MECANISMES FISIOPATOLÒGICS DE LESIÓ MITOCONDRIAL SECUNDÀRIA: ANTIRETROVIRALS, ANTIBIÒTICS, ANTIPSICÒTICS I MONÒXID DE CARBONI.

Tesi Doctoral

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Tesi Doctoral presentada per

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per aspirar al grau de **Doctora per la Universitat de Barcelona.**

Mecanismes fisiopatològics de lesió mitocondrial secundària: antiretrovirals, antibiòtics, antipsicòtics i monòxid de carboni.

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Barcelona, 29 de Setembre del 2008

Al meu pare,

que es mereixia alguna cosa millor. Perquè és el més similar que mai escriuré a un llibre, i d'haver-lo escrit, te l'hagués dedicat.

I a la meva mare, i al Lluís.
I al Lluís, i a la meva mare.

'Deixem de témer allò que hem après a entendre'

Marie Sklodowska (Marie Curie)

1867 Varsovia (Polònia)-1934 Sancellemoz (França)

Primera persona en rebre dos premis Nòbel (en física al 1903 i en química al 1911).

PRESENTACIÓ

La present tesi doctoral s'ha realitzat al **Laboratori de Funcionalisme Mitocondrial** del **Grup de Recerca Muscular** del **Servei de Medicina Interna**, ubicat a l'Institut d'Investigacions Biomèdiques August Pi i Sunyer (**IDIBAPS**) de la **Facultat de Medicina** de la **Universitat de Barcelona** annexa a l'**Hospital Clínic de Barcelona**.

Es va iniciar al març del 2003 amb el suport econòmic de **La Marató de TV3** (02-0210) i posteriorment amb els del **Fons d'Investigació Sanitària** (PI/041239) i els **Contractes de Suport per a la Investigació de l'Institut Carlos III** (CA07/00092), sota la direcció dels Drs. **Francesc Cardellach López i Òscar Miró i Andreu**.

Aquesta tesi pretén aprofundir en els mecanismes de toxicitat mitocondrial de determinats fàrmacs i tòxics mitocondrials d'ús comú per explicar part dels efectes secundaris que es manifesten sota tractament o la base etiopatològica dels símptomes clínics associats a la seva administració. Sota el títol '**MECANISMES FISIOPATOLÒGICS DE LESIÓ MITOCONDRIAL SECUNDÀRIA: ANTIRETROVIRALS, ANTIBIÒTICS, ANTIPSICÒTICS I MONÒXID DE CARBONI**' es recullen 6 treballs d'investigació que han donat lloc a les 5 publicacions i al manuscrit en preparació que conformen la present tesi doctoral.

ÍNDEX TEMÀTIC

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ABREVIATURES

ADN: Àcid desoxiribonucleic

ADNc: Àcid desoxiribonucleic complementari (retrotranscrit a partir del RNA; ADN sense introns)

ADNmt: Àcid desoxiribonucleic mitocondrial

ADNn: Àcid desoxiribonucleic nuclear

ADNpolγ: ADN polimerasa-γ

ADP: Adenosina difosfat

AIF: Factor inductor de l'apoptosi ('*apoptosis inducing factor*')

ARN: Àcid ribonucleic

ARNm: Àcid ribonucleic missatger

ARNmt: Àcid ribonucleic mitocondrial

ARNr: Àcid ribonucleic ribosòmic

ARNt: Àcid ribonucleic de transferència

ATP: Adenosina trifosfat

ATPasa: ATP sintasa o complex V

CI: Complex I o NADH-CoQ oxidoreductasa o NADH-deshidrogenasa mitocondrial

CII: Complex II o Succinat-CoQ oxidoreductasa

CIII: Complex III o $CoQH_2$-Cit c oxidoreductasa

CIV: Complex IV o Citocrom c oxidasa (COX)

CV: Complex V o ATP sintasa (ATPasa)

Cadena H: Cadena pesada ('*heavy*') de l'ADNmt

Cadena L: Cadena lleugera ('*light*') de l'ADNmt

CCR5: Correceptor de la cèl·lula hoste que reconeix el VIH

CD4: Receptor de la cèl·lula hoste que reconeix el VIH

Cit c: Citocrom C

CMI: Concentració mínima inhibitòria

CMSP: Cèl·lules mononuclears de sang perifèrica

CO: Monòxid de carboni

COHb: Carboxihemoglobina

C-HDL: Colesterol unit a lipoproteïnes d'alta denistat ('*high-density lipoprotein cholesterol*')

C-LDL: Colesterol unit a lipoproteïnes de baixa denistat ('*low-density lipoprotein cholesterol*')

CoQ: Coenzim Q (oxidat) o ubiquinona

$CoQH_2$: Coenzim Q (reduït) o ubiquinol

COX: Citocrom c oxidasa o complex IV

COX-II: Subunitat II de la COX codificada al mitocondri

COX-IV: Subunitat IV de la COX codificada al nucli

CRABP-1: Proteïna tipus 1 d'unió a l'àcid retinoic cel·lular ('*cellular retinoic acid binding protein 1*')

CRM: Cadena respiratòria mitocondrial

CS: Citrat sintasa

CXCR4: Correceptor de la cèl·lula hoste que reconeix el VIH

D-loop: '*Displacement loop'*

d4T: Estavudina

ddI: Didanosina

Deoxinucleòtids: Nucleòtids que formen part de l'ADN (A/T/C/G)

Dideoxinucleòtids: Anàlegs de nucleòtids que aturen la replicació de l'ADN (ITIAN)

FAD: Flavina adenina dinucleòtid (oxidat)

$FADH_2$: Flavina adenina dinucleòtid (reduït)

H^+: Protó

H_20_2: Peròxid d'hidrogen

HSP_1 o HSP_2: Promotor per la transcripció de la cadena pesada de tipus 1 o 2 ('*heavy strand promoter 1 or 2*')

IF: Inhibidors de la fusió

IP: Inhibidors de la proteasa

ITIAN: Inhibidors de la transcriptasa inversa anàlegs de nucleòsid o nucleòtid

ITINAN: Inhibidrors de la transcriptasa inversa no-anàlegs de nucleòsid o nucleòtid

LDH: Lactat deshidrogenasa

LHON: Neuropatia òptica hereditària de Leber ('*Leber's hereditary optic neuropathy*')

LRP: Proteïna associada al receptor de la lipoproteïna de baixa densitat ('*low-density lipoprotein receptor-related protein*')

LSP: Promotor per la transcripció de la cadena lleugera ('*Light strand promoter*')

MELAS: Síndrome d'encefalomiopatia mitocondrial, acidosi làctica i episodis recidivants d'isquèmia cerebral transitòria ('*myoencephalopathy, lactic acidosis and stroke-like episodes syndrome*')

MERRF: Síndrome d'epilèpsia mioclònica i fibres vermelles-desestructurades ('*myoclonic epilepsy and ragged-red fiber syndrom*e')

MHC-I: Complex major d'histocompatibilitat de classe I

MNGIE: Síndrome d'encefalopatia neurogastrointestinal mitocondrial ('*myo-neurogastrointestinal encephalopathy*')

mtSSB: Proteïna mitocondrial d'unió al ADNmt de cadena simple ('*mitochondrial DNA single-strand binding protein*')

mTERF: Factor de terminació de la transcripció mitocondrial ('*mitochondrial transcription terminator factor*')

mtTFA o TFAM: Factor de transcripció mitocondrial A ('*mitochondrial transcription factor A*')

NAD^+: Nicotinamida adenina dinucleòtid (oxidat)

NADH: Nicotinamida adenina dinucleòtid (reduït)

NARP: Debilitat muscular neurogènica, atàxia i retinitis pigmentosa ('*neurogenic muscle weakness, ataxia and retinitis pigmentosa*')

ND2: Subunitat II del complex I de la CRM (NADH-deshidrogenasa mitocondrial)

NO: Òxid nítric

Nucleòsid: Nucleòtid no fosforilat

Nucleòtid: Nucleòsid fosforilat

NVP: Nevirapina

O_2: Oxigen

O_2^-: Anió súperòxid

OH^-: Anió hidroxil

O_H: Origen de replicació de la cadena pesada ('*heavy*') de l'ADNmt

OHB: Oxigen hiperbàric

O_L: Origen de replicació de la cadena lleugera ('*light*') de l'ADNmt

ONB: Oxigen normobàric

$ONOO^-$: Anió peroxinitrit

pb: parells de bases

PCR: Reacció en cadena de la polimerasa ('*polymerase chain reaction*')

PDH: Piruvat deshidrogenasa

PKB: Proteïna cinasa B

Ribonucleòtids: Nucleòtids que formen part de l'ARN (A/U/C/G)

ROS: Espècies reactives de l'oxigen ('*reactive oxygen species'*)

RRF: Fibres rojes desestructurades ('*ragged-red fibers*')

SIDA: Síndrome de la immunodeficiència adquirida ('*AIDS*' en anglès)

Sistema OXPHOS: Sistema de la fosforilació oxidativa

Smac/Diablo: Segon activador de caspases derivat del mitocondri (smac: '*second mitochondria-derived activator of caspases*', i Diablo és la forma homòloga murina)

SQV/rit: Saquinavir potenciat amb ritonavir

SREBP-1: Proteïna tipus 1 d'unió a l'element regulador de l'esterol ('*sterol regulatory element-binding protein 1*')

T-20: Enfuvirtide

TARGA: Tractament antiretroviral de gran activitat

TDF: Tenofovir diproxil fumarat

TFAM o mtTFA: Factor de transcripció mitocondrial A ('*mitochondrial transcription factor A*')

TFB1M: Factor de transcripció mitocondrial B1 ('*mitochondrial transcription factor B1*')

TFB2M: Factor de transcripció mitocondrial B2 ('*mitochondrial transcription factor B2*')

USA FDA: Agència de l'alimentació i el medicament d'Estats Units d'Amèrica ('*Food and Drug Administration of the United States of America*')

V-DAC: Porina del canal aniònic voltatge-dependent mitocondrial ('*voltage-dependent anion channel porin*'), de codificació nuclear.

VHC: Virus de l'hepatitis C ('*HCV*' en anglès)

VIH: Virus de la immunodeficiència humana ('*HIV*' en anglès)

$\Delta\psi_{mt}$: Potencial de membrana mitocondrial

1. INTRODUCCIÓ

1.1. EL MITOCONDRI

El mitocondri és un orgànul d'herència materna present en la majoria de cèl·lules eucariotes. Es transmet de mares a fills perquè en el procés de fecundació l'oòcit és l'única cèl·lula germinal que aporta el citoplasma del futur zigot, i en cas de que l'espermatozou aportés algun mitocondri, es postula que seria eliminat per ubiquitinització (Sutovsky P et al, *Nature* 1999).

El nombre de mitocondris presents per cèl·lula és variable i depèn dels requeriments energètics de l'organisme i del teixit considerat; en general els teixits metabòlicament i energèticament més actius solen ser més rics en mitocondris.

Tenen la morfologia i les dimensions similars a les d'un bacteri tipus bacil, amb una mida d'aproximadament 1 μm de diàmetre per 10 μm de llargària, tot i que aquests paràmetres dependran del organisme i tipus cel·lular considerat.

És el principal centre productor d'energia química en forma d'adenosina trifosfat (ATP) de la cèl·lula, però també participa en molts d'altres processos; intervé en l'homeòstasi del calci (Ca^{2+}), la producció de calor, la síntesi de molts metabòlits cel·lulars (grups hemo, aminoàcids, nucleòtids o fosfolípids, entre d'altres), i darrerament s'ha descobert que estan implicats en el procés d'apoptosi o mort cel·lular programada.

És l'únic orgànul de la cèl·lula eucariota animal que disposa de material genètic propi (ADN mitocondrial o ADNmt), i maquinària transcripcional i traduccional autònoma, que permet la síntesi d'algunes de les proteïnes que participen en la cadena respiratòria mitocondrial (CRM).

1.1.1. Història

El mitocondri fou descobert per R. Altman que el va visualitzar per primera vegada mitjançant microscopia òptica al 1890, però no va ser fins a finals del S. XIX que C. Benda el va batejar amb el nom que ara és conegut, que deriva dels mots grecs *mitos* (filament) i *chondros* (gràmul).

A mitjans del S. XX A. Lehninger i E. Kennedy van demostrar que les principals rutes metabòliques cel·lulars (cicle de Krebs, β-oxidació i fosforilació oxidativa) es localitzen en aquest orgànul i B. Ephrussi proposà l'existència de material genètic al mitocondri (Ephrussi B et al, *Ann Inst Pasteur* 1949), que no es va constatar fins als anys seixanta (Nass MMK et al, *J Cell Biol* 1963).

L'any 1961 P. Mitchell proposa la *Teoria Quimiosmòtica* que postula que en la membrana interna del mitocondri, la síntesi d'ATP s'acobla al transport d'electrons i al consum d'oxigen en la CRM, en l'anomenada fosforilació oxidativa, en un procés que utilitza el poder reductor que s'obté en la degradació dels aminoàcids, carbohidrats o àcids grassos per reduir l'oxigen a aigua i sintetitzar ATP.
R. Luft i col·laboradors descriuen al 1962 la primera malaltia causada per una disfunció d'aquest orgànul (Luft R, *J Clin Invest* 1962), i des d'aleshores, cada vegada són més les funcions mitocondrials que coneixem i les malalties associades a l'alteració del seu funcionament. Cal destacar a principis dels anys 80 la seqüenciació del primer genoma mitocondrial humà (Anderson S et al, *Nature* 1981) com una eina absolutament necessària per a l'estudi de les malalties mitocondrials genètiques causades per mutacions en l'ADNmt.
Recentment, s'ha descobert la implicació mitocondrial en el fenomen d'apoptosi (Kroemer et al, *Nat Med* 2000 i Wang X, *Genes and Development* 2001), que actualment centra gran part de l'interès científic entorn a aquest orgànul.

1.1.2. Origen evolutiu

La *Teoria Endosimbiòtica* postula que els mitocondris de les actuals cèl·lules eucariotes deriven evolutivament d'un procariota aeròbic primitiu (una α-proteobacteria) que fou absorbit per la cèl·lula eucariota anaeròbia ancestral en qualitat d'endosimbiont fa uns dos mil milions d'anys (Lang BF et al, *Annu Rev Genet* 1999). El bacteri li va conferir a la cèl·lula eucariota la capacitat d'emprar metabòlicament l'oxigen i va acabar transferint part de la seva informació genètica al genoma cel·lular. Aquesta teoria explica per què és l'únic orgànul de la cèl·lula eucariota animal que disposa de material genètic propi i per què és sensible a gran part dels antibiòtics. A més a més, bacteris i mitocondris comparteixen moltes característiques genètiques i bioquímiques.

1.1.3. Estructura

El mitocondri està embolcallat per una doble membrana que delimita la matriu mitocondrial, que conté: el material genètic de l'orgànul, la maquinària que li permet la replicació, transcripció i traducció de la informació que s'hi troba codificada i la maquinària enzimàtico-

metabòlica que participa en les vies anabòliques i catabòliques característiques d'aquest orgànul (Figura 1).

La membrana externa és permeable a la majoria de soluts i permet l'intercanvi de molècules amb el citoplasma, mentre que la membrana interna és impermeable, rica en cardiolipina, i es troba replegada formant les anomenades crestes mitocondrials, que augmenten la seva superfície, i concentren els complexos enzimàtics que integren la CRM. Entre les dues membranes es localitza l'espai intermembranal, on es concentren les molècules mitocondrials que participen en l'apoptosi.

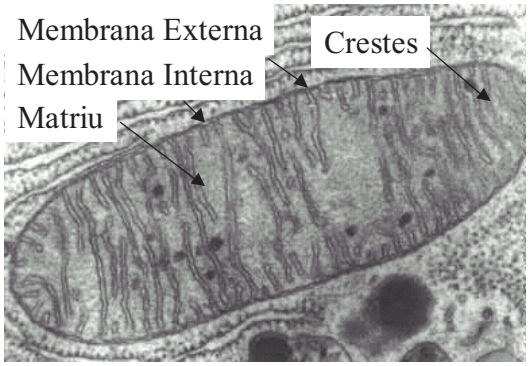


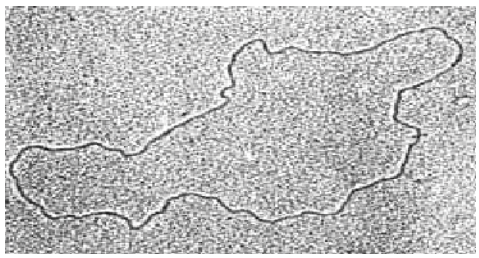
Figura 1: Micrografia electrònica d'un mitocondri humà (Fawcett 1994).

1.1.4. Material genètic

El mitocondri és l'únic orgànul de la cèl·lula eucariota animal que disposa de material genètic propi; es localitza a la matriu mitocondrial, possiblement ancorat a la membrana mitocondrial interna (Albring M et al, *Proc Natl Acad Sci USA* 1977), i la seva probable estructura supramolecular és en forma de nucleoid (múltiples còpies d'ADNmt i proteïnes associades) (Kaufman BA et al, *Proc Natl Acad Sci USA* 2000).

La seva mida i estructura difereix entre les diferents espècies. En l'ésser humà es tracta d'una molècula d'ADN de 16.569 parells de bases (pb) de doble cadena, circular i covalentment tancada (dsADNccc), que adopta topologia superhelicoidal en alguns dominis (Figura 2), i que representa el 0'5-1% de l'ADN total de la cèl·lula eucariota.

Figura 2: Micrografia electrònica de l'ADN mitocondrial (Fawcett 1994). Es tracta d'una molècula de doble cadena, circular i covalentment tancada.



Les dues cadenes s'anomenen pesada o H (*Heavy*) i lleugera o L (*Light*) en funció de la seva composició en nucleòtids (guanina i citosina vs adenina i timina o G+C vs A+T) i per tant en funció del seu pes, fet que permet separar-les per centrifugació diferencial en un gradient de densitat en condicions desnaturalitzants.

L'ADNmt té caràcter poliploide, doncs la majoria de cèl·lules eucariotes contenen cents de mitocondris i cada mitocondri múltiples còpies d'ADNmt per orgànul (2-10) (Shuster RC et al, *Biochem Biophys Res Commun* 1988). El nombre de còpies d'ADNmt és variable, a nivell interindividual i intraindividual, fins i tot en una mateixa cèl·lula o teixit, doncs les molècules es segreguen aleatòriament quan el mitocondri es divideix. Al mateix temps els mitocondris es segreguen aleatòriament entre les cèl·lules filles durant la divisió cel·lular, i ambdós tipus de segregació (mitocondrial i genètica), a l'atzar, donen lloc al fenòmen d'heteroplàsmia, o representació heterogènia d'un determinat genoma mitocondrial mutant (diferent del salvatge) entre les cèl·lules filles. Aquesta heteroplàsmia requereix que s'assoleixi un determinat nivell llindar ('*threshold effect'*) en el percentatge de molècules mutants per a la manifestació fenotípica de la malaltia mitocondrial genètica associada, que habitualment ha de ser bastant elevat (d'entre el 60-80%), per assolir manifestacions patològiques (Lightowlers RN et al, *Trends Genet* 1997).

La taxa de mutació de l'ADNmt és entre 10 i 20 vegades superior a la nuclear (Brown WM et al, *Proc Natl Acad Sci USA* 1979) perquè es localitza en una atmosfera rica en les espècies reactives d'oxigen (ROS) que es generen en el propi mitocondri, està menys protegit per proteïnes, no conté introns, i a més a més, els mecanismes de reparació d'errors són molt pobres en aquest orgànul.

La seva estructura és altament compacta; doncs a part de no contenir introns, la distribució dels diferents gens és contigua i en alguns casos fins i tot solapant (Anderson S et al i Montoya J et al, *Nature* 1981) i molts codons de terminació d'ARNm no estan codificats en l'ADNmt sinó que s'afegeixen per poliadenilació post-transcripcional (Ojala D et al, *Nature* 1981) (Tzagoloff 2001, Figura 3).
Els gens mitocondrials es troben asimètricament distribuïts en l'ADNmt, sent més abundants els que es localitzen en la cadena pesada (Figura 3). La gran majoria però dels gens que codifiquen per proteïnes mitocondrials es localitzen en el genoma nuclear, i els seus productes han de ser importats cap al mitocondri (Walter N, *Annu Rev Biochem* 1997).

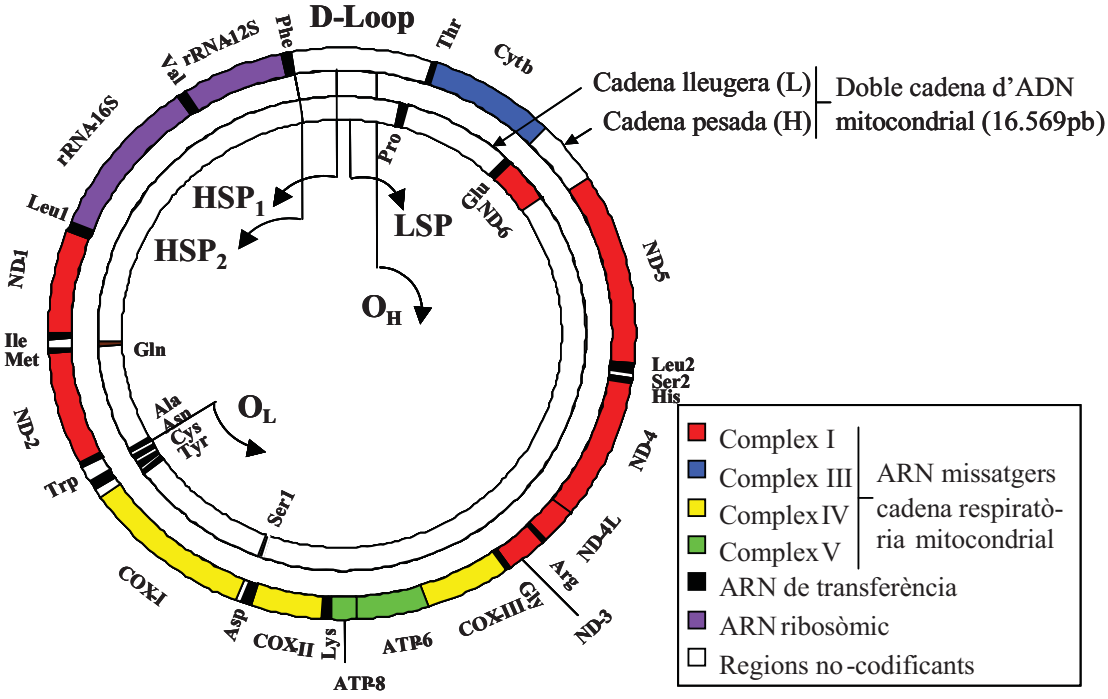


Figura 3: Distribució de la informació genètica que codifica l'ADN mitocondrial humà. O_H: Origen de replicació de la cadena pesada; O_L: Origen de replicació de la cadena lleugera; LSP: Promotor per la transcripció de la cadena lleugera; HSP_1 i HSP_2: Promotors per la transcripció de la cadena pesada de tipus 1 i 2.

Els 37 gens codificats en l'ADNmt es divideixen funcionalment en dos tipus: els 22 que donaran lloc a ARN ribosòmics (ARNr) i 2 de transferència (ARNt), que codifiquen per la maquinària que possibilita la síntesi de proteïnes mitocondrials, i els que codifiquen pels 13 ARN missatgers (ARNm) que es tradueixen a subunitats proteiques que formen part dels diferents complexos de la CRM, a excepció del complex II, exclusivament codificat en el nucli (Taula I).

Taula I: Subunitats proteiques codificades en el genoma mitocondrial (ADNmt) o nuclear (ADNn) respecte al total de subunitats que integren els diferents complexos de la cadena respiratòria mitocondrial.

Subunitats	C I	C II	C III	C IV	CV	Total
Totals	~ 40	4	11	13	14	>82
ADNmt	7	0	1	3	2	13
ADNn	~ 33	4	10	10	12	>69

Els ARNt es troben regularment intercalats entre els ARNr i/o ARNm per facilitar la maduració dels diferents tipus de RNA durant la transcripció a partir del transcrit policistrònic.

En l'ADNmt existeix un domini no-codificant anomenat *Displacement Loop* (D-Loop), on es concentra l'origen de replicació de la cadena pesada (O_H) i els promotors per la transcripció d'ambdues cadenes (LSP o *Light Strand Promoter* i HSP_1 i HSP_2 o *Heavy Strand Promoter* tipus 1 i 2). Aquest domini sovint es troba estructurat en forma de triple hèlix com un heterohíbrid format per la doble cadena d'ADN mitocondrial i un fragment de ARN que correspon al transcrit en síntesi. La seqüència del D-loop és altament variable i sovint es fa servir per a realitzar estudis genètics poblacionals, evolutius, de migracions o de parentesc, bàsicament per dues raons: per una banda per ser una regió no-codificant encara més permissiva a l'acúmul relatiu de mutacions, ja de per si elevades en l'ADNmt, i per l'altra, l'especial caràcter d'herència materna propi de l'ADNmt (Stoneking M, *J Bioenerg Biom embr* 1994).

Les variacions (conjunt de polimorfismes) presents en el genoma mitocondrial es coneixen amb el nom d'haplogrups i fins ara se n'han establert de 18 tipus (Torroni A et al, *Am J Hum Genet* 1993).

1.1.5. Replicació mitocondrial

A l'igual que la divisió de l'orgànul, la replicació, transcripció i traducció de l'ADNmt són temporalment independents (asincròniques) a la cel·lular, però la pràctica totalitat de molècules que hi intervenen estan codificades al nucli i per això és fonamental una bona comunicació nucli-mitocondri (també anomenada comunicació intergenòmica).

L'ADN polimerasa gamma (ADNpolγ) és l'únic enzim responsable de la síntesi d'ADNmt. Està codificada per un gen nuclear (anomenat POLG) i s'importa al mitocondri on exerceix la seva activitat polimerasa (de replicació) i exonucleasa (de correcció d'errors) de l'ADNmt. Però en la replicació de l'ADNmt hi intervenen molts d'altres factors, molts d'ells directament relacionats amb la transcripció mitocondrial, doncs l'inici de la replicació de l'ADNmt requereix l'existència d'encebadors de RNA originats per la digestió del transcrit del mateix material genètic. Entre d'altres factors intervenen: la RNA polimerasa i l'endoribonucleasa que sintetitzen i tallen, respectaivament, l'encebador de RNA necessari per iniciar la replicació, el factor de transcripció mitocondrial A (TFAM, mtTFA o '*mitochondrial transcription factor A*') que permet l'inici de la transcripció, l'helicasa (Twinkle) i les topoisomerases I i II necessàries per afegir o relaxar la torsió característica de l'ADNmt a mesura que avança la forca de replicació, i les proteïnes mitocondrials d'unió a

ADNmt de cadena simple (mtSSB o '*mitochondrial single-strand binding protein*'), entre d'altres, el paper de molts dels quals, és encara objecte d'estudi.

S'han descrit com a mínim tres models teòrics que expliquen la replicació de l'ADNmt, anomenats: '*strand-displacement'* o *'strand-asymetric'* (Clayton DA, *Cell* 1982), '*strand-coupled leading- and lagging-strand synthesis'* (Holt IJ et al, *Cell* 2000) i '*ribonucleotide incorporation throughout the lagging-strand'* (conegut com a RITOLS; Yang MY et al, *Cell* 2002 i Yasukawa T et al, *EMBO J* 2006). El primer model (anomenat 'desplaçament de la cadena' o 'cadena asimètrica'), inicialment proposat per Clayton i col·laboradors (Clayton DA, *Cell* 1982) i fins ara acceptat, considera que ambdues cadenes es sintetitzen de manera unidireccional i contínua en sentit 5'→3' (com ho fan la majoria de procariotes) mentre que les altres dues teories proposen un model de replicació bidireccional (anàleg al del nucli eucariota) en el que la cadena pesada es sintetitza de manera contínua (5'→3') i la cadena lleugera de manera discontínua (3'→5'), emprant encebadors de RNA per a la síntesi dels fragments d'Ogazaky de la cadena lleugera. La diferència entre els dos últims models, ambdós proposats pels mateixos investigadors, és que el model de '*strand-coupled leading-and lagging-strand synthesis'* descrit a l'any 2000 proposa el nou model de síntesi d'ADNmt bidireccional en coexistència amb el model tradicional descrit per Clayton, metres que la darrera teoria (RITOLS), afirma que el model bidireccional és l'únic existent per la replicació de l'ADNmt.

L'ADNmt conté dos orígens de replicació, localitzats en diferents posicions i en diferents cadenes (O_H i O_L). El model clàssic de Clayton i col·laboradors postula que la replicació de l'ADNmt comença amb la replicació de la cadena pesada, que s'inicia a l'O_H situat al D-loop i procedeix unidireccionalment i en sentit 5'→3' fins a dos terços del genoma mitocondrial, on el segon origen de replicació, el de la cadena lleugera (O_L), es veu exposat pel desplaçament de la cadena pesada. La replicació de la cadena lleugera comença llavors des de l' O_L i també en sentit 5'→3', sempre de manera contínua. Perquè s'iniciï la replicació de la cadena pesada es fa servir com a encebador un fragment del transcrit de la cadena lleugera en forma de ARN que l'ADNpolγ elonga a base de replicar la cadena pesada. Es tracta doncs d'una replicació asincrònica, asimètrica, unidireccional i contínua, on cada cadena antiparal·lela inicia la replicació a diferent temps i des de diferents orígens i on cada forca de replicació copia una sola cadena que es sintetitza de manera contínua i en sentit 5'→3' (Clayton DA, *Cell* 1982).

Com hem dit anteriorment, aquest model està sent actual motiu de controvèrsia i caldrà esperar noves aportacions en aquest camp que permetin establir un model de replicació nou o reforçat.

1.1.6. Transcripció mitocondrial

La transcripció mitocondrial és temporalment independent de la nuclear, però moltes de les molècules que hi intervenen (com l'ARN polimerasa o els factors de transcripció) estan codificades a l'ADN nuclear, de manera que la comunicació intergenòmica nucli-mitocondri és fonamental.

La iniciació de la transcripció requereix, com a mínim, l'activitat de l'ARN polimerasa mitocondrial (Tiranti V et al, *Hum Mol Genet* 1997) i del factor de transcripció mitocondrials A (mtTFA o TFAM) (Fisher RP et al, *J Biol Chem* 1985) amb combinació o bé amb el factor de transcripció mitocondrial B1 o bé amb el B2 (TFB1M i TFB2M, respectivament) (Falkenberg M et al, *Nat Genet* 2002), i per a la finalització del procés, és necassària la intervenció del factor terminador de la transcripció mitocondrial (mTERF) (Daga A et al, *J Biol Chem* 1993).

El genoma mitocondrial, a diferencia del nuclear, es transcriu de manera simètrica, completa i contínua a partir dels promotors de la transcripció per la cadena lleugera (*LSP)* o pesada *(HSP_1* i *HSP_2)* (Figura 3 i 4) localitzats al D-loop (Montoya J et al, *Proc Natl Acad Sci USA* 1982). Es generen en el procés 3 transcrits policistrònics que contenen el producte de la transcripció de tots els gens codificats en de la cadena lleugera (a partir del promotor LSP), tots els gens codificats a la cadena pesada (a partir del promotor HSP_1), o bé exclusivament els dos primers ARNr codificats en la cadena pesada de l'ADNmt (quan la transcripció s'inicia a partir del HSP_2). Un cop processats, els transcrits policistrònics donaran lloc als diferents tipus d'ARN mitocondrials: 13 missatgers, 22 de transferència i 2 ribosòmics. Com s'ha dit anteriorment, la cadena pesada es transcriu mitjançant dues unitats superposades que comencen i acaben a diferents nivells i que es transcriuen a partir de diferents promotors (HSP_1 i HSP_2). D'aquesta manera els ARNr codificats a la cadena pesada poden generar-se conjuntament amb altres ARN (a partir del HSP_1) o bé de manera exclusiva (a partir del HSP_2), sent aquesta unitat transcripcional molt més freqüent (fins a 20 vegades) que la primera. En canvi la cadena lleugera sempre es transcriu de manera completa.

En la maduració del transcrit policistrònic és essencial la distribució dels gens que codifiquen pels diferents tipus d'ARNmt, que s'organitzen de manera alternant per tal de que els gens

que codifiquen per ARNr o ARNm estiguin separats per gens que codifiquen per ARNt (Figura 3 i 4). En el transcrit policistrònic les molècules d'ARNt són identificades per enzims que tallen i separen els ARNt, alliberant els ARNm i ARNr que s'hi troben intercalats, que després seran modificats per produir transcrits madurs, en un model anomenat '*tRNA punctuation*' (Ojada D et al, *Nature* 1981).

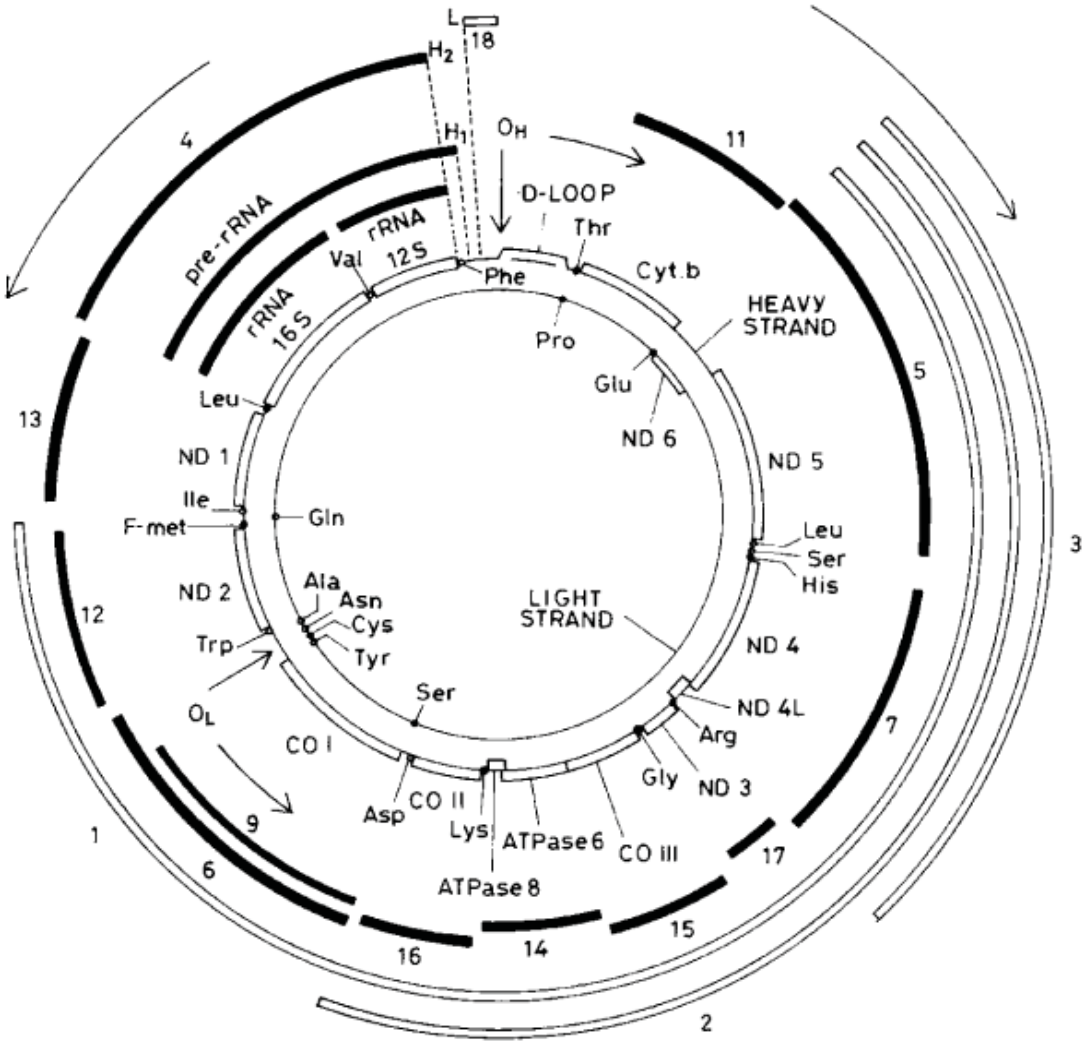


Figura 4: Mapa genètic i transcripcional de l'ADNmt. Els 2 cercles interns representen l'ADNmt i els gens codificats. Els dos cercles externs representen els ARNmt transcrits des de la cadena pesada (línies negres) o des de la cadena lleugera (línies blanques). Els ARNt mitocondrials s'indiquen amb el codi de l'aminoàcid pel que codifiquen. H_1, H_2 i L indiquen els punts d'inici de la transcripció per les cadenes pesada (H_1 i H_2) i lleugera (L). O_H i O_L indiquen l'origen de replicació de la cadena pesada i lleugera, respectivament. Les fletxes indiquen els sentits de la replicació i transcripció. Figura extreta de l'article de P. Fernández-Silva et al. 'Repliaction and transcription of mammalian mitochondrial DNA'. *Exp Physiol* 2003; 88: 41-56.

Aquest procés de maduració requereix quatre activitats enzimàtiques: la digestió endonucleotídica pels extrems 3' i 5' dels ARNt, l'addició de la seqüència CCA a l'extrem 3' dels ARNt i la poliadenilació a l'extrem 3' dels ARNr i ARNm (Fernandez-Silva P et al, *Exp Physiol* 2003). La majoria de gens estructurals (ARNm) no presenten codó de terminació codificat en l'ADNmt, sinó que aquest es genera per poliadenilació post-transcripcional de l'extrem 3'(Ojala D et al, *Nature* 1981), no presenten la càpsula característica de l'extrem 5' (Montoya J et al, *Nature* 1981) que s'agrega als ARNm del genoma nuclear dels eucariotes ni tampoc tenen les *'untranslated regions'* (UTR) característiques dels extrems 3' i 5' dels mARN nuclears.

1.1.7. Traducció mitocondrial

La traducció mitocondrial també és independent de la cel·lular i precisa d'una bona comunicació intergenòmica, com la majoria de processos mitocondrials.

La traducció mitocondrial, com la cel·lular, precisa del funcionament coordinat de ribosomes, ARNt, aminoàcids i els factors d'iniciació, elongació i finalització, propis de la traducció mitocondrial.

Els ribosomes mitocondrials, al igual que els citoplasmàtics, són complexos ribonucleoproteics constituïts per una sèrie de proteïnes ribosomals associades a ARNr que s'estructuren en dues subunitats funcionals. Cadascuna d'aquestes subunitats està formada per multitud de proteïnes ribosomals i un sol tipus de ARNr: el ARNr 16S forma part de la subunitat ribosomal major i el ARNr 12S forma part de la petita. Aquests ARNr es troben codificats en el genoma mitocondrial (Figura 3), però a diferència del nucli, els gens ribosomals es presenten en una sola còpia. En canvi, les aproximadament 80 proteïnes que constitueixen els ribosomes mitocondrials i els factors de traducció estan codificats en el genoma nuclear, es tradueixen en ribosomes citoplasmàtics i després s'importen al mitocondri (O'Brien et al, *Metods Enzymol* 1996). N'hi ha de diferents tipus segons formin part de l'estructura ribosomal o bé dels complexos d'iniciació (IF-2mt), elongació (EF-Tu mt, EF-Ts mt, EF-G mt) o finalització de la traducció (Schwartzbach et al, *Metods Enzymol* 1996).

El codi genètic mitocondrial és lleugerament diferent al nuclear (anomenat *universal*) en alguns codons, que són específicament reconeguts pels ARNt que ell mateix codifica (Taula II). A més a més, una versió simplificada de l'aparellament codó-anticodó per 8 dels aminoàcids mitocondrials basat en el reconeixement de només les dues primeres bases entre

ARNm i ARNt mitocondrial permet que la traducció es desenvolupi en aquest orgànul emprant només 22 ARNt (Attardi G et al, *Annu Rev Cell Biol* 1988).

Taula II: Codons que difereixen entre el codi genètic nuclear (universal) i el mitocondrial.

Codó	ADNn	ADNmt
UGA	STOP	Trp
AGG, AGA	Arg	STOP
AUA, AUU	Ile	Met i INICI
AUG	Met i INICI	INICI

Els ribosomes mitocondrials presenten d'altres peculiaritats en la seva estructura i funcionament que els diferencien dels citoplasmàtics i en canvi fan que comparteixin una gran homologia amb els ribosomes procariotes. Per aquest motiu els antibiòtics que exerceixen la seva acció antibacteriana bloquejant la síntesis proteica del bacteri, sovint també inhibeixen la síntesi de proteïnes mitocondrials i poden ser els responsables en l'aparició de síndromes tòxiques d'origen mitocondrial. Per exemple els aminoglucòsids poden provocar l'aparició de sordesa (Leroux L, *Ann Otolaryngol* 1950), el cloramfenicol l'anèmia aplàsica (Rich ML et al, *Ann Intern Med* 1950) o el linezolid hiperlactatèmia (Palenzuela L et al, *Clin Infect Dis* 2005), manifestacions adverses possiblement potenciades per la susceptibilitat genètica individual a presentar determinades mutacions o polimorfismes, especialment en els gens relacionats amb la traducció mitocondrial.

1.1.8. Cadena respiratòria mitocondrial. Fosforilació oxidativa

El mitocondri sintetitza (metabolisme anabòlic) i degrada (metabolisme catabòlic) els substrats cel·lulars segons les necessitats de cada moment.

Les vies catabòliques mitocondrials permeten la obtenció d'energia en forma d'ATP a partir de la degradació diferents metabòlits cel·lulars (àcids grassos, carbohidrats o aminoàcids). Aquestes rutes són específiques per la naturalesa del substrat fins que convergeixen en la producció d'acetilcoenzim A, que entra en el cicle de Krebs (cicle dels àcids tricarboxílics) per ser degradat a diòxid de carboni (CO_2) i aigua (H_20). Aquests processos catabòlics que tenen lloc a la matriu mitocondrial generen poder reductor en forma d'uns intermediaris anomenats nicotinamida adenina dinucleòtid (NADH) i flavina adenina dinucleòtid ($FADH_2$), que en la seva forma reduïda cedeixen electrons a la CRM localitzada a la

membrana interna del mitocondri perquè es sintetitzi ATP a través de la fosforilació oxidativa o sistema OXPHOS (Figura 5).

La CRM està integrada per 4 complexos enzimàtics (I, II, II i IV) i 2 transportadors mòbils d'electrons anomenats coenzim Q o ubiquinona (situat entre els complexos I, II i III) i citocrom c (localitzat entre el complexos III i IV). Els electrons són cedits des del NADH al complex I o des del $FADH_2$ al complex II, respectivament, i des d'aquests complexos al coenzim Q, que posteriorment els cedeix al complex III, i des del complex III al IV, a través del citocrom c.

Els electrons flueixen a través de la CRM mitjançant reaccions d'oxidoreducció que finalitzen en el complex IV, on l'oxigen actua com a acceptor final d'electrons per reduir-se a aigua. El transport d'aquests electrons al llarg de la CRM és un procés exergònic, doncs es realitza des d'un estat d'energètic superior a un de menor energia, de manera que l'energia alliberada s'aprofita en els complexos I, III i IV per exportar protons (H^+) en contra de gradient des de la matriu mitocondrial fins a l'espai intermembrana. Quan el complex V (ATPasa) retorna aquests protons a l'interior de la matriu, ho fa a favor de gradient, possibilitant la síntesi d'ATP.

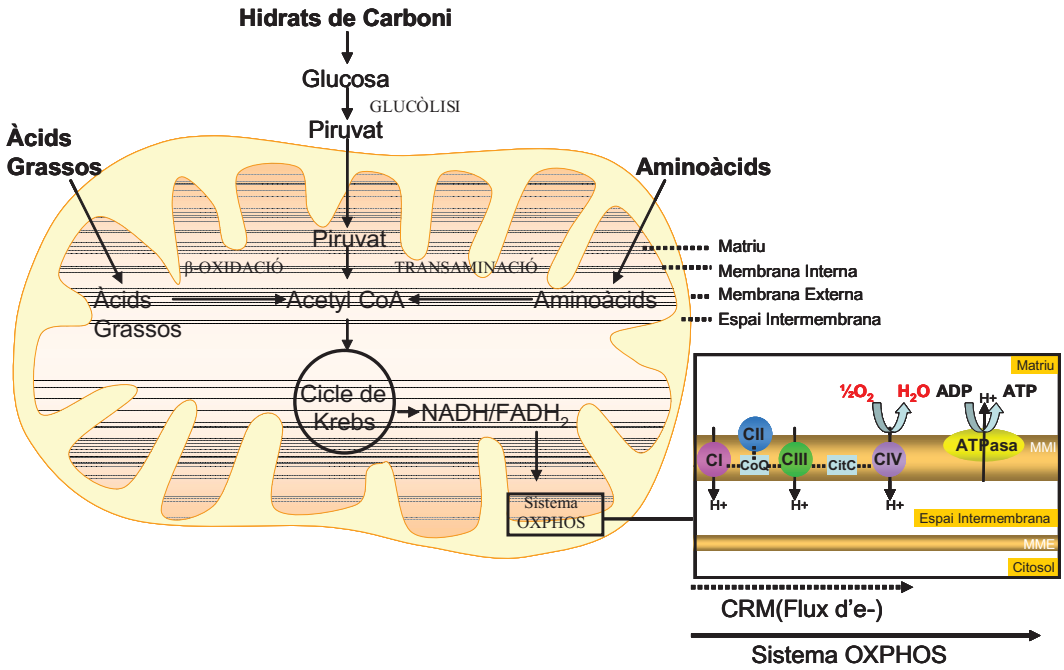


Figura 5: Esquema dels compartiments i metabolisme mitocondrials. CI-CIV: Complexos I-IV; MMI i MME: membrana mitocondrial interna i externa; CRM: cadena respiratòria mitocondrial; OXPHOS: sistema de la fosforilació oxidativa; e^-: electró/ns; H^+: protó/ns.

En els darrers temps s'ha qüestionat la idea fins ara establerta de que els diferents complexos que integren la CRM es distribueixen aleatòriament per la membrana mitocondrial interna fins a constituir una unitat funcional i s'ha proposat la teoria de que tots ells s'ensamblen de manera coordinada i ordenada en aquesta membrana per formar estructures supramoleculars (els anomenats 'super-complexos'), que constituirien de manera conjunta l'anomenat 'respirasoma' (Schägger H et al, *EMBO J* 2000).

1.1.9. Consum mitocondrial d'oxigen

El mitocondri és l'orgànul cel·lular responsable de consumir oxigen (per sintetitzar ATP en la fosforilació oxidativa) i generar CO_2 (últim producte del metabolisme catabòlic). Aquests gasos arriben (oxigen) o surten (CO_2) del mitocondri per difusió simple fins al torrent sanguini i és fonamental el seu equilibri en sang per la vida.

En una situació d'hipòxia o de funcionament mitocondrial deficient, el mitocondri no consumeix oxigen i les vies metabòliques es reajusten per tal d'utilitzar el poder reductor i produir energia de manera anaeròbica. En aquest cas, el piruvat procedent del processament dels hidrats de carboni no serà importat al mitocondri per dur a terme la glucòlisi aeròbica i la fosforilació oxidativa, sinó que serà transformat a lactat a través de l'enzim citosòlic lactat deshidrogenasa (Figura 6).

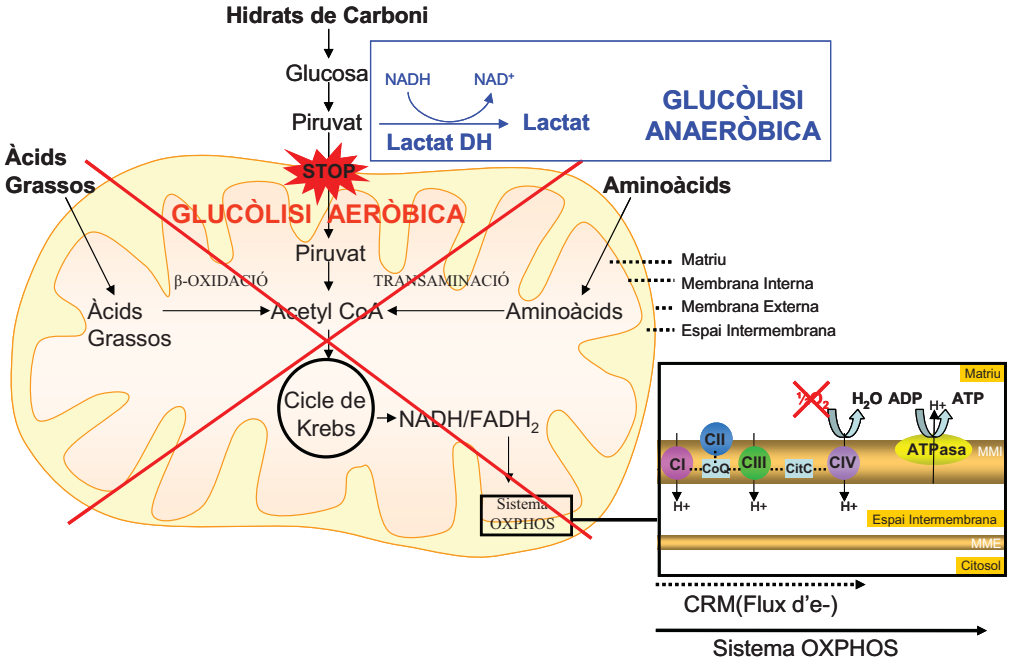


Figura 6: Metabolisme anaeròbic en situacions d'hipòxia o disfunció mitocondrial.

CI-CIV: Complexos I-IV; MMI i MME: membrana mitocondrial interna i externa; CRM: cadena respiratòria mitocondrial; OXPHOS: sistema de la fosforilació oxidativa; e^-: electró/ns; H^+: protó/ns.

La concentració de lactat en sang és el resultat de la producció de lactat per part de les cèl·lules de tot l'organisme (però especialment múscul esquelètic, fetge, teixit nerviós i limfoide) i del seu aclariment plasmàtic (per conversió del lactat a piruvat a través de la gluconeogènesi, que principalment té lloc al fetge, i en menor mesura al ronyó). En condicions de normalitat la concentració de lactat oscil·la entre els 0'5-2'4 mmols/L, però en situacions d'hipòxia o disfunció mitocondrial, augmenta la síntesi de lactat en els teixits productors i disminueix la gluconeogènesi en els teixits que el degraden, de manera que la concentració sanguínia de lactat augmenta i disminueix el pH sanguini, doncs el lactat és un àcid orgànic que a pH fisiològic (7'3) es desprotona (allibera H^+ al medi) acidificant la sang (Figura 7).

Per aquest motiu moltes malalties o intoxicacions amb base mitocondrial es caracteritzen per un augment plasmàtic de la concentració en lactat, a vegades acompanyat per la disminució del pH sanguini.

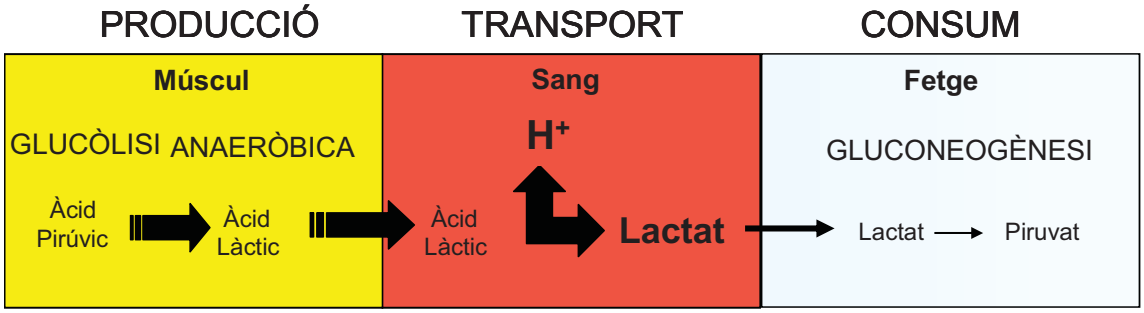


Figura 7: Metabolisme del lactat en situacions d'hipòxia o disfunció mitocondrial.

1.1.10. Espècies reactives de l'oxigen

Les espècies reactives de l'oxigen (ROS) són subproductes derivats de l'oxigen que majoritàriament es produeixen en el mitocondri quan la fosforilació oxidativa no funciona correctament. Són espècies oxidants considerades radicals lliures perquè presenten electrons desaparellats, altament inestables, que poden reaccionar i danyar la majoria d'estructures cel·lulars (proteïnes, lípids, hidrats de carboni i material genètic) i darrerament s'ha descobert que poden intervenir en processos de senyalització cel·lular. Existeixen diferents radicals: els ions superòxid (O_2^-) i peròxid d'hidrogen (H_2O_2) són relativament estables, però l'anió hidroxil (OH^-) i el peroxinitrit ($ONOO^-$) són molt més reactius. Tots ells deriven de l'anió superòxid, que es forma principalment a nivell dels complexos I i III de la CRM a través de

reaccions d'oxidoreducció de la ubiquinona (localitzada entre el complexos I-II i I-III), que en la seva forma semireduïda (de semiquinona) pot autoxidar-se per tornar a la forma oxidada (d'ubiquinona) a base de cedir un electró i reduir de manera monovalent l'oxigen molecular a anió superòxid.

Existeixen enzims cel·lulars antioxidants com la superòxid dismutasa que catalitza la conversió de O_2^- a H_2O_2, les catalases o peroxidases que detoxifiquen el H_2O_2 a H_2O o la glutatió peroxidasa que redueix el H_2O_2 a H_2O, així com els hidroperòxids (R-OOH) i els peròxids lipídics (fruit de l'atac de les ROS als lípids de membrana). Per la superòxid dismutasa i la glutatió peroxidasa existeixen 2 isoformes, i una és de localització mitocondrial. En canvi la catalasa només es troba als peroxisomes. També existeixen molècules antioxidants no-enzimàtiques com la vitamina E, la vitamina C, els carotenoids, les quinones, el glutatió i molts elements metàl·lics (seleni, zinc, ferro, coure...) que redueixen els nivells de ROS.

Aquests mecanismes antioxidants mantenen els nivells d'estrès oxidatiu dins dels rangs de normalitat fins que existeix una disfunció mitocondrial; llavors la producció de ROS s'incrementa per damunt dels nivells que poden ser detoxificats. Aquests radicals lliures poden atacar qualsevol estructura cel·lular, però el mitocondri, per ser el principal centre productor de ROS, resulta especialment vulnerable, tant el seu material genètic, que no està massa protegit per proteïnes, com els lípids i proteïnes de membrana, per exemple les que integren la CRM.

La producció mitocondrial de ROS pot estar motivada per una disfunció mitocondrial o pot tenir un altre origen (per exemple la disminució dels mecanismes de defensa antioxidant), però el que és segur és que la presència de ROS causarà disfunció mitocondrial, si no n'hi havia, o incrementarà la present.

1.1.11. Potencial de membrana

El flux exergònic d'electrons al llarg dels diferents complexos de la CRM allibera una energia que s'utilitza per bombejar protons a través dels complexos I, III i IV, des de la matriu a l'espai intermembrana (Figura 5). En conseqüència els protons s'acumulen a l'espai intermembrana en contra de gradient generant: 1) la força proto-motriu que impulsa la síntesi d'ATP a través del complex V (per entrada a favor de gradient d'aquests protons de nou cap a la matriu), i 2) una diferència de potencial elèctric a banda i banda de la membrana mitocondrial interna de -180mV que es coneix amb el nom de potencial de membrana de

l'orgànul ($\Delta\psi$). El gradient electroquímic de protons generat a l'espai intermembranal del mitocondri és la suma d'aquest potencial elèctric ($\Delta\psi$) generat per la diferent distribució de càrregues i del potencial químic ($\Delta\mu$) generat per la diferent distribució d'espècies químiques a través de la membrana mitocondrial interna.

Parlem de despolarització mitocondrial quan es perd part o la totalitat d'aquest potencial de membrana (quan el valor és superior als -180mV). La despolarització sol precedir l'alliberació de factors apoptogènics des de l'espai intermembranal del mitocondri (citocrom C, Smac / Diabolo, AIF -*Apoptosis Inducing Factor*-, endonucleasa G i altres), que desencadenen la mort cel·lular per apoptosi (Luo X et al, *Cell* 1998; Espositi MD, *Apoptosis* 2002).

1.1.12. Biogènesi

La població mitocondrial d'una determinada cèl·lula, teixit o organisme és dinàmica i s'adapta a les necessitats energètiques i metabòliques de cada moment. Els mitocondris varien la seva mida, número (mitjançant processos de fusió i fissió), estructura (per exemple la quantitat de crestes mitocondrials) i massa al llarg de les diferents etapes del desenvolupament, de la diferenciació cel·lular i en resposta a determinades situacions fisiològiques o patològiques (Goffart S et al, *Exp Physiol* 2003).

La biogènesi mitocondrial és la suma de la proliferació (augment del nombre de mitocondris per cèl·lula) i diferenciació mitocondrial (adquisició de les característiques estructurals i funcionals necessàries) (Nisoli E et al, *Biochem Pharmacol* 2004).

La biogènesi mitocondrial està regulada per nombrosos factors: factors cel·lulars propis de les circumstàncies, expressió i replicació del genoma mitocondrial, així com l'expressió i transport al mitocondri de les proteïnes mitocondrials codificades al nucli necessàries per dur a terme aquest procés.

A vegades la proliferació anòmala de mitocondris és sinònim de disfunció mitocondrial, doncs el mitocondri tendeix a compensar la deficiència funcional a base d'augmentar el nombre de mitocondris que realitzen aquella funció, i és un fenomen característic d'algunes malalties mitocondrials primàries clàssiques. Per aquest motiu, és important conèixer la capacitat que presenta un determinat teixit o cèl·lula de realitzar una determinada funció mitocondrial, però també el nombre de mitocondris que la realitzen; parlem de les capacitats funcionals mitocondrials relatives.

1.2. MITOCONDRIOPATIES

El correcte funcionament mitocondrial és essencial per a la viabilitat cel·lular i la seva disfunció ocasiona l'aparició de les anomenades malalties mitocondrials, o mitocondriopaties, que es classifiquen com a primàries o secundàries, en funció de si són degudes (secundàries) o no (primàries) al contacte amb algun agent tòxic.

Les malalties mitocondrials van començar a estudiar-se als anys seixanta amb l'observació d'acúmuls de mitocondris en pacients amb intolerància a l'exercici, gràcies al desenvolupament de tècniques morfològiques d'estudi ultraestructural i histoquímic del teixit muscular com la tinció pel tricròmic de Gomori, que posa de manifest les fibres rojes desestructurades (*ragged-red fibers* o RRF), fibres musculars que acumulen de manera anòmala mitocondris subsarcolèmics (Andreu AL et al, *Neurología* 2004).

S'ha avançat molt en el coneixement d'aquestes malalties, i darrerament, es comencen a plantejar tractaments destinats a minimitzar-ne les manifestacions (com les vitamines, cofactors o acceptors finals d'electrons) (Andreu AL et al, *Neurología* 2004; Pedrol E et al, *Med Clin* 2005; Artuch R et al; *J Neurol Sci* 2006).

1.2.1. Mitocondriopaties primàries

Les mitocondriopaties primàries (no tòxiques) es divideixen en aquelles que són congènites (heretades) o s'han adquirit espontàniament.

Luft i col·laboradors van ser els primers en descriure el 1962 la primera alteració mitocondrial que donava lloc a una clínica patològica (Luft R, *J Clin Invest* 1962) i al 1988 es descriuen les primeres mutacions a l'ADNmt responsables d'algunes mitocondriopaties primàries com les miopaties (Holt IJ et al, *Nature* 1988) o la neuropatia òptica hereditària de Leber (Wallace DC et al, *Science* 1988). Des de llavors s'han caracteritzat més de 50 tipus de disfuncions d'origen mitocondrial que donen lloc a mitocondriopaties primàries: MELAS, MERRF, MNGIE, NARP o LHON són algunes de les més conegudes (Andreu AL et al, *Methods Mol Biol* 2003).

Aquestes alteracions mitocondrials poden tenir lloc a diferents nivells. A nivell d'ADN mitocondrial, s'han descrit més de 155 mutacions puntuals i multitud de delecions, duplicacions o insercions, així com diferents graus de depleció, associats a disfunció mitocondrial i fenotip patològic (Andreu AL et al, *Neurología* 2004). En general, les

mitocondriopaties primàries degudes a una alteració en l'ADNmt poden classificar-se segons afectin a gens que intervenen en la síntesi de proteïnes (els que codifiquen per ARNr o ARNt mitocondrials) o directament als gens que codifiquen per a proteïnes (ARNm mitocondrials que codifiquen per les 13 subunitats de la CRM codificades a l'ADNmt) (DiMauro S et al, *Brain Pathol* 2000) (Figura 8). En aquests tipus d'alteracions mitocondrials, malgrat l'etiologia sigui la mateixa (el desordre genètic responsable), existeix un enorme ventall de possibles manifestacions clíniques. Aquesta variabilitat fenotípica es deu, entre d'altres motius, a l'heteroplàsmia (coexistència de molècules salvatges i mutants), l'efecte llindar (la necessitat de que hi hagi un cert grau de molècules mutants per a l'expressió del fenotip), la segregació mitòtica (distribució aleatòria de les molècules mutants i salvatges entre les cèl·lules filles) i el context genètic nuclear (per a la resta de gens que codifiquen per proteïnes implicades en el funcionament de l'orgànul), i tots aquests paràmetres, són sovint característics de cada individu (Andreu AL et al, *Neurología* 2004).

Les mitocondriopaties primàries també poden deure's, però, a defectes en gens nuclears que codifiquen per proteïnes amb funció mitocondrial, ja sigui les que intervenen en la intercomunicació genòmica nucli-mitocondri o les que participen en la biogènesi mitocondrial o en el funcionament transcripcional, traduccional o enzimàtic de l'orgànul. Es tracta d'alteracions de molt diversa índole que cursen amb una gran varietat de quadres clínics.

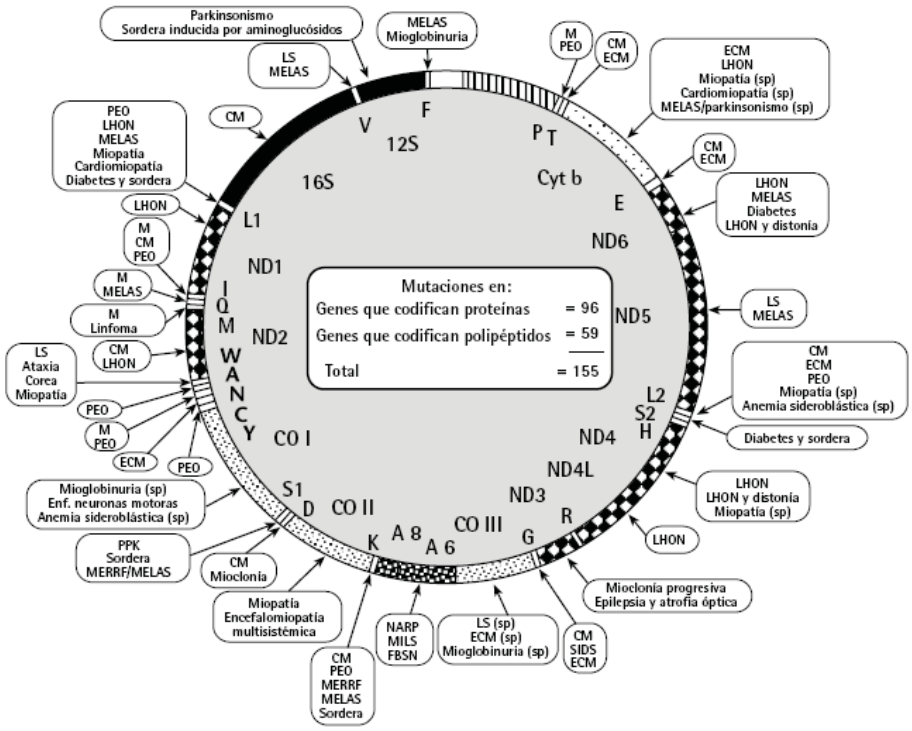


Figura 8: Malaties mitocondrials originades per una mutació en l'ADNmt. Figura extreta de l'article d'AL Andreu et al. 'Las enfermedades mitocondriales: una clasificación para el S. XXI'. *Neurologia* 2004; 19: 15-22.

1.2.2. Mitocondriopaties secundàries

Són aquelles degudes a la toxicitat mitocondrial d'un determinat agent etiològic, ja sigui de tipus natural (compostos presents en la natura) o artificial (substàncies sintetitzades per l'ésser humà).

Donada la similitud entre els efectes secundaris d'alguns tractaments, les manifestacions clíniques de certes intoxicacions o malalties i la simptomatologia de les mitocondripaties primàries, cada vegada són més els autors i les hipòtesi experimentals que associen la toxicitat mitocondrial d'un determinat tractament farmacològic o d'un tòxic com el mecanisme fisiopatològic que explica l'existència dels efectes secundaris de la teràpia o les manifestacions clíniques de la intoxicació o malaltia.

La lesió mitocondrial pot ser induïda per diversos agents i pot exercir-se a diferents nivells, dependent de la funció mitocondrial que s'alteri. Alguns dels nivells més elementals de funcionament mitocondrial que poden veure's afectats són els següents:

a) Replicació de l'ADNmt

b) Transcripció de l'ARNmt

c) Traducció de les proteïnes mitocondrials

d) Activitat enzimàtica dels complexos que integren la CRM

e) Consum mitocondrial d'oxigen

f) Producció de ROS

g) Potencial de membrana de l'orgànul

h) Biogènesi mitocondrial

L'alteració de qualsevol d'aquests paràmetres mitocondrials pot començar com un efecte directe aïllat fruit de l'acció mitotòxica d'un determinat agent que exerceix un mecanisme de lesió específic, però si el tòxic no es retira ràpidament, sol desencadenar l'alteració de la resta de paràmetres i una disfunció mitocondrial global que pot comprometre la viabilitat cel·lular, la funció del teixit i, eventualment, la vida del pacient.

1.3. TÒXICS MITOCONDRIALS

Existeixen molts agents capaços d'induir disfunció mitocondrial i poden tenir diferent naturalesa; alguns són fàrmacs que s'empren en la pràctica clínica diària (com els antivírics, anestèsics, neurolèptics, quimioteràpics, antiinflamatoris no-esteroidals, glucocorticoids, antibiòtics, anticolesterolèmics, etc) o d'altres són tòxics (com alguns insecticides, certs herbicides, el tabac, l'alcohol, el monòxid de carboni -CO- o l'òxid nítric -NO-). Aquests agents presenten diferents mecanismes de toxicitat, molts dels quals no estan encara ben establerts.

A continuació s'introduiran els tòxics mitocondrials l'estudi dels quals centra l'interès de la present tesi doctoral.

1.3.1. Fàrmacs antiretrovirals i virus de la immunodeficiència humana

Els fàrmacs antiretrovirals aprovats en l'actualitat per tractar la infecció causada pel virus de la immunodeficiència humana (VIH) es classifiquen en 6 grans famílies segons la seva naturalesa i l'etapa del cicle viral que bloquegin per frenar l'avanç de la infecció (Figura 9).

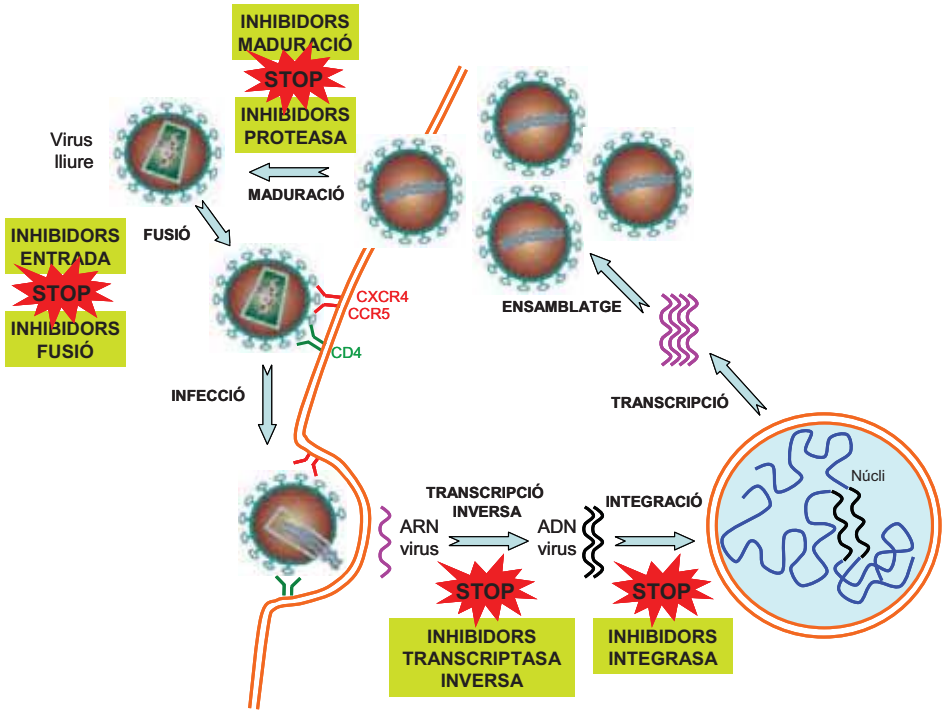


Figura 9: Etapes del cicle del VIH i els diferents fàrmacs antiretrovirals segons l'etapa del cicle viral que bloquegen. CD4, CCR5 i CXCR4: receptor (CD4) i correceptors (CCR5 i CXCR4) de la cèl·lula hoste que reconeix el VIH; ARN: Àcid ribonucleic; ADN: Àcid desoxiribonucleic.

Es tracta dels: inhibidors de la transcriptasa inversa del virus (que poden ser anàlegs o no de nucleòsid o nucleòtid), els inhibidors de la proteasa del VIH, els inhibidors de la fusió del virus amb la cèl·lula hoste, els inhibidors de la entrada (o inhibidors del correceptor CCR5) i els inhibidors de la integrasa del VIH.

La recerca i la indústria farmacèutica estan contínuament desenvolupant noves opcions terapèutiques, algunes de les quals es troben en fases avançades d'investigació, que pertanyen a les famílies antiretrovirals fins ara establertes, o bé a noves classes d'antiretrovirals (com els inhibidors de la maduració del virus, entre d'altres) (Figura 9).

Actualment existeixen més de 20 fàrmacs antiretrovirals per tractar el VIH, que combinats ofereixen múltiples possibilitats terapèutiques, especialment per aquells pacients que presenten resistències o toxicitat en front d'algun dels seus components (Taula III).

Taula III: Fàrmacs antiretrovirals comercialitzats al setembre del 2008. ITIAN: Inhibidors de la transcriptasa inversa anàlegs de nucleòsid o nucleòtid; ITINAN: Inhibidors de la transcriptasa inversa no anàlegs de nucleòsid o nucleòtid; IP, IF, IE, II: Inhibidors de la proteasa, de la fusió, de l'entrada (correceptor CCR5) i de la integrasa; *USA FDA*: *Food and Drug Administration of the United States of America* (agència de l'alimentació i el medicament d'Estats Units d'Amèrica).

Família	Nom Genèric	Nom Abreviat	Nom Comercial	Data Aprovació *FDA*
ITIAN	Zidovudina	AZT o ZVD	Retrovir	Març 1987
	Didanosina	ddI	Videx	Octubre 1991
	Zalcitabina	ddC	Hivid	Juny 1992
	Estavudina	d4T	Zerit	Juny 1994
	Lamivudina	3TC	Epivir	Novembre 1995
	Abacavir Sulfat	ABC	Ziagen	Desembre 1998
	Tenofovir Diproxil Fumarat	TDF	Viread	Octubre 2001
	Emtricitabina	FTC	Emtriva	Juliol 2003
		3TC+AZT	Combivir	Setembre 1997
		ABC+3TC	Kivexa	Desembre 1998
		ABC+3TC+AZT	Trizivir	Novembre 2000
		FTC+TDF	Truvada	Agost 2004
		EFV+TDF+FTC	Atripla	Juliol 2006
ITINAN	Nevirapina	NVP	Viramune	Juny 1996
	Delavirdina mesilat	DLV	Rescriptor	Abril 1997
	Efavirenz	EFV	Sustiva	Setembre 1998
IP	Saquinavir mesilat	SQV	Invirase	Desembre 1995
	Indinavir sulfat	IDV	Crixivan	Març 1996
	Ritonavir	RTV	Norvir	Març 1996
	Nelfinavir	NFV	Viracept	Març 1997
	Saquinavir	SQV	Fortovase	Novembre 1997
	Amprenavir	APV	Agenerase	Abril 1999
	Lopinavir+Ritonavir	LPV+RTV	Kaletra	Setembre 2000
	Atazanavir	ATA	Reyataz	Juny 2003
	Fosamprenavir	FPV	Telzir	Octubre 2003
	Tipranavir	TPV	Aptivus	Juny 2005
	Darunavir	TMC-114	Prezista	Juny 2006
IF	Enfuvirtide	T20	Fuzeon	Març 2003
IE	Maraviroc	MVC	Selzentry	Agost 2007
II	Raltegravir	RAL	Isentress	Octubre 2007

Aquest ampli arsenal terapèutic ha permès, des de 1996, tractar la infecció per VIH emprant combinacions d'aquests fàrmacs (habitualment 3 d'ells) en l'anomenat tractament antiretroviral de gran activitat (TARGA). El TARGA ha substituït la monoteràpia i ha demostrat ser molt més eficaç per anular la càrrega viral del pacient de manera prolongada i minimitzar així el deteriorament immunològic i l'aparició de resistències (Mallolas J, *Enferm Infecc Microbiol Clin* 1999).

El TARGA ha augmentat l'esperança i qualitat de vida dels infectats pel VIH que tenen accés al tractament, però la toxicitat mitocondrial dels seus components es proposa la principal responsable de la aparició de gran part dels efectes adversos associats a la teràpia com la miopatia, la lipodistròfia, la hiperlactatèmia, l'acidosis làctica, la polineuropatia o la pancreatitis (Grau JM et al, *Ann Neurol* 1993 i *Neurology* 1994; Lewis W et al, *Nat Med* 1995; Casademont J et al, *Brain* 1996; Masanés F et al, *J Neurol Sci* 1998; Cherry CL et al, *J Antimicrob Chemother* 2003). A més a més aquest efecte mitotòxic del tractament podria veure's incrementat per la pròpia infecció pel VIH, doncs el propi virus podria estar alterant ja de base algun d'aquests paràmetres mitocondrials, com el contingut en ADNmt, contribuint així a l'aparició dels efectes secundaris que es manifesten sota tractament (Côté H et al, *N Engl J Med 2002*; Miró Ò et al, *CID* 2004). Aquest efecte es veuria reforçat en aquells pacients co-infectats pel virus de la hepatitis C, que també s'ha demostrat que indueix depleció d'ADNmt (Bäuerle J et al, *HIV Med* 2005), en els que la toxicitat mitocondrial és un esdeveniment freqüent (Walker UA, *Hepatology* 2004; Laguno M et al, *Antivir Ther* 2005).

Per aquest motiu les estratègies terapèutiques del TARGA cada vegada tenen més en compte la toxicitat mitocondrial d'un tractament crònic, que ha d'administrar-se al llarg de tota la vida del pacient, i per això és tan important aprofundir en el coneixement dels mecanismes de toxicitat mitocondrial antiretroviral.

A continuació es detallen les principals alteracions mitocondrials que causen els antiretrovirals de les famílies que es troben actualment comercialitzades.

1.3.1.1. Inhibidors de la transcriptasa inversa anàlegs de nucleòsids/nucleòtids

La transcriptasa inversa o transcriptasa reversa és un enzim present en el VIH (i tots els retrovirus) que retrotranscriu l'ARN de cadena simple del virus a ADN proviral de cadena doble, que pot insertar-se en el material genètic de la cèl·lula hoste i quedar-s'hi en estat latent molt de temps, fins que s'activa la formació de noves partícules virals (Figura 9). Els fàrmacs que inhibeixen aquest enzim han demostrat tenir una gran eficàcia antiretroviral.

