVs, especially d4T, are more toxic to mitochondria, the differences among different HAART schedules detected in the analysis of longitudinal studies are not so clear. This kind of study in previously untreated individuals suggests that the greatest mtDNA loss appears at the beginning of the treatment. Conversely, in ARV-experienced patients, the potential beneficial effects of HAART switches in terms of affecting mtDNA content remain controversial and must be further investigated. Functional studies accompanying genetic investigations are needed for the correct pathogenic interpretation of mtDNA abnormalities.

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- SÍNTESI DE L'ESTUDI

El present treball, de recent publicació, consisteix en una revisió sobre quin és el grau d'evidència actual envers la participació mitocondrial en la síndrome de LD com a un dels efectes adversos més greus que manifesten molts pacients infectats pel VIH que reben TARGA de manera crònica. Hem considerat quines són les principals dificultats dels estudis que s'han realitzat fins el moment, i que han limitat l'obtenció de resultats homogenis i concloents.

RESULTATS EN PACIENTS ASSIMPTOMÀTICS INFECTATS PEL VIH

5.2. RESULTATS EN PACIENTS ASSIMPTOMÀTICS INFECTATS PEL VIH

5.2.1. Efecte dels antiretrovirals sobre els mitocondris de cèl·lules mononuclears de sang perifèrica

Aquest apartat fa referència als resultats que es deriven dels estudis realitzats en pacients infectats pel VIH que no presenten cap evidència clínica de sida ni de redistribució de greix corporal o hiperlactatèmia associats al tractament ARV. Així, els treballs d'investigació que es presenten han estat específicament dirigits a determinar els efectes que pot produir el tractament ARV sobre els mitocondris de les CMSP en aquests pacients.

MITOCHONDRIAL EFFECTS OF ANTIRETROVIRAL THERAPIES IN ASYMPTOMATIC PATIENTS

ANTIVIRAL THERAPY

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Mitochondrial effects of antiretroviral therapies in asymptomatic patients

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Background: A decrease in the mitochondrial (mt) DNA to nuclear DNA ratio has gained acceptance as a marker of mitochondrial toxicity in treated HIV-infected patients, but the functional meaning of this alteration is unclear.
Methods: We assessed mtDNA content, mitochondrial content and function in peripheral blood mononuclear cells (PBMCs) of consecutive asymptomatic HIV-infected patients. Patients selected had been receiving a first-line highly active antiretroviral therapy (HAART) regimen for at least 6 months, consisting of zidovudine plus lamivudine or stavudine plus didanosine plus either nelfinavir or nevirapine, or were antiretroviral-naive. The mtDNA content was assessed by quantitative real-time PCR, mitochondrial content by citrate synthase activity, enzyme activity of complexes III and IV (both partially encoded by mtDNA) of the electron transport chain by spectrophotometry, oxygen consumption by polarography, and oxidative damage in cell membranes by monitoring cis-parinaric acid fluorescence.
Results: Mitochondrial content was significantly lower in all treated groups. Patients receiving stavudine plus didanosine had mtDNA depletion and a decrease in complex IV activity. However, oxygen consumption capacity and lipid peroxidation were unaffected in all groups.
Conclusion: Long-term HAART may induce mitochondrial abnormalities in PBMC mitochondria, which do not necessarily translate into functional abnormalities, at least in asymptomatic patients.

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

Introduction

Antiretroviral therapies containing nucleoside reverse transcriptase inhibitors (NRTIs) may cause toxic effects in mitochondria. NRTIs inhibit HIV replication but can also inhibit human DNA polymerase-γ [1,2] and thus, replication of mitochondrial DNA (mtDNA), leading to depletion of mtDNA and drug toxicity [3,4]. This has justified the investigation of mtDNA to nuclear DNA ratio as a marker of NRTI-related mitochondrial toxicity. The development of clinical manifestations of mitochondrial toxicity in HIV-infected patients receiving NRTI-containing antiretroviral therapy [5] has proven to be consistently associated with a decrease in this ratio. Fortunately, clinical manifestations of mitochondrial toxicity may not develop in a substantial proportion of HIV-infected patients receiving NRTI-containing antiretroviral therapy [6]. In the absence of clinical manifestations of mitochondrial toxicity, it is not clearly known whether the mtDNA to nuclear DNA ratio may also be decreased [7]. In addition, even in cases where the mtDNA to nuclear DNA ratio has decreased, in asymptomatic HIV-infected patients, the functional meaning of such a disturbance has not been established. Therefore, it is necessary to demonstrate whether patients with mtDNA depletion present any functional disturbance, since the only phenotypic expression of mtDNA deficiency is the dysfunction of the mitochondrial respiratory chain (MRC) [8]. For all these reasons, we simultaneously studied the mtDNA to nuclear DNA ratio along with the mitochondrial content and function in peripheral blood mononuclear cells (PBMCs) from asymptomatic HIV-infected patients receiving different first-line antiretroviral regimens. PBMCs were chosen for this study because of

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

Materials and methods

Patients

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

Methods

Sample obtaining

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

Mitochondrial DNA quantification

For each DNA extract, the housekeeping r18S nuclear gene and the highly conserved mitochondrial ND2 gene were quantified separately by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals®, Germany) [12]. The results were expressed as the ratio of the mean values of duplicate measurements of each gene (mtDNA/nuclear DNA).

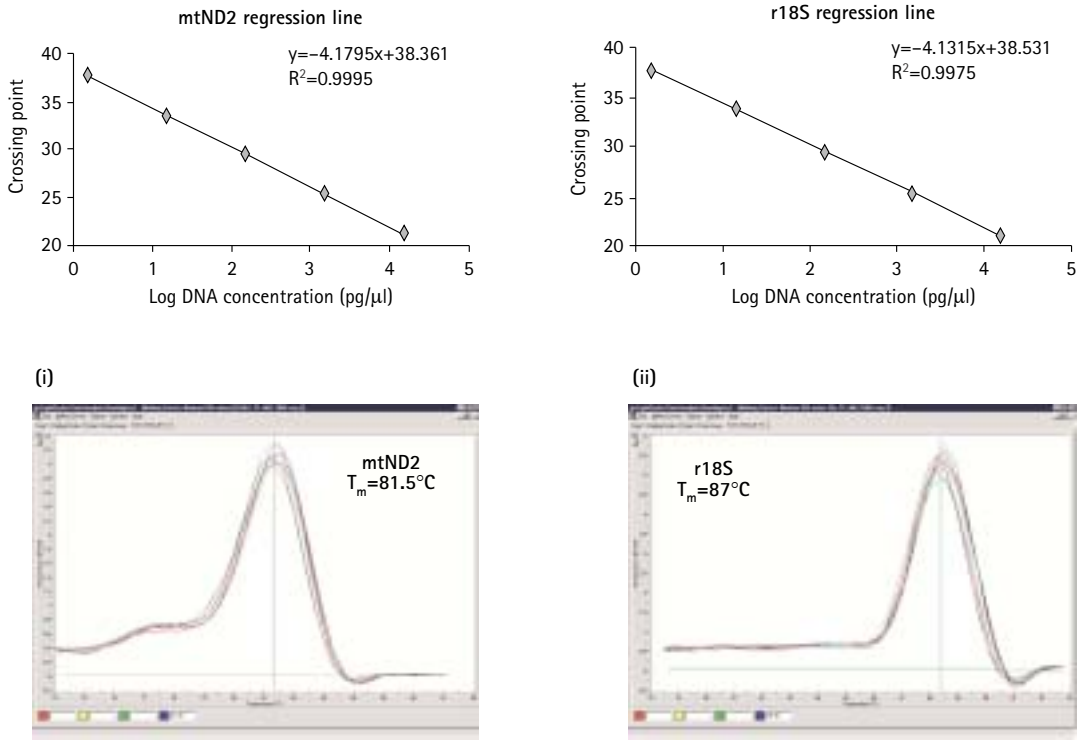
The PCR amplification of a 500 bp fragment length of the nuclear r18S gene was performed by using the forward 5′-ACGGACCAGAGCGAAAGCAT-3′ and the reverse 5′-GGACATCTAAGGGCATCACAGAC-3′ primers. For the mitochondrial ND2 gene, the forward 5′-GCCCTAGAAATAAACATGCTA-3′ and the reverse 5′-GGGCTATTCCTAGTTTTATT-3′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

The PCR reactions for the mitochondrial gene amplification contained 3 mM $MgCl_2$, 0.25 pmols/µl of each primer and 10 ng of the DNA extract in 20 µl of final volume. The PCR reactions for the nuclear gene amplification contained 2 mM $MgCl_2$, 0.3 pmols/µl of each primer and 10 ng of the DNA extract in 20 µl of final volume. The PCR amplification programme consisted of a single denaturation-enzyme-activation step of 10 min at 95°C, followed by 35 cycles (for the r18S gene) and 29 cycles (for the ND2 gene). Each cycle consists of a denaturation step (2 s at 95°C, for the r18S gene and 0 s at 94°C, for the ND2 gene), an annealing step (10 s at 66°C, for the r18S gene and 10 s at 53°C, for the ND2 gene), and an extension step (20 s at 72°C, for the r18S gene and 10 s at 72°C, for the ND2 gene), with a temperature-transition rate of 20°C/s. The fluorescent product was detected at the last step of each cycle by single acquisition. A double-stranded DNA dye (SYBR Green I) was used to monitor product formation continuously [13]. The sensitivity of SYBR Green I detection is limited by non-specific product formation, which is monitored by fluorescence acquisition at temperatures at which only specific products are double-stranded. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C, cooling at 20°C/s to 72°C for ND2 and 76°C for r18S, and slowly heating it at 0.2°C/s to 94°C with continuous fluorescence collection. Melting curves, as shown in Figure 1, were used to determine the specificity of the PCR products [14].

Biochemical studies

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

Figure 1. Real-time PCR



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

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

Oxidative activity by PBMCs (expressed as nmol of oxygen consumed per min per mg protein): measured polarographically with a Clark oxygen electrode in a water-jacketed microcell, magnetically stirred, at 37°C (Hansatech Instruments Ltd, Norfolk, UK) in 250 µl of respiration buffer (RB) consisting of 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, PH 7.4 and 1 mg/ml of bovine serum albumin (BSA), which should be freshly added to RB. Under these conditions, we determined the oxygen consumption rate by intact cells, as well as after permeabilization of the cellular membrane by incubation with digitonin, followed by the addition of different substrates, thus stimulating electron transfer through the enzymatic complexes that are partially encoded by the mitochondrial genome. The following assays were performed in PBMCs using two polarographic chambers in order to avoid contamination by rotenone:

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

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

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

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

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

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

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

Statistical analysis

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

Results

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

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

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

Table 1. Clinical and demographic data of patients

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

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

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

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

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

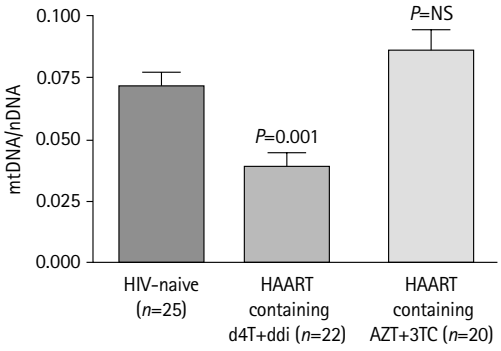
Similarly, we did not detect an increase in the oxidative damage of PBMC membranes due to lipid peroxidation reactions in treated patients, irrespective of the type of antiretroviral regimen when compared with the control group (Figure 6).

Conclusions

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

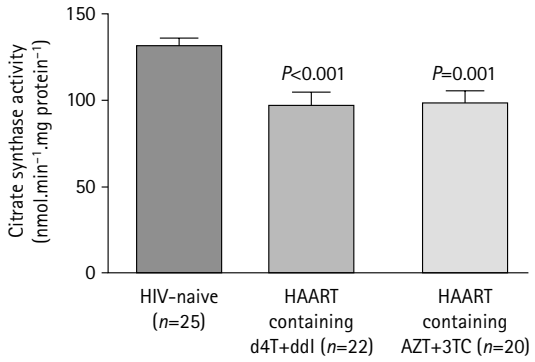
To our knowledge, these findings have not been previously reported. Different approaches have been used to study mitochondrial function during the clinical use of HAART, with most being addressed to investigate the role of mitochondria in patients with lipodystrophy [21–31] or hyperlactataemia [8,30–35]. In these studies, however, either a group of patients on HAART without symptoms is lacking [8,24–27,30,32–35] or the number of patients included is limited [21–23,28,29,31]. Moreover, they were not specifically designed to investigate the mitochondrial effects of HAART. Only one previous study has specifically addressed this issue and failed to demonstrate mtDNA depletion in 10 asymptomatic patients on different HAART schedules compared with HIV-negative patients. However, the heterogeneity of antiretroviral schedules received by these patients could hypothetically mask the existence of mtDNA depletion for a specific HAART regimen [7]. In contrast with the data reported by Henry *et al.*, our

Figure 2. Mitochondrial DNA (mtDNA) content



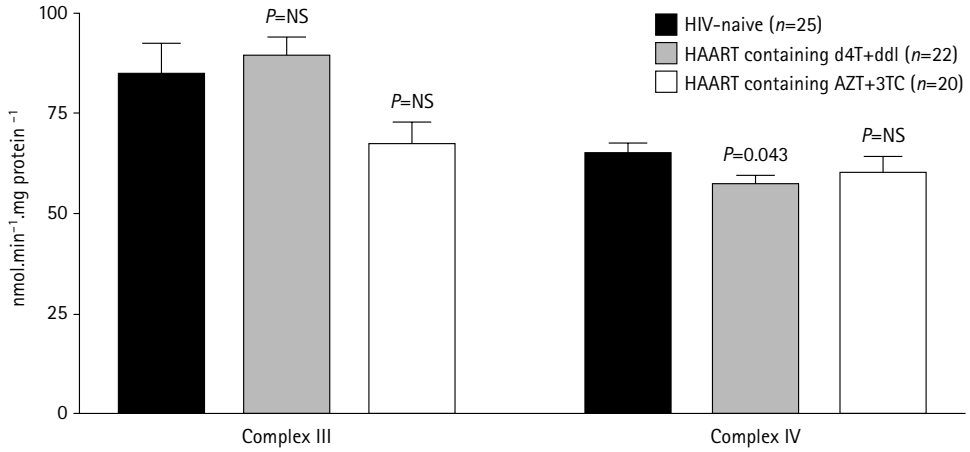
HAART, highly active antiretroviral therapy; d4T, stavudine; ddI, didanosine; AZT, zidovudine; 3TC, lamivudine.

Figure 3. Mitochondrial content



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

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



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

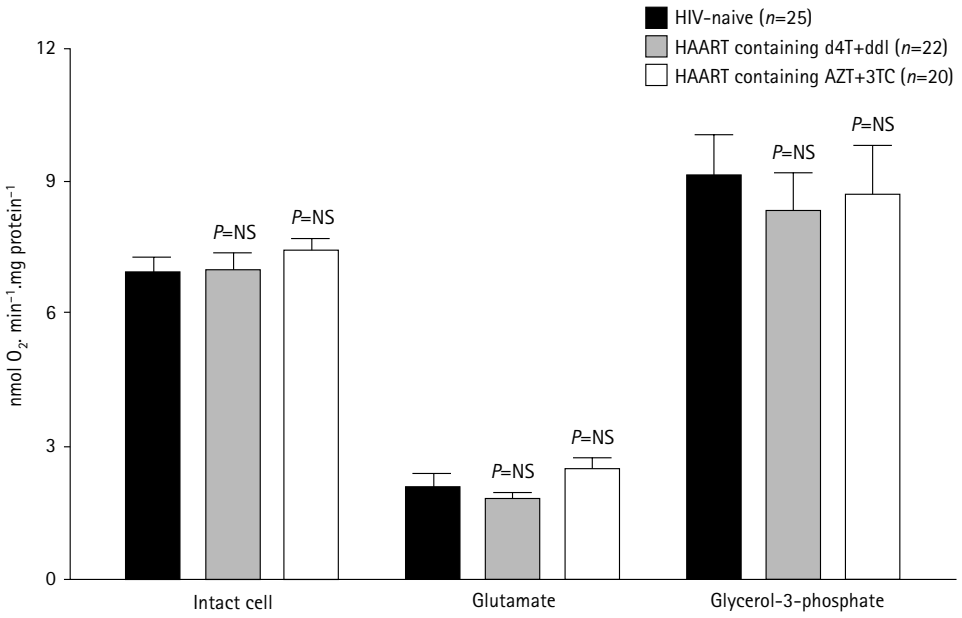
cohort of asymptomatic patients on HAART was larger and more homogenous, and undoubtedly showed mitochondrial disturbances.

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

A decrease in the mtDNA to nuclear DNA ratio in this study was detected exclusively in patients receiving d4T plus ddI. This finding is in accordance with previous studies *in vitro* demonstrating that ddI and d4T have a high potency to inhibit polymerase-γ [37] and, additionally, the triphosphated form of d4T is incorporated into replicating DNA more readily than other currently used NRTI and exerts the greatest inhibition on the human polymerase-γ [2]. This may explain the apparent association between clinical data of mitochondrial toxicity and the concomitant use of ddI and d4T. Our results confirm the relatively higher mitochondrial toxicity, in terms of a decrease in the mtDNA to nuclear DNA ratio, *in vivo* in patients receiving antiretroviral therapy containing d4T plus ddI. These results are concordant with those of another study in which the combination of nucleosides and a third drug (either a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor) were periodically changed [38]. In that study, there was an asymptomatic depletion of mtDNA in PBMCs in all treatment arms, but the depletion was greater with the regimen containing d4T plus ddI plus efavirenz than with AZT plus 3TC plus nelfinavir or the alternation of the two regimens.

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

Figure 5. Respiratory activity

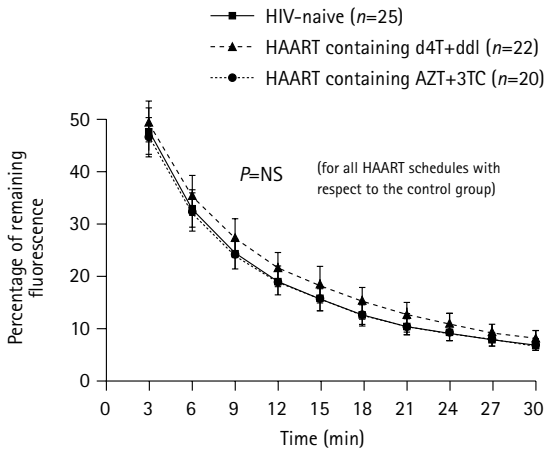


HAART, highly active antiretroviral therapy; d4T, stavudine; ddI, didanosine; AZT, zidovudine; 3TC, lamivudine; Glutamate, glutamate oxidation; Glycerol-3-P, Glycerol-3-phosphate oxidation.

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

However, decreases in mitochondrial and mtDNA content do not reach the threshold necessary to cause a significant decay in the overall respiratory capacity of MRC. It is possible that cumulated doses of antiretrovirals may not be sufficient to induce a degree of mtDNA depletion and lead to a decline in respiratory capacity. In fact, all of the patients remained asymptomatic at the time of inclusion in the study and in this setting it is foreseeable that the main MRC function, that is, to oxidize substrates, should remain unaltered. This does not exclude the fact that defects could already be present in other tissues more dependent on oxidative phosphorylation than PBMCs. In this sense, PBMCs are constantly removed from and introduced to peripheral blood. Thus, despite the fact that the HIV-infected patients included had received antiretroviral therapy for a mean of more than 1 year, it is possible that the cells studied were not exposed to antiretroviral therapy for their whole life cycle, and therefore, the drugs were not able to induce significant mtDNA damage to lead to electron transport chain dysfunction. Moreover, since PBMCs do not contain more than 15–20 mitochondria per cell and their metabolic rate is low in the absence of activation, the incorporation of damaging drugs into mtDNA is slow. It is important to note that at this preclinical stage, an antiretroviral withdrawal should probably be followed by the disappearance of all the reported abnormalities. This potential reversibility is supported by previous experience with AZT myopathy [44] and also with hyperlactataemia [45] caused by antiretrovirals, in

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



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

which molecular and biochemical disturbances normalized after drugs withdrawal.

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

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- SÍNTESI DE L'ESTUDI

En el present estudi hem volgut determinar si la toxicitat mitocondrial demostrada en els pacients infectats pel VIH que han desenvolupat algun dels efectes secundaris greus associats al TARGA és detectable en CMSP de manera precoç, abans de que els efectes adversos es manifestin clínicament.

L'estudi l'hem realitzat en les CMSP de pacients assimptomàtics infectats pel VIH que reben diferents combinacions TARGA de primera línia. Hem comparat els resultats amb un grup de pacients VIH positus que mai han rebut TARGA.

- CONCLUSIONS

Les CMSP representen un bon model d'estudi de la toxicitat mitocondrial associada al TARGA, inclús abans de que apareguin els efectes clínics adversos, doncs és possible detectar-hi alteracions mitocondrials.

Totes les combinacions TARGA incloses en l'estudi exerceixen algun efecte tòxic sobre els mitocondris, amb diferents patrons de toxicitat (depleció de l'ADNmt, disminució del contingut mitocondrial i/o disminució de l'activitat del C IV, depenent de l'esquema TARGA que s'estigui administrant). En conjunt, els resultats del treball suggereixen l'existència de diversos mecanismes patogènics deguts al TARGA.

Malgrat la disminució del contingut d'ADNmt present en pacients assimptomàtics, la funció mitocondrial roman inalterada i el dany oxidatiu no es troba augmentat.

Aquest treball subratlla la importància de valorar la repercussió funcional de la depleció del genoma mitocondrial mitjançant un estudi simultani genètic i funcional.

LONGITUDINAL STUDY ON MITOCHONDRIAL EFFECTS OF DIDANOSINE-TENOFOVIR COMBINATION

AIDS RESEARCH AND HUMAN RETROVIRUSES

2005, IN PRESS

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-----Mensaje original-----

De: Prof Bill Powderly [mailto:bpowderly@mater.ie]

Enviado el: viernes, 15 de julio de 2005 14:50

Para: MIRO, OSCAR (URGENCIES)

CC: Adrian Smith (E-mail)

Asunto: RE: AIDS Research and Human Retroviruses A-3229

July 15, 2005

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Villarroel 170, 08036 Barcelona, Catalonia, SPAIN

Re:. A-3229. LONGITUDINAL STUDY ON MITOCHONDRIAL EFFECTS OF DIDANOSINE-TENOFOVIR COMBINATION.

Dear Dr. Miro

I am happy to inform you that the above manuscript has been accepted for publication in AIDS Research and Human Retroviruses. The editorial office will contact you shortly with regard to galleys, reprints etc.

Sincerely

William G. Powderly MD

Section Editor, Opportunistic Infections

LONGITUDINAL STUDY ON MITOCHONDRIAL EFFECTS OF DIDANOSINE-TENOFOVIR COMBINATION

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Tenofovir disoproxil fumarate (TDF) has been reported to be free of adverse effects on mitochondria. We evaluate the effects of the introduction of TDF in a didanosine (ddI)-based highly active antiretroviral therapy (HAART) on mitochondrial DNA (mtDNA) content, mitochondrial mass (MM) and cytochrome c oxidase (COX) activity of the oxidative phosphorilation (OXPHOS) system over a 12 month period. Forty-four asymptomatic HIV patients with undetectable viral load receiving a ddI-based HAART were recruited and switched to ddI plus TDF (ddI+TDF) and nevirapine (n=22) or maintained with the same baseline ddI-based HAART scheme (n=22). Peripheral blood mononuclear cells were obtained at 0, 6 and 12 months. COX activity and MM were determined by spectrophotometry and the mtDNA content by quantitative real-time PCR. The mtDNA content showed a progressive decrease over the 12-month period of the study for the two groups with respect to baseline, being such a decrease statistically significant only in the ddI+TDF group (55% decrease, $p<0.001$). In addition, the decrease of mtDNA content over time was statistically different between both groups ($p<0.001$). Consistently, MM and COX activity decreased significantly at 12 months with respect to baseline only in the ddI+TDF group (28% decrease for MM, $p<0.05$; 47% decrease for COX activity, $p<0.001$). We conclude that switching to a HAART regimen containing ddI+TDF is associated with evolutive mitochondrial damage expressed as mtDNA depletion, loss of MM and decrease in COX efficiency. The particular relevance of either ddI, TDF or any interaction between them in such mitochondrial dysfunction remains to be established.

INTRODUCTION

Long-term use of nucleoside reverse transcriptase inhibitors (NRTIs) to treat HIV infection has been accompanied by severe secondary effects associated with mitochondrial toxicity. Among them, a prominent class-effect is their capacity to inhibit the synthesis of mitochondrial DNA (mtDNA), by means of direct inhibition of DNA polymerase gamma (DNA pol γ, the only polymerase responsible for the replication of mtDNA) and by acting as chain terminators of the growing DNA strand [1-3]. When mtDNA depletion reaches a certain threshold, dysfunction of the oxidative phosphorilation (OXPHOS) system occurs. This NRTIs-associated OXPHOS dysfunction is believed to play a role in the etiology of diverse clinical syndromes in HIV-infected individuals, including myopathy, cardiomyopathy, neuropathy, pancreatitis, lactic acidosis and lipodystrophy [4,5].

These mitochondrial side effects have prompted the pharmaceutical industry to develop and evaluate new antiviral agents with potent anti-HIV activity and less mitochondrial toxicity. Accordingly, tenofovir disoproxil fumarate (TDF), a prodrug of the nucleotide analogue tenofovir, is the first nucleotide analogue reverse transcriptase inhibitor (NtRTI) approved for use in the treatment of HIV infection. TDF has been associated with great efficacy, high tolerability and a favourable lipid profile and it has been proposed to be exempt of mitochondrial toxicity [6,7]. With respect to the latter, TDF has been tested to evaluate its effects on mtDNA content on *in vitro* experiments with a human hepatoblastoma cell line (HepG2 cells) and it was found to not induce mtDNA depletion at concentrations significantly exceeding its effective antiviral concentration and the therapeutically relevant levels in plasma [8]. Moreover, it has been shown that TDF does not exert any inhibitory effect on cellular expression of the COX-II (mtDNA-encoded)

and COX-IV (nuclear DNA-encoded) subunits of the cytochrome c oxidase (COX; the complex IV of the OXPHOS system) and does not increase lactate production [8]. The above data, together with a favourable *in vivo* tolerability profile, support the fact that TDF-associated mitochondrial dysfunction should be low compared to other NRTIs such as zalcitabine (ddC), didanosine (ddI), stavudine (d4T) and zidovudine (AZT) [9,10].

However, there are no data available on *in vivo* studies evaluating mitochondrial function in HIV-infected patients receiving TDF. In addition, the abovementioned experimental data were obtained using TDF as an isolated drug and not in combination with other antiretrovirals. In this sense, it has been reported that TDF increase plasma concentrations of ddI when they are intake concomitantly [7], and this aspect could eventually limit the favourable mitochondrial profile of TDF. In the present study, we evaluate in vivo the effects of the introduction of TDF in a highly active antiretroviral therapy (HAART) combination containing enteric coated ddI on various parameters of peripheral blood mononuclear cell (PBMC) mitochondria of HIV-infected patients.

METHODS

Study design: The present trial is a retrospective analysis of mitochondrial parameters with samples prospectively collected throughout 48 weeks of a group of patients belonging to a larger clinical trial, the *QD Study* [11]. This was a comparative, multicentre, prospective study, in which patients on twice-daily HAART regimens, visited consecutively, were offered to simplify therapy and switch to ddI+TDF+nevirapine (NVP), once-daily. Patients willing to simplify were switched, while the remainder of patients continued their existing twice-daily regimens.

Study population: Candidates for the mitochondrial study were all those subjects from the mentioned clinical study who were receiving a ddI-containing regimen at baseline and with available stored PBMCs at baseline and at months 6 and 12. Additionally, both groups studied were matched according to the HAART combination received at inclusion, defined as ddI+d4T and ddI+other NRTIs, in order to make groups homogeneous regarding antiretroviral therapy. Forty-four HIV-infected patients, on stable HAART regimens, all of which contained enteric coated ddI (400 mg) in the nucleoside backbone, were included in the study. Twenty-two of these subjects were switched to a new HAART scheme also containing 400 mg enteric coated ddI plus 300 mg TDF and 400 mg NVP, given once a day with food (ddI+TDF group) and the other 22 individuals maintained the same baseline ddI-based HAART regimen (control group). All patients were asymptomatic regarding HIV infection or antiretroviral therapy and maintained an undetectable viral load throughout the 12-month study period. None of the patients required either treatment interruptions or changes in antiviral therapy during the study.

Assessment: Clinical and epidemiological data were recorded, as was the antiretroviral history, specifically compiling the time receiving ddI, ddI+d4T and time receiving any NRTIs, in order to take into account the different background cytotoxic effect potentially produced by different antiretroviral combinations including NRTIs. Mitochondrial studies in PBMCs were performed at 0 (baseline), and at 6 and 12 months after the inclusion in the study. All patients provided written informed consent and the protocol was approved by the Ethics Committee of the Germans Trias i Pujol Hospital.