Distingim dos tipus d'inhibidors de la transcriptasa inversa del VIH, segons la seva naturalesa i mecanisme d'acció: els anàlegs o no anàlegs de nucleòsid o nucleòtid.

Els inhibidors de la transcriptasa inversa anàlegs de nucleòsid o nucleòtid (ITIAN) són compostos sintètics molt similars als nucleòsids o nucleòtids naturals (salvatges), però a diferència d'aquests, presenten una petita modificació estructural que impedeix l'elongació de la cadena proviral d'ADN en síntesi, un cop són incorporats (Figura 10).

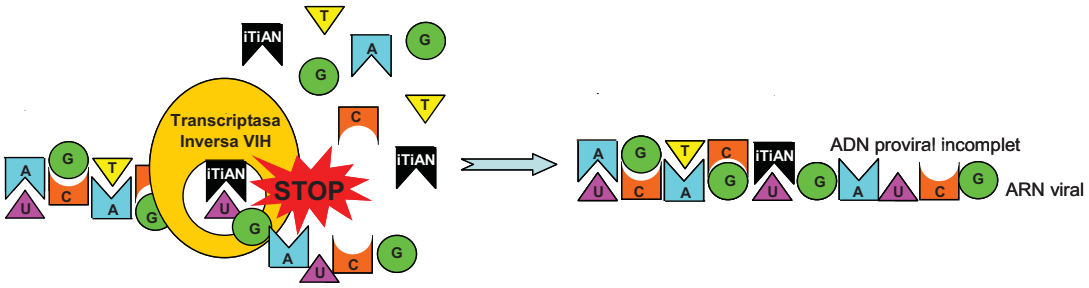


Figura 10: Bloqueig de la retrotranscipció de l'àcid ribonucleic (ARN) del VIH a àcid desoxiribonucleic (ADN) proviral per part dels inhibidors de la transcriptasa inversa anàlegs de nucleòsid o nucleòtid (ITIAN). Deoxinucleòtids (nucleòtids que formen part de l'ADN): A (Adenina), G (Guanina), T (Timina) i C (Citosina); Ribonucleòtids (nucleòtids que formen part de l'ARN): A (Adenina), U (Uracil), T (Timina) i C (Citosina).

La modificació estructural que presenten els ITIAN consisteix en la substitució del grup hidroxil de la posició 3' del sucre (la desoxiribosa) per un altre radical, de manera que quan s'incorpora l'ITIAN a la cadena d'ADN en síntesi el grup 5' fosfat del següent nucleòtid no pot formar l'enllaç fosfodiéster amb el grup 3' hidroxil de l'ITIAN i es bloqueja la transcriptasa inversa, l'elongació de la cadena i el cicle viral (Figura 11).

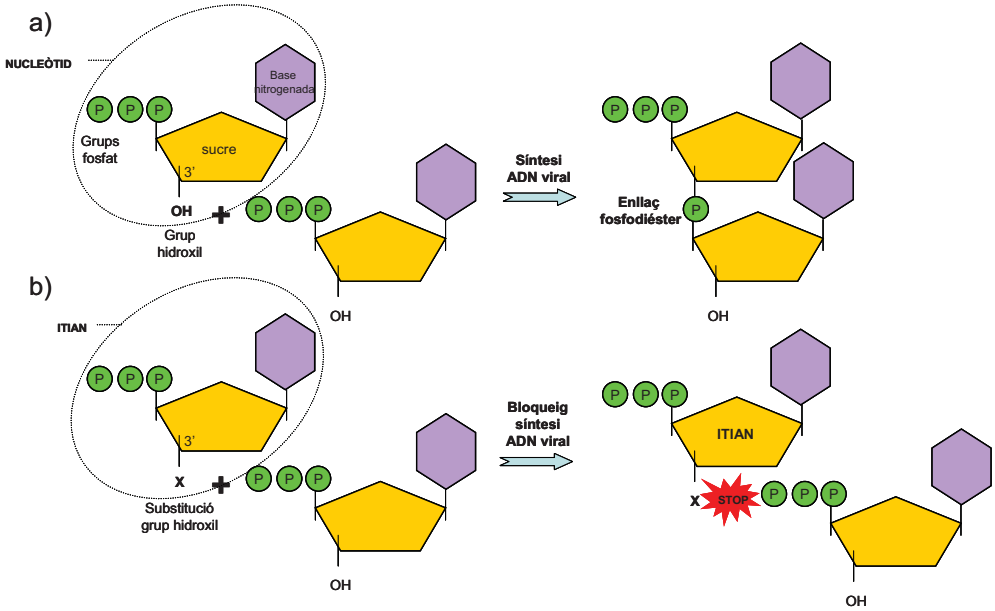


Figura 11: Estructura i síntesi de l'àcid desoxiribonucleic (ADN) entre nucleòtids naturals (a) o inhibidor de la transcriptasa inversa anàleg de nucleòtid -ITIAN- i nucleòtid salvatge (b). OH: Grup hidroxil; P: Grup fosfat.

Els nucleòsids que constitueixen l'ADN s'anomenen deoxinucleòsids i n'hi ha de 4 tipus naturals: adenosina (A), timidina (T), citidina (C) i guanosina (G). Cadascun d'aquests deoxinucleòsids fisiològics disposa com a mínim d'un anàleg denominat dideoxinucleòsid: la didanosina (ddI) es anàleg de la inosina que es transforma en adenosina; la zidovudina (AZT o ZDV) i l'estavudina (d4T) són anàlegs de la timidina; la zalcitabina (ddC), lamivudina (3TC) i emtricitabina (FTC) són anàlegs de la citidina; i l'abacavir (ABC) de la guanosina.
La unitat estructural necessària per sintetitzar l'ADN són els nucleòtids portadors de tres grups fosfat. Nucleòsids i anàlegs de nucleòsids hauran de ser fosforilats fins a tres vegades en l'interior de la cèl·lula per cinases cel·lulars per a poder-se incorporar en el material genètic en forma de nucleòtids o anàlegs de nucleòtids. Els nucleòtids són, doncs, nucleòsids parcial o totalment fosforilats. Actualment només existeix un anàleg de nucleòtid comercialitzat anomenat Tenofovir Diproxil Fumarat (TDF), anàleg de l'adenosina 5'-monofosfat, que només haurà de ser fosforilat 2 vegades per a poder-se incorporar en la cadena d'ADN en síntesi.

Els ITIAN constitueixen sense dubte la base de qualsevol teràpia TARGA, i habitualment són dos dels seus tres components fonamentals.
A més a més van ser els primers fàrmacs desenvolupats per tractar la infecció; la zidovudina (AZT o ZDV), en un principi concebuda com a fàrmac anticancerígen, es va començar a administrar com a monoteràpia antiretroviral per tractar la infecció per VIH al 1987. Durant molts anys fou l'únic fàrmac disponible i va suposar un gran avenç contra la infecció, però la seva administració crònica va produir efectes secundaris com la miopatia (Grau JM et al, *Ann Neurol* 1993; Grau JM et al, *Neurology* 1994; Dalakas MC et al, *Ann Neurol* 1994).
Els efectes secundaris dels ITIAN es deuen, majoritàriament, a la toxicitat mitocondrial que exerceixen, principalment, mitjançant la inhibició de la replicació del material genètic del mitocondri (Brinkman K et al, *AIDS* 1998). Els ITIAN, que no tenen efectes en el nucli cel·lular, s'incorporen en el material genètic del VIH frenant així la replicació viral, però també s'insereixen en el material genètic del mitocondri. Això es deu a que els ITIAN inhibeixen, a més a més de la transcripció inversa del virus, l'acció d'altres ADN polimerases cel·lulars. Dels 5 tipus d'ADN polimerases que disposa la cèl·lula eucariota (α i δ per la replicació del material genètic nuclear, β i ε per la reparació dels errors produïts en la duplicació de l'ADN nuclear i l'ADN polimerasa γ per la replicació i reparació de l'ADNmt), els ITIAN inhibeixen les ADN polimerases cel·lulars β i γ. L'acció de l'ADN polimerasa β

és redundant (doncs també l'exerceix l'ADN polimerasa ε) i es desconeixen efectes fisiopatològics derivats de la inhibició selectiva d'aquest enzim. En canvi, l'ADN polimerasa γ és l'únic enzim responsable de la replicació (i cert grau de reparació) del material genètic del mitocondri, i el bloqueig de la seva activitat té majors conseqüències, doncs condiciona la disminució del contingut en ADNmt de l'orgànul que pot arribar a comprometre'n el seu correcte funcionament. Per aquest motiu, els ITIAN es postulen els principals causants de la depleció d'ADNmt i la conseqüent disfunció mitocondrial associada al tractament antiretroviral, responsable de gran part dels efectes adversos associats al tractament (Brinkman K et al, *AIDS* 1998; Lim SE et al, *J Biol Chem* 2001; Carr A et al, *Lancet* 2000).

El mecanisme d'inhibició de l'ADN polimerasa γ mitocondrial per part dels ITIAN presenta múltiples efectes que s'esquematitzen a la Figura 12.

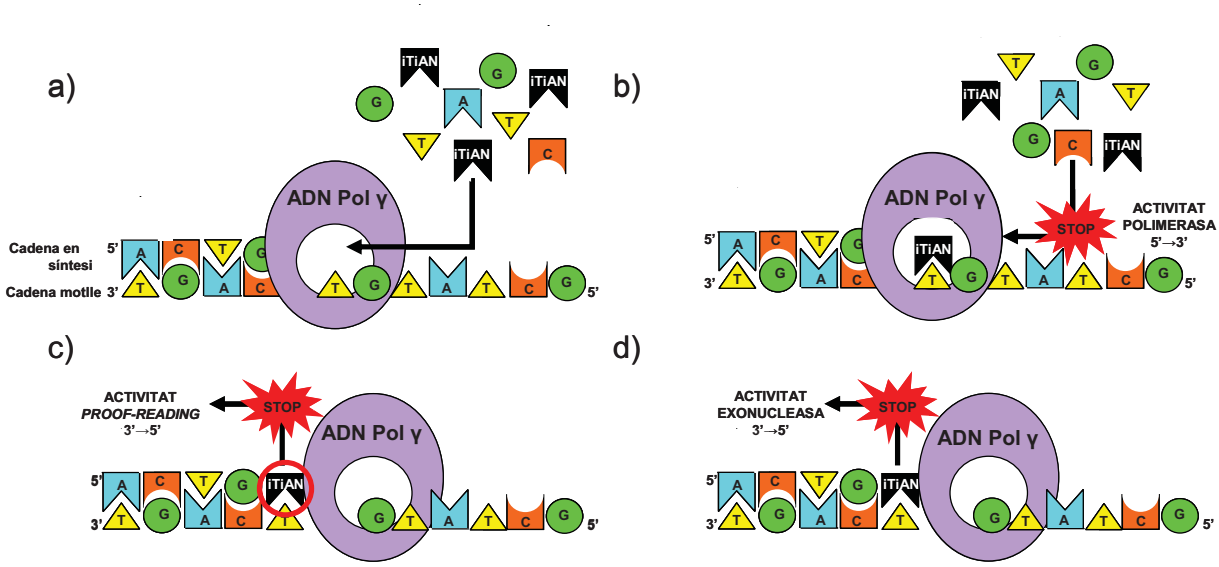


Figura 12: Bloqueig de l'ADN Polimerasa γ (ADN Pol γ) mitocondrial per part dels inhibidors de la transcriptasa inversa análegs de nucleòsid o nucleòtid (ITIAN). En primer lloc els ITIAN exerceixen una inhibició competitiva sobre aquest enzim en competir amb els nucleòtids fisiològics per incorporar-se a la cadena d'ADNmt naixent (a); actuen com a terminadors prematurs de l'elongació de la cadena d'ADNmt en síntesi perquè un cop incorporats inhibeixen l'activitat polimerasa del enzim promovent la generació d'espècies incompletes amb delecions (b); augmenten la taxa d'errors puntuals en la cadena d'ADNmt sintetitzada en inhibir l'activitat *proof-reading* de l'ADN polimerasa γ mitocondrial que reconeix els nucleòtids mal aparellats (c); i inhibeixen també l'activitat exonucleasa de l'enzim que corregeix els errors de fidelitat en la còpia produïts durant la síntesi de la nova cadena d'ADNmt (d). Deoxinucleòtids (nucleòtids que formen part de l'ADN): A (Adenina), G (Guanina), T (Timina) i C (Citosina).

D'aquesta manera els ITIAN disminueixen la qualitat i quantitat d'ADNmt augmentant la taxa de mutacions puntuals, delecions i depleció en les molècules d'aquest material genètic.

Per altra banda, s'ha descrit que l'alteració en la proporció relativa dels acúmuls endògens dels diferents nucleòtids ('*pools*') i l'alteració de les rutes mitocondrials i cel·lulars per a la síntesi d'aquests substrats poden també ser responsables de causar depleció de l'ADNmt (Ashley N et al, *Hum Mol Genet* 2007), i l'administració d'un ITIAN, podria causar aquest imbalanç. Donat que l'ADNmt codifica per 13 subunitats proteiques que integren complexos de la CRM, alteracions genètiques a nivell d'ADNmt poden comportar deficiències en el funcionament energètic i metabòlic de l'orgànul que poden arribar a comprometre la viabilitat cel·lular.

Kakuda va classificar els ITIAN segons la potencia que presentaven *in vitro* per inhibir l'ADN polimerasa γ mitocondrial en aquesta escala decreixent: ddC > ddI > d4T >>> AZT > 3TC > ABC = TDF (Kakuda T, *Clin Ther* 2000), que seria anàloga a l'escala de toxicitat mitocondrial per a aquests fàrmacs en el supòsit que els ITIAN només exercissin toxicitat mitocondrial a través de la inhibició d'aquest enzim.

Poden existir mecanismes que afavoreixin aquesta toxicitat. Per exemple el TDF presenta una baixa toxicitat mitocondrial *in vitro* (Birkus G et al, *Antimicrob Agents Chemother* 2002), associada a escassa toxicitat clínica mitocondrial *in vivo* (Lyseng-Williamson KA et al, *Drugs* 2005; Barditch-Crovo P et al, *Antimicrob Agents Chemother* 2001). En canvi s'ha observat que quan el TDF s'administra conjuntament amb didanosina (ddI), de reconeguda capacitat mitotòxica, potencia els efectes adversos d'aquest últim ITIAN per interacció farmacocinètica que augmenta la concentració plasmàtica i biodisponibilitat de ddI (León A et al, *AIDS* 2005 gener i octubre). Les actuals directrius de teràpia antiretroviral recomanen l'administració de la dosi reduïda de ddI quan aquest fàrmac es combina amb TDF, però existeixen poques dades mitocondrials dels efectes d'aquesta combinació reduïda.

Però darrerament s'han descrit efectes mitocondrials dels ITIAN en absència de depleció d'ADNmt. Això suggereix l'existència de mecanismes lesius pel mitocondri independents doncs a la inhibició de l'ADN polimerasa γ. Entre aquests mecanismes s'ha descrit, en el cas concret de la zidovudina (AZT o ZDV), la inhibició del transport de substrats del metabolisme energètic del mitocondri (succinat i ADP/ATP), la inhibició del complex IV (citocrom c oxidasa o COX) de la CRM o la disminució del contingut mitocondrial en carnitina (Dalakas MC et al, *Ann Neurol* 1994; Tomelleri G et al, *Ital J Neurol Sci* 1992; Barile M et al, *Gen Pharmacol* 1998), així com per a aquest i d'altres ITIAN, la inhibició *in vivo* (Mallon PW et al, *J Infect Dis* 2005) o *in vitro* (Galluzi L et al, *Antivir Ther* 2005) de la transcripció mitocondrial. Per altra banda s'han descrit mecanismes homeostàtics mitocondrials a nivell transcripcional o traduccional que *in vivo* han demostrat ser capaços de

compensar una depleció d'ADNmt causada per ITIAN com la ddI i la d4T (Miró Ò et al, *J Acquir Immune Defic Syndr* 2004). Existeixen, doncs, efectes dels ITIAN sobre l'ADNmt que no tindran conseqüències sobre el funcionament mitocondrial, i efectes d'aquests fàrmacs sobre el mitocondri que no es manifesten en una depleció d'ADNmt.

A més a més, els ITIAN s'han proposat com a fàrmacs inductors d'apoptosi a través d'exercir una lesió mitocondrial crònica que ocasiona la generació d'estrès oxidatiu. D'aquesta manera la cèl·lula arriba a una situació de compromís energètic i metabòlic (especialment per dèficit en ATP) que pot desencadenar la mort cel·lular programada a través de la via intrínseca (Opii WO et al, *Exp Neurol* 2007; Mallal SA et al, *AIDS* 2000; Brinkman K et al, *Lancet* 1999; Petit F et al, *TRENDS Pharmacol Sci* 2005; Viora M et al, *Int J Immunopharmacol* 1997). Aquest fenomen és especialment negatiu quan afecta a limfòcits T $CD4^+$ (essencials en la lluita contra la infecció pel VIH) o a cèl·lules del teixit adipós lipoatròfic (implicades en la síndrome de lipodistròfia).

1.3.1.2. Inhibidors de la transcriptasa inversa no anàlegs de nucleòsids

La transcriptasa inversa del VIH també pot bloquejar-se administrant els antiretrovirals inhibidors de la transcriptasa inversa no anàlegs de nucleòsid o nucleòtid (ITINAN).

Amb el mateix efecte que els ITIAN, presenten un mecanisme d'acció completament diferent. Els ITINAN no són anàlegs dels nucleòsids o nucleòtids fisiològics ni bloquegen la retrotranscipció del material genètic viral quan són incorporats en la cadena en síntesi, sinó que interaccionen directament amb la transcriptasa inversa del virus i bloquegen la seva activitat enzimàtica (Figura 13). S'uneixen a aquest enzim induint un canvi conformacional que bloqueja el seu centre catalític i la seva activitat i, a diferència dels ITIAN, no han de ser activats (per exemple mitjançant fosforilació) per exercir la seva activitat antiretroviral.

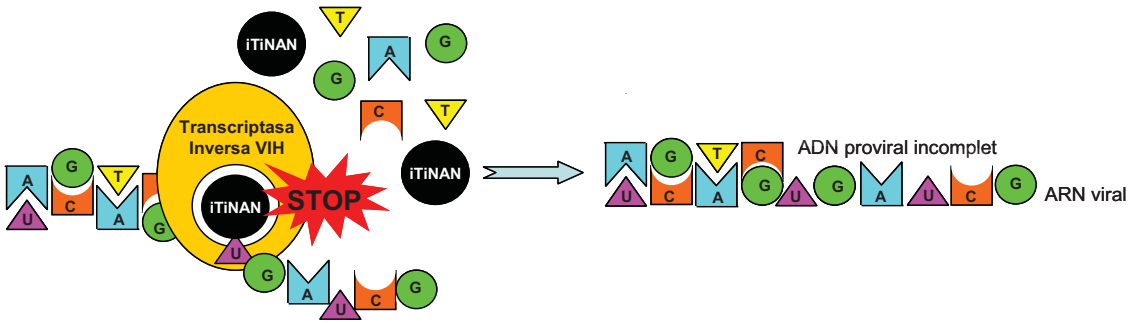


Figura 13: Bloqueig de la retrotranscipció de l'àcid ribonucleic (ARN) del VIH a àcid desoxiribonucleic (ADN) proviral per part dels inhibidors de la transcriptasa inversa no anàlegs de nucleòsid o nucleòtid (ITINAN). Deoxinucleòtids (nucleòtids que formen part de l'ADN): A (Adenina), G (Guanina), T (Timina) i C (Citosina); Ribonucleòtids (nucleòtids que formen part de l'ARN): A (Adenina), U (Uracil), T (Timina) i C (Citosina).

L'administració dels ITINAN comença el 1996 amb la comercialització de la Nevirapina (NVP) i actualment formen part de molts esquemes TARGA en combinació amb altres fàrmacs inhibidors de la transcriptasa inversa tipus ITIAN.

Aquests antiretrovirals, a diferència dels ITIAN, no inhibeixen cap ADN polimerasa cel·lular, ni tan sols la mitocondrial (γ), i el seu ús continuat només s'ha associat, potser, a un lleu increment dels fenòmens apoptòtics. Aquest possible efecte inductor d'apoptosi s'ha documentat especialment per l'ITINAN efavirenz (EFV), que també podria alterar la proliferació de limfòcits T (Pilon AA et al, *Antimicrob Agents Chemother* 2002) i la diferenciació *in vitro* dels adipòcits que du a terme el factor de transcripció SREBP-1c (Hadri KE et al, *J Biochem Chem* 2004). En canvi, un altre ITINAN com la nevirapina (NVP), que s'ha associat a hepatotoxicitat (Mallolas J, *AIDS Rev* 2006), podria tenir l'efecte apoptòtic contrari (Rodríguez de la Concepción ML et al, *Antivir Ther* 2005). En qualsevol cas, els efectes mitocondrials i fins i tot apoptòtics dels ITINAN estan poc estudiats i es suposen de baixa intensitat.

1.3.1.3. Inhibidors de la proteasa

Els inhibidors de la proteasa (IP) basen la seva activitat antiretroviral en inhibir la proteasa del VIH responsable de generar les proteïnes estructurals i els enzims virals (transcriptasa inversa, proteasa i integrasa) derivats de la traducció dels gens virals Gag i Pol, respectivament, essencials per a l'ensamblatge de nous virions infectius (Figura 9).

Quan s'activa el cicle lític en la cèl·lula infectada, l'ADN proviral inserit en el genoma de la cèl·lula hoste es transcriu a un ARNm que tindrà com un dels productes proteics inicials la poliproteïna Gag-Pol que haurà de ser processada proteolíticament (tallada) per la aspartil-proteasa del virus en les unitats proteiques estructurals i funcionals bàsiques que ensamblen els nous virions en el denominat procés de maduració. Els IP bloquegen l'activitat d'aquest enzim (Figura 14).

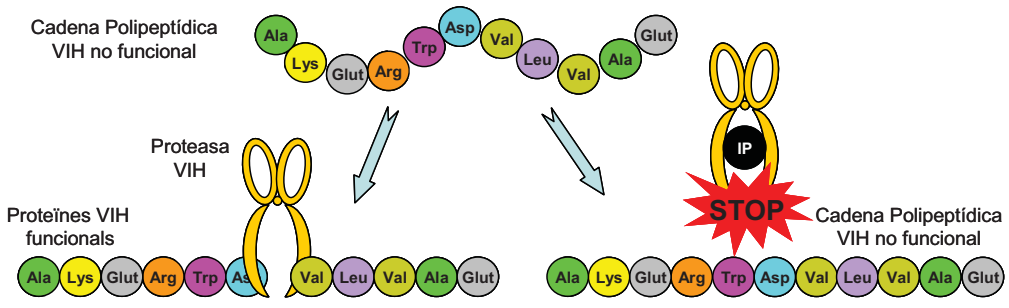


Figura 14: Bloqueig de la proteòlisi de les proteïnes del VIH per part dels inhibidors de la proteasa del virus (IP).

Es tracta de bloquejar la replicació del VIH en una etapa avançada del cicle viral, quan aquest ja ha infectat la cèl·lula i ha començat el seu cicle lític. Aquest cicle lític, en el cas de las cèl·lules immunitàries, sol coincidir amb la seva activació. Per tant són fàrmacs actius contra cèl·lules que ja es troben infectades i són portadores de l'ADN proviral en el seu genoma, de manera crònica, i poden actuar com a reservoris.

Però a més a més de l'activitat terapèutica antiretroviral, als IP se'ls ha assignat efectes secundaris de tipus metabòlic, mitocondrial i apoptòtic, sent aquests 2 últims actual motiu de controvèrsia.

Els efectes adversos metabòlics dels IP poden explicar-se per la homologia estructural que existeix entre la proteasa del virus i certes proteïnes cel·lulars, a les que podria inhibir de la mateixa manera que inhibeix a l'enzim viral. Concretament s'ha descrit homologia de la proteasa del virus amb CRABP-1 (proteïna tipus 1 d'unió a l'àcid retinoic cel·lular o *cellular retinoic acid binding protein 1*), que intervé en la diferenciació adipocitària, així com amb la proteïna LRP (associada al receptor de la lipoproteïna de baixa densitat o *low-density lipoprotein receptor-related protein*), receptor hepàtic i de l'endoteli capil·lar que degrada els triglicèrids circulants a àcids grassos aptes per l'acumulació adipocitària. L´alteració de la funció d'aquestes proteïnes té un efecte directe sobre el metabolisme lipídic per inhibició de la formació de cèl·lules adipocitàries i per afavorir l'acumulació de lípids en sang, respectivament.

Els IP també alteren el metabolisme de la glucosa. Alguns d'aquests fàrmacs (com nelfinavir i indinavir) s'ha demostrat que inhibeixen la translocació o activació dels transportadors de glucosa que importen aquest metabòlit a l'interior de la cèl·lula, com GLUT1 o GLUT4, ja sigui per interacció directa (Nolte LA et al, *Diabetis* 2001; Rudich A et al, *Diabetologia* 2003), o a través d'intermediaris com la proteïna cinasa B (PKB), que intervé en la supervivència cel·lular i fa de mitjancera dels efectes de la insulina envers el metabolisme cel·lular, o per interacció amb el factor de transcripció SREBP-1 (proteïna tipus 1 d'unió a l'element regulador de l'esterol o *sterol regulatory element-binding protein 1*), que intervé en la diferenciació d'adipòcits i incrementa la captació de glucosa (Ben-Romano R et al, *AIDS* 2003).

El resultat d'aquestes interaccions comporta l'augment de la concentració plasmàtica de glucosa i triglicèrids i la disminució de la seva disponibilitat cel·lular. Aquesta deprivació pot promoure un dèficit energètic que pot ocasionar la mort cel·lular per apoptosi. A més a més afavoreix el desenvolupament de la resistència a la insulina, que juntament amb l'alteració del metabolisme glucídic i lipídic, constitueixen la denominada síndrome metabòlica.

En canvi no existeix consens sobre els efectes mitocondrials dels IP, tot i que en cas de ser deleteris, se suposen de menor potència que els induïts pels ITIAN. Alguns autors apunten a que els IP podrien prevenir la pèrdua del potencial de membrana d'aquest orgànul, que a més a més de ser necessari pel seu correcte funcionament, precedeix l'alliberació de factors apoptogènics cap al citoplasma (Phenix BN et al, *Blood* 2001). Aquesta hipòtesi atribuiria efectes anti-apoptòtics i de protecció mitocondrial a aquests fàrmacs, malgrat altres autors afirmen que a dosis superiors, el seu efecte seria el contrari i actuarien promovent la despolarització de l'orgànul i la conseqüent entrada de la cèl·lula en apoptosi (Estaquier J et al, *J Virol* 2002). A favor de l'efecte mitotòxic dels IP s'ha descrit un altre mecanisme de lesió mitocondrial indirecta, especialment per ritonavir, que actuaria inhibint l'activitat de la glicoproteïna-p (Drewe J et al, *Biochem Pharmacol* 1999), encarregada d'expulsar els ITIAN fora de la cèl·lula, i el citocrom p-450 (Hsu A et al, *Agents Chemother* 1998), que detoxifica alguns fàrmacs antiretrovirals. La combinació d'IP amb ITIAN, característica de la majoria de règims TARGA, potenciaria el dany mitocondrial que exerceixen aquests últims.

D'igual manera, existeix una gran controvèrsia entorn dels efectes cel·lulars que exerceixen els IP: alguns autors asseguren que són anti-apoptòtics (Badley AD et al, *Blood* 2000; Andre P et al, *Proc Natl Acad Sci USA* 1998; Sloand EM et al, *Blood* 1999 i 2000; Phenix BN et al, *AIDS Res Hum Retrov* 2000) i d'altres pro-apoptòtics (Dowell P et al, *J Biol Chem* 2000; Bastard JP et al, *Lancet* 2002; Matarrese P et al, *J Immunol* 2003), tot i que la majoria afirmen que el seu efecte dual dependria de la dosi (Estaquier J et al, *J Virol* 2002; Sloand EM et al, *Blood* 1999 i 2000).

Les teories que reforcen els efectes anti-apoptòtics d'aquests antiretrovirals es basen en la capacitat que presenten aquests fàrmacs per modular l'activitat dels proteasomes i la presentació d'antígens per part del complex major d'histocompatibilitat de classe I (MHC-I), que disminuirien així l'activació immunològica i l'apoptosi derivada d'aquesta activació (Andre P et al, *Proc Natl Acad Sci USA* 1998). També hi ha autors que descriuen la disminució en l'expressió de proteïnes pro-apoptòtiques com la caspasa-1 (Sloand EM et al, *Blood* 1999 i 2000; Phenix BN et al, *AIDS Res Hum Retrov* 2000), malgrat existeix molta polèmica al respecte (Lu W et al, *Blood* 2000; Chavan S et al, *Blood* 2001).

A favor del caràcter pro-apoptòtic dels IP està el paper que se'ls assigna en la pèrdua d'adipòcits en les regions lipoatròfiques de la síndrome de lipodistròfia per alteració de la diferenciació d'aquestes cèl·lules mitjançant la interacció amb la proteïna CRABP-1 (Dowell P et al, *J Biol Chem* 2000) o el factor de transcripció SREBP-1 (Bastard JP et al, *Lancet* 2002), anteriorment citats. També s'han associat *in vitro* a la reducció de la proliferació de

cèl·lules T (Estaquier J et al, *J Virol* 2002; Matarrese P et al, *J Immunol* 2003), a la mort per apoptosi de cèl·lules endotelials exposades a concentracions plasmàtiques d'aquests fàrmacs (Zhong DS et al, *Arterioscler Thromb Vasc Biol* 2002) o a l'activitat anticancerígena per inducció de mort cel·lular descrita per a alguns IP (Pajonk F et al, *Cancer Res* 2002; Gaedicke S et al, *Cancer Res* 2002; Gills JJ et al, *Clin Cancer Res* 2007).

En resum, els IP s'associen a la alteració del metabolisme dels carbohidrats i dels lípids que caracteritza la síndrome metabòlica derivada de l'ús del TARGA, però els seus efectes sobre l'estat mitocondrial i apoptòtic no estan del tot establerts. La majoria d'estudis que reporten propietats protectores per al mitocondri i anti-apoptòtiques per part dels IP estan realitzats *in vitro* (Phenix BN et al, *Blood* 2001; Sloand EM et al, *Blood* 1999 i 2000; Phenix BN et al, *AIDS Res Hum Retrov* 2000) amb concentracions d'aquests fàrmacs inferiors a les plasmàtiques, especialment quan es troben potenciats per ritonavir. Per contra, els estudis realitzats amb concentracions d'IP similars a les fisiològiques destaquen els efectes pro-apoptòtics i mitotòxics (per pèrdua del potencial de membrana, dèficit energètic o potenciació dels efectes adversos dels ITIAN) (Petit F et al, *TRENDS Pharmacol Sci* 2005).

En qualsevol cas, estudis *in vivo* realitzats pel nostre grup amb concentracions plasmàtiques de l'IP nelfinavir demostren que el grau d'apoptosi que pogués induir un TARGA que inclogués un IP, si és que n'indueix, és molt menor al grau d'apoptosi que indueix el propi virus, doncs el tractament amb aquest TARGA s'associa a un grau d'apoptosi inferior al d'aquells individus infectats que no reben tractament (Miró Ò et al, *Antivir Ther* 2005).

1.3.1.4. Inhibidors de la fusió

La família antiretroviral dels inhibidors de la fusió (IF) del virus amb la cèl·lula hoste és de recent creació i només presenta un representant comercialitzat al 2003 anomenat enfuvirtide (T-20). Aquest fàrmac s'uneix a les proteïnes víriques que formen part de l'envolta lipídica del virus, impedeix la seva interacció amb els receptors i correceptors de la cèl·lula hoste i bloqueja així l'entrada del virus i la infecció de la cèl·lula hoste (Figures 9 i 15).

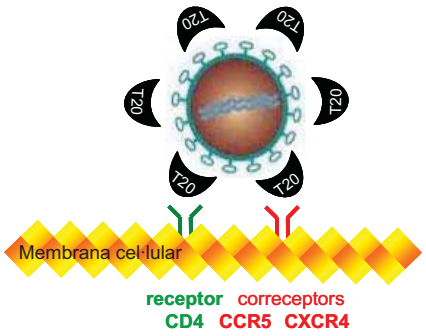


Figura 15: Bloqueig de la fusió del VIH amb la cèl·lula hoste per part dels inhibidors de la fusió (IF). T20: enfuvirtide; CD4, CCR5 i CXCR4: receptor (CD4) i correceptors (CCR5 i CXCR4) de la cèl·lula hoste que reconeix el VIH.

Es tracta doncs de bloquejar el cicle viral en la seva etapa més inicial; abans de que pugui infectar la cèl·lula.

Com que la interacció del fàrmac amb la cèl·lula hoste és exclusivament extracel·lular, s'espera nul·la toxicitat mitocondrial per aquest tipus de fàrmacs. Malgrat existeixen pocs estudis experimentals al respecte, les evidencies clíniques no associen efectes secundaris d'origen mitocondrial al tractament amb aquest fàrmac (Cooper DA et al, *Lancet Infect Dis* 2004; Bottaro EG, *Medicina (B Aires)* 2007).

Actualment enfuvirtide sol formar part de pautes de rescat perquè la seva via d'administració és una injecció subcutània i està sotmesa a rigoroses pautes d'administració (Ribera E et al, *Enferm Infecc Microbiol Clin* 2007), però la indústria farmacèutica ja treballa en la creació d'IF de nova generació que s'administrin via oral.

1.3.1.5. Inhibidors de l'entrada

Una altra família d'antiretrovirals recentment creada és la dels inhibidors del correceptor CCR5 o inhibidors de l'entrada del virus a la cèl·lula hoste (IE), entre els quals, actualment, només hi ha un representant comercialitzat; es tracta d'un fàrmac anomenat maraviroc (Taula III).

Aquests antiretrovirals impedeixen que el virus entri a la cèl·lula (Figura 9), però a diferència del inhibidors de la fusió, per impedir-ho no s'uneixen al virus, sinó a la cèl·lula hoste. El VIH necessita interaccionar amb el receptor CD4 de la cèl·lula hoste, i aquesta unió promou un canvi conformacional que possibilita la posterior interacció amb el correceptor cel·lular CCR5 o CXCR4, segons el tropisme del virus, i només així el virus podrà entrar i infectar a una nova cèl·lula. Com hem dit anteriorment, es poden diferenciar dos tropismes virals segons el correceptor que empri el VIH per infectar la cèl·lula, que al mateix temps determinarà el tipus de cèl·lula que infecta. Els inhibidors del correceptor CCR5 (com maraviroc) només impediran la infecció dels virus que emprin aquest correceptor (virus R5), que s'estima que representen entre un 50-70% del total de virus, i no tindrien cap efecte sobre els virus amb tropisme per correceptor CXCR4, estirps virals emergents que s'han associat a la formació de sincitis cel·lulars, progressió ràpida de la malaltia i depleció de limfòcits T $CD4^+$.

Degut a la recent incorporació d'aquest fàrmac al mercat, no existeix cap estudi que testi la seva toxicitat mitocondrial, però com que exerceix la seva activitat antiretroviral a nivell extracel·lular (com els inhibidors de la fusió), es preveu que presenti una baixa capacitat de

lesió mitocondrial (fins i tot nul·la). Els únics dubtes generats entorn dels possibles efectes secundaris associats als antagonistes del correceptor CCR5 es deuen a que a diferència dels inhibidors de la fusió i de la resta de famílies antiretrovirals, són els únics fàrmacs que per combatre la infecció interaccionen amb la cèl·lula hoste en lloc de l'agent patògen, i caldrà comprovar que aquesta interacció no sigui perjudicial a llarg termini.

1.3.1.6. Inhibidors de la integrasa

Els inhibidors de la integrasa (II) del virus són una altra nova classe d'antiretrovirals recentment llençada al mercat (Taula III), que presenten un mecanisme d'acció completament diferent al de les famílies fins ara existents. L'únic fàrmac fins ara comercialitzat es diu raltegravir (RAL), però n'hi ha d'altres en actual fase de desenvolupament.

Aquests fàrmacs inhibeixen un enzim viral anomenat integrasa, que possibilita la inserció de l'ADN proviral retrotranscrit a partir del material genètic del virus al material genètic de la cèl·lula hoste (Figura 9). Es tracta d'una nova diana terapèutica per frenar l'avanç de la infecció per VIH i per tant una teràpia que serà molt útil en aquells pacients que ja han pres altres tractaments antiretrovirals i fins i tot presenten resistències envers alguns d'aquests fàrmacs.

En tractar-se d'una família antiretroviral tan nova en el mercat no es coneixen els possibles efectes adversos cel·lulars o moleculars (mitocondrials per exemple) per aquests fàrmacs.

1.3.2. Antibiòtics

El tractament antibiòtic constitueix una de les teràpies que ha salvat més vides en la història de la medicina moderna. Són fàrmacs que tenen naturalesa química i mecanisme d'acció molt divers, però una mateixa finalitat: aturar la infecció bacteriana. El mecanisme d'acció a través del qual l'antibiòtic exerceix la seva acció antibacteriana és un dels criteris de classificació. Entre els diferents antibiòtics, els que aturen la traducció bacteriana són els que presenten una toxicitat mitocondrial més evident. Aquest efecte advers es deu a la similitud que existeix entre els ribosomes procariotes i els mitocondrials, que segons la teoria endosimbiòtica comparteixen un origen evolutiu comú.

El linezolid pertany a una família d'antibiòtics, les oxazolidinones, que inhibeixen la síntesis proteica bacteriana per unió al ARNr 23S que forma part de la subunitat gran del ribosoma

procariota, i impedeix així que les subunitats 30S i 50S s'uneixin i formin el complex d'inici de la traducció. És un antibiòtic molt útil, especialment per tractar les infeccions causades per bacteris cocs gram-positius o els estafilococs, enterococs o estreptococs coagulasa-negatius, que com que a més a més assoleix elevades concentracions als diferents teixits, resulta molt eficaç per tractar infeccions derivades de la implantació de cossos estranys a l'organisme. Però la seva administració prolongada en determinades circumstàncies clíniques, per un període superior als 28 dies aconsellats per la *USA FDA* (Lee E et al, *Clin Infect Dis* 2003; Bressler AM et al, *Lancet Infect Dis* 2004), està associada a l'aparició d'efectes adversos com alteracions hematològiques (trombocitopènia i anèmia), neuropatia perifèrica, hiperlactatèmia i acidosi làctica, manifestacions característiques de malalties mitocondrials primàries.

In vitro alguns autors han demostrat que oxazolidinones com l'eperezolid inhibeixen la síntesi de proteïnes mitocondrials en línies cel·lulars (Nagiec EE et al, *Antimicrob Agents Chemother* 2005). En models animals, el linezolid s'ha demostrat que disminueix la quantitat de proteïnes mitocondrials (De Vriese AS et al, *Clin Infect Dis* 2006) i inhibeix la síntesi proteica mitocondrial (McKee EE et al, *Antimicrob Agents Chemother* 2006), però existeixen molt poques dades referents a estudis *in vivo* o *ex vivo* en l'ésser humà. La bibliografia només documenta la disminució d'activitats oxidatives i/o enzimàtiques mitocondrials en múscul esquelètic, fetge i ronyó (De Vriese AS et al, *Clin Infect Dis* 2006) o en cèl·lules mononuclears de sang perifèrica (CMSP) (Soriano A et al, *N Engl J Med* 2005) de pacients en tractament amb linezolid, però sense determinar en el mecanisme causal. Cap d'aquests estudis transversals analitza la possible reversibilitat dels efectes.

1.3.3. Antipsicòtics

Els neurolèptics, antipsicòtics o tranquil·litzants majors són uns fàrmacs de gran heterogeneïtat química però amb mecanisme d'acció comú; el bloqueig dels receptors dopaminèrgics cerebrals D_2, malgrat molts antipsicòtics presenten activitat sobre receptors d'altres neurotransmissors, com els de la serotonina.

S'empren majoritàriament en la clínica psiquiàtrica per tractar l'esquizofrènia, que es caracteritza per 2 tipus de signes: els positius (deliri, al·lucinacions, agitació i descontrol emocional, associats a hiperactivitat del sistema dopaminèrgic) i els negatius (falta de reaccions emocionals, desconnexió del medi, incapacitat d'experimentar plaer i falta d'energia, sense al·lucinacions o deliris greus).

La utilitat del primer d'aquests fàrmacs, la clorpromazina, va ser descoberta accidentalment a mitjans del S.XX. Al 1958 Janssen va descobrir les propietats antipsicòtiques de l'haloperidol, malgrat un temps després també en descrivia les manisfestacions extrapiramidals associades (Schillevoort I et al, *Ann Pharmacother* 2001), i des de llavors s'han seguit explorant els usos antipsicòtics de substàncies similars.
Els usos terapèutics dels antipsicòtics no es limiten al tractament de l'esquizofrènia, malgrat és el més habitual, doncs els psiquiatres també els prescriuen en els quadres clínics de paranoia, psicosis orgàniques i funcionals. S'han emprat pel tractament del deliri i la demència, la mania i la depressió, a dosis baixes com a ansiolítics, en casos de singlot intractable per contrarestar la nàusea i el vòmit, o en síndromes més rares com el de Gilles de la Tourette o la corea de Huntington. A més a més l'haloperidol i la clorptomazina s'administren també per aturar els estats alterats de consciència produïts per les anomenades drogues al·lucinògenes.

Els antipsicòtics bloquegen els receptors dopaminèrgics que es distribueixen al llarg del sistema nerviós central i perifèric, i per tant presenten efectes a molts diversos nivells, des de reduir el reflex del vòmit (acció antiemètica) fins a augmentar la secreció de prolactina, amb tots els efectes endocrins derivats.

Tots els antipsicòtics tenen efectes col·laterals a nivell central i perifèric.

A nivell de sistema nerviós central els antipsicòtics poden causar efectes adversos com crisis convulsives, acatísia (impossibilitat d'estar tranquil), distonies (contraccions musculars involuntàries que poden manifestar-se com gesticulació, discinèsies, torticoli o moviments oculars exagerats, des del principi del tractament, que es controlen eficaçment amb els anticolinèrgics que s'administren per tractar el Parkinson), síndrome parkinsoniana (per alentiment dels moviments -bradicinèsia-, rigidesa muscular -hipertonia- i tremolor, signes similars als d'un parkinsonisme essencial o idiopàtic), discinèsia tardana (es produeix després de mesos de tractament, com moviments involuntaris, estereotipats i repetitius de la boca, llavis i llengua, de les extremitats i l'adopció de posicions estranyes amb contractures musculars prolongades i que empitjoren amb fàrmacs antiparkinsonians) o la síndrome neurolèptica maligna (crisis greus de parkinsonisme, catatonia, tremolor, alteracions de la freqüència cardíaca i la pressió arterial, augment de la temperatura corporal, etc, amb desenllaç fatal en el 10% dels casos).

En el sistema nerviós perifèric els antipsicòtics poden causar constipació, sequedat de boca, congestió nasal, visió borrosa, dilatació pupil·lar, fotofòbia, taquicàrdia, arítmia cardíaca, retenció urinària, al·lèrgies cutànies i vertigen, augment del pes corporal, alteracions sanguínies (anèmia i destrucció de cèl·lules), obstrucció hepàtica amb icterícia, augment de la temperatura corporal, contraccions musculars involuntàries i parkinsonisme (tremolor d'extremitats, tronc, cap i llengua que ocasiona alteracions en la coordinació motriu fina).

Els antipsicòtics actualment es divideixen en 2 tipus segons el nivell funcional i terapèutic en el que actuïn: els clàssics (com l'haloperidol) o els atípics (com la risperidona o la clozapina). Aquests últims (els atípics) són els que bloquegen simultàniament els receptors dopaminèrgics D_2 i els receptors serotoninèrgics $5HT_2$ i es caracteritzen perquè s'associen a efectes extrapiramidals mínims o nuls (Kane JM et al, *J Clin Psychiatry* 2003), tenen acció sobre els símptomes negatius (a més a més dels positius) de l'esquizofrènia i posseeixen major eficàcia en els quadres refractaris.

El conjunt d'efectes extrapiramidals que ocasionen alteracions en el moviment d'aquests pacients és un dels efectes secundaris més greus associat a aquests fàrmacs i es coneix amb el nom de manisfestacions extrapiramidals en el moviment. El temps en tractament i l'edat del pacient són 2 dels factors independents de risc que s'associen al desenvolupament d'aquest trastorn (Goff DC et al, *Am J Psychiatry* 1995; Wolfarth S et al, *Prog Neuropsychopharmacol Biol Psychiatry* 1989), però també s'ha suggerit que juguen un paper important els polimorfismes en el citocrom p450 que detoxifica aquests fàrmacs (Shillevoort I et al, *Pharmacogenetics* 2002) o el nombre i sensibilitat dels receptors de dopamina (Pickar D, *Lancet* 1995). Aquests paràmetres però, no poden explicar totalment la susceptibilitat individual per patir aquesta alteració. Per això, s'han proposat mecanismes alternatius de toxicitat mitocondrial per explicar l'aparició de les manisfestacions extrapiramidals, i una de les més acceptades és la inhibició del complex I de la CRM per part d'aquests fàrmacs (Jackson-Lewis V et al, *Soc Neurosci Abstr* 1992; Burkhardt C et al, *Ann Neurol* 1993; Maurer I et al, *Mol Cell Biochem*1997). Com que aquest efecte secundari al tractament a vegades és irreversible malgrat la retirada del fàrmac, alguns autors proposen una alteració permanent en l'ADNmt com a base fisiopatològica de l'alteració (Burkhardt C et al, *Ann Neurol* 1994), que altres estudis no confirmen (Barrientos A et al, *J Neurosci Res* 1998).

1.3.4. Monòxid de carboni

La intoxicació per monòxid de carboni (CO) és la causa més freqüent d'intoxicació per gasos asfixiants i constitueix la causa més comú d'inhalació mortal d'origen ocupacional (Raub JA et al, *Toxicology* 2000; Kales SN et al, *N Engl J Med* 2004). La seva incidència és més gran durant l'hivern, perquè la majoria d'exposicions deriven de fugues del gas per avaries en els sistemes de calefacció o per una combustió anòmala de sistemes o vehicles que afavoreixen l'acumulació del gas en compartiments o vivendes que no disposen d'una adequada ventilació (García-Arroyo I et al, *Rev Clin Esp* 2003). El número de persones intoxicades per CO al llarg de l'any al nostre país està xifrat entorn els 2.500 casos, dels quals un centenar tindrien un desenllaç fatal, el que suposa, conjuntament amb la sobredosi per drogues d'abús, la principal causa de mort d'origen tòxic (Nogué S et al, *Med Clin* 2005).

Els símptomes de la intoxicació per CO s'han atribuït generalment a la hipòxia tissular deguda a l'elevada afinitat del CO per l'hemoglobina, que desplaça l'oxigen que transporta, i que impedeix que aquest arribi als teixits (Miró Ò et al, *Med Clin* 2000; Dueñas-Laita A et al, *Med Clin* 2006). Efectes d'hipòxia similar s'han descrit per altres gasos asfixiants com el sulfur d'hidrogen (Campanyà M et al, *Med Lav* 1989; Sanz-Gallen P et al, *An Med Interna* 1994; Nogué S et al, *Revista de Toxicología* 2007) o l'àcid cianhídric (Dueñas-Laita A et al, *Med Clin* 2006), així com per productes químics, que un cop metabolitzats, generen algun d'aquests gasos (Mondóna S et al, *Med Clin* 2005; Trullàs JC et al, *Rev Toxicol* 2005). En el cas del CO, aquests símptomes inclouen cefalea, palpitacions, nàusees, vòmits, pèrdues de coneixement, confusió, arítmia, isquèmia, síncope i coma (Choi IS, *J Korean Med Sci* 2001; Hampson NB et al, *Headache* 2002). Però existeix també l'evidència d'una toxicitat directa causada pel CO en els teixits, doncs alguns símptomes apareixen fins i tot en absència de carboxihemoglobina (COHb) en sang (Gorman D et al, *Toxicology* 2003). Especialment, aquesta toxicitat podria donar-se a nivell de sistema nerviós central, doncs alguns dels símptomes més característics, com la migranya i seqüeles de major gravetat, poden persistir o desenvolupar-se setmanes després de la desaparició de la COHb de la sang. Com que oxigen i CO competeixen per la unió als anells porfirínics que contenen ferro, s'ha suggerit que l'efecte histotòxic del CO estaria mediat per la unió del CO a aquelles proteïnes amb grups hemo no-hemoglobínics, com la mioglobina, el citocrom P450 i la citocrom c oxidasa (COX) mitocondrial de la CRM, interacció que bloquejaria la funció d'aquestes proteïnes. Estudis en animals han establert la inhibició de la funció de la COX mitocondrial per unió del CO al citocrom aa3 d'aquest enzim (Piantadosi C, *J Hyper Med* 1987), i el nostre grup ha demostrat

que aquesta inhibició està present el les CMSP de pacients afectats per una intoxicació aguda por CO (Miró Ò et al, *Pharmacol Toxicol* 1998, *Toxicol Lett* 1999 i *Med Clin* 2004).

El fum del tabac conté CO, entre molts d'altres components tòxics, i de manera anàloga al que passa als intoxicats, s'ha proposat al CO com a principal responsable de les alteracions mitocondrials presents en les cèl·lules mononuclears d'individus fumadors (Miró Ò et al, *Carcinogenesis* 1999; Cardellach F et al, *Rev Neurol* 1998 i *J Toxicol Clin Toxicol* 2003), en CMSP de voluntaris no-fumadors exposats a la inhalació de fum de tabac (Alonso JR et al, *Eur Respir J* 2004) i en estudis *in vitro* que analitzen directament l'efecte del CO sobre mitocondris aïllats (Alonso JR et al, *Pharmacol Toxicol* 2003). Aquests treballs descriuen una inhibició de l'activitat enzimàtica de la COX en fumadors crònics (Miró Ò et al, *Carcinogenesis* 1999; Cardellach F et al, *Rev Neurol* 1998 i *J Toxicol Clin Toxicol* 2003) i aguts (Alonso JR et al, *Eur Respir J* 2004), mentre que el dany oxidatiu derivat de la disfunció de la CRM només estaria present en els fumadors crònics.

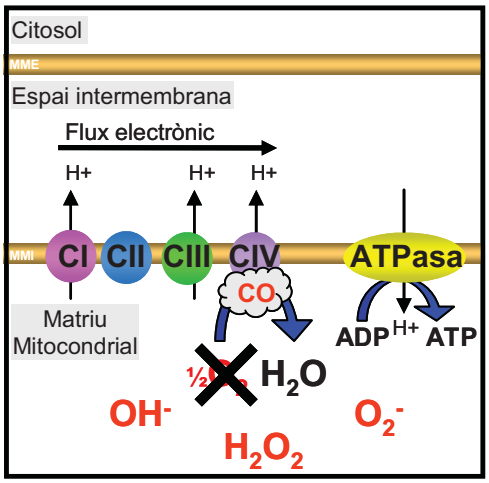


Figura 16: Mecanisme lesiu del monòxid de carboni (CO) sobre la funció mitocondrial. MME: membrana mitocondrial externa; MMI: Membrana mitocondrial interna; CI, II, III i IV: complex I, II,III i IV de la cadena respiratòria mitocondrial; ADP: adenosina difosfat; ATP: adenosina trifosfat; OH^-: anió hidroxil; H_2O_2: peròxid d'hidrogen; O_2^-: anió súperòxid.

Aquests resultats confirmen el dany que pot exercir el CO sobre el mitocondri, com una via alternativa de lesió independent de la hipòxia tissular derivada de la presència de COHb en sang i la falta de transport d'oxigen als teixits.

La gravetat dels símptomes i l'aparició de seqüeles tardanes derivades de la intoxicació per CO depenen de la intensitat i duració de l'exposició, i el valor de la COHb en sang sovint no serveix per establir pronòstics acurats (Gorman D et al, *Toxicology* 2003). Quan la concentració de CO a l'aire és del 0,01% i la duració de l'exposició prolongada, la saturació

de COHb en sang és d'entre 0-10%, concentracions de CO entre 0,01-0,02% correspon a valors de COHb del 10-20%, concentracions de CO entre 0,02-0,03% més curtes (5-6 hores) es manifesten en valors de COHb entre 20-30% i concentracions d'entre 0,04-0,06% de CO durant 4-5 hores poden correspondre's amb valors de COHb del 30-40%. En qualsevol cas, independentment de la duració i temps d'exposició, i per tant de forma no exempta de discussió, nivells de COHb en sang superiors al 20% es consideren que corresponen a una intoxicació greu, i COHb inferiors es consideren d'intoxicació lleu, sempre i quan no s'acompanyi d'una clínica neurològica (pèrdua de consciència) o cardiològica (arítmies, signes d'isquèmia), que automàticament cataloga la intoxicació com a greu.

En general, el tractament fonamental per la intoxicació aguda per CO es basa en l'administració d'oxigen a alta concentració, a pressió ambiental (oxigen normobàric) o bé a dues o tres atmosferes de pressió (oxigen hiperbàric). La forma d'administració més adequada fisiopatològicament seria mitjançant cambra hiperbàrica (oxigenoteràpia hiperbàrica, OHB), perquè aquesta modalitat d'oxigenoteràpia redueix la vida mitja de dissociació de la COHb a 23 minuts, quan en condicions ambientals seria de més de 4 hores o amb oxigenoteràpia normobàrica seria de 45 minuts (Scheinkestel CD et al, *Med J Aust* 1999). Això facilita que la hemoglobina s'uneixi de nou a l'oxigen, el transport d'oxigen als teixits es normalitzi al final de la sessió (de duració variable segons el tractament aplicat) (Hampson NB et al, *J Hiperb Med* 1992) i millori substancialment la situació d'hipòxia cel·lular (Tibbels PM et al, *N Engl J Med* 1996). Malgrat això, és més senzill i freqüent que l'oxigen s'administri en condicions de normobària.

Però la respiració cel·lular no només depèn de l'aportació sanguínia d'oxigen i també s'ha vist inhibida de manera directa per la unió del CO a la COX mitocondrial (Tibbels PM et al, *N Engl J Med* 1996; Miró Ò et al, *Pharmacol Toxicol* 1998; Gorman D et al, *Toxicology* 2003), efecte que podria perdurar més en el temps. En 3 pacients intoxicats per CO, el nostre grup va observar que la funció de la COX es mantenia bloquejada fins i tot 10 dies després de normalitzar els valors de COHb en sang (Miró Ò et al, *Pharmacol Toxicol* 1998). La persistència de tal inhibició pot ocasionar un dèficit en la fosforilació oxidativa i, al mateix temps, un augment dels radicals lliures derivats de l'oxigen per interferència en el normal funcionament de la CRM (Miró Ò et al, *Pharmacol Toxicol* 1998; Miró Ò et al, *Carcinogenesis* 1999, Gorman D et al, *Toxicology* 2003) que, al mateix temps, contribuiria al ja anormal funcionament de la cadena. Altres efectes del CO són l'activació de leucòcits polimorfonuclears i l'augment de l'òxid nítric (NO). Tots aquests efectes del CO a nivell

cel·lular i, sobretot, subcel·lular, podrien ajudar a explicar alguns dels símptomes precoços i tardans.

L'administració d'OHB, a més a més de separar el CO de la hemoglobina, afavoreix la dissociació del CO de la COX mitocondrial (Brown SD et al, *Adv Exp Med Biol* 1989*, J Appl Physiol* 1990 i *J Clin Invest* 1992), efecte que hauria de redundar en la millora de la simptomatologia. En canvi, malgrat els efectes beneficiosos de la OHB des del punt de vista fisiològic, àmpliament evidenciats, encara no està clarament establert si aquesta modalitat terapèutica presenta efectes igualment rellevants sobre la supervivència dels pacients o sobre els símptomes i signes tardans, quan es compara amb l'oxigenoteràpia normobàrica (ONB) (Myers RA et al, *Ann Emerg Med* 1985; Raphael JC et al, *Lancet* 1989; Thom SR et al, *Ann Emerg Med* 1995; Ducasse JL et al, *Undersea Hyperb Med* 1996; Mathieu DW et al, *Undersea Hyperb Med* 1996; Scheinkestel CD et al, *Med J Aust* 1999; Jones AL et al, *Br Med J* 1999; Raub JA, *Toxicology* 2000; Waever LK et al, *N Engl J Med* 2002). Existeix molta polèmica al respecte de quin tractament és millor aplicar. Mentre que Scheinkestel i col·laboradors (*Med J Aust* 1999) desaconsellen l'ús d'OHB en la intoxicació aguda per CO, doncs alguns pacients poden empitjorar la simptomatologia, els grups de Kehat (*Undersea Hyper Med* 2000) o Gorman (*Toxicology* 2003) arriben a la conclusió contrària, i Weaver i col·laboradors (*N Engl J Med* 2002) consideren que tres sessions d'OHB en un període de 24 hores només redueix el risc d'aparició de seqüeles cognitives tardanes. Una multianàlisi de la Fundació Cochrane que inclou tots els estudis comparatius entre ONB i OHB conclou que els pacients intoxicats per CO que reben tractament amb OHB presenten una *odds ratio* (OR) de 0'78 a desenvolupar seqüeles neurològiques tardanes respecte als que reben ONB, però aquesta menor incidència no seria significativa (p=0'18), segurament degut a la heterogeneïtat metodològica i estadística dels estudis analitzats. Per tant, amb les dades disponibles, segons els autors, no és possible assegurar l'eficiència en la reducció de seqüeles neurològiques tardanes per tractament amb OHB (Juurlink DN et al, *Cochrane Database Syst Rev* 2005). A més a més, i en concret dins de la teràpia amb OHB, el nombre i duració de les sessions a aplicar també és motiu de controvèrsia (Piantadosi CA, *UMH* 2004).

En definitiva, ni la patogènia de las lesions induïdes pel CO ni el paper de l'OHB en front de la ONB en el tractament de la intoxicació aguda per CO estan encara suficientment clars.

La determinació de la concentració de COHb en sang, malgrat que és absolutament vigent i necessària en la intoxicació aguda per CO, no constitueix un paràmetre analític de suficient fiabilitat per a la predicció de la gravetat dels símptomes ni pel desenvolupament de seqüeles (Raub JA et al, *Toxicology* 2000), que sovint apareixen quan s'han normalitzat els seus

nivells. El mitocondri i la inhibició de la COX podrien estar implicats en el desenvolupament dels símptomes i/o les seqüeles. Per tant, la determinació de la COX en algun moment de l'evolució de les intoxicacions agudes per CO podria ser un indicador addicional de la gravetat de la intoxicació, de l'efectivitat real (cel·lular) del tractament i un element a tenir en compte a l'hora de decidir el règim terapèutic més adequat. Fins i tot, podria tenir valor en la predicció de lesions tardanes.

2. HIPÒTESI

La present tesi doctoral es va fonamentar en la següent **HIPÒTESI DE TREBALL**:

El mecanisme de toxicitat de determinats fàrmacs o agents lesius presenta una base mitocondrial caracteritzada per una lesió que es pot exercir a diferents nivells de l'orgànul. El coneixement dels paràmetres mitocondrials que resulten alterats pot ajudar a entendre el mecanisme de toxicitat de l'agent lesiu. La toxicitat mitocondrial d'aquestes substàncies pot manifestar-se en forma d'una sèrie de símptomes clínics, a vegades similars als de les malalties mitocondrials primàries.

La toxicitat mitocondrial podria ser detectada emprant tests d'esforç dissenyats pel diagnòstic de mitocondriopaties primàries, i en funció del mecanisme lesiu i la intensitat de la lesió induïda, la toxicitat mitocondrial i les seves manifestacions clíniques podrien ser reversibles en retirar, substituir o reduir la dosi d'aquests agents tòxics. Les manifestacions mitocondrials adverses haurien de ser proporcionals a la capacitat d'inducció de lesió mitocondrial que presentin els agents tòxics i la recuperació de la lesió mitocondrial podria servir per avaluar l'eficiència dels tractaments destinats a reduir els efectes deleteris de la toxicitat.

Les cèl·lules mononuclears podrien resultar un model d'estudi adequat per avaluar la toxicitat mitocondrial d'aquestes substàncies, a més a més dels teixits diana, perquè presenten mitocondris i estan en contacte amb l'agent tòxic, i constitueixen addicionalment una aproximació menys invasiva.

L'aprofundiment en el coneixement de la lesió i la possible recuperació mitocondrial que ha estat induïda pels agents tòxics hauria de tenir implicacions tant preventives com terapèutiques que evitessin la seva aparició i/o reduïssin els símptomes clínics derivats.

Per al desenvolupament de la hipòtesi plantejada s'han portat a terme els 6 estudis que configuren la present tesi doctoral.

3. OBJECTIUS

Per tal de comprovar la hipòtesi plantejada, els **OBJECTIUS D'ESTUDI** han estat els següents:

OBJECTIU 1

Avaluar la toxicitat mitocondrial dels **antiretrovirals** emprats per tractar la infecció contra el VIH per determinar:

a. L'aplicació d'anàlisis ideades per diagnosticar mitocondriopaties primàries en la detecció de disfuncions mitocondrials d'origen tòxic (com la hiperlactatèmia induïda per antiretrovirals) i la seva potencial reversibilitat. Comprovar si els resultats del test alternatiu són paral·lels als dels tests enzimàtics i moleculars convencionals realitzats en teixits diana (múscul) i CMSP, així com a l'evolució clínica dels pacients.

b. La potencial reversibilitat dels efectes mitocondrials en substituir tòxics de contrastada potència mitotòxica per d'altres amb un teòric efecte menys lesiu.

c. La possibilitat de reduir o revertir els efectes adversos mitocondrials disminuint la dosi dels agents mitotòxics.

OBJECTIU 2

Determinar el mecanisme de toxicitat mitocondrial de l'**antibiòtic** linezolid emprat pel tractament de la infecció bacteriana en pacients que presenten hiperlactatèmia associada a la seva administració i determinar la reversibilitat de l'alteració mitocondrial (en cas que existeixi) en retirar la teràpia.

OBJECTIU 3

Determinar el mecanisme de toxicitat mitocondrial dels **antipsicòtics** emprats pel tractament de l'esquizofrènia i la correlació de la seva potència mitotòxica amb els efectes clínics adversos associats.

OBJECTIU 4

Avaluar la base mitocondrial de la toxicitat per **monòxid de carboni**, la correlació entre la gravetat de la intoxicació i el grau de disfunció mitocondrial, i comparar l'eficiència dels diferents tractaments amb oxigen per revertir els efectes mitotòxics.

4. MATERIAL I MÈTODE

4.1. PACIENTS

La inclusió de pacients s'ha efectuat des de diversos centres hospitalaris d'arreu del territori català (Hospital Clínic de Barcelona, Hospital de Palamós, Hospital Germans Trias i Pujol de Badalona, Fundació Hospital-Asil de Granollers i Hospital Joan XXIII de Tarragona).

Tots els pacients i controls que han participat en els presents estudis ho han fet de manera voluntària i donant el seu consentiment informat.

Tots aquells pacients i controls que presentaven antecedents familiars o clínica compatible amb alguna malaltia mitocondrial primària han estat exclosos de l'anàlisi, així com aquells que presentaven alguna patologia addicional o prenien algun fàrmac potencialment tòxic pel mitocondri (estatines, aminoglucòsids, oxizolidinones...).

Per als estudis que analitzen l'efecte d'un determinat tractament sobre la funció mitocondrial, s'han exclòs aquells pacients que han introduït o canviat algun component de la seva teràpia a més a més del fàrmac considerat, així com aquells que han interromput el tractament o no l'han seguit adequadament. Concretament, en els estudis sobre antiretrovirals, s'han exclòs aquells pacients que han rebut alguna teràpia concomitant amb immunosupresors (hidroxiurea, interferó o ribavirina) o immunomoduladors (com la interleuquina-2).

Com a controls s'han utilitzat individus no fumadors per excloure l'efecte del fum del tabac (sobretot a través del monòxid de carboni) envers la funció mitocondrial.

4.2. MOSTRES

Els presents treballs avaluen la funció mitocondrial *ex vivo* en dos tipus de teixit, muscular i sanguini, que s'han processat en fresc fins a la obtenció de les mostres d'estudi: homogenat de múscul esquelètic (Casademont J et al, *Med Sci Monit* 2004) i CMSP (Prilutskii AS et al, *Lab Delo* 1990). La metodologia detallada pot consultar-se en l'apartat de material i mètode corresponent a cadascun del treballs inclosos.

De manera general, el múscul esquelètic s'ha obtingut per biòpsia muscular del deltoides i en aquest tipus de mostres s'ha quantificat el contingut en ADNmt d'un fragment del teixit i les activitats enzimàtiques de la CRM en l'homogenat de múscul. L'homogeneïtzació del múscul esquelètic s'ha realitzat al 5% (w/v) emprant uns 30 mg de teixit resuspesos en una solució isotònica amb albúmina sèrica bovina lliure d'àcids grassos al 1% (w/v) (Figura 17).

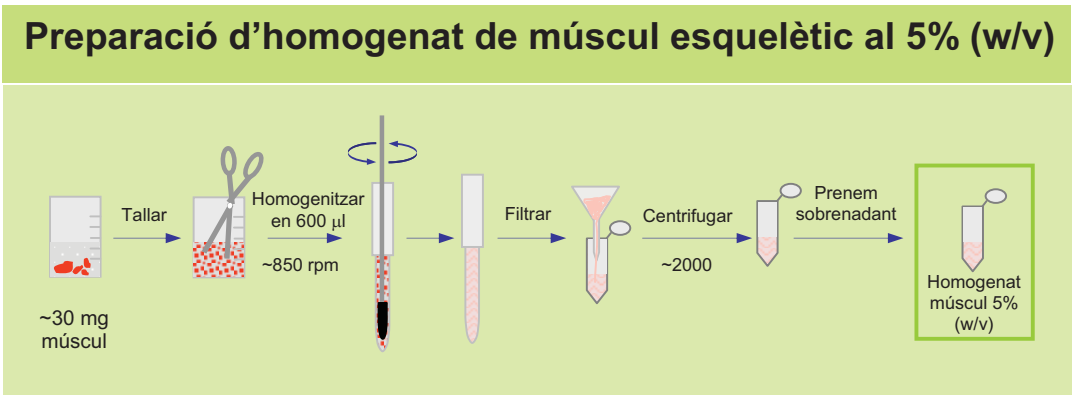


Figura 17: Preparació d'homogenat a partir de múscul esquelètic. mg: mil·ligram; rpm: revolucions per minut; µl: microlitre; w/v: *weigh/volume* o pes/volum.

Les CMSP s'han obtingut per centrifugació diferencial en un gradient de ficoll, prèvia centrifugació inicial de la sang sencera per minimitzar el contingut en plaquetes (que alteren la quantificació d'ADNmt perquè contenen mitocondris però no nucli) (Figura 18).

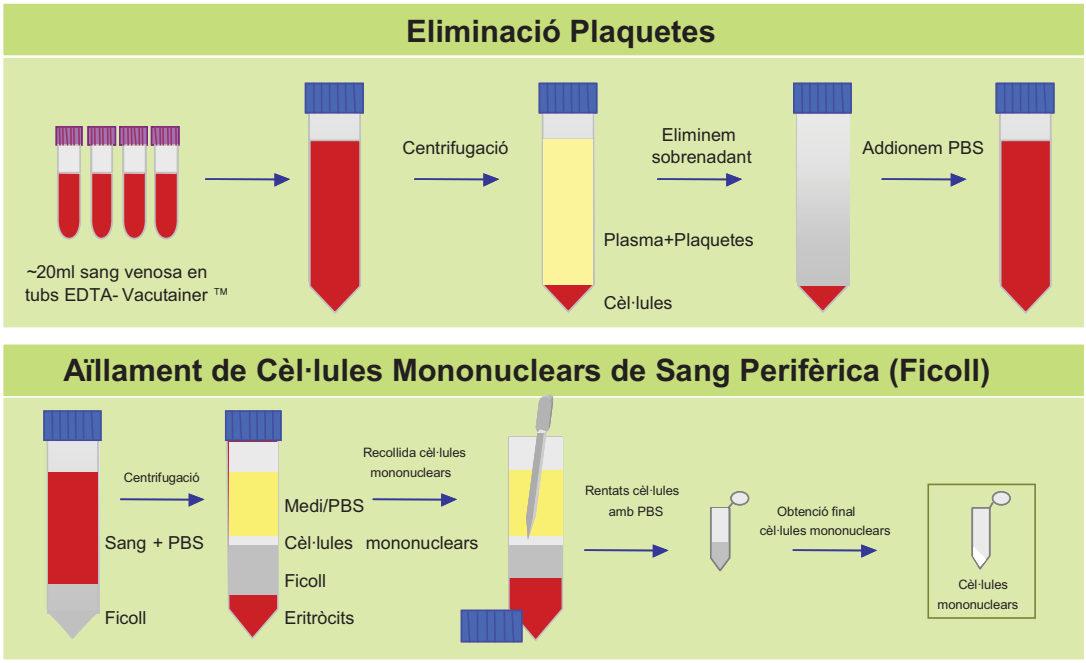


Figura 18: Aïllament de cèl·lules mononuclears a partir de sang perifèrica pel mètode del ficoll (Histopaque®-1077) prèvia minimització de la contaminació per plaquetes. ml: mil·lilitre; PBS: tampó fosfat salí.

Les cèl·lules fresques s'han emprat per quantificar les activitats oxidatives de la CRM i el potencial de membrana de l'orgànul i la mostra congelada per analitzar les activitats

enzimàtiques de la CRM, el dany oxidatiu, el contingut en ADNmt i la quantitat de subunitats proteiques de la CRM.

En totes les mostres s'ha mesurat el contingut proteic mitjançant el mètode de Bradford (Bradford M, *Anal Biochem* 1976) adaptat per l'ús de microvolums, per tal de relativitzar els paràmetres mitocondrials segons el contingut en proteïna de cada mostra.

4.3. MESURA DELS PARÀMETRES MITOCONDRIALS

4.3.1. Replicació de l'ADNmt

S'ha extret l'ADN total de la mostra mitjançant el mètode del fenol-cloroform i s'ha quantificat el contingut en ADNmt mitjançant PCR quantitativa a temps real (Miró Ò et al, *Antivir Ther* 2003) (Figura 19).

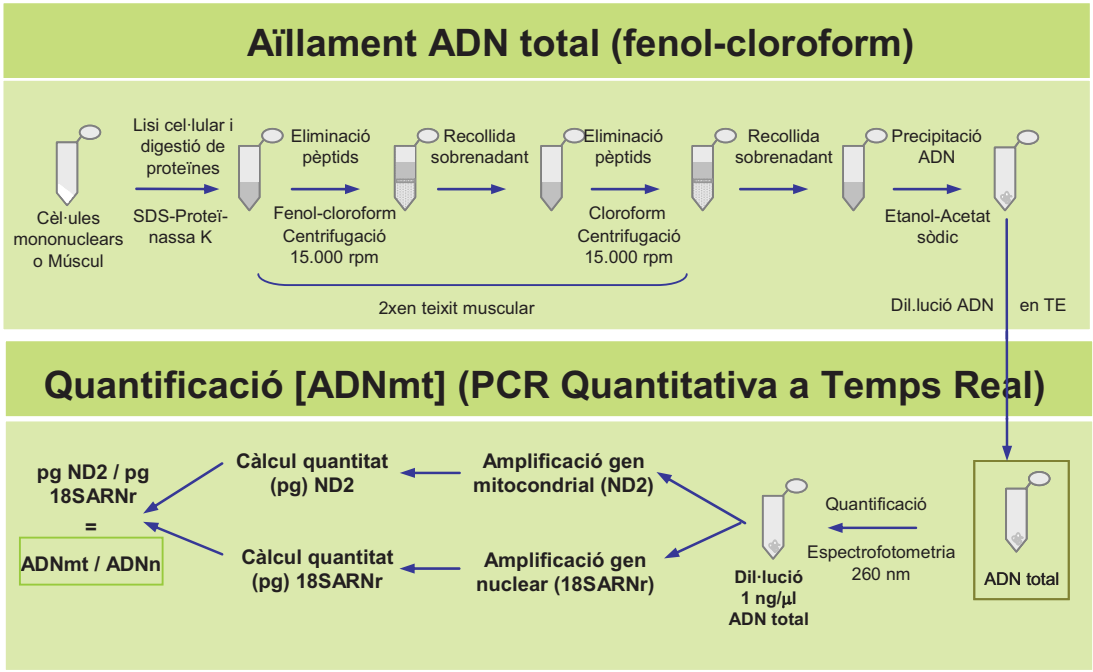


Figura 19: Aïllament (per fenol-cloroform) i quantificació (per PCR a temps real) de l'ADNmt. ADNmt o ADNn: Àcid desoxiribonucleic mitocondrial o nuclear; SDS: sodyum dodecyl sulfate; rpm: revolucions per minut; TE: Tampó tris-EDTA; nm: nanòmetre; ng: nanogram; µl: microlitre; ND2: Gen mitocondrial codificant per la subunitat II del complexe I de la cadena respiratòria mitocondrial (NADH-deshidrogenasa); 18SrARN: Gen nuclear codificant per l'ARNr (àcid ribonucleic ribosòmic)18S; PCR: reacció en cadena de la polimerasa.

Concretament, s'ha amplificat per separat i en duplicat un fragment del gen mitocondrial ND2 que codifica per la subunitat II del complex I (NADH-deshidrogenasa) de la CRM i un fragment del gen *housekeeping* nuclear que codifica pel ARNr18S. El contingut en ADNmt s'expressa com la taxa d'ADNmt respecte ADNn (contingut en ADN que codifica per ND2/ARNr18S).

4.3.2. Transcripció de l'ARNmt

S'ha extret l'ARN total per columna d'afinitat, s'ha retrotranscrit a ADNc mitjançant encebadors aleatoris i s'ha quantificat el contingut en ARNmt mitjançant PCR quantitativa a temps real (Guallar JP et al, *Mol Genet Metab* 2006). Concretament, s'ha amplificat per separat i en duplicat un fragment del retrotranscit mitocondrial COX-II (subunitat II del complex IV de la CRM) i un fragment del retrotranscrit *housekeeping* nuclear ARNr18S (Figura 20). El contingut en ARNmt s'expressa com la taxa d'ARNmt (cADNmt) respecte ARNn (cADNn) (contingut en COX-II/ARNr18S).