Obtaining PBMCs: Twenty millilitres of peripheral venous blood, collected in Vacutainer™ EDTA tubes, were used to perform all the studies. PBMCs were obtained by separation on Ficoll density gradient centrifugation (Histopaque®-1077, Sigma Disgnostics, St. Louis, MO) and cryopreserved in liquid nitrogen. Platelet contamination must be limited to as minimum as possible (ideally, to less than 5 platelets per PBMC) in order to do not overestimate mtDNA content, because platelets are cellular fragments that contain mitochondria (mtDNA) but not nuclei (nuclear DNA). For this reason we confirmed a platelets count below 25 per PBMC in all patients coming from both groups. PBMCs were re-suspended in phosphate buffered saline (PBS) for biochemical and genetic determinations. Protein content was measured by means of Bradford's protein-dye binding principle [12].

MtDNA content: An aliquot of PBMCs was used for the extraction of total DNA by standard phenol-chloroform procedures. For mitochondrial DNA quantification, the nuclear housekeeping 18S rRNA gene and the highly conserved mitochondrial ND2 gene were amplified separately by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals, Mannheim, Germany) as reported extensively elsewhere [13-15]. The results were expressed as the mtDNA to nuclear DNA ratio from the mean values of duplicate measurements of each gene.

Mitochondrial mass (MM): The quantity of mitochondria was estimated by the spectrophotometric measurement of citrate synthase activity (CS, enzyme code (EC) 4.1.3.7), a mitochondrial matrix enzyme of the Krebs' cycle, at 37ºC and 412 nm (U-2001 UV/Vis Spectrophotometer, Hitachi Instruments, Inc., San Jose, CA). CS is considered to be a reliable marker to estimate the MM [16-18]. CS activity was expressed as nanomols of reduced substrate per minute and per milligram of cell protein (nmols/min/mg protein). The complete methodology has been described previously [15].

Cytochrome c oxidase (COX; EC 1.9.3.1) activity: We chose COX as a representative enzyme of OXPHOS system, because it is partially encoded by the mitochondrial genome. The measurement of the specific enzyme activity was performed spectrophotometrically (U-2001 UV/Vis Spectrophotometer, Hitachi Instruments, Inc., San Jose, CA) at 37ºC and 550 nm, according to Rustin et al. [19], and slightly modified for minute amounts of biological samples [15,20]. COX activity was expressed as nanomols of oxidated substrate per minute and per milligram of cell protein (nmols/min/mg protein).

Statistical analysis: The clinical and epidemiological characteristics of all patients at baseline were expressed as mean±SD (quantitative data) and percentages (qualitative data), and comparisons were carried out by using the unpaired Student's t test and chi square test, respectively. Normality of the distribution of all the variables was ascertained by the Kolmogorov-Smirnov test. The results of the mitochondrial studies in PBMCs at 6 and 12 months were referred as percentages of the baseline values (100%). Two-way ANOVA for repeated measurements was used to determine significant differences with respect to baseline values and between ddI+TDF and control groups. Linear regression analysis was performed to establish any association among mitochondrial parameters. In all the cases, p values less than 0.05 were considered statistically significant.

RESULTS

All patients remained asymptomatic regarding HIV infection or antiretroviral therapy, and no TDF or ddI-associated adverse events were reported during the 12 months of follow-up. The clinical and epidemiological characteristics at baseline did not differ between ddI+TDF and control groups (table 1).

The content of mtDNA, MM and COX activity did not differ between the two groups at baseline (p=NS, for each mitochondrial parameter; figure 1). In addition, no differences were found for any of the mitochondrial parameters studied at baseline between ddI+TDF and control groups according to the fact that they were on treatment with ddI+d4T or ddI+other NRTIs (data not shown).

The content of mtDNA showed a mild decrease at 6 months with respect to baseline in both ddI+TDF group (22% decrease, p<0.01) and control group (11% decrease, p=NS). MM and COX activity were normal at this time for the two groups. The decrease in the content of mtDNA was more pronounced at 12 months for the two groups with respect to baseline, being such a decrease statistically significant only in the ddI+TDF group (55% decrease, p<0.001) (figure 2). In addition, the decrease of the mtDNA content over time was statistically different between the two groups (p<0.001). Similarly, MM and COX activity showed a significant decrease compared to the baseline value at the end of the study only in the group of patients that had switched to receive the ddI+TDF combination (28% decrease for MM, p<0.05; and 47% decrease for COX, p<0.001) but not in the patients who maintained the same baseline ddI-based HAART regimen without the addition of TDF during the study period (controls) (figure 2). The decrease observed over time in MM and COX activity was statistically different between the two groups (p<0.05).

We also evaluated whether the mitochondrial parameters studied differed in patients who switched to ddI+TDF combination according to the fact that they had been receiving ddI+d4T or ddI+other NRTIs at baseline. As shown in figure 3, the two subgroups exhibited comparable outcomes with respect to mtDNA content, MM and COX activity, with no significant differences between these subgroups over the study time (p=NS). The decrease over time of the mtDNA content and COX activity in each subgroup was also statistically significant in each case at 12 months when compared to baseline (p<0.001 for both mtDNA content and COX activity, for each subgroup).

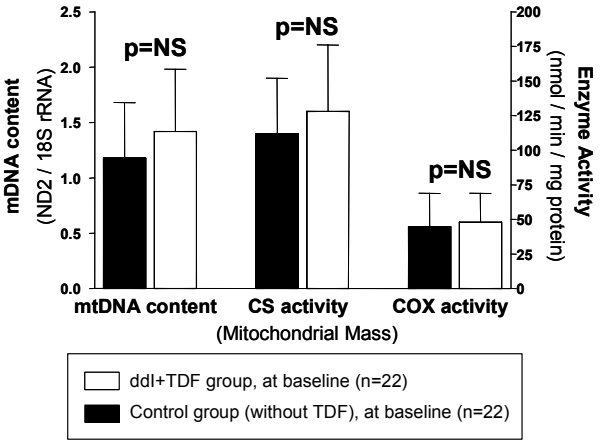

Figure 1. Comparison of baseline mitochondrial parameters between ddI+TDF and control groups. The result for the mtDNA content is expressed as the ratio between the mitochondrial gen (ND2) and the nuclear gene (18S rRNA) quantification. The results for the enzyme activities are expressed as nanomols per minute and per milligram of cell protein. (mtDNA: mitochondrial DNA; COX: cytochrome c oxidase; CS: citrate synthase; NS: not significant).

Table 1: Baseline clinical and epidemiological characteristics.

	ddI+TDF group (n=22)	Control group (without TDF) (n=22)	p value
Age (years), ±SD	43±10	40±7	NS
Male gender (%)	68	88	NS
CD4$^+$ lymphocyte count (cells/mm^3), ±SD	715±272	720±235	NS
Viral load (RNA copies per mL)	<80 in all cases	<80 in all cases	NS
Patients on ddI+d4T at baseline (%)	50	50	NS
Patients on ddI+other NRTIs at baseline (%)	50	50	NS
Months on treatment at baseline			
- receiving NRTIs, ±SD	74±46	69±42	NS
- receiving ddI, ±SD	33±20	39±21	NS
- receiving ddI+d4T, ±SD	10±17*	9±12*	NS

*Only calculated for the 11 patients of each group receiving ddI+d4T.
SD: standard deviation.
NS: not significant.
ddI: didanosine; d4T: stavudine; NRTIs: nucleoside analogue reverse transcriptase inhibitors.

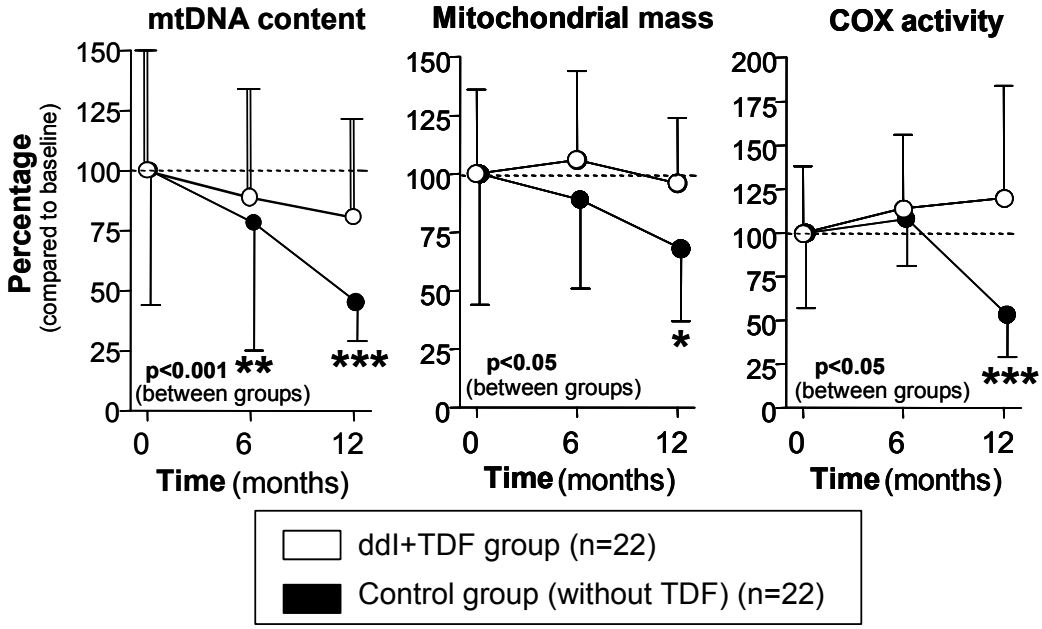

Figure 2: Modification of the mitochondrial parameters in both ddI+TDF and control groups during the 12-month period of study. The results are expressed as percentage compared to baseline measurements (100%) for the two groups. (mtDNA: mitochondrial DNA; COX: cytochrome c oxidase; TDF: tenofovir disoproxil fumarate; *:p<0.05; **:p<0.01; ***:p<0.001, with respect to baseline).

However, MM did not reach statistical significance when the ddI+TDF group was stratified, probably because the size of each subgroup was now smaller.

Linear regression analysis did not show significant correlation between the mitochondrial parameters evaluated in present study. As the only remark, we observe a mild not significant association between the depletion of mtDNA and the decrease of COX activity (R=0.28; p=0.08) which was more evident for patients of control group that for patients of ddI+TDF group (figure 4).

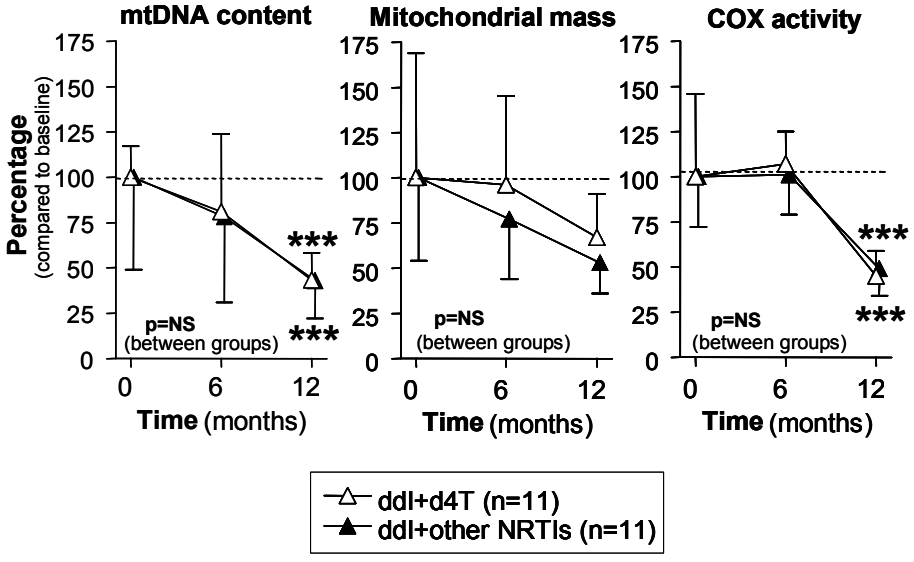

Figure 3: Modification of the mitochondrial parameters during the 12-month period of study in the group of patients switched to TDF according to the fact that they received ddI+d4T or ddI+other NRTIs at baseline. The results are expressed as percentage compared to baseline measurements (100%) for the two subgroups. (mtDNA: mitochondrial DNA; COX: cytochrome c oxidase; TDF: tenofovir disoproxil fumarate; ddI: didanosine; d4T: stavudine; NRTIs: nucleoside analogue reverse transcriptase inhibitors; NS: not significant; *:p<0.05; **:p<0.01; ***:p<0.001, with respect to baseline).

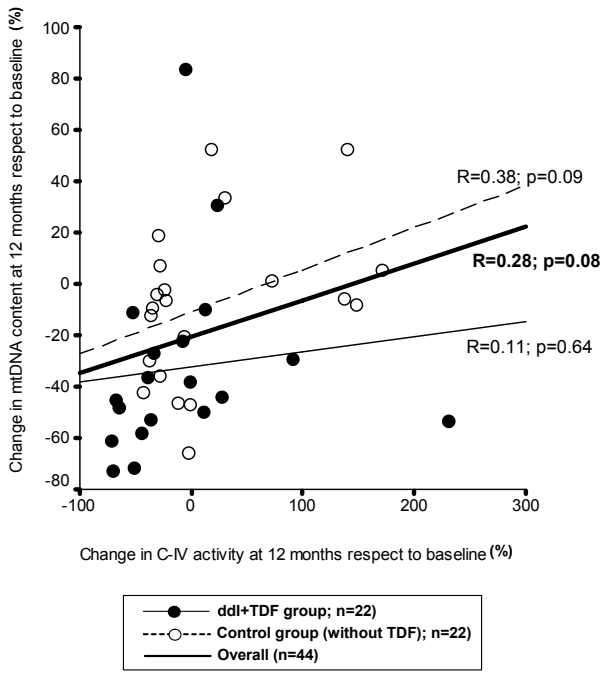

Figure 4: Linear regression **analysis between the changes of mtDNA and COX activity at the end of study (12 months) respect to baseline (mtDNA: mitochondrial DNA; COX: cytochrome c oxidase).**

DISCUSSION

Although previous *in vitro* studies in cultured cells [8] and animals [21] have shown that TDF has very low affinity for DNA pol γ, suggesting that little *in vivo* mitochondrial toxicity may be expected in long-term clinical use of TDF, pharmacokinetic studies in blood samples from healthy volunteers have demonstrated that the co-administration of 300 mg TDF in combination with 400 mg ddI in a HAART regimen results in 40-64% increase in Cmax and area under the curve (AUC) of ddI with no effects on the pharmacokinetic characteristics of TDF [10,22-26]. The interaction between TDF and ddI has been proposed, based on *in vitro* studies, to be mediated by phosphorylated metabolites of tenofovir, which may directly inhibit the purine nucleoside phosphorylase-dependent degradation of ddI, resulting in increased level of exposure to ddI [27]. In the present study, we have evaluated *in vivo* the cellular consequences of this drug-drug interaction by means of the assessment of the mitochondrial effects of the introduction of TDF in a ddI-based HAART regimen on PBMCs of HIV-infected patients. Our data shows that the combination of TDF and ddI at doses of 400 mg is associated with a progressive and statistically significant decrease of the mtDNA content at 12 months. Our study also shows that the mtDNA depletion found at 12 months of study in patients on ddI+TDF is accompanied of increased mitochondrial damage, which is expressed as loss of MM and decrease in COX efficiency of the OXPHOS system.