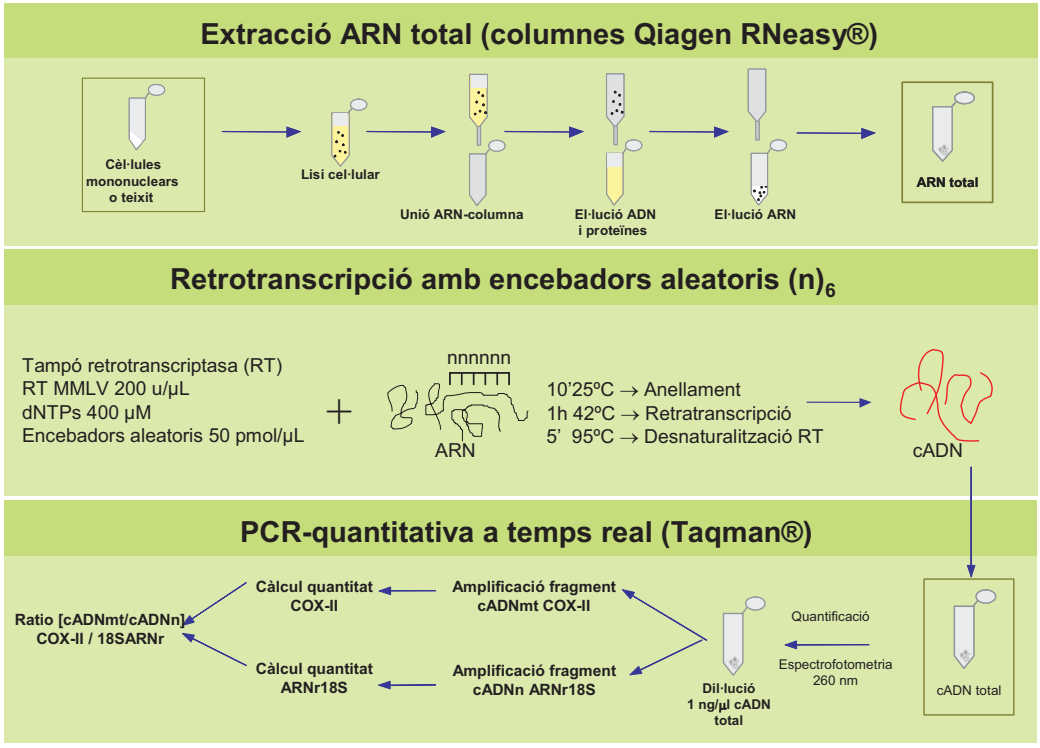


Figura 20: Aïllament (per columna), retrotranscripció (per RT-PCR) i quantificació (per PCR a temps real) de l'ARNmt. ARN: Àcid ribonucleic; cADN: ADN complementari; dNTPs: deoxinucleòtids trifosfat; nm: nanòmetre; ng: nanogram; µl: microlitre; COX-II: Transcrit o cADN mitocondrial codificant per la subunitat II del complex IV de la cadena respiratòria mitocondrial (citocrom c oxidasa); 18SrARN: Transcrit o cADN nuclear codificant per l'ARNr (àcid ribonucleic ribosòmic)18S; PCR: reacció en cadena de la polimerasa.

4.3.3. Traducció de les proteïnes mitocondrials

La taxa d'expressió de proteïnes mitocondrials s'ha mesurat per *western blot* (Miró Ò et al, *J Acquir Immune Defic Syndr* 2004). Concretament, s'ha immunoquantificat el contingut en la subunitat proteica mitocondrial de la CRM COX-II (codificada, transcrita i traduïda al mitocondri) respecte tres proteïnes codificades, transcrites i traduïdes a nivell cel·lular: la subunitat proteica mitocondrial de la CRM COX-IV, la proteïna mitocondrial de tipus estructural V-DAC (porina del canal aniònic voltatge-dependent mitocondrial) i la proteïna cel·lular de tipus estructural β-actina (Figura 21). La taxa d'expressió de proteïnes mitocondrials es pot expressar a nivell mitocondrial com el contingut relatiu entre COX-II/COX-IV o COX-II/V-DAC o a nivell cel·lular com la taxa entre COX-II/β-actina.

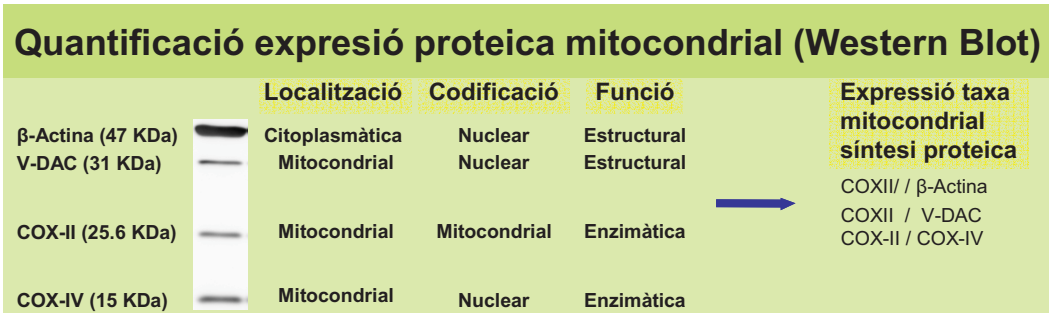


Figura 21: Quantificació de la taxa d'expressió de proteïnes mitocondrials per *western blot*. KDa: kiloDalton; COX-II: subunitat II de la proteïna citocrom c oxidasa de localització i codificació mitocondrial; COX-IV: subunitat IV de la proteïna citocrom c oxidasa amb localització mitocondrial però codificació nuclear; V-DAC: porina del canal aniònic voltatge-dependent mitocondrial de localització mitocondrial però codificació nuclear; β-actina: proteïna de localització citoplasmàtica i codificació nuclear.

4.3.4. Activitat enzimàtica dels complexos que integren la cadena respiratòria mitocondrial

Les activitats enzimàtiques dels diferents complexos que integren la CRM es mesura a 37ºC per espectrofotometria quantificant el consum dels substrats o la generació dels productes específics per a cada reacció enzimàtica que du a terme cadascun dels complexos que integren la CRM. L'activitat específica de cada complex es calcula restant l'activitat inespecífica residual que persisteix després d'inhibir el complex considerat i es relativitza segons el contingut en proteïna de la mostra (Rustin P et al, *Clin Chim Acta* 1994; Miró Ò et al, *J Neurosci Methods* 1998). Les activitats enzimàtiques específiques s'expressen en nanomols de substrat consumit o producte generat/minut/mil·ligram de proteïna (Figura 22).

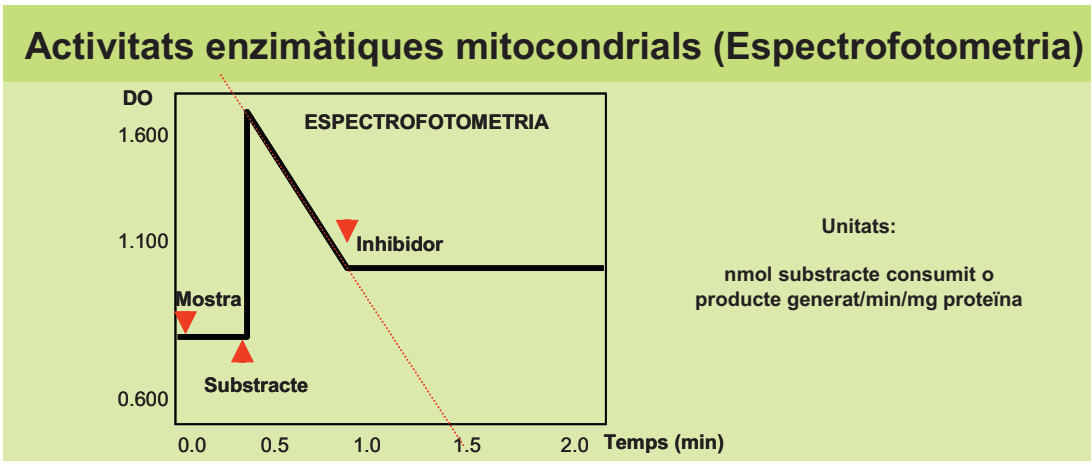


Figura 22: Quantificació de les activitats enzimàtiques específiques de la cadena respiratòria mitocondrial. DO: densitat òptica; min: minuts; nmol: nanomol; mg: mil·ligram.

L'activitat enzimàtica del complex I de la CRM només es pot mesurar en CMSP després de realitzar un tractament amb percoll i digitonina que redueix considerablement l'activitat inespecífica contaminant (Chretien D et al, *Biochem Biophys Res Commun* 2003).

4.3.5. Consum mitocondrial d'oxigen

El consum d'oxigen mitocondrial es mesura a 37ºC i en agitació constant per polarografia emprant un elèctrode de Clark i estimulant els diferents complexos de la CRM amb els substrats pertinents (Figura 23). Per determinar les activitats oxidatives específiques s'ha restat l'activitat inespecífica residual després d'inhibir cadascun dels complexos de la CRM considerats i s'ha relativitzat el consum d'oxigen segons el contingut en proteïna de la mostra (Rustin P et al, *Clin Chim Acta* 1994). Les activitats oxidatives específiques s'expressen en nanomols d'oxigen consumit/minut/mil·ligram de proteïna.

L'activitat oxidativa cel·lular en CMSP es mesura quantificant el consum d'oxigen endogen de la mostra sense estimular la CRM amb cap tipus de donador d'electrons. Per determinar les activitats oxidatives específiques estimulades a través dels diferents complexos de la CRM, s'ha d'administrar el donador d'electrons i l'inhibidor específic per a cada complex; l'activitat oxidativa a través del complex I es mesura estimulant el consum d'oxigen amb piruvat, malat i glutamat i inhibint-lo amb rotenona, la respiració a través del complex II afegint succinat com a estimulador i malonat com a inhibidor i la activitat oxidativa del complex III s'estimula addicionant glicerol-3-fosfat i s'inhibeix amb antimicina.

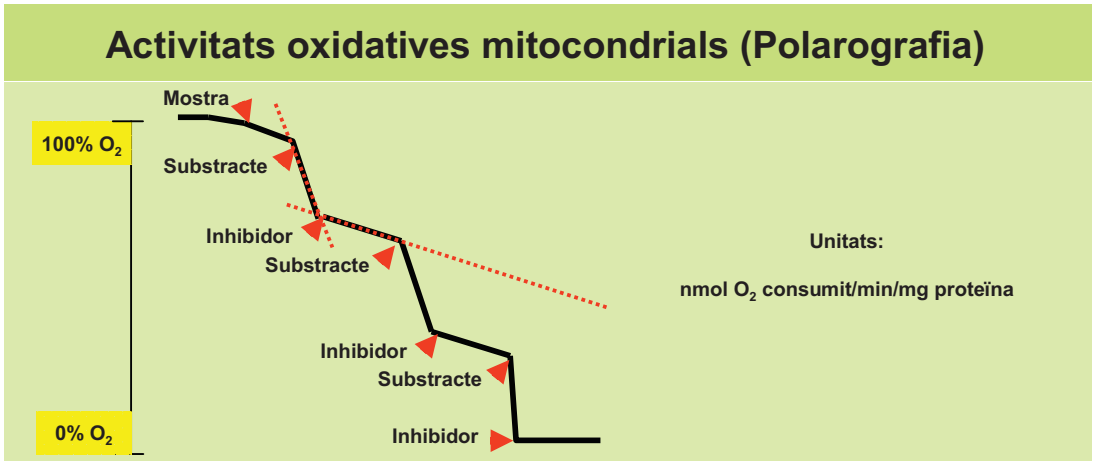


Figura 23: Quantificació de les activitats oxidatives específiques de la cadena respiratòria mitocondrial. O_2: oxigen; min: minuts; nmol: nanomol; mg: mil·ligram.

4.3.6. Producció d'espècies reactives de l'oxigen

El dany oxidatiu lipídic l'hem mesurat segons 2 metodologies.

L'àcid *cis*-parinàric és una molècula fluorescent que s'intercala a les membranes cel·lulars. Per quantificar el grau de peroxidació lipídica al que són sotmesos els lípids de membrana en ser atacats per les espècies ROS, s'incuba sempre la mateixa quantitat de mostra amb àcid *cis*-parinàric a 37ºC i, en agitació, monitoritzem per fluorimetria la pèrdua de la seva fluorescència al llarg del temps a mesura que és atacat per les molècules ROS (Figura 24). Quanta més fluorescència es perd, més ROS conté la mostra i més estrès oxidatiu associat (Miró Ò et al, *Clin Infect Dis* 2004). Els resultats s'expressen com el percentatge de fluorescència residual present a la mostra al llarg del temps respecte a la fluorescència inicial.

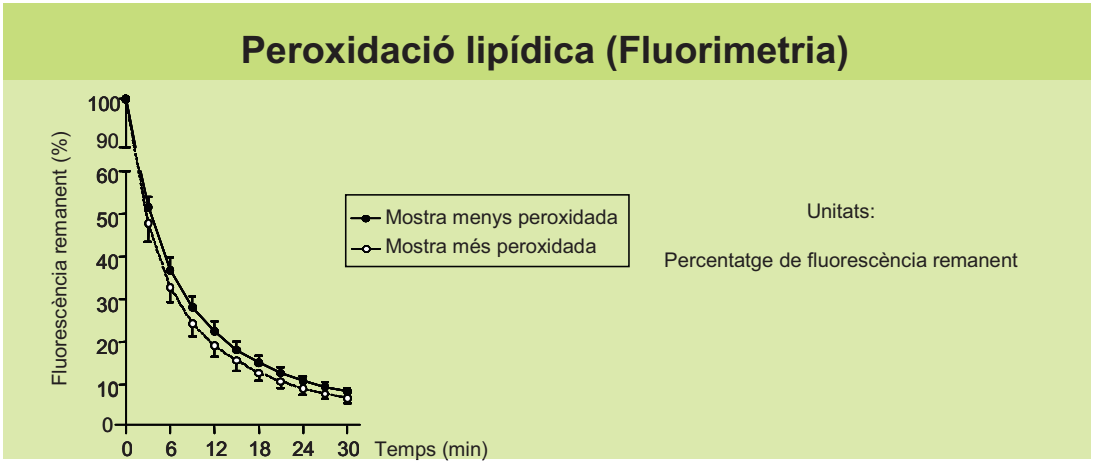


Figura 24: Quantificació del dany oxidatiu per peroxidació lipídica mitjançant fluorimetria. min: minuts.

Al llarg d'aquesta tesi s'ha posat a punt al nostre laboratori un segon mètode alternatiu per mesurar la peroxidació lipídica que consumeix molta menys mostra i temps (BIOXYTECH LPO-586® d'Oxys Research). Consisteix en mesurar l'estrès oxidatiu lipídic per espectrofotometria mitjançant la quantificació a 586 nm del contingut en malondialdehid (MDA) i 4-hidroxialquenal (4-HA), ambdós productes derivats de la descomposició dels peròxids d'àcids grassos, emprant una corba estàndard de 1, 1, 3, 3-tetrametoxipropà (Figura 25). La concentració d'aquests productes es normalitza pel contingut en proteïna per relativitzar l'estrès oxidatiu lipídic segons la quantitat de mostra. Els resultats s'expressen com la concentració micromolar de MDA i 4-HA per mil·ligram de proteïna cel·lular (µM MDA i 4-HA/mg proteïna).

Figura 25: Quantificació del dany oxidatiu per peroxidació lipídica mitjançant espectrofotometria. nm: nanòmetres.

4.3.7. Potencial de membrana de l'organul

El potencial de membrana mitocondrial es mesura per citometria de flux mitjançant un fluorocrom anomenat JC-1 que canvia el seu estat de conformació i la longitud d'ona d'emissió en funció del potencial de membrana del mitocondri. Quan el potencial de membrana mitocondrial és l'estàndard (de -180mV), JC-1 es troba en estat agregat i emet llum taronja quantificable a través del canal FL2 i, quan el mitocondri es despolaritza, JC-1 es disgrega en monòmers que emeten llum verda quantificable a través del canal FL1. Quantificant la intensitat de fluorescència verda i taronja per cadascuna de les cèl·lules presents en una mostra, podem determinar el percentatge de cèl·lules que han perdut el potencial de membrana mitocondrial estandar (Cossarizza A et al, *Current Protocols in Cyometry* 2000). Els resultats s'expressen com el percentatge de CMSP que presenten

mitocondris reversiblement o irreversiblement despolaritzats respecte al total de cèl·lules analitzades (Figura 26).

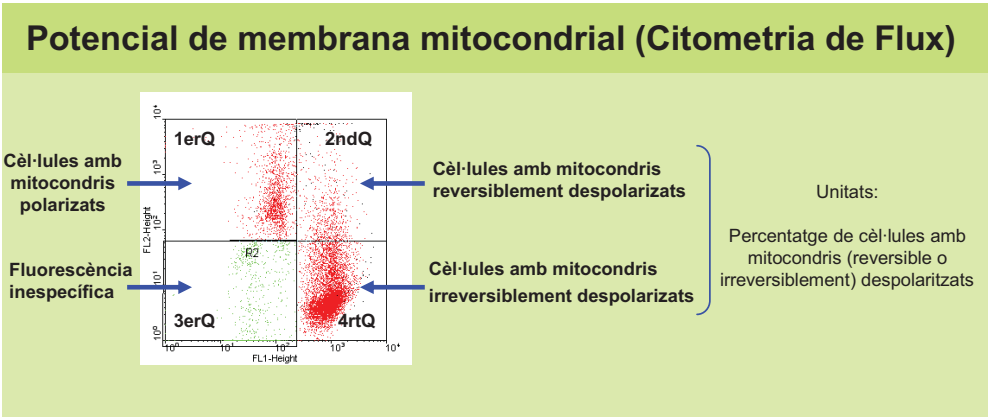


Figura 26: Quantificació del potencial de membrana mitocondrial per citometria de flux emprant la tinció amb JC-1. 1er, 2on, 3er i 4rt Q: primer, segon, tercer i quart quadrant.

4.3.8. Biogènesi mitocondrial

La quantitat de mitocondris presents a la mostra s'ha determinat mitjançant 2 metodologies diferents.

La citrat sintasa (EC: 4.1.3.7) és un enzim del cicle de Krebs que es localitza a la matriu mitocondrial. Es considera que la seva activitat enzimàtica és constant en el mitocondri (Barrientos A, *Methods* 2002; Rustin P et al, *Clin Chim Acta* 1994; Pallotti F et al, *Methods Cell Biol* 2001) i per aquest motiu es considera un bon marcador del contingut mitocondrial. La seva activitat enzimàtica es mesura a 37ºC en presència dels substrats adequats i s'expressa en nanomols de producte generat/minut/mil·ligram de proteïna (Figura 27).

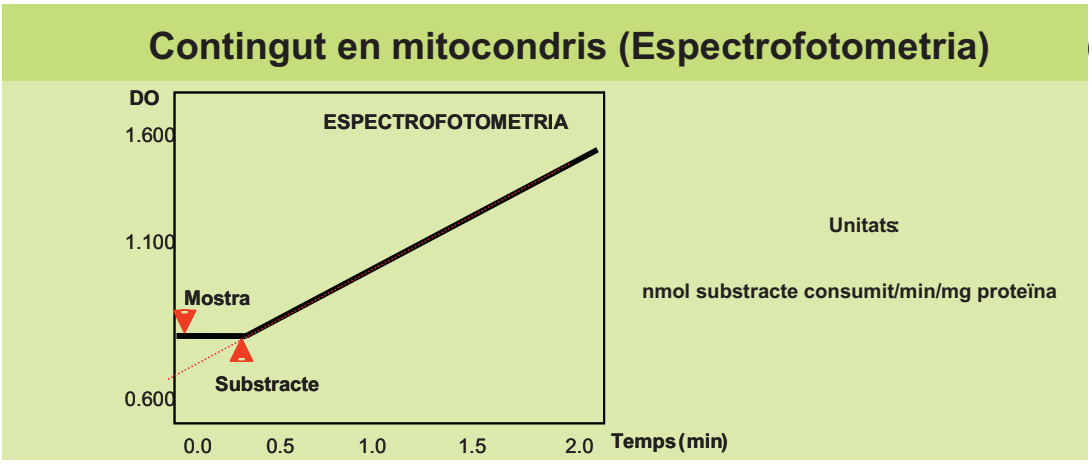


Figura 27: Quantificació del contingut en mitocondris mitjançant la mesura de l'activitat citrat sintasa per espectrofotometria. DO: denistat òptica; nmol: nanomol; min: minut; mg: mil·ligram.

Mitjançant *western blot* també podem immunoquantificar el contingut en la proteïna estructural del mitocondri V-DAC respecte la proteïna estructural citoplasmàtica β-actina, ambdues de codificació nuclear, com un indicador del contingut en mitocondris (Miró Ò et al, *J Acquir Immune Defic Syndr* 2004). S'expressa com la taxa del contingut en la proteïna mitocondrial respecte la citoplasmàtica (V-DAC/β-actina) (Figura 21).

5. RESULTATS

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

CLINICAL INFECTIOUS DISEASES 2006; 42: 584-585

G Garrabou, E Sanjurjo, Ò Miró, E Martínez, AB Infante, S López, F Cardellach, JM Gatell and J Casademont

El **PRIMER** dels treballs inclosos en la present tesi doctoral planteja com a:

HIPÒTESI

Un tòxic mitocondrial que es distribueix per tot l'organisme, i en particular un antiretroviral, pot causar una lesió mitocondrial secundària que cursi amb una clínica similar a la de les disfuncions mitocondrials primàries (com la hiperlactatèmia) i que podria ser detectable mitjançant un test dissenyat per al diagnòstic d'aquestes. Si la lesió mitocondrial és reversible un cop superat l'episodi de toxicitat, els resultats del test alternatiu haurien de correlacionar l'estat clínic dels pacients i els dels tests convencionals que detecten les alteracions mitocondrials moleculars i bioquímiques, que haurien de poder ser detectades, a part de en els teixits diana, en CMSP.

OBJECTIU

Avaluar la utilitat d'un test d'esforç aeròbic sobre l'avantbraç, inicialment dissenyat pel diagnòstic de les disfuncions mitocondrials primàries, en pacients VIH-positius sota TARGA que desenvolupen un episodi d'hiperlactatèmia, determinar si la lesió mitocondrial es pot detectar a nivell del contingut en ADNmt i en l'activitat enzimàtica del complex IV de la CRM en CMSP (a més a més de en el múscul esquelètic) i si totes aquestes possibles alteracions reverteixen un cop superat l'episodi.

titis activity in patients with chronic hepatitis is always preceded by an increase in the serum HBV load [8, 9]. Taken together, the findings from the studies mentioned above show that, if we are to identify cases of HBV reactivation sooner after transplantation, it is paramount to monitor the dynamics of the serum HBV DNA level.

In summary, the significance of detecting occult HBV infection cannot be overlooked in bone marrow transplant recipients. Occult HBV infection can be manifested in different serological profiles. In the future, further studies should focus both on the serological profile associated with the risk of HBV reactivation and on the best time to initiate antiviral therapy to avoid such a catastrophic event.

Acknowledgments

Financial support. The study was supported by grants from the National Taiwan University Hospital; the National Science Council, Department of Health, Executive Yuan, Taiwan; and the National Health Research Institutes, Taiwan.

Potential conflicts of interest. All authors: no conflicts.

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Clinical Infectious Diseases 2006; 42:583–4

1058-4838/2006/4204-0032$15.00

Noninvasive Diagnosis of Mitochondrial Dysfunction in HAART-Related Hyperlactatemia

Sir—It has been suggested that hyperlactatemia that develops in patients who are receiving HAART is caused by mitochondrial dysfunction [1–6]. This hypothesis is based on the occurrence of mtDNA depletion and/or mitochondrial respiratory chain dysfunction in liver [1, 2], skeletal muscle [3, 4], and/or PBMCs [5, 6]. However, these techniques are laborious and time consuming, and some require invasive biopsies.

Noninvasive methods have been developed for the screening of mitochondrial function in subjects with primary myopathies [7–9], but these methods have not been applied to the study of HAART-mediated mitochondrial damage. We tested the utility of the forearm aerobic exercise test (FAET) [8] in 2 HIV-infected patients during an episode of HAART-related hyperlactatemia and after resolution of the episode. To determine mitochondrial function, we simultaneously measured mtDNA content by quantitative real-time PCR, and we determined complex IV mitochondrial respiratory chain enzymatic activity by spectrophotometry; homogenate skeletal deltoid muscle biopsy specimens and PBMCs were used, as previously described [10, 11]. Results for HIV-infected subjects were compared with those for 10 healthy HIV-uninfected control subjects.

The FAET measures venous oxygen saturation in forearm blood before aerobic exercise (time 0), during aerobic exercise (minutes 1, 2, and 3), and after 1 min of resting (minute 4), to monitor oxygen saturation and to detect cellular oxygen uptake. The aerobic exercise consists of an intermittent static handgrip exercise at 33% of the intended maximum voluntary contraction force determined for each subject. In primary mitochondrial disorders, there is less oxygen desaturation in venous blood during aerobic exercise, indicating oxidative phosphorilation impairment [8].

Both HIV-infected patients, who had been infected before 1997, were receiving treatment with didanosine and stavudine (patient A was also receiving tenofovir, and patient B was also receiving efavirenz). The patients had been admitted to the hospital because of fatigue, as well as anorexia and weight loss for patient A. The serum lactate levels were 3.26 and 2.69 mmol/L (normal range, 0.4–2 mmol/L), the CD4 cell counts were 624 and 439 cells/mm^3, and the HIV-1 RNA levels were 198 copies/mL and undetectable for patients A and B, respectively. The patients were both asymptomatic after experiencing either a 6-week interruption of HAART (patient A) or a 10-week treatment switch to lamivudine, tenofovir, and efavirenz (patient B).

The mitochondrial analysis performed on PBMCs and muscle homogenate specimens during the hyperlactatemic phase confirmed the occurrence of mitochondrial dysfunction, with a 20%–80% reduction in complex IV activity and a 65%–80% depletion of mitochondrial DNA, depending on tissue specimens. These deficiencies returned to normal levels after clinical resolution of hyperlactatemia (figure 1). At the same time, both patients

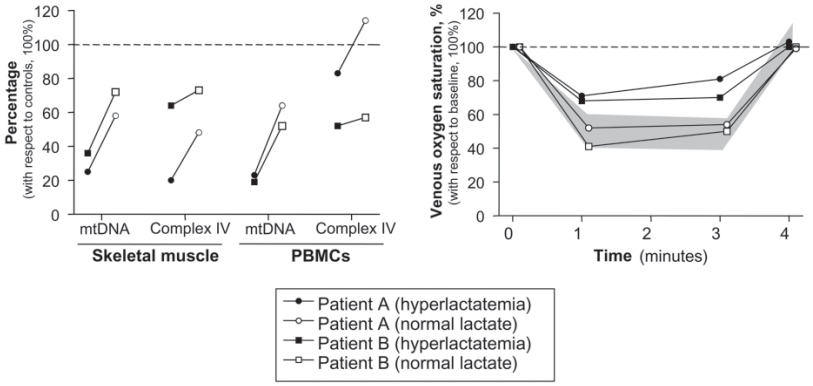

Figure 1. *Left,* Mitochondrial respiratory chain complex IV enzymatic activity and mtDNA content in muscle homogenate and PBMCs, compared with values for control subjects. *Right,* Forearm aerobic exercise test results, compared with baseline values. Black circles and boxes denote data for the 2 HIV-infected patients during symptomatic hyperlactatemia, and white circles and boxes denote the values 6 months after recovery. The shaded grey area represents the normal control ranges.

exhibited a marked decrease of oxygen utilization during the symptomatic phase, as determined by FAET, because venous oxygen saturation only decreased by 30% from the baseline level (100%), compared with the 50% decrease found in control subjects, indicating reduced blood oxygen uptake [8]. After clinical recovery, the values for both HIV-infected patients matched the values for healthy persons (figure 1).

Results of mitochondrial studies and FAET findings were consistent with the clinical manifestations in these 2 HIV-infected patients during symptomatic HAART-related hyperlactatemia and after clinical recovery. The possibility that use of antiretroviral drugs (i.e., didanosine or stavudine) and not hyperlactatemia was the direct cause of abnormal FAET results seems improbable, in view of previous metabolic studies [12]. The FAET, accordingly, seems to be a useful noninvasive tool for the detection of mitochondrial dysfunction developed during HAART-induced hyperlactatemia.

Acknowledgments

Financial support. Fundació la Marató de TV3 (02/0210 and 01/0710), Redes de Investigación en Mitocondrias (V2003-REDC06E-0) y SIDA (Redg 173), and Suports a Grups de Recerca de la Generalitat de Catalunya (2001/SGR/00379).

Potential conflicts of interest. All authors: no conflicts.

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Clinical Infectious Diseases 2006; 42:584–5

RESUM DEL PRIMER ESTUDI

El present treball avalua l'eficàcia diagnòstica d'un test d'esforç aeròbic de l'avantbraç inicialment dissenyat per Jensen i col·laboradors (Jensen T et al, *Neurology* 2002) per diagnosticar disfunció mitocondrial primària, en 2 pacients VIH-positius sota TARGA que desenvolupen una disfunció mitocondrial secundària que es manifesta en forma de crisi d'hiperlactatèmia. Paral·lelament, s'estudia la funció mitocondrial d'aquests 2 pacients quantificant el contingut en ADNmt i l'activitat enzimàtica del complex IV (COX) de la CRM en un dels teixits diana responsables de l'augment del lactat en sang (el múscul) i en CMSP, per confirmar l'existència de la disfunció mitocondrial a nivell molecular i bioquímic i comprovar si les CMSP suposen un model d'estudi adequat per l'anàlisi de la disfunció mitocondrial d'origen tòxic. Aquests estudis es realitzen quan els pacients presenten un episodi d'hiperlactatèmia i després de la recuperació clínica i normalització dels nivells de lactat, i es comparen amb els valors control de 10 individus no-infectats.

El test d'esforç assajat detecta una capacitat disminuïda per consumir l'oxigen del torrent sanguini en els pacients VIH-positius en TARGA que desenvolupen la crisi d'hiperlactatèmia mentre porten a terme un exercici aeròbic de l'avantbraç i les anàlisis bioquímiques i moleculars detecten una important disminució del contingut d'ADNmt (65-80%) i de l'activitat enzimàtica del complex IV de la CRM (20-80%), tant en múscul com en CMSP. Aquests valors retornen a la normalitat després de la recuperació clínica dels pacients i quan els nivells de lactat es normalitzen.

CONCLUSIONS DEL PRIMER ESTUDI

1. El test d'esforç aeròbic de l'avantbraç, inicialment ideat per diagnosticar mitocondriopaties primàries, es pot aplicar per determinar la disfunció mitocondrial en pacients VIH-positius que manifesten un episodi d'hiperlactatèmia.

2. La disfunció mitocondrial detectada en el test d'esforç es confirma amb l'existència de lesions mitocondrials bioquímiques i genètiques en CMSP (fins llavors només descrites en la bibliografia a nivell d'ADNmt), i aquestes alteracions són igualment presents en el teixit muscular.

3. Aquestes anomalies mitocondrials es normalitzen quan el pacient supera l'episodi d'hiperlactatèmia, fet que suggereix que la toxicitat del tractament antiretroviral només s'associa a disfunció mitocondrial quan existeix la manifestació clínica i que són efectes que reverteixen quan el tractament se suprimeix o es canvia a una pauta menys mitotòxica (sense ddI ni d4T).

METABOLIC AND MITOCHONDRIAL EFFECTS OF SWITCHING ANTIRETROVIRAL-EXPERIENCED PATIENTS TO ENFUVIRTIDE, TENOFOVIR AND SAQUINAVIR/RITONAVIR

ANTIVIRAL THERAPY 2006; 11: 625-630

Ò Miró, **G Garrabou**, S López, E Deig, I Vidal, AB Infante, F Cardellach, J Casademont and E Pedrol

El **SEGON** dels treballs inclosos en la present tesi doctoral planteja com a:

HIPÒTESI

Existeixen tòxics, i específicament antiretrovirals, amb diferent potència de lesió mitocondrial i aquesta lesió sol revertir en retirar el tòxic, de manera que la substitució d'un fàrmac de gran potència mitotòxica per un agent menys nociu ha de reflectir-se en la millora dels paràmetres mitocondrials.

OBJECTIU

Determinar els paràmetres immunovirològics, metabòlics i sobretot l'estat mitocondrial global (contingut en ADNmt i activitats enzimàtiques dels complexos III i IV -COX- de la CRM, taxa d'expressió proteica mitocondrial de COX-II i activitat oxidativa del complex I, tots ells parcialment codificats al mitocondri) en CMSP de pacients VIH-positius amb lipodistròfia en els que l'augment de la càrrega viral motiva la substitució durant 6 mesos d'un TARGA de major potència mitotòxica (que inclou com a mínim un dideoxinucleòsid) per un tractament de rescat teòricament menys nociu consistent en T20+TDF+SQV/rit (lliure de dideoxinucleòsids).

Antiviral Therapy 11:625–630

Short communication

Metabolic and mitochondrial effects of switching antiretroviral-experienced patients to enfuvirtide, tenofovir and saquinavir/ritonavir

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Objective: Investigate the metabolic and mitochondrial effects of switching a highly active antiretroviral therapy (HAART) regimen with a high mitochondrial toxicity profile to a HAART with a theoretically low mitochondrial toxicity.
Patients and methods: Six consecutive HAART-experienced patients receiving at least one dideoxy-nucleoside reverse transcriptase inhibitor (NRTI) switched to enfuvirtide plus tenofovir plus saquinavir/ritonavir (T20+TDF+SQV/r). Blood samples were collected at baseline, 12 and 24 weeks after the switch, and viral load (VL) and lymphocyte $CD4^+$ T-cell count were determined. Metabolic parameters consisted of fasting serum triglycerides, cholesterol (total and fractions), glucose, insulin, C-peptide and lactate. Mitochondrial assessment consisted on mitochondrial DNA (mtDNA) quantification, COX-II mitochondrial protein expression rate, mitochondrial respiratory chain complex III and IV activities, and oxygen consumption in peripheral blood mononuclear cells. For baseline mitochondrial comparisons, we included six HIV-infected patients naive for ART.
Results: Switched patients exhibited a mean increase of 26 $CD4^+$ T-cells/mm^3 and a mean decrease of 1.1 log in VL (P=NS for both). Lactate, lipids and glycaemia remained stable during the study; only insulin levels increased significantly ($P<0.05$). Switched patients exhibited, at baseline, low mitochondrial measurements, being significant only for complex III and IV activities with respect to naive patients ($P<0.05$ for both). MtDNA content did not rise significantly during the study. However, we observed increases in COX-II mitochondrial protein synthesis (124%, $P<0.05$), complex III activity (127%, $P<0.05$), complex IV activity (86%, $P=0.37$) and oxygen consumption (194%, $P<0.05$).
Conclusion: Switching a HAART-containing dideoxy-NRTI to T20+TDF+SQV/r minimally alters metabolic parameters and exerts beneficial effects on mitochondrial function at 24 weeks. Mitochondrial improvement should be considered as an additional advantage of this rescue therapy.

Introduction

Mitochondrial dysfunction is an adverse effect linked to the long-term use of highly active antiretroviral therapy (HAART), which mainly relies on the nucleoside analogue reverse transcriptase inhibitors' (NRTIs) capacity to inhibit mitochondrial DNA (mtDNA) γ-polymerase leading to a reduction of mtDNA content, mitochondrial respiratory chain (MRC) dysfunction and energy failure. Some NRTIs, especially dideoxynucleosides (dideoxy-NRTIs), are particularly prone to causing γ-polymerase inhibition [1]. Moreover, NRTIs can also inhibit mtRNA transcription in the absence of mtDNA depletion either *in vitro* [2] or *in vivo* [3]. In addition, zidovudine (AZT) can reduce the succinate transport, ADP/ATP antiport, cytochrome *c* oxidase activity and carnitine content, all in the absence of significant mtDNA reduction [4–6].

According to this rationale, switching to a NRTI-sparing HAART or to a HAART containing NRTIs with low mitochondrial toxicity should translate into an improvement of mitochondrial function [7,8]. This theoretical effect has been, to date, poorly explored and it is not clear if the hypothetical beneficial effects are circumscribed to mtDNA recuperation and/or affect other mitochondrial parameters. This could be of interest

because, in addition to the advantages of immunological and virological response linked to switching, understanding the mitochondrial and metabolic profile of a particular HAART combination could help when choosing different rescue strategies. In the present study we evaluate, from a multidisciplinary approach, the metabolic and mitochondrial effects of switching experienced patients from their current dideoxy-NRTI-based HAART regimen to a new HAART schedule with a low mitochondrial toxicity profile consisting of enfuvirtide, tenofovir and saquinavir/ritonavir (T20+TDF+SQV/r). To evaluate the effects, we used peripheral blood mononuclear cells (PBMCs) because they are widely considered to be a reliable marker for monitoring mitochondrial toxicity of antiretrovirals *in vivo*, and some mitochondrial parameters, such as mtDNA content, are currently usually measured in PBMCs in clinical trials [8–11].

Patients and methods

Patients

We prospectively included six consecutive patients (five men and one woman, mean age 49 ±13 years) on HAART with clinically diagnosed lipodystrophy (five lipoatrophy and one mixed syndrome) who had been switched to a rescue therapy consisting of T20+TDF+SQV/r (T20: 90 mg/12 h subcutaneously, TDF: 245 mg/24 h orally, and SQV/r: 1000 mg/100 mg/12 h orally) because of immunovirological failure. They were antiretroviral (ART)-experienced patients (111 ±57 months) with a heavy background of ART (Table 1). They had received at least one uninterrupted dideoxy-NRTI for at least 6 months (10.5 ±2.5 months) immediately before their inclusion in the study. All of them were recruited at the HIV unit of the Hospital de Granollers (Barcelona, Spain), which takes care of about 400 HIV-infected people. As a control group, we included six HIV-infected patients naive for ART (five men and one woman, mean age 53 ±15 years) who were just about to initiate HAART due to immunological decay and/or uncontrolled HIV infection, in order to be comparable with patients from a virological point of view. All patients gave informed consent to be enrolled in the study.

Table 1. Clinical and epidemiological data of the patients switched to T20+TDF+SQV/r

Case	Age/gender	Suspected mechanism of transmission	Previous ART (duration of treatment, months)	HAART schedule immediately before switching (duration of treatment, months)	Total duration of ART, months	Total time on dideoxy-NRTI prior to inclusion in the study, months
1	38/F	Htx	AZT (45); 3TC (19); ddI (16); d4T (9); TDF (14); ABV (20); NVP (19); LPV (51); RTV (51); NFV (13)	d4T+TDF+NFV (9)	63	13
2	58/M	Hmx	AZT (25); 3TC (40), d4T (47); ddI (18); TDF (7); ABV (18); NVP (25);NFV (25); IDV (87); LPV (8); RTV (8); SQV (1)	ddI+NVP+LPV/r (8)	105	34
3	70/M	Htx	AZT (38); 3TC (13); ddI (20); d4T (47); ddC (10); ABV (9): TDF (11); NVP (3); EFV (8); IDV (44); NFV(5); RTV (13); SQV (3); LPV (36); APV (9)	d4T+TDF+IDV/r (11)	122	24
4	37/M	Hmx	AZT (39); 3TC (20); ddI (41); ddC (11); d4T (14); TDF (19); ABV (9); NVP (10); EFV (8); LPV (8); RTV (53); NFV (11); SQV (2); IDV (31); APV (4)	d4T+TDF+NFV (11)	100	14
5	48/M	Parenteral drug user	AZT (39); 3TC (16), ddI (18), TDF (26); ABV (49); NVP (3); EFV (20); LPV (26); RTV (27); NFV(18); SQV (1)	ddI+TDF+LPV/r (9)	58	9
6	44/M	Hmx	AZT (15); 3TC (42); ddI (80); ddC (67); d4T (17); TDF (15); ABV (25); EFV (15); IDV (65)	ddI+TDF+EFV (15)	216	39

3TC, lamivudine; ABV, abacavir; APV, amprenavir; ART, antiretroviral therapy; AZT, zidovudine; d4T, stavudine; ddC, zalcitabine; ddI, didanosine; EFV, efavirenz; F, female; HAART, highly active antiretroviral therapy; Hmx, homosexual; Htx, heterosexual; IDV, indinavir; LPV, lopinavir; M, male; NFV, nelfinavir; NRTI, nucleoside reverse transcriptase inhibitor; NVP, nevirapine; RTV, ritonavir; SQV/r, saquinavir/ritonavir; T20, enfuvirtide; TDF, tenofovir.

Analytical determinations

Blood samples were collected at baseline (prior to switching) and at 12 and 24 weeks after switching to T20+TDF+SQV/r. We determined the plasma HIV viral load (VL) and the lymphocyte CD4⁺ T-cell count, as well as fasting triglycerides, cholesterol [total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) fractions], glucose, insulin, C-peptide and lactate by means of standard automatized biochemical procedures.

Mitochondrial studies

For the mitochondrial assays, 20 ml of peripheral venous blood was used to obtain PBMCs. DNA extraction was performed by the standard phenol-chloroform procedure and mtDNA quantification was then carried out using quantitative real-time PCR methodology. The mitochondrial protein synthesis rate was assessed as the expression of the cytochrome *c* oxidase II (COX-II) subunit of the MRC complex IV (COX-II is entirely encoded, transcribed and translated in mitochondria) by Western blot and read by enhanced chemiluminescence, and protein β-actin was used to normalize for equal cell protein loading. MRC complex III and IV COX activities were measured by spectrophotometry. Finally, oxygen consumption was measured polarographically using malate plus pyruvate as a substrate, (complex I substrate, electrons flow through complexes I, III and IV of MRC). All methodological aspects are described in detail elsewhere [6,12]. In all cases, results were expressed as percentages with respect to either the control group or baseline, which were arbitrarily assigned 100%.

Statistical analysis

Results were expressed as mean ±SEM, and ANOVA for independent or for repeated measures (as required) were used to search for differences.

Results

The baseline lymphocyte CD4⁺ T-cell count for switched patients was 132 ±51 per mm^3, while the VL was 4.9 ±0.2 log_{10} HIV RNA copies/ml. The control group had a better baseline immunological status (429 ±120 CD4⁺ lymphocytes per mm^3; $P<0.05$) but the VL was very similar (5.0 ±0.3 log_{10} HIV RNA copies/ml; $P=0.92$).

No significant changes in CD4⁺ T-cell count were observed in the switching group during the study (158 ±49 at 24 weeks, that is, a mean increase of 26 cells/mm^3; $P=0.26$). Conversely, four out of six patients exhibited a decrease of >0.5 log in serum VL, the mean VL being 3.8 ±0.5 at the end of the study (a mean decrease of 1.1 log; $P=0.07$).

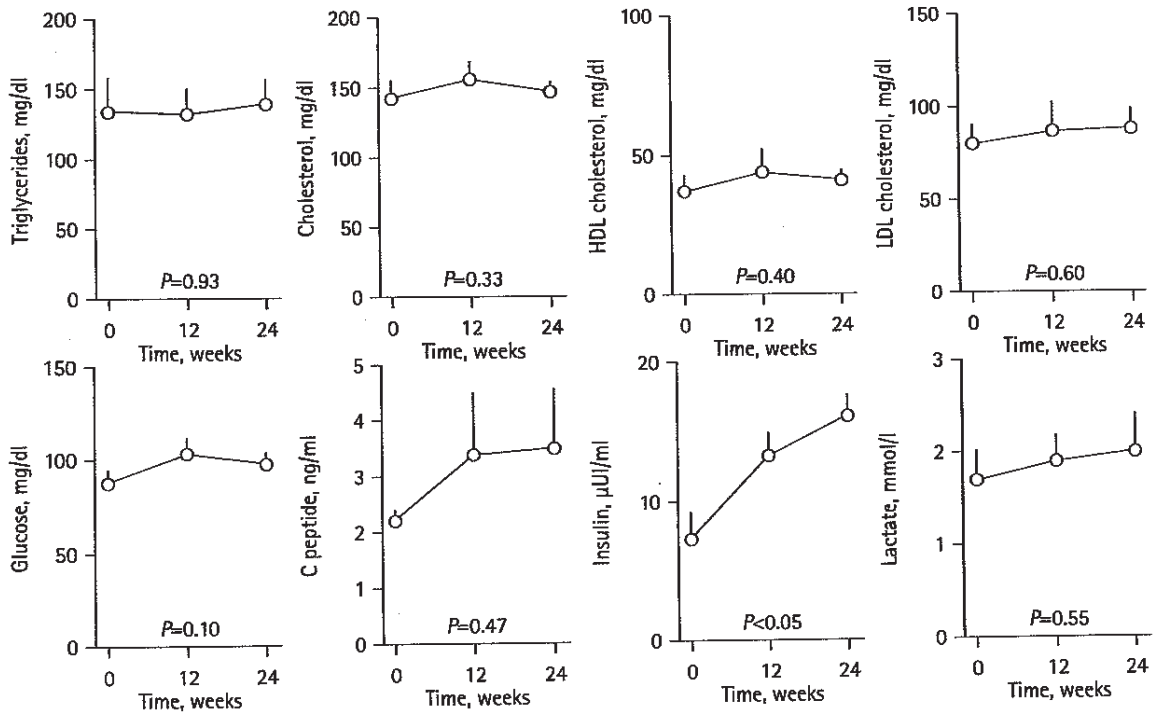
Two switched patients had mild hypertriglyceridemia at baseline; the rest of the metabolic parameters were within normal values. No significant changes occurred in the serum lipid profile during the study since no clinical changes were observed in lipodystrophy patterns. Glycaemia did not change either, although insulin levels rose significantly. Finally, lactate remained practically unchanged during the study (Figure 1).

Switched patients exhibited, at baseline, lower values for mitochondrial parameters than controls, significant only for complex III and IV activities ($P<0.05$ for both; Figure 2). We did not observe significant changes in mtDNA content after patients switched to T20+TDF+SQV/r (Figure 2). Conversely, progressive improvements for the rest of the mitochondrial parameters were observed after switching, and at the end of study COX-II expression had increased 124% ($P<0.05$), complex III activity 127% ($P<0.05$), complex IV activity 86% ($P=0.37$) and oxygen consumption with pyruvate as substrate 194% ($P<0.05$; Figure 2).

Discussion

The present study has two limitations. The sample size is rather small mainly due to the low percentage of patients using T20+TDF+SQV/r as a rescue therapy and this fact could imply a type-II error in some of the statistical analyses performed. Additionally, the time of follow-up was relatively short, especially when taking into account that most patients had previously been receiving HAART regimens for more than 5 years consecutively. It is therefore possible that we had not achieved a complete washout of previous mitochondrial side effects caused by these HAART regimens before the therapy switch. However, despite these study design constraints, we believe that some conclusions can be drawn from our data. The switching to the T20+TDF+SQV/r combination has been associated with a trend to favourable immunovirological response and a safe lipidic and hydrocarbonate metabolic profile. No patient developed dyslipaemia or diabetes mellitus during treatment, a progressive increase of serum insulin being the only significant change. With respect to mitochondrial effects, patients included in the switching group initially had a wide spectrum of mitochondrial disarrangements that cannot be attributed to the effects of the HIV *per se*, because the control group was made up of naive patients with uncontrolled HIV infection with nearly identical VL to the switching group. The longitudinal follow-up has shown that, despite the fact that the switching strategy was not able to improve mtDNA content, the mtDNA-encoded protein expression increased markedly, and this increase was associated

Figure 1. Metabolic parameters of the switching group along the study



HDL, high-density lipoprotein; LDL, low-density lipoprotein.

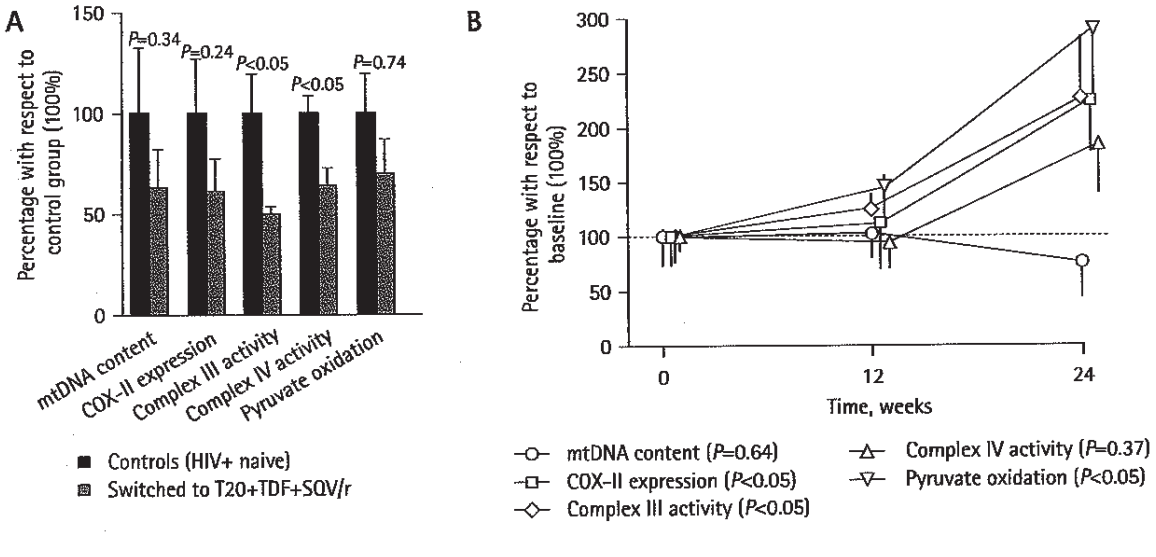
with the recovery of MRC enzyme activities and the oxidative capacity of PBMCs.

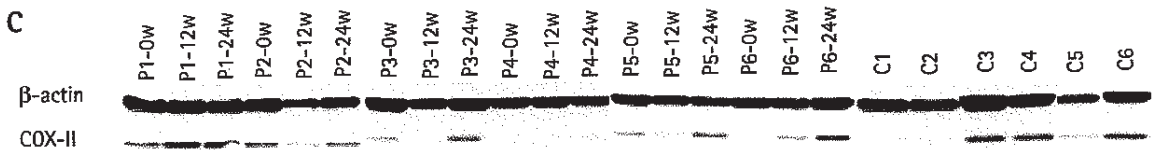
Data regarding metabolic effects of T20+TDF+SQV/r are scarce and effects of this specific combination have not been explored to date. Previous data on T20 coming from TORO trials suggest that it does not modify fat distribution or adversely affect lipid or glycaemic parameters as is the case with other antiretrovirals [13]. On the other hand, TDF has a low impact on lipid profile and actually seems to be able to partly revert dyslipaemia in patients receiving other NRTIs such as stavudine (d4T) [14,15]. Finally, protease inhibitors (PIs) have been consistently linked to the development of dyslipaemia and diabetes mellitus, although small differences among different drugs exist. With respect to those used in the present study, while SQV exhibits a more favourable effect on the ratio between total and HDL cholesterol with respect to other PIs, ritonavir exerts negative effects on this ratio as well as causing greater increases in total cholesterol and triglycerides than the other PIs [16]. Our study suggests that, when used in combination, T20+TDF+SQV/r does not cause lipid and hydrocarbonate metabolism to deteriorate during the first 24 weeks of treatment.

The switching from a dideoxy-NRTI-containing HAART to a NRTI-sparing HAART or to a HAART containing NRTIs with a low mitochondrial toxicity profile is expected to improve mitochondrial function at least in part via the increase of mtDNA abundance [7,8]. Few studies, however, have addressed this hypothesis. McComsey *et al.* [10] demonstrated that the substitution of d4T with abacavir (ABV) or AZT improves mtDNA content in either muscle, adipose tissue or PBMCs at week 48 after the switching, and similar findings have been reported in fat tissue by Nolan *et al.* [17]. Conversely, Hoy *et al.* [11] failed to demonstrate significant changes of mtDNA abundance in PBMCs of patients switched from a thymidine analogue to ABV. Similarly, we have observed only a small and insignificant improvement of mtDNA content after 48 weeks of switching from a two-NRTI-based HAART to an NRTI-sparing HAART [8]. Results of the present study agree with these latter studies because, despite the discontinuation of dideoxy-NRTI, mtDNA content did not recover at 24 weeks.

In contrast with the absence of effects on mtDNA, the expression of COX-II (a subunit of COX that is encoded by mtDNA, and transcribed and translated entirely in mitochondria) significantly increased with the switch to T20+TDF+SQV/r. This fact raises the question on the

Figure 2. Mitochondrial parameters of patients on switching therapy at baseline and during the study





(A) Baseline values. (B) Evolution of patients switched to T20+TDF+SQV/r. (C) Western blot assays of COX-II and β-actin. mtDNA, mitochondrial DNA; SQV/r, saquinavir/ritonavir; T20, enfuvirtide; TDF, tenofovir.

mechanism(s) by which such an improvement is achieved. One possibility is that previous HAART schedules have damaged both mtDNA replication capacity and mitochondrial transcriptional and/or post-transcriptional machinery. It may be that after switching to a less toxic HAART (from a mitochondrial point of view), transcriptional and/or post-transcriptional mechanisms recover faster than γ-polymerase inhibition. In agreement with this hypothesis, Cossarizza's group have recently reported that when HAART is interrupted, mtDNA content recovers very slowly during the first 6 months and that this increases up to fivefold after 6 months [18]. Additionally, mitochondria can adapt transcription of mtRNA to meet altered OXPHOS requirements independently of regulation by nuclear factors or changes in mtDNA abundance [19,20]. In fact, cells depleted of mtDNA maintain expression of mtRNA and mitochondrial protein synthesis [6], whereas such isolated mtDNA depletion is insufficient to induce up-regulation of nuclear-encoded mtDNA replication factors [4,5,19]. In any case, the increase in COX-II expression observed in the present study was of a great enough magnitude to be associated with a partial restoration of complex IV activity. A significant increase was observed in MRC complex III activity, although we have not investigated if such an increase was due to improved expression of cyt b gene (the only complex III subunit codified by mtDNA). Whatever the mechanism(s) involved, the improvement of MRC complex III and IV activities led to a significant recovery of the PBMCs capacity to use oxygen to metabolize pyruvate-malate, a substrate combination that induces the electron flow across MRC complexes I, III and IV, all of them partly encoded by mtDNA.

Our patients had received high cumulative doses of NRTIs with a high potential for mitochondrial toxicity. Therefore, it is feasible that the remaining mitochondrial adverse effects observed in PBMCs 24 weeks after the switch could be, at least in part, the consequence of the previous HAART schedules. In fact, given the relatively short half-life of circulating mononuclear cells, the results better express toxic effects of antiretrovirals within the bone marrow rather than only within peripheral blood. In support of this theory, persistence of mitochondrial dysfunction has been described in children whose

mothers were on treatment with NRTIs during pregnancy [21]. Of note, and similar to that observed by us in the present study, defects in mitochondrial function in these children were observed without significant depletion of mtDNA.

An unresolved question of the present study is what antiretroviral(s) is the main contributor to the observed changes in mitochondrial metabolism. No studies regarding the effects of fusion inhibitors (T20) on mitochondria are currently available. On the other hand, TDF was marketed to be exempt of adverse mitochondrial effects. However, in recent years a decrease in mtDNA content has been reported in PBMCs of patients treated with TDF in combination with didanosine (ddI) at doses of 400 mg [22]. Although this side effect has been ultimately attributed to an increase in ddI plasma concentrations when given together with TDF, the complete TDF mitochondrial effects are now being redefined. Finally, some PIs interfere with apoptotic processes [23] and, therefore, it could be hypothesized that SQV/r modulates apoptotic pathways and is, at least in part, responsible for the mitochondrial changes observed during the study. In any case, our study demonstrates once again that when assessing the antiretroviral impact on mitochondria, the isolated measurement of mtDNA content probably underestimates the whole effects of such drugs on mitochondrial function. Our study also suggests that, in addition to other advantages linked to switching, the improvement of mitochondrial functionality should be also be considered as an advantage of rescue therapy including T20+TDF+SQV/r.

Acknowledgements

We are indebted to Dr Esteban Martínez for his critical review of draft versions of this manuscript. This study was supported by the foollowing grants: Fundació La Marató de TV3 (020210), Redes de Investigación en Mitocondrias (V2003-REDC06E-0) y Sida (Redg 173) and Suport a Grups de Recerca de la Generalitat de Catalunya (2005/SGR/0300).

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Accepted for publication 6 March 2006

RESUM DEL SEGON ESTUDI

El present treball avalua els efectes immunovirològics, metabòlics i mitocondrials de canviar la pauta antiretroviral de 6 pacients VIH-positius afectes de lipodistròfia, amb gairebé 10 anys d'experiència en diferents TARGA de contrastada potència mitotòxica i en els que l'augment de la càrrega viral motiva el canvi a un tractament de rescat teòricament menys nociu consistent en T20+TDF+SQV/rit. En aquests pacients s'analitza l'estat immunovirològic (nombre de limfòcits T $CD4^+$ i càrrega viral), el perfil metabòlic de lípids i glúcids (nivells de triglicèrids, colesterol -total, HDL i LDL-, glucosa, pèptid c, insulina i lactat) i la funció mitocondrial en CMSP (contingut en ADNmt, activitats enzimàtiques dels complexos III i IV -COX- de la CRM, taxa d'expressió proteica mitocondrial de COX-II i activitat oxidativa a través del complex I) a nivell basal i a les 12 i 24 setmanes després del canvi de tractament. Els resultats s'han comparat amb els d'un grup control de 6 individus infectats que no reben tractament antiretroviral per descartar els efectes causats pel propi virus.

Després de 24 setmanes del canvi de pauta antiretroviral, s'observa a nivell immunològic un lleuger increment en el nombre de limfòcits T $CD4^+$ (26 cèl·lules/mm^3), però una reducció molt important de la càrrega viral en 4 dels 6 pacients. A nivell metabòlic, el canvi de pauta TARGA no altera els paràmetres del metabolisme lipídic i glucídic estudiats, excepte els nivells d'insulina, que augmenten. Abans del canvi de tractament, els pacients presenten un pitjor estat mitocondrial que els controls, estadísticament significatiu pels complexos III i IV de la CRM. Com que ambdós grups són d'individus infectats, sembla que l'experiència antiretroviral acumulada, i no a la infecció per VIH, és la responsable d'aquesta lesió mitocondrial. El canvi de TARGA comporta la millora de tots els paràmetres mitocondrials estudiats, especialment passades 24 setmanes (augmenta un 127% l'activitat del complex III, un 86% l'activitat del complex IV, un 124% l'expressió de COX-II i un 194% l'activitat oxidativa a través del complex I), excepte l'ADNmt. La manca de recuperació del contingut en ADNmt pot deure's a que 24 setmanes no són suficients per observar canvis en aquest paràmetre (Mussini C et al, *AIDS* 2005) o a que el canvi de TARGA no pot eliminar la totalitat de la toxicitat mitocondrial de tractament acumulada, que ja no afectaria a la majoria de cèl·lules sanguínies analitzades, sinó als seus precursors en la medul·la òssia (de vida mitja molt superior).

CONCLUSIONS DEL SEGON ESTUDI

1. En pacients amb una llarga història de tractament amb TARGA, el canvi d'un TARGA de contrastada potència mitotòxica per un de teòricament menys nociu consistent en T20+TDF+SQV/rit resulta satisfactori després de 24 setmanes des del punt de vista immunovirològic, metabòlic i mitocondrial.

2. La recuperació mitocondrial no és immediata i els canvis en l'evolució dels paràmetres mitocondrials s'observen a partir de les 24 setmanes.

3. La recuperació mitocondrial resulta significativa a nivell funcional (activitats enzimàtiques i oxidatives i taxa d'expressió proteica) però no a nivell genètic (contingut en ADNmt).

4. La mesura del contingut en ADNmt no s'hauria d'utilitzar de manera aïllada per efectuar el diagnòstic de l'estat mitocondrial, ni tan sols quan s'estudia l'efecte de tòxics que alteren el funcionament de l'ADN polimerasa γ, doncs la capacitat de recuperació sembla estar retardada.

PARTIAL IMMUNOLOGICAL AND MITOCHONDRIAL RECOVERY AFTER REDUCING DIDANOSINE DOSES IN PATIENTS ON DIDANOSINE AND TENOFOVIR-BASED REGIMENS

ANTIVIRAL THERAPY 2008; 13(2): 231-240

E Negredo*, **G Garrabou***, J Puig, S López, C Morén, R Bellido, R Ayen, F Cardellach, Ò Miró and B Clotet

*(primer i segon autors figuren com a primer autor)

El **TERCER** dels treballs inclosos en la present tesi doctoral planteja com a:

HIPÒTESI

Els efectes mitotòxics, en particular de certs antiretrovirals, són dosi-dependents i la lesió mitocondrial sol revertir en retirar el tòxic. Per tant, la simple reducció en la dosi d'exposició a un fàrmac mitotòxic hauria de traslladar-se també en la millora de l'estat mitocondrial.

OBJECTIU

Avaluar l'estat immunològic i mitocondrial general (contingut en ADNmt, activitat enzimàtica del complex IV -COX- de la CRM i la taxa d'expressió proteica mitocondrial per COX-II, codificada al mitocondri) en CMSP de pacients VIH-positius asimptomàtics amb una prolongada història en tractament antiretroviral en els que es redueix durant 14 mesos la dosi d'un dels components del TARGA (ddI de 400 a 250 mg/dia), sense canviar cap altre component de la pauta antiretroviral (NVP i TDF).

Antiviral Therapy **13:**xxxx–xxxx

Correction

Partial immunological and mitochondrial recovery after reducing didanosine doses in patients on didanosine and tenofovir-based regimens

Eugènia Negredo[1†], Glòria Garrabou[2,3†], Jordi Puig[1], Sònia Lòpez[2,3], Constanza Morén[2,3], Rocio Bellido[1], Rafaela Ayen[1], Francesc Cardellach[2,3], Óscar Miró[2,3] and Bonaventura Clotet[1]*

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Antiviral Therapy 2007; **12:**231–240.

The authors would like to report an error that appeared in the authorship of the above article. The first and second authors - Eugènia Negredo and Glòria Garrabou – should have been noted as making an equal contribution to this work.

Original article

Partial immunological and mitochondrial recovery after reducing didanosine doses in patients on didanosine and tenofovir-based regimens

Eugènia Negredo[1], Glòria Garrabou[2,3], Jordi Puig[1], Sònia Lòpez[2,3], Constanza Morén[2,3], Rocio Bellido[1], Rafaela Ayen[1], Francesc Cardellach[2,3], Óscar Miró[2,3] and Bonaventura Clotet[1]*

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Background: Tenofovir disoproxil fumarate (TDF) has a safe toxicity profile; however, administration together with didanosine (ddI) increases ddI levels causing mitochondrial damage and CD4+ T-cell decline. We assessed whether a simple reduction of the ddI dose in patients receiving ddI (400 mg/day) and TDF could revert this side effect.

Methods: Immunological and mitochondrial changes were analysed in 20 patients at baseline, after 14 months of receiving ddI (400 mg/day), TDF (300 mg/day) and nevirapine (NVP; 400 mg/day) and 14 months after a ddI dose reduction to 250 mg/day. Immunological analyses measured CD4+ and CD8+ T-cell counts and mitochondrial studies in peripheral blood mononuclear cells assessed mitochondrial DNA content by quantitative real-time PCR, cytochrome c oxidase (COX) activity by spectrophotometry and mitochondrial protein synthesis (COX-II versus β-actin or COX-IV expression) by western blot.

Results: Treatment with TDF, ddI (400 mg/day) and NVP for 14 months produced significant decreases in mitochondrial parameters and CD4+ T-cell counts. The reduction in ddI dose resulted in mitochondrial DNA recovery; however, the remaining mitochondrial parameters remained significantly decreased. Levels of CD4+ T-cells were partially restored in 35% of patients. Subjects presenting a significant reduction in CD4+ T-cells during the high ddI dose period showed greater mitochondrial impairment in this stage and better mitochondrial and immunological recovery after drug reduction.

Conclusions: Administration of high ddI doses together with TDF was associated with mitochondrial damage, which may explain the observed CD4+ T-cell decay. A reduction of the ddI dose led to mitochondrial DNA recovery, but was not sufficient to recover baseline CD4+ T-cell counts. Other mitochondrial toxicity in addition to DNA γ-polymerase inhibition could be responsible for CD4+ T-cell toxicity.

Introduction

Mitochondrial dysfunction has been proposed as the etiopathogenic mechanism of many adverse events induced by nucleoside reverse transcriptase inhibitors (NRTIs) [1,2]. Inhibition by nucleoside analogues of DNA polymerase γ (DNA pol-γ), the only enzyme responsible for mitochondrial DNA (mtDNA) replication, leads to a reduction in mtDNA production (mtDNA copy number) and quality by increasing the number of point mutations and deletions [3–5]. The mtDNA encodes for components of the mitochondrial oxidative phosphorylation (OXPHOS) system, such as subunits I, II and III of the cytochrome *c* oxidase complex (COX-I, COX-II and COX-III) and, consequently, nucleoside analogues can induce the dysfunction of OXPHOS complexes like COX. Nonetheless, alternative mechanisms of mitochondrial damage other than DNA pol-γ inhibition have also been described for NRTIs, such as mitochondrial transcription impairment [6,7], inhibition of the transport of energetic substrates (succinate or

ADP/ATP), depletion of mitochondrial carnitine content or direct inhibition of COX activity [8–10].

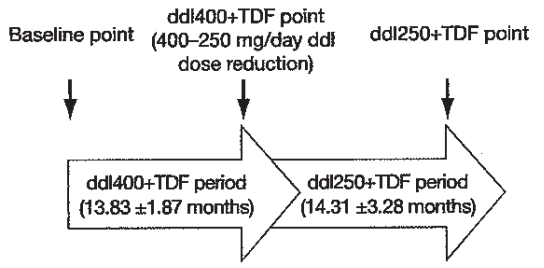
Pancreatitis, lipoatrophy, peripheral neuropathy, lactic academia, steatosis and myopathy are some of the mitochondrial-related toxicities observed. These secondary effects have prompted the modification of highly active antiretroviral therapy (HAART) schedules, including the substitution of highly mitochondrial toxic drugs with other antiretroviral combinations comprising less toxic nucleoside analogues.

One of these 'new' HAART schedules comprises the combination of didanosine (ddI) and tenofovir disoproxil fumarate (TDF) – the only nucleotide analogue marketed and which has been reported to be free of adverse effects on mitochondria [11–13]. However, our group has described a surprising and marked mitochondrial toxicity [14] and a risk of dramatic CD4$^+$ T-cell decline [15] related to the combination of these two adenosine analogues with ddI doses of 400 mg/day. These findings are supported by the results of other investigators [16]. The reduction in ddI degradation, which occurs when purine nucleoside phosphorylase (PNP) is inhibited by phosphorylated metabolites of tenofovir [17], could explain the marked elevation of plasma and intracellular levels of ddI during co-administration with TDF [18–20]. The maintenance of increased ddI levels achieved in regimens that include high doses of ddI together with TDF could favour ddI-driven mitochondrial damage, despite the favourable mitochondrial toxicity profile of TDF [14].

Pancreatitis can occur in many well-known classical primary mitochondrial diseases and increased pancreatic toxic effects have been described with the combined use of ddI and TDF [21,22]. Moreover, as mentioned earlier, our group and others have also reported a risk of CD4$^+$ T-cell decline with the use of this antiretroviral combination [15,16], but to our knowledge no study has established a possible mitochondrial basis for this deleterious effect.

Pharmacokinetic studies support the need to reduce ddI doses with the administration of this combination. It is currently recommended to prescribe 250 mg of ddI for subjects weighing >60 kg who also receive TDF. In patients receiving this dosage, plasma and intracellular concentrations of ddI diminish to levels similar to those achieved when ddI is administered without TDF [15,23,24]. However, changes in mitochondrial function after ddI dose reduction on administration together with TDF have not been described. The objective of the present study was to investigate the correlation between immunological and mitochondrial changes, measured in peripheral blood mononuclear cells (PBMCs), in patients receiving a ddI+TDF regimen, before and after reducing ddI from 400 mg to 250 mg.

Figure 1. Study design: time points and study periods



ddI, didanosine; TDF, tenofovir disoproxil fumarate.

Methods

Study design and participants

We selected all HIV-infected subjects from our clinical unit who had received ddI (400 mg/day), TDF (300 mg/day) and NVP (400 mg/day) for at least 1 year, and who remained virologically suppressed for at least 12 months after reducing the ddI dose to 250 mg/day. Patients requiring changes in some component of their antiretroviral regimen, other than a ddI dose reduction, were excluded from the study as were those presenting viral failure while using these regimens or receiving concomitant therapies with immune suppressors (hydroxyurea, interferon and ribavirin) or immune modulators (interleukin-2). Other exclusion criteria were changes or discontinuations of therapy, lack of adherence or a follow-up of less than 3 months after the switch. A total of 20 patients met the inclusion criteria.

Mitochondrial and immunological analysis were performed in all 20 patients at three time points: immediately before starting the ddI 400 mg/day plus TDF-based regimen (baseline point); after at least 1 year of this HAART regimen and immediately before the ddI reduction (ddI400+TDF point); and after at least 12 months of having reduced ddI to 250 mg/day (ddI250+TDF point) (Figure 1). In between these time points we analysed changes during two periods of time: the minimum 12-month period while receiving ddI at 400 mg/day plus TDF and NVP between the baseline point and the ddI400+TDF point (ddI400+TDF period); and during the minimum 1-year period of ddI at 250 mg/day plus TDF and NVP between the ddI400+TDF point and ddI250+TDF point (ddI250+TDF period) (Figure 1).

Patients were divided into two groups depending on CD4$^+$ T-cell evolution during the ddI400+TDF period: those presenting a significant decay >50 cells/mm^3 (CD4$^+$ T-cell declined group) and those maintaining or even increasing the T-cell count (CD4$^+$ T-cell preserved group).

Data collection

Epidemiological data and antiretroviral history were recorded retrospectively.

Immunological analyses

To assess changes in immunological parameters the absolute and perceptual number of $CD4^+$ and $CD8^+$ T-cells were collected at baseline and at the ddI400+TDF and ddI250+TDF time points.

Mitochondrial parameters

At the same three study points of the follow up (see Figure 1), 20 ml of venous peripheral blood were obtained for the isolation of PBMCs with Ficoll density gradient centrifugation. Different mitochondrial parameters were measured in these cells.

The mtDNA content was measured by quantitative real-time PCR. A fragment of the mitochondrial-encoded ND2 gene was amplified separately and levels monitored with respect to a fragment of the nuclear-encoded housekeeping 18S rRNA gene: the result was expressed as the ND2/18S rRNA ratio [25].

Cytochrome *c* oxidase (COX) or mitochondrial respiratory chain (MRC) complex IV (EC 1.9.3.1) enzymatic activity were measured by spectrophotometry and expressed as nmols/min/mg protein [26,27].

Mitochondrial protein synthesis was measured by western blot quantification of COX-II subunit abundance (mitochondrially encoded, transcribed and translated) with respect to the amount of two nuclear-encoded proteins, either mitochondrially (COX-IV subunit) or cytoplasmatically located (β-actin) [28] and expressed as COX-II/COX-IV or COX-II/β-actin ratios.

Study endpoints

Immunological endpoints

Changes in the immunological parameters were analysed during three study time points – the ddI400+TDF period, the ddI250+TDF period, and between baseline and the ddI250+TDF point – to determine changes before and after exposure to the whole combination.

Mitochondrial endpoints

Changes in mitochondrial parameters were investigated during the ddI400+TDF and ddI250+TDF periods. Variations during the ddI400+TDF period have already been partially published by our group [14]. The current manuscript includes complementary information regarding this period, including the analysis of mitochondrial protein synthesis (COX-II expression study) and mitochondrial parameter changes depending on $CD4^+$ T-cell evolution, in order to correlate mitochondrial dysfunction with immunological deterioration. All these studies were also performed during the ddI250+TDF period and have not been previously published.

Statistical analysis

Qualitative variables are expressed as a percentage of change or as a percentage of the remaining content with respect to the baseline measurement, which was considered 100%. Quantitative variables are expressed as mean (±SD). For these quantitative variables, differences for each immunological or mitochondrial parameter between time-points for all studied patients (Figures 2 and 3) or for all patients in the same group (Figures 4 and 5) were assessed using the paired-samples t-test for comparison of the mean of repeated measures. By contrast, differences between groups on the same time-point (Figures 4 and 5) were assessed using the independent-samples t-test for the comparison of the mean for independent measures. Associations between quantitative variables were assessed by means of linear regression (Figures 4 and 5). In all cases, *P*-values are quoted in the text and the respective figures and were considered statistically significant when *P*-values were <0.05.

Results

A total of 20 HIV-infected men with a mean age of 37 ±6 years were included in the study. At inclusion, all patients had an undetectable viral load and a mean $CD4^+$ T-cell count of 733 ±243 cells/mm^3. All were antiretroviral-experienced individuals with a mean time of exposure of 68 ±43 months to NRTIs, 42 ±18 months specifically to ddI and 9 ±13 months to a combination including stavudine (d4T)+ddI. The epidemiological characteristics and antiretroviral history of the patients are described in Table 1.

The ddI400+TDF period

Immunological outcome

Considering all patients included, the mean absolute $CD4^+$ T-cell count decreased during the ddI400+TDF period from 733 ±243 to 626 ±213 cells/mm^3 ($P=0.05$; Figure 2) and the mean percentage of change in $CD4^+$ T-cell count was a significant -15%.

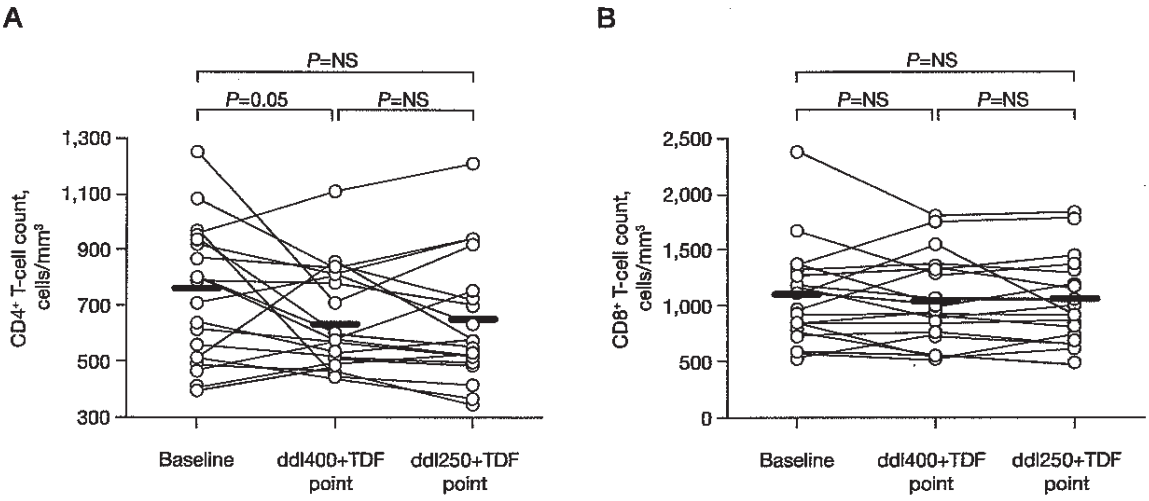
The $CD4^+$ T-cell declined group was composed of half of the subjects (10/20) who showed a significant -36% decline in $CD4^+$ T-cell count ($P=0.001$) compared with the $CD4^+$ T-cell preserved group (10/20) who presented a mean increase of 15% in $CD4^+$ T-cell count. The statistical analysis was considered not significant [NS] when *P*-values were >0.05.

$CD8^+$ T-cell count did not vary significantly during this period (from 1,084 ±434 to 1,046 ±395 at the ddI400+TDF point; *P*=NS).

Mitochondrial outcome

A general decline in all mitochondrial parameters was observed during this period (Figure 3). With respect to baseline (considered as 100%), treatment with

Figure 2. Immunological parameters measured throughout the study for all patients



(A) CD4+ T-cell count and (B) CD8+ T-cell count. ddI, didanosine; NS, not significant ($P>0.05$); TDF, tenofovir disoproxil fumarate.

TDF+ddI400+NVP was associated with a significant decrease in mtDNA (63% content; $P=0.001$), COX activity (53%; $P=0.01$) and mitochondrial protein synthesis (measured by the COX-II/β-actin or COX-II/COX-IV ratio, 32% and 69% expression, respectively; $P=0.006$ in both cases).

Mitochondrial parameter status differed depending on CD4+ T-cell evolution throughout the ddI400+TDF period (Figure 4). Mitochondrial impairment was higher in the CD4+ T-cell declined group who showed a significant reduction in mtDNA content, COX activity, COX-II//β-actin and COX-II/COX-IV ratio (51% [$P=0.007$], 37% [$P=0.020$], 19% [$P=0.013$] and 57% [$P=0.041$] content, respectively). In the CD4+ T-cell preserved group only the decrease in the COX-II/COX-IV ratio achieved statistical significance (76% expression; $P=0.016$).

Nonetheless, when we compared mitochondrial parameters between the two groups of patients at the ddI400+TDF point, only COX activity was found to be significantly decreased in the CD4+ T-cell declined group (37% versus 76% COX activity; $P=0.039$).

When we analysed mitochondrial changes with respect to CD4+ T-cell count change, COX activity again showed a direct (positive) and significant association with CD4+ T-cell evolution (Pearson's correlation significance of $P=0.004$).

The ddI250+TDF period

Immunological outcome

Considering all the subjects included, the mean CD4+ T-cell count at the ddI250+TDF point did not vary from the ddI400+TDF point (626 ±213 cells/mm^3 to 638 ±223 cells/mm^3; $P=0.696$). Only 35% of the total number of patients included (7/20) recovered >50 CD4+ T-cells/mm^3 during the ddI250+TDF period and only the same percentage of individuals (35% or 7/20) achieved CD4+ T-cell counts similar to those at baseline (established as a difference <50 cells/mm^3 compared with original counts).

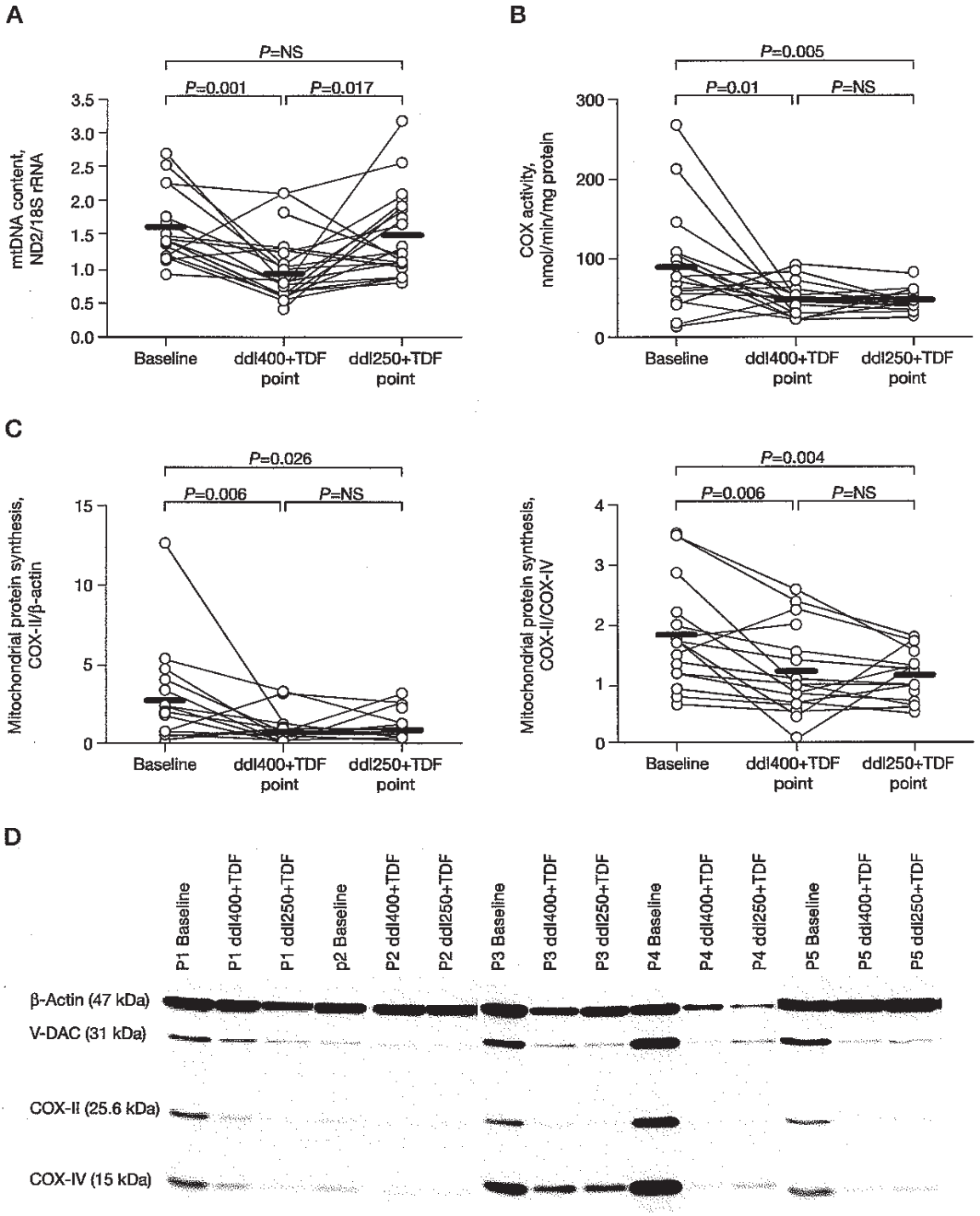
When we considered the CD4+ T-cell declined group, the mean CD4+ T-cell value at the ddI250+TDF point increased almost to statistical significance (from 558 ±199 cells/mm^3 to 619 ±197; $P=0.1$) compared with the CD4+ T-cell preserved group of patients (693 ±215 versus 657 ±255; $P=0.4$). Among the CD4+ T-cell declined group, 50% of patients recovered >50 CD4+ T-cells/mm^3 during the ddI250+TDF period (5/10) and 20% achieved baseline CD4+ T-cell counts (1/5).

The mean CD8+ T-cell count at the ddI250+TDF point was 1,030 ±387 cells/mm^3, with no differences with respect to the two previous points ($P=0.7$ and $P=0.315$, respectively).

Mitochondrial outcome

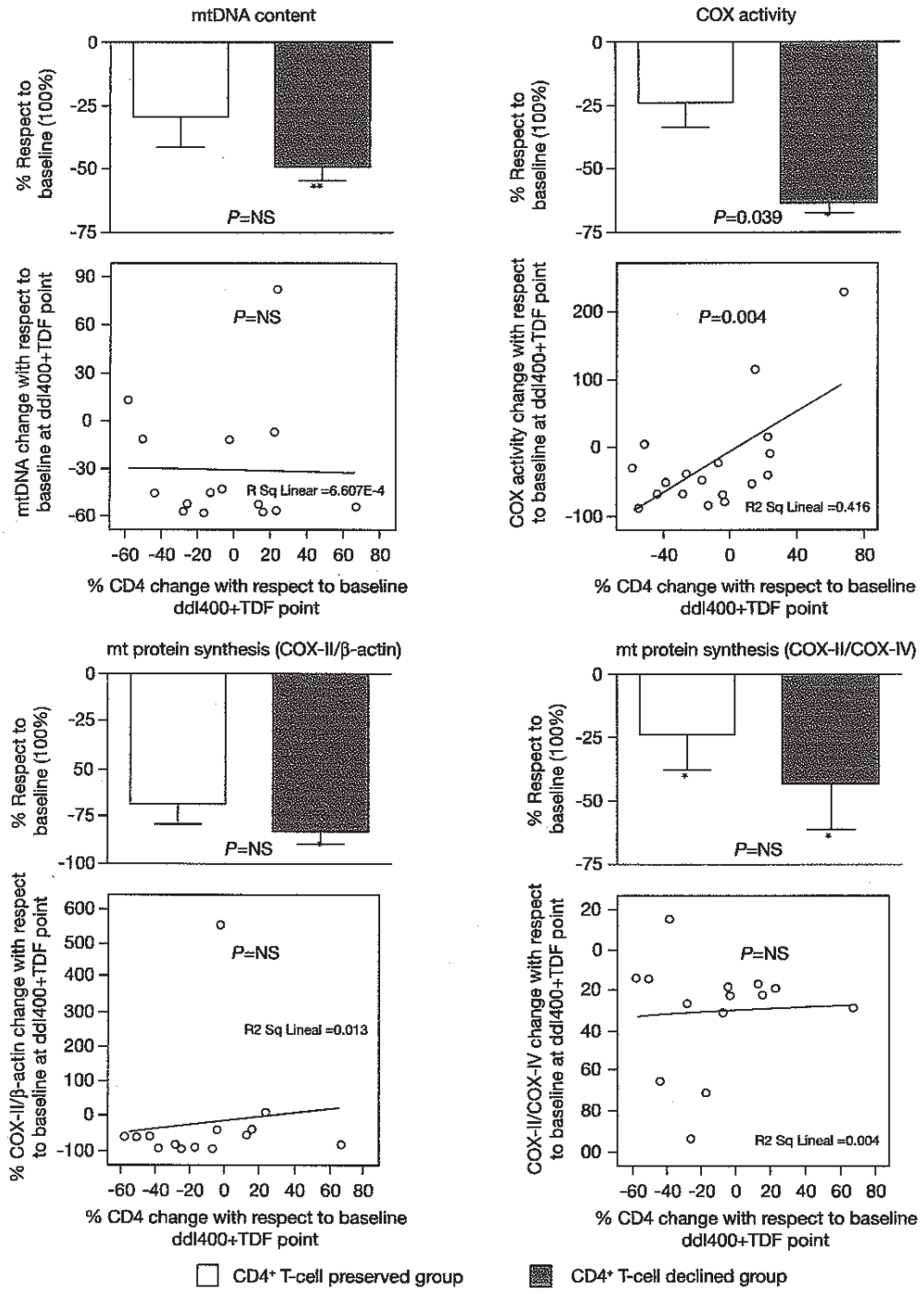
At the ddI250+TDF point, the mtDNA rose to 93% of its original value ($P=0.017$ compared with the ddI400+TDF point and P=NS with respect to baseline). Conversely, COX activity and mitochondrial protein synthesis maintained similar values to those observed at the ddI400+TDF point (-4% for COX activity, +4% for COX-II/β-actin content and -3% for COX-II/COX-IV quantity; P=NS in all cases). Consequently, except for mtDNA content, mitochondrial parameters

Figure 3. Changes in mitochondrial parameters for all the patients included in the study



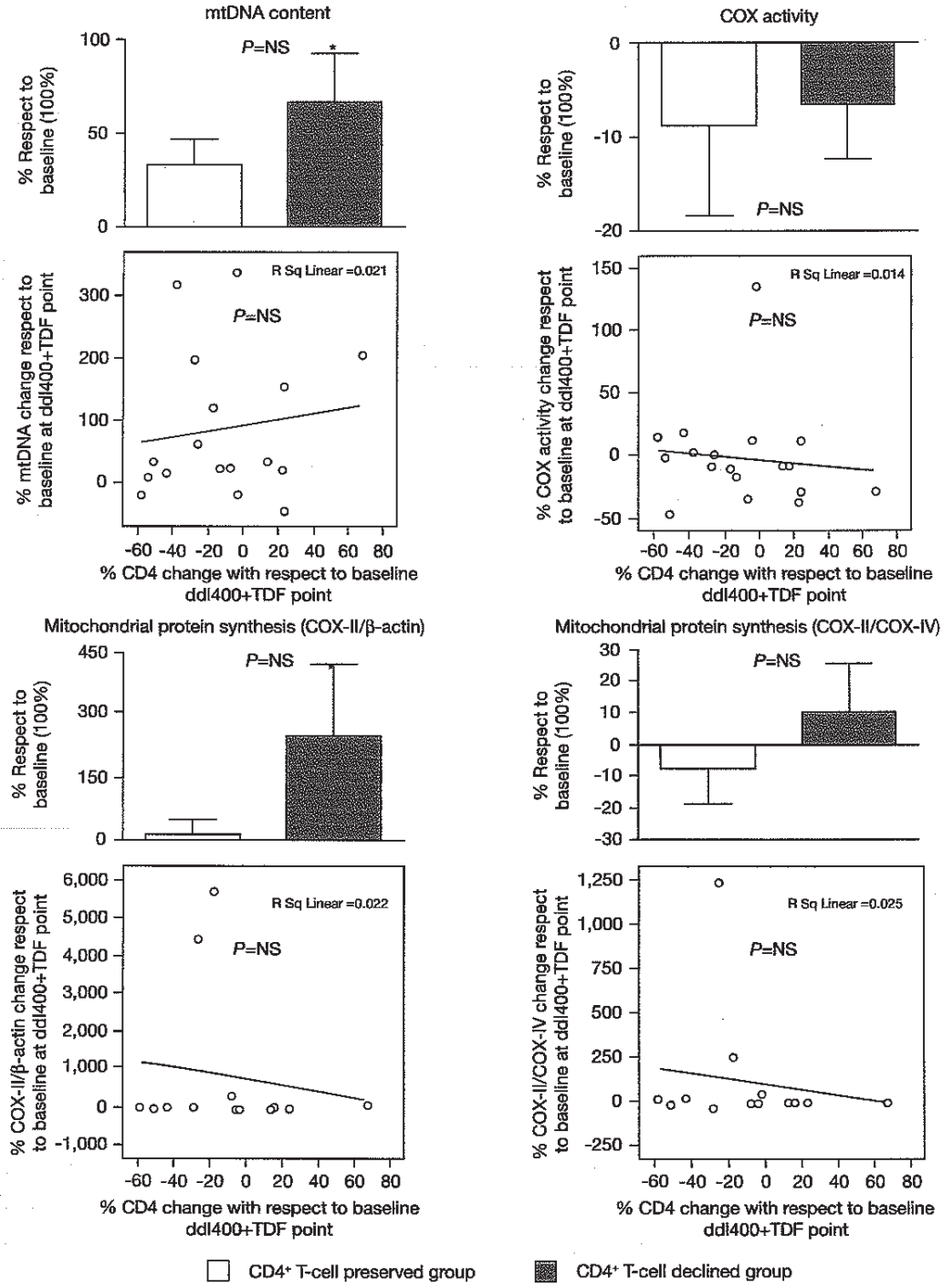
(A) Mitochondrial DNA (mtDNA) content, (B) cytochrome *c* oxidase (COX) activity, and (C) mitochondrial protein synthesis (mitochondrial-encoded COX-II subunit expression compared with nuclear-encoded and mitochondrially located COX-IV or nuclear-encoded and cytoplasmically located β-actin content) for all patients included in the study. (D) Western blot depicting expression of four proteins (β-actin, V-DAC, COX-II and COX-IV) at the three different time points (baseline, ddI400+TDF and ddI250+TDF). P1, P2, P3 refer to patients 1, 2, 3 and so on. ddI, didanosine; NS, not significant; TDF, tenofovir disoproxil fumarate; V-DAC, voltage-dependent anion channel.

Figure 4. Mitochondrial parameters measured during the ddI400+TDF period for the CD4⁺ T-cell declined and CD4⁺ T-cell preserved groups of patients



Percentage of mitochondrial change with respect to baseline (100%) depending on development or not of CD4⁺ T-cell decay (upper panels) and relationship between changes in CD4⁺ T-cell count and changes in mitochondrial parameters (lower panels). The differences between the ddI400+TDF point and baseline measurement for each group are indicated below each bar (*P<0.05; **P<0.001) and differences between the two groups of patients at the ddI400+TDF point are shown at the bottom of each upper panel. In lower panels data fitting to the linear regression model are shown by R2 Sq lineal coefficients and association between parameters by the statistical signification P-value. COX, cytochrome *c* oxidase; ddI, didanosine; mtDNA, mitochondrial DNA; NS, not significant (P>0.05); TDF, tenofovir disoproxil fumarate.

Figure 5. Mitochondrial parameters during the ddI250+TDF period for the CD4⁺ T-cell declined and CD4⁺ T-cell preserved groups of patients



Percentage mitochondrial change with respect to baseline (100%) depending on development or not of CD4⁺ T-cell decay (upper panels). Relationship between changes in CD4⁺ T-cell count and changes in mitochondrial parameters (lower panels). The differences between the ddI400+TDF point and ddI250+TDF point for each group are indicated at the bottom of each bar (*$P<0.05$) and differences between the two groups of patients at the ddI250+TDF point is shown at the bottom of each upper panel. In lower panels data fitting to the linear regression model are shown by R2 Sq lineal coefficients and association between parameters by the statistical signification P-value. COX, cytochrome c oxidase; ddI, didanosine; mtDNA, mitochondrial DNA; NS, not significant ($P>0.05$); TDF, tenofovir disoproxil fumarate.

remained significantly or almost significantly decreased at the ddI250+TDF point with respect to baseline measurement (49% COX activity, P=0.005; 37% COX-II/β-actin content, P=0.026; and 66% COX-II/COX-IV quantity, P=0.004; Figure 3).

Again mitochondrial evolution was different in patients belonging to the CD4⁺ T-cell declined group or CD4⁺ T-cell preserved group (Figure 5). Mitochondrial parameters showed a stronger trend towards amelioration for the CD4⁺ T-cell declined group of patients, although statistical significance was only achieved by mtDNA content.

Discussion

A reduction in ddI dose from 400 mg to 250 mg per day has been recommended when using TDF in the same HAART schedule [19,23,24] as the use of both analogues at high ddI dosage has been shown to be associated with serious toxic effects [15,16,21], such as a decline in CD4⁺ T-cells [15,16]. Many authors have demonstrated that after this intervention both increased plasma and intracellular levels of ddI become similar to those observed when ddI is used without TDF [18,23,24]. At the same time, a decreased rate of related toxicities has also been reported when recommended doses of ddI are used together with TDF [29,30]. However, it has not been assessed whether the simple reduction of ddI doses is enough to recover CD4⁺ T-cells and mitochondrial parameters in patients who have shown a significant decline when using a previous HAART combination including ddI at 400 mg/day and TDF.

Our current findings confirm the previously reported immunological and mitochondrial toxicity of ddI at 400 mg/day plus TDF-based regimens [14–16], not only on genetic or enzymatic mitochondrial measurements but also on mitochondrial protein synthesis. Of patients on a mean 14-month course of this antiretroviral combination, 50% showed significant CD4⁺ T-cell decay while conserving CD8⁺ T-cell values, excluding virological causes of immunological failure. All the mitochondrial parameters evaluated showed an important decrease during this antiretroviral treatment period, especially in patients showing a significant CD4⁺ T-cell decline. Mitochondrial and CD4⁺ T-cell evolution were consequently matched during this period. Patients presenting a greater CD4⁺ T-cell depletion also showed increased mitochondrial lesion and mitochondrial function was better preserved in patients who conserved CD4⁺ T-cell counts, suggesting that the underlying mechanism of CD4⁺ T-cell damage may be mitochondrial toxicity of nucleoside analogues.

Our results also demonstrate a certain improvement in CD4⁺ T-cell count after 14 months of ddI dose reduction from 400 to 250 mg/day in patients presenting a relevant CD4⁺ T-cell decline while receiving ddI at 400 mg/day plus TDF and NVP, but they did not achieve total recovery. Among the patients included, only 50% of those showing a previous significant reduction in CD4⁺ T-cell count during the period on high ddI dose recovered >50 cells/mm^3 after at least 1 year of ddI dose reduction, and only 20% achieved baseline CD4⁺ T-cell values. These immunological results are further supported by mitochondrial data. Although a total recovery of mtDNA was seen after ddI dose modification, the remaining mitochondrial parameters did not improve, suggesting that mechanisms other than DNA pol-γ inhibition (classically believed as being responsible for NRTI-mediated mitochondrial toxicity) could be responsible for maintaining mitochondrial damage and T-cell CD4⁺ cytotoxicity.

The existence of alternative antiretroviral-mediated mechanisms to regulate or alter mitochondrial gene expression, independently of the quantity of mtDNA, has been previously reported, both *in vivo* [6,28] and *in vitro* [31] and at the transcriptional and translational level. In the present study, mitochondrial protein expression correlated with mtDNA content decrease during the ddI400+TDF period. This data could indicate the absence of upregulatory mechanisms to compensate for mtDNA depletion [28] and suggests the presence of extensive mitochondrial damage throughout the different stages of the mitochondrial protein homeostasis system, which finally affects mitochondrial function at a biochemical level.

The etiopathogenic mechanism of cytotoxicity and CD4⁺ T-cell decline in HIV-infected patients receiving full doses of ddI plus TDF is currently better understood. It is most likely that mitochondrial dysfunction induced by some antiretroviral drugs, other than the mitochondrial-driven apoptosis

Table 1. Antiretroviral history and baseline epidemiological, virological and immunological data of patients included in the study

Characteristic	Value
Age, years (±SD)	37 (±6)
Male gender, *n* %	20 (100)
CD4⁺ T-cell count, cells/mm^3 (±SD)	733 (±243)
HIV RNA <50 copies/ml, *n* (%)	20 (100)
Patients on ddI+d4T at baseline, *n* (%)	7 (35)
Patients on ddI+other NRTIs at baseline, *n* (%)	15 (75)
Time on treatment at baseline	
Receiving NRTIs, months (±SD)	68 (±43)
Receiving ddI, months (±SD)	42 (±18)
Receiving ddI+d4T, months (±SD)	9 (±13)

ddI, didanosine; NRTI, nucleoside reverse transcriptase inhibitor, d4T, stavudine.

caused by HIV itself, plays an important role [32,33]. Antiretroviral-mediated mitochondrial toxicity could affect different types of body cells (if not all), but HIV tropism preferentially chooses immunological cells, including CD4+ T-cells. We demonstrate a direct positive correlation between mitochondrial damage and CD4+ T-cell toxicity in these patients: those presenting a negative effect on CD4+ T-cell counts during the nucleoside combination showed a significantly higher decrease in all the PBMC mitochondrial parameters measured than those who did not. Therefore, the cytotoxic effect on CD4+ T-cells could be directly related to the mitochondrial toxicity of the antiretrovirals, although HIV-induced damage could preferentially and specifically locate this deleterious effect in CD4+ T-cells. A similar combined mechanism has been proposed in adipocytes as the etiopathogenic basis of lipoatrophy [34]. The shorter CD4+ T-cell life and increased cell replacement rate with respect to CD8+ T-cells could also make CD4+ T-cells more vulnerable to toxicity. Different mitochondrial effects have been previously reported among CD4+ and CD8+ T-cell populations [35]. This finding should motivate clinicians to replace those antiretrovirals with a high mitochondrial toxicity profile with less toxic drugs, to avoid or delay long-term toxicities, which may otherwise be difficult to revert.

An interesting finding was that immunological or mitochondrial changes did not appear immediately after drug intervention. Likewise, the decline in CD4+ T-cells and mitochondrial parameters was not detected until at least 24 weeks on ddI400+TDF [15]. Indeed, many subjects continued presenting a CD4+ T-cell decline during the first weeks after ddI dose reduction. These findings support the theory that this phenomenon might be attributed to an accumulative toxicity [15]. The long-term intracellular half-life of tenofovir diphosphate, which can be greater than 4 weeks [18], and of ddI [36] may explain the slow CD4+ T-cell recovery over time after reduction of the ddI dose or ddI substitution [37]. Indeed, most mitochondrial parameters require a lengthy time to change status after toxic disruption [35,38,39], and if mitochondrial toxicity was the basis of CD4+ T-cell decline this would explain the slow change in immunological parameters. It is necessary to perform complete mitochondrial toxicity studies based on measuring different mitochondrial parameters to better understand mitochondrial toxicity mechanisms.

In conclusion, ddI dose reduction can help to improve CD4+ T-cell count and mitochondrial function in NRTI-experienced HIV-infected patients receiving ddI and TDF-based regimens; however, this intervention does not allow a total recovery of immunological or mitochondrial parameters, as previously reported using other interventions to reduce nucleoside-related mitochondrial damage [40]. Patients starting an antiretroviral treatment at recommended ddI doses together with TDF, who have never received high doses of ddI and TDF before, should also be studied. A long-term follow up is necessary to ascertain whether this recommended combination is totally free of adverse mitochondrial and immunological effects. Mitochondrial and CD4+ T-cell toxicity of the ddI plus TDF combination has been demonstrated to be a long-term effect, and the delay in toxic manifestations could disguise the toxic effects of this schedule, even at low ddI doses.

Finally, new antiretroviral drugs or HAART combinations should not only take antiviral efficiency into consideration, but should also consider the lack of side effects for mitochondria and cell toxicity to avoid long-term treatment of chronic toxicities that become difficult to revert.

Acknowledgements

This work was supported by grants from the Foundation for Research and Prevention of AIDS in Spain (FIPSE 36612-06 and FIPSE 36572-06), Fondo de Investigaciones Sanitarias (FIS 40381/04), Support to Research Groups (2005/SGR/0300) and CIBER de Enfermedades Raras (CIBERER, Barcelona, Spain).

Disclosure statement

The authors declare no conflicts of interest.

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Accepted for publication 21 October 2007

RESUM DEL TERCER ESTUDI

El TDF és un antiretroviral de baixa toxicitat mitocondrial, però la interacció farmacocinètica amb ddI augmenta la disponibilitat i els efectes nocius del ddI. Estudis previs de la combinació ddI a 400 mg/d+TDF han associat aquest règim a una important toxicitat mitocondrial (López S et al, *AIDS Res Hum Retrov* 2006) i una reducció dramàtica en el nombre de limfòcits T $CD4^+$ al cap d'uns mesos de tractament amb aquesta pauta (Negredo E et al, *AIDS* 2004; León A et al, *AIDS* 2005 gener i octubre). L'objectiu del present estudi és avaluar els mecanismes de toxicitat mitocondrial d'aquest règim i determinar els efectes immunològics i mitocondrials de reduir la dosi de ddI de 400 a 250 mg/d en 20 pacients VIH-positius asimptomàtics sota tractament amb ddI+TDF+NVP. En aquests pacients s'ha analitzat l'estat immunològic (nombre de limfòcits T $CD4^+$i T $CD8^+$) i mitocondrial en CMSP (contingut en ADNmt, activitat enzimàtica del complex IV -COX- de la CRM i taxa d'expressió proteica mitocondrial de COX-II) a nivell basal (abans d'iniciar aquest esquema terapèutic), després de 14 mesos en tractament amb ddI400 mg/d+TDF+NVP i 14 mesos després de reduir la dosi de ddI a 250 mg/d.

A nivell immunològic, durant el període amb ddI a 400 mg/d, s'observen 2 subgrups de pacients clarament diferenciats segons l'evolució de limfòcits T $CD4^+$: en un 50% de pacients decau el nombre de cèl·lules (una mitja del -36%) i en l'altre 50% es manté o puja (una mitja del +15%). A nivell mitocondrial observem que durant el període amb ddI a 400mg/d decauen significativament tots els paràmetres estudiats quan analitzem tots els pacients conjuntament (63% contingut en ADNmt, 53% activitat COX, 32% expressió COX-II/β-Actina i 69% COX-II/COX-IV). Però si els dividim en els 2 subgrups que presenten diferent evolució de limfòcits T $CD4^+$, observem major disfunció mitocondrial pel subgrup que presenta major depleció cel·lular (51% contingut ADNmt, 37% activitat COX, 19% expressió COX-II/β-Actina i 57% COX-II/COX-IV), on tots els paràmetres disminueixen significativament. En canvi en el subgrup amb limfòcits T $CD4^+$ mantinguts, només la taxa d'expressió COX-II/COX-IV disminueix significativament un 76%.

Després de 14 mesos amb ddI a 250 mg/d, només milloren el recompte de cèl·lules T $CD4^+$ en el subgrup de pacients que n'havia perdut més, que són els que presenten una millora més accentuada de l'estat mitocondrial, però no completa. De fet, el contingut en ADNmt és l'únic paràmetre que es recupera totalment. El nombre de limfòcits T $CD8^+$ es manté estable al llarg de tot l'estudi.

CONCLUSIONS DEL TERCER ESTUDI

1. Els paràmetres mitocondrials i el nombre de limfòcits T $CD4^+$ presenten una evolució paral·lela que reforça la idea de que l'estat mitocondrial està relacionat amb la viabilitat cel·lular, que possiblement es veu amenaçada per l'estreta relació existent entre mitocondri i apoptosi.

2. La reducció de dosi de ddI de 400 a 250 mg/d en un règim consistent en ddI+TDF+NVP s'associa a la millora (però no la completa recuperació) de l'estat immunològic i mitocondrial.

3. La restauració dels nivells d'ADNmt no es tradueixen en la millora global de l'estat mitocondrial.

4. La manca total de recuperació de la funció mitocondrial fa pensar que la toxicitat antiretroviral acumulada en aquests pacients durant molts anys de tractament i la potència mitotòxica dels 14 mesos de tractament amb ddI400mg/d+TDF+NVP és massa gran per poder ser revertida amb una intervenció menor com pot ser la reducció de la dosi d'un dels components del TARGA.

5. De nou, destacar la necessitat de realitzar estudis de funcionalisme mitocondrial complets que no es limitin a la mesura del contingut en l'ADNmt, doncs la funció i la genètica mitocondrial no es comporten de nou de manera paral·lela.

REVERSIBLE INHIBITION OF MITOCHONDRIAL PROTEIN SYNTHESIS DURING LINEZOLID-RELATED HYPERLACTATEMIA

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY 2007; 51(3); 962-967

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El **QUART** dels treballs inclosos en la present tesi doctoral planteja com a:

HIPÒTESI

El tractament amb nous antibiòtics que eviten la proliferació bacteriana inhibint la traducció procariota presenta efectes secundaris (com la hiperlactatèmia) similars als de les mitocondriopaties primàries. Donada la similitud entre ribosomes mitocondrials i bacterians, l'alteració en el funcionament de la traducció mitocondrial podria ser la base d'aquests efectes adversos, que haurien de revertir en retirar el tòxic.

OBJECTIU

Determinar el mecanisme causal de la toxicitat manifesta en forma d'hiperlactatèmia en pacients sota tractament amb l'antibiòtic linezolid, inhibidor de la síntesi proteica bacteriana, mitjançant l'anàlisi de la funció mitocondrial global (contingut en mitocondris i en ADNmt, activitat oxidativa global i potencial de membrana, taxa de transcripció i expressió proteica i activitat del complex IV -COX- de la CRM), així com determinar la reversibilitat dels seus efectes.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2007, p. 962–967 Vol. 51, No. 3
0066-4804/07/$08.00+0 doi:10.1128/AAC.01190-06

Reversible Inhibition of Mitochondrial Protein Synthesis during Linezolid-Related Hyperlactatemia▽

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Received 22 September 2006/Returned for modification 2 November 2006/Accepted 18 December 2006

The objective of the present study was to determine the mitochondrial toxicity mechanisms of linezolid-related hyperlactatemia. Five patients on a long-term schedule of linezolid treatment were studied during the acute phase of hyperlactatemia and after clinical recovery and lactate normalization following linezolid withdrawal. Mitochondrial studies were performed with peripheral blood mononuclear cells and consisted of measurement of mitochondrial mass, mitochondrial protein synthesis homeostasis (cytochrome *c* oxidase [COX] activity, COX-II subunit expression, COX-II mRNA abundance, and mitochondrial DNA [mtDNA] content), and overall mitochondrial function (mitochondrial membrane potential and intact-cell oxidative capacity). During linezolid-induced hyperlactatemia, we found extremely reduced protein expression (16% of the remaining content compared to control values [100%], $P < 0.001$) for the mitochondrially coded, transcribed, and translated COX-II subunit. Accordingly, COX activity was also found to be decreased (51% of the remaining activity, $P < 0.05$). These reductions were observed despite the numbers of COX-II mitochondrial RNA transcripts being abnormally increased (297%, $P = 0.10$ [not significant]) and the mitochondrial DNA content remaining stable. These abnormalities persisted even after the correction for mitochondrial mass, which was mildly decreased during the hyperlactatemic phase. Most of the mitochondrial abnormalities returned to control ranges after linezolid withdrawal, lactate normalization, and clinical recovery. Linezolid inhibits mitochondrial protein synthesis, leading to decreased mitochondrial enzymatic activity, which causes linezolid-related hyperlactatemia, which resolves upon discontinuation of linezolid treatment.

Linezolid belongs to a family of antibiotics (oxazolidinones) that inhibit bacterial protein synthesis by binding to 23S rRNA in the large ribosomal subunit and preventing the fusion of 30S and 50S ribosomal subunits and the formation of the translation initiation complex (1). It has shown excellent efficacy against gram-positive cocci, including *Staphylococcus aureus*, coagulase-negative staphylococci, enterococci, and streptococci, with MICs ranging from 0.5 to 4 mg/liter (16). Furthermore, linezolid has 100% oral bioavailability and reaches high concentrations at different sites (skin, synovial fluid, bone, cerebrospinal fluid, or lung), thus being a good alternative for the long-term treatment of orthopaedic implant infections, ventriculo-peritoneal shunts, and other infections related to foreign bodies in which gram-positive cocci are the main pathogens.

However, a major concern with this antibiotic is its safety profile, especially when it is administered for more than the 28-day period approved by the U.S. Food and Drug Administration (6, 15). Adverse events of linezolid include hematological disturbances (especially thrombocytopenia and anemia), peripheral neuropathy, hyperlactatemia, and metabolic acidosis (1, 2, 4, 6, 11, 12, 15, 23, 30). Some of these events could be related to the capacity of linezolid to interfere with mitochondrial function (25, 29) due to similarities between bacterial and mitochondrial ribosomes. Experimental reports support this hypothesis. Nagiec et al. reported the inhibition of mitochondrial protein synthesis in human erythroleukemia cells exposed to eperezolid, another oxazolidinone (24). More recently, other authors published reports of decreased numbers of mitochondrial respiratory chain subunits (10) or mitochondrial protein synthesis (19) in different tissue homogenates or isolated mitochondria, respectively, for rats treated with linezolid. All these studies also suggest that this effect is time and concentration dependent, although their cross-sectional experimental design did not allow direct information regarding the capacity of mitochondrial recovery after discontinuation of linezolid treatment to be obtained. Moreover, there are very few data for humans receiving treatment with linezolid that confirm experimental hypotheses. We recently reported a decrease in mitochondrial enzyme activities in peripheral blood mononuclear cells (PBMCs) from three patients on linezolid therapy during the acute phase of symptomatic hyperlactatemia (29). In addition, De Vriese et al. reported decreased enzyme activities for some mitochondrial respiratory chain proteins partially synthesized by mitochondrial ribosomes in the skeletal muscle, liver, and kidney of a patient with severe hyperlactatemia (10). In none of these four cases were data on a mitochondrial outcome after hyperlactatemia resolution presented. We have conducted a prospective longitudinal study to assess the homeostasis of mitochondrial protein synthesis and function in selected patients developing linezolid-related hy-

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▽ Published ahead of print on 28 December 2006.

perlactatemia both during treatment and after discontinuation of linezolid treatment.

MATERIALS AND METHODS

Patients. In our hospital, all patients who receive linezolid for more than 1 month at the standard oral dosage (600 mg/12 h) are closely followed. Clinical symptoms of infection and linezolid-related or possibly related adverse events (anemia, thrombocytopenia, gastrointestinal discomfort, etc.) were monitored, and a hemogram was performed weekly. For the present study, blood lactate (as a surrogate marker of mitochondrial toxicity) was determined at least once a month or when hyperlactatemia was suspected based on clinical findings. When the patients developed hyperlactatemia, with or without clinical symptoms of metabolic acidosis, a blood sample was obtained for mitochondrial studies. In order to avoid confounders of mitochondrial toxicity, critically ill patients and those taking other potentially toxic drugs for mitochondria (i.e., aminoglycosides or antiretrovirals) were excluded from the study. Linezolid was withdrawn when a severe adverse event was observed (a platelet count of $<100{,}000$ cells/mm^3, a hemoglobin concentration of <9 g/liter, or severe gastrointestinal manifestations). An asymptomatic increase in serum lactate was not a criterion for stopping linezolid treatment. For those patients who developed hyperlactatemia, a second sample was obtained for mitochondrial studies once linezolid treatment was discontinued, the patient became asymptomatic, and lactate levels returned to normal. For mitochondrial studies, six healthy volunteers matched by age and gender were included as controls. All individuals signed written consents to be included in the protocol.

Sample collection. For mitochondrial analysis, we obtained 20-ml samples of venous blood during the hyperlactatemic episode during linezolid use and after linezolid discontinuation and hyperlactatemia resolution. PBMCs were isolated by Ficoll's gradient (27), and protein content was measured according to the Bradford protein-dye binding-based method (5).

Determination of mitochondrial mass. The quantity of mitochondria was estimated by two different methodologies. One was spectrophotometric measurement of the activity of citrate synthase (EC 4.1.3.7, nmol/min/mg protein), 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 (3, 26, 28, 31). The other method for measurement of mitochondrial mass was Western blot analysis of V-DAC (a nuclear-encoded mitochondrial structural protein) amount, corrected by β-actin (a nuclear-encoded cytoplasmic protein) content (22).

Studies on mitochondrial protein synthesis homeostasis. (i) Mitochondrial respiratory chain complex IV (COX, EC 1.9.3.1) enzyme activity. Enzyme activity was measured spectrophotometrically according to the methodology of Rustin et al. (28), slightly modified for complex IV for minute amounts of biological samples (20). Cytochrome *c* oxidase (COX) activity was expressed in absolute values (nmol/min/mg protein) as well as in values relative to mitochondrial mass (by dividing absolute values by citrate synthase activity values).

(ii) Mitochondrial protein synthesis. We assessed the mitochondrial protein synthesis of the COX-II subunit (mitochondrially encoded, transcribed, and translated) by Western blot analysis (22). This expression was normalized by the content of the β-actin signal to establish the relative COX-II abundance per overall cell protein as well as per V-DAC content to establish the relative COX-II abundance per mitochondrion.

(iii) mtRNA quantification. Total RNA was obtained by an affinity column-based procedure (RNeasy; QIAGEN). RNA (1 μg/sample) was reverse transcribed (TaqMan reverse transcriptase; Applied Biosystems) using random primers. The real-time PCR was performed using TaqMan universal master mix (Applied Biosystems) in an ABI PRISM 7700 sequence detection system, in a total reaction volume of 25 μl. Quantification of COX-II mRNA was performed using the amplification conditions indicated by the supplier (Assay-by-Design; Applied Biosystems); the primers were CAAACCACTTTCACCGCTACAC (forward) and GGACGATGGGCATGAAACTGT (reverse), and the 6-carboxyfluorescein-labeled probe was AAATCTGTGGAGCAAACC. As a reference control, housekeeping nuclear-encoded 18S rRNA abundance was determined using a premade kit (Assay-on-Demand, Hs99999901_s1; Applied Biosystems). Absolute COX-II mitochondrial RNA (mtRNA) content was expressed as the ratio of the mean COX-II mRNA value to the mean 18S rRNA value (13), while COX II mtRNA content relative to mitochondrial mass was calculated by dividing absolute values by citrate synthase activity values.

(iv) mtDNA quantification. Total DNA was obtained by a standard phenol-chloroform extraction procedure. Fragments of the mitochondrial ND2 gene and the nuclear rRNA 18S gene were amplified in duplicate and separately by quantitative real-time PCR as previously reported (21). The absolute content of mtDNA was expressed as the mtDNA-to-nuclear-DNA ratio (ND2 mtDNA/18S

TABLE 1. Clinical characteristics of individuals included in the study

Patient	Age (yr)/sex[d]	Comorbidity	Infection location	Etiology of infection	Serum lactate level[a]	No. of days on linezolid[b]	Adverse event(s)	Linezolid treatment stopped due to adverse event	Serum lactate level[c]
1	25/M	Knee osteosarcoma	Knee prosthesis	Vancomycin-resistant *Enterococcus faecium*	59	90	None	No	14
2	75/M	Waldenstrom macroglobulinemia	Central nervous system	*Nocardia otitidiscaviarum*	28	80	Anemia, moderate asthenia	Yes	19
3	65/M	Diabetes mellitus	Knee prosthesis	Methicillin-resistant *Staphylococcus epidermidis*	44	35	None	No	15
4	74/F	Sd. Sjögren	Hip prosthesis	Methicillin-resistant *Staphylococcus aureus*	29	44	Mild asthenia	No	17
5	83/M	None	Knee prosthesis	Methicillin-resistant *Staphylococcus epidermidis*	43	30	Severe gastrointestinal discomfort	Yes	17

[a] The highest value during linezolid therapy (mg/liter).
[b] At the time of the first sample collection for mitochondrial studies.
[c] At least 15 days after discontinuation of linezolid (mg/liter).
[d] M, male; F, female.

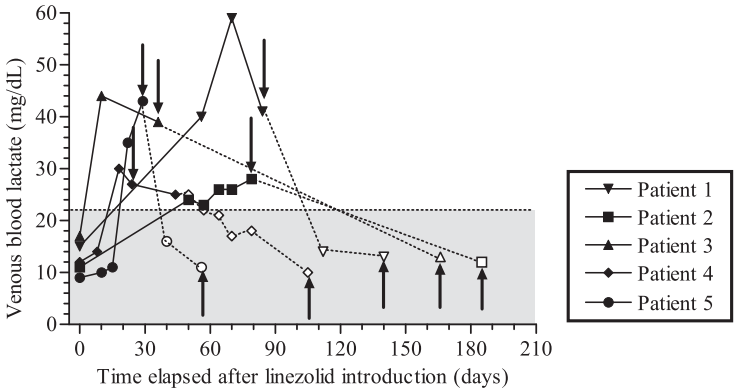

FIG. 1. Lactate levels (lines) and sample collection for mitochondrial studies (downward arrows, first study; upward arrows, second study) for the patients developing linezolid-related hyperlactatemia. Dashed lines for patient lactate evolution correspond to the interval period between mitochondrial studies, and the shaded square area corresponds to the lactate normality level.

nuclear DNA), and mtDNA content relative to mitochondrial mass was obtained after absolute values were divided by citrate synthase activity values.

Studies on overall cellular function. (i) Mitochondrial membrane potential. Fresh PBMCs (750,000) were stained with 2.5 μg/ml of JC-1 probe (T-3168; Molecular Probes, Eugene, OR) in 10% fetal bovine serum-complete RPMI medium. The resulting cells were washed and analyzed by flow cytometry (FACScan cytometer; Becton Dickinson, San Jose, CA) on FL1 and FL2 channels (9). The results were expressed as percentages of PBMCs with depolarized mitochondria with respect to the total number of PBMCs analyzed.

(ii) Spontaneous mitochondrial oxidative activity. Oxygen usage was measured polarographically using a Clark electrode in a water-jacketed cell magnetically stirred at 37°C (Hansatech Instruments Limited, Norfolk, England) in 250 μl of respiration buffer containing 100 to 200 μg of fresh cells. Intact-cell endogenous substrate oxidation was assessed as spontaneous PBMC oxygen uptake. The complete methodology is reported elsewhere (21, 28).

Statistical analysis. Results were expressed as means ± standard deviations (SD) and/or the percentages compared to control values (which were arbitrarily assigned values of 100%). The *t* test for independent or paired measures (as needed) was used to search for differences.

RESULTS

From January to December 2005, 5 out of 13 patients receiving linezolid therapy for more than 1 month developed hyperlactatemia and were included in the present study. Mitochondrial enzymatic activity and spontaneous cell oxidation during the acute phase of hyperlactatemia in two patients have previously been reported (29). In those two cases, results for mitochondrial DNA, RNA, and protein synthesis as well as complete mitochondrial functionality studies were added during and after hyperlactatemia resolution. The clinical characteristics and serum lactate values determined during and after discontinuation of linezolid treatment are shown in Table 1 and Fig. 1. As can be seen, three patients were symptomatic, and in two, linezolid treatment was prematurely interrupted due to adverse events.

The mitochondrial masses were similar irrespective of the methodology employed and were decreased during the hyperlactatemic phase (59% of control values for the citrate synthase method [$P < 0.01$] and 60% of control values for the V-DAC method [$P < 0.05$]). After linezolid discontinuation and hyperlactatemia resolution, a trend toward normalization of mitochondrial mass was observed (from 59% and 60% to 80%, respectively; $P = 0.17$ for both methods).

We found a marked reduction in COX activity (a mitochondrial respiratory chain complex which is partially encoded by mtDNA) during the acute phase of hyperlactatemia in patients receiving linezolid compared to what was found for controls (51% with respect to control values, $P < 0.05$) (Fig. 2) and an even greater reduction was observed for the expression of COX-II protein (one of the COX subunits encoded by mtDNA and translated by mitochondrial ribosomes), which was reduced to 16% of control values when assessed per cell ($P < 0.001$) (Fig. 2). These changes were observed despite an increase in COX-II mtRNA expression (297% of control values, $P = 0.1$ [not significant]) and no change in mtDNA levels (82% of control values, $P = 0.57$) during the hyperlactatemic phase (Fig. 2). After linezolid discontinuation and normalization of lactate levels, mitochondrial abnormalities showed a significant trend toward normalization (from 51% to 130% for COX activity [$P < 0.01$], from 16% to 72% for COX-II expression per cell [$P < 0.05$], and from 297% to 210% for COX-II mtRNA abundance [$P < 0.05$]) (Fig. 2 and 3). It is important to note that mitochondrial parameters conserved tendencies even after the correction for the decrease in mitochondrial mass present in patients developing linezolid-related hyperlactatemia (Fig. 3).

No significant changes were found in the overall PBMC function, irrespective of whether it was considered by spontaneous cell oxidation capacity or by mitochondrial membrane potential (Fig. 4).

DISCUSSION

In the present study, we evaluated the impacts of standard dosages of 600 mg/12 h of linezolid on mitochondrial activities by using lactic acid serum levels as a surrogate marker. We studied five patients developing hyperlactatemia, in whom we demonstrated reductions in COX activity and COX-II protein synthesis in PBMCs, despite an increase in COX-II mtRNA production. All these abnormalities tended to disappear after

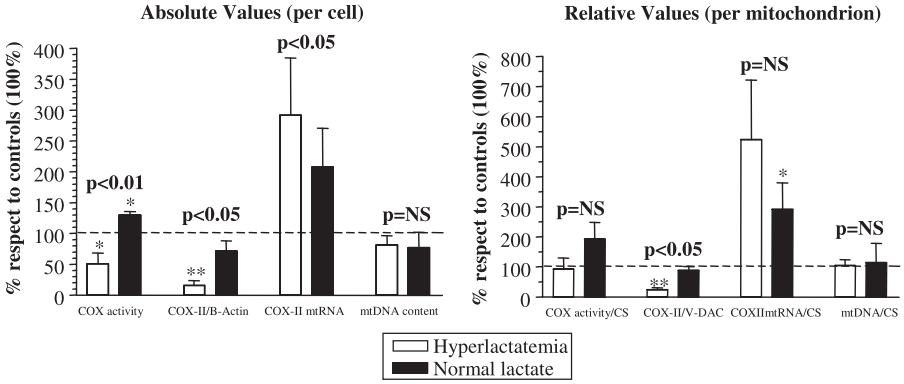

FIG. 2. Comparison of mitochondrial parameters between controls (100%) (dashed line) and patients developing linezolid-related hyperlactatemia (columns) (means ± standard errors of the means). *P* values correspond to comparisons between values measured during and after linezolid treatment. Asterisks correspond to significant differences (*, $P < 0.05$; **, $P < 0.01$) between values for each column and the control values. NS, not significant; CS, citrate synthase.

linezolid treatment was discontinued and serum lactate levels returned to normal.

Our findings for humans offer a complete picture of what occurs in mitochondria during linezolid-related hyperlactatemia and corroborate previous experimental data suggesting the inhibitory action of linezolid on mitochondrial protein synthesis. Few previous partial data regarding the in vivo effects of linezolid therapy had been reported to date. De Vriese et al. recently reported abnormally reduced enzymatic mitochondrial respiratory chain activity in the liver, kidney, and muscle of one patient under severe-symptomatology treatment after a 4-month course of linezolid treatment (10). All of our hyperlactatemic patients were studied before a 3-month linezolid treatment and presented null or mild clinical manifestations. De Vriese et al. performed no further studies to determine the causes of such a reduction of COX activity (10). In this case, no mitotoxic effects were found in PBMCs. Although some authors have argued against the validity of mitochondrial studies on PBMCs, mainly because these cells are not the typical target of mitochondrial disturbances, in our experience, most mitochondrial defects can reliably be demonstrated in PBMCs (17, 21, 22). In addition, very recently, McKee et al. (19) demonstrated equal mitochondrial ribosomal structures and antibiotic effects in all studied tissues. We believe that to find mitochondrial disturbances in PBMCs, it may be necessary to control crude results for mitochondrial

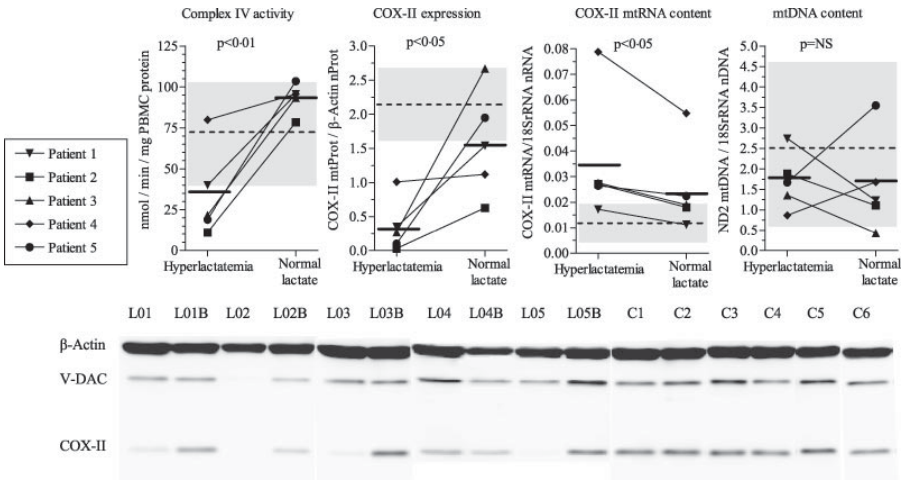

FIG. 3. (Top) Individual values of mitochondrial protein synthesis homeostasis parameters measured in the study. Dashed lines correspond to the means for the controls and shaded squares the normality interval (±2 SD). *P* values refer to the statistical significance of the changes observed in patients developing linezolid-related hyperlactatemia after discontinuation of linezolid treatment (n, nuclear). (Bottom) Western blot analysis of the mitochondrially coded cytochrome *c* oxidase subunit II (COX-II) expression with respect to both nuclear-coded porin V-DAC (mitochondrial marker) and β-actin (cellular marker) (L denotes patients receiving linezolid, C denotes control individuals, and B corresponds to the second sample after hyperlactatemia resolution).

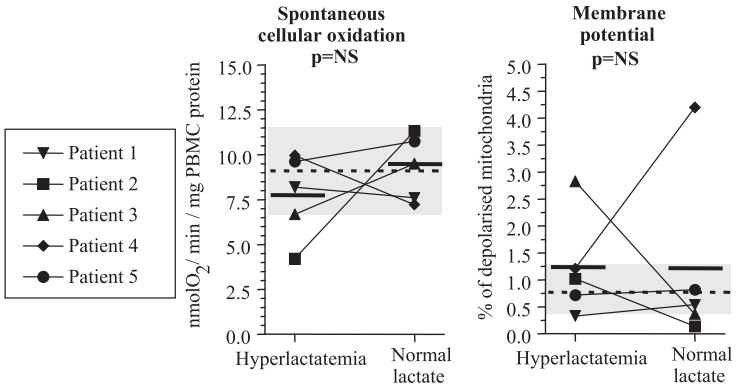

FIG. 4. Overall mitochondrial function of PBMCs of patients developing linezolid-related hyperlactatemia. Dashed lines correspond to the means for controls and shaded squares to the normality interval (±2 SD). *P* values refer to the statistical significance of the changes observed in patients developing linezolid-related hyperlactatemia after discontinuation of linezolid treatment. NS, not significant.

content, which may increase or decrease in response to functional defects. Actually, we also found important reductions in PBMC mitochondrial masses in all the hyperlactatemic patients analyzed, irrespective of the methodology employed. These reductions in mitochondrial mass may not be the cause of the mitochondrial dysfunctions observed in these patients. Conversely, we believe that the reductions could be the consequence of linezolid mitochondrial protein synthesis inhibition, since mitochondrial parameters remained abnormal (or, alternatively, showed a clear tendency to be lower than control values) when they were normalized by the mitochondrial mass. In this sense, a reduction of mitochondrial mass could suggest that linezolid protein synthesis inhibition is limiting mitochondrial biogenesis. Finally, it is important to note that mitochondrial disturbances identified in the PBMCs of patients with linezolid-related hyperlactatemia could be even more pronounced in other tissues located in more-central places of lactate homeostasis, such as the liver and kidney, which are, in the end, the main organs responsible for lactate level increases in plasma.

Additionally, in these linezolid-induced-hyperlactatemia patients, mtRNA abundance is increased, probably to compensate for the linezolid inhibition of mitochondrial protein synthesis. Up-regulation of mtRNA levels in response to experimental inhibition of mitochondrial protein synthesis by thiamphenicol has previously been reported (8, 14). Nevertheless, a reduced enzymatic activity of the partially mitochondrion-coded COX was detected in our patients, suggesting that the up-regulatory mechanisms leading to increased mitochondrial DNA-encoded transcripts are not able to compensate for linezolid-mediated mitochondrial translation inhibition.

Linezolid exerts a time-dependent antimicrobial action, but an influence of linezolid concentration on adverse effects has previously been suggested by hematological alterations in patients with renal failure (18), which is provably associated with linezolid or metabolite accumulation (10). Although serum linezolid concentration was not measured, no hepatic or renal impairment which could influence linezolid pharmacokinetics was present among the studied patients. Therefore, individual susceptibilities to mitochondrial toxicity could be related to mtDNA mutations on mitochondrial rRNA genes coding for mitochondrial ribosomal subunits (25) or particular linezolid metabolism. McKee et al. (19) reported that the linezolid concentration that inhibits 50% of the mitochondrial protein synthesis (IC_{50}) in rat and rabbit heart and liver mitochondria is between 3.37 and 5.26 mg/liter. Considering that linezolid trough serum concentrations range from 1 to 6 mg/liter, we hypothesize that patients with trough levels above the IC_{50} could maintain permanently inhibited mitochondrial protein syntheses and could be especially vulnerable to mitochondrial toxicity with respect to those patients with trough levels below the IC_{50}. Since linezolid has a 100% oral bioavailability and achieves trough serum levels above the MIC for most susceptible gram-positive microorganisms, it is necessary to further evaluate whether guiding linezolid dosage by using the linezolid "trough level/MIC ratio" (7, 10) could prevent the development of the adverse events related to mitochondrial protein synthesis inhibition while maintaining the clinical efficacy, especially in patients receiving prolonged courses of linezolid treatment.

ACKNOWLEDGMENTS

This work was supported by Fundació La Marató de TV3 (020210 and 020631), Redes de Investigación en Mitocondrias (V2003-REDC06E-0) y SIDA (Redg173), Fondo de Investigaciones Sanitarias (FIS 40381/04), Suport a Grups de Recerca de la Generalitat de Catalunya (2005/SGR/0300), and Instituto de Salud Carlos III (CIBER 06/03).

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RESUM DEL QUART ESTUDI

Les oxazolidinones com el linezolid són antibiòtics que exerceixen la seva acció bactericida inhibint la traducció bacteriana. L'ús perllongat d'aquest antibiòtic s'associa a l'aparició d'efectes secundaris característics de mitocondriopaties primàries, com l'augment del lactat en sang. Els ribosomes procariotes i mitocondrials són molt similars i la inhibició de la síntesi proteica mitocondrial podria ser la base de l'aparició de toxicitat secundària al tractament.

L'objectiu del present estudi és determinar el mecanisme de toxicitat del linezolid i la possible reversió dels seus efectes un cop suspès el tractament i normalitzats els nivells de lactat. S'ha realitzat un estudi mitocondrial de quantificació de la massa mitocondrial, dels nivells d'ADNmt i ARNmt, de la taxa d'expressió de proteïnes mitocondrials com la COX-II i de l'activitat enzimàtica del complex IV (COX) de la CRM en CMSP de 5 pacients sotmesos a tractament perllongat amb linezolid que desenvolupaven hiperlactatèmia, així com després d'interrompre la teràpia i normalitzar el lactat. Els resultats s'han comparat amb els de 6 controls que no han pres l'antibiòtic.

El tractament perllongat amb linezolid en pacients que desenvolupen hiperlactatèmia secundària al tractament està associat a la disminució significativa d'un 84% de l'expressió proteica mitocondrial de COX-II/β-Actina, d'un 49% de l'activitat de COX i d'un 60% de la massa mitocondrial, respecte als valors control. Totes aquestes disminucions es mantenen en normalitzar els paràmetres pel contingut en mitocondris, suggerint que la disminució en el contingut mitocondrial seria la conseqüència i no la causa del bloqueig traduccional, que podria estar limitant la biogènesi mitocondrial. El nivell d'ADNmt, la capacitat oxidativa i el potencial de membrana resten inalterats, contrastant uns nivells incrementats de manera no significativa d'ARNmt del 197%. Aquest augment podria ser un intent infructuós de compensar el bloqueig de la síntesi proteica mitocondrial per normalitzar l'expressió proteica, ja descrit per altres antibiòtics (Chrzanowska-Lightowlers ZM et al, *J Biol Chem* 1994; Kuzela S et al, *Biochim Biophys Acta*1988). Després de la interrupció del tractament i la recuperació dels nivells estàndard de lactat, tots els paràmetres mitocondrials retornen a la normalitat. La toxicitat del linezolid podria dependre de la seva concentració (De Vriese A et al i Mateu de Antonio J et al, *Clin Infect Dis* 2006) i la variabilitat individual a l'hora de metabolitzar-lo faria que aquells pacients que mantinguessin les concentracions del fàrmac,

fins i tot en els moments de concentració-vall, per damunt de la concentració que inhibeix la síntesi proteica mitocondrial, manifestessin efectes adversos al tractament abans que la resta.

CONCLUSIONS DEL QUART ESTUDI

1. El linezolid sembla que exerceix la seva toxicitat a través d'inhibir la síntesi proteica mitocondrial, com d'altres antibiòtics, inhibició que no compensa l'augment observat en la transcripció d'aquest orgànul. Aquesta inhibició alteraria el funcionament dels complexos respiratoris i podria limitar la biogènesi mitocondrial, sense que se'n ressentís el contingut en ADNmt, la capacitat oxidativa o el potencial de membrana.

2. Totes aquestes alteracions mitocondrials retornen a la normalitat quan els nivells de lactat es normalitzen al cap d'unes setmanes de suspendre's el tractament amb linezolid.

NEUROLEPTIC TREATMENT EFFECT ON MITOCHONDRIAL ELECTRON TRANSPORT CHAIN
Peripheral Blood Mononuclear Cells Analysis in Psychotic Patients

JOURNAL OF CLINICAL PSYCHOPHARMACOLOGY 2007; 27(3); 284-288

J Casademont, **G Garrabou**, Ò Miró, S López, A Pons, M Bernardo and F Cardellach

El **CINQUÈ** dels treballs inclosos en la present tesi doctoral planteja com a:

HIPÒTESI

Si els efectes secundaris d'un tractament amb diferents fàrmacs d'una mateixa família, i en concret els antipsicòtics, tenen base mitocondrial i presenten diferent grau de manifestacions clíniques, el grau de toxicitat mitocondrial també hauria de ser diferent segons el fàrmac emprat.

OBJECTIU

Comparar la toxicitat mitocondrial (contingut en mitocondris, dany oxidatiu, activitats enzimàtiques dels complexos I, II, III i IV i activitat oxidativa global i dels complexos I, II i III de la CRM), de diferents fàrmacs antipsicòtics (clozapina, ripseridona i haloperidol) en CMSP de pacients asimptomàtics en tractament, per tal de determinar el mecanisme causal d'aquesta toxicitat i la correspondència entre lesió mitocondrial i incidència d'efectes adversos en forma de manifestacions extrapiramidals.

Neuroleptic Treatment Effect on Mitochondrial Electron Transport Chain

Peripheral Blood Mononuclear Cells Analysis in Psychotic Patients

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Abstract: A limitation in the use of classic neuroleptic drugs is the eventual appearance of extrapyramidal symptoms. Some studies, mainly based on experimental situations, have related these symptoms with a defect in the mitochondrial electron transport chain (ETC), especially with complex I (CI). One of the advantages of the ''atypical'' neuroleptics is a lower incidence of movement disorders. We studied peripheral blood mononuclear cells from naive schizophrenic patients (n = 25) and patients under chronic treatment with 1 ''typical'' neuroleptic (haloperidol, n = 15) or 1 ''atypical'' neuroleptic (risperidone, n = 23; or clozapine, n = 21). Patients were on standard clinical situation, on treatment for at least 28 months, and did not present signs or symptoms of extrapyramidal dysfunction. Absolute enzyme activities of ETC complexes I to IV were spectrophotometrically quantified, and oxygen consumption with substrates of different complexes was measured polarographically. As an indirect measure of oxidative damage, we quantified membrane lipid peroxidation through the loss of *cis*-parinaric acid fluorescence. We found differences among groups when comparing the activity of CI, which was decreased in patients receiving neuroleptics, either assessed enzymatically or through oxygen consumption. This effect was lower for atypical neuroleptics than for haloperidol. Haloperidol was also associated with a significant increase of peripheral blood mononuclear cell membrane peroxidation. We conclude that antipsychotics given at clinical standard doses, either typical or atypical, inhibit CI of the ETC. It remains to be established if this finding in a nontarget tissue for antipsychotics may account for the lower incidence of movement disorders observed in patients on atypical agents.

(*J Clin Psychopharmacol* 2007;27(3):284–8)

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Received July 12, 2006; accepted after revision February 28, 2007.

Supported by Fundació La Marató de TV3 (1710/01), Redes de Investigación en Mitocondrias (V2003-REDG011 and V2005-FSO50301), Fondo de Investigaciones Sanitarias (FIS PI 04/0381), and Suport a Grups de Recerca de la Generalitat de Catalunya (2005/SGR/0300).

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ISSN: 0271-0749/07/2703-00
DOI: 10.1097/JCP.0b013e318054753e

Chronic treatment with neuroleptic drugs has a series of adverse effects, the most important being the appearance of extrapyramidal movement disorders (EMDs).[1] Time on treatment and age of patients are the most frequent independent risk factors for their development,[2,3] but other factors, including some polymorphisms of cytochrome P450, are also important.[4] Experimental studies have suggested that a modification in the number or sensitivity of dopamine receptors is critical in the pathogenesis of EMD (see Ref. [5] for review). However, certain dissociation between the susceptibility to present EMD and the modification of the number of dopamine receptors suggests that other mechanisms may be involved in this relation.[6]

In 1992, it was reported that neuroleptics inhibit electron transport chain (ETC) complex I (CI) activity in rat brain mitochondria,[7] a finding which was later confirmed by other authors.[8] Similar results were also described in an in vitro model based on postmortem human brain.[9] As an abnormal cellular energy state may account for alterations in neuronal function, the ETC inhibition was proposed as a putative mechanism contributing to the extrapyramidal side effects of these drugs. It has even been postulated that the potential irreversible nature of some forms of EMD could be related to the appearance of mtDNA damage,[10] data not confirmed in other studies.[11]

In recent years, ''atypical'' neuroleptics with properties different from classic neuroleptics and significantly fewer extrapyramidal side effects have been introduced into clinical use.[12] Some of these drugs have already been tested in cellular models and animals from an ETC point of view,[8,13,14] but it is difficult to translate some experimental situations into clinical practice. The most important problem is that target tissues for neuroleptics (ie, brain) are not easily available. In recent years, a series of methodological improvements has led to reliable mitochondrial biochemical studies on peripheral blood mononuclear cells (PBMCs), including the analysis of isolated CI activity,[15] allowing a noninvasive study of patients in their usual clinical situation.

PATIENTS AND METHODS

This study was designed in a cross-sectional manner. Psychiatrists of our institution recruited all patients with schizophrenia according to *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*. Naive patients were included consecutively after diagnosis and before regimen of any antipsychotic medication was begun. Patients

on treatment were stable patients followed up in the outpatient clinic, without any sign of EMD according to Simpson and Angus scale. All followed chronic monotherapy treatment either with risperidone, clozapine, or haloperidol for a minimum of 28 months at doses considered clinically effective by their physicians, which were highly homogeneous (risperidone, 5.2 ± 0.8 mg/d; clozapine, 317 ± 57 mg/d; or haloperidol, 13 ± 2.3 mg/d). All were at least 18 years of age, and either them or their legal representatives gave informed consent to be included in the study. The protocol has been carried out in accordance with the Declaration of Helsinki and approved by the ethical committee of our institution.

Thirty milliliters of peripheral venous blood was used to perform all the studies. Peripheral blood mononuclear cells were obtained by separation on Ficoll density gradient centrifugation (Histopaque-1077; Sigma Diagnostics, St Louis, Mo). Biochemical methods are specified in detail elsewhere.[15-18] In brief, enzyme activities of complexes I to IV were measured spectrophotometrically (U-2001 UV/VIS spectrophotometer; Hitachi Instruments, Inc, San Jose, Calif), and oxygen utilization was measured polarographically with a Clark electrode (Hansatech Instruments Ltd, Norfolk, England). To measure CI, PBMCs were treated with Percoll and digitonin to minimize contaminating nonmitochondrial nicotinamide adenine dinucleotide−quinone oxidoreductase enzymatic activity.[15] Citrate synthase (CS) activity, an enzyme located in the mitochondrial matrix integrated into the Krebs cycle, was used as marker of mitochondrial PBMC content.[17] As a measure of oxidative damage, PBMCs were labeled with *cis*-parinaric acid (5 μM) (Molecular Probes, Eugene, Ore). Loss of parinaric acid fluorescence during 30 minutes is considered to be a reliable method to measure the chemical process of lipid peroxidation.[18]

Proportions were compared by means of χ^2 test with Yates correction. A 1-way analysis of variance (ANOVA) was used to test the differences between groups. If a variable (such as time on treatment) might predict the dependent variable (eg, activity of 1 complex) but was not the direct interest of the study, it was introduced in the analysis as a covariate. A 2-way ANOVA for repeated measures considering only the between-subjects factor was used to compare the curves of *cis*-parinaric acid loss of fluorescence. If the ANOVA was significant, a Tukey post hoc test was applied to determine between which pair of groups the difference(s) lay. Analyses were performed using SPSS 14.0. A P value of less than 0.05 was considered statistically significant. Results are given as mean value ± SD.

RESULTS

Eighty-four patients were included in the study. Sixty-eight were male, and 16 were female. Mean age was 30 ± 8 years, and time on treatment in the treated groups was 43.8 ± 10.9 months. As it can be noted in Table 1, there were no statistical differences between groups according to age and sex, but there were differences according to time on treatment. Patients treated with haloperidol had been on treatment for a mean of 10 months more than patients on risperidone (P = 0.008 in the Tukey post hoc analysis). Because of this fact, comparisons of mitochondrial activities among groups were controlled by time on treatment considered as a covariate.

We only found differences among the 4 groups in those experiments in which CI was involved, but not for the rest of

TABLE 1. Age, Sex, Time on Treatment, and Mitochondrial Activities of Patients According to the Type of Treatment

	Treatment Groups				
	Naive	Clozapine	Risperidone	Haloperidol	*P*
No. patients	25	21	23	15	NS
Age (yrs)	28 ± 8	31 ± 8	29 ± 9	34 ± 6	NS
Sex (female/male)	3/22	6/15	5/18	2/13	NS
Months on treatment	—	44.5 ± 10.5	39.3 ± 8.5	49.9 ± 12.4	0.011
CI	25.8 ± 4.6	23.0 ± 9.2	19.4 ± 5.0	8.2 ± 4.4	<0.001
CII	32.9 ± 13.8	36.7 ± 12.9	31.8 ± 11.3	33.1 ± 8.8	NS
CIII	98.4 ± 33.1	87.2 ± 27.5	102.6 ± 32.7	91.5 ± 22.0	NS
CIV	90.4 ± 34.9	99.4 ± 31.1	93.5 ± 34.3	88.9 ± 20.5	NS
CS	130.7 ± 23.2	135.0 ± 35.2	129.5 ± 30.5	132.6 ± 12.4	NS
Spontaneous cell oxidation	7.2 ± 1.9	7.3 ± 2.0	7.7 ± 1.8	6.3 ± 1.3	NS
Pyruvate-malate oxidation	5.8 ± 1.6	5.3 ± 1.7	4.9 ± 2.3	4.1 ± 1.0	<0.05
Succinate oxidation	4.6 ± 2.2	4.5 ± 1.6	4.8 ± 2.5	4.1 ± 1.6	NS
G3P-oxidation	15.5 ± 3.9	16.7 ± 4.7	15.9 ± 3.5	16.2 ± 3.5	NS

Results are expressed as (mean ± SD).

Enzymatic activities are expressed as nanomoles per minute per milligrams of protein (nmol $\cdot min^{-1} \cdot mg^{-1}$ protein). Oxidations are expressed as nanomoles of oxygen consumed per minute per milligram of protein (nmol O_2 consumed$\cdot$ $min^{-1} \cdot$ mg^{-1} protein).

NS indicates statistically not significant; G3P, glycerol-3-phosphate.

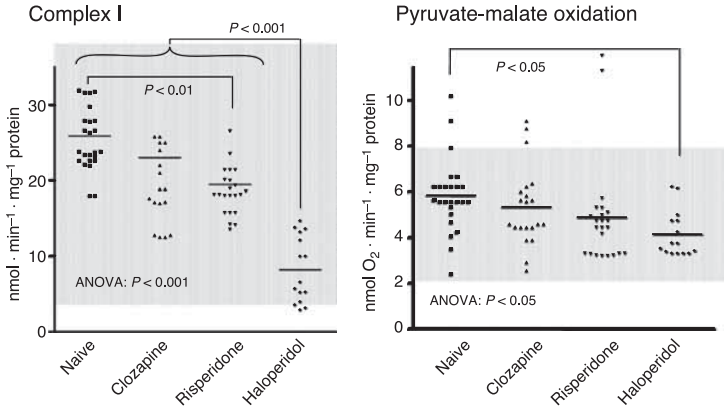

FIGURE 1. Left, Enzymatic activities of CI. Patients on haloperidol had lower activity than the rest of the groups, although the values fell within the reference ranges of our laboratory (shadowed area) except for 3 patients. Patients on risperidone also disclosed lower activities than naive patients. Right, Oxygen consumption using pyruvate-malate (substrates of CI) also demonstrated that patients on haloperidol had lower respiratory rates than other groups, being the significance in respect to naive patients.

complexes or CS. Complex I enzymatic activities of patients treated with haloperidol were statistically lower than activities in naive patients or patients treated with atypical antipsychotics, whereas CII, CIII, and CIV activities did not differ significantly (Table 1 and Fig. 1). Patients treated with risperidone also had a lower activity of CI when compared with naive patients but not when compared with clozapine-treated patients. When results were normalized by CS activity to control for an eventual variation in the number of mitochondria, results were very similar (data not shown).

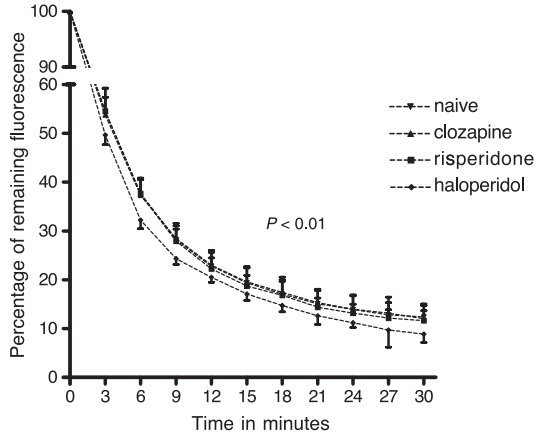

FIGURE 2. Studies of lipid peroxidation measured as loss of *cis*-parinaric acid fluorescence over time. Patients on haloperidol had greater and faster loss of fluorescence, compared with naive patients or patients on atypical antipsychotics, denoting an increase in lipid peroxidation of PBMC membranes. Results are expressed as the percentage of the remaining fluorescence (± SD) at 3-minute intervals.

Similarly, oxidation of pyruvate-malate (a CI substrate) was also different among groups, the differences lying between naive and patients treated with haloperidol (Fig. 1).

Finally, the loss of parinaric acid fluorescence as a measure of oxidative damage was higher in PBMCs from patients on haloperidol than in the other groups, without differences among them (Fig. 2).

DISCUSSION

The present study demonstrates that stable schizophrenic patients treated with antipsychotics have a decreased activity of ETC CI in PBMCs. This is demonstrated either by directly measuring the CI enzymatic activity or through the polarographic measure of oxygen consumption using pyruvate-malate as a substrate of CI. The decrease is particularly evident in patients treated with a classic neuroleptic (haloperidol), but patients treated with newer ''atypical'' neuroleptics also have, although less marked, a decreased CI activity. Among patients treated with the tested atypical antipsychotics, it seems that risperidone produces more inhibition than does clozapine (Fig. 1).

The inhibitions, although statistically significant, did not reach our laboratory's inferior normal limits, except for the enzymatic activity of CI in 3 patients on haloperidol. This is particularly important because inhibition of mitochondrial activities has been proposed as the mechanism underlying the appearance of EMD in patients treated with neuroleptics. In our series, patients were recruited among those without signs of extrapyramidal dysfunction. This was because of the fact that, currently, most patients are treated with atypical antipsychotics, whose proneness to cause extrapyramidal side effects is considered to be low. On this basis, the inclusion of a sufficient number of patients with movement disorders to draw conclusions would have needed a very prolonged period of time. Taking this into account, the

demonstrated decrease in CI activity could be considered a risk factor for the development of EMD that, eventually, may appear with a longer period of treatment or with other unknown adjuvant factors. In any case, the inclusion of patients with EMD presumably would have demonstrated greater levels of CI inhibition.

Neuroleptic drugs block dopamine receptors, which may interfere with the metabolism and turnover of dopamine and favor the formation of hydrogen peroxide through the activity of monoamine oxidase.[19] Reactive oxygen species may cause damage near the site of their formation, that is, ETC itself, and further impair the function of the respiratory machinery. This would, in turn, promote the generation of more oxygen free radicals and damage of membrane phospholipids and proteins in a sort of catastrophic vicious cycle.[20] This view is consistent with the observation that CI is particularly sensitive to free radical attack.[21] Furthermore, free radical damage can be abolished by pretreatment with antioxidants.[22] We have herein found that the group of patients on haloperidol, which is also the group with more inhibition of CI activity, presents a significant increase in oxidative damage assessed through membrane lipid peroxidation.

Oxidative damage could thus explain changes in ETC function and, eventually, contribute to EMD.[23,24] Unfortunately, the ''catastrophic free radical vicious cycle hypothesis,'' although very attractive and very frequently invoked in neurodegenerative disorders and other circumstances, including normal aging, has never been clearly demonstrated.

There are a series of limitations in the present study. One is that it is currently difficult to find patients on chronic treatment with classic antipsychotics. Most of them have been switched to atypical neuroleptics because of their better safety profile. The long period of inclusion of this study has been due precisely to this fact. It may be that patients still on classic neuroleptics are specially well adapted to them and not representative of what could have been found in a less selected population. The findings of the present study probably underestimate the real degree of inhibition of CI activities, and for this reason, we believe that this selection bias does not invalidate the significant differences found.

Another limitation is that patients on haloperidol had been on treatment for a more prolonged period than had other groups of patients. One could argue that the inhibition is more related to time on treatment than to type of treatment. To overcome this problem, time on treatment was considered as a covariate and biochemical activities controlled by this factor. Furthermore, tardive dyskinesia, one of the most worrisome forms of EMD, seems to have its highest incidence early in treatment, but after 3 years, it decreases to approximately 1% per year or less, which is near the spontaneous rate.[25]

Finally, it is clinically uncertain the meaning of a decrease in CI activity in a nontarget tissue for antipsychotics such as PBMCs. The generalization of that effect on other cell types, that is, neurons, is probable but difficult to demonstrate in this particular clinical setting. In other scenarios, such as lipoatrophy related to antiretroviral use (in which the target tissue—liver—is more accessible to study), it has been found that PBMC mitochondrial DNA levels have a good correlation with mitochondrial DNA levels in the liver.[26]

In conclusion, we herein demonstrate that neuroleptic drugs used in standard clinical conditions for a prolonged period inhibit the CI of the ETC. This is particularly true with classic antipsychotics. Considering that ETC defects have been considered to play a role in the cerebral dysfunction found in neurodegenerative diseases, including some untreated schizophrenics patients,[27,28] it seems reasonable to recommend to be very cautious in their use.

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RESUM DEL CINQUÈ ESTUDI

La teràpia antipsicòtica està associada a l'aparició d'efectes adversos com les manifestacions extrapiramidals, especialment en el cas dels neurolèptic clàssics com l'haloperidol, respecte els fàrmacs atípics de darrera generació com la clozapina i la risperidona. Aquestes alteracions podrien tenir una base mitocondrial.