The mechanism for mitochondrial dysfunction is not investigated in present study. We hypothesise that, since COX enzymatic complex is partially encoded by the mitochondrial genome, a decrease of COX mtDNA-encoded protein subunits due to mtDNA depletion could contribute to the decrease in COX activity. This mitochondrial dysfunction would imply that up-regulatory mechanisms that mitochondria develop to compensate mtDNA depletion [28] are insufficient in patients receiving ddI+TDF for 12 months. Alternatively, since only a weak (and not significant) correlation between the mtDNA depletion and the loss of COX activity was found in present study, it is also possible that mechanisms other than mtDNA depletion could be contributing to the decrease of COX efficiency.

We expected to observe certain mtDNA depletion over time after TDF introduction, because TDF was added to a ddI-based HAART regimen and ddI is a NRTI with a well-recognised capacity of causing mtDNA depletion [1]. In fact, a mild and not significant mtDNA depletion over time was observed in control group formed by patients maintained in a ddI-based HAART without the addition of TDF. However, the great increase in the deleterious mitochondrial effects following the introduction of TDF has been a little bit surprising. Whether these side mitochondrial effects (mtDNA depletion, loss of MM and decrease in COX efficiency of the OXPHOS system) are caused by TDF or by increased concentrations of ddI remain to be investigated. However, we believe that such mitochondrial damage may reflect the cytotoxic effects of ddI, due to the increase of exposure to ddI caused by the interaction with TDF when the two drugs are co-administered.

The clinical relevance of the mitochondrial abnormalities found in this study is unknown. Very recently, Martínez et al. have reported increased pancreatic toxic effects associated with the co-administration of ddI+TDF [29], being pancreatitis a well-known disorder associated with classical mitochondrial diseases [30]. In addition, several groups recommend to avoid the co-administration of ddI+TDF because of the increased risk of virologic failure recently reported [31,32] and the decline of CD4+ cell count observed in patients receiving ddI+TDF despite viral supression [24]. Thus, although several experimental studies have established a mitochondrial safety profile for TDF, additional mitochondrial damage to that one already observed in HIV-patients on HAART containing ddI, could be present after several months of therapy with ddI and TDF in combination and contribute to the development of such mitochondrial-related disorders.

In any case, the present study constitutes the first report of functional data of PBMC mitochondria from HIV-infected patients receiving TDF in combination with standard doses of ddI as antiviral therapy. Although it has some limitations (non-randomised, potential selection bias, low statistical power), we believe that our data indicates that the clinical use of these two antiviral agents in combination is not completely free of mitochondrial adverse effects, at least when high doses of ddI are given with TDF for 12 months. Further studies are needed to elucidate whether reduced doses of ddI are able to limit these

adverse mitochondrial effects and whether there is any relationship between mitochondrial abnormalities and the adverse effects observed with ddI+TDF co-administration. Until then, the use of both drugs in combination should be cautiously recommended. Reduced doses of ddI may be used with TDF only in patients with viral suppression and when more appropriate nucleoside combinations are not available, always followed of a close monitorization.

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- SÍNTESI DE L'ESTUDI

En aquest estudi hem volgut determinar els efectes que produeix una combinació TARGA basada en la combinació de ddI (400 mg) + TDF (300 mg) sobre els mitocondris de les CMSP en pacients infectats pel VIH.

Hem realitzat un estudi longitudinal durant un període de 12 mesos en un grup de pacients assimptomàtics infectats pel VIH que reben una combinació TARGA amb ddI, a la que s'ha incorporat TDF. Hem comparat els resultats amb un grup de pacients VIH positus que reben la mateixa combinació TARGA amb ddI i que han continuat amb el mateix esquema ARV sense afegir-hi TDF.

- CONCLUSIONS

La combinació TARGA que inclou una dosi de 400 mg de ddI i una dosi de 300 mg de TDF produeix un efecte tòxic mitocondrial superior a l'efecte que produeix una pauta ARV que inclou 400 mg de ddI sense TDF.

Aquest efecte de toxicitat mitocondrial atribuït a l'administració de ddI + TDF (amb les dosis anteriorment descrites) pot ser conseqüència de l'efecte sinergístic que produeixen els dos fàrmacs en combinació, doncs s'ha determinat que el TDF incrementa la concentració plasmàtica del ddI, la qual cosa podria incrementar la toxicitat associada al ddI.

UPREGULATORY MECHANISMS COMPENSATE FOR MITOCHONDRIAL DNA DEPLETION IN ASYMPTOMATIC INDIVIDUALS RECEIVING STAVUDINE PLUS DIDANOSINE

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Upregulatory Mechanisms Compensate for Mitochondrial DNA Depletion in Asymptomatic Individuals Receiving Stavudine Plus Didanosine

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Summary: Nucleoside analogue use is often related to mitochondrial DNA (mtDNA) depletion, but mitochondrial function is preserved in most asymptomatic patients. We determined whether homeostatic mechanisms are able to compensate for this mtDNA depletion in patients receiving stavudine plus didanosine (d4T + ddI), an antiretroviral combination with great in vitro and in vivo capacity to decrease mtDNA. We included 28 asymptomatic HIV-infected individuals: 17 subjects (cases) on a first-line antiretroviral regimen consisting of d4T + ddI as the nucleoside backbone plus nevirapine or nelfinavir for at least 6 months (mean: 16 ± 8 months) and 11 naive subjects (controls). We assessed the following in peripheral blood mononuclear cells: mitochondrial mass by citrate synthase activity, mtDNA content by real-time polymerase chain reaction, cytochrome c oxidase subunit II (COX-II) expression by Western blot analysis, and COX activity by spectrophotometry. The mitochondrial mass and mtDNA content of cases decreased when compared with controls, whether normalized per cell or per mitochondrion. Conversely, COX-II expression and COX activity were similar in cases and controls. COX-II expression was constant and independent of the mtDNA content, whereas it was closely related to COX activity. We concluded that treatment with dd4T + ddI is associated with decreased mitochondrial mass and mtDNA content but that COX-II expression and COX activity remain unaltered. These data suggest that upregulatory transcriptional or posttranscriptional mechanisms compensate for mtDNA depletion caused by d4T + ddI before profound mtDNA depletion occurs.

Key Words: peripheral blood mononuclear cells, mitochondrial DNA, cytochrome c oxidase subunit II, nucleoside reverse transcriptase inhibitors, stavudine, didanosine, DNA γ-polymerase

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Mitochondrial DNA (mtDNA) depletion is a secondary effect inherent to most of the nucleoside reverse transcriptase inhibitors (NRTIs). The main reason for this fact is that NRTI triphosphates are also able to inhibit DNA γ-polymerase, the only enzyme that replicates mtDNA.[1] Some reports have attributed a pathogenic role to mtDNA depletion in the long-term adverse effects associated with the use of NRTIs, especially hyperlactatemia[2,3] and lipodystrophy.[4–8] However, the cumulated lifetime doses of NRTIs at which these adverse effects develop have not been established. In fact, the reasons why some people do not develop these adverse effects, despite large doses of NRTIs, remain unknown, and few studies have evaluated the functional relevance of mtDNA depletion at the level of the mitochondrial respiratory chain (MRC). Thus, many questions arise as to the real role of mtDNA depletion in these adverse effects.[9–11]

One factor that may contribute to the inconsistent presentation of adverse effects, despite the constant presence of mtDNA depletion, may be the development of homeostatic mechanisms to compensate for this depletion. At least before profound mtDNA depletion occurs, these mechanisms may act at transcriptional or posttranscriptional levels to compensate for the mild to moderate decrease of the mtDNA content. The final objective of these hypothetic changes would be to maintain the MRC capacity unaltered, because all the mtDNA-encoded genes are devoted to the synthesis of proteins of MRC complexes. To extend this mechanism, we assessed the effects of the antiretroviral therapy beyond mtDNA content by determination of the expression of the human cytochrome c oxidase subunit II (COX-II, 1 of the 3 subunits of COX encoded by mtDNA) and COX activity. We chose HIV-infected individuals undergoing treatment with stavudine and didanosine

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(d4T + ddI) as the nucleoside backbone of a highly active antiretroviral therapy (HAART) regimen because of the great in vitro[12] and in vivo[13,14] capacity of this antiretroviral combination to decrease the mtDNA content.

METHODS

We designed a cross-sectional and observational study including HIV-infected patients on a first-line HAART regimen consisting of d4T + ddI as the nucleoside backbone (plus nelfinavir or nevirapine) for at least 6 months. A control group consisting of antiretroviral-naive HIV-infected patients was also studied. All patients were symptom-free regarding HIV disease and/or antiretroviral therapy at the time of inclusion in the study. None of the individuals receiving treatment had clinical data of lipodystrophy. Clinical and demographic data for each patient, including age, gender, $CD4^+$ T-cell count, HIV-1 RNA copies, and duration of HAART, were recorded at the time of inclusion. Patients with a personal or familial history suggestive of mitochondrial disease or neuromuscular disorder were excluded. As reference values for all the mitochondrial experiments, we used data from 20 healthy individuals matched by age and gender who were not infected with HIV and had been previously assayed in our laboratory. The protocol was approved by the institutional ethics committee of each hospital, and all the patients provided written informed consent.

Obtaining Samples

A total of 20 mL of venous blood was extracted from each patient, and peripheral blood mononuclear cells (PBMCs) were immediately isolated by Ficoll density gradient centrifugation (Histopaque-1077; Sigma Diagnostics, St. Louis, MO). The platelet count after the PBMC extraction procedure was confirmed to be less than 200 per PBMC in each case. Total DNA was obtained by a standard phenol-chloroform extraction procedure from an aliquot of PBMCs and was used for genetic studies. The remaining PBMCs were resuspended in 100 to 150 μL of phosphate-buffered saline (PBS) and used for Western blot studies and enzyme assays. Protein content was measured according to the protein-dye binding principle of Bradford.[15]

Mitochondrial Mass

The quantity of mitochondria was estimated by means of spectrophotometric measurement at 412 nm (UVIKON 922; Kontron, Basel, Switzerland) of the citrate synthase (CS) activity (Enzyme Code (EC) 4.1.3.7), a mitochondrial matrix enzyme of the Krebs cycle, which remains highly constant in mitochondria and is considered to be a reliable marker of mitochondrial content.[16–18] CS activity was expressed as nanomoles of reduced substrate per minute and per milligram of cell protein. The complete methodology has been described elsewhere.[19]

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 (PCR; LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany). Detailed conditions of the experiments have been reported extensively elsewhere.[19] The results were expressed as the ratio of the mean mtDNA value of duplicate measurements to the mean nuclear DNA value (nDNA) of duplicate measurements (mtDNA/nDNA).[20,21] The results of mtDNA content using the methodology described are related to cells. To normalize these results by the cellular mitochondrial content, we also calculated mtDNA content per mitochondria by dividing the mtDNA/nDNA ratio by CS activity.

Measurement of the Cytochrome C Oxidase Subunit II of Cytochrome C Oxidase

Crude protein extracts containing 20 μg of protein were mixed with a 1:5 volume of a solution containing 50% glycerol, 10% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 0.5% bromophenol blue, and 0.5 M of Tris (pH 6.8), incubated at 90°C for 5 minutes and electrophoresed on 0.1% SDS/13% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were probed with a monoclonal antibody for the mtDNA-encoded human COX-II (A-6404; Molecular Probes, Eugene, OR) as well as with antibodies against voltage-dependent anion carrier (VDAC) or porin (Calbiochem Anti-Porin 31HL; Darmstadt, Germany) as a marker of mitochondrial protein loading and against β-actin (Sigma clone AC-15; St. Louis, MO) as a marker of overall cell protein loading. Immunoreactive material was detected by the enhanced chemiluminescence detection system and resulted in a 25-kd band for COX-II, a 31-kd band for VDAC, and a 47-kd band for β-actin as expected (Fig. 1). The intensity of signals was quantified by densitometric analysis (Phoretics 1D Software; Phoretics International LTD, Newcastle, England). The content of the COX-II subunit was normalized by the content of β-actin signal to establish the relative COX-II abundance per overall cell protein and by the nuclear-encoded mitochondrial

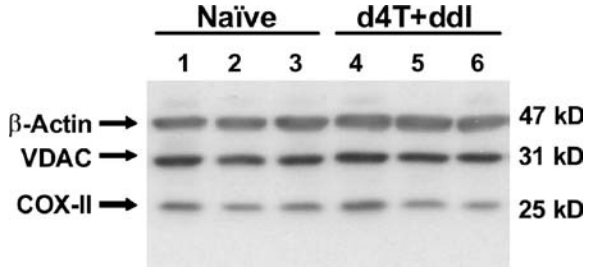

FIGURE 1. Example of immunoblot analysis of cytochrome c oxidase subunit II (COX-II) protein abundance in protein extracts from peripheral blood mononuclear cells of naive (1–3) and stavudine plus didanosine (d4T + ddI)–treated (4–6) patients. Each lane corresponds to 20 μg of protein, and arrows indicate the specific immunoreactive signals for COX-II, voltage-dependent anion channel (VDAC), and β-actin proteins. The COX-II/β-actin and COX-II/VDAC ratios were calculated using densitometric analysis of the chemoluminescence specific signal obtained in Western blots probed with the specific antibodies (see Methods section).

protein VDAC to establish the relative COX-II abundance compared with overall mitochondria.

Cytochrome C Oxidase (Enzyme Code (EC) 1.9.3.1) Activity

The measurement of the specific enzyme activity was performed spectrophotometrically (UVIKON 922) at 37°C according to Rustin et al[22] and was slightly modified for minute amounts of biologic samples.[18,23] COX activity was expressed as nanomoles of oxidated substrate per minute and per milligram of cell protein. It was also calculated per mitochondrion by dividing absolute COX activity by CS activity.

Statistical Analysis

Qualitative data were expressed as percentages and quantitative data as mean ± SD. Comparisons were carried out by using the χ^2 test and unpaired Student *t* test for qualitative and quantitative variables, respectively. In the Student *t* test, normality of the distribution was ascertained using the Kolmogorov-Smirnov test before applying parametric tests. Linear regression analysis was performed to evaluate the relation between quantitative variables. In all cases, *P* values less than 0.05 were considered statistically significant.

RESULTS

We included 11 consecutive HIV-infected treatment-naive individuals (controls) and 17 consecutive HIV-infected individuals (cases) receiving a first-line HAART regimen containing d4T + ddI as the nucleoside backbone (9 of them receiving nevirapine and 8 of them taking nelfinavir as the third drug of the antiretroviral combination). The clinical characteristics are shown in Table 1. The 2 groups only differed in viral load, which was greater in untreated individuals.

CS activity was 126 ± 19 nmol/min/mg of protein for naive individuals and 92 ± 31 nmol/min/mg of protein for individuals receiving treatment (73% of control activity; $P < 0.001$), indicating decreased mitochondrial mass in patients on d4T + ddI. Individuals receiving d4T + ddI also showed a significant decrease in mtDNA content, which was expressed per cell (60% of control content; $P < 0.01$) or per mitochondrion (72% of control content; $P < 0.05$). Conversely, the expression of the COX-II subunit of COX (encoded by mtDNA) was similar in the 2 groups, regardless of expression

TABLE 1. Clinical Characteristics of Individuals Included in the Study

	Naive (n = 11)	d4T + ddI (n = 17)	*P*
Age (y), ±SD	39 ± 10	42 ± 12	NS
Male gender (%)	91	77	NS
$CD4^+$ lymphocyte count (cells/mm^3), ±SD	304 ± 196	446 ± 206	NS
Log_{10} viral load (copies/mL)†, ±SD	5.2 ± 0.5	2.0 ± 0.6	<0.001
Months on treatment, ±SD	—	16 ± 8	—

*$P < 0.05$ was considered to be statistically significant with respect to the controls.
†Viral load <50 copies/mL was considered to be 49 copies/mL.
NS indicates not significant.

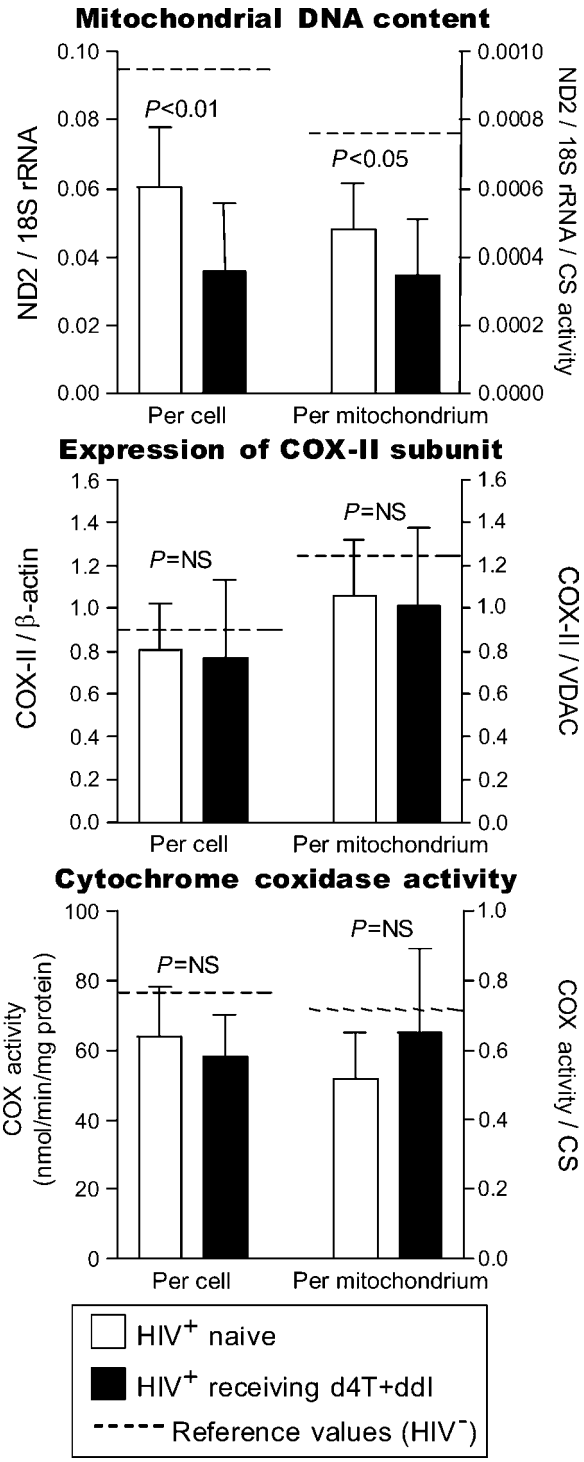

FIGURE 2. Mitochondrial DNA content and expression of cytochrome c oxidase subunit II (COX-II) and COX activity assessed in peripheral blood mononuclear cells of naive and stavudine plus didanosine (d4T + ddI)–treated patients. CS indicates citrate synthase; VDAC, voltage-dependent anion channel.

per cell or per mitochondrion. Similarly, patients receiving d4T + ddI did not show a decrease in COX activity expressed per cell or per mitochondrion (Fig. 2). When we analyzed the cases according to the treatment that they were receiving, nevirapine or nelfinavir in combination with d4T + ddI, we did not find differences between the 2 subgroups for any of the studied mitochondrial parameters (data not shown). It is remarkable that although mtDNA content decreased in HIV-positive treatment-naive patients in comparison to uninfected individuals, the expression of COX-II and COX activity did not differ between the 2 groups.

Expression of the COX-II subunit was independent of the amount of mtDNA for the 2 groups of individuals, and this absence of a relation was confirmed per cell and per mitochondrion. In contrast, expression of the COX-II subunit and COX activity showed a close relation, being stronger in treatment-naive individuals than in subjects undergoing treatment with d4T + ddI (Fig. 3).

The time on antiretroviral treatment was associated with a significant decrease in mtDNA content, whereas the expression of COX-II was mild and not statistically significantly decreased and the activity of COX remained unaltered over time (Fig. 4). Interestingly, when we assessed the effects of HIV infection itself (by means of viral load) and immunologic status (by means of $CD4^+$ T-cell count) on COX-II expression in the absence of antiretrovirals, we found a significantly greater decrease in the expression of COX-II in patients with greater viremia and poorer immunologic status (Fig. 5).

DISCUSSION

The present study demonstrates that although mtDNA depletion is clearly present in asymptomatic HIV-infected individuals treated with antiviral regimens containing d4T + ddI, this depletion is not associated with a decrease in expression of the COX-II subunit (encoded by mtDNA) or with a decrease in COX activity (complex IV of the MRC). Identical conclusions are achieved if the results are normalized per cell or per mitochondrion, which reasonably excludes any masking effect caused by changes in the whole mitochondrial content of the cell as a result of antiretroviral drugs. Thus,

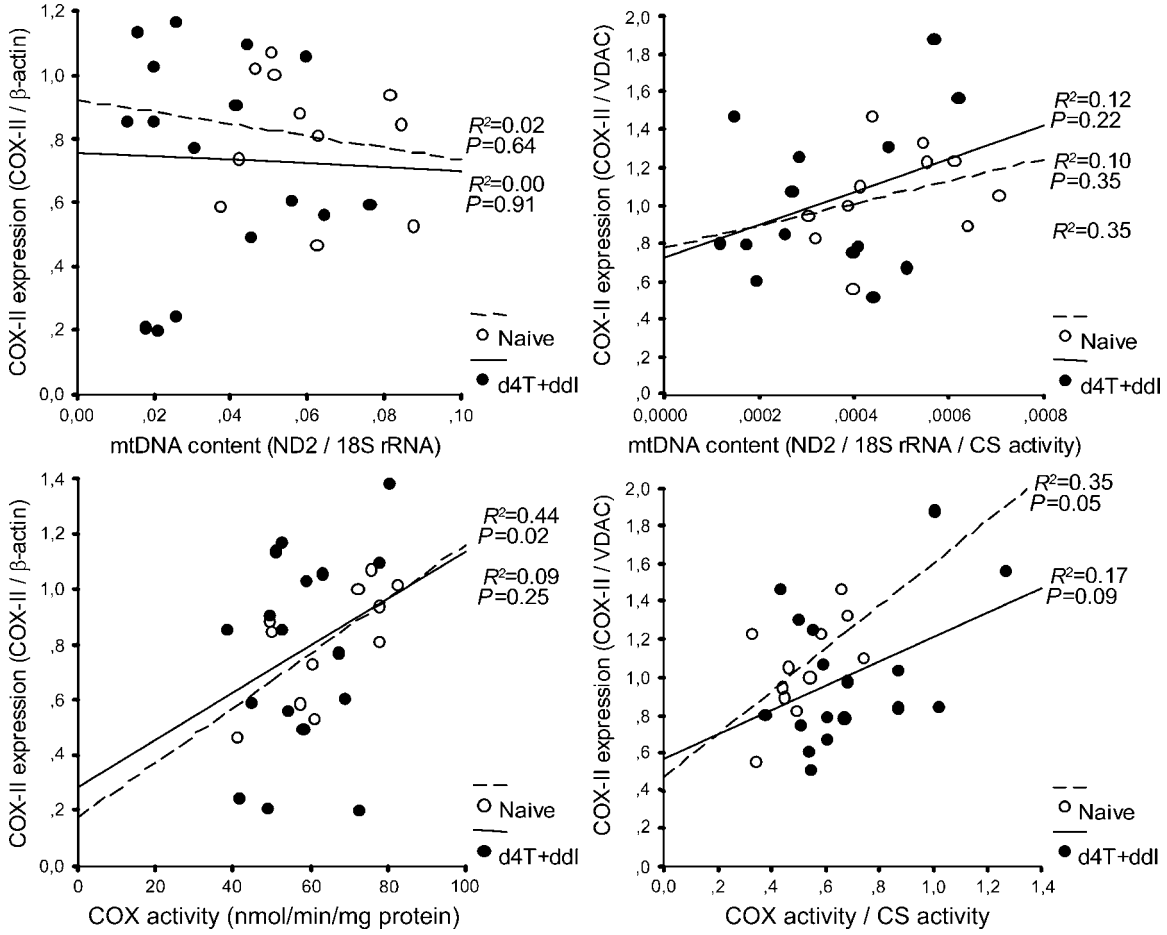

FIGURE 3. Relation between the expression of cytochrome c oxidase subunit II (COX-II) and mitochondrial DNA (mtDNA) content (upper) and COX activity (lower) in peripheral blood mononuclear cells of naive and stavudine plus didanosine (d4T + ddI)–treated patients. In all cases, these relations are assessed per cell (left) and per mitochondrion (right). CS indicates citrate synthase; VDAC, voltage-dependent anion channel.

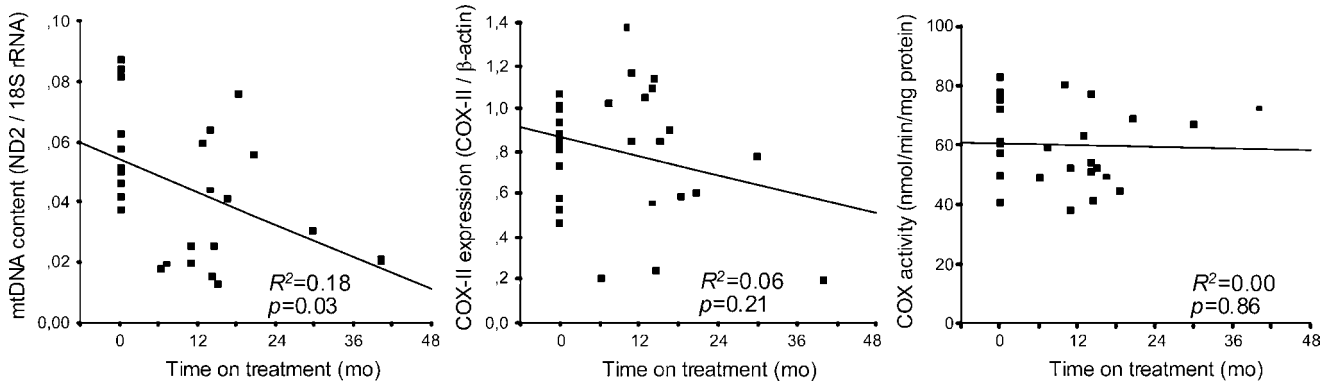

FIGURE 4. Relation between time receiving antiretroviral therapy, including stavudine plus didanosine (d4T + ddI), and mitochondrial DNA (mtDNA) content (left), expression of cytochrome c oxidase subunit II (COX-II; center), and COX activity (right).

these data suggest that transcriptional (increased transcription of mRNA from mtDNA) or posttranscriptional (increased synthesis of protein from mRNA) mechanisms act to compensate for the loss in the number of mtDNA copies. Figure 2 is highly illustrative of the supposition that the amount of COX-II is maintained irrespective of the mtDNA content.