L'objectiu d'aquest estudi és determinar el mecanisme de toxicitat mitocondrial d'aquests fàrmacs i avaluar si existeix una correspondència entre la lesió mitocondrial i la capacitat d'induir efectes adversos. Per això, s'ha realitzat una completa anàlisi mitocondrial en CMSP de 15 pacients diagnosticats d'esquizofrènia en tractament amb haloperidol, 21 amb clozapina i 23 amb risperidona, tots ells asimptomàtics en la manifestació d'efectes secundaris, respecte 25 controls diagnosticats del mateix trastorn que encara no prenien antipsicòtics. L'anàlisi mitocondrial ha consistit en la mesura del contingut en mitocondris, el dany oxidatiu, les activitats enzimàtiques dels complexos I, II, III i IV, l'activitat oxidativa global i l'estimulada a través del complexos I, II i III.

En els pacients tractats amb antipsicòtics, el complex I de la CRM presenta una activitat disminuïda, tant a nivell enzimàtic com oxidatiu. Aquesta disfunció és més evident en aquells pacients que es troben en tractament neurolèptic amb haloperidol (fàrmac clàssic associat a major incidència de manisfestacions extrapiramidals) que en aquells pacients tractats amb clozapina o risperidona (antipsicòtics atípics amb menor incidència d'efectes adversos), sent la risperidona lleugerament més mitotòxica que la clozapina. La disfunció mitocondrial en la majoria de pacients es troba dins dels rangs de normalitat del nostre laboratori, potser perquè tots aquests pacients són asimptomàtics i la toxicitat mitocondrial és encara massa incipient per manifestar-se en forma d'efecte advers. A més a més, els pacients tractats amb haloperidol presenten un increment significatiu del dany oxidatiu mitocondrial.

Els neurolèptics incrementen l'estrès oxidatiu generat per l'enzim monoamina oxidasa (Lohr JB et al, *Arch Gen Psychiatry* 1991). Les ROS són especialment nocives pel mitocondri, per ser el seu lloc de formació, i el complex I resulta especialment vulnerable a l'atac oxidatiu (Zhang Y et al, *J Biol Chem*1990), de manera que generaria més ROS, en una espècie de cercle viciós (Wallace DC et al, *Nat Genet* 1998) que podria propiciar l'aparició dels efectes adversos.

CONCLUSIONS DEL CINQUÈ ESTUDI

1. Els antipsicòtics inhibeixen l'activitat oxidativa i enzimàtica del complex I de la CRM.

2. La potència d'inducció de lesió mitocondrial pels fàrmacs estudiats (haloperidol > risperidona > clozapina) es correspon amb la incidència de manifestacions extrapiramidals definida per altres autors en pacients sota tractament amb aquests 3 fàrmacs.

3. Els pacients tractats amb haloperidol presenten, a més a més de la inhibició del complex I, un increment significatiu del dany oxidatiu mitocondrial, que no s'observa amb el tractament amb els altres antipsicòtics.

MITOCHONDRIAL INJURY IN ACUTE CARBON MONOXIDE POISONING: THE EFFECT OF OXYGEN TREATMENT

MANUSCRIT EN PREPARACIÓ

G Garrabou, JM Inoriza, G Oliu, MJ Martí, C Morén, J Casademont, Ò Miró and F Cardellach

El **SISÈ** dels treballs inclosos en la present tesi doctoral planteja com a:

HIPÒTESI

La intoxicació per CO produeix alteracions en la funció mitocondrial per l'afinitat d'aquest gas pel citocrom aa3 de la COX. Aquesta alteració podria tenir relació amb la gravetat de la intoxicació i amb la intensitat de les manifestacions clíniques. Així mateix, la reversibilitat d'aquesta alteració podria estar condicionada pel tipus de tractament administrat.

OBJECTIU

Estudiar la base mitocondrial de la intoxicació per CO (activitat del complex IV -COX- de la CRM i estrès oxidatiu lipídic), determinar la correlació de la gravetat de la intoxicació amb la disfunció mitocondrial i comparar l'eficiència dels diferents tractaments d'oxigenoteràpia per revertir els efectes mitocondrials.

MITOCHONDRIAL INJURY IN ACUTE CARBON MONOXIDE POISONING: THE EFFECT OF OXYGEN TREATMENT

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Background: Symptoms of acute carbon monoxide (CO) poisoning (ACOP) are generally attributed to hypoxia due to carboxihemoglobin (COHb). But there is also a persistent effect on cells due to the inhibition of mitochondrial complex IV (mitCIV), essential for energy supply and the main resposible for oxidative stress production. COHb rapidly decreases after oxygen (normobaric -NBO- or hyperbaric -HBO-) treatment, but normalization of mitCIV activity takes longer and could be a more sensible marker of ACOP severity and treatment efficacy. The objective of the present study is to establish mitCIV activity and oxidative stress in ACOP, correlate mitochondrial parameters with the severity of intoxication and determine its recovering depending on NBO or HBO treatment.

Patients and methods: 52 subjects with ACOP were distributed as: a) severe (n=29, COHb>20% and/or clinical symptoms), randomly treated with one or two sessions of HBO (SHBO1, n=18, and SHBO2, n=11), and b) moderate (n=23, COHb:10-20%), randomly treated with NBO (MNBO, n=10) or one session of HBO (MHBO1, n=13). 30 Controls were obtained from non-smoker blood donors. Clinical manifestations, COHb, mitCIV activity and lipid peroxidation of mononuclear cells were analysed before and immediately after treatment, as well as at 24 hours, 1 month and 3 months after it.

Results: Before and immediatey after-treatment mitCIV activities were decreased in both severe and moderate ACOP compared to controls ($p<0.001$). We did not find differences in after-treatment mitCIV activities or mitCIV evolution along time among groups, which was completely restored after the three-month follow-up. Lipid peroxidation was not increased in ACOP compared to control values and there were no-differences on oxidative stress level among groups or along the time. All treatments had similarly effectiveness in normalizing blood COHb and acute neurological symptoms, which were more serious in severe ACOP, normalised and disapeared after treatment.

Conclusions: In ACOP mitochondrial dysfunction involves mitCIV inhibition without effects on oxidative stress. Baseline mitCIV activity is decreased but absolutely restored after treatment and a three-month follow-up, independently to treatment and intoxication severity. In severe ACOP patients one single session of HBO is enough effective in restoring mitCIV activity and in moderate ACOP subjects NBO is effective-enough to recover mitCIV function. mitCIV seems to be a good marker of ACOP symptomatology, recovery and treatment effectiveness. Clinical implications of these results remain to be established.

BACKGROUND

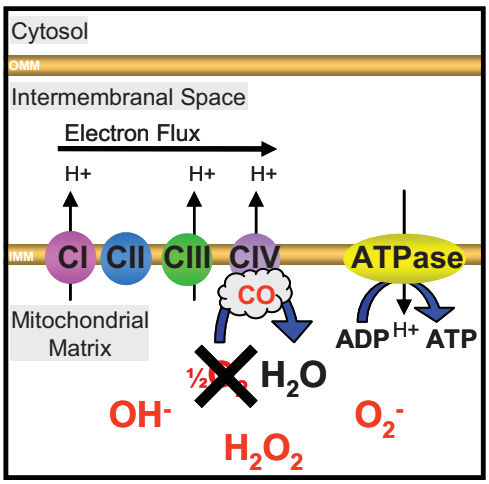
Carbon monoxide (CO) poisoning is the most frequent cause of intoxication by suffocating gases and the biggest cause of death by occupational inhalation in many countries [1, 2]. Its incidence increases on winter because of abnormal combustion on heating systems, responsible for CO accumulation in non-ventilated areas [3].

Symptoms of acute carbon monoxide poisoning (ACOP) include cephalea, palpitations, nausea, vomit, fading, arrhythmia, ischemia, confusion and coma [4, 5]. These manifestations are

generally attributed to tissular hypoxia due to carboxihemoglobin (COHb) formation, which is generated when CO expels oxygen out of hemoglobin because its higher affinity for this iron-containing gas carrier. ACOP gravity depends on intensity and duration of CO exposition and in clinical practice COHb levels, although controversial [6], are used as a marker of CO intoxication severity. COHb levels below 20% indicate moderate CO poisoning and COHb content above 20% or neurological or cardiac manifestations, independent to COHb levels, are classified as severe CO poisoning. But some clinical symptoms appear without COHb formation [6] and many of them appear as later sequels several months after COHb normalisation, suggesting the existance of a secondary damage mechanism independent to COHb.

CO competitive interaction with other oxygen-binding proteins containing iron-porfirinic rings (like myoglobin, cytochrome p450 or the mitochondrial complex IV -mitCIV-), have also been described. Among them, CO inhibition of the mitCIV has been proposed an alternative mechanism of persistent CO injury independent to hypoxia. mitCIV (also called cytochrome c oxidase or COX) is the last component of the electron transport chain located in the inner membrane of the mitochondria. It participates in the oxidative phosphorilation system responsible for cellular energy supply (Figure 1), but it is also one of the main oxidative stress inducers, especially when mitochondria is impaired and mitCIV can not reduce oxygen into water, driving electron flow trough reactive oxygen species (ROS) production. Experimental models have demonstrated that CO binds to mitCIV cytochrome aa3 subunit [7] and studies on human peripheral blood mononuclear cells (PMBC) of patients under ACOP demonstrate CO inhibitory effect on mitCIV enzymatic activity and consequent cellular respiration inhibition [8], which could be responsible for the oxidative damage described on these cells [9]. Similar studies on smoker patients have corroborated CO deleterious effects on mitCIV function [10-13].

Figure 1: Carbon monoxide (CO) effects on mitochondria.



OMM: Outer mitochondrial membrane; IMM: Inner mitochondrial membrane; CI, CII, CIII and CIV: Compexes I, II, III and IV of the electron transport chain; H^+: Proton; OH^-, H_2O_2 and O_2^-: Hidroxyl anion, hydrogen peroxide and superoxide anion (all three reactive oxygen species); O_2 and H_2O: Oxygen and water; ADP and ATP: adenosine di and triphosphate.

ACOP treatment consists on oxygen administration, but multiple therapy possibilities are available. Normobaric oxygen (NBO) treatment consists on pure oxygen administration at standard pressure and hyperbaric oxygen (HBO) therapy consists on

administrating this pure oxygen at 2'5 atmospheres. Both interventions, especially HBO treatment, increase CO-hemoglobin dissociation rate [14], oxygen transport to tissues and hypoxia resolution [15]. But the best oxygenotheraphy option remains still to be established; authors disagree about the beneficial effects of the different treatment possibilities. Some studies abrogate for NBO use [14] while some others support HBO treatment [16], without achieving consensus on time of oxygen treatment or number of administered sessions [17].

COHb rapidly decreases after oxygen treatment, whatever the therapy applied is, but normalization of mitCIV activity takes longer [8] and could be a more sensible marker of ACOP severity, treatment efficacy and sequels predictive marker.

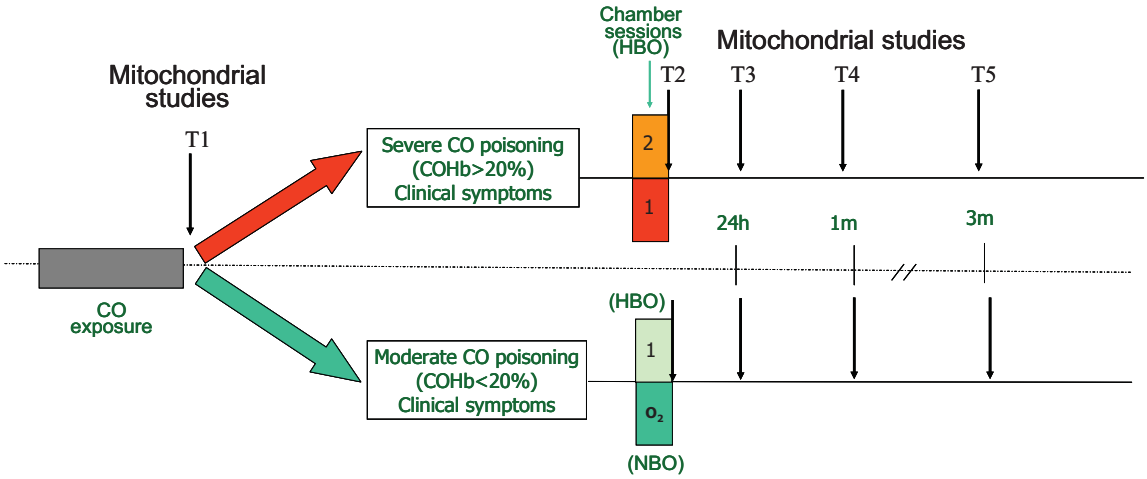
The objective of the present study is to establish the activity of mitCIV and oxidative stress in PBMC of patients suffering from ACOP, correlate both mitochondrial parameters with the severity of intoxication and determine the level of mitochondrial recovery depending on the treatment administered (NBO vs. HBO).

PATIENTS AND METHODS

Patients: 52 subjects with ACOP were distributed (Figure 2) as: a) severe poisoned patients, when COHb levels were above 20% (n:29) or they presented neurological or cariac manifestations, which were randomly treated with one (n:18) or two (n:11) sessions of HBO (SHBO1 and SHBO2), or b) moderate poisoned patients, when COHb content was 10-20% (n:23), which were randomly treated with NBO (MNBO) (n:10) or one session of HBO (MHBO1) (n:13). Controls were obtained from 30 non-smoker blood donors matched by age and gender with included patients. Neither patients nor controls had familiar antecedents for mitochondrial disease or were taking any toxic drug for mitochondria. Written informed consent was obtained in all cases, and the protocol was approved by the Ethical Committee of our hospitals.

Methods: For all included patients we registered clinical manifestations, COHb percentage, mitCIV enzymatic activity and lipid peroxidation levels on mononuclear cells before (T1) and immediately after-treatment (T2), as well as at 24 hours (T3), 1 month (T4) and 3 months (T5) after therapy (Figure 2).

Figure 2: Study design; mitochondrial analysis and groups of treatment.



Mitochondrial analysis was performed in mononuclear cells isolated from 20 mL of peripheral blood by centrifugation on a ficoll density gradient [18]. In each sample protein content was measured by the Bradford protein-dye binding-based method [19] and mitCIV specific enzymatic activity (COX, EC 1.9.3.1) was quantified spectrophotometrically according to Rustin et al. methodology [20], slightly modified for mitCIV measurement in minute amounts of biological sample [21], and expressed in nanomols of consumed substrate per minute and milligram of protein (nmols/min/mg protein). Lipid peroxidation levels were mesured using the Kit Oxys Research of Deltaclone by the spectrophotometric measurement at 586 nm of malondialdehyde (MDA) and 4-hydroxyalkenal concentration (HAE), both products of fatty acid peroxide decomposition, using an standard curve of 1, 1, 3, 3-Tetramethoxypropane and normalising MDA and HAE levels by protein content (μM/mg protein).

Statistical analysis: We performed different statistical tests to search for: differences in mitochondrial parameters at baseline (T1) between severe and/or moderate CO-poisoned patients with respect control subjects (T test for independent measures), for each treatment group just before and after treatment (T1-T2, using the T test for repeated mesures), in the immediately after-treatment measurement (T2) between treatment groups (T test for independent measures), as well as the evolution (T1-T2-T3-T4-T5) of mitCIV, lipid peoxidation levels and COHb levels, for each treatment group along the study (analysis of variance for repeated mesures). We have tested: 1) an analysis for intention-to-treat principle depending on the assignment of groups; 2) the comparison of MNBO-MHBO1, SHBO1-SHBO2 and MHBO1- SHBO1; 3) the analysis of variance for repeated measures to search for inter and intra groups differences; 4) the correlation between COHb, peroxidation levels and mitCIV by linear regression analysis.

Results for each parameter are expressed as mean value and standard error mean (SEM), and p values below 0.05 have been considered significant.

RESULTS

The mitCIV enzymatic activity of all included CO-poisoned patients was decreased at baseline (T1), just after CO exposure and before oxygenotherapy. Both severe and moderated CO-exposed groups showed a significant decrease in the mitCIV activity value with respect control measurements of non-intoxicated and non-smoker subjects (62.16±3.82 and 63.55±5.40 vs. 95.29±4.20, $p < 0.001$ in both cases) (Figure 3, panel 1 and 2), but no-statistical significant differences were found between both groups of ACOP subjects depending on severity of intoxication.

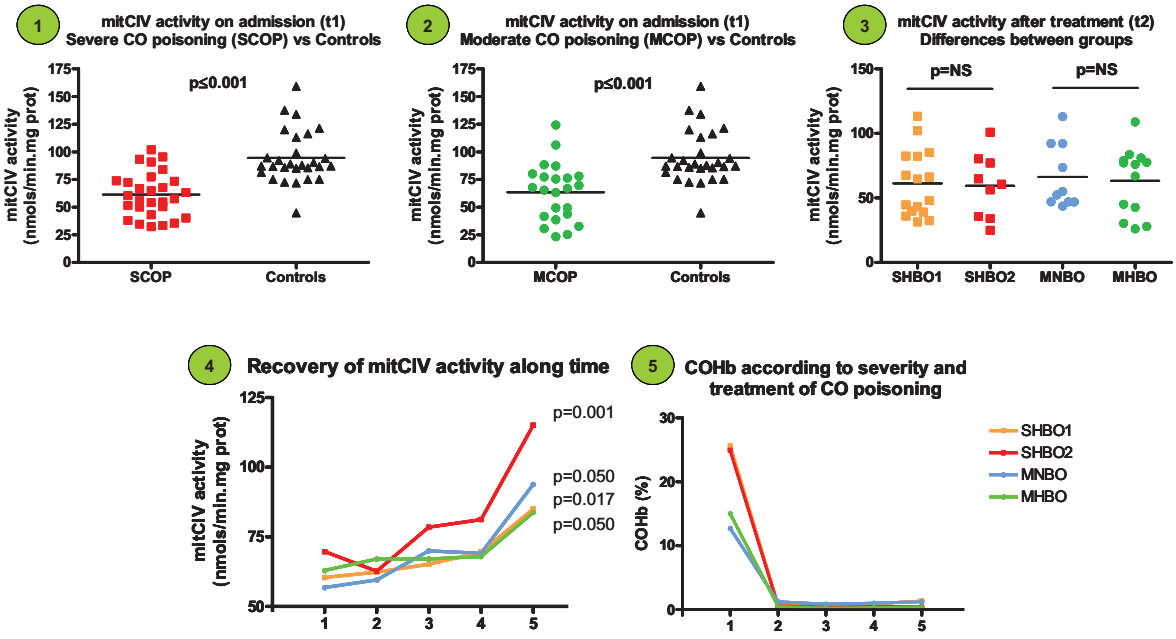
We found no-differences on the mitCIV enzymatic activity before and immediately after-treatment measurements (T1 vs. T2) in any of the studied treatment groups.

When we considered the after-treatment mitCIV activities (T2) among the different oxygen treatment groups, we did not find differences in moderated ACOP between normobaric oxygen (MNBO) and one session of hyperbaric oxygen (MHBO1) therapy (66.31±7.84 vs. 63.27±7.20, p=NS), as well as in severe ACOP between one (SHBO1) and two (SHBO2) sessions of hyperbaric oxygen (61.13±6.46 vs. 59.42±8.25, p=NS) (Figure 3, panel 3).

Recovery of mitCIV enzymatic activity along the study (T1-T2-T3-T4-T5) was statistically

significant in all treatment groups of ACOP, independent to treatment option (SHBO1 p=0.017, SHBO2 p=0.001 and MHBO1 and MNBO p=0.050) (Figure 3, panel 4).

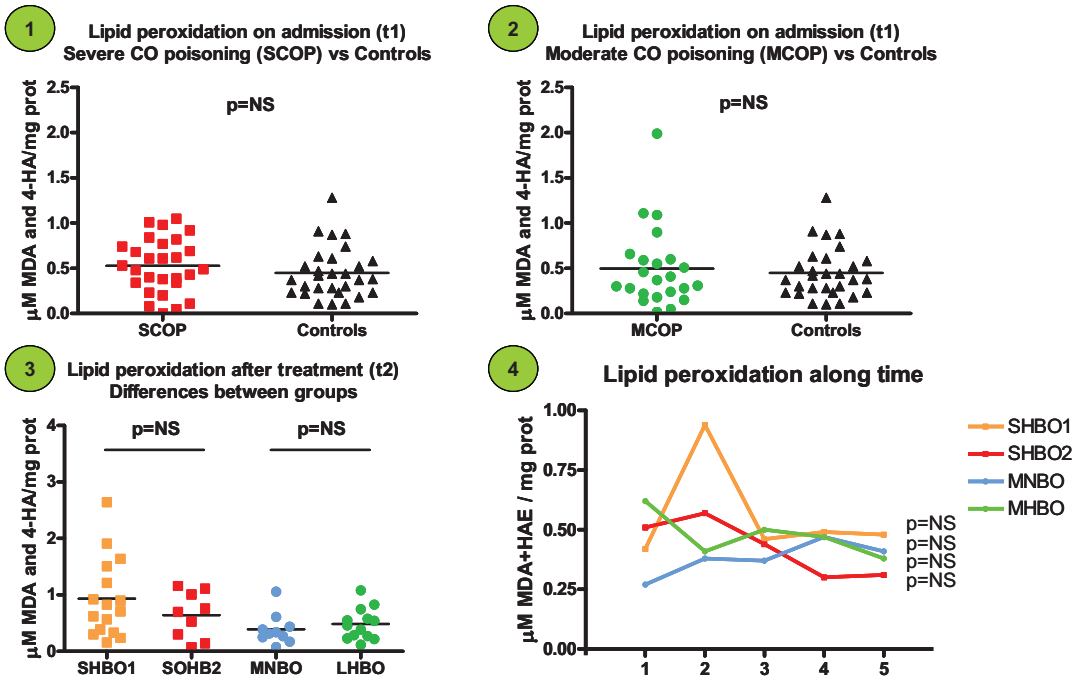
Figure 3: Enzymatic mitochondrial complex IV (mitCIV) activity values and carboxihemoglobin (COHb) levels for the different oxygen treatment groups. Baseline (T1) mitCIV measurement between severe (1) or moderate (2) CO poisoned patients and non-intoxicated controls; Immediately-after treatment values (T2) of mitCIV among treatment groups (3); Evolution of mitCIV along the study (T1-T5) for the different treatment options (4); Evolution of COHb along the study (T1-T5) for the distinct therapy groups. SHBO1 and SHBO2: severe CO poisoned patients treated with one or two sessions, respectively, of hyperbaric oxygen; MNBO and MHBO1: moderate CO poisoned patients treated with normobaric oxygen or just one session of hyperbaric oxygen, respectively.



Lipid peroxidation baseline levels (T1) were not significantly increased in either severe or moderate ACOP patients with respect controls (0.51±0.06 and 0.49±0.09 vs. 0.047±0.06, p=NS) and they showed no-statistical significant differences between them (Figure 4, panel 1 and 2).No-differences were found between the baseline and immediately after-treatment lipid peroxidation values (T1-T2) for none of the studied groups (Figure 4, panel 3) and lipid peroxidation evolution along time (T1-T2-T3-T4-T5) remained unchanged, independently to the severity intoxication and group assignment (Figure 4, panel 4).

In all groups the treatment was similarly effective in normalizing blood COHb values immediately-after oxygen therapy (T2, Figure 3, panel 5).

Figure 4: Lipid peroxidation levels for the different oxygen treatment groups. Baseline (T1) lipid peroxidation measurement between severe (1) or moderate (2) CO poisoned patients and non-intoxicated controls; Immediately-after treatment values (T2) of lipid peroxidation among treatment groups (3); Evolution of lipid peroxidation along the study (T1-T5) for the different treatment options (4). SHBO1 and SHBO2: severe CO poisoned patients treated with one or two sessions, respectively, of hyperbaric oxygen; MNBO and MHBO1: moderate CO poisoned patients treated with normobaric oxygen or just one session of hyperbaric oxygen, respectively.



DISCUSSION

Hemoglobin and mitCIV are both oxygen-binding proteins that contain iron-porfirinic rings which show higher affinity for CO than for oxygen do. Inhaled CO gas crosses all the biological membranes by passive diffusion and binds to all the iron porfirinic-containing structures. Lack of oxygen transport into tissues has been proposed the ethiopathogenical mechanism that underlays clinical symptoms of CO-poisoning, but mitCIV inhibition has been proposed an alternative and more persistent mechanism of biological damage, independent to hypoxia, that could explain unresolved questions, like clinical manifestations independent to COHb levels.

As it is reported on the bibliography, carbon monoxide inhibits mitCIV enzymatic activity [7-9]. The present study corroborates this data in mononuclear cells of both severe and moderate ACOP patients. The use of PBMC to study CO-mediated effects on mitochondrial function has been clearly validated in the past [8, 9, 10, 13]. Our work found similar decreased mitCIV values at baseline for all studied ACOP, independently to their COHb levels, showing that deleterious CO effects at the mitochondrial or cellular level can be developed in moderate CO-intoxicated subjects when CO exposition is not still big or prolonged enough to completely saturate hemoglobin (10-20% COHb content) and hypoxic conditions can still get worse.

Oxygen therapy has been clearly recommended to treat CO-poisoning, although no-consensus has been achieved about the best therapeutic choice [14, 16, 17].

Our study confirms that oxygenotherapy immediately reverts COHb content, independently to the treatment option chosen, normalising oxygen transport into surrounding tissues and restoring the previous hypoxic status [14, 15]. But after oxygen treatment mitCIV activity remains still decreased, whatever the oxygen treatment applied is, confirming the previously reported slow-movement recovery of this parameter [8]. At the immediately post-treatment stage, no-significant differences have been found on mitochondrial function recovery among the different oxygen treatment groups.

But although time-delayed, these differences have been found in our ACOP patients as long as 3 months after oxygen therapy, when we have analysed the global mitCIV activity evolution along the time. These results, accordingly to Brown and Piantadosi [22-24], show that oxygen treatment not only promotes CO-hemoglobin dissociation, but also mitCIV-CO separation. Our study demonstrates additionally the effectiveness on mitCIV function recovery depending on the oxygen treatment administered. The present three-month-study monitoring mitCIV function demonstrates that from the mitochondrial point of view normobaric treatment is effective-enough in promoting the recovery of mitochondrial function for moderate ACOP patients and that compared to non-CO exposed controls, one single session of hyperbaric oxygen is enough effective to restore mitCIV normal activity values in severe ACOP individuals. All our ACOP patients showed acute neurological symptoms, especially those with severe intoxication, but all these manifestations disappeared quickly after treatment and none of them showed late-sequels, confirming the amelioration found on mitochondrial function. Clinical implications of our mitochondrial-guidelines for ACOP treatment remain to be established.

Carbon monoxide immediately inhibits mitCIV activity after gas-exposure even when hemoglobin is not completely saturated and its effects take longer than hypoxia to resolve once oxygen treatment is applied. mitCIV could be more sensitive than hemoglobin to CO, which would impair mitochondrial function in smaller doses and make a stronger binding to mitCIV core. Accordingly, mitochondria rather than hypoxia could better explain early or late clinical manifestations. The early mitCIV CO-sensitiveness could explain some clinical symptoms of CO-poisoning that appear without COHb formation [6], and the time-extended mitCIV inhibition could explain late-happening sequels which appear several months after COHb normalisation. Unfortunately, the early saturation of mitCIV by CO limits its usefulness as a severity maker.

In our series mitochondrial acute CO-mediated lesion has been independent to oxidative stress, corroborating previous studies done on acute smokers. CO is one of the toxic substances present on the smoke which has been demonstrated to inhibit mitCIV activity in both chronic [10-12] and acute [13] smokers, although oxidative stress has only been found to be increased on chronic addicts patients. An acute CO-exposition (through smoke or gas exposure) would not be able to impair oxidative stress, or in case it was, ROS detoxification system would act quickly resolving ROS

accumulation, unless there was a chronic production.

mitCIV could be a good marker for ACOP treatment effectiveness and real biological recovery, and maybe will be able to explain long term damage after CO intoxication. Further studies have to be done to elucidate if this prolonged mitochondrial injury could stand at the basis of long term sequels and if mitochondrial therapeutic drugs could help in avoiding mitochondrial-mediated damage.

Acknowledgments: This study has been supported by Fondo de Investigaciones Sanitarias (FIS 40381/04, PI041239), Foundation for Research and Prevention of AIDS in Spain (FIPSE 36612-06 and FIPSE 36572-06), Support to Research Groups 2005/SGR/0300 and CIBER de Enfermedades Raras (CIBERER, Barcelona, Spain).

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RESUM DEL SISÈ ESTUDI

Els símptomes de la intoxicació aguda per monòxid de carboni (CO) s'atribueixen a la hipòxia derivada de la formació de carboxihemoglobina (COHb), però existeixen altres efectes més persistents del CO a nivell cel·lular, derivats de la seva afinitat pels grups hemo, com els que indueix la seva unió al complex IV de la CRM (COX). No sempre existeix correspondència entre símptomes clínics i COHb; existeixen símptomes que apareixen en absència de COHb i d'altres que es manifesten mesos després de la normalització dels nivells de COHb. La oxigenoteràpia restaura ràpidament els nivells de COHb, però no els de COX, i existeix polèmica sobre quin és el millor tractament a aplicar: oxigen normobàric (ONB) o hiperbàric (OHB), i el nombre de sessions a administrar.

L'objectiu del present estudi és determinar l'activitat de la COX en intoxicats per CO, correlacionar la COX amb amb la gravetat de la intoxicació i avaluar la recuperació de la COX segons el tractament d'oxigenoteràpia aplicat. Per aquest motiu s'ha analitzat la funció mitocondrial de 52 pacients que pateixen una intoxicació aguda de tipus moderat (COHb<20%) o greu (COHb>20%), segons els diferents tipus de tractament aplicat (comparació d'una sessió d'ONB vs. OHB en intoxicats moderats i una o dues sessions de OHB en intoxicats greus), al llarg de 3 mesos (abans i immediatament, 24hores, un mes i 3 mesos després del tractament), respecte 30 individus control.

Tots els intoxicats per CO presenten l'activitat enzimàtica de la COX inhibida. La COX s'inhibeix de manera immediata en intoxicats moderats i greus, de manera independent dels nivells de COHb assolits en sang, i la seva funció no es restaura immediatament després de la oxigenoteràpia, com ho fa la COHb. El retard en la restauració del correcte funcionament mitocondrial podria explicar les manifestacions clíniques adverses independents als nivells de COHb. L'activitat de la COX només es normalitza quan es monitoritza l'evolució d'aquest paràmetre al llarg dels 3 mesos posteriors a l'administració del tractament d'oxigenoteràpia, i ho fa per a tots els intoxicats, independentment de la gravetat de la intoxicació o del tractament aplicat.

L'estrès oxidatiu, en canvi, no es veu alterat per la intoxicació ni es modifica al llarg del seguiment d'aquests pacients.

Cap dels pacients tractats ha patit seqüeles tardanes que permetin veure l'evolució diferencial dels paràmetres mitocondrials en aquest tipus d'intoxicats.

CONCLUSIONS DEL SISÈ ESTUDI

1. Tots els intoxicats per CO presenten l'activitat enzimàtica de la COX inhibida, independentment de la gravetat de la intoxicació. Per tant la COX és un bon marcador d'intoxicació per CO, però no un bon marcador de la gravetat de la intoxicació.

2. L'activitat de la COX no es normalitza immediatament després d'aplicar el tractament d'oxigenoteràpia, com ho fa la COHb, sinó que requereix més temps per retronar als valors de normalitat. Aquest fet podria explicar les manifestacions clíniques adverses un cop restaurants els nivells basals de COHb.

3. La COX es recupera en tots els pacients que han rebut oxigenoteràpia, independentment del tractament aplicat. Per tant, des del punt de vista mitocondrial, la oxigenoteràpia normobàrica és suficientment eficient per restaurar la funció mitocondrial en els intoxicats lleus i una sola sessió d'oxigenoteràpia hiperbàrica per als intoxicats greus.

4. L'activitat de la COX podria ser un bon marcador de l'eficiència del tractament d'oxigenoteràpia aplicat i de la recuperació real, a nivell cel·lular, dels pacients intoxicats per CO. Seria de gran interès poder avaluar el paper de la lesió mitocondrial en el desenvolupament de lesions neurològiques tardanes en els intoxicats per CO.

5. L'estrès oxidatiu no sembla incrementar-se en la intoxicació aguda per CO, doncs possiblement requereix una lesió crònica per manifestar-se, i això és similar al que passa amb el fum del tabac, ric en CO, que inhibeix la COX en fumadors crònics i aguts, però només augmenta l'estrès oxidatiu en els crònics.

6. DISCUSSIÓ GENERAL

La present tesi doctoral comprèn 6 estudis científics que han donat lloc a les publicacions aquí recollides sobre l'anàlisi dels mecanismes de toxicitat mitocondrial de determinats fàrmacs d'ús habitual en la pràctica clínica, sovint responsables de l'aparició d'alguns dels efectes secundaris associats al tractament, així com de tòxics mitocondrials que poden explicar la etiopatogènia de les manifestacions clíniques associades a la intoxicació.

Aquests estudis han suposat l'anàlisi de 498 mostres i han requerit la inclusió de més de 221 voluntaris, entre pacients i controls, que de manera consentida i desinteressada han volgut participar en els nostres protocols, així com nombrosos professionals clínics i científics de l'àmbit hospitalari (Hospital Clínic de Barcelona, Hospital de Palamós, Hospital Germans Trias i Pujol de Badalona, Fundació Hospital-Asil de Granollers i Hospital Joan XXIII de Tarragona) i de la recerca bàsica (Departament de Bioquímica i Biologia Molecular de la facultat de Biologia de la UB), amb els que ens trobem en deute. Per a tots els individus inclosos s'han recollit les dades clíniques, epidemiològiques i demogràfiques necessàries i s'ha realitzat una completa anàlisi de la funció mitocondrial en diferents tipus de mostra (múscul esquelètic o CMSP), a vegades complementat amb estudis immunovirològics o metabòlics. La possibilitat de mesurar els més de 26 paràmetres del funcionament mitocondrial que portem a terme en el nostre laboratori permet l'avaluació global de la biologia mitocondrial i l'obtenció de la extensa informació que resulta imprescindible per determinar l'estat mitocondrial, realitzar diagnòstics de disfunció i establir els mecanismes de toxicitat d'un determinant agent causal, de manera acurada. Aquests estudis mitocondrials genètics i bioquímics s'han realitzat *ex vivo*, sempre utilitzant mostres procedents d'individus que es trobaven sota tractament amb algun d'aquests fàrmacs/tòxics mitocondrials; mai s'han utilitzat models animals o línies cel·lulars. Les anàlisis *ex vivo* presenten l'avantatge d'obtenir informació de primera mà de què està passant en l'organisme, la cèl·lula o el mitocondri, sense la possibilitat de que el model experimental dissenyat sigui artefactual, però introdueix la variabilitat biològica interindividual, que fa necessari treballar amb mostres poblacionals com més grans millor, a més a més dels imprescindibles grups control.

El **PRIMER dels treballs** que recull aquesta tesi doctoral mostra com les malalties mitocondrials, ja siguin genètiques o tòxiques, que impliquin un funcionament anòmal del mitocondri amb repercussions clíniques (com la miopatia o la hiperlactatèmia),

poden diagnosticar-se emprant la mateixa metodologia de treball. Aquesta idea que ja s'aplica en les tècniques de laboratori, no s'havia provat encara amb el test d'esforç aeròbic de l'avantbraç inicialment dissenyat per Jensen i col·laboradors (Jensen T et al, *Neurology* 2002) com una variant del test d'esforç isquèmic de l'avantbraç, que dona resultats molt satisfactoris per diagnosticar les miopaties primàries d'origen mitocondrial causades per defectes en el metabolisme anaerobi del glucogen (com la malaltia de McArdle) (Sanjurjo E et al, *Med Clin* 2004). Nosaltres hem aplicat el test d'esforç aerobi de l'avantbraç amb resultat satisfactori en 2 individus VIH-positius sota TARGA durant un episodi d'hiperlactatèmia, en els que la disfunció mitocondrial es manifesta a nivell molecular com una depleció del contingut en ADNmt i a nivell bioquímic com una disminució de l'activitat del complex IV (COX) de la CRM. Aquesta nova aplicació del test d'esforç pot ser útil per diagnosticar la disfunció mitocondrial en tots aquells pacients VIH-positius sota TARGA que presenten uns nivells elevats de lactat en sang, i en els que no sigui possible realitzar un diagnòstic molecular i bioquímic del funcionalisme mitocondrial (per exemple perquè no es disposa del utillatge o personal especialitzat). Les alteracions genètiques i bioquímiques s'han trobat, a més a més del teixit muscular, en CMSP. Les alteracions en CMSP que fins ara s'havien comunicat en la bibliografia per a aquest tipus de pacients només referien dades del contingut en ADNmt (Côté H et al, *N Engl J Med* 2002; McComsey GA et al, *AIDS* 2005). Aquest estudi reforça la hipòtesi de la base mitocondrial de la hiperlactatèmia associada al TARGA, i valida l'ús de CMSP per l'estudi del funcionalisme mitocondrial, a més a més dels teixits diana (en el cas de la hiperlactatèmia múscul i fetge), que habitualment resulten de més difícil accés. En aquest cas, la retirada o substitució del tractament antiretroviral per un de menys mitotòxic comporta la recuperació clínica dels pacients (que superen l'episodi d'hiperlactatèmia) i la millora clínica correlaciona amb els resultats del test aeròbic de l'avatbraç i els resultats enzimàtics i moleculars mitocondrials realitzats en múscul i CMSP.

Per aquest motiu, la resta d'estudis mitocondrials s'han realitzat en CMSP, doncs aquestes han resultat ser un bon model d'estudi de mitocondriopaties d'origen tòxic i suposen, sense dubte, un teixit de més fàcil obtenció.

El **SEGON i TERCER dels treballs** inclosos en aquesta tesi, també realitzats en CMSP de pacients infectats pel VIH i sota TARGA, indiquen que la toxicitat mitocondrial dels antiretrovirals és dosi i temps-dependent. Actualment, el disseny del TARGA idoni per a cada pacient, a més a més de tenir en compte l'eficàcia immunovirològica del tractament, les resistències i l'experiència antiretroviral prèvia, comença a introduir el concepte de procurar minimitzar la toxicitat mitocondrial d'una teràpia, que és crònica i s'acumula al llarg de tota la vida del pacient. Els pacients inclosos en aquests 2 treballs portaven molt de temps amb tractament antiretroviral abans de ser inclosos en l'estudi, especialment els que redueixen la dosi de didanosina de 400 a 250 mg/d, respecte a aquells que canvien a una pauta antiretoviral teòricament menys mitotòxica i sense dideoxinucleòtids consistent en T20+TDF+SQV/rit. En ambdós casos s'observa que abans de la intervenció que suposa la reducció de la exposició als tòxics mitocondrials, el TARGA acumulat, a més a més dels efectes mitocondrials que pot estar exercint la pròpia infecció per VIH (Côté H et al, *N Engl J Med* 2002; Miró Ò et al, *CID* 2004), indueix una disfunció mitocondrial que es detecta a nivell molecular (disminució del contingut en ADNmt) i/o bioquímic (decrement de l'activitat enzimàtica dels complexos III i IV de la CRM i de la taxa d'expressió proteica mitocondrial per COX-II). En el cas del treball que avalua el canvi de pauta antiretroviral, podríem afirmar que els efectes mitocondrials observats previs a la intervenció es deuen exclusivament al TARGA, doncs els resultats es comparen amb un grup d'individus infectats pel VIH que no reben tractament, en els que s'estarien valorant els possibles efectes mitocondrials que estaria exercint el propi virus.

Un altre tret en comú entre tots dos treballs és que els resultats globals de la reducció d'exposició als tòxics mitocondrials (en termes de dosi o potència) indiquen que millora l'estat d'alguns dels paràmetres mitocondrials, però en cap d'ells s'assoleix una recuperació mitocondrial completa. Aquesta recuperació mitocondrial parcial que només afecta a alguns dels paràmetres estudiats també l'hem observat en altres treballs que avaluen l'efecte de petites intervencions en el TARGA (com la reducció de 40 a 30 mg/d de didanosina) en el TARGA de pacients amb llarga experiència antiretroviral (Milinkovic A, *Antivir Ther* 2007). Tots dos estudis comparteixen plantejament i conclusions globals similars. Ara bé, en comparar els resultats concrets de la millora del funcionalisme mitocondrial per a cadascun d'ells, veiem que existeixen diferències; els paràmetres mitocondrials que responen a la reducció de la toxicitat mitocondrial en els 2 estudis són diferents. En el canvi de TARGA a un tractament teòricament menys nociu

consistent en T20+TDF+SQV/rit s'observa al cap de 6 mesos la recuperació de tots els paràmetres mitocondrials a excepció de l'ADNmt, mentre que en l'estudi de la reducció de dosi de ddI en pacients que mantenen TARGA amb ddI+TDF+NVP, transcorreguts 14 mesos, l'únic paràmetre mitocondrial que es recupera és precisament el contingut en ADNmt.

En l'estudi que avalua el canvi d'un TARGA mitotòxic amb dideoxinucleòsids al tractament de rescat compost per T20+TDF+SQV/rit, es pot considerar que permet, al cap de 6 mesos, la recuperació de la funció mitocondrial (entesa com l'activitat bioquímica de l'orgànul en tant que activitat enzimàtica dels complexos III i IV de la CRM, activitat per consumir oxigen a través del complex I o la taxa de síntesi proteica mitocondrial), i potser no reverteix la depleció en ADNmt perquè és un paràmetre que requereix més temps per canviar el seu estat. La lenta recuperació dels nivells d'ADNmt durant els 6 primers mesos que segueixen a una important depleció ha estat descrita en la bibliografia per grup del Dr. Cossarizza (Mussini C et al, *AIDS* 2005). Aquests autors demostren que la taxa de recuperació en el contingut d'ADNmt s'incrementa fins a 5 vegades un cop superats els 6 primers mesos. Hi ha autors que han vist la millora d'aquest paràmetre en substituir components de la pauta antiretroviral al cap d'un any de la intervenció (McComsey GA et al, *AIDS* 2005; Nolan D et al, *Antivir Ther* 2003), mentre que d'altres no han observat la millora d'aquest paràmetre ni tan sols passat aquest temps (Hoy JF et al, *J Infect Dis* 2004; Miró Ò et al, *Antivir Ther* 2005). El nostre estudi presenta la limitació d'un seguiment longitudinal de 6 mesos que potser ha resultat insuficient per detectar la recuperació en el contingut d'ADNmt, malgrat no es pot descartar que la intervenció no hagi estat capaç de revertir totalment la toxicitat mitocondrial acumulada al llarg de tot el tractament antiretroviral previ, i que els resultats haguessin estat els mateixos si el seguiment hagués estat més llarg. En alguns casos, la lesió mitocondrial acumulada pot resultar tan profunda que afecta als precursors de les cèl·lules sanguínies de la medul·la òssia (de vida mitja molt superior), i encara que es suprimeixi l'agent mitotòxic, les cèl·lules sanguínies circulants de nova generació descendent del llinatge afectat podran heretar els efectes induïts pel tòxic. En qualsevol cas, aquest treball permet concloure que el canvi de TARGA a aquesta pauta antiretroviral de rescat és beneficiós pel mitocondri, a més a més d'eficaç a nivell immunovirològic, i segur des del punt de vista metabòlic. A més a més, es tracta d'un dels pocs estudis que avaluen la toxicitat mitocondrial *in vivo* de l'inhibidor de la fusió del virus amb la cèl·lula hoste enfuvirtide. La interacció del fàrmac amb la cèl·lula hoste

exclusivament extracel·lular i la manca d'evidències clíniques que indiquin el contrari (Cooper DA et al, *Lancet Infect Dis* 2004; Bottaro EG, *Medicina (B Aires)* 2007) fan pensar que la seva toxicitat mitocondrial és nul·la. Però aquest estudi és el primer que ho analitza experimentalment. Un altre estudi *in vivo* realitzat al nostre laboratori amb cèl·lules mononuclears de voluntaris no-infectats als que se'ls administra durant una setmana enfuvirtide confirma l'efecte nul d'aquest fàrmac sobre el contingut en ADNmt (dades no publicades).

L'estudi que avalua la reducció de dosi d'un dels antiretrovirals del TARGA, concretament ddI, quan aquest es combina amb TDF i NVP, mostra que al cap de 14 mesos la funció mitocondrial es pot considerar que continua alterada (entesa com la capacitat bioquímica del mitocondri d'expressar i fer funcionar la CRM), i l'únic paràmetre mitocondrial que es recupera és l'ADNmt. El ddI és un dels ITIAN que presenta una capacitat manifesta més gran per inhibir *in vitro* l'ADNpolγ mitocondrial (ddC > ddI > d4T >>> AZT > 3TC > ABC = TDF, Kakuda T, *Clin Ther* 2000), i la interacció famacocinètica amb el TDF augmenta la seva concentració plasmàtica i biodisponibilitat, augmentant també la seva potència mitotòxica (Martinez E et al, *Lancet* 2004). És lògic doncs esperar una recuperació del contingut en ADNmt quan es redueix la presència d'aquest fàrmac durant més dels 6 mesos necessaris per observar canvis en aquest paràmetre. Ara bé, el fet de que la recuperació en els nivells d'ADNmt no es tradueixi en una millora de la funció mitocondrial transcorreguts aquests 14 mesos només pot deure's al fet que la llarga història de tractament antiretroviral d'aquests pacients (superior per exemple a la dels pacients sotmesos al canvi de pauta antiretroviral) és massa gran per revertir-se amb una intervenció tan simple com aquesta, o bé perquè un o més dels components de la pauta antiretroviral (ddI, TDF o NVP) presenten toxicitat mitocondrial independent a la inhibició de la replicació mitocondrial. Pel que fa a la recuperació dels paràmetres mitocondrials en estudis que avaluen la simple reducció de dosi durant 6 mesos d'un altre component del TARGA, en un dels treballs realitzats al nostre laboratori que monitoritza l'efecte mitocondrial de reduir la dosi d'estavudina de 40 a 30 mg/d sense canviar cap altre component del tractament, també van detectar una millora mitocondrial parcial (Milinkovic A, *Antivir Ther* 2007), que només afectava alguns paràmetres. Concretament, la reducció de dosi d'estavudina durant 6 mesos permetia la recuperació de l'activitat del complex IV de la CRM, però no del contingut en ADNmt. Potser la manca de recuperació en el contingut

d'ADNmt també es deu al curt seguiment efectuat per aquests pacients al llarg del temps, com en el cas de l'estudi del canvi de pauta antiretroviral a T20+TDF+SQV/rit. En qualsevol cas, encara que parcial, la reducció de ddI és beneficiosa pel mitocondri i manté l'eficàcia virològica del tractament, permetent que el 35% dels pacients recuperi parcialment els seus nivells de cèl·lules $CD4^+$ disminuïdes durant l'etapa amb elevades dosi de ddI (400 mg/d) i TDF, decrement prèviament descrit en la bibliografia (Negredo E et al, *AIDS* 2004; León A et al, *AIDS* 2005 gener i octubre). Precisament l'evolució en els nivells de cèl·lules $CD4^+$ dels pacients sí que correlaciona positivament amb la recuperació mitocondrial (tan funcional com molecular). Durant l'etapa amb ddI a 400 mg/d els individus que perden més cèl·lules $CD4^+$ són els que presenten major disfunció mitocondrial (51% contingut ADNmt, 37% activitat COX, 19% expressió COX-II/β-Actina i 57% COX-II/COX-IV), respecte aquells que manténen els nivells de limfòcits T- $CD4^+$ estables (que només redueixen la taxa d'expressió COX-II/COX-IV), i durant l'etapa de reducció de la dosi de ddI, només milloren el recompte de cèl·lules T $CD4^+$ en el subgrup de pacients que n'havia perdut més, que són els que presenten una millora més accentuada de l'estat mitocondrial, però no completa. Totes aquestes dades reforcen la idea de que existeix una clara relació entre disfunció mitocondrial i apoptosi (Kroemer et al, *Nat Med* 2000 i Wang X, *Genes and Development* 2001), i fins i tot poden fer-nos pensar que la base etiopatològica de la toxicitat immunològica descrita per la combinació d'aquests dos fàrmacs (ddI i TDF) (Negredo E et al, *AIDS* 2004; León A et al, *AIDS* 2005 gener i octubre) sigui de tipus mitocondrial.

La conclusió global que podem extraure del segon i tercer estudi és que existeixen mecanismes de toxicitat mitocondrial independents i dependents a la inhibició de l'ADNpolγ, característica dels ITIAN, que sempre s'ha considerat responsable dels efectes adversos del TARGA. Els efectes dels antiretrovirals ADNpolγ-dependents potser són majoritaris, però no determinen de manera directa la funcionalitat de l'orgànul, que pot restar inalterada per exemple en front de deplecions importantíssimes d'ADNmt com del 80-90% (demostrat en les mitocondriopaties primàries). De fet, existeix una línia cel·lular que conté mitocondris sense material genètic (cèl·lules *rho zero*), que pot viure suplementada només per piruvat i uridina. L'orgànul presenta mecanismes per suplir la deficiència en ADNmt a base de reajustar les taxes de transcripció i traducció mitocondrial, l'activitat enzimàtica dels diferents complexos, i d'altres mecanismes homeostàtics compensatoris, fins que aquesta depleció es fa molt

important i assoleix un determinat valor llindar, característic de cada teixit i individu, a partir del qual s'alterarà el funcionalisme mitocondrial i se'n deriven conseqüències clíniques. Alguns d'aquests mecanismes homeostàtics compensatoris mitocondrials transcripcionals o traduccionals han demostrat *in vivo* ser capaços de compensar una depleció d'ADNmt causada per ITIAN com la ddI i la d4T (Miró Ò et al, *J Acquir Immune Defic Syndr* 2004). En canvi darrerament s'han descrit efectes mitocondrials dels ITIAN en absència de depleció d'ADNmt i per tant s'ha posat de manifest l'existència de mecanismes lesius pel mitocondri independents doncs a la inhibició de l'ADNpolγ. Entre aquests mecanismes s'ha descrit, en el cas concret de la zidovudina (AZT o ZDV), la inhibició del transport de substrats del metabolisme energètic del mitocondri (succinat i ADP/ATP), la inhibició del complex IV (Citocrom C Oxidasa o COX) de la CRM o la disminució del contingut mitocondrial en carnitina (Dalakas MC et al, *Ann Neurol* 1994; Tomelleri G et al, *Ital J Neurol Sci* 1992; Barile M et al, *Gen Pharmacol* 1998), així com per a aquest i d'altres ITIAN, la inhibició *in vivo* (Mallon PW et al, *J Infect Dis* 2005) o *in vitro* (Galluzi L et al, *Antivir Ther* 2005) de la transcripció mitocondrial. Existeixen doncs efectes dels ITIAN sobre l'ADNmt que no tindran conseqüències sobre el funcionament mitocondrial, i efectes d'aquests fàrmacs sobre el mitocondri que no es manifesten en una depleció d'ADNmt. Aquests últims, els efectes ADNpolγ-independents, minoritaris o no, determinen de manera directa la funcionalitat mitocondrial en tant que capacitat metabòlico-enzimàtica per consumir oxigen i produir ATP, funcions que determinen de manera directa i immediata la viabilitat cel·lular.

D'aquests estudis s'extrau la conclusió de que la mesura del contingut en ADNmt com a únic paràmetre per avaluar l'estat mitocondrial pot resultar insuficient, en la nostra opinió, com a mínim per 3 motius: 1) La mesura d'un sola variable mitocondrial, sigui quina sigui, pot induir a l'elucubració de conclusions errònies, perquè només ens permet obtenir una visió parcial de la situació i pot donar-se el cas que el paràmetre en qüestió presenti una evolució contraposada a la de la resta de variables, especialment cert per la toxicitat antiretroviral i l'ADNmt, doncs existeixen mecanismes de lesió mitocondrial ADNpolγ-dependents i independents; 2) És un paràmetre de recuperació lenta, doncs existeixen retards de més de 6 mesos en observar una millora en el contingut d'ADNmt després d'una depleció important (Mussini C et al, *AIDS* 2005); i 3) l'ADNmt no ens

dóna una idea real de la funció mitocondrial, doncs existeixen mecanismes d'homeòstasi mitocondrials molt potents per contrarestar deplecions prou importants.

En qualsevol cas, és difícil avaluar l'efecte del canvi en la dosi o composició d'un dels elements del TARGA quan, primer, el TARGA presenta 3 components i segon, els pacients en els que s'avalua el canvi presenten una perllongada història de tractament antiretroviral acumulada per la vida mitja dels fàrmacs (que supera les 4 setmanes per exemple en el cas del TDF) i perquè pot haver exercit una lesió mitocondrial profunda, si es tracta de CMSP, que no només afecta a les cèl·lules circulants, sinó també en els seus precursors en la medul·la òssia (de vida mitja molt superior).

Per altra banda, la toxicitat mitocondrial s'ha demostrat que és present fins i tot quan no hi ha manifestacions adverses. Aquells estudis que avaluen la funció mitocondrial en individus asimptomàtics en tractament amb tòxics mitocondrials (anàlisi de la toxicitat antiretroviral per reducció de dosi de ddI i estudi de toxicitat antiretroviral per antipsicòtics) mostren cert grau de lesió mitocondrial en les CMSP analitzades, que presumiblement s'incrementaria quan augmentés l'exposició al tòxic fins a fer-se manifesta clínicament.

Un altre mecanisme de toxicitat mitocondrial ben diferent és el que hem establert en el **QUART dels estudis** inclosos en aquesta tesi, que mostra que la inhibició de la traducció mitocondrial és, possiblement, la causa de l'aparició dels efectes secundaris associats a l'administració prolongada de l'antibiòtic linezolid. Aquesta inhibició, de fins el 84% de l'expressió proteica mitocondrial, comporta la disminució de l'activitat enzimàtica del complex IV (COX) de la CRM, que no compensa ni tan sols mitjançant l'augment enregistrat en els nivells de transcrits mitocondrials. Aquesta regulació a l'alça de la transcripció mitocondrial és una resposta que forma part dels mecanismes d'homeòstasi mitocondrial i que ja s'ha descrit per altres antibiòtics inhibidors de la síntesi proteica procariota (Chrzanowska-Lightowlers ZM et al, *J Biol Chem* 1994; Kuzela S et al, *Biochim Biophys Acta*1988), però també per tòxics antiretrovirals (Miró Ò et al, *J Acquir Immune Defic Syndr* 2004). El bloqueig de la traducció mitocondrial es deu, possiblement, a la similitud entre ribosomes procariotes i mitocondrials, responsable de la toxicitat mitocondrial descrita per altres antibiòtics que exerceixen la seva acció antibacteriana bloquejant la síntesi de proteïnes bacterianes. Aquest bloqueig

mitocondrial és tan efectiu que causa una toxicitat aguda molt ràpida i pronunciada, que es manifesta al cap d'un mes d'inici del tractament i pot comportar greus conseqüències si no se suspèn la teràpia. La limitació en la disponibilitat de proteïnes mitocondrials bloqueja el funcionament mitocondrial però, en la nostra opinió, també limita la biogènesi de l'orgànul, que redueix així el seu nombre. Aquesta conclusió es desprèn en observar com la tendència en el decrement dels paràmetres mitocondrials es manté en normalitzar-los pel contingut en mitocondris, de manera que la disminució de la massa mitocondrial seria la conseqüència i no la causa de la disfunció mitocondrial. Una troballa interessant d'aquest estudi ha estat la detecció de la toxicitat mitocondrial per linezolid en CMSP, que discrepa de l'única dada publicada al respecte per aquest tipus de mostra en éssers humans (De Vriese AS, *Clin Infect Dis* 2006). De Vriese i col·laboradors, sense aprofundir en les causes, descriuen activitats enzimàtiques mitocondrials reduïdes en fetge, ronyó i múscul d'un pacient que després de quatre mesos de tractament amb linezolid mostrava simptomatologia clínica adversa associada a la teràpia, a més a més de la hiperlactatèmia, però en canvi, troben la funció mitocondrial inalterada en CMSP. El nostre estudi, en canvi, detecta la lesió mitocondrial en les CMSP de tots els pacients estudiats (cinc), que havien estat tractats amb linezolid menys de tres mesos, i que, a més a més, en la majoria dels casos, presentaven manifestacions clíniques nul·les o mínimes, addicionals a la hiperlactatèmia secundària al tractament. Malgrat alguns autors no són massa favorables a l'ús de CMSP per a la realització d'estudis mitocondrials, principalment perquè aquestes cèl·lules no són la diana etiopatològica de les manifestacions clíniques associades a les mitocondriopaties, segons la nostra experiència, la majoria d'alteracions mitocondrials són demostrables en aquest tipus de teixit (López S et al, *Antivir Ther* 2004 i Miró Ò et al, *Antivir Ther* 2003 i *J Acquir Immune Defic Syndr* 2004). A favor d'aquest argument, com a mínim per a l'estudi de la toxicitat deguda a alteracions en la traducció mitocondrial, McKee i col·laboradors, poc abans del nostre estudi, van posar de manifest l'analogia estructural dels ribosomes mitocondrials i dels efectes antibiòtics en tots els teixits que havien estudiat (McKee EE et al, *Antimicrob Agents Chemotherm* 2006).

Però la toxicitat mitocondrial presenta susceptibilitat individual a l'hora de manifestar-se, i en el cas concret de la derivada de l'administració de linezolid, podria deure's a polimorfismes o mutacions, com a mínim, en 2 tipus de gens: els que codifiquen pels

ARNr que conformen el ribosoma mitocondrial i els que codifiquen pels enzims que participen en el metabolisme d'aquest fàrmac. Tal vegada per això, només 5 dels 13 pacients que mantenen el tractament amb aquest antibiòtic més de 30 dies incrementen els nivells de lactat. Destacar que cap dels 5 pacients amb toxicitat manifesta per l'antibiòtic presenta una insuficiència renal que pugui explicar un increment de la toxicitat derivada de l'acumulació d'aquest fàrmac per problemes renals. És possible que els pacients que no manifesten clínicament la toxicitat mitocondrial (els asimptomàtics) també presentin parcialment inhibit el funcionament ribosòmic mitocondrial, però no fins al punt de comprometre la funció mitocondrial, tot i que en el present estudi no es van avaluar aquest tipus de pacients. L'acció antibacteriana del linezolid és temps-dependent, però la seva toxicitat podria ser concentració-dependent, com indica la presència prematura d'alteracions hematològiques en pacients amb insuficiència renal que tendeixen a acumular-lo (De Vriese AS et al i Mateu de Antonio J et al, *Clin Infect Dis* 2006). El metabolisme interindividual d'aquest antibiòtic fa variar les concentracions plasmàtiques de linezolid entre 1-6 mg/L en els períodes de concentració mínima (valls), per damunt de la CMI (0,5-4 mg/L). Aquesta variabilitat metabòlica interindividual podria fer que alguns pacients mantinguessin innecessàriament elevada la concentració de l'antibiòtic per damunt de la concentració que inhibeix el 50% de la síntesi proteica mitocondrial (estimada entre 3,37-5,26 mg/L en models animals, McKee EE et al, *Antimicrob Agents Chemother* 2006), de manera que inhibiria de forma permanent la síntesi proteica mitocondrial i causaria l'aparició d'efectes secundaris adversos. Per contra, en altres pacients les concentracions, com a mínim en els moments-vall, es mantindrien per sota d'aquesta concentració crítica mitocondrial que permetria una funció mitocondrial mínima però efectiva. Potser es podrien prevenir aquests efectes adversos ajustant la taxa d'antibiòtic fins a nivells propers a la CMI, com a mínim en els moments-vall.

L'estudi dels efectes mitocondrials que exerceixen els diferents fàrmacs antipsicòtics en les CMSP de pacients asimptomàtics en tractament es mostra en el **CINQUÈ dels treballs** que recull aquesta tesi. Aquest estudi ha posat de manifest que el mecanisme de toxicitat mitocondrial que exerceixen aquests fàrmacs consisteix en la disminució de l'activitat enzimàtica i oxidativa del complex I de la CRM i, en el cas concret de l'haloperidol, la inducció d'estrès oxidatiu. Aquests efectes mitocondrials podrien ser la base per a l'aparició de les manifestacions extrapiramidals associades a la teràpia, ja

documentades en models animals i en teixit *post-mortem* cerebral humà (teixit diana per a l'estudi de l'acció d'aquests fàrmacs) (Burkhardt C, *Ann Neurol* 1993; Modica-Napolitano JS, *Arch Pharm Res* 2003; Prince JA, *J Pharmacol Exp Ther* 1997). L'anàlisi mitocondrial realitzat denota diferent capacitat per induir lesió mitocondrial per part dels diferents fàrmacs antipsicòtics assajats (haloperidol > risperidona > clozapina) i aquesta gradació d'activitat mitotòxica precisament concorda amb la seva incidència a l'hora d'induir l'aparició dels efectes clínics secundaris associats a la teràpia. La lesió mitocondrial induïda a través de la alteració del correcte funcionament del complex I de la CRM per part dels antipsicòtics podria estar retroalimentada, és a dir; la disfunció del complex I incrementaria la formació de ROS, que al mateix temps incrementaria la disfunció del complex I. Els neurolèptics bloquegen els receptors de dopamina i afavoreixen la formació de ROS a través de l'enzim monoamina oxidasa (Lohr JB et al, *Arch Gen Psychiatry* 1991). L'estrès oxidatiu és especialment nociu pel mitocondri, per ser el seu lloc de formació, i el complex I s'ha demostrat que resulta especialment vulnerable a l'atac oxidatiu (Zhang Y et al, *J Biol Chem*1990). La disfunció mitocondrial produïda quan les ROS ataquen el complex I genera encara més ROS, en una espècie de cercle viciós (Wallace DC et al, *Nat Genet* 1998) que podria propiciar l'aparició dels efectes adversos. D'aquesta manera la inhibició del complex I en les CMSP dels pacients asimptomàtics analitzats, que només és inferior als rangs de normalitat establerts al nostre laboratori en 3 dels 84 casos, podria incrementar-se segons aquesta teoria al llarg del temps en tractament, fins arribar a creuar el nivell de disfunció mitocondrial necessari (llindar) per manifestar-se en forma d'efecte secundari al tractament en aquells pacients, ara sí, simptomàtics. En qualsevol cas, una limitació del present estudi és no disposar d'individus amb manifestacions clíniques, que per altra banda i sortosament cada vegada són menys nombrosos després de la incorporació dels neurolèptics atípics (com la risperidona i la clozapina) en el tractament antipsicòtic, que presenten una incidència molt menor d'efectes adversos associats.

El **SISÈ dels treballs** inclosos en la present tesi doctoral estudia la lesió mitocondrial induïda per un tòxic, i no per un agent farmacològic; es tracta de la inhibició del complex IV induïda pel CO. El mecanisme de toxicitat mitocondrial d'aquest gas es coneix des de fa temps (Piantadosi C, *J Hyper Med* 1987), però el nostre estudi és el primer en avaluar l'eficiència del tractament d'oxigenoteràpia aplicat segons la millora de la funció mitocondrial. Així s'ha arribat a la conclusió de que totes les teràpies

aplicades possibiliten la recuperació mitocondrial dels intoxicats al llarg del seguiment, independentment de la gravetat de la intoxicació. Des del punt de vista mitocondrial podem concloure que la recuperació mitocondrial s'assoleix tractant als intoxicats moderats (COHb 10-20% que no estigui acompanyada de simptomatologia clínica neurològica o cardíaca) amb oxigen normobàric i als intoxicats greus (COHb superior al 20%, o inferior, si està acompanyada per clínica neurològica o cardíaca) amb una sola sessió d'oxigenoteràpia hiperbàrica.

Per altra banda, segons les dades obtingudes en aquest estudi, la base etiològica de la disfunció mitocondrial en la intoxicació aguda per CO es limitaria a la disminució de l'activitat de la COX, sense que això comportés un augment de l'estrès oxidatiu lipídic en aquests pacients. Estudis previs realitzats pel nostre grup de recerca han demostrat que la toxicitat mitocondrial pel fum de tabac, ric en CO, podria ser anàloga a la intoxicació pel gas CO. En els fumadors crònics i aguts es troba present la disfunció de la COX (Miró Ò et al, *Carcinogenesis* 1999; Cardellach F et al, *Rev Neurol* 1998 and *J Toxicol Clin Toxicol* 2003; Alonso et al, *Eur Respir J* 2004), però la peroxidació lipídica només es troba alterada en els fumadors crònics.

En cap dels intoxicats aguts per CO inclosos en el nostre estudi s'han desenvolupat seqüeles tardanes. Aquestes seqüeles es manifesten setmanes després de la intoxicació, quan els nivells de COHb fa temps que estan normalitzats (per sota del 3%), la qual cosa suggereix que la etiopatologia de base per aquestes manifestacions hauria de ser diferent a la hipòxia, resolta molt temps abans. La disfunció mitocondrial, que es manifesta de manera immediata a la intoxicació, no es normalitza després de la oxigenoteràpia tan ràpidament com ho fa la COHb, sinó que requereix molt més temps per retornar a valors de normalitat. Aquesta lesió, potser millor que la hipòxia, o de manera complementària a aquesta, podria explicar l'existència de símptomes persistents i/o seqüeles tardanes. De fet, l'activitat enzimàtica de la COX s'ha vist inhibida en tots els intoxicats per CO estudiats, fins i tot aquells que tenien valors més baixos de COHb, demostrant que es tracta d'un paràmetre molt sensible al tòxic, que potser podria alterar-se quan la hipòxia i els nivells de COHb encara no són patològics, en alguns casos. Aquesta elevada sensibilitat al tòxic, però, dificultaria l'ús de la COX com a marcador de la gravetat de la intoxicació.

En qualsevol cas, la monitorització de la funció mitocondrial a través de la mesura del complex IV al llarg del temps podria ser, segons els nostres resultats, una forma alternativa de valorar la recuperació de la intoxicació o l'eficiència d'un tractament, i

podria resultar d'utilitat per realitzar el seguiment d'aquells pacients amb intoxicació greu que es preveu puguin patir lesions, sobretot neurològiques, tardanes. Aquest és un camp en el que pensem que pot ser molt interessant aplicar el model d'estudi assajat, per comprovar si la mesura de la lesió mitocondrial evoluciona paral·lelament a la clínica o fins i tot si es pot emprar com a valor pronòstic en aquells intoxicats que puguin presentar complicacions a mitjà o llarg termini.

Per últim, i **de manera global per a TOTS ELS TREBALLS**, voldríem comentar que els paràmetres mitocondrials quan s'analitzen *ex vivo* presenten una enorme variabilitat interindividual que dificulta les anàlisis transversals i fa difícil establir un valor numèric que serveixi com a valor llindar per determinar expressió fenotípica patològica. Per superar aquesta limitació els estudis inclosos en aquesta tesi s'han realitzat fent un seguiment longitudinal dels mateixos pacients, per disposar del valor de referència abans i després d'introduir el canvi mitocondrial a valorar, excepte en l'estudi de la toxicitat per antipsicòtics, en el que s'ha procurat augmentar el nombre de pacients inclosos per cada tractament estudiat, tenint en compte la dificultat de reunir pacients d'aquestes característiques. Aquestes anàlisis longitudinals confirmen que en tots els casos inclosos en la present tesi doctoral la toxicitat mitocondrial és reversible quan es retira, substitueix o disminueix l'exposició a l'agent mitotòxic/fàrmac, encara que només sigui de manera parcial o a llarg termini.

Per altra part, i com a valor afegit, creiem que els treballs inclosos en la present tesi doctoral aporten llum no només als diferents mecanismes fisiopatològics de la lesió mitocondrial secundària, sinó també a la bondat de les CMSP com a eina per a l'estudi d'aquestes mitocondriopaties. La validació d'aquest tipus de teixit com a model d'estudi és veritablement important per a la recerca mitocondrial en éssers humans per evitar l'ús de teixits que suposin aproximacions al malalt més cruentes.

7. CONCLUSIONS FINALS

Les **CONCLUSIONS** extretes a partir dels resultats obtinguts dels diferents treballs que composen la present tesi doctoral són les següents:

CONCLUSIÓ 1

El test d'esforç aeròbic de l'avantbraç pot ser útil per demostrar la disfunció mitocondrial present en pacients VIH-positius que sota tractament antiretroviral desenvolupen un episodi d'hiperlactatèmia, sense necessitat de dur a terme anàlisis de laboratori de funcionalisme mitocondrial. Aquesta lesió mitocondrial es caracteritza a nivell molecular per una disminució en el contingut d'ADNmt i a nivell bioquímic per una reducció de l'activitat del complex IV (COX) de la CRM, tant en múscul (un dels teixits diana) com en CMSP (mostra de fàcil obtenció). Totes aquestes alteracions reverteixen quan el pacient supera la crisi d'hiperlactatèmia.

CONCLUSIÓ 2

Els pacients VIH-positius amb una llarga història de tractament antiretroviral i afectats de lipodistròfia que són sotmesos a 6 mesos de substitució d'un TARGA de major potència mitotòxica per un tractament de rescat lliure de dideoxinucleòsids i teòricament menys nociu pels mitocondris (consistent en T20+TDF+SQV/rit) milloren el seu funcionalisme mitocondrial (activitat enzimàtica dels complexos III i IV de la CRM, taxa d'expressió proteica mitocondrial per COX-II i activitat oxidativa estimulada pel complex I) sense augmentar el contingut en ADNmt.

CONCLUSIÓ 3

Els pacients VIH-positius amb una llarga història de tractament antiretroviral que reben TARGA consistent en ddI+TDF+NVP i són sotmesos a 14 mesos de reducció de la dosi de ddI (de 400 a 250 mg/d) incrementen el contingut en ADNmt sense que millori la funció mitocondrial (activitat enzimàtica del complex IV de la CRM o taxa d'expressió proteica mitocondrial per COX-II). L'evolució dels paràmetres mitocondrials i el contingut en limfòcits T $CD4^+$ correlaciona positivament, segurament per l'estreta relació existent entre mitocondri i apoptosi.

CONCLUSIÓ 4 La utilització mantinguda durant setmanes de l'antibiòtic linezolid en aquells pacients que desenvolupen hiperlactatèmia s'associa a una taxa disminuïda d'expressió proteica mitocondrial per COX-II, fet que suggereix que l'antibiòtic linezolid, com d'altres antibiòtics, inhibeix la traducció mitocondrial. Possiblement el descens de la síntesi proteica mitocondrial i la disminució de l'activitat enzimàtica del complex IV de la CRM estan limitant la biogènesi mitocondrial, malgrat l'augment de transcripció mitocondrial. Aquests efectes mitocondrials reverteixen quan es normalitzen els nivells de lactat en retirar el fàrmac.

CONCLUSIÓ 5 En pacients esquizofrènics asimptomàtics per l'aparició de manifestacions extrapiramidals els fàrmacs antipsicòtics presenten la capacitat d'inhibir l'activitat enzimàtica i oxidativa del complex I de la CRM i concretament l'haloperidol, a més a més, d'induir estrès oxidatiu. Els diferents fàrmacs antipsicòtics presenten diferent grau d'inducció de lesió mitocondrial (haloperidol > risperidona > clozapina) i aquesta gradació d'activitat mitotòxica coincideix amb la classificació d'aquests fàrmacs segons la incidència d'aparició de manisfestacions extrapiramidals secundàries associades a la teràpia, descrita per altres autors.

CONCLUSIÓ 6 El CO presenta la capacitat immediata d'inhibir el complex IV de la CRM en pacients amb intoxicació de tipus greu i moderada sense alterar el nivell d'estrès oxidatiu. L'oxigenoteràpia restaura immediatament els valors de COHb, però el complex IV triga més temps en normalitzar-se, i ho fa en tots els pacients (greus i lleus), independentment del tractament aplicat, al cap d'unes setmanes. Per tant, pel que fa a la recuperació mitocondrial, n'hi ha prou amb aplicar una sessió d'oxigenoteràpia hiperbàrica en els intoxicats greus i oxigen normobàric en els moderats.

CONCLUSIÓ 7 Les cèl·lules mononuclears suposen un bon model d'estudi per a l'anàlisi de les mitocondriopaties secundàries i a més a més requereixen una aproximació mínimament invasiva per al pacient.

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9. ANNEX

9.1. PUBLICACIONS ADDICIONALS

A continuació s'adjunten les 13 **publicacions addicionals** (12 articles i un capítol d'un llibre) en les que la doctoranda ha participat al llarg d'aquest doctorat, independents a les que estructuren el cos d'aquesta tesi, que tracten sobre la toxicitat mitocondrial del tractament antiretroviral.

▪ Mitochondrial effects of HIV infection on peripheral blood mononuclear cells of HIV-infected patients never treated with antiretrovirals

▪ Up-regulatory mechanisms compensate mitochondrial DNA depletion in asymptomatic individuals receiving stavudine plus didanosine

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

▪ Short communication: HIV infection, antiretrovirals, and apoptosis: studies on skeletal muscle

▪ Diagnosis of mitochondrial dysfunction in HIV-infected patients under highly active antiretroviral therapy: possibilities beyond the standard procedures

▪ Treatment of symptomatic hyperlactatemia and lactic acidosis in HIV+ patients under nucleoside reverse transcriptase inhibitors

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

▪ Longitudinal study on mitochondrial effects of didanosine-tenofovir combination

▪ The impact of reducing stavudine dose versus switching to tenofovir on plasma lipids, body composition and mitochondrial function in HIV-infected patients

- HIV-1-infected long-term non-progressors have milder mitochondrial impairment and lower mitochondrially-driven apoptosis in peripheral blood mononuclear cells than typical progressors

- Mitochondrial DNA depletion in oocytes of HIV-infected antiretroviral-treated infertile women

- Mild improvement of mitochondrial function after three years of antiretroviral treatment interruption in spite of mitochondrial DNA impairment

- Capítol 'Mitocondria y antiretrovirales' del llibre 'Infección por VIH. Tratamiento antiretroviral y mitocondria'

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

CLINICAL INFECTIOUS DISEASES 2004; 39(5): 710-716.

Ò Miró, S López, E Martínez, E Pedrol, A Milinkovic, E Deig, **G Garrabou,** J Casademont, JM Gatell, F Cardellach.

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

Received 12 December 2003; accepted 27 March 2004; electronically published 13 August 2004.

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Clinical Infectious Diseases 2004; 39:710–6

1058-4838/2004/3905-0016$15.00

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.

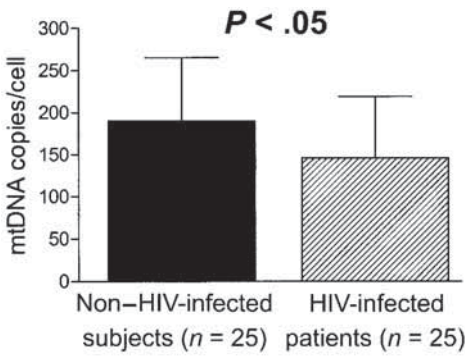

Figure 1. Quantification of mtDNA. Bars denote the results (expressed as mean values ± SD) for each group. Antiretroviral-naive HIV-infected individuals had a significant decrease in the number of mtDNA copies per PBMC, compared with healthy (non–HIV-infected) individuals. Comparison between groups was 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

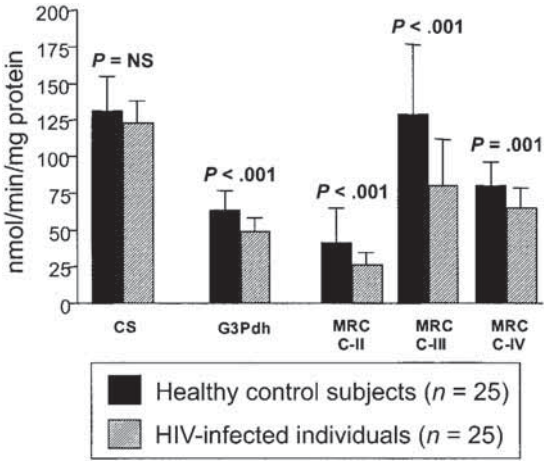

Figure 2. Enzyme activities, expressed as nanomoles of reduced or oxidized substrate per minute per milligram of total cell protein, of citrate synthase (CS), glycerol-3-phosphate dehydrogenase (G3Pdh), and mitochondrial respiratory chain (MRC) complex II (C-II), MRC complex III (C-III), and MRC complex 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.

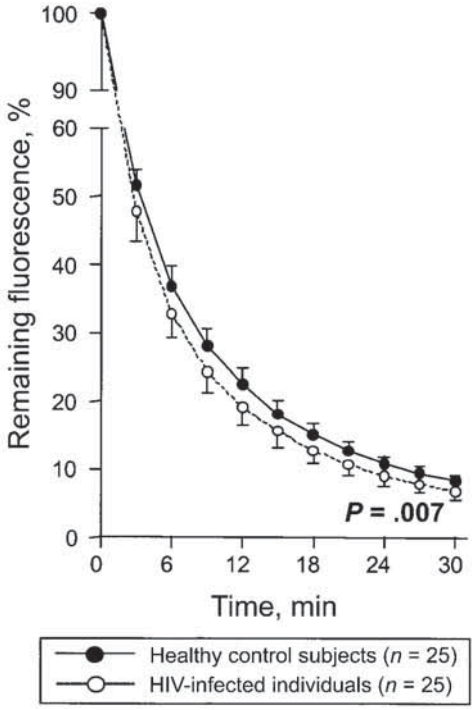

Figure 3. Studies of lipid peroxidation measured as loss of *cis*-parinaric acid fluorescence loss over time. Results are expressed as the percentage of the remaining fluorescence (± SD) at 3-min intervals. Untreated HIV-infected individuals had greater and faster loss of fluorescence, compared with 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

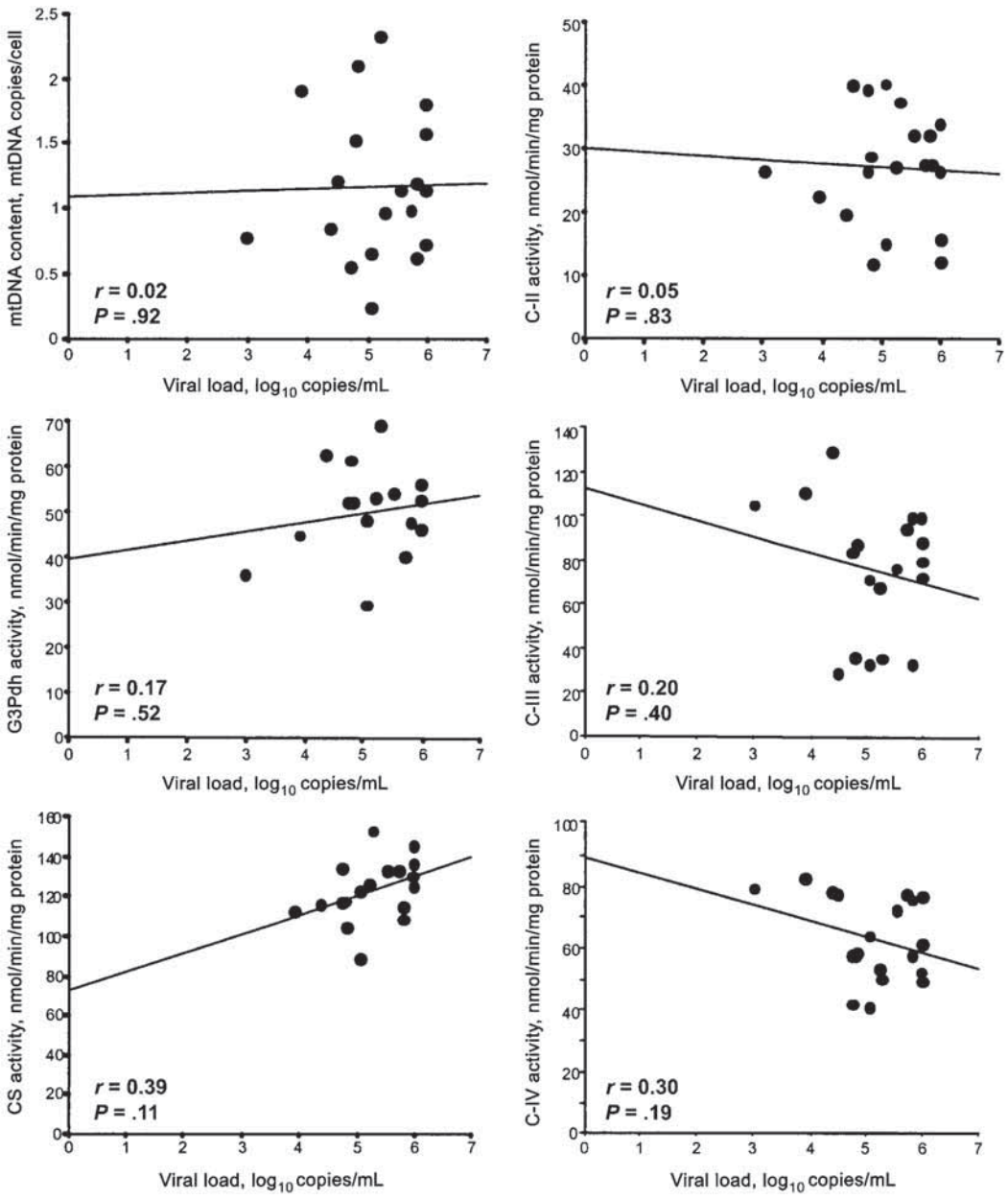

Figure 4. Association between viral load and mitochondrial parameters in antiretroviral-naive HIV-infected individuals, as established by a linear regression model. There was no significant association with any of the parameters assessed (mtDNA; glycerol-3-phosphate dehydrogenase [G3Pdh] activity; citrate 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.

Acknowledgments

We thank Anna Beato for her technical laboratory support; Emma Hammond, from the Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital, and Murdoch University (Perth, Western Australia), for providing us with the set of "international" standards for standardization of mtDNA quantification; and Pierre Rustin, from the Unité de Recherches sur les Handicaps Génetiques de l'Enfant, Hospital Necker des Enfants Malades (Paris, France), for his helpful counseling.

Financial support. Fundación para la Investigación y la Prevención del SIDA en España (FIPSE; grant 3102-00), Fundació La Marató de TV3 (grant 02/0210), Redes de Investigación en Mitocondrias (grant V2003-REDC06E-0) y SIDA (grant Redg173), and Suport a Grups de Recerca Consolidats de la Generalitat de Catalunya (grant 2001/SRG/00379).

Conflict of interest. All authors: No conflict.

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UP-REGULATORY MECHANISMS COMPENSATE MITOCHONDRIAL DNA DEPLETION IN ASYMPTOMATIC INDIVIDUALS RECEIVING STAVUDINE PLUS DIDANOSINE

JOURNAL OF ACQUIRED IMMUNE DEFICIENCY SYNDROMES 2004; 37(5): 1550-1555.

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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

(*J Acquir Immune Defic Syndr* 2004;37:1550–1555)

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

Received for publication April 16, 2004; accepted July 6, 2004.

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Supported by the following grants: Fundación para la Investigación y la Prevención del Sida en España (FIPSE 3102-00 and 316l/00A), Fundació La Marató de TV3 (020210), Redes de Investigación en Mitocondrias (V2003-REDC06E-0) y Sida (Rg-173), and Suport a Grups de Recerca 2001/SGR/00379.

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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

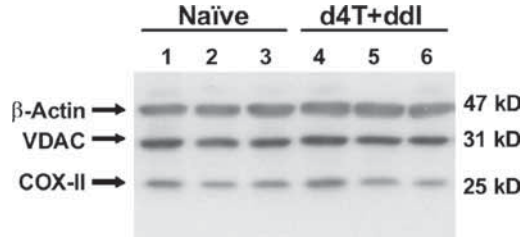

FIGURE 1. Example of immunoblot analysis of cytochrome c oxidase subunit II (COX-II) protein abundance in protein extracts from peripheral blood mononuclear cells of naive (1–3) and stavudine plus didanosine (d4T + ddI)–treated (4–6) patients. Each lane corresponds to 20 μg of protein, and arrows indicate 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.

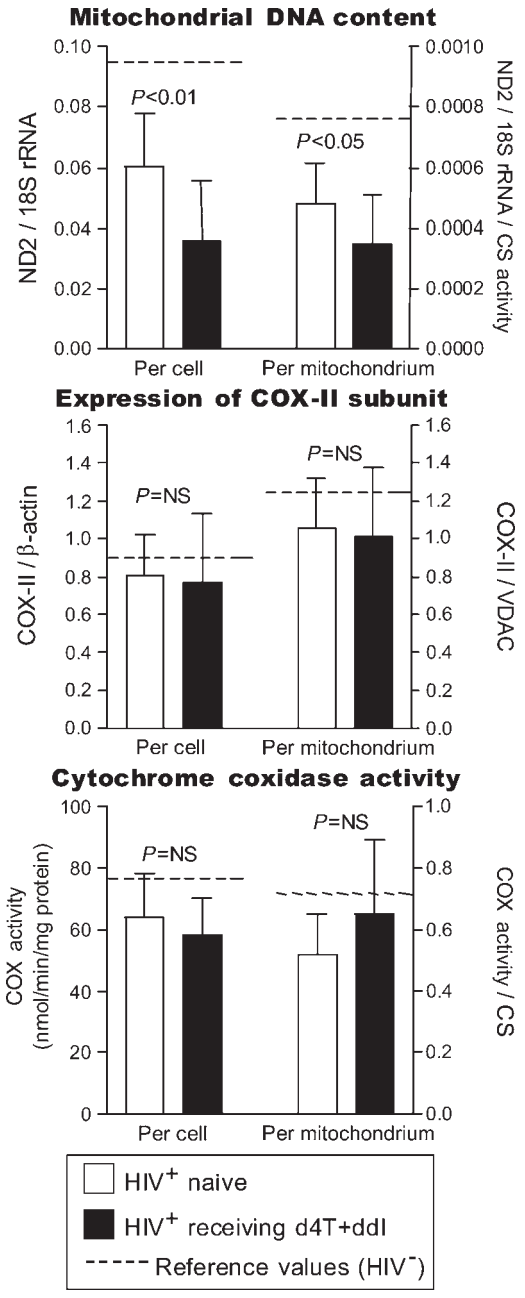

FIGURE 2. Mitochondrial DNA content and expression of cytochrome c oxidase subunit II (COX-II) and COX activity assessed in peripheral blood mononuclear cells of naive and stavudine plus didanosine (d4T + ddI)–treated patients. CS indicates citrate synthase; VDAC, voltage-dependent anion channel.

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,

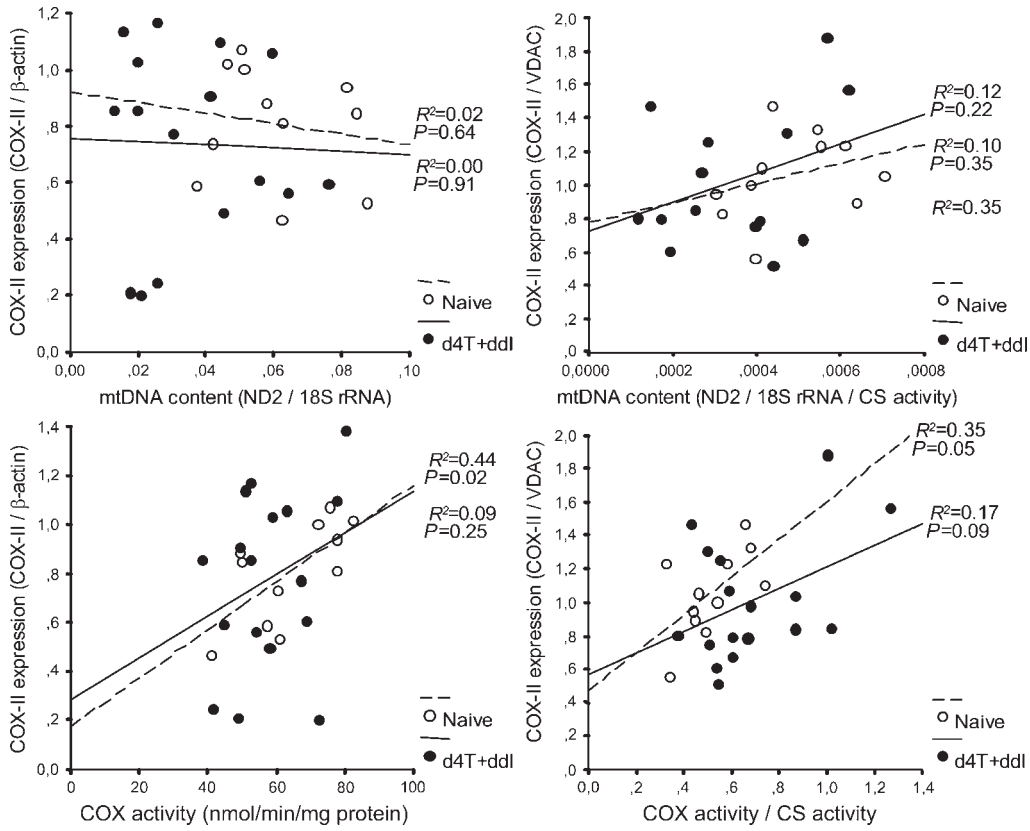

FIGURE 3. Relation between the expression of cytochrome c oxidase subunit II (COX-II) and mitochondrial DNA (mtDNA) content (upper) and COX activity (lower) in peripheral blood mononuclear cells of naive and stavudine plus didanosine (d4T + ddI)–treated patients. In all cases, these relations are assessed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

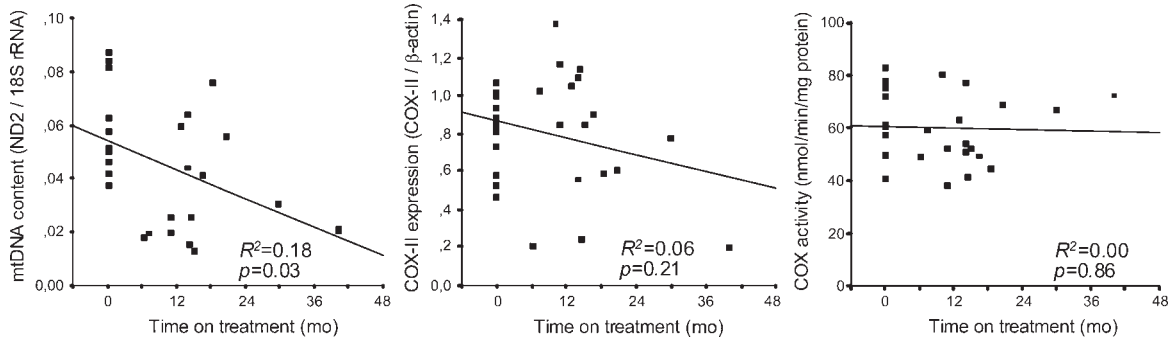

FIGURE 4. Relation between time receiving antiretroviral therapy, including stavudine plus didanosine (d4T + ddI), and mitochondrial DNA (mtDNA) content (left), expression of cytochrome c oxidase subunit II (COX-II; center), and COX activity (right).

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

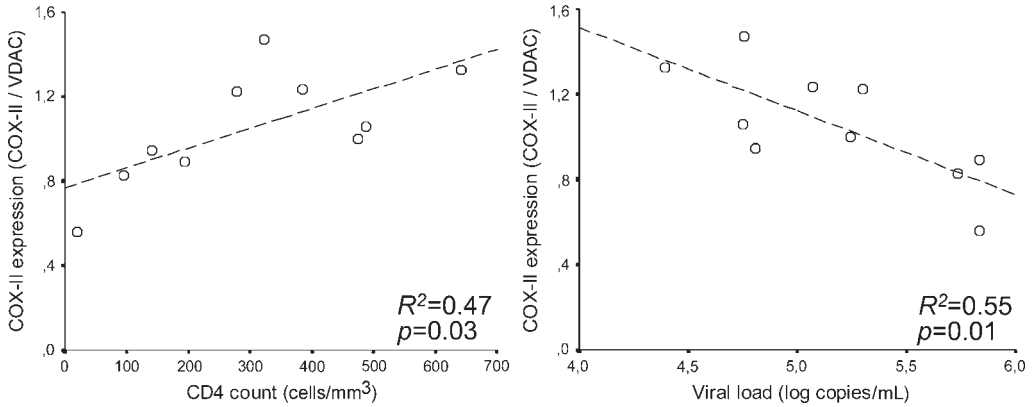

FIGURE 5. Relation between the expression of cytochrome c oxidase subunit II (COX-II) and $CD4^+$ T-cell count (left) and viral load (right) in the absence of antiretroviral therapy (naive patients). VDAC indicates voltage-dependent anion channel.

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

ANTIVIRAL THERAPY 2004; 9(6): 969-977.

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

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

Introduction

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

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

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

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

Materials and methods

Study design

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

Study population

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

Treatment groups

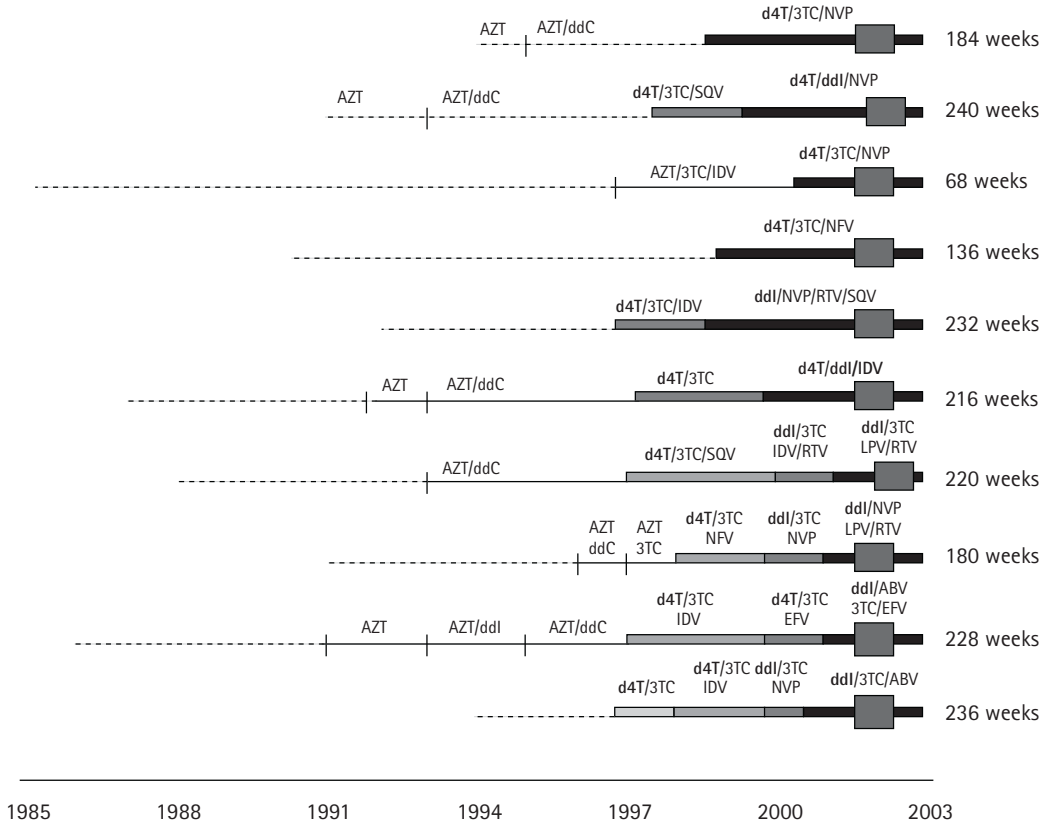
The HCV-treated patients were recruited and screened from a group of 27 patients enrolled in a trial of directly observed treatment against HCV with PEG-IFN α-2b (1.5 μg/kg/weekly) and a fixed dose of RBV (800 mg/daily) [18]. For the present study, patients were selected if they fulfilled the entry criteria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

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

Mitochondrial function evaluation

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

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



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

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

Mitochondrial DNA quantification. For each DNA extract, the housekeeping r18S nuclear gene and the highly conserved mitochondrial ND2 gene were quantified separately by quantitative real-time polymerase chain reaction (LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany). The results were expressed as the ratio of the mean of duplicate measurements of mtDNA to the mean of duplicate measurements of nuclear DNA (mtDNA/nDNA).

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

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

Clinical and anthropometric evaluation

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

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

Biochemical analyses

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

Statistical analyses

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

Results

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

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

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

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

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

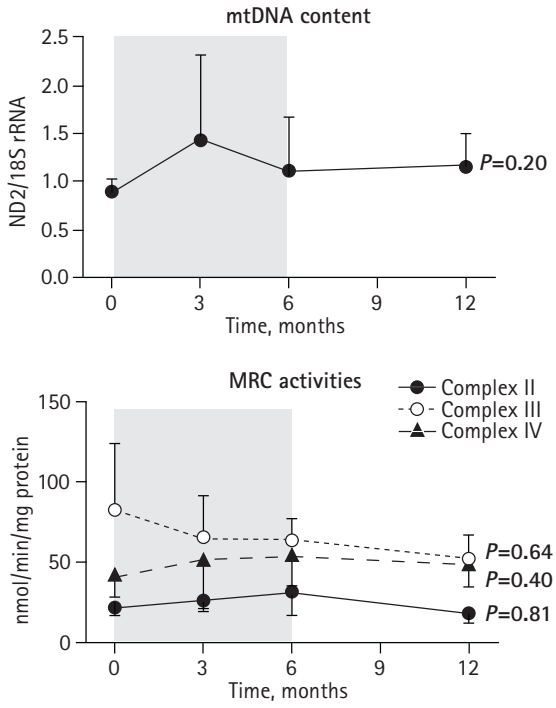
Table 2. Mitochondrial data at baseline and after 6 months in the HCV-treated group and the control group

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

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

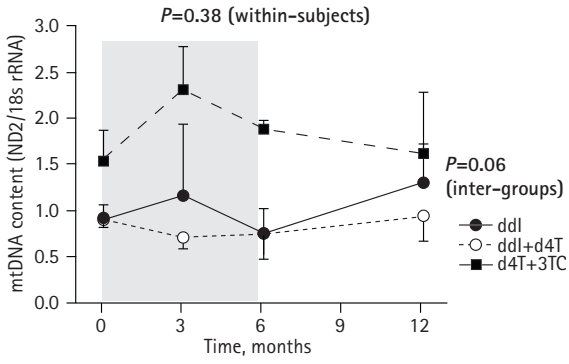
Although all HCV-treated patients received 800 mg of RBV daily, exposure adjusted by weight was different in every patient. Moreover, the mean daily RBV dosage by weight increased significantly between the determinations at baseline and 24 weeks (12.3 ±1.5 to 13.1 ±1.4 mg/kg; *P*=0.002) of HCV therapy.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

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



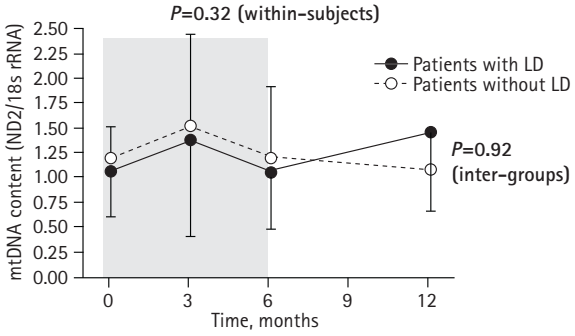
P values were calculated using the general linear model for repeated measures. Points denote the means and bars denote the SDs. Shadowed areas represent the time on treatment for HCV infection.

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



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

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



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

Conclusions

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

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

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

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

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

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

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

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

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

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

Acknowledgements

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

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

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Received 12 May 2004, accepted 30 September 2004

SHORT COMMUNICATION: HIV INFECTION, ANTIRETROVIRALS, AND APOPTOSIS: STUDIES ON SKELETAL MUSCLE

AIDS RESEARCH AND HUMAN RETROVIRUSES 2005; 21(8): 702-705.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 21, Number 8, 2005, pp. 702–705

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.

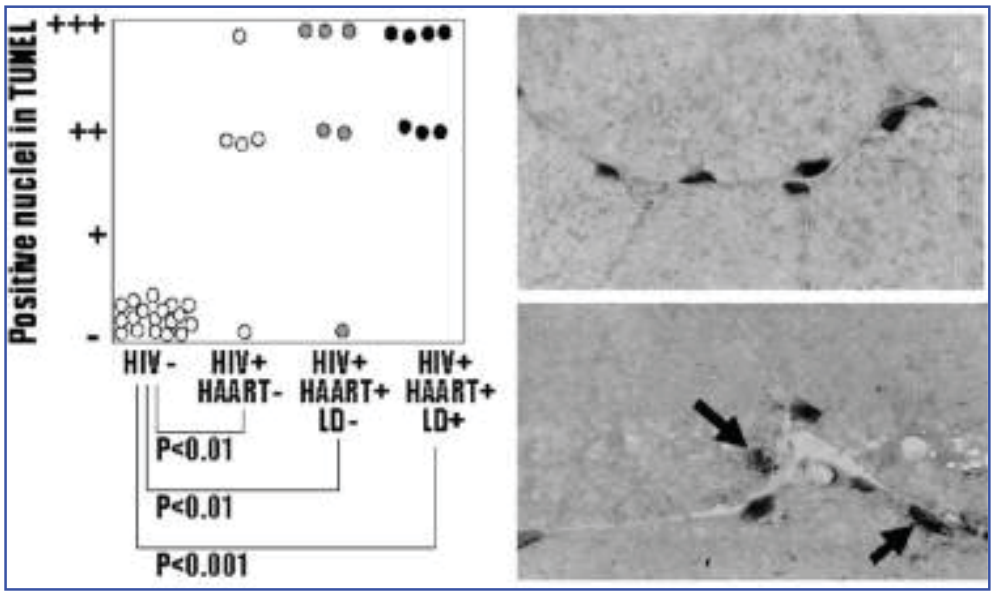

FIG. 1. Results of studies using TUNEL immunoreaction activity on skeletal muscle (left). Negative nuclei in an uninfected HIV individual (group A, upper right) and abnormal nuclei surrounded by positive TUNEL reaction (arrows) in an untreated HIV-infected individual (group B, right, down). The differences 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.

ACKNOWLEDGMENTS

This study has been supported by Fundación para la Investigación y la Prevención del Sida en España (FIPSE 3102-00), Fundació La Marató de TV3 (02/0210 and 01/1710), Suport als Grups de Recerca (2001/00379), and Redes de Investigación en Mitocondrias (V-2003-REDG011-O) y en Sida (Rg-173) del Fondo de Investigaciones Sanitarias.

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DIAGNOSIS OF MITOCHONDRIAL DYSFUNCTION IN HIV-INFECTED PATIENTS UNDER HIGHLY ACTIVE ANTIRETROVIRAL THERAPY: POSSIBILITIES BEYOND THE STANDARD PROCEDURES

ANTIVIRAL THERAPY 2005; 10 Suppl 2: M65-M71.

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Diagnosis of mitochondrial dysfunction in HIV-infected patients under highly active antiretroviral therapy: possibilities beyond the standard procedures

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Introduction

Mitochondrial (mt) disorders are a complex group of diseases due to malfunctions of the mitochondrial respiratory chain (MRC). As mitochondria are ubiquous within body cells, clinical manifestations of mt diseases are very polymorphous; classically, they have been considered primary or secondary. Primary mt defects are considered to be those caused by mutations in genes encoding subunits of the MRC [1]. Because mitochondria have a dual genetic control, these defects include mutations of mtDNA as well as nuclear DNA (nDNA) genes. Mitochondrial dysfunctions not related to mutations in genes encoding subunits of the MRC are considered secondary mt disorders [2,3]. They include defects of nuclear-encoded mt proteins responsible for the maintenance of MRC subunits and mtDNA biogenesis, or those involved in many other mt biochemical pathways. They also include non-genetic defects that produce derangements of mt homeostasis that have an impact on MRC function. This last situation is generally due to exposure to an environmental toxin or drug, which may interfere with MRC either directly or through a series of mechanisms including secondary genetic defects, which may appear as a consequence of mt derangement.

Since the introduction of highly active antiretroviral therapy (HAART) the prognosis of HIV infection has changed dramatically, shifting the concept of HIV disease from that of a highly mortal disease to that of a chronic illness. However, the chronic use of HAART has also been associated with an increase in adverse drug effects, such as hyperlactataemia, polyneuritis and lipodystrophy. It has been proposed that some of these effects could be mediated by mt toxicity and, actually, a decrease in mtDNA copy number and a MRC dysfunction has been repeatedly described [4–12].

A frequent problem in clinical practice is that the diagnosis of mt dysfunction is not easy to establish. A classic and relatively simple way to approach this issue is the measurement of ketone body ratios. An enzymatic defect of MRC produces a modification of redox potential status due to the accumulation of reduced equivalents (NADH and $FADH_2$). This accumulation partially inhibits the Krebs cycle that, in turn, favours the production of β-hydroxybutyrate with respect to acetoacetate inside mitochondria, and lactate with respect to pyruvate in cytoplasm. These ratios were measured some years ago in children with perinatal HIV infection treated with zidovudine without any significant result, possibly because its sensitivity is very low [13]. To our knowledge, its measurement has not been reported in HIV infection since. A series of more sophisticated techniques have been used since then, including morphological, molecular genetic and enzymatic studies. The most important findings using these approaches are the presence of ragged-red fibres on muscle histochemical studies [4], a depletion of mtDNA and a dysfunction of MRC in diverse tissues [5–12]. A problem with these techniques is that they are usually not routine. Moreover, to establish a diagnosis of mt dysfunction, a combination of these studies is frequently required, and only a few medical centres in each country are equipped for thorough mt evaluation. The suspicion of a possible mt dysfunction in the context of HAART treatment is, consequently, difficult to prove and frequently only suspected. This may have serious implications because the suspicion of mt toxicity often induces a change in HAART regime with the consequent discontinuation of useful antiretrovirals.

In this review, we analyse a series of techniques that may suggest the existence of a mt dysfunction not based on pathology, biochemical tissue or molecular genetic analyses. Several of these techniques have been used for the evaluation of classic mt diseases and, with some modifications, could be applied to the diagnosis of HAART-associated mt dysfunction.

Most have not been used for this purpose, their utility being speculative.

Breath tests

Breath tests are simple, cost-effective and safe. For these reasons, they have been proposed for many 'dynamic' evaluations, especially related to liver disease either of genetic or acquired origin. The rationale for their use to assess mt function is that oxidative metabolism of some substrates, such as methionine and ketoisocaproic acid, need the integrity of the hepatocyte electron transport chain and/or ATP synthesis for decarboxylation [14,15]. Methionine is the best-studied carbon-labelled substrate; the isotope usually used is ^{13}C, which is non-radioactive, although ^{14}C can also be used. The procedure consists of measuring the exhalation of $^{13}CO_2$ after administration of 2 mg/kg body weight of [methyl-^{13}C]-labelled methionine. Breath $^{13}CO_2$ is measured with a mass spectrometer at baseline and every 15 min thereafter for 120 or 180 min [15]. What the study reflects is hepatic mt function, although to date there is still no general consensus as to its usefulness in the clinical setting.

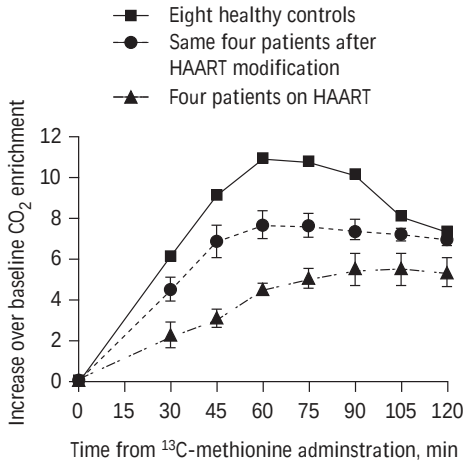
In the absence of liver disease, the ^{13}C-methionine breath test has been used to assess mt function in patients under HAART. Decreased intramitochondrial decarboxylation capacity has been reported in HIV-infected patients with antiretroviral drug-related hyperlactataemia compared with healthy controls [16,17]. Drug discontinuation or regimen modification led to a recovery of this capacity (Figure 1).

The most important limitation in the evaluation of HIV-infected patients is the high prevalence of coinfection with hepatitis C and B viruses, as well as possible alcohol abuse or hepatotoxicity from drugs, making it difficult to completely exclude an undiagnosed hepatic disease. Another minor problem in the use of breath tests is the need for mass spectrophotometry. However, this problem can be easily overcome with the development of a kit similar to those used to diagnose *Helicobacter pylori* infection. This kit uses ^{13}C-urea as a substrate and breath-exhaled samples are collected into bags and mailed to a reference laboratory [18]. The general impression is that this is a young but promising field and that further studies are needed to assess the suitable substrate or substrates required for the evaluation of the complex mt metabolism.

Cardiopulmonary exercise tests

Cardiopulmonary exercise testing is an objective method for evaluating exercise limitations either of cardiac, pulmonary or metabolic origin, in which subjects exercise, preferably on a bicycle ergometer.

Figure 1. $^{13}CO_2$ breath test exhalation curves of four patients with drug-related hyperlactataemia during therapy and after therapy modification



The second breath test was performed between 3 weeks and 2 months after HAART modification. Results are compared with a series of eight healthy controls (continuous line). Modified from Milazzo *et al.* [16]. HAART, highly active antiretroviral therapy.

During exercise they breathe through a mouthpiece that is a miniaturized pressure differential pneumotachygraph. The inspired and expired gas is continuously sampled and O_2 uptake and CO_2 elimination are computed [19]. Typical recordings also include cardiac output, dynamic flow volume loops and ventilation-perfusion measurements performed at rest and during an incremental workload exercise test. Serum lactate can also be measured. A series of derived parameters can be obtained and correlated with workload, measured in watts. The respiratory exchange ratio (RER) is the quotient of the amount of CO_2 produced to the amount of O_2 consumed. Peak oxygen consumption (VO_2max) in ml oxygen/kg body weight/min denotes cardiovascular or 'aerobic fitness'. The final workload (peak watts/kg) achieved by patients is considered their 'peak work capacity'. The point during exercise at which anaerobic metabolism is used to supplement aerobic metabolism as a source of energy is termed the 'anaerobic threshold'. It normally occurs at >40% of VO_2max [19,20].

In mt myopathies, exaggerated circulatory and ventilatory responses to exercise are governed by skeletal muscle oxidative capacity, in which more severely impaired oxidative phosphorylation elicits more active systemic responses. Exercise capacity varies widely, which is attributable to different levels of oxidative impairment, but in general, the average peak work capacity and VO_2max are lower than in control

subjects [21,22]. Low anaerobic threshold in a subject with unimpaired cardiovascular fitness provides additional evidence of mt dysfunction [22].

In a group of HIV-positive patients on HAART, classified according to their level of venous lactate levels, there were no significant differences with respect to peak work capacity, expired volume/min or VO_2max when compared with a group of controls. However, patients with abnormal venous lactate levels had a higher RER at the peak of exercise and tended to have a lower anaerobic threshold [23]. In another group of HIV-positive patients on HAART, lactate production was higher in patients with lipodystrophy than in patients without lipodystrophy at the same level of VO_2max. In the same way, peak work capacity on the cycloergometer was reduced in the lipodystrophic group. The anaerobic threshold occurred earlier in the lipodystrophic group [24]. Finally, a group of HIV-infected patients with lipodystrophy and elevated P-lactate levels had a significantly lower peak work capacity and a trend towards reduced VO_2 max compared with controls [25].

One problem in interpreting these results is that a series of HIV-positive patients analysed in the late 1980s before the introduction of HAART, also exercised to a significantly lower peak work capacity compared with a group of non-infected patients. The ventilatory anaerobic threshold values and VO_2max were also significantly lower [26]. There are actually several possible mechanisms to explain exercise limitations in HIV-positive patients aside from mt dysfunction: cardiac, ventilatory, peripheral nerve or muscle abnormalities, anaemia, smoking, deconditioning, decreased motivation resulting from chronic disease or a combination of these factors [27]. The extreme difficulty in excluding all these circumstances in HIV-positive patients, together with the need for well-trained personnel to interpret the results and the relatively sophisticated material used, makes cardiopulmonary exercise testing far from being an easy method to implement in usual clinical practice.

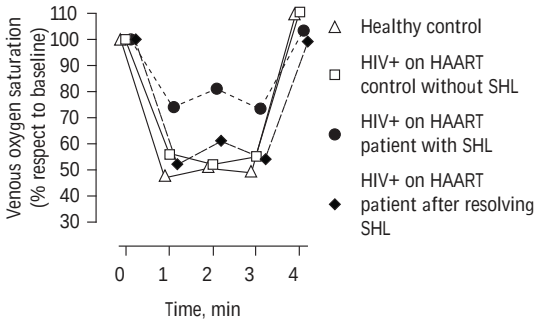
Forearm exercise tests

Functional blood tests

The best known is the *ischaemic forearm exercise test* introduced with the specific aim of screening for abnormalities of muscle glycogen metabolism such as myophosphorylase deficiency or McArdle's disease [28,29]. Although not designed for the study of mt defects, it has been suggested that the rate of lactate clearance in the post-exercise period may be significantly slower in patients with mt myopathies than in healthy subjects [30,31]. To our knowledge, there are no systematic studies that sustain this hypothesis. Furthermore, in a study not designed to study the rate of lactate clearance, the authors did not find any difference between HIV-infected patients with lipodystrophy and elevated P-lactate levels and a group of controls [25]. This possibly indicates that the test is not useful in the analysis of mitochondria in patients under HAART.

An *aerobic forearm exercise test* specifically developed for the study of mt performance [32] seems more interesting for the study of HAART-related mt dysfunction. The protocol consists of intermittent handgrip exercise (squeeze 1 sec, rest 1 sec) for 3 min at 33% of intended maximal voluntary contraction (MVC) force, which is determined 30 min before initiation of data collection. Oxygen saturation is analysed in blood samples collected from the median cubital vein of the exercised arm. Patients with a mt myopathy do not develop a significant desaturation during exercise compared with healthy subjects or patients with other muscular diseases [32]. We recently had the opportunity to study a patient with symptomatic hyperlactataemia due to HAART [33]. During the acute phase of hyperlactataemia, O_2 saturation did not decrease as expected in response to exercise. Antiretroviral therapy was initially stopped and afterwards modified. The patient clinically recovered and after 6 months, the *aerobic forearm test* was completely normal (Figure 2). Although promising and relatively easy to implement in clinical practice, more studies are required to determine the sensitivity and specificity of this method.

Figure 2. Venous oxygen saturation before a *forearm aerobic exercise test* (minute 0), during aerobic exercise (minutes 1, 2 and 3) and after resting (minute 4)



A patient on HAART who developed symptomatic hyperlactataemia did not present a normal venous desaturation during exercise as compared with healthy subjects and patients on HAART without hyperlactataemia. This is an indication of poor O_2 utilization during exercise. After 6 months and once the patient was clinically recovered, the aerobic forearm test was normal. SHL, symptomatic hyperlactataemia; HAART, highly active antiretroviral therapy.

Near-infrared spectroscopy (NIRS)

NIRS is a non-invasive optical method for continuous monitoring of oxygenation and haemodynamics in tissue. It is based on the capacity of light in the near-infrared range to penetrate tissues to a depth of several centimetres and on absorption characteristics of oxyhaemoglobin plus oxymyoglobin, compared with deoxyhaemoglobin plus deoxymyoglobin, with differential near-infrared light spectrometry. A series of optical fibres are placed on top of the muscles of the exercising limb and the difference in the absorption at 760 and 850 nm estimates the relative change in oxyhaemoblobin versus deoxyhaemoglobin. The sum of these spectra provides an estimate of the blood volume. Thus, the net extraction of oxygen from oxyhaemoglobin relative to oxygen delivery by blood circulation can be determined over the area of the tissue sampled by the device, and the effect of exercise on muscle oxygenation can be assessed [34]. Since data can be collected continuously, this device provides unique information regarding the kinetics of oxygen utilisation relative to delivery in the transition from rest to exercise, during sustained exercise and during recovery. This technique has been used to assess patients with classic mt diseases. It seems that these patients present a decrease in O_2 consumption compared with controls in both low-intensity exercise and at rest [34,35]. To our knowledge, NIRS has not been used to assess the possible mt toxicity in HAART-treated patients. The advantages of the non-invasiveness of the procedure are possibly counterbalanced by the difficulties in interpreting the results. It seems, nonetheless, to be a technique with potential utility in this setting.

Imaging techniques

Imaging studies have changed our approach to medical diagnosis in the last 20 years. Many modalities may contribute toward the diagnosis of mt diseases. Table 1 summarizes the most important methods [36]. Among them, magnetic resonance spectroscopy (MRS) seems the most promising for the diagnosis of mild mt dysfunction due to its ability to detect metabolic changes. MRS can be used to monitor tissue bioenergetic changes in both the brain and the skeletal muscle.

Proton-MRS is useful in studying bioenergetic changes in the brain. The main resonances observed in the spectrum arise from N-acetylaspartate (a neuronal marker), lactate, creatine and choline. Proton-MRS is less useful in the investigation of skeletal muscle because of the large signal from the protons in the subcutaneous fat, which can obscure other signals of interest [36]. For this reason, proton-MRS seems to be of low utility in the investigation of the effects of HAART regimes.

The spectra of phosphorus-MRS (P-MRS) contain several resonances: three arise from the γ, α and β phosphate groups of ATP, one from phosphocreatine (PCr) and one from inorganic phosphate (Pi) (Figure 3); two additional smaller resonances can sometimes be observed from phosphomonoesters and phosphodiesters. The area under each resonance is proportional to the amount of the corresponding metabolite. The spectral distance between Pi and PCr provides information about intracellular pH.

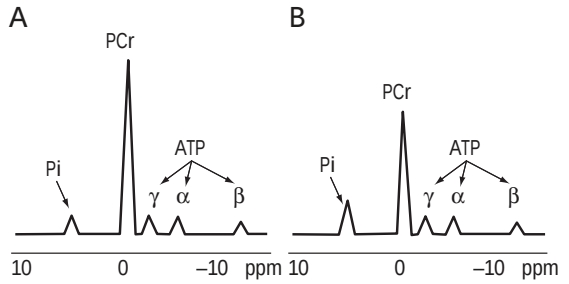
In normal exercising muscle, there is a PCr decrease linked to Pi accumulation, while the ATP signal remains

Table 1. Clinical imaging techniques useful in the diagnosis of mitochondrial diseases

Imaging modality	Contribution to diagnosis	Comments
Magnetic resonance imaging (MRI)	Structural changes	No insight into tissue function
Computed tomography (CT)	Structural changes	No insight into tissue function
Diffusion weighted imaging	Differentiation between ischemic and MELAS stroke	Limited relevant studies to date
Single-photon-emission computed tomography (SPECT)	Changes in central blood flow	Low spatial resolution
Positron emission tomography (PET)	Detection of glucose/oxygen and oxygen/blood flow ratios	Limited availability
Phosphorous and proton magnetic resonance spectroscopy (MRS)	Metabolic changes	Measurement of metabolite concentrations at rest or under exercise

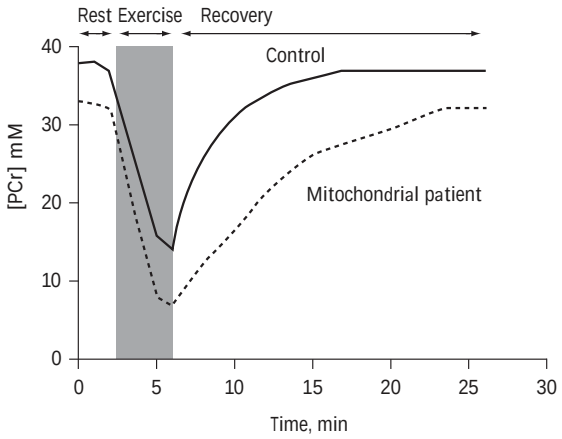
MELAS, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes. Modified from Parry & Matthews [35].

Figure 3. Representation of a muscle phosphorous magnetic resonance spectra from two hypothetical subjects: (A) healthy individual and (B) patient with a mitochondrial myopathy



Spectra arise from the phosphate groups γ, α and β of ATP, PCr and Pi. The spectrum from the patient with mitochondrial myopathy has a reduced PCr and an increased Pi concentration in comparison to the spectrum from the healthy individual. ppm, parts per million; Pi, inorganic phosphate; PCr, phosphocreatine.

Figure 4. Muscle dynamic P-MRS study. Scheme of the variations of PCr during a rest-exercise-recovery protocol



Patients with a mitochondrial myopathy have a rapid rate of PCr depletion and a prolonged rate of PCr recovery, which is a direct consequence of slower oxidative rephosphorylation of ADP to ATP. Modified from Mattei *et al.* [39]. PCr, phosphocreatine; P-MRS, phosphorous magnetic resonance spectroscopy.

unchanged due to the continuous resynthesis of ATP through different metabolic pathways. After exercise, mt oxidative phosphorylation remains the main source of ATP, which continues for a while at an accelerated rate to replenish the high-energy phosphates utilised during physical activity. Thus, during this period of recovery, PCr gradually increases, Pi and ADP decrease, and pH returns to its resting level. The initial rate of PCr recovery provides a measure of maximal oxidative rate in the tissue and the recovery of ADP is now considered as one of the most sensitive and reliable indexes of mt dysfunction *in vivo* [37].

P-MRS of muscle is particularly interesting in the evaluation of patients with mt diseases, which present, at rest, an increase in Pi and, less often, a decrease in PCr resulting in a low PCr/Pi (Figure 3). Abnormalities in resting skeletal muscle and in brain can be detected in patients even with relatively mild disease and normal serum lactate, or no clinical evidence of CNS involvement [38]. Muscle dynamic P-MRS studies during exercise can increase the specificity of the examination (Figure 4). Patients with mt myopathies display a rapid rate of PCr depletion. After stopping exercise, there is a prolonged rate of PCr recovery, which is a direct consequence of slower oxidative rephosphorylation of ADP to ATP, and faster than normal pH recovery [39]. These changes do not seem to be specific for primary mt diseases. They have also been described to occur secondary to traumatic muscle injury or in inflammatory myopathies [40,41]. It is conceivable that P-MRS may be a useful tool for the evaluation of HIV-infected patients on HAART, although no studies have been reported to date.

Conclusions

There is currently a general belief that mt dysfunction plays a pivotal role in some adverse effects observed in patients receiving HAART. The demonstration of this hypothetical dysfunction is complex and relies on a combination of pathological, biochemical and molecular genetic studies that are far from being considered routine in most general clinical settings. In the present review we have analysed some further diagnostic procedures that may help in deciding whether mitochondria are really affected in a given patient. Although some of these procedures are expensive and also limited to a few high technology centres (for example, MRS), others can be easily performed in more conventional outpatient clinics (for example, functional forearm exercise tests). The major inconvenience of all these methods is that they have either been introduced relatively recently or their use in diagnosing mt dysfunction is only a proposition. For these reasons their sensitivity, specificity and positive and negative predictive values remain to be elucidated in the diagnosis of HAART-related mt toxicity.

Acknowledgements

To Fundació La Marató de TV3 (01/1710 and 02/0210), SGR 2001/00379 and V2003-REDG011-0.

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Received 10 January 2005, accepted 14 April 2005

TREATMENT OF SYMPTOMATIC HYPERLACTATEMIA AND LACTIC ACIDOSIS IN HIV+ PATIENTS UNDER NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

MEDICINA CLINICA (Barcelona) 2005; 125(6): 201-204.

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ORIGINALES

Tratamiento de la hiperlactatemia sintomática y de la acidosis láctica en pacientes con infección por el virus de la inmunodeficiencia humana en tratamiento con inhibidores de la transcriptasa inversa análogos de los nucleósidos

Localizador web
Artículo 96.778

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FUNDAMENTO Y OBJETIVO: **Conocer la efectividad de un tratamiento para la hiperlactatemia sintomática y la acidosis láctica secundarias al tratamiento con antirretrovirales dirigido a corregir la toxicidad mitocondrial.**
PACIENTES Y MÉTODO: **Se reclutó de forma consecutiva a pacientes infectados por el virus de la inmunodeficiencia humana (VIH) a los que se les diagnosticó hiperlactatemia secundaria o acidosis láctica. Se consideró hiperlactatemia con concentraciones por encima de 2,2 mmol/l. El tratamiento consistía en la administración diaria de L-carnitina, tiamina, vitamina B_6, hidroxicobalamina y vitamina C, además de interrumpir la administración de glucosa intravenosa y el tratamiento antirretroviral de forma inmediata.**
RESULTADOS: **Se incluyó a 9 pacientes, a los que se les diagnosticó de hiperlactatemia sintomática (n = 4) o acidosis láctica (n = 5) entre enero de 2001 y septiembre de 2002. Todos eran pacientes con sida y habían recibido inhibidores de la transcriptasa inversa análogos de los nucleósidos (ITIAN) durante una media de 5 años: 7 habían recibido didanosina, 5 estavudina, 3 zidovudina, 2 lamivudina y 1 abacavir. Los síntomas que más frecuentemente se presentaron fueron taquipnea, febrícula, dolor abdominal, náuseas, vómitos y diarreas. Todos los pacientes tuvieron un buen pronóstico tras administrar L-carnitina y el complejo vitamínico descrito, así como tras la interrupción del tratamiento antirretroviral y de la perfusión de glucosa. Los síntomas desaparecieron a los 7 días. Después de una media (desviación estándar) de 15 (5) meses de seguimiento, no se ha observado recurrencia de esta complicación.**
CONCLUSIÓN: **La administración de L-carnitina, tiamina, vitamina B_6, hidroxicobalamina y vitamina C junto a la suspensión del tratamiento antirretroviral podría desempeñar un papel en el tratamiento de la acidosis láctica por ITIAN en pacientes infectados por VIH.**

Palabras clave: Acidosis láctica. Hiperlactatemia sintomática. Tratamiento antirretroviral. Sida.

Treatment of symptomatic hyperlactatemia and lactic acidosis in HIV+ patients under nucleoside reverse transcriptase inhibitors

BACKGROUND AND OBJECTIVE: **We intended to find out the effectiveness of lactic acidosis therapy for mitochondrial toxicity.**
PATIENTS AND METHOD: **HIV-patients receiving nucleoside reverse transcriptase inhibitors (NRTIs), hospitalized with lactic acidosis or symptomatic hyperlactatemia. Venous hyperlactatemia was considered at > 2.2 mmol/l. Treatment consisted of a daily vitamin regime of L-carnitine, thiamine, vitamin B_6, hydroxicobalamine, and vitamin C; any glucose intake was discontinued. NRTIs treatment was stopped immediately.**
RESULTS: **Nine patients on current therapy were identified who had symptomatic hyperlactatemia (n = 4) or lactic acidosis (n = 5) from 1/2001 to 9/2002. All were patients with AIDS, receiving NRTIs with a mean duration of 5 years: ddI (n = 7), d4T (n = 5), AZT(n = 3), 3TC(n = 2), abacavir (n = 1). Most common symptoms were tachypnea, slight fever, abdominal pain, nausea, vomiting and diarrhea. All patients had a favourable prognosis after administration of L-carnitine and vitamin complexes, with discontinuation of NRTIs and glucose intake. Clinical features lasted 7 days. After 15 (5) months of follow up, none had a recurrence of the syndrome.**
CONCLUSION: **The application of this therapy could play a role in the treatment of NRTI - related lactic acidosis.**

Key words: Lactic acidosis. Symptomatic hyperlactatemia. Antirretroviral therapy. AIDS.

En los últimos años, el tratamiento antirretroviral de gran eficacia (TARGA) ha determinado un cambio en la supervivencia y la calidad de vida de los pacientes infectados por el virus de la inmunodeficiencia humana (VIH)[1]. Sin embargo, su uso ha comportado a la vez la aparición de efectos indeseables que en algunos casos pueden comprometer la vida del paciente[2].

Las combinaciones de fármacos del TARGA suelen contener de forma invariable inhibidores de la transcriptasa inversa análogos de los nucleósidos (ITIAN), que pueden inducir disfunción mitocondrial, al inhibir la función de la ADN-γ-polimerasa del ADN mitocondrial (ADNmt), ya que ésta es la única enzima causante de la replicación de éste[3-6]. Esta inhibición parece ser la principal causa de la depleción del ADNmt y, por tanto, del consiguiente cambio en la síntesis de proteínas mitocondriales, lo que da lugar a diversas situaciones clínicas similares a las encontradas en las enfermedades mitocondriales congénitas[7].

Entre los variados síntomas de estas disfunciones atribuibles al uso de ITIAN se encuentran la polineuropatía, miopatía, miocardiopatía, pancreatitis, pancitopenia, disfunción tubular renal, esteatosis hepática y acidosis láctica[8-10].

De estas complicaciones, la más grave y potencialmente mortal es la acidosis láctica (AL). Fue en 1991 cuando Lai et al[11] describieron el primer caso de muerte producida por una hepatitis aguda con AL en un paciente que recibía tratamiento con didanosina. Hasta entonces, sólo se habían descrito casos aislados, con una incidencia estimada de esta complicación del 0,8% de casos/año y por paciente tratado[12].

La hiperlactatemia es un síndrome con un amplio espectro clínico, puede cursar de forma asintomática –hiperlactatemia asintomática (HA)– o con diversos síntomas como astenia, pérdida de peso, debilidad, dolor abdominal, taquicardia y taquipnea –hiperlactatemia sintomática (HS).

Este estudio ha sido desarrollado bajo el auspicio de la Red Temática Cooperativa de Investigación en Sida (Red de grupos 173) del FISS y de la beca La Marató de TV3 02/0210.

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Recibido el 8-11-2004; aceptado para su publicación el 12-1-2005.

En algunos casos de AL puede producirse un fallo hepático grave, que conduce a la muerte al 57% de los pacientes que desarrollan esta complicación[13,14].

El tratamiento habitual de la AL consiste en la aplicación de medidas sintomáticas y de soporte vital, así como la interrupción del tratamiento antirretroviral. No se conoce un tratamiento específico para esta complicación atribuible a los ITIAN. En las enfermedades mitocondriales congénitas se han ensayado diversos fármacos, como cofactores esenciales, antioxidantes y aceptores de electrones de la cadena respiratoria mitocondrial, con éxito terapéutico diverso[15,16]. Aunque podemos encontrar en la bibliografía descripciones puntuales del uso de complejos vitamínicos y nutrientes en diversas combinaciones, no hay un tratamiento específico de la AL secundaria a ITIAN[17-19]. En este trabajo se presentan los resultados del modelo terapéutico aplicado a estos pacientes.

Pacientes y método

Se realizó un estudio prospectivo desde enero de 2001 a septiembre de 2002. Se incluyó a los pacientes infectados por el VIH mayores de 18 años que recibían tratamiento antirretroviral que incluía ITIAN y que presentaban HS o AL sin ninguna otra causa conocida a la que se le pudiera atribuir esta complicación[20]. Es decir, se excluyeron los casos de hipoxemia, deshidratación, insuficiencia cardíaca, isquemia miocárdica aguda, crisis convulsivas durante las 4 h previas, sepsis o infecciones activas, enfermedad hepática activa, consumo de psicofármacos, alcohol o estimulantes, intoxicación por monóxido de carbono, cianuro o sustancias anestésicas, embarazo, enfermedades mitocondriales congénitas, enfermedades neurodegenerativas (como enfermedad de Alzheimer, Parkinson o corea de Huntington) o diabetes mellitus.

TABLA 1

Régimen terapéutico

	Dosis	Vía[a]	Días
Vitamina C	1 g/12 h	Oral	7
Carnitina	1 g/12 h	Oral	7
Hidroxocobalamina	0,5 g/8 h	Oral	7
Piridoxina	250 mg/8 h	Oral	7
Tiamina	250 mg/8 h	Oral	7

[a]La administración por vía intravenosa de vitamina C y carnitina e intramuscular para los otros componentes, todos a las mismas dosis, es posible cuando la vía oral no lo es.

Los pacientes infectados por el VIH procedían de los 406 que se atienden de forma regular en nuestro hospital. Los valores de lactato se determinaron de forma automática mediante un método enzimático basado en la conversión del lactato a piruvato usando la L-lactato oxidasa (Boehringer, Mannheim, Alemania). Se consideró que había hiperlactatemia cuando las concentraciones de lactato eran iguales o superiores a 2,2 mmol/l, y AL cuando existía acidosis metabólica (pH inferior o igual a 7,35, o un exceso de bases igual o superior a 6) con concentraciones de ácido láctico superiores a 5 mmol/l[21,22].

Cuando se sospechaba clínicamente la presencia de HS o AL, se retiraban inmediatamente el tratamiento antirretroviral y la administración de glucosa por vía intravenosa, si la recibían. Se iniciaba a la vez tratamiento con carnitina, tiamina, hidroxicobalamina, piridoxina y vitamina C (tabla 1), que se mantenía hasta la desaparición de los síntomas.

Todos los pacientes y sus familiares otorgaron el consentimiento informado para poder participar en el estudio. Se recogieron los siguientes datos demográficos y epidemiológicos: edad, sexo, año de diagnóstico de la infección por el VIH, conductas de riesgo asociados al VIH, tratamiento antirretroviral actual y previo, duración de éste, y presencia o ausencia de otros cambios metabólicos que pudiesen haber sucedido durante el TARGA, como lipodistrofia, diabetes o dislipemia. También se registraron el estadio de infección según la clasificación de los Centers for Disease Control de 1993 y los datos clínicos asociados al episodio actual y el tratamiento concomitante recibido. Se excluyó a los pacientes con enfermedad hepática aguda y con otras causas conocidas de AL, tras realizar la historia clínica y las exploraciones complementarias pertinentes (a todos los pacientes se les realizó una analítica sanguínea, radiografía de tórax, tomografía computarizada abdominal y/o ecografía abdominal).

Respecto a los parámetros analíticos, se recogieron los recuentos de linfocitos CD4, carga viral, glucemia, insulinemia, péptido C, transaminasas, bilirrubina total, fosfatasa alcalina, gammaglutamiltransferasa, amilasas, lactatodeshidrogenasa, creatinina, colesterol total y sus fracciones, triglicéridos y creatincinasa. En todos los casos se realizaron serologías frente al virus de la hepatitis C y determinación del antígeno de superficie del virus de la hepatitis B.

La determinación de lactato se hizo de manera inmediata cuando existió una sospecha diagnóstica clínica y posteriormente se recogieron otras muestras al tercer y séptimo día, así como a la tercera semana y en controles posteriores de seguimiento.

La determinación en plasma de ácido láctico se realizó mediante extracción venosa heparinizada, obtenida sin torniquete ni compresión a primera hora de la mañana. Se transportaba de forma inmediata al laboratorio en hielo y se procesaba en los siguientes 30 min de la obtención sanguínea. El paciente no debía realizar ningún esfuerzo físico las horas previas a la extracción y permanecía tumbado en la cama los 10 min antes de realizarla[23].

Resultados

Se identificó a un total de 9 pacientes con concentraciones anormalmente altas de ácido láctico y síntomas clínicos. Cinco de ellos cumplían criterios de AL y 4 de HS (tabla 2).

Los valores medios (desviación estándar) de lactato fueron de 7,3 (1,7) mmol/l en pacientes con AL y de 3,35 (0,8) mmol/l en los casos de HS. Los datos epidemiológicos se muestran en la tabla 3. Ninguna de las 2 mujeres incluidas estaba embarazada.

Todos los pacientes mostraban un importante deterioro inmunológico con una media de linfocitos CD4 de 108 (93)/μl (límites 19-293) y una carga viral del VIH entre 1150 y 750.000 copias ARN/ml.

Los pacientes presentaban una media de evolución conocida de la infección por el VIH de 10 (5) años (límites: 2-16 años). El índice de masa corporal osciló entre 20 y 25 kg/m con una media de 23 kg/m^2. Un paciente había desarrollado lipodistrofia durante el tratamiento antirretroviral y 3 pacientes dislipemia; uno de ellos precisaba tratarse con gemfibrozilo. Ninguno de ellos presentaba hiperglucemia o diabetes. Todos fueron tratados con ITIAN durante una media de 5 años (límites: 8 meses-13 años), con una media de 3 cambios terapéuticos durante la evolución de su enfermedad (tabla 4).

Todos los pacientes estaban recibiendo un régimen que incluía ITIAN cuando presentaron la complicación (HL o AL) que obligó a hospitalizarlos. Siete tomaban didanosina, 5 estaduvina (de entre éstos, todos junto con didanosina), 3 zidovudina, 2 lamivudina y 1 abacavir (asociado con didanosina).

Los tratamientos concomitantes fueron en todos profilaxis para la neumonía por *Pneumocystis carinii* con trimetoprim-sulfametoxazol oral, excepto uno que la realizaba con pentamidina inhalada mensual. Un paciente recibía fluconazol como profilaxis para una candidiasis esofágica recurrente.

Todos los pacientes presentaban un cuadro clínico consistente en taquipnea y pérdida de peso el mes anterior a la consulta. Asimismo 7 pacientes referían dolor abdominal, náuseas, vómitos y diarreas la semana previa al diagnóstico (tabla 5). Ningún paciente presentaba síntomas ni

TABLA 2

Datos analíticos en los casos de hiperlactatemia sintomática y acidosis láctica

Paciente	Lactato basal (mmol/l)		pH	HCO_3 (μl)	LDH (U/l)	CK	ALT (U/l)	Bi (mg/dl)	Otros
	Diagnóstico	12 meses							
1	3,5	–	7,33	19	1.107	N	25	0,44	Hipocolesterolemia
2	7,9	–	7,34	18	5.105	N	27	0,45	–
3	5,06	1,40	7,34	12	790	N	58	0,27	–
4	2,34	1,7	7,32	17	411	N	41	0,42	Hipoalbuminemia
5	9,35	1,5	7,24	18	1.357	N	26	0,87	Hipoalbuminemia
6	4,1	2,03	7,33	19	896	N	137	0,18	–
7	8,04	1,98	7,27	18	367	N	34	0,81	Hipoalbuminemia
8	6,15	–	7,40	10,7	645	N	46	2,06	Hipoalbuminemia
9	3,46	1,88	7,30	16	1.291	N	39	0,62	Hipoalbuminemia

N: valor normal; LDH: lactatodeshidrogenasa; CK: creatincinasa; ALT: alaninoaminotransferasa; Bi: bilirrubina.
Valores normales: lactato, < 2,2 mmol/l; LDH, 240-480 U/l; CK, 0-195 U/l; ALT, 5-40 U/l; Bi, < 1 mg/dl.

TABLA 3

Datos epidemiológicos

Paciente	Edad (años)	Sexo	Estadio (CDC 1993)	Año del diagnóstico de VIH	CD4 (CD4/μl)	CV (ARN-VIH copias/ml)	Conducta de riesgo	Enfermedades concomitantes	LPD	DM	Dislipemia
1	40	V	C3	1988	22	750.000	ADVP	VHC	No	No	Sí
2	26	V	B3	2000	68	337.000	HMX	Ninguna	No	No	No
3	41	M	B3	1991	142	< 50	Transfusión	Ninguna	Sí	No	Sí
4	33	V	C3	1998	9	1.150	HMX	Ninguna	No	No	No
5	39	M	C3	1991	197	1.710	ADVP	VHC	No	No	Sí
6	32	V	C3	1998	141	16.400	ADVP	VHC	No	No	No
7	39	V	C3	1991	293	1.680	ADVP	VHC	No	No	No
8	34	V	C3	1986	50	379.400	ADVP	VHC	No	No	No
9	49	V	C3	1989	51	750.000	ADVP	VHC, HBsAG+	No	No	No

CDC: clasificación de los Centers for Disease Control; CV: carga viral; LPD: lipodistrofia; DM: diabetes mellitus; V: varón; M: mujer; VHC: virus de la hepatitis C positivo y CV-VHC > 600 UI/ml; HBsAg: antígeno de superficie del virus de la hepatitis B; VIH: virus de la inmunodeficiencia humana; HMX: homosexual; ADVP: adicto a drogas por vía parenteral.

TABLA 4

Tratamiento antirretroviral

Paciente	Tratamiento antirretroviral		Tiempo último régimen (meses)	Duración acumulada de tratamiento (meses)	Otros tratamientos
	Último régimen	Otros fármacos incluidos en combinaciones previas			
1	AZT + 3TC + NEV	IND	11	156	TMP/SMX; fluconazol
2	AZT + 3TC + IND	NFV	14	14	TMP/SMX
3	d4T + ddI + NEV	AZT, IND	7	120	Pentamidina
4	LPV/RTV + ddI + ABV	d4T	2	8	TMP/SMX; gemfibrozilo
5	d4T + ddI	AZT, ddC, 3TC	11	72	TMP/SMX
6	ddI + d4T + RTV + APV	AZT, 3TC	4	16	TMP/SMX
7	d4T + ddI + NFV	AZT, ddC, SQV	6	70	–
8	d4T + ddI + EFV	AZT, ddC, 3TC, RTV	1	90	TMP/SMX
9	ddI + AZT + ABV	3TC, d4T, IND, NEV	2	126	TMP/SMX

AZT: zidovudina; 3TC: lamivudina; IND: indinavir; NEV: nevirapina; NFV: nelfinavir; d4T: estaduvina; ddI: didanosina; LPV: lopinavir; ABV: abacavir; RTV: ritonavir; APV: amprenavir: SQV: saquinavir; TMP/SMX: trimetroprim-sulfametoxazol.

TABLA 5

Características clínicas

Paciente	Síntomas				Signos	Estancia en UCI	Evolución
	Dolor abdominal	Pérdida de peso	Náuseas y vómitos	Taquicardia, taquipnea temperatura < 38 °C			
1	No	Sí	Sí	Sí	Ninguno	No	Resolución
2	Sí	Sí	No	Sí	Hepatomegalia, respiración de Kussmaul	Sí	Resolución
3	Sí	No	Sí	Sí	Lipodistrofia	No	Resolución
4	No	Sí	NoSí	Hepatomegalia	–	Resolución	
5	Sí	No	Sí	Sí	Ninguno	Sí	Resolución
6	Sí	No	Sí	Sí	Hepatomegalia	No	Resolución
7	Sí	Sí	Sí	Sí	Hepatomegalia	No	Resolución
8	No	Sí	No	Sí	Hepatomegalia	No	Resolución
9	Sí	No	Sí	Sí	Ninguno	No	Resolución

UCI: unidad de cuidados intensivos.

signos clínicos ni analíticos de deshidratación. Un paciente fue diagnosticado durante su ingreso de un linfoma renal (estadio IV).

Seis de los 9 pacientes presentaban coinfección por el virus de la hepatitis C y 1 tenía positividad para el antígeno de superficie del virus de la hepatitis B. En 4 se palpaba hepatomegalia, todos ellos con ecografía indicativa de esteatosis hepática, y el resto tenía esta prueba de imagen normal. Sin embargo, ningún paciente presentaba valores de las enzimas hepáticas y amilasas anormalmente elevados.

A todos los pacientes se les aplicó el modelo terapéutico propuesto, que se continuó hasta la desaparición de los síntomas.

Los 9 pacientes presentaron una buena evolución clínica, con remisión de los síntomas una media de 7 días después del inicio del tratamiento protocolizado, que se consideraba así si se resolvían los síntomas y los criterios bioquímicos de acidosis.

La resolución analítica de los valores de lactato fue mucho más lenta, pues se normalizaron al cabo de uno y 3 meses. No se observaron efectos secundarios o intolerancia atribuible al tratamiento con cofactores o antioxidantes. Se reintrodujo el tratamiento antirretroviral un mes después de haberse producido la resolución clínica y analítica en 6 de los 9 pacientes, con esquemas terapéuticos que no incluían ITIAN, a excepción de abacavir y lamivudina.

Después de un período medio de seguimiento de 15 (5) meses, ninguno de los pacientes ha presentado recurrencia de este síndrome.

Discusión

La presencia de valores de lactato a 3 mmol/l es un fenómeno común y temporal entre los pacientes tratados con ITIAN, aunque sin ninguna correlación clínica clara. La frecuencia de este hecho es variable, entre un 5 y un 25% según las series analizadas[24]. En estos casos no se recomienda tratamiento y la monitorización de las concentraciones de lactato no se aconseja en la práctica clínica, ya que no

se ha demostrado que sean predictivas de un ulterior desarrollo de HS o AL[25].

Por lo contrario, la HS y la AL atribuible a ITIAN son complicaciones infrecuentes, pero de curso impredecible, que se asocian a una elevada mortalidad en el caso de la AL. No hay consenso en el tratamiento de la AL, a excepción del soporte hemodinámico y la retirada del tratamiento antirretroviral, por lo que parece necesaria la búsqueda de nuevos tratamientos alternativos.

Aunque la fisiopatología de esta complicación no está absolutamente bien definida, cada vez es más evidente que parece deberse a una disfunción mitocondrial que secundariamente produciría una disminución de la capacidad oxidativa de la cadena respiratoria mitocondrial. Por este motivo, parecería lógico que el aporte de cofactores esenciales, antioxidantes y aceptores artificiales de electrones de la cadena respiratoria mitocondrial ayudará a mejorar esta lesión.

El uso de estas sustancias *in vitro* ha demostrado ser beneficioso para corregir el daño producido en la cadena respiratoria mitocondrial. Por otro lado, el uso de L-carnitina, coenzima Q, riboflavina, tiamina y piridoxina, se ha probado con éxito diverso[15] en las enfermedades congénitas mitocondriales. En pacientes infectados por el VIH estos tratamientos se han aplicado en casos aislados de AL en diversas combinaciones que incluían principalmente riboflavina, tiamina y L-carnitina[26-29], ubiquinona o vitaminas C y E, la mayoría de las veces con resultados favorables. En la revisión de Falcó et al[14], su uso se asoció a un mejor pronóstico y a una menor mortalidad. En nuestro estudio, además del soporte hemodinámico y de la suspensión del tratamiento antirretroviral, también administramos tiamina y piridoxina[20-30], junto con L-carnitina, vitamina C e hidroxicobalamina durante unos 7 días, que fue el tiempo en el que desaparecieron los síntomas en todos los casos. A su vez, también se evitaba la administración de glucosa por vía intravenosa en los pacientes que precisaban soporte hemodimámico por su estado, debido a que de esta forma se impide en parte la conversión de piruvato a lactato, producto final del metabolismo de la glucosa. Por otro lado, se administraba hidroxicobalamina, que hasta el momento se ha usado a dosis altas en la intoxicación por cianuro[31], proceso que produce una AL grave por disfunción en la cadena respiratoria mitocondrial que impide la correcta utilización del oxígeno consumido durante la fosforilación oxidativa.

El uso concomitante de todas estas sustancias se ha asociado a una buena evolución en todos los pacientes de nuestra serie. Sin embargo, es importante destacar que esta mejoría pronóstica puede deberse al hecho de que con el diagnóstico de sospecha se retiraban los antirretrovirales y se iniciaba el tratamiento propuesto, es decir, se realizaba un diagnóstico muy temprano y probablemente debido a ello las concentraciones de lactato de nuestros pacientes no fueron superiores a 10 mmol/l (límite: 5,06-9,35 mmol/l) y, como se ha descrito, es a partir de este límite donde se observa un pronóstico peor[14].

También se excluía a los pacientes con insuficiencia hepática aguda, una causa reconocida de AL grave y, por tanto, de mal pronóstico, aunque durante la realización del estudio no se excluyó a ningún paciente por esta circunstancia.

A 6 pacientes se les reintrodujo el tratamiento antirretroviral con fármacos diferentes de los utilizados previamente, incluyendo por el común inhibidores de proteasas e ITIAN. Cuando esto no era posible, se usaron abacavir y lamivudina, fármacos a los que se les atribuye una menor afinidad por la ADN-γ-polimerasa mitocondrial[32]. Durante el seguimiento efectuado ningún paciente ha presentado recidiva de los síntomas.

En conclusión, el diagnóstico temprano es básico en el tratamiento de la AL asociada al uso de antirretrovirales. Una vez diagnosticada, deben interrumpirse los antirretrovirales y la perfusión de glucosa. Aunque el papel del uso de cofactores y antioxidantes aún no está claro, nuestro estudio, al igual que otros, indica que el tratamiento con L-carnitina y los complejos vitamínicos descritos se asocia a una evolución favorable en la HS y AL secundaria a ITIAN, y carece de efectos secundarios. Sin embargo, serían necesarios estudios aleatorizados más amplios para determinar el beneficio real de estos componentes, aunque su realización es difícil debido a la escasa frecuencia de esta complicación.

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

ANTIVIRAL THERAPY 2005; 10(8): 945-951.

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

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Background: ***In vitro*** **studies have reported controversial effects of protease inhibitors (PIs) on mitochondrially driven apoptosis. Additionally, since PIs in the clinical setting are almost always given in combination with nucleoside analogues, which may have negative effects on mitochondrial DNA (mtDNA), the impact of PI-containing highly active antiretroviral therapy (HAART) on apoptosis and mtDNA content is unclear.**

Patients and methods: A cross-sectional study was performed including 20 HIV-negative (HIV–) patients, 16 HIV-positive, antiretroviral-naive (HIV+) patients and 17 HIV-positive patients receiving the PI nelfinavir (NFV) plus zidovudine and lamivudine (AZT+3TC) or didanosine and stavudine (ddI+d4T) – collectively known as HIV+PI – as first-line antiretroviral treatment for at least 12 months. Peripheral blood mononuclear cells (PBMCs) were isolated. BCL2 expression (anti-apoptotic) and the levels of the cleaved, active form of caspase-9 (pro-apoptotic) were determined by western blot. An index of mitochondrially driven apoptotic activation was estimated calculating the ratio caspase-9:BCL2. Mitochondrial DNA content was measured by real-time PCR.

Results: BCL2 expression was lower in HIV+ than in HIV– patients (P<0.01), whereas levels of caspase-9 were higher (P=0.001). The caspase-9:BCL2 ratio was significantly increased in HIV+ compared with HIV– individuals (P<0.001). Mitochondrial DNA content was also decreased in HIV+ compared with HIV– patients (P<0.001). The HIV+PI group exhibited a trend to normalization for BCL2 expression and caspase-9 compared with the HIV+ group, whereas the caspase-9:BCL2 ratio significantly improved (decreased, P<0.05 compared with HIV+ group). The mtDNA content in the HIV+PI group was similar to that of the HIV+ group, although the results of mtDNA content differed depending on whether NFV was combined with AZT+3TC (preserved) or with ddI+d4T (depleted). Conversely, no differences were found in apoptotic markers between the two subgroups of HIV+PI.

Conclusions: NFV-based PI-containing HAART regimens may exert some beneficial effects counteracting the increased mitochondrially driven apoptosis present in HIV-infected people.

Introduction

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

On the other hand, the effects of protease inhibitors (PIs) on mitochondria continue to be controversial. It has been reported that these drugs can either inhibit or induce mitochondrially driven apoptosis in leukocytes and endothelial cells [7–13]. Additionally, a switch from apoptosis inhibition to induction is observed when high concentrations of PIs are used [8,12]. The PI nelfinavir (NFV) constitutes an example of these controversies. While some *in vitro* studies showed that NFV prevents mitochondrially driven apoptosis by

blocking transmembrane potential loss [11] and/or by inhibiting adenine nucleotide translocator pore function in mitochondria [14], others demonstrated that NFV induces apoptosis and, accordingly, it could be hypothetically used as an antitumour agent on the basis of this pro-apoptotic capacity [9,13].

Studies are scarce in the clinical setting, where PIs are given in combination with other antiretrovirals and especially with nucleoside analogues. It is unknown whether such combinations of antiretrovirals increase or decrease mitochondrial damage and/or mitochondrially driven apoptosis. Additionally, most *in vitro* investigations have been performed using the low concentration range of PIs required for viral inhibition [7–9]. We therefore evaluated the effects of HAART regimens containing the PI NFV on peripheral blood mononuclear cell (PBMC) levels of BCL2, a main anti-apoptotic mitochondrial protein, and of the active cleaved form of caspase-9, a major effector protein of mitochondrially driven apoptosis. We have also measured PBMC mtDNA content.