Our results are exclusively related to the conditions of the study (ie, to PBMCs of individuals receiving d4T + ddI for a mean of 16 months as a first-line therapy with no clinical evidence of drug-related long-term secondary effects). Therefore, our data do not mean that mtDNA depletion does not play a role in the adverse effects that may develop during long-term d4T + ddI treatment. PBMCs are not the target of such adverse effects; accordingly, they only represent a conservative estimate of what really occurs at target tissues. In addition, in patients receiving antiretrovirals for longer periods or those who have developed lipodystrophy or hyperlactatemia, the expression of mitochondrially encoded proteins and/or the activity of such proteins may be dramatically impaired. In fact, we found a tendency to a decline in COX-II expression in PBMCs in relation to the length of time on antiretroviral drugs. The results of the present study agree with the finding that in patients suffering from zidovudine myopathy, mtDNA depletion at the skeletal muscle level is accompanied by a clear decrease in COX-II expression in the sarcoplasm.[24] In vitro studies have also shown a time- and dose-dependent mtDNA depletion caused by d4T and ddI on human hepatocellular carcinoma cell line (HepG), which preceded or coincided with a decline in COX-II expression.[25] In any case, we believe that the absence of downstream effects caused by mtDNA depletion emphasizes the efficiency of mitochondria in compensating for antiretroviral toxicities, at least when mtDNA depletion is mild to moderate. This is in accordance with the hypothesis that only mtDNA defects involving more than 80% of the genetic material are able to induce MRC dysfunction.[26]

Compensatory mechanisms for mtDNA depletion have been proposed as an explanation for the lack of a close correlation between time on treatment and the probability of developing adverse effects. The intensity of such compensatory effects may vary from patient to patient according to risk

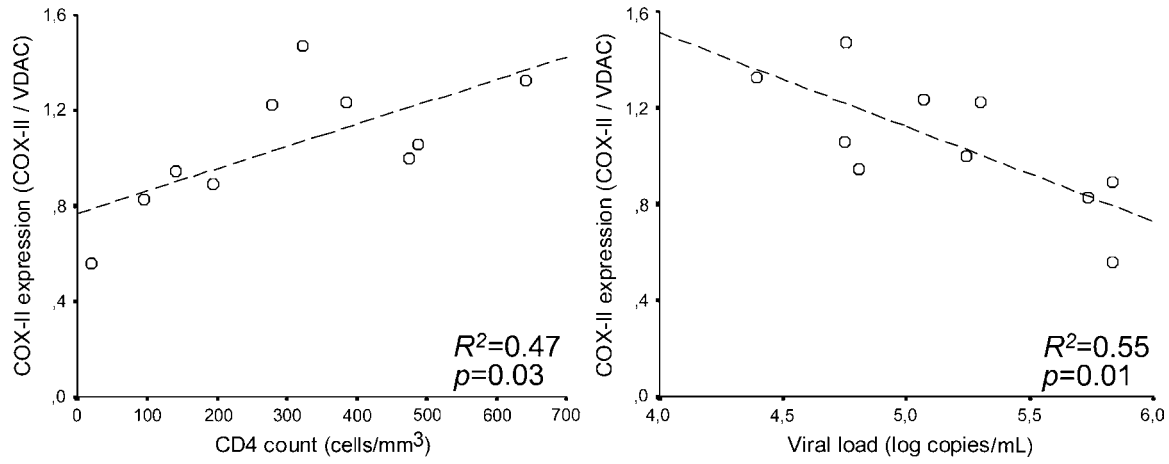

FIGURE 5. Relation between the expression of cytochrome c oxidase subunit II (COX-II) and $CD4^+$ T-cell count (left) and viral load (right) in the absence of antiretroviral therapy (naive patients). VDAC indicates voltage-dependent anion channel.

factors such as the time on antiretroviral therapy, immunologic status, and/or degree of viremia. Interestingly, we found that although the expression of COX-II only showed a weak correlation with the first factor (time on antiretroviral therapy), the latter 2 factors (immunologic status and degree of viremia) were significantly associated with this expression. These findings suggest that toxic mitochondrial effects are not only limited to the inhibition of DNA γ-polymerase caused by antiretrovirals but to the combined effects of being immune compromised and having HIV infection and that receiving antiretrovirals could have a cumulative effect. Nowadays, it has been widely reported that HIV itself is also increasingly implicated in diverse and extensive mitochondrial disturbances,[3,14,27–32] most of which are triggered by mitochondrially dependent apoptotic mechanisms. Consistent with those reports, the present study also postulates that HIV-infected patients naive to antiretrovirals have a decreased amount of mtDNA compared with uninfected people. Conversely, the expression of COX-II and COX activity seems to be less influenced than mtDNA content by the effects of HIV infection itself. Accordingly, adaptive mechanisms may be effective in withholding the decline in MRC function caused by mtDNA depletion as a result of administration of d4T + ddI in certain circumstances; however, in other cases, the collateral actions of antiretrovirals and/or HIV itself against mitochondria may be the final determinants leading to mitochondrial failure. In any case, the demonstration of up-regulatory mechanisms compensating for mtDNA depletion is an additional argument for the need to be cautious when using mtDNA quantification as the only tool to monitor the clinical relevance of the mitochondrial toxicity of antiretroviral drugs.

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- SÍNTESI DE L'ESTUDI

En el present estudi hem volgut comprovar si la depleció de l'ADNmt que sovint es detecta en les CMSP de pacients assimptomàtics infectats pel VIH que reben una combinació TARGA que conté d4T i ddI implica la disminució o no de les subunitats proteiques codificades pel genoma mitocondrial que conformen els complexos enzimàtics del sistema OXPHOS.

L'estudi l'hem realitzat en CMSP de pacients assimptomàtics infectats pel VIH que reben d4T i ddI com a part important de la combinació TARGA de primera línia. Hem comparat els resultats amb un grup de pacients VIH positus que mai han rebut TARGA.

- CONCLUSIONS

El tractament ARV amb d4T i ddI s'associa amb depleció del contingut d'ADNmt i una disminució del contingut mitocondrial.

Malgrat les alteracions mitocondrials trobades als pacients tractats amb d4T i ddI, el contingut de la subunitat COX-II i l'activitat del C IV mitocondrial mantenen uns nivells normals respecte els pacients que mai han rebut ARVs.

Aquests resultats suggereixen l'actuació de mecanismes reguladors que compensen la depleció del genoma mitocondrial als pacients tractats amb d4T i ddI.

5.2.2. Efecte de la infecció pel VIH sobre els mitocondris de cèl·lules mononuclears de sang perifèrica

Aquest apartat fa referència als resultats que es deriven dels estudis realitzats en pacients infectats pel VIH que no presenten cap evidència clínica de sida ni de redistribució de greix corporal o hiperlactatèmia associats al tractament ARV. Així, els treballs d'investigació que aquí es mostren han estat específicament dirigits a determinar els efectes que la pròpia infecció pel VIH pot produir sobre els mitocondris de CMSP.

MITOCHONDRIAL EFFECTS OF HIV INFECTION ON THE PERIPHERAL BLOOD MONONUCLEAR CELLS OF HIV-INFECTED PATIENTS WHO WERE NEVER TREATED WITH ANTIRETROVIRALS

CLINICAL OF INFECTIOUS DISEASES

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Mitochondrial Effects of HIV Infection on the Peripheral Blood Mononuclear Cells of HIV-Infected Patients Who Were Never Treated with Antiretrovirals

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To investigate the effects of HIV infection on mitochondrial DNA (mtDNA) content and other mitochondrial parameters, we used peripheral blood mononuclear cells (PBMCs) from 25 asymptomatic antiretroviral-naive human immunodeficiency virus (HIV)–infected patients and from 25 healthy control subjects. HIV-infected patients had significant decreases in mtDNA content (decrease, 23%; $P < .05$) and in the activities of mitochondrial respiratory chain (MRC) complex II (decrease, 41%; $P < .001$), MRC complex III (decrease, 38%; $P < .001$), MRC complex IV (decrease, 19%; $P = .001$), and glycerol-3-phosphate dehydrogenase (decrease, 22%; $P < .001$), along with increased lipid peroxidation of PBMC membranes ($P = .007$). Therefore, HIV infection is associated not only with mtDNA depletion, but also with extensive MRC disturbances and increased oxidative damage.

Antiretroviral therapy that contains nucleoside reverse-transcriptase inhibitors (NRTIs) may induce adverse effects due to mitochondrial toxicity. The main pathogenic mechanism suspected involves the inhibition of mtDNA polymerase γ (mtDNA γ-pol), which is the only enzyme responsible for the replication of mtDNA (a circular, double-stranded DNA molecule of 16.5 kb), which only encodes for some components of some mitochondrial respiratory chain (MRC) complexes. Depletion of mtDNA [1–6], deletions [6, 7], and point mutations [8] have been reported to occur in some tissues as a consequence of inhibition of mtDNA γ-pol by NRTIs. In some instances, these abnormalities may lead to an impairment of MRC function [1, 6].

Although previous studies that have assessed the mitochondrial effects of NRTIs have systematically included a group of untreated HIV-infected patients, they have essentially lacked a control group of non–HIV-infected people. Although this fact does not negate the conclusions reached by these studies regarding the harmful effects of NRTIs against mitochondria, the role (if any) of HIV in the diminishment of mtDNA content remains unclear. A recent study by Côté et al. [9] found that the mtDNA content in the buffy coats of 47 asymptomatic HIV-infected patients who had never received antiretroviral therapy was significantly reduced (56%), compared with that of 24 non–HIV-infected people (100%). This difference was not explained by the lower $CD4^+$ T lymphocyte count of the HIV-infected patients, compared with the non–HIV-infected subjects. Similarly, a very recent study by Miura et al. [10] showed a significant reduction in the mtDNA content (70%) of PBMCs from 46 antiretroviral-naive HIV-infected

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patients, compared with 29 healthy people (100%). In the study by Miura et al. [10], mtDNA content was positively correlated with $CD4^+$ T cell count and was inversely correlated with HIV load. Nonetheless, it currently has not been ascertained whether mtDNA depletion is an isolated finding or whether it is associated with impaired MRC function or, even, with more-extensive damage of mitochondrial enzyme capacity that leads to increased oxidative damage.

METHODS

For the present study, we recruited 25 asymptomatic HIV-infected patients who had never received antiretroviral therapy, as well as 25 non–HIV-infected people who were matched by age (± 5 years) and sex and who were considered to be control subjects. Written, informed consent was obtained from all subjects before their inclusion in the study. The clinical data for individuals included in the study are presented in table 1.

PBMCs were isolated, by means of Ficoll density-gradient centrifugation (Histopaque-1077; Sigma Diagnostics), from 20 mL of peripheral venous blood collected in Vacutainer EDTA tubes (BD Vacutainers Systems). After isolation, the PBMCs were resuspended in PBS and were frozen and stored at $-80°C$, until their use in biochemical and genetic determinations. Protein content was measured according to Bradford's methodology [11].

An aliquot of PBMCs was used for the extraction of total DNA by means of a standard phenol-chloroform procedure. For quantification of mtDNA, the nuclear housekeeping 18S rRNA gene and the highly conserved mitochondrial ND2 gene were amplified separately by use of quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals). The mtDNA content was first expressed as the ratio of mtDNA to nuclear DNA, by use of the LightCycler-based methodology (LightCycler System; Roche Diagnostics, Idaho Technology) [12], and it then was transformed to the number of mtDNA copies per cell, by use of a set of 4 "international" or "common" standards with known values of mtDNA copies/mL and nuclear DNA copies/mL (provided by E. Hammond, D. Nolan, and S. Mallal), to standardize mtDNA assays in an international collaborative approach [13]. This approach has allowed our group of investigators to calibrate our own set of working standards (LightCycler-Control Kit DNA; Roche Diagnostics, Applied Sciences) and to report, for the present study, concordant data regarding mtDNA copies per cell.

Another aliquot of PBMCs was used for spectrophotometrical analyses. We determined glycerol-3-phosphate dehydrogenase (G3Pdh) activity, citrate synthase (CS; a mitochondrial matrix enzyme representative of the Krebs cycle) activity, and the enzyme activities of MRC complexes II–IV (the 3 complexes are representative of the MRC function; the first complex is exclusively encoded by the nuclear genome, and the latter 2 complexes are partially encoded by mtDNA). All enzyme activities were measured using standard procedures described elsewhere [14, 15] and were expressed as nanomoles of reduced or oxidized substrate per minute per milligram of total cell protein, representing the absolute activities.

Another aliquot was used to determine the degree of oxidative damage, by means of assessment of *cis*-parinaric acid to measure the lipid peroxidation of PBMC membranes. *Cis*-parinaric acid, a fatty acid that contains 4 conjugated double bonds that render it naturally fluorescent, is attacked during lipid peroxidation reactions. Accordingly, *cis*-parinaric acid fluorescence is consumed in lipid peroxidation reactions. Because *cis*-parinaric acid is readily incorporated into PBMC membranes, its loss of fluorescence is used to indirectly monitor the degree of lipid peroxidation. For this purpose, 100 μg of PBMC protein were placed into 3 mL of nitrogenized PBS that contained *cis*-parinaric acid (5 μmol/L; Molecular Probes), and they were incubated in darkness at 37°C. Afterward, fluorescence was measured at 3-min intervals, for 30 min, by use of 318-nm excitation and 410-nm emission [14, 16]. The greater the lipid peroxidation, the less fluorescence is detected.

CS activity was used to estimate the mitochondrial content, to adjust for mtDNA content and the MRC enzyme activities due to hypothetical changes in the mitochondrial amount of PBMCs. CS seems to be a rather stable mitochondrial enzyme, the activity of which is not subjected to fluctuations and path-

Table 1. Clinical characteristics of individuals included in the present study.

Characteristic	Healthy control subjects (n = 25)	HIV-infected patients (n = 25)
Age, mean years ± SD	40 ± 12	37 ± 8
Male sex, % of patients	76	76
Duration of infection, mean months ± SD	…	44 ± 71
$CD4^+$ T cell count, mean cells/mm^3 ± SD	…	317 ± 215
Viral load,[a] mean $\log_{10}$ HIV RNA copies/mm^3 ± SD	…	5.0 ± 0.9

[a] Any viral load of <50 copies/mL was recorded as 49 copies/mL.

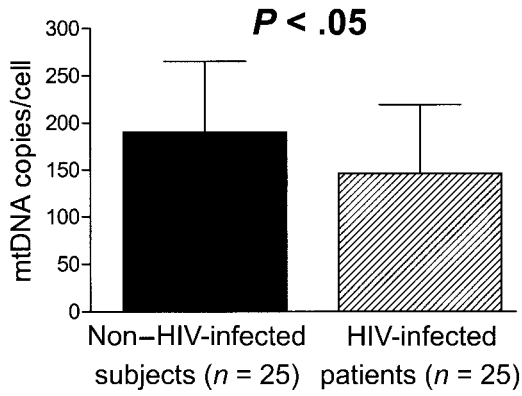

Figure 1. Quantification of mtDNA. Bars denote the results (expressed as mean values ± SD) for each group. Antiretroviral-naive HIV-infected individuals had a significant decrease in the number of mtDNA copies per PBMC, compared with healthy (non–HIV-infected) individuals. Comparison between groups was performed using Student's *t* test. $P < .05$ denoted statistical significance.

ological changes. For this reason, when homogenates or impure mitochondrial fractions have to be used for enzymatic determinations, activities are best compared when they are divided by CS activity, to prevent artifacts caused by differences in the content of pure mitochondria. Accordingly, the mtDNA content and the MRC enzyme activities were also estimated per organelle (mitochondria)–relative values, by dividing absolute values per CS activity [17–19].

The results for HIV-infected patients and control subjects are expressed as mean values ± SD, for quantitative variables, and they were compared using an unpaired Student's *t* test. Results are also expressed as percentages, for qualitative variables, and they were compared using the χ^2 test. Differences between groups are expressed as percentages with a 95% CI. A 2-way analysis of variance was used when measurements were repeated to compare *cis*-parinaric curves, and linear regression was used to search for any association between quantitative variables. For all cases, $P < .05$ was considered to be statistically significant.

RESULTS

The number of mtDNA copies per cell was significantly decreased in HIV-infected patients (by 23%; 95% CI, 4%–42%; $P < .05$), compared with healthy control subjects (figure 1). HIV-infected patients also exhibited a significant decrease in the MRC enzyme activities of complexes partially encoded by mtDNA. Specifically, complex III activity decreased by 38% (95% CI, 31%–51%; $P < .001$), and complex IV activity decreased by 19% (95% CI, 9%–29%; $P = .001$) (figure 2). On the other hand, the enzymatic activity of complex II, which is exclusively encoded by the nuclear DNA, was also found to be decreased in HIV-infected patients (decrease, 41%; 95% CI, 24%–58%; $P < .001$) (figure 2). When the activities of the representative enzymes of other metabolic pathways were determined, no differences in CS activity were found when HIV-infected patients were compared with healthy control subjects (mean CS activity [±SD], 123 ± 15 nmol/min/mg protein and 131 ± 24 nmol/min/mg protein, respectively; *P* is not significant). However, a significant decrease in G3Pdh activity was noted in HIV-infected patients, compared with healthy control subjects (decrease, 22%; 95% CI, 11%–33%; $P < .001$) (figure 2). All the differences in mitochondrial parameters found between HIV-infected individuals and healthy people remained significant, even when they were estimated per mitochondria (by dividing mtDNA content and enzyme activities per CS activity; data not shown).