Patients and methods

We designed a cross-sectional study including HIV-uninfected (HIV–) individuals, HIV-infected, antiretroviral-naive (HIV+) patients and HIV-infected patients receiving a first-line NFV-based PI-containing HAART regimen (HIV+PI). This latter group included HIV+PI individuals consecutively visited as outpatients, who were asymptomatic regarding HIV infection and antiretroviral adverse effects, with an uninterrupted duration of at least 12 months of first-line HAART. Concerning the nucleoside backbone, HIV+PI patients were receiving either zidovudine and lamivudine (AZT+3TC) or didanosine and stavudine (ddI+d4T). On the other hand, HIV– patients were recruited from voluntary blood donors and HIV+ patients from asymptomatic ambulatory patients. All patients were attended at the Hospital Clinic and Hospital de Granollers and gave informed consent. The Ethics Committees of both hospitals approved the protocol.

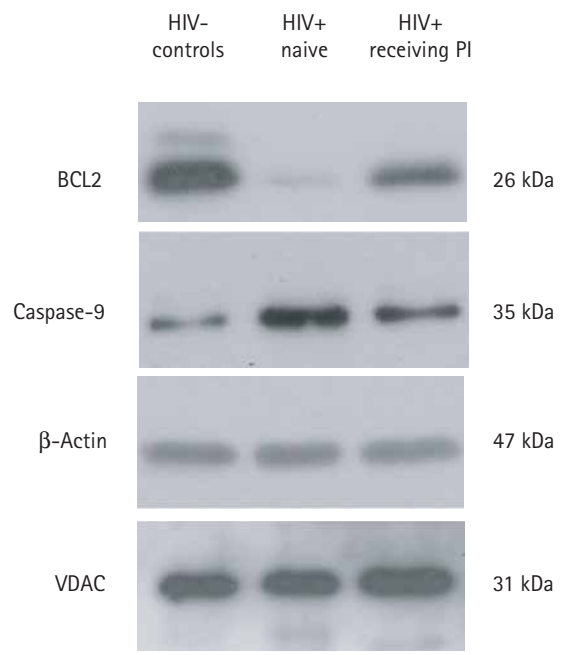
All patients were assessed in order to exclude any clinical evidence of lipodystrophy, and to confirm normal cholesterol, triglycerides, glucose and lactate plasma levels. We recorded clinical and demographic data. In all cases, 20 ml of venous blood were obtained to perform mitochondrial studies on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

An aliquot of PBMCs was used to assess parameters indicative of mitochondrially driven apoptosis. Crude protein extracts containing 20 μg protein were mixed with 1/5 vol of a solution containing 50% glycerol, 10% sodium dodecyl sulphate, 10% 2-mercaptoethanol, 0.5% bromophenol blue and 0.5 M Tris (pH 6.8), incubated at 90°C for 5 min and electrophoresed on 0.1% SDS/13% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, USA). Blots were probed with a mouse polyclonal antibody for human BCL2 (Santa Cruz Biotechnology sc-599, Santa Cruz, CA, USA; 1:500) and with a rabbit polyclonal antibody specific for the cleaved, active form, of human caspase-9 (Cell Signalling Technology 9505, Beverly, MA, USA; 1:1000). The monoclonal mouse antibody against β-actin (Sigma A5441, St Louis, MI, USA; 1:10.000) was used to normalize caspase-9 immunoreactive signals for equal cell protein loading. The mitochondrial protein voltage-dependent anion carrer (VDAC) or porin was determined using a monoclonal mouse antibody (Calbiochem Anti-Porin 31HL, Darmstadt, Germany; 1:1000) to refer changes in the mitochondrial protein BCL2 to the overall content of mitochondrial protein present in cell extracts. Immunoreactive material was detected with a horseradish peroxidase-coupled anti-mouse (Bio-Rad 170-6516, Hercules, CA, USA; 1:3000) or anti-rabbit (Santa Cruz Biotechnology sc-2004, Santa Cruz, CA, USA; 1:3000) secondary antibody and the enhanced chemiluminiscence (ECL) detection system (Amersham, Buckinghamshire, UK). Immunoblot analysis resulted in a 26-kD band for BCL2, a 35-kD band for the cleaved, active form of caspase-9, a 47-kD band for β-actin, and a 31-kD band for VDAC, as expected (Figure 1). The intensity of the signals was quantified by densitometric analysis (Phoretics 1D Softward, Phoretic International Ltd, Newcastle, UK). The quotient caspase-9:BCL2 expression was arbitrarily used as an 'apoptotic index' to quantify the extent of activation of PBMC mitochondrially driven apoptosis.

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

Results were expressed as percentages and mean ±standard deviation, and compared by means of χ^2 test and Mann–Whitney U test, respectively. Linear regression analysis was used to assess any relationship between quantitative variables. *P*-values less than 0.05 were considered statistically significant.

Figure 1. Example of immunoblot analysis of BCL2 and of the active, truncated form, of caspase-9



Each lane corresponds to 20 mg of protein extracts from peripheral blood mononuclear cells. Panels at the bottom correspond to immunoblot with markers of the overall cell protein loading (β-actin) and the mitochondrial protein content of samples (voltage-dependent anion carrier [VDAC]). The size of the specific immunoreactive signals is indicated on the right.

Results

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

As shown in Figure 2, BCL2 expression was lower in the HIV+ than in the HIV– group ($P<0.01$), whereas caspase-9 was higher ($P=0.001$). The levels of processed caspase-9 at a given amount of BCL2 (caspase-9:BCL2 ratio) were very significantly increased in HIV+ compared with HIV– individuals ($P<0.001$). HIV+PI patients exhibited a trend to normalize BCL2 expression as well as caspase-9 levels, whereas the caspase-9:BCL2 ratio was significantly improved (decreased, $P<0.05$ compared with the HIV+ group).

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

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

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

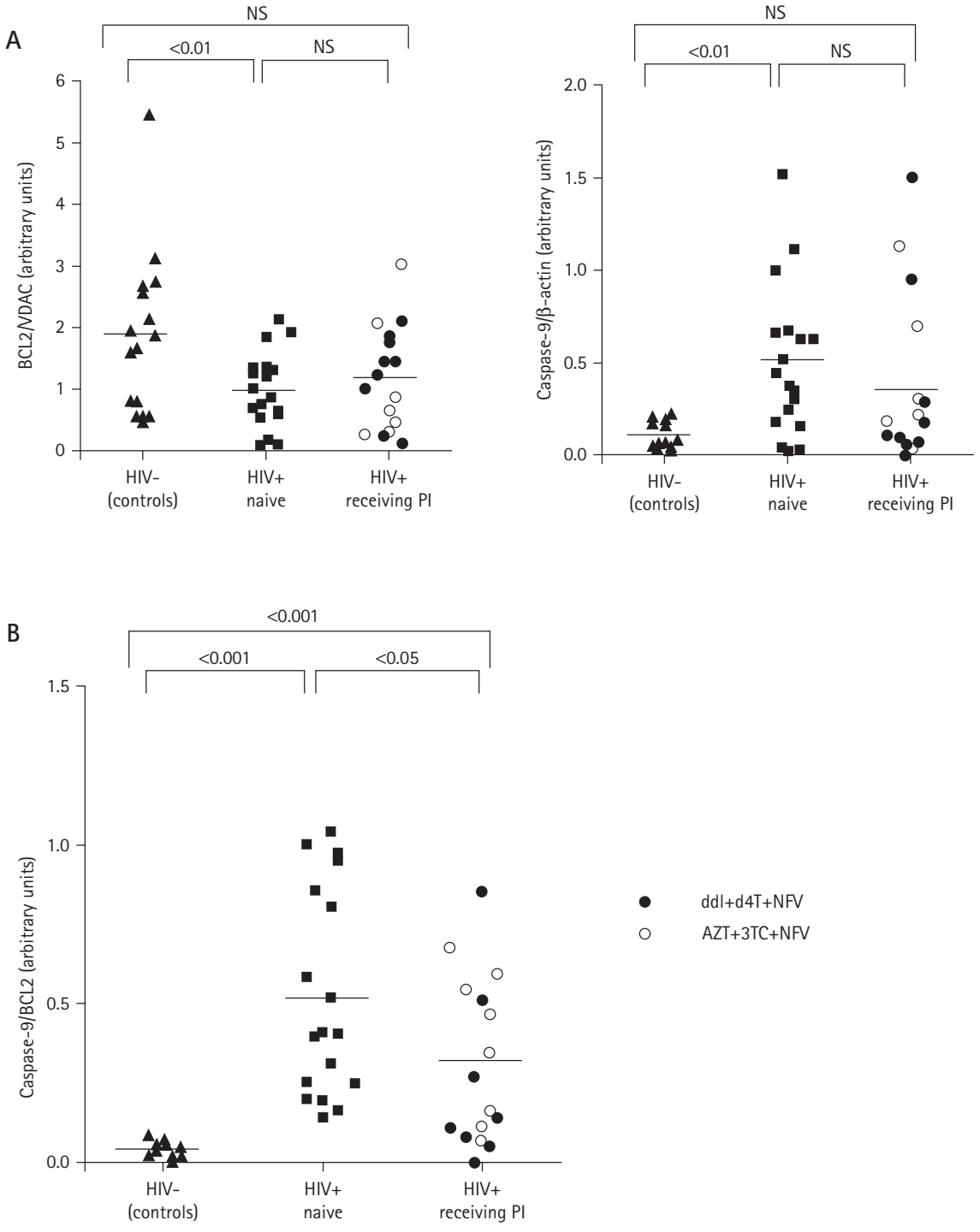
Discussion

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

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

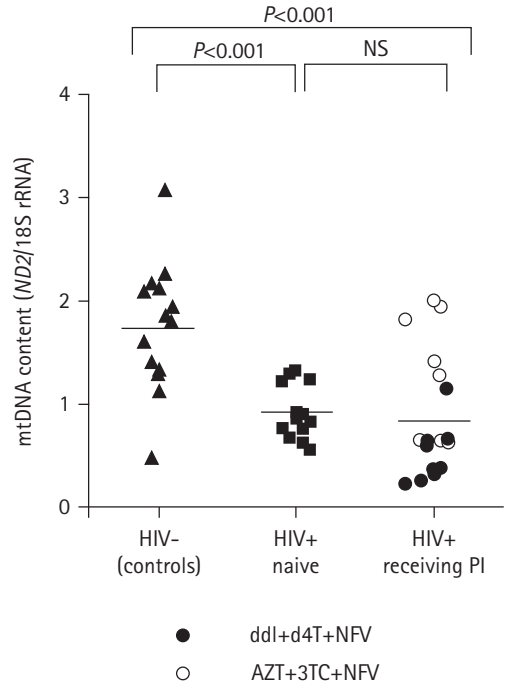
	HIV- (n=16)	HIV+ naive (n=20)	HIV+ receiving PI (n=17)	P-value
Age (years)	40 ±9	38 ±8	41 ±9	0.62
Gender male (%)	69	72	82	0.64
HIV viral load (log HIV RNA copies/μl)	–	4.9 ±0.8	2.1 ±0.9	<0.001
$CD4^+$ lymphocyte count (cells/μl)	–	303 ±185	395 ±195	0.40
Time receiving antiretrovirals (months)	–	–	18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

Figure 2. BCL2 and caspase-9 protein expression determined by western blot (**A**) and the apoptotic index of mitochondrially driven apoptosis activation (**B**)



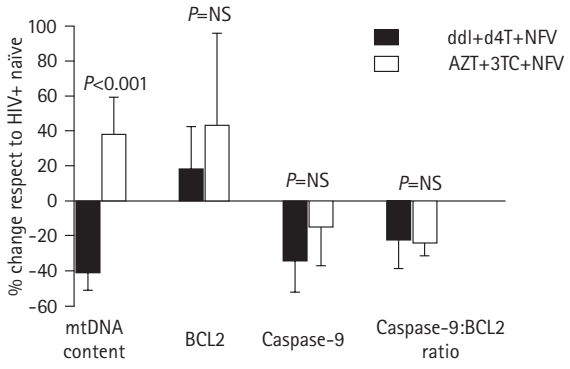
3TC, lamivudine; AZT, zidovudine; d4T, stavudine; ddI, didanosine; NFV, nelfinavir; NS, not significant; PI, protease inhibitor; VDAC, voltage-dependent anion carrier.

Figure 3. Mitochondrial DNA content



3TC, lamivudine; AZT, zidovudine; d4T, stavudine; ddI, didanosine; mtDNA, mitochondrial DNA; NFV, nelfinavir; NS, not significant; PI, protease inhibitor.

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



3TC, lamivudine; AZT, zidovudine; d4T, stavudine; ddI, didanosine; mtDNA, mitochondrial DNA;NFV, nelfinavir; NS, not significant; PI, protease inhibitor.

HAART, the two relevant mitochondrial disturbances described in HIV-infected patients (that is, enhancement of mitochondrially driven apoptosis and reduction in mtDNA levels) can be independently influenced by the clinical use of antiretroviral therapy.

Previous *in vitro* experiments have indicated that the anti-apoptotic action of PIs do not involve down-regulation of apoptosis-regulatory molecules [11,16]. The present *in vivo* study in PBMCs from HIV-infected patients confirms that NFV-based PI-containing HAART reduces apoptotic activation through mechanisms that do not involve an improvement in the HIV-infection-mediated reduction in BCL2 levels. The fact that, at similar levels of BCL2, caspase-9 levels were lowered in treated patients (reduced caspase-9:BCL2 ratio in treated versus untreated HIV+ patients) points to caspase-9 processing itself or other events upstream from this process as the potential targets of the anti-apoptotic action. Although a direct effect of PIs on caspase-9 processing can be hypothesized (caspase-9 activation is the result of pro-caspase-9 proteolysis), research in recent years has not demonstrated any direct inhibitory effects of PIs on proteolytic activation of cellular caspases [11,17], including caspase-9 [18]. Alternatively, other experimental studies have proposed that the anti-apoptotic action of NFV occurs at the level of mitochondria, downstream from the activation of pro-apoptotic members of the BCL2 family, and involving, as a primary event, the opening of the mitochondrial permeability transition pore and subsequent activation of caspase-9 [14]. The results of our study on PBMCs from treated patients, in which caspase-9 reduction by NFV-containing HAART occurs in the absence of BCL2 changes, are in accordance with this model of NFV action.

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

We believe that our results agree with the hypothesis of a double-positive effect of NFV when used in the treatment of HIV infection: one depending on their

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

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

Acknowledgments

Grants: Fundación para la Investigación y la Prevención del Sida en España (FIPSE 3102-00), Fundació La Marató de TV3 (020210 and 020631), Redes de Investigación en Mitocondrias (V2003-REDC06E-0) y Sida (Redg 173) and Suport a Grups de Recerca de la Generalitat de Catalunya (SGR/0300/2005).

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Received 11 September 2005, accepted 26 October 2005

LONGITUDINAL STUDY ON MITOCHONDRIAL EFFECTS OF DIDANOSINE-TENOFOVIR COMBINATION

AIDS RESEACRH AND HUMAN RETROVIRUSES 2006; 22(1): 33-39.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 22, Number 1, 2006, pp. 33–39

Longitudinal Study on Mitochondrial Effects of Didanosine–Tenofovir Combination

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ABSTRACT

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 phosphorylation (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, with 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 a 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 phosphorylation (OXPHOS) system occurs. This NRTI-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 favorable lipid profile and it has been proposed that it is exempt from mitochondrial toxicity.[6,7] With respect to the latter, TDF has been tested to evaluate its effects on mtDNA content on *in vitro* ex-

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periments 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 cytochrome *c* oxidase (COX)-II (mtDNA-encoded) and COX-IV (nuclear DNA-encoded) subunits of COX (complex IV of the OXPHOS system) and does not increase lactate production.[8] The above data, together with a favorable *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 above mentioned 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 increases plasma concentrations of ddI when they are taken concomitantly,[7] and this could eventually limit the favorable 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.

MATERIALS AND 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, multicenter, prospective study in which patients on twice-daily HAART regimens, visited consecutively, were give the opportunity 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 subjects from the above-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 to make the 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 any NRTIs, to take into account the different background cytotoxic effects potentially produced by different antiretroviral combinations including NRTIs. Mitochondrial studies in PBMCs were performed at 0 (baseline), and at 6 and 12 months after 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 milliliters of peripheral venous blood, collected in Vacutainer EDTA tubes, was 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 kept to a minimum (ideally, to less than 5 platelets per PBMC) so as not to overestimate mtDNA content, because platelets are cellular fragments that contain mitochondria (mtDNA) but not nucleus (nuclear DNA). For this reason, we confirmed a platelet count below 25 per PBMC in all patients from both groups. PBMCs were resuspended 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 ratio of mtDNA to nuclear DNA 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 37°C 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 nanomoles of reduced substrate per minute and per milligram of cell protein (nmol/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 the OXPHOS system, because it is partially encoded by the mitochondrial ge-

TABLE 1. BASELINE CLINICAL AND EPIDEMIOLOGICAL CHARACTERISTICS[a]

	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[b]	9 ± 12[b]	NS

[a]ddI, didanosine; d4T, stavudine; NRTIs, nucleoside analogue reverse transcriptase inhibitors; SD, standard deviation; NS, not significant.
[b]Calculated only for the 11 patients of each group receiving ddI + d4T.

nome. Measurement of the specific enzyme activity was performed spectrophotometrically (U-2001 UV/Vis Spectrophotometer, Hitachi Instruments, Inc., San Jose, CA) at 37°C 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 nanomoles of oxidated substrate per minute and per milligram of cell protein (nmol/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 χ^2 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 given as percentages of the baseline values (100%). A 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; Fig. 1). In addition, no differences were found for any of the mitochondrial parameters studied at baseline between ddI + TDF and control groups based on the fact that they were being treated 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 the ddI + TDF group (22% decrease, $p < 0.01$) and the 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, with the decrease statistically significant only in the ddI + TDF group (55% decrease, $p < 0.001$) (Fig. 2). In addition, the decrease in 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

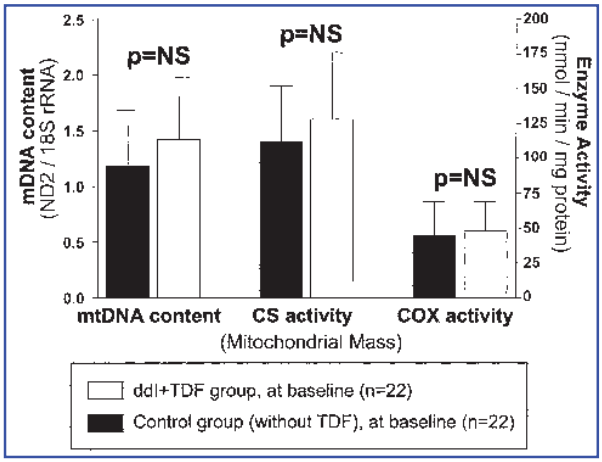

FIG. 1. Comparison of baseline mitochondrial parameters between ddI + TDF and control groups. The result for the mtDNA content is expressed as the ratio between the mitochondrial gene (ND2) and the nuclear gene (18S rRNA) quantification. The results for the enzyme activities are expressed as nanomoles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

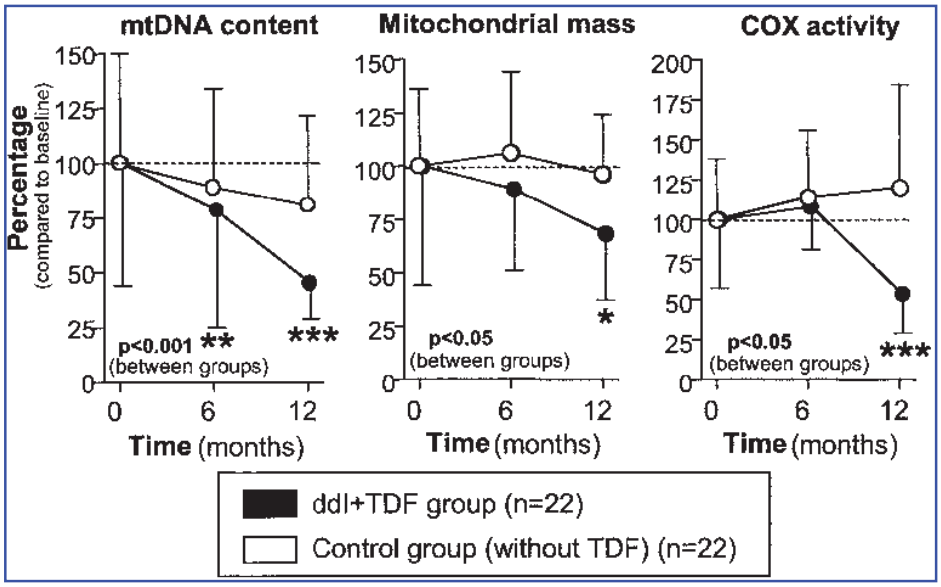

FIG. 2. Modification of the mitochondrial parameters in both ddI + TDF and control groups during the 12-month period of study. The results are expressed as percentage compared to baseline measurements (100%) for the two groups. mtDNA, mitochondrial DNA; COX, cytochrome *c* oxidase; TDF, tenofovir disoproxil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

patients that had switched to 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 ddI-based HAART regimen without the addition of TDF during the study period (controls) (Fig. 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 the ddI + TDF combination based on the fact that they had been receiving

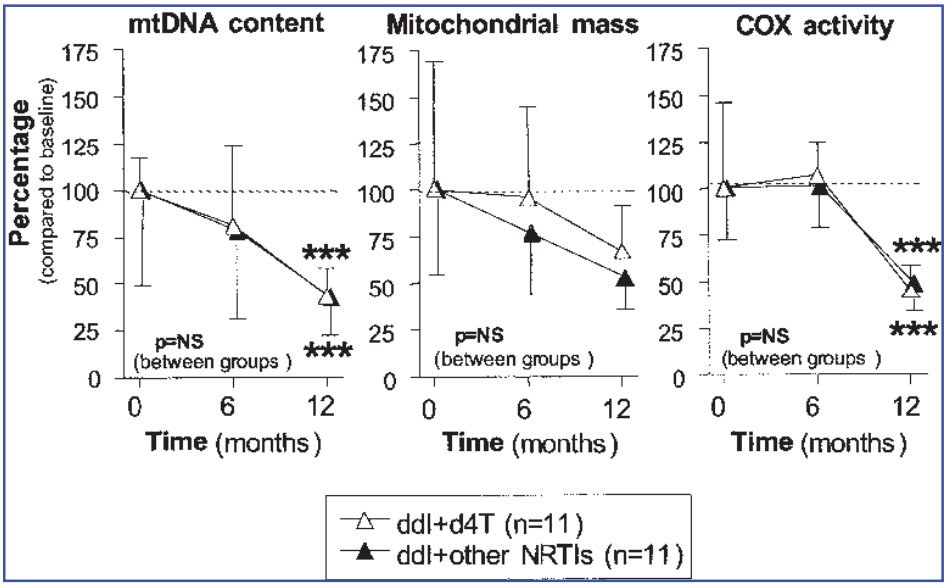

FIG. 3. Modification of the mitochondrial parameters during the 12-month period of study in the group of patients switched to TDF based on whether they received ddI + d4T or ddI + other NRTIs at baseline. The results are expressed as percentage compared to baseline measurements (100%) for the two subgroups.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

ddI + d4T or ddI + other NRTIs at baseline. As shown in Fig. 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 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). 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 the present study. However, we did 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 in the control group that in patients from the ddI + TDF group (Fig. 4).

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 coadministration of 300 mg of TDF in combination with 400 mg of ddI in a HAART regimen results in a 40–64% increase in C_{max} and the 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 an increased level of exposure to ddI.[27] In the present study, we 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 show that the combination of TDF and ddI at doses of 400 mg is associated with a progressive and statistically significant decrease in 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 by increased mitochondrial damage, which is expressed as loss of MM and decrease in COX efficiency of the OXPHOS system.

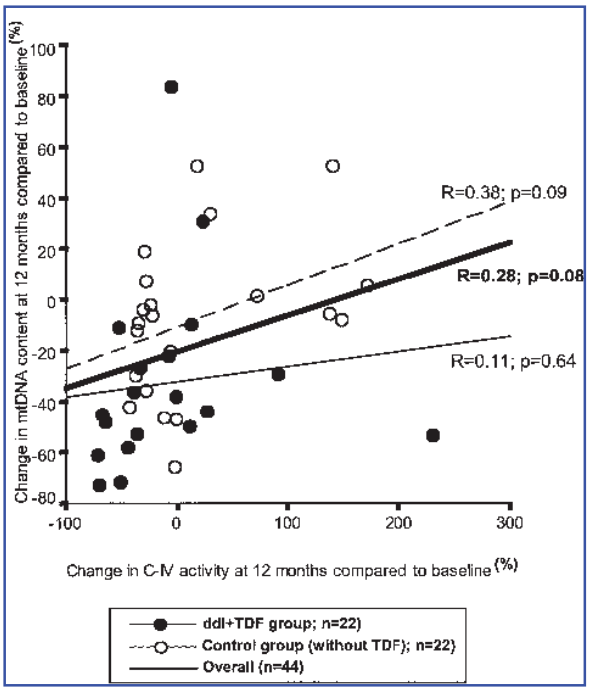

FIG. 4. Linear regression analysis between the changes of mtDNA and COX activity at the end of the study (12 months) with respect to baseline. mtDNA, mitochondrial DNA; COX, cytochrome *c* oxidase.

The mechanism for mitochondrial dysfunction is not investigated in the present study. We hypothesize that since the 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 for 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 the 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 some mtDNA depletion over time after TDF introduction, because TDF was added to a ddI-based HAART regimen and ddI is an NRTI with a well-recognized capacity for causing mtDNA depletion.[1] In fact, a mild and not significant mtDNA depletion over time was observed in the control group formed by patients maintained on 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 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 remains 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 coadministered.

The clinical relevance of the mitochondrial abnormalities found in this study is unknown. Very recently, Martínez *et al.* reported increased pancreatic toxic effects associated with the coadministration of ddI + TDF,[29] since pancreatitis is a well-known disorder associated with classical mitochondrial diseases.[30] In addition, several groups recommend avoiding the coadministration 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 suppression.[24] Thus, although several experimental studies have established a mitochondrial safety profile for TDF, mitochondrial damage in addition to the damage 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 (nonrandomized, potential selection bias, low statistical power), we believe that our data indicate 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 coadministration. 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 by close monitoring.

ACKNOWLEDGMENTS

Financial support was provided by Fundación para la Investigación y la Prevención del Sida en España (FIPSE 3102-00), Fundació La Marató de TV3 (020210), Redes de Investigación en Mitocondrias (V2003-REDC06E-0) y Sida (Redg 173), and Suport a Grups de Recerca SGR/0300/2005.

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THE IMPACT OF REDUCING STAVUDINE DOSE VERSUS SWITCHING TO TENOFOVIR ON PLASMA LIPIDS, BODY COMPOSITION AND MITOCHONDRIAL FUNCTION IN HIV-INFECTED PATIENTS

ANTIVIRAL THERAPY 2007; 12(3): 407-415.

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The impact of reducing stavudine dose versus switching to tenofovir on plasma lipids, body composition and mitochondrial function in HIV-infected patients

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Background: Stavudine (d4T)-containing regimens are associated with a potential for lipoatrophy and dyslipidaemia. We assessed the safety and efficacy of reducing the dose of stavudine compared with switching to tenofovir or maintaining the standard dose of d4T.

Methods: Clinically stable HIV-infected patients receiving antiretroviral therapy containing stavudine 40 mg twice daily with a plasma HIV RNA <200 copies/ml for at least 6 months were randomized to maintain stavudine 40 mg twice daily (d4T40 arm), to reduce to 30 mg twice daily (d4T30 arm), or to switch from d4T to tenofovir (TDF arm).

Results: Fifty-eight (93% male) patients were included: 22 in the d4T40 arm, 19 in the d4T30 arm and 17 in TDF arm. At baseline, median time on d4T was 6 years (interquartile range [IQR] 2.6–7.1), median age 43 years (IQR 36–51) and median $CD4^+$ T-cell count was 587/mm^3 (IQR 329–892). At week 24, median limb fat changes (g) were as follows: d4T40=–182 (95% CI: –469–-5); d4T30=527 (95% CI: –343–694); and TDF=402 (95% CI: 130–835; d4T40 versus TDF, P=0.0003). Significant differences between median values of laboratory parameters were detected: triglycerides (mg/dl): d4T40=19; d4T30=–23 and TDF=–79 (d4T40 versus TDF, P=0.03); and total cholesterol (mg/dl): d4t40=22, d4T30=–4, and TDF=–28 (d4T40 versus TDF, P=0.04). No significant difference was observed in mitochondrial function assessed in peripheral blood mononuclear cells.

Conclusions: Although both strategies were associated with a trend toward a decrease in plasma lipids and an increase in body fat, the only significant changes were observed among those who switched to tenofovir.

Introduction

Clinical experience with stavudine (d4T) has been extensive. It has a favourable resistance profile, good short-term tolerability, does not require dietary restrictions and, due to its relatively low cost, is still widely used. However, d4T has been associated with a higher risk of mitochondrial DNA (mtDNA) depletion and adipocyte metabolic dysfunction compared with other nucleoside reverse transcription inhibitors (NRTIs) [1], which may contribute to the pathogenesis of subcutaneous fat wasting, and the development of lipoatrophy and metabolic complications [2–6].

The following two main approaches have been used to reduce the toxic effects of d4T: replacement of d4T by potentially less toxic agents, usually tenofovir (TDF) [7,8] or abacavir (ABC) [9,10]; or more recently a d4T dose reduction [11–15]. Beneficial effects of d4T replacement with an alternative nucleoside reverse transcriptase inhibitor (NRTI; TDF or ABC) led to significant increase in peripheral fat mass and improvements in lipid profiles in studies with 24 weeks [7,9,16] or more [8,10] of follow-up. Results obtained mainly from cohort studies, show that a d4T dose reduction strategy may lead to a modest reversal of side effects [11–15]. To date, no controlled studies have been reported evaluating the effects of a d4T dose reduction compared with switching from d4T to TDF.

The objectives of this study were to evaluate and compare the impact of a d4T dose reduction or a switch from d4T to TDF, based on morphological changes, lipid measurements and mitochondrial parameters, in a population of virologically suppressed HAART-experienced HIV-infected people.

Methods

Study design

This was a single-centre, randomized, open-label study. Clinically stable HIV-infected patients receiving antiretroviral therapy containing d4T at the standard dose (40 mg twice daily), with undetectable viral load

(plasma HIV RNA <200 copies/ml) for at least six months were randomly assigned either to continue current therapy (d4T 40 mg twice daily; d4T40 arm), to reduce the dose of d4T from 40 mg twice daily to 30 mg twice daily (d4T30 arm), or to switch from d4T to TDF (TDF arm), while preserving the remaining concomitant drugs. Patients were followed at least 24 weeks after randomization. The randomization list was computer-generated in blocks. Additionally, patients were stratified according to current treatment (protease inhibitor [PI] or non-NRTIs).

Patient population

Study eligibility criteria included documented HIV infection, age ≥18 years, total body weight >60 kg, moderate to severe clinical lipoatrophy in at least one region upon physical examination [17,18], viral load <200 copies/ml for at least 6 months prior to inclusion in the study, a stable triple antiretroviral therapy including d4T 40 mg twice daily for at least the 6 preceding months, and no prior TDF use. Patients receiving anti-diabetic or lipid-lowering therapies before the study could be included, but any new prescriptions of these drugs were not allowed during the study. The Ethical Committee of our institution approved the study protocol. All patients signed an informed consent.

Laboratory, metabolic and body composition assessment

Fasting plasma glucose, triglycerides, total high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, venous lactate, $CD4^+$ T-cell count and viral load were assessed at all study visits (at baseline, and at weeks 4, 12 and 24). The plasma glucose level was measured by the glucose hexokinase method. Total cholesterol and triglyceride levels were determined using enzymatic reagents (Trinder; Bayer Diagnostics, Tarrytown, NY, USA) adapted to a Cobas Mira automated analyzer (Hoffmann-LaRoche, Basel, Switzerland). The HDL-cholesterol level was determined after precipitation with phosphotungstic acid and magnesium chloride; LDL-cholesterol levels were determined by direct measurement. $CD4^+$ T-cells were quantified by flow cytometry, and HIV-RNA by the Amplicor quantitative restriction transcriptase-PCR assay (Roche Diagnostic System, Branchburg, NJ, USA) with a lower limit of detection of 200 copies/ml. Dual-energy X-ray apsorptiometry (DEXA: Lunar DPXL, Madison, Wisconsin, USA) scanner running enhanced array whole body software (version 8.23a:3) and regional array software (version 8.26f:3) was used to asses bone mineral density (BMD) of the lumbar spine (L1–L4) and the proximal femur, and soft-tissue composition of the total body and major sub-regions at baseline and at week 24. Osteopenia was defined according to World Health Organization criteria by a BMD *t*-score between 1.0–2.5 SD below median normal (an average *t*-score in young men is 0, and average z-score in a matching age, sex, and racial group is also 0), and osteoporosis by a BMD *t*-score of >-2.5 SD below normal [19].

Mitochondrial studies

Sample. Twenty millilitres of venous blood was extracted from each patient and collected in Vacutainer™ EDTA tubes (Becton-Dickinson Ltd, Plymouth, UK). Peripheral blood mononuclear cells (PBMCs) were immediately isolated by Ficoll density gradient centrifugation (Histopaque®-1077, Sigma Diagnostics, St. Louis, MO, USA). Platelet contamination was negligible, since counting of cells and level of platelets was confirmed to be below 25 per PBMC in each case. Protein content was measured according to Bradford's protein-dye binding-based method [20].

Measurement of oxygen consumption. Overall mitochondrial function of PBMCs was determined by the measurement of the oxidative activity of these cells. The oxygen consumption of PBMCs, expressed as nanomols of oxygen consumed per minute and per milligram of cell protein, was measured polarographically with a Clark oxygen electrode in a water-jacketed micro cell, magnetically stirred, at 37°C (Hansatech Instruments Limited, Norfolk, England) in 250 µl of respiration buffer using 100–200 µg of cell protein. Under previously described experimental conditions [21], we determined the oxygen consumption rate of intact cells by following the oxidation of endogenous substrates [21,22].

Mitochondrial content. Mitochondrial content was estimated by spectrophotometric measurement of the citrate synthase activity (CS, enzyme code: 4.1.3.7). CS is a mitochondrial matrix enzyme of the Krebs cycle, which remains highly constant in mitochondria and is considered a reliable marker of mitochondrial content [21–24]. The specific activity was measured by following the increase in absorbance resulting from the enzymatic reduction of the substrate 5,5′-dithio-bis(2-nitrobenzoic acid) (DNTB). CS activity was expressed as nmol of reduced DNTB per min and per mg of cell protein [20].

Oxidative phosphorylation (OXPHOS) system enzyme activities. Mitochondrial function of PBMCs was also evaluated by the measurement of the specific enzyme activity of the OXPHOS system complex IV (partially encoded by mtDNA). The individual assay was performed spectrophotometrically (U-2001 UV/Vis Spectrophotometer, Hitachi Instruments, Inc., San Jose, CA, USA) at 37°C according to the methodology of Rustin *et al.* [22], which was slightly modified for Complex IV measurement [23,24].

mtDNA quantification. Total DNA was obtained by standard phenol-chloroform extraction procedure from an aliquot of PBMCs and used for mtDNA quantification. The amplification of both mtDNA and nuclear DNA (nDNA) was performed in duplicate and separately by quantitative real time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals®, Mannheim, Germany) using 10 ng of total DNA [20]. The content of mtDNA was expressed as an mtDNA/nDNA ratio.

Statistical analysis

The primary study endpoint was the median change in limb fat mass measured by DEXA at week 24. Secondary endpoints were median change in the following: total and central fat mass; lipid and mitochondrial parameters; viral load; and $CD4^+$ T-cell count.

The sample size of 20 patients in each group achieves 80% power to detect a difference of 400 g (from -200 g to 200 g) in mean limb fat (at week 24) with a significance level-α of 0.05 using a two-sided Mann–Whitney test (assuming the actual distribution is double exponential).

Analysis was by intention to treat using the policy of last-date-carried-forward analysis adopted for patients with missing data for the remaining follow-up period. Quantitative parameters were described with median and interquartile ranges, and qualitative parameters were described with frequencies and percentages. Comparisons between groups were made using Kruskal–Wallis and χ^2-squared tests for quantitative and qualitative characteristics, respectively.

Change over time in continuous variables was defined as the difference between the values at 4, 12 and 24 weeks, and the baseline value, which was described using median and non-parametric 95% confidence interval (95% CI) estimated using bootstrapping based on percentile method. The treatment effect was expressed as the difference between group means and its 95% CI. The hypothesis that the difference between group means was zero was tested with a *t*-test.

The effect of the baseline variables and the treatment assignment on the change in limb and total fat mass was assessed using a univariate and multivariate analysis, the former based on the Kruskal–Wallis test and the later, on a liner regression model. Baseline demographics, biochemical, body composition and immunological variables were assessed as predictors in addition of treatment assignment. Correlation between covariates was checked by χ^2 test.

Multiple comparisons were performed using the Bonferroni adjustment for significance, to confirm if the global comparisons were statistically significant. The McNemar test was used to detect an increase in the percentage of osteopenia at week 24 in each group and the Fisher's exact test to compare if the proportion of patients with a change in osteopenia differed over groups. Correlation between continuous variables was assessed by means the Spearman's correlation coefficient (ρ). All tests were two-sided with a 95% CI.

Results

Participants

From January to May 2004, 54 men and 4 women were included in the study. Twenty-two patients were randomized to continue their 40 mg twice-daily dose of d4T, 19 were randomized to a 30 mg twice-daily dose of d4T, and 17 to a TDF treatment arm. Baseline demographic, clinical and laboratory parameters did not differ as far as age, duration of HIV infection, antiretroviral treatment, duration of prior d4T use, $CD4^+$ T-cell count, peripheral and total fat, lean mass, or BMD (Table 1). Correlations between baseline covariates were only observed between total and peripheral fat.

Forty-five patients were receiving non-nucleoside reverse transcription inhibitor, eight were receiving PIs, and five patients were on triple NRTI regimen. Lamivudine was the second nucleoside in 46 patients, and didanosine (ddI) in 12 cases, six of whom were randomized into the d4T40 arm, five into the d4T30 arm, and one to the TDF arm (where the dose of ddI was reduced to 250 mg once daily according to current recommendations) [26]. None of the patients were receiving lipid-lowering and/or anti-diabetic therapy at baseline.

One patient was excluded due to pregnancy (TDF arm), four were lost to follow up (two from the d4T40 arm, one from the d4T30 arm and one from the TDF arm) and one was excluded due to symptomatic hyperlactataemia at baseline (d4T40 arm). Patient follow-up is summarized in Figure 1.

Metabolic parameters

Although triglycerides and cholesterol levels were within the normal range (<150 mg/dl and <247mg/dl, respectively) in most patients at baseline, median fasting triglycerides and cholesterol levels decreased from baseline in both the d4T30 and TDF groups during the study, reaching statistical significance at week 12 (total cholesterol) and week 24 (total cholesterol and triglycerides), when the d4T40 arm was compared to the TDF arm. Conversely in the d4T40 arm, triglycerides and cholesterol levels continued to rise. Similar trends were observed in HDL- and LDL-cholesterol. Fasting glucose remained unchanged in all groups (Table 2).

Body composition

DEXA scan data demonstrated an increase in peripheral and total fat in the d4T30 and TDF arms during the

Table 1. Patient baseline characteristics

Variable	d4T40 (n=22)*	d4T30 (n=19)*	TDF (n=17)*
Age, years	43 (36–49)	42 (39–51)	45 (42–48)
Duration of HIV infection, years	8.3 (6.8–12.1)	11.9 (7.2–15.5)	7.3 (2.7–11)
Duration of HAART, years	6.2 (6.1–7.6)	6.7 (5.2–6.8)	6.7 (2.7–7.1)
Duration of d4T, years	6.7(5.3–7.1)	6.6 (5.2–6.8)	5.8 (2.6–6.9)
CD4$^+$ T-cell count/μl	569 (417–811)	659 (388–892)	529 (336–810)
Glucose, mg/dl	84 (80–94)	89 (83–127)	93 (85–95)
Triglycerides, mg/dl	141 (122–313)	207 (124–229)	242 (137–404)
Total cholesterol, mg/dl	222(222–251)	227 (212–261)	205 (195–254)
HDL cholesterol, mg/dl	48(43–55)	47 (39–54)	47 (40–52)
LDL cholesterol, mg/dl	142 (130–153)	141 (126–167)	137 (131–144)
Lactate, mg/dl	13 (9–16)	15 (12–16)	13 (11–19)
Limb fat, g	3,204 (2,238–4,797)	3,295 (2,785–4,351)	4,439 (2,589–5,619)
Total fat, g	11,843 (8,674–15,061)	11,459 (9,851–16,990)	14,316 (10,673–18,848)
FFM total, g	55,008 (48,792–62,588)	53,896 (52,531–59,918)	56,134 (53,128–59,874)
BMD total	1.2 (1.1–1.2)	1.2 (1.2–1.3)	1.2 (1.1–1.3)

*All values are presented as medians with interquartile ranges. There is no statistically significant difference between groups in baseline characteristics in any parameter. BMD, bone mineral density; d4T, stavudine; d4T40, d4T 40 mg twice daily; d4T30; d4T 30 mg twice daily; FFM, fat free mass (lean mass); HAART, highly active antiretroviral therapy; HDL, high density lipoprotein; LDL, low density lipoprotein; TDF, tenofovir.

study, whereas peripheral and total fat decreased in the d4T40 arm. The change was significant between the d4T40 and the TDF arm. Median peripheral fat changes compared with baseline in g were as follows: d4T40 arm: -182 (95% CI: -469–-50); d4T30 arm: 527 (95% CI: -343–694); TDF arm: 402 (95%CI: 130–835); d4T40 versus d4T30 P=0.12; d4T30 versus TDF, P=0.94; d4T40 versus TDF, P=0.0003; and global P=0.006. Median total fat changes were as follows: d4T40 arm: -429 (95% CI: -949–58); d4T30 arm: 1355.5 (95% CI: -218.5–2,064); TDF arm: 1,190 (95% CI: 126–2,188); d4T40 versus d4T30, P=0.17; d4T30 versus TDF, P=0.99; d4T40 versus TDF, P=0.032; and global P=0.034. Absolute values of peripheral and total fat, assessed by DEXA at week 24, did not differ statistically between arms (Table 3).

An unexpected finding in the TDF arm was a statistically significant decrease in total and trunk lean mass, whereas in the d4T40 and d4T30 arms the values remained stable. No total body weight changes were observed in any of the treatment arms. Changes in truncal lean mass compared to baseline measured in g were as follows: d4T40 arm: 127 (95% CI: -543–423); d4T30 arm: -168.5 (95% CI: -896–480); TDF arm: -1,837 (95% CI: -3,016–-491); d4T40 versus d4T30, P=0.99; d4T30 versus TDF, P=0.009; d4T40 versus TDF, P=0.005; and global P=0.002. Changes in total lean mass compared with baseline were as follows: d4T40 arm: 816 (95% CI: -683–1,220); d4T30 arm: 394 (95% CI: -577–1,032); TDF arm: -1,432 (95% CI: -3,111–-403); d4T40 versus d4T30, P=0.99; d4T30 versus TDF, P=0.007; d4T40 versus TDF, P=0.013 and global P=0.002 (Table 3). No correlation was observed between fat gain and loss of lean mass in the TDF arm (ρ=-0.04; P=0.88).

The treatment effect was explored by calculating the difference between mean changes and the 95% CI (Table 4).

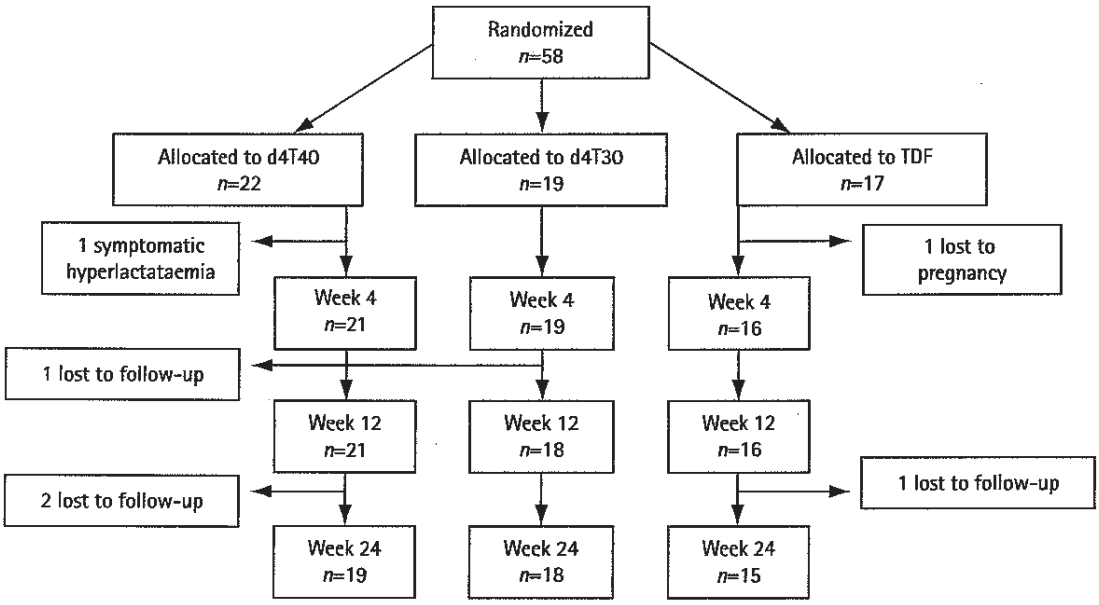
The change in limb and total fat mass were assessed by both univariate and multivariate analyses. A significant univariate association was found between an increase in limb fat mass and the treatment assigned (P=0.006). Also a significant univariate association was found between an increase in total fat mass and higher baseline values of limb fat (P=0.009), total fat mass (P=0.033) and assigned treatment (P=0.034). In the multivariate regression analysis, none of the baseline variables were significantly or independently associated with changes in body composition.

At the end of the study, no significant changes in lumbar spine or hip BMD were observed between arms, and there was no faster incidence of progression towards osteopenia or osteoporosis. No patients developed bone fractures during the study.

Mitochondrial function

No significant differences were found for any of the mitochondrial parameters at week 24 with respect to baseline values in any of the study arms, with the exception of Complex IV activity, which increased in both the d4T30 and TDF arms, but not in the d4T40 arm (Table 5). In the d4T40 arm lactate levels rose throughout the study, whereas in the d4T30 arm, they remained stable. Among TDF users, lactate levels initially decreased significantly (week 12: d4T40 versus TDF arm, P=0.015), but then rose again (Table 2),

Figure 1. Patient follow-up



d4T40, stavudine 40 mg twice daily; d4T30; stavudine 30 mg twice daily; TDF, tenofovir.

although at the end of the study lactate levels in the TDF arm were still below baseline levels. Lactic acidosis occurred in two patients (both in the d4T40 arm) and asymptomatic hyperlactataemia was detected during the study in four additional patients, all in the d4T40 arm. Four of these six patients were receiving concomitant ddI therapy.

Efficacy

In the TDF and the d4T30 arms, all patients maintained HIV RNA levels <200 copies/ml during the study. A virological rebound was seen in two patients in the d4T40 arm. No significant changes in $CD4^+$ T-cell counts were observed at any time point in any treatment arm.

Adverse events

There were two serious adverse events, symptomatic hyperlactataemia (lactate >22mg/dl), both in the d4T40 arm, at weeks 4 and 24. Both patients had been receiving d4T for more than 3 years. No other serious adverse events were observed in any treatment arm.

Discussion

Use of d4T has been associated with the occurrence of metabolic disorders, HIV-associated lipoatrophy, and mtDNA depletion [1–6]. This study evaluated two possible approaches for managing lipid and morphological changes in persons on d4T-containing antiretroviral regimens. Both approaches maintained virological control and there were no significant differences in $CD4^+$ T-cell count between arms at any time point. Overall, patients randomized to either TDF or the d4T30 arm had a recovery of limb fat, and a decrease in lipid and lactate levels, although there was no difference at week 24 in mitochondrial parameters compared with those in the d4T40 arm.

Consistent with previous reports [7,28], fasting triglycerides and total cholesterol values decreased significantly when the standard dose of d4T was replaced with TDF, and improved, although non-significantly, in the d4T30 arm. None of the approaches led to any significant changes in levels of glucose, HDL- or LDL-cholesterol, at any point during the study.

Benefits of a d4T dose reduction and/or switching to TDF were also observed by total body DEXA, showing a median increase in peripheral and total fat compared with baseline values. Although the median increase was higher in the d4T30 arm compared with the TDF arm, only those changes in the TDF arm reached statistical significance when compared with the d4T40 arm. Values in subcutaneous fat continued to decrease in the d4T40 arm, compared with baseline, but did not reach statistical significance. Since all patients included in the study had moderate to severe lipoatrophy at the baseline, and were receiving the d4T standard dose for a median of

Table 2. Changes in laboratory parameters from baseline

Outcome measures	d4T40*	d4T30*	TDF*	Global *P*-value
Glucose				
Week 4	5 (4–16)	7.5 (-3–11)	5 (-8–15.5)	0.82
Week 12	4 (2– 9)	0 (-10–11)	3 (-1–7)	0.35
Week 24	2.1(-7–9)	-1.5 (-11–4)	4 (-8–7)	0.60
Triglycerides				
Week 4	36 (5–74.3)	-6 (-40–90)	-20 (-71–-7)	0.13
Week 12	25 (0–41)	-3 (-46–48)	-31 (-136–34)	0.25
Week 24	11 (-21–51)	-16.5 (-62–18.5)	-69.5 (-231–19)	0.02†
Total cholesterol				
Week 4	0 (-26–13)	-2 (-18–21)	-35 (-61–1)	0.212
Week 12	-1 (-11–8)	-14 (-29–1)	-24 (-62–-8)	0.03‡
Week 24	-3 (-7–22)	1.5 (-7–22)	-32 (-54–-7)	0.02§
HDL-cholesterol				
Week 4	-3.5 (-14–1)	-3 (-8– 4)	-7 (-41–-1)	0.35
Week 12	-3.5 (-12–3)	-5 (-7–1.5)	-7 (-10.5–0)	0.78
Week 24	-2 (-7–3)	1(-5–6)	-6 (-8–0)	0.76
LDL-cholesterol				
Week 4	-9 (-13–9)	-15 (-31.5–17.5)	-26 (-34–-16)	0.31
Week 12	4 (-1–21)	-12 (-27–-4)	-6 (-54–10)	0.17
Week 24	2.5 (-2–29)	6.5 (-19–23.5)	-27 (-49–10)	0.08
Lactate				
Week 4	0.5 (-3–1)	-0.5 (-1–2)	-2.5 (-6–0.5)	0.32
Week 12	2 (0–4)	-1 (-2.5–2)	-3.3 (-5.5–0.5)	0.02¶
Week 24	3 (-1– 6)	0 (-3–4)	-1 (-3–3)	0.13

Other virological and immunological parameters not shown.*Results are presented as median (95% confidence interval). †d4T40 (stavudine 40 mg twice daily) versus d4T30 (stavudine 30 mg twice daily), *P*=0.63; d4T30 versus TDF (tenofovir), *P*=0.15; d4T40 versus TDF, *P*=0.03. ‡d4T40 versus d4T30, *P*=0.46; d4T30 versus TDF, *P*=0.34; d4T40 versus TDF, *P* =0.04. §d4T40 versus d4T30, *P*=0.93; d4T30 versus TDF, *P*=0.07; d4T40 versus TDF, *P*=0.02. ¶d4T40 versus d4T30, *P*=0.46; d4T30 versus TDF, *P*=0.34; d4T40 versus TDF, *P*=0.01. HDL, high-density lipoprotein; LDL, low-density lipoprotein.

6 years prior to inclusion in the study, we expected that the process of fat loss had reached a plateau and would not continue to decrease, as we found.

Generally, in studies performed in the 'HAART era', lean mass changes measured by DEXA are either not presented or, if so, lean body mass remains stable. Surprisingly, in our study population, a decrease in total fat free mass was observed in all patients treated with TDF, whereas total body mass remained stable, and the decrease did not correlate with fat mass changes. One explanation is that observed loss of lean mass in the TDF arm may be attributed to the limitations of DEXA in measuring the intramuscular lipid content, known to be increased in patients with HIV-associated lipoatrophy [29,30]. This finding once more confirms that DEXA cannot be considered the gold standard for measuring body fat changes. In this study, we found no evidence of accelerated bone loss in any of the treatment arms. These results may be due to the small study sample or short follow up, because in a previous study that included more than 200 patients receiving TDF, a decrease in BMD was observed between weeks 24 and 48 [3].

Mitochondrial parameters studied here show, in general terms, a slight trend to improvement with either the reducing or switching strategy, but only reached statistical significance for a restoration of Complex IV activity. Through what mechanism such improvement is achieved was not investigated in the present study, although it seems that a recovery in mtDNA content does not fully explain the increase in Complex IV activity. Alternatively, there is some evidence that other mitochondrial effects of NRTIs besides γ-polymerase inhibition and mtDNA depletion could play a relevant role in the development of adverse effects. It is well known that mitochondrial energetic homeostasis is regulated by a process called 'mitochondrial fine tuning' that allows it to adapt transcription of mtRNA to meet altered OXPHOS requirements independent of regulation by nuclear factors or changes in mtDNA abundance [31,32]. Recently published reports demonstrate *in vitro* [33] or *in vivo* [34] the existence of NRTI inhibition of mtRNA transcription in the absence of mtDNA depletion. In this scenario, it is possible that the beneficial effects on Complex IV activity reached via

Table 3. Changes in body composition over 24 weeks – changes in body composition from baseline to week 24

Outcome measures	d4T40*	d4T30*	TDF*	Global *P*-value[†]
Limb fat, g	-182 (-469–-50)	527 (-343–694)	402 (130–835)	0.006[†]
Truncal fat, g	-145 (-461–161)	937.5 (-336–1316)	-676 (-9–978)	0.14
Total fat, g	-429 (-949–-58)	1,355 (-218.5–2064)	1,190 (126–2188)	0.034[‡]
Limb FFM, g	339 (-380–685)	245.5 (-85–558.5)	128 (-617–534)	0.92
Truncal FFM, g	-127 (-543–423)	-168.5 (-896–480)	-1,837 (-3,016–-491)	0.002[§]
FFM total, g	816 (-683–1220)	394 (-577–1032)	-1,432 (-3,111–-403)	0.002[¶]
BMD total	-0.001 (-0.007–0.010)	0.007 (0.003–0.012)	0.003 (-0.016–0.003)	0.21

*Results are presented as median (95% confidence interval) [†]d4T40 (stavudine 40 mg twice daily) versus TDF (tenofovir), *P*=0.0003. [‡]d4T40 versus TDF, *P*=0.032. [§]d4T30 (stavudine 30 mg twice daily) versus TDF, *P*=0.009; d4T40 versus TDF, *P*=0.005. [¶]d4T30 versus TDF, *P*=0.007; d4T40 versus TDF, *P*=0.013. BMD, bone mineral density; FFM, fat free mass.

Table 4. Changes in body composition over 24 weeks – treatment effect on changes in body composition expressed as difference between mean and 95% CI

Outcome measures	d4T30-d4T40	TDF-d4T40
Limb fat, g	397.8 (2.5–793)	491 (8.8–974)
Truncal fat, g	371.6 (-363–1106)	137 (-943–1217)
Total fat, g	759 (-273–186.7)	661.7 (-884–2207)
Limb FFM, g	45.7 (-489–581)	-55 (-682–571)
Truncal FFM, g	-87.7 (-795–620)	-1666.6 (-2562–771)
FFM total, g	17.7 (-1136–1171)	-1882.7 (-3184–-582)
BMD total	-0.008 (-0.04–0.012)	-0.007 (-0.045–0.03)

BMD, bone mineral density; d4T40, stavudine 40 mg twice daily; d4T30, stavudine 30 mg twice daily; FFM, fat free mass.

the strategies tested in the present study were achieved independently of effects on mtDNA abundance, perhaps through changes at mtRNA transcriptional or post-transcriptional levels.

Our results in mitochondrial parameters studied in PBMCs did not differ among groups, even though a significant reversal of lipoatrophy and improvement of metabolic parameters was observed. The findings of our study are similar to those of MITOX, a 24-week sub-study that concluded that assays for the detection of mtDNA in PBMCs are less sensitive than results of a DEXA scan in assessing reversal of lipoatrophy [35]. Adequate biological material in which functionality of the mitochondria can be measured is still to be defined [36]. Our results support the previously established hypothesis that if body fat and metabolic changes are mediated by mitochondria, its function should not be measured in PBMCs, since drug toxicities are probably organ-specific [37–39].

Additionally, it is now known that restoration of mtDNA content in PBMCs is observed only at a time point beyond 24 weeks after HAART withdrawal, and is limited to some lymphocyte subpopulations [40]; thus, in switching strategies, changes in the mtDNA content of PBMCs require even a longer period to initiate any observable increase. It is also not clear if Complex IV recovery is the main cause of the fat and plasma lipid effects observed. This may suggest either that fat and plasma lipid effects are not mediated via mitochondria or that PBMCs are not sensitive enough to detect those changes [36,37,41].

More than half of all cases described in the literature of lactic acidosis are attributed to d4T [42]. In our study, we observed statistically significant decrease of lactate levels in the TDF arm after 12 weeks, stabilization in the d4T30 arm, whereas they continued to rise in the d4T40 arm throughout the study. We recommend routine determinations of lactate in patients receiving a standard but not reduced dose of d4T, since this potentially life-threatening adverse event may be relatively common.

A dose-reduction strategy is neither new nor exclusive for d4T. In the historical evolution of the first antiretroviral, zidovudine (AZT), a change in prescription guidelines was done after several studies showed that what was once called the 'standard dose' of AZT (1,200 mg/day) was far more toxic than a reduced dose ('half dose') of AZT, now the standard dose of 600 mg/day [43–46]. During the Phase II dose-finding studies for d4T, a dose lower than the current 'standard' was seen to be virologically and immunologically effective [47,48]. Our study, along with recent observational studies, suggests that a reduced dose of d4T can be as effective as the standard dose, with less side-effects [11–15].

Although the easiest way to avoid d4T-associated side effects is to not use d4T, this may not always be feasible. Given the ever-increasing incidence and prevalence of HIV in resource-poor settings, the need for cost-effective anti-HIV therapy is evident. Fifty percent of antiretroviral treatment combinations in developing countries are d4T-based. One approach to reduce the cost of antiretroviral therapy in settings with limited resources would be the use of a safe yet effective lower dose of drugs. In our study, a dose

Table 5. Evolution of mitochondrial parameters (% change from baseline) in week 24

Outcome measures	d4T40*	d4T30*	TDF*	Global *P*-value
Mitochondrial mass	4.5 (-25.9–31.5)	23 (5–52.4)	7.9 (-15.9–46.5)	0.49†
mtDNA content	18.5 (-8.1–44.8)	2.2 (-16.6–47.5)	0.7 (-24–36.2)	0.53‡
Complex IV activity	14.95 (-0.9–43.3)	29.5 (2.7–86.7)	33.5 (-0.9–66.8)	0.681§
Spontaneous cell oxidation	5.7 (-27.3–48.1)	1.2 (-16–11)	1.2 (-18–37.6)	0.69¶

Individual *P*-values shows changes from baseline for each treatment arm. *Results presented as median (95% confidence interval). †d4T40 (stavudine 40 mg twice daily) , *P*=0.28; d4T30 (stavudine 30 mg twice daily), *P*=0.85; and TDF (tenofovir), *P*=0.30. ‡d4T40, *P*=0.18; d4T30, *P*=0.07; and TDF, *P*=0.47. §d4T40, *P*=0.12; d4T30, *P*=0.03; and TDF, *P*=0.02. ¶d4T40, *P*=0.76; d4T30, *P*=0.21; and TDF, *P*=0.57. mtDNA, mitochondrial DNA.

reduction of d4T of 25% maintains efficacy while reducing toxicity. Our finding may lead to a more intelligent use of d4T, especially in settings where dose reduction can make treatment more accessible.

Our results demonstrate that in the actual context of HAART usage in HIV-infected patients, substitution of the standard daily dose of d4T to a reduced dose of 30 mg twice daily, or to TDF once daily are equally safe and effective, although the study was not powered to asses the virological efficacy of these approaches. Both options seems to have minor, if any effects on the overall mitochondrial parameters assessed in PBMCs. Although both strategies increase peripheral fat and are associated with a certain decrease of lipid parameters, only switching to TDF led to a significant reversal of peripheral lipoatrophy. Longer follow-up studies and studies of an even greater d4T dose reduction are warranted and should be performed.

Acknowledgments

Thanks to Rob Camp, Treatment Action Group, New York, for reading this manuscript with a critical eye. We also acknowledge the efforts of Javier Fernandez from the Nuclear Medicine Department for his work on body composition studies, and Ana B. Infante for her technical laboratory support. We are especially grateful to the patients who participated in the study.

Grants

This study was supported in part by grants: PI02590 from Fondo de Investigaciones Sanitarias; RIS G03/173 from Red Tematica Cooperativa de Investigacion en SIDA, Ministerio de Sanidad y Consumo; Fundació La Marató de TV3 (020210), Redes de Investigación en Mitocondrias (V2003-REDC06E-0); and by Grups de Recerca de la Generalitat de Catalunya (SGR 0300/2005). The funding sources played no role in the design of the study, data collection, data analysis, data interpretation, or writing of the report.

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Accepted for publication 30 November 2006

HIV-1-INFECTED LONG-TERM NON-PROGRESSORS HAVE MILDER MITOCHONDRIAL IMPAIRMENT AND LOWER MITOCHONDRIALLY-DRIVEN APOPTOSIS IN PERIPHERAL BLOOD MONONUCLEAR CELLS THAN TYPICAL PROGRESSORS

CURRENT HIV RESEARCH 2007; 5(5): 467-473.

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HIV-1-Infected Long-Term Non-Progressors have Milder Mitochondrial Impairment and Lower Mitochondrially-Driven Apoptosis in Peripheral Blood Mononuclear Cells than Typical Progressors[§]

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Abstract: Mitochondrial parameters in peripheral blood mononuclear cells (PBMC) and their relationship with mitochondrially-driven PBMC apoptosis were investigated in a group of HIV-1-infected long-term nonprogressors (LTNP) and compared with untreated asymptomatic HIV-1 infected typical progressors (TP) and uninfected healthy controls (HC). Twenty-six LTNP, 27 TP and 31 HC were evaluated. Studies were performed in PBMCs. Mitochondrial DNA content (mtDNA) was assessed by quantitative real-time PCR. Activities of mitochondrial respiratory chain complexes (MRC) II, III and IV were determined by spectrophotometry. Caspase-3 activity was assessed by fluorimetry, and caspase-9 activation and Bcl-2 levels were assessed by immunoblotting. mtDNA abundance ($p<0.05$), MRC complex II ($p<0.001$), complex III ($p<0.01$) and complex IV ($p=0.01$) were lower in the TP group than in the HC group. In the LTNP group these parameters were similar to those of the HC group except for complex II, which was decreased ($p<0.01$). The PBMC of TP showed the highest overall apoptotic activation, since their caspase-3 activity was greater than that of HC ($p<0.05$) and LTNP. In the case of LTNP, however, the difference was non-significant. Caspase-9 and the caspase-9/Bcl-2 ratio were both over-expressed in TP compared to HC ($p<0.01$) and LTNP ($p<0.05$). Both of these measurements indicate that mitochondrially-driven apoptosis in TP is greater than in LTNP and HC. A relationship between mitochondrial damage and apoptotic activation was found in TP. Mitochondrial damage is associated with increased PBMC apoptosis in patients with active HIV-1 replication (TP). These abnormalities are slight or not present in LTNP.

Keywords: HIV-1 infection, mitochondrial DNA, caspases, apoptosis, long-term nonprogressors, peripheral blood mononuclear cells.

INTRODUCTION

HIV-1 infection can induce mitochondrial derangement in peripheral blood mononuclear cells (PBMC). A seminal study by Côté *et al*. found that the mitochondrial DNA (mtDNA) content in PBMC was lower in asymptomatic HIV-1-infected patients naive for antiretroviral therapy than in healthy controls [10]. This finding has been confirmed in further studies [6,9,24,26]. Some of our group have also reported that the decrease in PBMC mtDNA content is accompanied by an extensive mitochondrial dysfunction, involving both mitochondrial respiratory chain (MRC)-related and unrelated enzymes [24]. The fact that HIV-1 reduces PBMC mtDNA content and its bioenergetic function has therefore been convincingly demonstrated [6,9,10,24,26].

Whether this mitochondrial impairment has any consequence on the integrity of PBMC *in vivo* is, however, unknown. Given the pivotal role of mitochondria as a key regulator of cell apoptosis, it is of paramount importance to investigate the possible relationship between HIV-1-induced mitochondrial derangement and apoptosis of PBMC. We hypothesized that increased PBMC apoptosis induced by HIV may be linked with diffuse mitochondrial dysfunction. To test this hypothesis, in this study we have measured several mitochondrial and apoptosis parameters in HIV-1-infected patients and attempted to correlate the findings. We have selected two groups of HIV-1-infected patients who clearly differ in the extent of apoptosis activation. On the one hand, we studied untreated asymptomatic typical progressors (TP) who have already progressed and who show immunological decline and a high HIV-1 viremia. In this group, apoptotic-mediated cell death (even without direct viral infection) is responsible, at least in part, for the CD4+ T-cell decline over time [31,46]. On the other hand, we stud-

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[§]Presented in part at the 45th annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington 2005 (abstract nº ICAAC05-A-1582-ASM) and at the 7th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV, Dublin 2005 (Abstract nº 71).

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ied HIV-1-infected patients who were long-term non-progressors (LTNP). These patients have self-control of the infection and a low HIV-1 viral burden and it is widely accepted that resistance to HIV-1-induced apoptosis is one of the mechanisms behind the maintenance of high CD4+ T-cell counts [15].

PARTICIPANTS AND METHODS

Design, criteria and participants: We performed a cross-sectional study which involved three categories of participants: healthy controls (HC), HIV-1-infected LTNP and HIV-1-infected TP. All categories were matched by age (±5 years). Patients were recruited from a prospectively collected cohort of almost 5000 HIV-1-infected patients followed-up at the HIV outpatient clinic of the four participating hospitals. Criteria for LTNP were: asymptomatic HIV-1 infection with a known duration of over 15 years, a stable CD4+ T-cell count persistently over 500 cells/ml and plasma HIV-1 viral load repeatedly under 5,000 copies/ml, in the absence of any antiretroviral treatment [20]. Untreated TP were individuals whose HIV-1 infection had progressed (i.e. if they had an HIV-1 viral load of over 35,000 copies/ml and a progressively declining CD4+ T-cell count over time that had gone below 350 cells/ml at least twice during the first eight years of infection) and who had not previously received or were not currently receiving antiretroviral drugs, were free of symptoms and had not suffered opportunistic infections until recruitment. For a few patients whose date of infection was unavailable, this was assumed to be the midpoint between the first positive and the last negative HIV-1 blood test [19]. We carefully checked that both HIV-1-infected patients and healthy controls had no conditions known to damage mitochondria, such as smoking, alcohol abuse or liver disease, and also that they were not taking drugs known to cause mitochondrial derangement [7]. Informed consent was obtained from each participant. The project was approved by the local ethical research committees.

We have already reported studies on the host genetic determinants of the LTNP state [40-43]. Our cohort is currently made up of 76 carefully chosen individuals who fulfill the criteria for LTNP mentioned above. Of these, 26 also satisfied the requirements for mitochondrial studies and agreed to participate. With respect to previously untreated TP, we randomly selected 27 patients who satisfied our predefined criteria for TP and the requirements for mitochondrial studies and who agreed to participate in the present study. As a control group we studied a sample of 31 healthy subjects recruited from voluntary blood donors.

Collection of blood samples: After an overnight fast, 20 mL of blood obtained from a peripheral vein was collected in Vacutainer EDTA tubes. PBMCs were isolated by means of Ficoll density gradient centrifugation (Histopaque -1077, Sigma Diagnostics, St. Louis, MO). After isolation, PBMC were resuspended in phosphate-buffered saline (PBS) and stored frozen at 80°C until used in biochemical and genetic determinations. In our experience, less than 30 platelets per cell are present in the final suspension. Protein content was measured according to Bradford´s method [5].

HIV-1 infection: This was diagnosed by a positive enzymoimmunoanalysis and confirmed by a positive Western-Blot test.

Plasma HIV-1 viral load: This was determined by the Cobas Amplicor HIV-1 Monitor Test v 1.5 using the CO-BAS AMPLICOR system (Roche Diagnostics®, Basel, Switzerland).

Assessment of blood CD4+ T-cell count: Samples were analyzed in a flow cytometer FAC Scan (Becton Dickinson Immunocytometry Systems®, San Jose, CA, USA). The data acquired were analyzed using the Multiset program.

PBMC mtDNA content: An aliquot of PBMC was used for extracting total DNA by a standard phenol-chloroform procedure. For mtDNA 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®, Germany). The complete methodology use in our laboratory has been reported elsewhere [17].

Mitochondrial enzyme activities: Another aliquot of PBMC was used for the spectrophotometric analyses. Currently, isolated MRC complex I and complex V activities cannot be assessed in whole cells, because the former is not activated with decylubiquinone and because of the strong oligomycin-insensitive ATPase activity of the latter [17]. Hence, we only determined the enzyme activities of complexes II (exclusively encoded by nuclear DNA), III and IV (both partially encoded by mtDNA) following standard procedures proposed by Rustin *et al*. [34] slighly modified for complex IV assessment [21]. In order to correct the enzyme activities for any eventual difference in cellular mitochondrial content, absolute activities were corrected by citrate synthase activity, which is considered to be a good marker of the mitocondrial content [4,17,24,29].

Studies of apoptotic parameters in PBMC: To assess overall (mitochondrial and extramitochondrial-dependent) PBMC apoptotic activation, caspase-3 activity was measured using the fluorimetric CaspACE Assay System (Promega, USA). This was based on the fluorimetric detection of amino-4-trifluoromethyl coumarin following proteolytic cleavage of the synthetic substrate DEVD-amino-4-trifluoromethyl coumarin [16]. PBMC were homogeneized by 15 strokes of a 22G syringe in cell "lysis buffer" (25 mM Hepes, 5 mM MgCl2, 5mM EDTA, 5 mM DTT, 2mM PMSF, 10 g/ml pepstatin A and 10 g/ml leupeptin) followed by 3 cycles of freezing/thawing. The cytosolic extracts to be assayed were then collected after 10,000 x g centrifugation for 20 min. Four hundred g protein, as determined by the Bradford Assay Kit (Bio-Rad), was used for the assays, which were performed according to the supplier. Assay specificity was assessed using 50 M M Z-VAD-FMK, a fluoromethyl ketone-derivatized peptide inhibiting caspase-3 (Promega, USA), which in all the assays led to a residual activity of less than 5% of the total activity detected. Results were expressed as fluorescence relative units/mg protein. We also analysed apoptotic activation through mitochondrial pathways by measuring the cleaved, active form of caspase-9 with a specific immunoblot assay [37]. Crude protein extracts containing 20 mg protein were mixed with 1/5 vol of a solution containing 50% glycerol, 10% SDS, 10% 2-mercaptoethanol, 0.5% bromophenol blue and 0.5 M Tris (pH 6.8), incubated at 90ºC for 5 min and electrophoresed on 0.1% SDS/13% polyacrylamide gels. Proteins were trans-

ferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, USA). Blots were probed with a rabbit polyclonal antibody specific for the 35 kDa cleaved, active form, of human caspase-9 (Cell Signaling Technology 9505, Beverly, MA, USA)(1:1000) and further probed with mouse polyclonal antibodies for human Bcl-2 (Santa Cruz Biotechnology sc-599, Santa Cruz, CA, USA)(1:500) and for -actin (Sigma A5441, St Louis, MI, USA) (1:10.000). Quantitative densitometric analysis of the active caspase-9 (35kDa), -actin (47 kDa) and Bcl-2 (26 kDa) immunoblot signals was performed (Phoretics 1D Softward, Phoretic International Ltd, Newcastle, UK). Active caspase-9 immunoreactive signals were expressed either by -actin to normalize for equal cell protein loading or by Bcl-2 to quantify the extent of activation of PBMC mitochondrially-driven apoptosis for a given amount of Bcl-2. This constitutes a kind of apoptotic index because it is expressed as a ratio between an apoptotic marker (caspase 9) and an antiapoptotic one (Bcl-2) as has already been reported [25].

Statistical analysis: In all experiments, TP and LTNP data were presented as relative values with respect to those of HC, which were arbitrarily assigned a value of 100%. The results in HIV-1-infected patients and controls were expressed as mean±SEM and compared using the unpaired Student's T test, the one-way analysis of variance (ANOVA) test for normally distributed data, and the Kruskal-Wallis test when non-parametric tests were necessary. A linear regression was also performed to find relationships between the quantitative variables. In all cases, a *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Characteristics of the Participants

We studied 84 individuals, of whom 53 were HIV-1-infected (26 LTNP and 27 TP) and 31 HC. None had a personal or family history suggesting neuromuscular or mitochondrial disorders or were taking drugs known to provoke mitochondrial derangement. All subjects had normal acid-base equilibrium. The main demographic and clinical characteristics of the groups are shown in Table **1**. Men predominated in the HC and TP groups, while in the LTNP group the gender distribution was balanced. This was of low significance because the mtDNA content and MRC complexes for male and female patients were similar in the three groups defined (data not shown). As expected by the case definition of LTNP and TP, these groups differed in duration of infection, CD4+ cell count and plasma HIV-1 viral load.

mtDNA content and mitochondrial enzyme activities: With respect to HC, the mtDNA content of TP decreased ($p<0.05$), while that of LTNP remained unchanged (Fig. **1**). TP exhibited a decrease in MRC enzyme activities with respect to healthy controls. This was statistically significant for complex II ($p<0.001$) and complex III ($p<0.01$) (Fig. **1**). In contrast, the MRC enzyme activities of LTNP were not significantly different from those of HC, with the exception of complex II, which was 75% of that found in HC ($p<0.05$) (Fig. **1**).

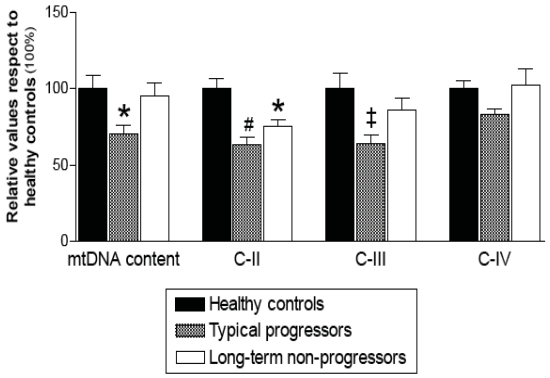

Fig. (1). Mitochondrial parameters evaluated in present study. Results are expressed in mean ± SEM. mtDNA: mitochondrial DNA content; C-II: complex II activity, C-III: complex III activity; C-IV: Complex IV activity. *: $p<0.05$ respect to healthy controls (HC); ‡: $p<0.01$ respect to HC; #: $p<0.001$ respect to HC.

PBMC apoptotic studies: A sample of PBMC useful for performing studies of apoptotic parameters, was available for 40 of the 53 HIV-1-infected patients (19 LTNP and 21 TP), and for 20 HC. The main characteristics of these patients did not differ from those of the group as a whole. We detected a marked increase in caspase-3 activity in TP, which indicated increased overall apoptosis activation (Fig. **2**). There was a similar increase in apoptotic activation involving mitochondrial pathways in TP because the cleaved, active form of caspase-9 (a major effector of mitochondrially driven apoptosis), was four times higher in TP than in HC ($p<0.01$ with respect to HC and $p<0.05$ with respect to LTNP, Fig. **2**).