The oxidative damage to the PBMC membranes was significantly increased in HIV-infected patients, compared with healthy control subjects. As shown in figure 3, a greater loss of *cis*-parinaric acid fluorescence over time was noted among HIV-infected patients, a finding that indicates increased lipid peroxidation ($P = .007$).

Correlation of the mitochondrial parameters with immunological status (as assessed by the $CD4^+$ T lymphocyte count) was performed for the HIV-infected patients. The mitochondrial parameters were also correlated with the severity of the

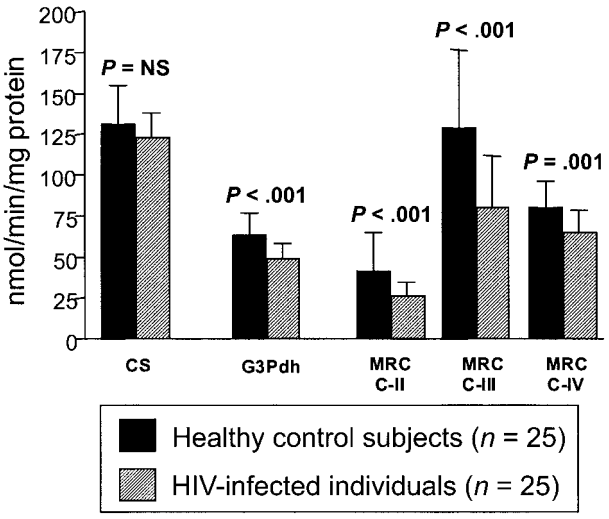

Figure 2. Enzyme activities, expressed as nanomoles of reduced or oxidized substrate per minute per milligram of total cell protein, of citrate synthase (CS), glycerol-3-phosphate dehydrogenase (G3Pdh), and mitochondrial respiratory chain (MRC) complex II (C-II), MRC complex III (C-III), and MRC complex IV (C-IV). Bars denote the results (expressed as mean values ± SD) for each group. PBMCs from untreated HIV-infected individuals showed a significant decrease in all enzyme activities (with the exception of CS activity), compared with PBMCs from healthy (non–HIV-infected) individuals. Student's *t* test was used for comparison between groups. $P < .05$ denoted statistical significance. NS, not significant.

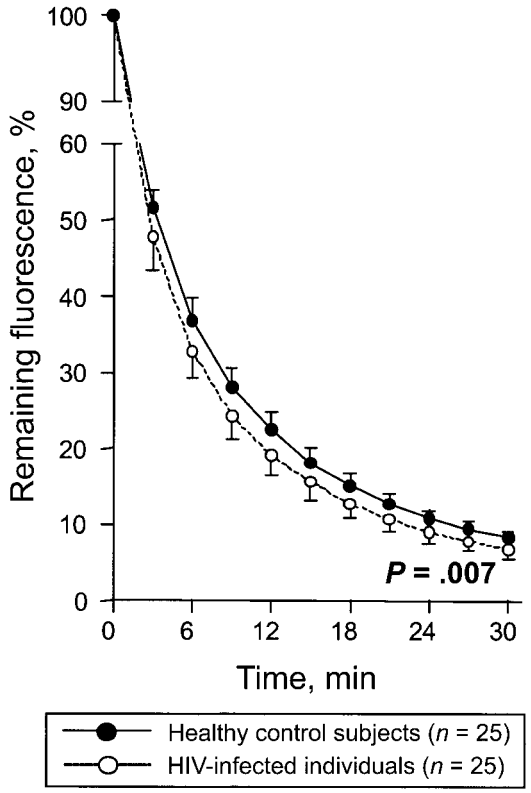

Figure 3. Studies of lipid peroxidation measured as loss of *cis*-parinaric acid fluorescence loss over time. Results are expressed as the percentage of the remaining fluorescence (± SD) at 3-min intervals. Untreated HIV-infected individuals had greater and faster loss of fluorescence, compared with healthy (non–HIV-infected) individuals, denoting an increase in lipid peroxidation of PBMC membranes. Comparison of *cis*-parinaric acid curves was performed using 2-way analysis of variance. $P < .05$ denoted statistical significance.

HIV infection (as assessed by plasma HIV load). We did not find any statistically significant association between either the $CD4^+$ T lymphocyte count or the viral load in HIV-infected patients, with respect to any of the altered mitochondrial parameters. However, although not significant, a trend was noted toward a decrease in MRC complex IV activity, along with an increase in CS activity, in association with viral load (figure 4).

DISCUSSION

Previous studies have noted that some degree of mtDNA depletion is present in the PBMCs [10, 20, 21], skeletal muscle [2], adipose tissue [3], liver [22, 23], or placenta [24] of HIV-infected patients. However, these data were indirectly obtained in studies with main objectives that did not focus on the effects of HIV on mitochondria. In addition, to date, no studies demonstrating mitochondrial dysfunction associated with mtDNA depletion in human PBMCs have been reported. Therefore, the results of the present study show that HIV-infected patients who have never been treated with antiretrovirals have decreased mtDNA levels, along with decreased enzyme activity of the MRC complexes and other metabolic pathways, as well as increased oxidative damage of the PBMC membranes. This is the first direct evidence that HIV is associated with extensive functional mitochondrial damage, which does not seem to only be limited to MRC complexes encoded by mtDNA.

The mechanism by which HIV causes this mtDNA depletion is currently elusive. Nonetheless, the coexistence of a generalized impairment that affects both mtDNA- and nuclear DNA–encoded MRC complexes, as well as G3Pdh activity, indicates that mtDNA depletion may be better interpreted as resulting from generalized mitochondrial damage rather than from a specific mechanism of the mtDNA lesion induced by HIV. This hypothesis agrees with the findings of recent studies that have reported signs of mitochondrial necrosis in HIV-infected cells [25]. Moreover, a main feature of HIV pathogenesis is cell death of $CD4^+$ T lymphocytes as a result of apoptosis, and, currently, it is well known that several HIV-1–encoded proteins (Env, Vpr, Tat, and PR) are directly or indirectly associated with the dissipation of mitochondrial membrane potential, thereby causing apoptotic cell death [26–32]. In fact, the appearance of morphologic mitochondrial abnormalities, along with increased apoptosis, has been demonstrated in both ex vivo studies of individuals with seroconversion [33] and in vitro experiments involving acutely infected monocytoid and lymphoblastoid cells [25]. Therefore, it is conceivable that, in addition to contributing to $CD4^+$ T lymphocyte depletion, proapoptotic effects of virion proteins also have a role in the mtDNA depletion and the mitochondrial functional disturbances observed in the present study. This hypothesis, which probably is better addressed to cultured HIV-infected cells, revolves around the adverse effects of the viral gene products against mitochondria.

Although some authors have suggested that the intensity of HIV infection could correlate with the degree of mitochondrial damage, other authors have reported discordant data. In this sense, although Miura et al. [10] reported that mtDNA levels in HIV-infected individuals have a direct correlation with the $CD4^+$ T cell count and an inverse correlation with the number of HIV RNA copies, Côté et al. [9] did not identify such an association. In our series, no significant association was found between markers of severity of infection and mitochondrial function; only a tendency toward an increase in the mitochondrial content in patients with a higher number of circulating HIV copies was remarkable. This fact could reflect that the classically known mitochondrial proliferation observed in the skeletal muscle in primary MRC defects is a form that responds to toxic insults.

The combined effects of HIV and antiretrovirals on mitochondria should be considered, because mitochondriotoxicity

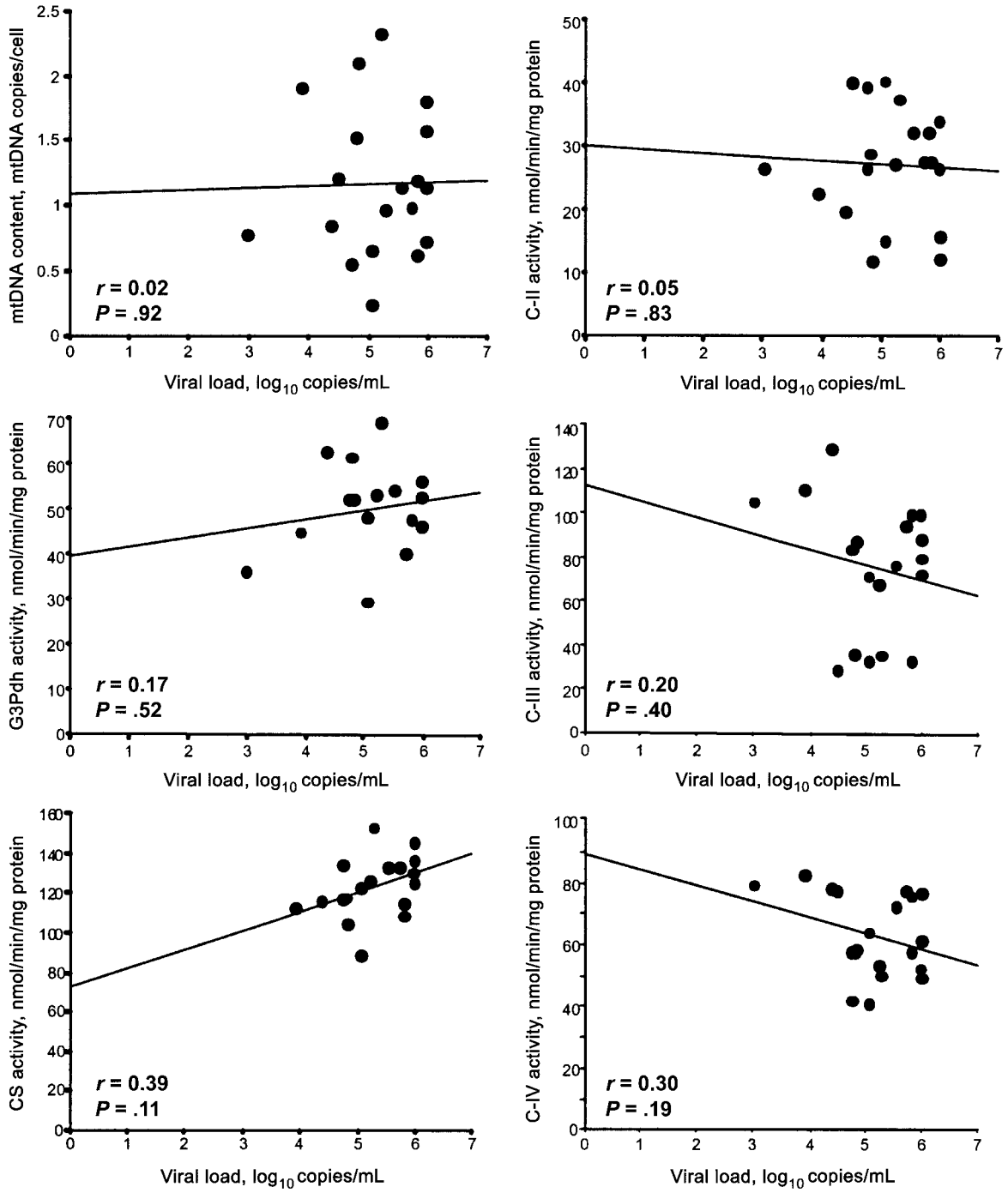

Figure 4. Association between viral load and mitochondrial parameters in antiretroviral-naive HIV-infected individuals, as established by a linear regression model. There was no significant association with any of the parameters assessed (mtDNA; glycerol-3-phosphate dehydrogenase [G3Pdh] activity; citrate synthase [CS] activity; and mitochondrial respiratory chain [MRC] complex II [C-II], MRC complex III [C-III], and MRC complex IV [C-IV] activity). $P < .05$ denoted statistical significance.

is a well known side effect of antiretrovirals. On one hand, the negative effects of HIV infection, per se, on mtDNA could render HIV-infected patients more susceptible to the mitochondrial toxicities of NRTIs, compared with the general population, because HIV-infected patients can reach the "threshold" for clinically relevant adverse effects faster. As an example, we have found that untreated HIV-infected patients with greater viremia showed less complex IV activity; this finding, although not statistically significant, suggests that HIV-infected patients may be more susceptible than non–HIV-infected individuals to

the mitochondrial toxic effects of antiretrovirals. In addition, NRTIs also induce apoptotic death in several cell types [34]. Conversely, protease inhibitors exert antiapoptotic effects, which seem to be relevant for their clinical benefit, in a way different from that achieved by means of their antiviral activity [35, 36]. Thus, the net effect of HIV and antiretrovirals on mitochondrial function may differ from one patient to another, and it may explain, at least in part, the existence of discordant results of studies of the mitochondrial toxicity of antiretrovirals.

Whatever the mechanism involved in the effects of HIV on mitochondria, we believe that the findings of the present study support the hypothesis that the effects of HIV on mtDNA content are nonspecific and would be better reflected in a scenario of more diffuse mitochondrial damage, probably in association with apoptotic changes caused by HIV. Moreover, our results should also be taken into account in the design of further studies evaluating the mitochondrial toxic effects of antiretrovirals, because untreated individuals with HIV infection should be included in the control group of such studies.

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Conflict of interest. All authors: No conflict.

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- SÍNTESI DE L'ESTUDI

En el present estudi hem volgut determinar si el VIH pot exercir efectes tòxics sobre els mitocondris de les CMSP, independentment dels efectes que pugui produir el tractament ARV.

L'estudi l'hem realitzat en CMSP de pacients infectats pel VIH que mai han rebut ARVs. Hem comparat els resultats amb un grup control format per individus sans no infectats pel virus.

- CONCLUSIONS

La infecció pel VIH produeix depleció de l'ADNmt, increment del dany oxidatiu cel·lular i disminució de les activitats enzimàtiques dels complexos codificats parcialment pel genoma mitocondrial i dels complexos codificats únicament pel nucli. Per tant, el VIH produeix un efecte tòxic cel·lular generalitzat, que no està exclussivament limitat a la toxicitat mitocondrial. Es suggereix que l'activació de l'apoptosi causada pel VIH podria ser, al menys en part, la responsable d'aquestes alteracions mitocondrials difuses.

HIV INFECTION, ANTIRETROVIRALS AND APOPTOSIS: STUDIES ON SKELETAL MUSCLE

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Short Communication

HIV Infection, Antiretrovirals, and Apoptosis: Studies on Skeletal Muscle

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ABSTRACT

Increased apoptosis in CD4$^+$ T lymphocytes plays an important role in the pathogenesis of HIV infection and it has also invoked some HIV-related as well as antiretroviral-related adverse events. We assessed whether increased apoptosis is also present in the skeletal muscle of HIV-infected patients. We included 36 consecutive individuals, 18 without (group A) and 18 with HIV infection. The latter group consisted of five asymptomatic antiretroviral-naive HIV-infected individuals (group B), six asymptomatic HIV-infected individuals on highly active antiretroviral therapy (HAART, group C), and seven HIV-infected individuals on HAART with lipodystrophy (group D). Immunohistochemical reaction using deoxyribonucleotidyltransferase-mediated-dUTP-biotin nick-end labeling (TUNEL) was performed on skeletal muscle samples. None of the uninfected patients (group A) showed data of increased apoptosis, while 16 out of 18 infected patients did ($p < 0.001$). All subgroups of infected subjects (groups B–D) showed a significant increase of apoptosis in TUNEL with respect to uninfected individuals, but the comparison between the different subgroups of infected patients did not reveal significant differences. We conclude that skeletal muscle of HIV-infected patients exhibits increased apoptosis compared with that of uninfected patients, but the role of HAART in inducing apoptosis remains to be established.

Apoptosis is the key in cell biogenesis and tissue homeostasis and purges unnecessary, aged, or damaged cells. Abnormal resistance to apoptotic cell death is the basis of developmental malformations, autoimmune diseases, and many cancers, whereas enhanced susceptibility of cells to apoptotic signals participates in tissue damage secondary to acute infectious diseases, ischemia–reperfusion damage, and chronic pathologies including neurodegenerative and neuromuscular diseases.[1] Apoptosis is also related to the pathogenesis of HIV infection. Increased apoptosis of CD4$^+$ T lymphocytes is considered of crucial relevance in lymphocyte depletion caused by HIV infection.[2,3] It is also present in cardiomyocytes from individuals developing HIV-related cardiomyopathy,[4] as well as in neurons from patients with HIV-related encephalopathy.[5] On the other hand, increased apoptosis is the presumed mechanism of some adverse effects of highly active antiretroviral therapy (HAART), such as osteopenia or lipodystrophy (LD).[6] But the precise role of antiretrovirals and HIV itself has not been completely elucidated. It is interesting to note that while studies of apoptosis using lymphocytes are abundant, studies on tissues are scarce. Therefore, we designed the present study to ascertain whether apoptosis is present in skeletal muscle of HIV-infected individuals as well as whether there are differences according to the existence of antiretroviral treatment and LD.

We included 18 consecutive asymptomatic uninfected (control group, group A), and 18 consecutive HIV-infected indi-

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viduals. The controls were recruited from individuals who underwent skeletal muscle biopsy because of muscle complaint in whom skeletal muscle histology was thereafter normal. The cases were from two cohorts of HIV-positive outpatients from the Hospital Clínic of Barcelona and the Hospital of Granollers. HIV-infected patients were naïve with respect to antiviral treatment ($n = 5$, group B), receiving HAART, and asymptomatic ($n = 6$, group C), and on HAART with LD ($n = 7$, group D). Group B patients had just been diagnosed with HIV infection, and were free of active opportunistic infection or neoplasia at the time of inclusion in the study. The individuals in groups C and D had been receiving HAART at least for the previous 12 months and, in group D, LD was the only clinical abnormality at the time of initiating the study, as defined by the managing physician. The Ethical Committee of both hospitals approved the protocol, and an informed consent was obtained from all patients included.

An open-biopsy specimen was taken from the deltoid muscle of the nondominant arm of each patient and control, processed as usual, and incubated with deoxyribonucleotidyltransferase-mediated-dUTP-biotin nick-end labeling (TUNEL) using the In Situ Cell Death Detection Kit (Boehringer, Manheim, Germany) as reported elsewhere.[7] Negative control slides were performed in all procedures using the same process without reagent incubation, and positive control slides were performed by using palatinal amygdalar tissue, in which lymphoid cells take high TUNEL. The specimens were coded with random numbers and read by three independent, blind observers (O.M., J.F.S., J.C.). The presence of apoptotic cells was quantified by means of an apoptotic index (Ai) that was calculated by dividing the total number of positive staining myocyte nuclei in the TUNEL assay by the total number of the myocyte nuclei, and multiplying this value by 100 as previously reported by Narula *et al.*[8] Stained cell nuclei at the edges of the tissues or in the interstitium were not counted. The count was performed on at least five different fields (magnification 250×) of each sample, with at least 500 nuclei per field, according to the method described by Sandri *et al.*[9] The results were consigned in a semiquantitative scale as follows: − if less than 1 per 1000 of positive nuclei were present, + if less than 1%, ++ if less than 5%, and +++ if more than 5%. The Student's *t* test was used to compare quantitative variable and the Fisher's exact test (with the approximation of Woolf when necessary) was used for the comparison of qualitative variables. For all statistics, *p* values less than 0.05 were considered significant.

The only clinical differences among the groups were observed in $CD4^+$ lymphocyte depletion and viral load, which were significantly greater in group B than in groups C and D (Table 1). None of the 18 HIV-negative controls exhibited data of increased apoptosis in TUNEL. Conversely, 16 out of 18 HIV-infected patients showed varying degrees of increased apoptosis in TUNEL ($p < 0.001$). A detailed distribution for each subgroup of cases (B–D) of findings in TUNEL is depicted in Fig. 1. As shown, all subgroups of HIV-infected patients presented a significant increase in positive nuclei in TUNEL compared to the control group, while no differences between the HIV subgroups (B–D) are found.

Detection of apoptotic cells in tissue sections currently relies mainly on the TUNEL assay, which works by labeling single DNA strand breaks or nicks.[10] The final cardinal event of apoptosis is nuclear fragmentation, which definitely leads to cell death in mononucleated cells. Multinucleated muscle cells are highly resistant to apoptosis and in the general population less than 1 per 1000 positive nuclei for TUNEL is present in skeletal muscle.[7] Using this approach, we demonstrated that all subgroups of HIV-infected patients, irrespective of whether they had received HAART or had developed LD, exhibit increased apoptosis in skeletal muscle compared to uninfected individuals, a finding that has not previously been reported to date.

Increased DNA fragmentation in skeletal muscle from naïve patients is in accordance with the well-demonstrated proapoptotic effects of HIV. The mechanisms by which HIV infection leads to increased apoptosis are not clear. Direct infection of

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS INCLUDED IN THE STUDY[a]

	HIV^- controls (group A) (n = *18*)	*HIV^+ naive (group B)* (n = *5*)	*HIV^+ on HAART, LD^- (group C)* (n = *6*)	*HIV^+ on HAART, LD^+ (group D)* (n = *7*)
Age (years) (mean ± SD)	40 ± 13	41 ± 21	37 ± 7	41 ± 12
Females (%)	17	20	17	14
Viral load (log_{10} copies) (mean ± SD)	—	5.10 ± 0.60	2.80 ± 1.31[b]	3.08 ± 0.92[c]
Lymphocyte $CD4^+$ count (per mm^3) (mean ± SD)	—	60 ± 60	241 ± 186	314 ± 146[b]
Time on HAART (months) (mean ± SD)	—	—	31 ± 14	31 ± 3
HAART containing d4T, but not PI (%)	—	—	50	57
HAART containing PI, but not d4T (%)	—	—	100	57
HAART containing d4T, and PI (%)	—	—	50	29

[a]HAART, highly active antiretroviral therapy; LD^-, without lipodystrophy; LD^+, with lipodystrophy; d4T, stavudine; PI, protease inhibitor.

[b]$p < 0.01$ compared to group B.

[c]$p = 0.001$ compared to group B.

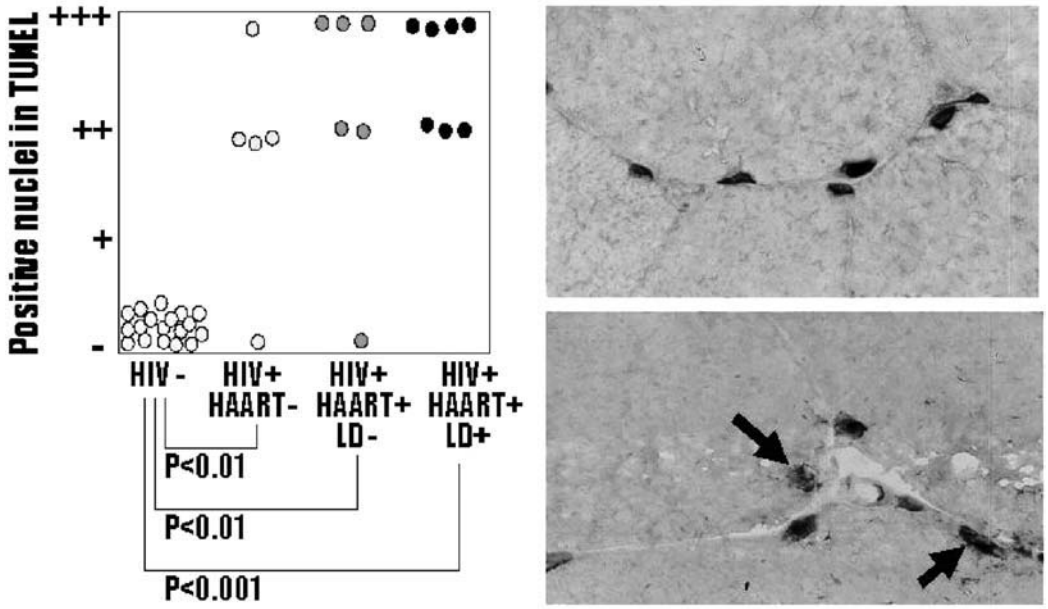

FIG. 1. Results of studies using TUNEL immunoreaction activity on skeletal muscle (left). Negative nuclei in an uninfected HIV individual (group A, upper right) and abnormal nuclei surrounded by positive TUNEL reaction (arrows) in an untreated HIV-infected individual (group B, right, down). The differences between controls (HIV^- individuals) and cases (altogether HIV^+ individuals) rendered a p value of less than 0.001.

the cell by HIV is not always required for the induction of an HIV-mediated increase of apoptosis,[11] and the indirect mechanisms initiated by viral proteins (such as Vpr, PR, Tat, or Env) leading to a cytokine-mediated inflammatory cell response should therefore also play an important role in inducing apoptosis to the surrounding cells.[2,11–17] Our study supports the idea that the increase of apoptosis caused by HIV infection is not an HIV effect circumscribed to a particular cell or tissue, but it should be considered a more generalized process. As a peculiarity, the syncytial nature of skeletal muscle cells makes apoptosis in this tissue a localized event that leads to fiber atrophy of the sarcoplasma surrounding affected nuclei rather than to cellular death.[18] Indeed, in the preantiretroviral era, the natural history of HIV infection included the development of a wasting syndrome characterized by the loss of lean body mass, which affected up to 10% of infected patients, and increased apoptosis mediated by tumor necrosis factor-α had been suggested as a participating mechanism leading to skeletal muscle fiber atrophy.[19] A mechanism that has been confirmed in other circumstances.[20]

We did not find gross differences in the degree of nuclear fragmentation between treated and untreated HIV-infected people. These results could reflect the dual role of HAART in apoptosis balance because, while protease inhibitors prevent the loss of mitochondrial membrane potential and seem to exert beneficial effects in preventing apoptosis,[21–23] nucleoside analogues (especially zidovudine) act as apoptotic inducers.[24] This fact could explain the absence of differences between treated and untreated individuals found in the current study. Similarly, the role of apoptosis in the development of LD remains unclear. We did not find an increase in apoptosis in HIV individuals with LD receiving HAART compared with the remaining HIV-treated individuals. Domingo *et al.*[6] found increased adipocyte apoptosis in HIV-infected patients developing HAART-related LD. However, since samples of asymptomatic infected patients were not analyzed in this work, it is not clear if these changes corresponded to the development of LD or were merely due to the presence of HIV infection. The same group also reported that subcutaneous adipocyte apoptosis continues to occur in such patients despite switching to a less lipodystrophic HAART regimen, which also points to a potential role of HIV itself in the apoptotic phenomena.[25] However, since apoptosis behaves differently on different cell lineages of the same organism, skeletal muscle could exhibit a different expression than adipocytes. In addition, adipocytes could be more sensitive or more prone to undergo apoptosis in patients with LD because fat tissue is the target in this syndrome. Whatever the cause, apoptosis by HIV itself must be carefully considered when studying the toxic mitochondrial effects of antiretrovirals. Antiretrovirals alone seem to be insufficient to explain all the secondary effects and the combined mechanisms of HIV and antiretrovirals against mitochondria may provide a better explanation for these effects.

In conclusion, our findings indicate that skeletal muscle of HIV-infected individuals has increased apoptosis when compared with uninfected subjects. It is not possible, however, to identify clear differences in the degree of apoptosis regarding the different treatment regimens or the development of LD. This may indicate that HIV itself is the most important factor in the induction of apoptotic phenomenon in skeletal muscle of HIV-infected patients.

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- SÍNTESI DE L'ESTUDI

En aquest treball hem volgut determinar si el grau d'apoptosi es veu incrementat al múscul esquelètic per l'efecte de la pròpia infecció pel VIH i/o per l'efecte del TARGA. De forma addicional, hem volgut determinar si el grau d'apoptosi és superior als pacients que manifesten evidències clíniques de LD en comparació amb els pacients sense LD.

L'estudi l'hem realitzat en tres grups de pacients infectats pel VIH: pacients assimptomàtics que mai han rebut TARGA, pacients assimptomàtics que reben TARGA i pacients que reben TARGA i presenten LD. Hem comparat els resultats amb un grup control format per pacients no infectats pel VIH.

Hem portat a terme un estudi immunohistoquímic a través del qual hem pogut valorar en múscul esquelètic la fragmentació de l'ADN cel·lular, que representa una característica inconfusible del procés de mort cel·lular programada.

- CONCLUSIONS

El propi VIH exerceix un efecte proapoptòtic al múscul esquelètic, doncs tots els subgrups de pacients infectats presenten un grau d'apoptosi significativament superior en comparació amb els individus sans no infectats.

El TARGA no sembla exercir cap efecte proapoptòtic addicional (ni tampoc antiapoptòtic) sobre el múscul esquelètic dels pacients infectats pel VIH, dons no hem detectat un increment significatiu de l'apoptosi al grup de pacients que reben TARGA en comparació amb els que mai han rebut tractament ARV.

El paper de l'apoptosi en el desenvolupament de la LD resulta poc clar, al menys en aquest model de múscul esquelètic, doncs no hem detectat un increment significatiu de l'apoptosi al grup de pacients amb LD en comparació amb la resta de grups de pacients infectats pel VIH.