Table 1. Demographic and Clinical Characteristics of the Participants

	Healthy controls	TP	LTNP
Number of patients	31	27	26
Age in years (mean ± SD)	41 ± 11	38 ± 12	42 ± 9
Males : Females (nº)	22:9	21:6	13:13
Duration of infection in years (mean ± SD)		5.7±1.7 [a]	16.9 ± 1.3 [a]
CD4+T cell count/mm3 (mean ± SD)		267 ± 92 [a]	763 ± 176 [a]
log_{10} HIV RNA copies/mL (mean ± SD)		4 ± 0.9 [a]	2.5 ± 0.8 [a]

TP: HIV-1-infected patients usual progressors.
LTNP: HIV-1-infected patients long-term non-progressors.
[a]$p < 0.0001$.

Similar results were obtained when caspase-9 activity was corrected by a given amount of Bcl-2 levels. Conversely, although for LTNP the quotients of all apoptotic markers were higher than those of HC, they were located midway between HC and TP values and did not reach statistical significance (Fig. **2**).

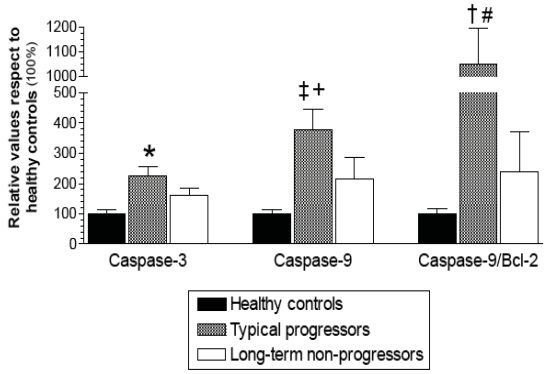

Fig. (2). PBMC apoptotic parameters evaluated in the present study. Results correspond to caspase-3 activity (absorbance units/mg protein) and to active caspase-9 (densitometric scanning of the 35 kDa immunoblot signal for the cleaved caspase-9 referred to the 47 kDa -actin signal or to the 26 kDa Bcl-2 signal). Data are expressed as percentages of control values and are mean±SEM. *: $p<0.05$ with respect to healthy controls (HC); ‡: $p<0.01$ with respect to HC; †: $p<0.001$ with respect to HC; +: $p<0.05$ with respect to long-term non-progressors (LTNP); #: $p<0.01$ with respect to LTNP.

In correlation analysis involving all HIV-1-infected patients, we found a relationship between markers of mitochondrial damage and markers of apoptosis, which was more manifest in the TP subgroup of patients than in LTNP subgroup of patients. Fig. **3** illustrates one of these studies, and shows an inverse relationship betweem mtDNA content and caspase-9. Likewise, we found a mild correlation between HIV-1 replication (measured as plasma viral load) and the increase in mitochondrial damage (measured as both mtDNA content and complex III activity) and apoptosis activation (Fig. **4**).

DISCUSSION

This study confirms that PBMC from HIV-1 infected patients with uncontrolled infection (typical progressors) have diffuse mitochondrial damage and show increased apoptosis which at least involves the mitochondrial-dependent pathway of apoptosis. It also shows that PBMC from LTNP have more preserved mitochondria and lower apoptotic activation than untreated TP despite the much longer duration of their HIV-1 infection. Our data also indicate that, in HIV-1 infected patients, PBMC apoptosis mediated by mitochondria correlates positively with mitochondrial damage and with the HIV-1 viral burden. Altogether, our results suggest that there is a relationship between PBMC mitochondrial damage and apoptotic activation, and offer indirect evidence that such disturbances could be influenced by the amount of viral burden. We recognize, however, some limitations to our study. First, it is a cross-sectional design, so we are unable to determine cause-effect relationships and, second, our findings in PBMC as a whole may not necessarily reflect what occurs in CD4+ T-cells. Moreover, we have chosen a set of experiments that closely represent what happens in mitochondrial function, and take into account the previously demonstrated effects of HIV-1 on such organelles, i.e. mtDNA depletion and decreased MRC activities irrespective of whether they are or are not encoded by mitochondrial DNA [22]. We acknowledge, however, that a complete evaluation of mitochondrial functions was not carried out, since we did not assess substrate oxidation, mitochondrial membrane potential or ATP production. Finally, the apoptosis analysis relied on caspase measurements rather than apoptosis itself, but this enabled us to distinguish between overall apoptotic activation (shown by caspase-3 activity) and mitochondria-dependent activation of apoptosis (shown by caspase-9 activation).

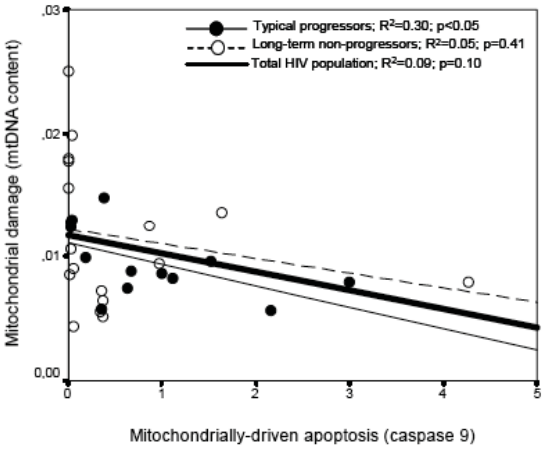

Fig. (3). Dots diagram and regression lines assessing the relationship between caspase-9, as a surrogate marker of mitochondrially-driven apoptosis, and mtDNA content, as a surrogate marker of HIV-1-mitochondrial damage. Data show a relationship between greater caspase-9 activity and lower mtDNA content. This relationship is only statistically significant for typical progressors.

At present, there is a great deal of evidence to suggest that HIV-1 facilitates the apoptosis of affected cells, leading to various sorts of organ damage [3,8,14,33,38]. It has also been proven that HIV-1-related mtDNA depletion and mitochondrial impairment are present even in PBMC of untreated asymptomatic patients who already have a decreased CD4+ T-cell count [10,24]. Many HIV-1 proteins have been proposed as being responsible for this. Among them, gp120, Tat, Nef and Vpr have been shown to cause dysregulation of the Bcl-2 family members expression, increase in mitochondrial-membrane permeabilization, cytochrome *c* release, and loss of mitochondrial transmembrane potential, all of which lead to cell apoptosis [1-3,13,30,33,39]. It is reasonable to assume that there is a relationship between both processes and the negative relationship between apoptotic activity and mtDNA content agrees with this hypothesis. Additionally, the negative correlation between mtDNA content and apoptosis is greater for TP than for LTNP (Fig. **3**), which also suggests that apoptosis has a role in the development of mitochondrial damage. It should also be noted that an emerging

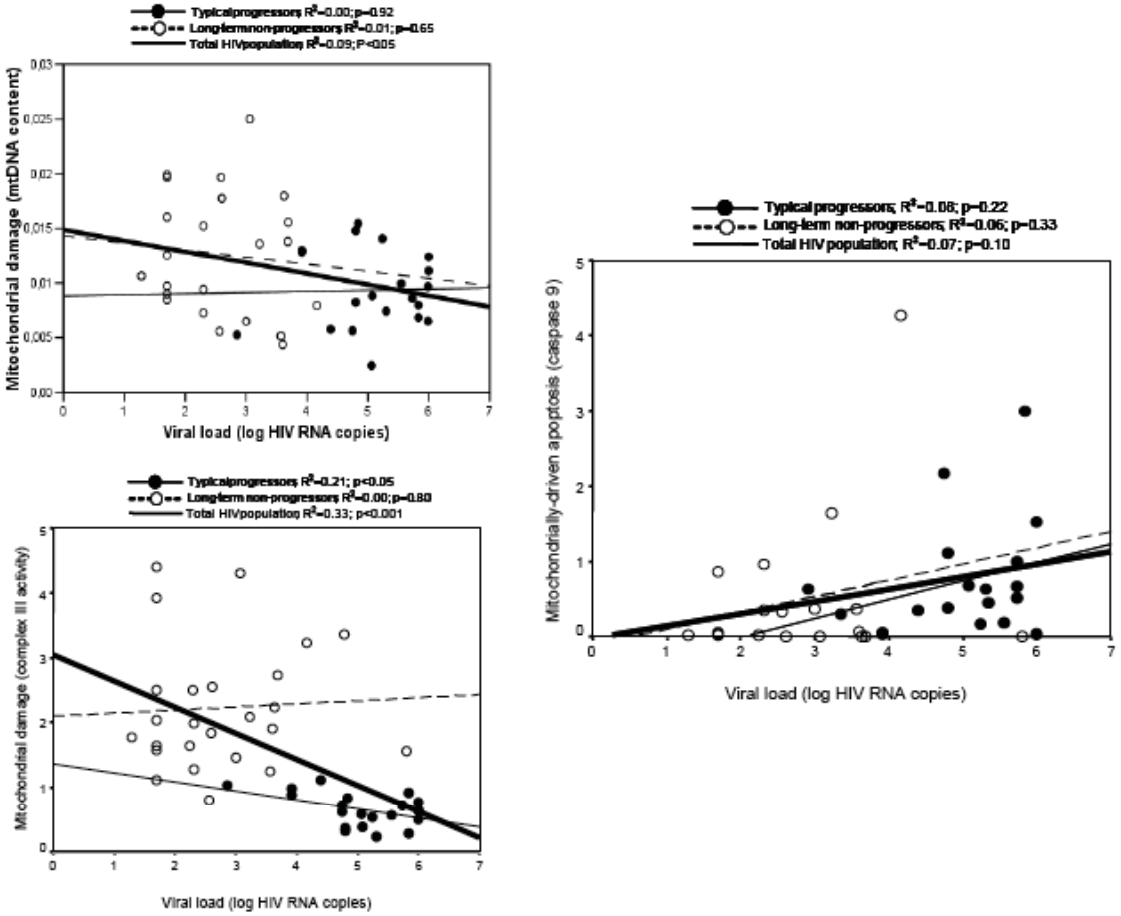

Fig. (4). Dots diagram and regression lines assessing the relationship between plasma viral load, as a surrogate marker of viral replicative activity, and mtDNA content (top left) and complex III (bottom left), both as surrogate markers of mitochondrial damage, and caspase-9 activation, as a surrogate marker of mitochondrially-driven apoptosis (right). Data show a relationship between greater viral load, lower mtDNA content and complex III activity, and greater caspase-9 activity. This relationship is only statistically significant for mitochondrial damage when the HIV-1-infected population is considered as a whole.

body of literature suggests a link between LTNP and apoptosis dysregulation, and mutations within Vpr impact on the mitochondriotoxic effects of this HIV-1-related protein [18,32] and can confer LTNP status.

With respect to caspase-9 activity, it has previously been shown that the HIV-1 protein Vpr induces apoptosis of PBMC through down-regulation of Bcl-2 and over-induction of caspase [28]. The Bcl-2 amount in mitochondria is more indicative of the sensitivity of cells to activate apoptosis in response to insults through pathways involving mitochondria rather than the mitochondrially-driven apoptotic activity per se, which is more clearly evidenced by other parameters such as caspase-9 activity. Our results agree with this scenario since we found that activation of caspase-9 was higher in TP than in LTNP (the viral replication and mass of the former are greater) and that in our patients there was a trend towards a relationship between mitochondrially-driven apoptosis and viral load.

Our results may also eventually explain the different immunologic slope of TP and LTNP over time. LTNP are a subset of patients that show self-control of the disease, a low HIV-1 viral burden and persistently elevated CD4+ T-cell counts over many years [20]. The LTNP group presented here were patients who had been living with HIV-1 for up to 15 years or even more, and had therefore been exposed to HIV-1 products for a long time, though at low levels of intensity. It should be noted that we provide evidence that the HIV-1 viral mass probably determines mitochondrial damage since low-invasive HIV-1 infection, which is what is found in the LTNP, produces less mitochondrial derangement than that of TP (which have a markedly greater viral burden). Apart from primary mitochondriopathies [11], most of the acquired toxic [12] and drug-induced [23,35,36,44] mitochondrial disorders are thought to be related to the amount of inoculum and the duration of exposure. In this respect, an earlier study of several mitochondrial functional

data in LTNP found that superoxide generation in these patients was decreased, and that the mitochondrial transmembrane potential was preserved. These data were consistent with a well-preserved MRC function, which in turn was associated with a decreased rate of CD4+ and CD8+ T-cell apoptosis [27]. In this previous study, however, the comparator group was made of patients who had progressed to AIDS and were receiving zidovudine, so it can not be ruled out that this drugs had a cumulative deleterious (and therefore confusing) effect on mitochondrial parameters [45]. Despite this bias, our results are consistent with those of Moretti *et al*. [27].

It can be speculated that LTNP probably do not reach the threshold of cumulative insults required to produce significant PBMC mitochondrial damage. Irrespective of the final mechanisms involved in these changes, we can conclude that HIV-1-infected patients LTNP have lower PBMC mitochondrially-driven apoptosis and better preserved mitochondria than TP.

ACKNOWLEDGEMENTS

This work was supported by the Fundación para la Investigación y la Prevención del Sida en España (FIPSE 3102/00, 3161/00 and 36610/06); Fundació La Marató de TV3 (02/1830 and 02/0210); Red de Investigación en Mitocondrias (V2003-REDC06E-0); Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Red de Sida; Fondo de Investigación Sanitaria (FIS 02/1282, 05/1501, 05/1591 and 07/0976) and Suport a Grups de Recerca de la Generalitat de Catalunya (2005/SGR/0300). The comments and criticisms of the two anonymous reviewers are highly appreciated. John Bates kindly improved the English text.

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Received: March 1, 2007 **Revised: May 22, 2007** **Accepted: May 27, 2007**

MITOCHONDRIAL DNA DEPLETION IN OOCYTES OF HIV-INFECTED ANTIRETROVIRAL-TREATED INFERTILE WOMEN

ANTIVIRAL THERAPY 2008 (en premsa)

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27-Jun-2008

Dear Mrs. Lopez (on behalf the rest of authors):

It is a pleasure to accept your resubmitted manuscript entitled "Mitochondrial DNA depletion in oocytes of HIV-infected antiretroviral-treated infertile women" in its current form for publication in Antiviral Therapy. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of Antiviral Therapy, we look forward to your continued contributions to the Journal.

Sincerely,

Dr. Peter Reiss
Section Editor,
Antiviral Therapy
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Reviewer(s)' Comments to Author:

Reviewer: 1
Comments to the Author
No specific comments.

Reviewer: 2
Comments to the Author
There are no comments

MITOCHONDRIAL DNA DEPLETION IN OOCYTES OF HIV-INFECTED ANTIRETROVIRAL-TREATED INFERTILE WOMEN

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Objective: HIV-infected women under highly active antiretroviral therapy (HAART) undergoing *in vitro* fertilization (IVF) have a lower pregnancy rate than non-infected controls, which depends on oocyte-related factors. We hypothesize that mitochondrial toxicity caused by antiretrovirals could be the underlying mechanism of such disturbance.

Methods: We have studied 16 and 19 previously frozen-thawed oocytes obtained after oocyte retrieval IVF cycles from 8 and 14 infertile HIV-infected and uninfected women, respectively, matched by age. At inclusion, HIV-positive women had been infected for more than 13 years and had received HAART for more than 9 years, including at least one NRTI. All of them had undetectable HIV-viral load and a good immunological status. Mitochondrial DNA (mtDNA) content was determined by quantitative real-time PCR in each individual oocyte.

Results: HIV-infected infertile women on HAART showed significant oocyte mtDNA depletion when comparing with uninfected controls (32% mtDNA decrease; $p<0.05$). This oocyte mtDNA depletion was even greater on those HIV-infected women who failed to become pregnant when comparing with controls (39% mtDNA decrease; $p=0.03$). No significant correlation was found between mtDNA oocyte content and cumulative doses of antiretrovirals or the immunological status of HIV-patients.

Conclusions: Oocytes from infertile HIV-infected HAART-treated women show decreased mtDNA content and this could explain their poor reproductive outcome.

INTRODUCTION

The ability of highly active antiretroviral therapy (HAART) to decrease viral load up to undetectable levels and to increase $CD4^+$ T lymphocytes has drastically reduced mortality and morbidity among HIV-infected patients [1,2]. As a consequence, HIV-infection has become a chronic disease.

Many adverse effects have been described and associated with long-term use of antiretrovirals. Most of them (miopathy, lipoatrophy, lactic acidosis, polineuropathy or pancreatitis) have been related to mitochondrial toxicity caused by

nucleoside analogues reverse transcriptase inhibitors (NRTIs) [3-7].

The main underlying mechanism described for mitochondrial toxicity is the capacity of NRTIs of inhibiting the mtDNA synthesis by both direct inhibition of the human DNA polymerase-γ (DNA pol-γ, the only polymerase responsible for the mtDNA replication) and by acting as chain terminators of the growing DNA strand. Consequently, NRTIs lead to the generation of abnormal mitochondria, with decreased number of mtDNA molecules per organelle (depletion), as well as an increase of mutations (deletions and/or point mutations) in the mtDNA genome [8-11]. MtDNA is a 16.6-kb double-stranded molecule which only encodes for 13 proteins of the mitochondrial respiratory chain associated with the oxidative phosphorilation process, 2 mitochondrial ribosomal RNA (mt rRNA) and 22 mitochondrial transfer RNA (mt tRNA). In this context, a depletion of the mtDNA levels can finally lead to mitochondrial dysfunction and energetic cell impairment.

According to epidemiological data, HIV-infected women have a lower spontaneous fertility rate than uninfected women [12-14]. Recently, we reported the same observation when these women underwent *in vitro* fertilization (IVF) with their own oocytes. Furthermore, no significant reduction in the pregnancy rate was found when healthy donated oocytes were used. These results suggest that the reduced pregnancy rate observed among HIV-infected women on HAART could be attributed to the oocyte [15]. Although the underlying mechanism of this decreased fertility is unknown, it has been shown that sufficient energy production from mitochondria seems to be relevant in oocytes viability and in the development of embryos, so any mitochondrial defect in oocytes could eventually lead to cell dysfunction and infertility. In this sense, it is especially noteworthy the fact that *a priori*, the toxic effects of NRTIs can affect any cell containing mitochondria, since they have not been specifically designed to enter a particular cellular type, and that oocytes are post-mitotic cells, with no ability to remove damaged mtDNA. We hypothesize that mtDNA content is depleted in oocytes of infertile HIV-infected women under HAART treatment.

PATIENTS AND METHODS

A total of 8 HIV-infected women all under HAART and 14 controls (non-infected women) undergoing *in vitro* fertilization (IVF) for infertility treatment at Clinica Eugin' matched by age and IVF indication were included. All women provided informed consent to participate in the study. Duration of, number of previous ART-cycles, and total dose of FSH for ovarian hyperstimulation were similar in both groups.

A total of 16 oocytes non-suitable from 8 HIV-infected women and 19 non suitable oocytes from 14 HIV-negative women were obtained after ovarian hyperstimulation performed using recombinant FSH or human menopausal gonadotrophin and hypophysary suppression was obtained using either GnRH agonists or antagonists in a short-stimulation protocol. Oocytes were recovered 36 h after the administration of recombinant HCG.

During an IVF cycle, a proportion of the retrieved oocytes are immature, either at the metaphase-I (absence of both a germinal vesicle and a first polar body) or at the germinal vesicle

(GV) stage. On day 1 after sperm insemination a proportion of metaphase II (mature) oocytes are not fertilized (NF). All of them are defined as non-suitable for assisted reproduction and therefore, the study did not limit the likelihood of achieving a pregnancy.

All the women from both groups had viable embryos, which were transferred.

Human oocytes can only be assessed during an IVF cycle. Given that mature oocytes have to be used for fertility purposes, the assessment of non-suitable oocytes and non-fertilized oocytes from the same cycle from both infected and uninfected women is the best model available, although it may have some limitations. Nevertheless, it is currently the only ethically acceptable approach.

The mean age of women included in the study was 362 years in the HIV group and 37,44 in the control group. At the time of the IVF cycle, all the HIV-infected women had undetectable HIV viral load and a good immunological status. The mean time after HIV diagnosis was more than 13 years (153±57 months). All patients were on HAART (mean duration of 101±58 months), which included NRTIS in all cases. These clinical and epidemiological characteristics of all the patients are summarized in Table 1.

Oocytes were obtained after a standard IVF cycle (hormonal-stimulated poliovulation and ultrasound guided oocyte retrieval) and treated with hyaluronidase to remove cumulus cells. Mature oocytes were inseminated by intracytoplasmic sperm injection (ICSI) and observation of non-fertilization was performed on day 1 (18-20 hours after ICSI). Selected oocytes were individually rinsed in 0.5 ml of PBS 1x (Dulbecco's Phosphate Buffered Saline Solution without Calcium and Magnesium Salts; Irvine-Acientific, USA) and placed into a DNAse/RNAse free cryotube which were frozen in liquid nitrogen.

DNA isolation and mtDNA quantification

Lysis of each individual oocyte was done in the appropiate buffer containing 125 µl of proteinase K solution (2 mg/ml proteinase K - Roche Diagnostics GmbH, Mannheim, Germany-, SDS 1% and 2 mM EDTA pH 8.0), 50 µl of SDS 10% and 750 µl of lysis solution (10 mM Tris-HCl pH 7.5, 400 mM NaCl, 2 mM EDTA pH 8.0) and incubated for 60 min at 55°C and for 10 min at 100°C. After lysis, we added to each sample 1 µl of pellet paint (Pellet Paint® Co-Precipitant, Novagen, Merck KgaA, Darmstadt, Germany) and 10 µg of glycogen (Roche Applied Science, Mannheim, Germany) as carriers. Total DNA from each sample was isolated by standard phenol-chloroform procedure adapted to microvolumes (1:1 phenol:chloroform:isoamyl alcohol 25:24:1, Sigma-Aldrich, Inc., Sant Louis, MO) and then precipitated with 0.1 volumes of 3 M sodium acetate pH 5.3 and 2.5 volumes of absolute ethanol previously kept at -20°C. The pellet of DNA was dissolved in 40 µl of TE 10:1 buffer (Tris 10 mM / EDTA 1 mM; pH 7.5) and homogenized at 37°C for 2 hours. For each DNA extract, the highly conserved mitochondrial ND2 gene was quantified by quantitative real-time PCR (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals®, Germany) in duplicate using the sequences of the primers and the conditions of the PCR reaction previously reported [16]. The results for the mtDNA content were expressed as picograms (pg) of the

ND2 gene. The mtDNA content of controls was considered to be 100%.

Statistical analysis

Clinical and epidemiological characteristics of all women included in the study were expressed as mean ±SD (quantitative data) and percentages (qualitative data). One-way ANOVA was used to detect significant differences between groups. When ANOVA was significant and comparisons included three groups, the Bonferroni post-hoc analysis was used to uncover between which pair of groups the difference laid. Linear regression analysis was performed to establish any association among mtDNA content and cumulated doses of antiretrovirals or immunovirological parameters. In all cases, p values less than 0.05 were considered statistically significant.

RESULTS

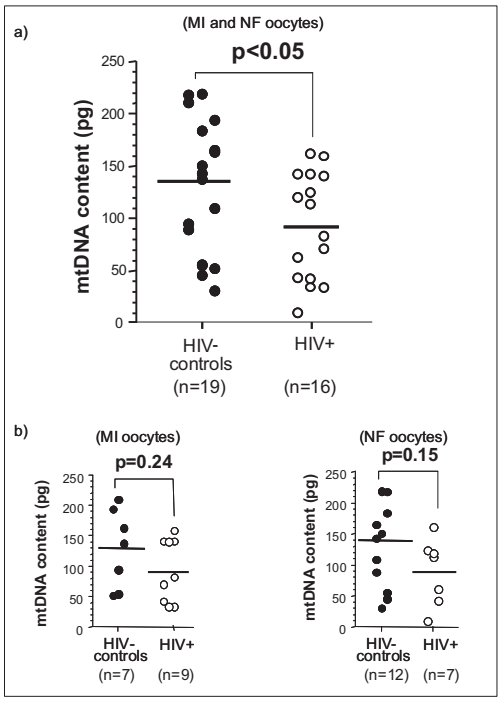
Clinical and epidemiological characteristics of HIV-infertile women and controls included in the study are detailed in table 1.

Table 1: Clinical and epidemiological characteristics of infertile women included in the study.

	Cases	Controls
Patients (n)	8	14
Age (years ±	36 ± 2	37 ±4
CD4 (cells/mm^3 ±SD)	781 ± 298	
Viral load <200 (%)	100	
Months (± SD) from HIV diagnosis	153 ± 57	
Months (± SD) on HAART	101 ± 58	
Months (± SD) on NRTIs	100 ± 59	
Patients on NRTIs at the time of IVF (%)	100	
Total oocytes analysed (n)	16	19

Infertile HIV-infected women on HAART had 32% lower oocyte mtDNA content than infertile controls (92±51 vs 136±71, respectively; p<0.05) (Figure 1-a). When mtDNA content was analyzed stratifying by the type of oocytes (MI or NF), differences between infected and uninfected women were no longer statistically significant (Figure 1-b). Nevertheless, as shown in Figure 1-b, a consistent trend persisted in the two types of oocytes of infected patients, with 27% depletion for MI oocytes and 36% depletion for NF oocytes when comparing with controls (94±52 vs 129±64 for MI oocytes; p=NS, and 90±53 vs 140±77 for NF oocytes; p=NS). Lack of statistical differences could be attributable to the small number of oocytes analyzed in each subgroup.

Figure 1: Oocyte mitochondrial DNA (mtDNA) content (expressed as picograms of the ND2 gene) for controls (uninfected women) and cases (HIV-infected women), a) when analysed both MI and NF oocytes all together, and b) when analysed according to the type of oocyte (MI or NF). (MI: metaphase I; NF: non-fertilized).

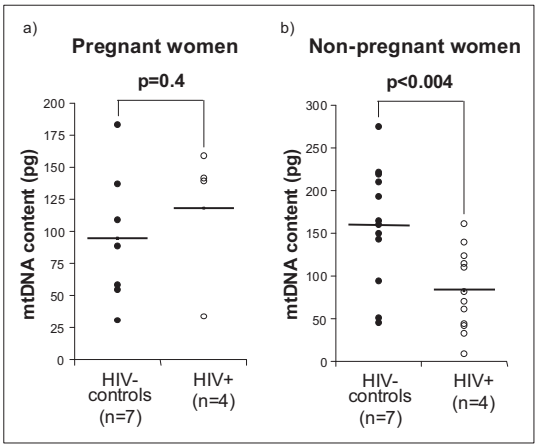


Mitochondrial DNA levels were evaluated according to pregnancy outcome of the same IVF cycle. Pregnancy was established if at least one intrauterine sac revealed by ultrasonography approximately 5 weeks after embryo transfer.

Pregnancy rate per cycle was 25% (4/16) among infertile HIV-infected women on HAART and 37% (7/19) in the control group. Among HIV-infected women, oocytes from a non-pregnancy (n=12) had 30% depletion of mtDNA content with respect to oocytes from a pregnancy cycle (n=4) (8348 vs 11957, respectively; p=0.237). When comparing with the control group (n=19; 100%), oocytes from a non-pregnancy (n=12) showed 39% decrease of the mtDNA levels (p=0.03), while oocytes from a pregnancy cycle (n=4) only showed 13% decrease of the mtDNA content (p=0.653). In addition, when comparing HIV-infected women oocytes from a non-pregnancy (n=12) and uninfected control oocytes from a non-pregnancy (n=12) the former showed 48% decrease of the mtDNA (8348 vs 16170, respectively; p=0.004) (Figure 2). However, no differences were found when comparing HIV+ and HIV- oocytes from a successful pregnancy (11957 vs 9453, respectively; p=NS)(Figure 2).

No significant correlation was found between oocyte mtDNA content and cumulated antiretroviral doses or the immunovirological status of HIV-patients (data not shown).

Figure 2: Oocyte mitochondrial DNA (mtDNA) content (expressed as picograms of the ND2 gene) for controls (uninfected women) and cases (HIV-infected women) according to the pregnancy outcome of the same *in vitro* fertilization (IVF) cycle: a) oocyte mtDNA content analysed in pregnant women, and b) oocyte mtDNA content analysed in non-pregnant women.



DISCUSSION

The oocyte is the largest human cell (300 times bigger in average than the rest of somatic cells) and contains large amount of mitochondria that represent at least the 23% of the ooplasm [17].

Mitochondria are double-membrane intracellular organelles and the main source of the high-energy phosphate molecule adenosine triphosphate, wich is essential for all active intracellular processes [18]. Oocytes are packed with mitochondria, and disorders of mitochondrial function may cause reproductive failure. Mitochondrial DNA copy number per mature human oocyte is about 100.000-600.000 molecules, compared with the 500-10.000 molecules for the rest of the cells [19 20]. Furthermore, the oocyte approximately contains a single mtDNA molecule per mitochondrion to avoid heteroplasmy segregate through the maternal lineage [21], and differs from the 2-10 mtDNA copies for the rest of human somatic mitochondria [17]. This fact makes oocyte mitochondria especially vulnerable to mtDNA depletion and oocyte especially sensitive to mtDNA depleting factors (drugs, toxins, infections, etc.).

Mitochondrial dysfunction has been associated with reproductive outcome since their functionalism influences the viability of both sperm and oocytes. Accordingly, low mtDNA content in both male [22] and female gametes [17, 23, 24] has been associated with infertility. In addition, mutations in the mtDNA genome have been also described in spermatozoa with declined motility and fertility [25]. Other investigations relate the generation of abnormal sperm mtDNA molecules (with multiple mtDNA deletions) to long-term antiretrovirals intake in HIV-infected patients [26]. Moreover, it has been suggested that HIV infection and NRTIs have negative effects on semen parameters (semen volume, percentage of progressive motile spermatozoa, total sperm count, polynuclear cell count, pH, spermatozoa anomalies), compromising male fertillity.

In the oocyte, mitochondria contribute to fertilization and embryonic development. Cohen et al. reported that ooplasm transfer (including mitochondria) from a young donor oocyte partially restores the reproductive capacity in oopausic oocytes [27]. Both Reynier et al. [23] and Santos et al. [17] established an association between the mtDNA content and the probability of oocyte fertilization. The latest suggested that the mtDNA content could be an oocyte quality and fertility marker. Another study suggests that low mtDNA content is associated with the impaired oocyte quality observed in ovarian insufficiency [24]. These studies suggest that mitochondria are critical to fertilization outcome and embryonic development [27]. Nevertheless, there are no published studies assessing mtDNA levels in oocytes of HIV-infected women.

Epidemiological and clinical data suggest that HIV-infected women have a lower spontaneous pregnancy rate. We recently described that infertile HIV-infected women on HAART undergoing *in vitro* fertilization (IVF) had lower pregnancy than uninfected women (16.2% vs 39.2%). However, the effect of HIV infection was not observed in women undergoing oocyte donation (36% vs 45.1%).

This is the first study that addresses the underlying mechanism that could explain a low pregnancy rate in HIV-infected women. Our data suggest that oocytes from infertile HIV-infected women on HAART have decreased mtDNA levels compared with infertile uninfected controls.

Two possible mechanisms could explain the mtDNA depletion found in our study. One of them is related to the secondary effects of antiretrovirals and oocyte characteristics. HAART combinations against HIV, specifically those containing NRTIs, may cause mitochondrial toxicity. *A priori*, the toxic effects of NRTIs can affect any cell containing mitochondria, since they have not been specifically designed to enter a particular cellular type. Therefore, oocytes may be exposed to cumulated therapeutic doses of these drugs. In addition, oocytes are post-mitotic cells with no ability to eliminate damaged mitochondria and with high dependence on the oxidative phosphorilation system. Moreover, oocytes contain large number of mitochondria with only one molecule of mtDNA per organelle, so they are more sensitive to mtDNA depleting factors. In this scenario, oocytes would be especially prone to decrease the mtDNA levels. On the other hand, HIV may induce unspecific mitochondrial damage in other tissues. HIV may cause disruption of the mitochondria-mediated apoptotic mechanisms and thus, may indirectly deplete oocyte mtDNA levels [28]. We did not find an association between mtDNA content and the length of antiretroviral therapy and immunovirological status of patients and, therefore, we cannot further demonstrate the contribution of each mechanism. However, we believe that HIV should not have a direct impact on the human oocyte because no receptors for HIV have been described on either the cumulus cells or on the surface of the oocyte.

According to the above mentioned, the most plausible hypothesis is that the underlying mechanism that cause oocyte mtDNA depletion and reduced fertility among HIV-infected women on HAART is the antiretroviral therapy. However, we can not give a firm conclusion, since all HIV-infected women included in our study were on HAART. In this context, it is

currently not possible to discriminate whether these results are attributable to HIV-infection or to HAART. No data are available on mtDNA content of oocytes from HIV-infected non-HAART-treated women.

Interestingly, among HIV-infected women, mtDNA content in the non-viable oocytes from women that did not become pregnant was lower than among women who achieved a pregnancy, although differences where not significant (83448 vs 119557, respectively; p=0.237). However, this mtDNA depletion was significant when comparing HIV-infected women that did not become pregnant with uninfected controls (39% depletion; p=0.03). Lack of statistically significance for the first comparison could be attributed to the small size of patients in the subgroups analysed. These results could explain the low pregnancy rate after IVF observed among HIV-infected women.

Human oocytes can only be assessed during an IVF cycle. Given that mature oocytes will be used for fertility purposes, the assessment of non-viable oocytes and non-fertilized oocytes from the same cycle from both infected and uninfected women is the best model available.

According to our results, the lower fertility rate observed in IVF cycles among HIV-infected women under antiretroviral therapy may be explained by oocyte mitochondrial impairment secondary to mitochondrial toxicity of HAART. However, we can not rule out the potential effects that HIV could indirectly have on fertilization outcome and on mtDNA content through other unknown biological causes. Further studies are required to confirm these results.

ACKNOWLEDGEMENTS

We are indebted to all women who have participated in the study. Supported by grants from Fundació La Marató de TV3 (020210 and 020631)', Fondo de Investigaciones Sanitarias (FIS 40381/04)', Suport a Grups de Recerca de la Generalitat de Catalunya (2005/SGR/0300)' and CIBER de Enfermedades Raras (CIBERER). The 'CIBER de Enfermedades Raras' is an initiative of the Instituto de Salud Carlos III' (ISCIII).

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MILD IMPROVEMENT OF MITOCHONDRIAL FUNCTION AFTER THREE YEARS OF ANTIRETROVIRAL TREATMENT INTERRUPTION IN SPITE OF MITOCHONDRIAL DNA IMPAIRMENT

JOURNAL OF ANTIMICROBIAL CHEMOTHERAPY (Enviat)

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MILD IMPROVEMENT OF MITOCHONDRIAL FUNCTION AFTER 3 YEARS OF ANTIRETROVIRAL TREATMENT INTERRUPTION IN SPITE OF MITOCHONDRIAL DNA IMPAIRMENT

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Objective: To evaluate whether a prolonged antiretroviral treatment interruption could reverse mitochondrial toxicity, a prospectively defined sub-study of the TIBET study (a CD4 cell-guided antiretroviral treatment interruption trial) was performed.

Patients and Methods: We included those patients from the TIBET study who were followed for ≥96 weeks and whose peripheral blood mononuclear cells (PBMCs) had been collected at baseline and throughout the study period. Out of the total number of 201 patients included in the TIBET study, 38 patients were selected for the mitochondrial sub-study; 18 patients had discontinued antiretroviral therapy for ≥96 weeks and 20 had maintained therapy. Mitochondrial DNA (mtDNA) was measured in PBMCs by real-time polymerase chain reaction and mitochondrial function was estimated through a cytochrome c oxidase and citrate synthase (COX/CS) ratio.

Results: Whereas mtDNA content showed a similar progressive decrease throughout the study period in both study arms, the COX/CS ratio significantly improved in patients who interrupted antiretroviral therapy in comparison with those who did not. The univariate and multivariate analysis performed showed that only CD4+ T-cell value at the time of initiation of antiretroviral therapy and time with viral suppression before the study were associated with the change of the COX/CS ratio.

Conclusions: Mitochondrial function improved during a prolonged antiretroviral treatment interruption in spite of a decrease in mtDNA levels in PBMCs. The absence of correlation between mitochondrial parameters suggests the existence of a mitochondrial transcriptional or translational upregulation mechanism or the reversion of a mitochondrial toxicity through a DNA polymerase gamma-independent way.

INTRODUCTION

It is well-known that the use of more intensive antiretroviral therapies in HIV-infected patients has allowed to reduce substantially the morbidity and mortality rates of these patients in the previous

years [1]. Current guidelines for the management of HIV-infected patients recommend the combination of at least three agents of which usually two of them are nucleoside reverse transcriptase inhibitors (NRTIs), and the third one could be a protease inhibitor (PI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), or even another NRTI [2]. Despite of the positive effects of antiretroviral drugs on HIV infection, their long-term use have revealed the appearance of a wide variety of serious adverse effects, many of which have been directly related with the mitochondrial function in association with NRTIs [3]. In fact, the administration of nucleosides is probably the factor with higher detrimental effect on mitochondrial function, although other factors, such as the virus itself, can play also a complementary role [4].

Previous studies have showed that NRTIs affect the function of DNA polymerase beta and gamma among the 5 cellular enzymes that repair and/or replicate DNA. The effects of DNA polymerase beta on nuclear DNA (nDNA) reparation are redundant, but it is the effect on DNA polymerase gamma, the unique enzyme that replicates mitochondrial DNA (mtDNA), which lead to mtDNA depletion and/or mutation, insufficient energy generation and subsequent cellular dysfunction [3]. The clinical presentation of mitochondrial dysfunction generally occur after several months of NRTI treatment and could be very heterogeneous as it will depend on the organ that is specifically affected [5]. The most dramatic clinical presentation of mitochondrial toxicity is acute lactic acidosis with hepatic failure. The estimated incidence is around 1.3 per 1000 person-years and is fatal in most cases [6]. Other clinical presentations of mitochondrial toxicities could include lipoatrophy, peripheral neuropathy, myopathy, nephrotoxicity and pancreatitis, although the incidences reported in literature vary widely [7].

Some options to manage mitochondrial toxicity and its clinical manifestations have already been proposed. Supplements such as acetyl-carnitine, riboflavin, thiamine and coenzyme-Q are able to give some benefit in these situations [8-11]. Other options include the substitution of the probable causative NRTIs by an alternative less toxic nucleoside analogue [12-15] or NRTI-sparing approaches in which a PI is combined with a NNRTI or monotherapies with boosted PIs [16], as well as the uridine supplementation [17].

Finally, the complete interruption of the antiretroviral regimen may be even a better strategy to resolve, or at least to minimize, not only mitochondrial toxicity but also other antiretroviral-related toxicities. This is especially true in the case of PI- or NNRTI-associated adverse events, such as gastrointestinal disorders, hypersensitivities, hepatotoxicity or even lipid abnormalities. However, controversial data has been published regarding the reversibility of mitochondrial-related toxicity during antiretroviral treatment interruptions among HIV-infected patients [18, 19].

In order to address these concerns, we performed a prospectively defined sub-study of the TIBET study [20], a CD4 cell-guided antiretroviral treatment interruption trial, to evaluate in chronically HIV-1 infected patients the effect of prolonged antiretroviral therapy interruptions on mitochondrial parameters.

METHODS

Study design

This was a prospectively defined mitochondrial sub-study of the TIBET study [20], a trial designed to investigate the impact of CD4-guided treatment interruption on clinical, virological and immunological parameters.

The TIBET study was a randomized, open-label trial that was performed from May 2001 to January

2002 in five hospitals of Spain and Italy. Participants were randomized to interrupt all drugs simultaneously (CD4 guided-treatment interruption [GTI] arm) or to continue the current antiretroviral treatment (control arm). Patients allocated to the GTI arm restarted antirretroviral treatment if any of the following situations were observed: an AIDS-defining illness, a severe or prolonged acute retroviral syndrome occurred, CD4 cell counts ≤ 350/μl and/or plasma HIV-RNA level ≥ 5.0 $\log_{10}$ copies/ml.

The study was approved by the Institutional Review Board of each participating site and all patients provided their written inform consent.

Objectives

The primary objective of this sub-study was to evaluate the effect of prolonged antiretroviral therapy interruption on mitochondrial parameters in chronically HIV-1 infected patients.

Secondary objectives included to identify those factors which may have an impact on mitochondrial function and to assess whether clinical prospective and retrospective covariates had any effect on mtDNA and mitochondrial function.

Selection of patients

Apart from the selection criteria defined in the TIBET study (i.e. basically being on intensive antiretroviral therapies for more than one year without interruptions, absolute CD4 cell counts ≥ 500/μl during at least six months and never below 50/μl, HIV-RNA < 400 copies/ml during at least one years and < 50 copies/ml at study entry, and no previous opportunistic infections), patients had to stay in the TIBET study for at least 96 weeks and had to have peripheral blood mononuclear cells (PBMC) stored throughout the study period to participate in the mitochondrial sub-study.

Study procedures

In the mitochondrial sub-study, biochemical data was determined in PBMCs stored at baseline, at week 96 and at week 144. Briefly, 10-20 ml of whole blood was collected in ethylenediaminetetraacetic acid-Vacutainer tubes (BD, Madrid, Spain) and processed immediately after collection for plasma and PBMC isolation. Plasma was recovered after centrifugation at 1200x g for 10 min and immediately cryopreserved and stored at -80ºC until used. PBMC were obtained by separation on Ficoll-Hypaque (Atom Reactiva, Barcelona, Spain) density gradient and cryopreserved in a mixture of DMSO/FCS (10:90) in liquid nitrogen for further determinations.

Mitochondrial DNA analysis

Total DNA was obtained by a standard phenol-chloroform extraction procedure from an aliquot of PBMCs and used for genetic studies. Quantification of mitochondrial NADH dehydrogenase subunit 2 (ND2) and nuclear ribosomal RNA 18S reference genes was performed in a similar strategy as previously described [21]. Amplification and detection for each gene were carried out in separated reactions using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Primer and probe sequences were ND2-F (5'-CATCTTTGCAGGCA-CACTCATC), ND2-R (5'-TGGTTAGAACTGGA-ATAAAAGCTAGCA), ND2-P (5'-AGCGCTA-AGCTCGCACTGATTTTTTACCTGA) for the mitochondrial gene and 18S-F (5'-AGTGGAGCC-TGCGGCTTAAT), 18S-R (5'- ACCCACGGAAT-CGAGAAAGAG), 18S-P (5'-CCGGACACGGA-CAGGATTGACAGAT) for the nuclear gene.

Before analysing GTI and control subjects, amplification with optimised PCR conditions of the two genes was performed with a 6-fold serial DNA dilution to measure the PCR efficiency for both genes. Following these considerations, DNA of GTI and control groups was analysed in triplicates and a DNA reference sample was always included in each

performed PCR throughout the study to assess the inter-experiment variation.

As the efficiency value remained similar for mtDNA and nuclear genes when analysing both amplification curves generated from DNA dilutions, the use of the comparative CT method ($2^{-\Delta\Delta CT}$) was allowed for ratio calculation and comparisons between groups [22].

Citrate Synthase (CS) and Cytochrome C Oxidase (COX) enzymatic activity

The quantity of mitochondria was assessed by the spectrophotometric measurement of CS activity (EC: 4.1.3.7), a mitochondrial matrix enzyme of the Krebs' cycle, which remains highly constant in mitochondria and is considered to be a reliable marker of mitochondrial content (expressed as nmol/min/mg protein) [23-26]. COX, also known as mitochondrial complex IV, is an essential enzyme in the mitochondrial respiratory chain. COX activity was measured spectrophotometrically (EC 1.9.3.1) according to the Rustin et al methodology [25], slightly modified for minute amounts of biological samples [27], and expressed in absolute values (nmol/min/mg protein), as well as in relative values per mitochondria by dividing absolute COX values by mitochondrial mass (CS activity), COX/CS ratio.

Statistical analysis

Statistical summaries for the main retrospective and baseline variables before antiretroviral interruption, as well as a pictorial and tabular description of mitochondrial function (COX activity) and mtDNA measurements were prepared. The statistical significance of the longitudinal changes and group differences in mitochondrial function and mtDNA measurements were assessed by means of Wilcoxon, Mann-Whitney, Chi-square or Fisher's exact tests for all the patients and distinguishing between study arms. A linear mixed model with a random intercept was used for each patient to study the relationship between the COX/CS ratio and the plasma viral load, absolute $CD4^+$ T-cell counts and the retrospective and baseline variables while off-treatment. The equation for the suggested model indicated that the average value of the COX/CS for a given patient "*i*" at time "*t*" was linearly related to the considered covariates [$COX/CS_i(t)$= (Intercept + b_i) +α Covariate i + βt + $\epsilon_i(t)$], where the random effect b_i identified individual characteristics, and in particular showed how the specific patient differed from the average subject with respect to course of disease. It was assumed that b_i followed a multivariate normal distribution centred at zero and with unstructured variance-covariance for model flexibility. The variable *t* was expressed in weeks and only took values 0, 96 and 144; $\varepsilon_i(t)$ was the within-patient measurement error and was assumed to follow a multivariate normal distribution centred at zero. An F-statistic was used to test the hypotheses about the fixed α and β effects. The model specification was validated by the Akaike Information Criterium (AIC). A model adding a quadratic term of t was also considered.

The data consisted of all the COX/CS measurements from the moment that a patient interrupted antiretroviral therapy until therapy was restarted. For each patient at least two and at the most three measurements were available.

Statistical analyses were performed with the use of SPSS software (version 11.5, SPSS, Inc., Chicago, Illinois, USA) and S-PLUS 2000 (version 2000 Professional Release 3, SPSS, Inc., Chicago, Illinois, USA).

RESULTS

Baseline characteristics

From 201 chronically HIV-1 infected patients included in the TIBET study, 38 (19%) stayed at least 96 weeks and had PBMC stored throughout the study period; so they were selected to participate in the mitochondrial sub-study. Of them, 18 patients belonged to the GTI arm and 20 patients to the control arm. In fact, 30 out of 38 patients (79%) remained in the study until week 144 (17 in the GTI arm and 13 in control arm). Baseline characteristics of these patients are described in Table 1. No differences in baseline epidemiological or HIV-related parameters were observed between both study arms.

Table 1. Baseline epidemiological and HIV-related characteristics of patients (N = 38)

Characteristic	Control arm	GTI arm	*P value*
	n = 20	n = 18	
Age, years	38 (32; 42)	38 (34; 41)	0.704
Male, n (%)	13 (65)	14 (78)	0.264
Route of HIV transmission, n (%)			
Men's sex with men	10 (50)	11 (61)	
Injecting drug users	6 (30)	4 (22)	0.771
Heterosexual sex	4 (20)	3 (17)	
Time with HIV infection, months	87.8 (50.6; 110.7)	66.9 (38.5; 90.0)	0.143
Time on ART, months	47.7 (41.6; 55.9)	46.3 (40.3; 56.0)	0.264
Type of previous ART, n (%)			
ddI	5 (25)	3 (17)	0.380
d4T	11 (55)	11 (61)	0.552
Baseline CD4 cell count, percentage	41 (33; 48)	32 (29; 40)	0.245
Time on VL<50 copies/ml, months	29.4 (25.7; 41.5)	31.5 (17.2; 37.9)	0.413

GTI: Guided-treatment interruption arm; IQR: Interquartile range; ART: Antiretroviral treatment; VL: Viral load

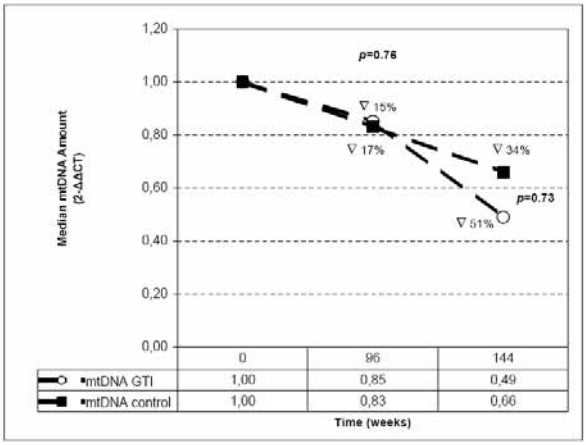
All data has been expressed as median and IQR except when was a percentage

Mitochondrial DNA analysis (2-ΔΔCT)

MtDNA content showed a progressive decrease when each study arm was independently analysed throughout the follow-up period (Figure 1). From baseline to week 96, both study arms showed a similar decay in mtDNA (15% in GTI arm and 17% in control arm; p=0.76 between both arms). The median value of mtDNA [interquartile range] in GTI arm decreased from 1.00 [1.00-1.00, reference value] at baseline to 0.85 [0.59-1.01] at week 96 (p=0.10). In the control arm, mtDNA decreased to 0.83 [0.56-1.07] at week 96 (p=0.03) (Figure 1).

Surprisingly, the percentage of change from baseline to week 144 seemed to be higher in the GTI arm (51%) than in the control arm (34%, p=0.73 between both study arms). Median values decreased from 1.00 [1.00-1.00, reference value] to 0.49 [0.21-1.26] (p=0.31) in the GTI arm and to 0.66 [0.33-0.79] (p=0.01) in the control arm (Figure 1).

Figure 1. Analysis of mitochondrial DNA (mtDNA, 2-ΔΔCT).



Mitochondrial function analysis

No statistically significant differences between GTI and control arm were observed in the baseline mitochondrial function (median [interquartile range] COX/CS ratio) (0.31 [0.23-0.66] versus 0.56 [0.33-0.68] respectively; p=0.18).

Throughout the follow-up period, the COX/CS ratio did not vary in the control arm. Considering the baseline value as 1 (100%), the percentage of change was 2% at week 96 and 2% at week 144 with respect baseline values (p=0.71 and p=0.11 respectively) (Figure 2).

In contrast, we observed a significant increase in the COX/CS ratio in the GTI arm at week 96 (130%, p=0.28) and at week 144 (80%, p=0.50) from baseline (Figure 2).

No statistically significant differences were observed in the change of COX/CS ratio between both arms at week 144 (p=0.42), although a trend was observed at week 96 (p=0.06).

Modelling analysis

Previous modelling, the time effect on the COX/CS ratio was assessed. Different baseline factors were included in the fixed part of the model. The univariate approach revealed that CD4+ T-cell percentage at baseline and the time with undetectable viral load previous to study entry affected the expected mean value of COX/CS ratio. Higher baseline CD4+ T-lymphocyte values and longer time with undetectable viral load previous to study entry were associated with greater improvement of COX/CS ratio.

The multivariate model was constructed including all the covariates that reached a p value of less than 0.10 in the univariate model. The multivariate model showed that both, the percentage of CD4+ T-cell count at the time of beginning antiretroviral therapy and the time with viral suppression before treatment interruption were associated with the change of the COX/CS ratio. Complete data of these models is shown in Table 2.

According to this data, the expected value of mitochondria function marker for a patient who was 32 months with undetectable viral load and who had 32% of CD4+ T-cells previous to antiretroviral therapy would be 0.477 units. The effect of 96 weeks without treatment would represent an increase of 0.144 units.

The mean number of COX/CS units increased by 0.2 with each increase of 10% CD4 T-lymphocytes previous to antiretroviral therapy. The effect of being six additional months with undetectable viral load before treatment interruption increased the expected value of COX/CS by 0.1 units. Furthermore, every six months without treatment, the COX/CS ratio increased 0.04 units.

Figure 2. Changes in mitochondrial function (Delta COX/CS ratio)

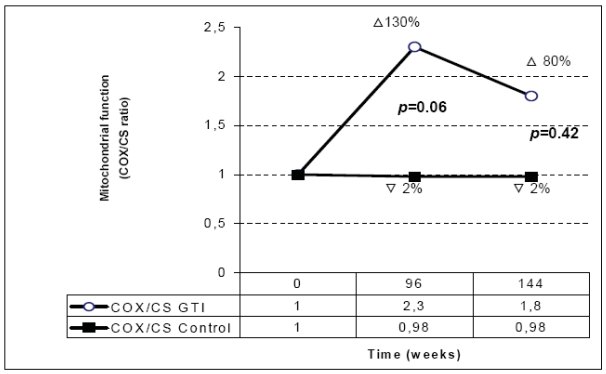


Table 2. Multivariate model for COX/CS dynamics during HIV treatment discontinuation.

Covariates	Coefficient	SE (Coefficient)	*p value*
Intercept	-0,5444	0,3246	0,10
Week	0,0015	0,0010	0,13
CD4 percentage Pre-HAART	0,0169	0,0078	0,05
Days undetectable VL	0,0005	0,0002	0,01

$b_0 \sim N(0, 0.000005)$; $e_{ij} \sim N(0, 0.384539)$

Discussion

We have prospectively evaluated the effect of a prolonged antiretroviral therapy interruption on mitochondrial parameters such as mtDNA content and mitochondrial function (COX/CS ratio) in chronically HIV-1 infected patients. Our results indicate that, whereas mtDNA content showed a similar progressive decrease throughout the study period in both study arms, the mitochondrial function significantly improved only in the GTI arm in comparison with the control arm.

Currently, there is an important concern regarding which is the optimal method to diagnose and evaluate nucleoside-induced mitochondrial toxicity. Although many authors recognize that the gold standard would be to perform a liver or a muscle biopsy [18, 19], other investigators have tested the suitability of adipose tissue to clearly show changes in mtDNA or mtRNA [28-31]. However, none of

those methods seems to be practical for routine screening and follow-up. It has been speculated that the quantification of mtDNA in PBMCs [18, 19, 32], could be a reliable predictor of mitochondrial toxicity. Cote el al concluded that mtDNA levels in PBMCs were significantly decreased in patients with symptomatic, nucleoside-related hyperlactatemia and that this effect could be reversed after antiretroviral therapy discontinuation [18]. Mussini et al showed in a platelet-free highly purified lymphocyte population that mtDNA content increased in CD8+ T lymphocytes after six months of treatment interruption, although it did not changed in CD4+ T lymphocytes [32]. Nonetheless, other authors have found opposite results. In a prospective evaluation of mtDNA in PBMCs as a marker of toxicity in 157 consecutively recruited HIV-positive patients, a similar decrease of mtDNA was observed in both, naïve and antiretroviral treated patients [19]. These findings suggest a direct mitochondrial toxicity of the virus itself, but they did not find any link between the mtDNA/nDNA ratio in PBMCs and any clinical symptoms or lactate level. Consequently, some authors have indicated the necessity of analysing the mitochondrial function, together with the quantification of mtDNA, in order to achieve a more deep knowledge of mitochondrial damage and changes [13]. This should be also specially useful considering that mitochondrial toxicity of antiretroviral drugs has been demonstrated in the absence of mtDNA depletion [31, 33-36]. In the present work, the study of quantification of mtDNA content has been completed with the enzymatic analysis of mitochondrial function in order to obtain a more deep and reliable vision of the real mitochondrial status .

Based on our data, we observed a similar progressive decrease of mtDNA content in both study arms. An explanation for these findings would be the role of HIV itself on mitochondrial damage, i.e. the re-emergence of HIV in all of these patients who interrupted therapy after a long-term viral suppression. In fact, the in vitro and in vivo association between HIV itself and mitochondrial damage has already been described [4, 18, 37]. Unfortunately, we were unable to demonstrate in our study the influence of HIV itself on mitochondrial parameters. This was due to the low viral replication rate observed along with the fact that none of the participants in the GTI arm needed to reinitiate treatment during the follow-up period of the study according to the protocol guidelines.

Nonetheless, we observed a significant recovery of mitochondrial function after two or three years of antiretroviral treatment interruption in comparison with the control arm, although no statistical differences were observed between both arms, probably due to the wide range of COX/CS ratio values.

The absence of correlation between mitochondrial parameters such as mtDNA and mitochondrial function can be explained, in our opinion, at least by two different hypothesis. One could be the existence of an upregulatory mechanism, in which an increase of the mitochondrial transcriptional (that synthesise RNA from DNA) or of the post-transcriptional (which translates RNA into proteins) rates could compensate the acute mtDNA depletion after antiretroviral therapy interruption and virus re-emergence. This mechanism has been suggested before in the context of antiretroviral-mediated damage [36]. The other possibility could be that the antiretroviral treatment interruption favours the reversibility of a previously antiretroviral-induced mitochondrial damage, independently of DNA polymerase gamma function, that would have directly affected mitochondrial function. Then, treatment interruption would improve mitochondrial function without being able to revert

those mitochondrial lesions which depend on DNA polymerase activity. Recently, previous reports have found improvements in all functional mitochondrial parameters except for mtDNA content when high-mitochondrial-toxic antiretroviral therapy was reduced [13, 38]. These data suggest the existence of alternative mitochondrial toxicity mechanisms to DNA polymerase gamma inhibition, which could respond independently to mtDNA content. Conversely, in the control arm, the additive mitochondrial toxicity of the continued antiretroviral therapy during the study period would explain the more stable loss of mtDNA content throughout the 3 years of follow-up and the lack of a homeostatic mechanism that compensates mtDNA depletion.

Apart from that, the modelling analysis of our study showed that only the value of CD4+ T-cell count at the time of beginning antiretroviral therapy and time with undetectable viral load before the study could influence in some way the COX/CS ratio at baseline; those patients who presented higher CD4 values and had registered longer time with viral suppression had a higher COX/CS ratio. As well, the time without therapy increased the mean value of the ratio throughout the study. No other epidemiological factors nor HIV-related factors were associated with the change of the mitochondrial function.

The design of the study did not contemplate the possibility of recording clinical changes in fat distribution or other mitochondrial-related toxicities. Therefore, the association between the variation of mitochondrial parameters and more relevant clinical changes remain to be elucidated. Indeed, we should have evaluated the real benefits obtained with the prolonged antiretroviral therapy interruption since they could not compensate the risks associated with the deleterious virological and immunological outcome along with the quicker clinical progression described recently [39].

In summary, two main conclusions may be obtained from this study. The first one is that the mitochondrial function is improved during a prolonged antiretroviral treatment interruption in spite of the progressive decrease of mtDNA content in PBMCs, probably related to the re-emergence of the virus. The second conclusion, which is derived from the first, is to highlight the real usefulness of mtDNA quantification in PBMCs if there is not a certain correlation with mitochondrial function. The existence of mitochondrial homeostatic mechanisms to preserve mitochondrial function albeit genetic disturbances or alternative antiretroviral toxicity mechanisms to DNA polymerase gamma inhibition could explain the lack of correlation. Other questions like which is the best strategy to achieve a recovery of mitochondrial toxicity, whether this recovery could be total or partial, or the real influence of mitochondrial changes in more relevant clinical features like fat distribution remain to be answered.

Acknowledgment

This work was partially supported by the Spanish AIDS network “Red Temática Cooperativa de Investigación en SIDA” (RD06/0006) and CIBERER (Barcelona, Spain).

Transparency declaration

The authors do not have any financial conflicts of interest and the founders have not played any decision-making role in the research.

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Capítol: **'MITOCONDRIA Y ANTIRETROVIRALES'**
Autors: **G Garrabou**, Ò Miró

Llibre: **'INFECCIÓN POR VIH. TRATAMIENTO ANTIRETROVIRAL Y MITOCONDRIA'**

Mitocondria y antirretrovirales

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LOS FÁRMACOS antirretrovirales aprobados para su uso clínico en la actualidad pertenecen a 4 grandes familias en función de su naturaleza y la etapa del ciclo viral que bloqueen para frenar el avance de la infección del VIH: los inhibidores de la transcriptasa inversa del virus, que pueden ser análogos o no de nucleósido o nucleótido, los inhibidores de la proteasa del VIH y los inhibidores de la fusión del virus con la célula huésped. (**Tabla 1**).

Actualmente se están desarrollando nuevos fármacos, algunos de los cuáles se encuentran en avanzadas fases de investigación, que pertenecen a nuevas familias antirretrovirales: son los inhibidores de la integrasa del VIH, los inhibidores de la entrada (o inhibidores del correceptor CCR5) o los inhibidores de la maduración del virus, entre otros. (**Figura 1**).

TABLA 1.
Fármacos antirretrovirales comercializados en septiembre de 2007

Familia	Nombre genérico	Nombre abreviado	Nombre comercial	Fecha aprobación FDA
ITIAN	Zidovudina	AZT o ZDV	Retrovir	Marzo 1987
	Didanosina	ddI	Videx	Octubre 1991
	Zalcitabina	ddC	Hivid	Junio 1992
	Estavudina	d4T	Zerit	Junio 1994
	Lamivudina	3TC	Epivir	Noviembre 1995
	Abacavir sulfato	ABC	Ziagen	Diciembre 1998
	Tenofovir Diproxil Fumarato	TDF	Viread	Octubre 2001
	Emtricitabina	FTC 3TC+AZT ABC+3TC ABC+3TC+AZT FTC+TDF	Emtriva Combivir Kivexa Trizivir Truvada	Julio 2003 Septiembre 1997 Diciembre 1998 Noviembre 2000 Agosto 2004
ITINAN	Nevirapina	NVP	Viramune	Junio 1996
	Delavirdina mesilato	DLV	Rescriptor	Abril 1997
	Efavirenz	EFV	Sustiva	Septiembre 1998
IP	Saquinavir mesilato	SQV	Invirase	Diciembre 1995
	Indinavir sulfato	IDV	Crixivan	Marzo 1996
	Ritonavir	RTV	Norvir	Marzo 1996
	Nelfinavir	NFV	Viracept	Marzo 1997
	Saquinavir	SQV	Fortovase	Noviembre 1997
	Amprenavir	APV	Agenerase	Abril 1999
	Lopinavir+Ritonavir	LPV+RTV	Kaletra	Septiembre 2000
	Atazanavir	ATA	Reyataz	Junio 2003
	Fosamprenavir	FPV	Telzir	Octubre 2003
	Tipranavir	TPV	Aptivus	Junio 2005
	Darunavir	TMC-114	Prezista	Junio 2006
IF	Enfuvirtide	T20	Fuzeon	Marzo 2003

ITIAN: Inhibidores de la transcriptasa inversa análogos de nucleósido o nucleótido; ITINAN: Inhibidores de la transcriptasa inversa no análogos de nucleósido o nucleótido; IP: Inhibidores de la proteasa; IF: Inhibidores de la fusión; *FDA*: *Food and Drug Administration* (Agencia estadounidense de la Alimentación y el Medicamento).

Etapas del ciclo del VIH y los distintos fármacos antirretrovirales según la etapa del ciclo viral que bloquean

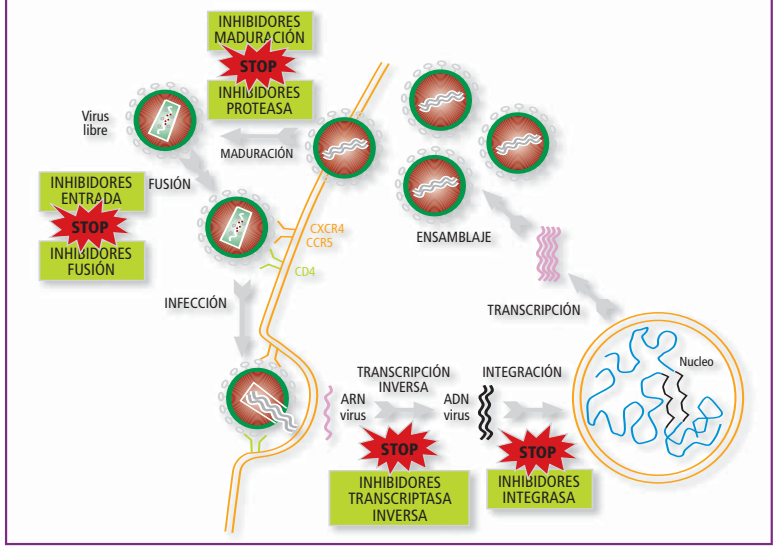

FIGURA **1.**
CD4, CCR5 y CXCR4: receptor (CD4) y correceptores (CCR5 y CXCR4) de la célula huésped que reconoce el VIH; ARN: Ácido ribonucleico; ADN: Ácido desoxirribonucleico.

Este amplio arsenal terapéutico ha permitido, desde 1996, tratar la infección por VIH utilizando combinaciones de 3 de estos fármacos en el denominado tratamiento antirretroviral de gran actividad (TARGA). El TARGA ha substituido la monoterapia y ha demostrado ser mucho más eficaz para anular la carga viral del paciente de manera prolongada y minimizar así la aparición de resistencias. El TARGA ha aumentado la esperanza y calidad de vida de los infectados por VIH que tienen acceso al tratamiento, pero la toxicidad mitocondrial de sus componentes se propone como la principal responsable de la aparición de gran parte de los efectos adversos asociados a la terapia (lipodistrofia, hiperlactatemia, acidosis láctica, miopatía, polineuropatía o pancreatitis)[1,2]. Además, este efecto mitotóxico podría verse incrementado por la propia infección por VIH ya que, como se ha discutido en el capítulo anterior, el propio virus podría estar alterando algunos parámetros mitocondriales, contribuyendo así a la aparición de los efectos secundarios que se manifiestan durante el tratamiento[3,4]. En este capítulo se discutirán las principales alteraciones mitocondriales que causan los antirretrovirales de las familias que se han comercializado hasta la fecha.

Inhibidores de la transcriptasa inversa análogos de los nucleósidos/nucleótidos

La transcriptasa inversa o transcriptasa reversa es una enzima presente en el VIH (y otros muchos retrovirus) para retrotranscribir el ARN de cadena simple del virus a ADN proviral de cadena doble, que puede insertarse en el material genético de la célula huésped y permanecer allí de forma latente mucho tiempo, hasta que se activa la formación de nuevas partículas virales. Los fármacos que inhiben esta enzima han demostrado tener una gran eficacia antirretroviral. Distinguimos dos tipos de inhibidores de la transcriptasa inversa del VIH, según su naturaleza y el mecanismo de acción: los análogos y los no análogos de nucleósido o nucleótido.

Los inhibidores de la transcriptasa inversa análogos de nucleósido o nucleótido (ITIAN) son compuestos sintéticos muy parecidos a los nucleósidos o nucleótidos naturales (salvajes), pero a diferencia de éstos,

Bloqueo de la retrotranscripción del ácido ribonucleico del VIH a ácido desoxirribonucleico proviral por parte de los inhibidores de la transcriptasa inversa análogos de nucleósido o nucleótido

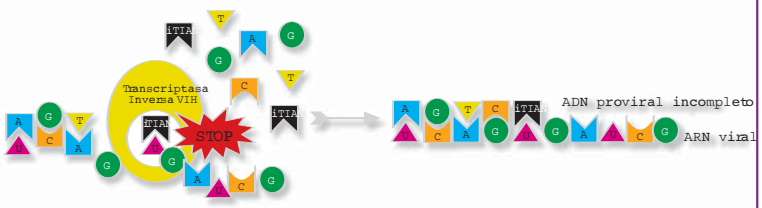

FIGURA **2.**
ADN: Ácido desoxirribonucleico; ARN: Ácido ribonucleico; ITIAN: Inhibidores de la transcriptasa inversa análogos de nucleósido o nucleótido; Deoxinucleótidos (nucleótidos que forman parte del ADN): A (Adenina), G (Guanina), T (Timina), C (Citosina); Ribonucleótidos (nucleótidos que forman parte del ARN): A (Adenina), U (Uracilo), T (Timina), C (Citosina).

presentan una pequeña modificación en su estructura que impide la elongación de la cadena proviral de ADN en síntesis, una vez son incorporados. (**Figura 2**).

La modificación estructural que presentan los ITIAN consiste en la substitución del grupo hidroxilo de la posición 3' del azúcar (la desoxirribosa) por otro radical, de manera que cuando se incorpora el ITIAN en la cadena de ADN en síntesis el grupo 5' fosfato del siguiente nucleótido no puede formar el enlace fosfodiéster con el grupo 3' hidroxilo del ITIAN y se bloquea la transcriptasa inversa, la elongación de la cadena y el ciclo viral. (**Figura 3**).

Los nucleósidos que forman parte del ADN se denominan deoxinucleósidos y existen 4 tipos naturales: adenosina (A), timidina (T), citidina (C) y guanosina (G). Cada uno de estos deoxinucleósidos fisiológicos dispone de al menos un análogo, también denominado dideoxinucleósido: la didanosina (ddI) es análogo de la inosina que se transforma en adenosina; la zidovudina (AZT o ZDV) y la estavudina (d4T) son análogos de la timidina; la zalcitabina (ddC), lamivudina (3TC) y emtricitabina (FTC) son análogos de la citidina; y el abacavir (ABC) de la guanosina. Nucleósidos y análogos de éstos deberán ser fosforilados hasta en 3 ocasiones en el interior de la célula por quinasas celulares para poderse incorporar en el material genético en forma de nucleótidos o análogos de éstos, que constituyen su unidad estructural.

Los nucleótidos son nucleósidos fosforilados. Actualmente sólo existe un análogo de nucleótido comercializado, el tenofovir diproxil fumarato (TDF), análogo de la adenosina 5'-monofosfato, que sólo

Estructura y síntesis del ácido desoxirribonucleico entre nucleótidos naturales (a) o inhibidor de la transcriptasa inversa análogos de nucleótido y nucleótido natural (b)

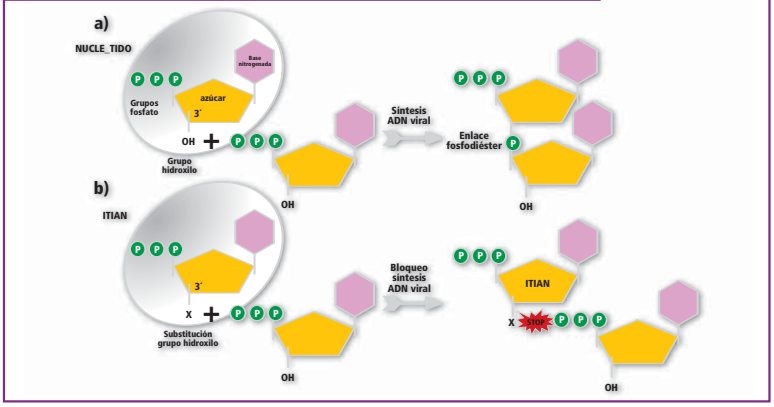

FIGURA **3.**
ADN: Ácido desoxirribonucleico; ITIAN: Inhibidores de la transcriptasa inversa análogos de nucleósido o nucleótido; OH: Grupo hidroxilo; P: Grupo fosfato.

deberá ser fosforilado en 2 ocasiones para poderse incorporar en la cadena de ADN en síntesis.

Los ITIAN constituyen la base de cualquier terapia TARGA, la cuál debe incluir al menos 2 de ellos como componentes fundamentales.

Los efectos secundarios de los ITIAN se deben, mayoritariamente, a la toxicidad mitocondrial que ejercen, principalmente, mediante la inhibición de la replicación del material genético de la mitocondria[5]. Los ITIAN, que no tienen efectos en el núcleo celular, logran incorporarse en el material genético del VIH frenando así la replicación viral, pero también se insertan el material genético de la mitocondria, el único orgánulo de la célula animal que posee ADN propio. Ésto se debe a que los ITIAN inhiben, además de la transcripción inversa del VIH, a otras ADN polimerasas celulares. De los 5 tipos de ADN polimerasas que dispone la célula eucariota (α y δ para la replicación del material genético nuclear, β y ε para la reparación de los errores producidos en la duplicación del ADN nuclear y la ADN polimerasa γ para la replicación del ADN mitocondrial -ADNmt-), los ITIAN inhiben las ADN polimerasas celulares β y γ. La acción de la ADN polimerasa β es redundante y no se conocen efectos fisiopatológicos inducidos por la inhibición selectiva de esta enzima. Sin embargo, la ADN polimerasa γ es el único enzima responsable de la replicación (y cierto grado de reparación) del material genético de la mitocondria, y el efecto del bloqueo de su actividad tiene consecuencias mayores, ya que condiciona la disminución del contenido en ADNmt[5-7], que puede llegar a comprometer la función mitocondrial cuando esta depleción es importante.

El mecanismo de inhibición de la ADN polimerasa γ mitocondrial por parte de los ITIAN se esquematiza en la **Figura 4**. En primer lugar, los ITIAN ejercen una inhibición competitiva sobre esta enzima al competir con los nucleótidos fisiológicos por incorporarse en la cadena de ADNmt naciente (a); actúan como terminadores prematuros de la elongación de la cadena de ADNmt en síntesis porque una vez incorporados inhiben la actividad polimerasa del enzima promoviendo la generación de especies incompletas con deleciones (b); aumentan la tasa de errores puntuales en la cadena de ADNmt sintetizada al inhibir la actividad *proof-reading* de la ADN polimerasa g mitocondrial que reconoce los nucleótidos mal apareados (c); e inhiben también la actividad exonucleasa del enzima que corrige los errores de fidelidad en la copia que se hayan producido durante la síntesis de la cadena naciente de ADNmt (d).

De esta manera los ITIAN disminuyen la calidad y cantidad de ADNmt aumentando la tasa de mutaciones puntuales, deleciones y depleción en las moléculas de este material genético. Dado que el ADNmt codifica para 13 subunidades proteicas que integran los complejos de la cadena de respiración mitocondrial, deficiencias genéticas en el ADNmt pueden comportar deficiencias en la síntesis de estas

Bloqueo de la ADN polimerasa gamma mitocondrial por parte de los inhibidores de la transcriptasa inversa análogos de nucleósido o nucleótido

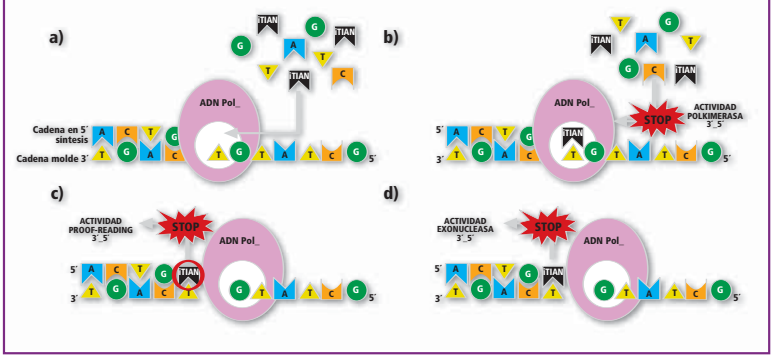

FIGURA **4.**

ITIAN: Inhibidores de la transcriptasa inversa análogos de nucleósido o nucleótido; ADN pol γ: ADN Polimerasa gamma; Deoxinucleótidos (nucleótidos que forman parte del ADN): A (Adenina), G (Guanina), T (Timina), C (Citosina).

unidades y finalmente problemas funcionales energéticos y metabólicos mitocondriales que pueden llegar a comprometer la viabilidad celular.

Kakuda clasificó los ITIAN según la potencia que presentaban in vitro para inhibir la ADN polimerasa γ mitocondrial en esta escala decreciente: ddC > ddI > d4T >>> AZT > 3TC > ABC = TDF[8], que sería análoga a la escala de toxicidad mitocondrial para estos fármacos en el supuesto que sólo ejercieran toxicidad mitocondrial a través de la inhibición de esta enzima.

Pueden existir mecanismos favorecedores de esta toxicidad. Por ejemplo el TDF, posee una baja toxicidad mitocondrial *in vitro*[9] asociada a escasa toxicidad clínica mitocondrial *in vivo*[10,11]. Sin embargo, se ha observado que si se administra conjuntamente con la didanosina (ddI), la cuál presenta una reconocida capacidad mitotóxica, potencia los efectos adversos de este último ITIAN al aumentar la concentración plasmática y biodisponibilidad de ddI. La depleción de ADNmt inducida bajo este régimen se revierte disminuyendo la dosis de ddI administrada de 400 a 250 mg/día[12].

Sin embargo durante los últimos años también se han descrito efectos mitocondriales de los ITIAN en ausencia de depleción de ADNmt. Ello sugiere que existen mecanismos lesivos independientes de la inhibición de la ADN polimerasa γ. Entre ellos se han sugerido, en el caso concreto de la zidovudina (AZT), la inhibición del transporte de substratos del metabolismo energético de la mitocondria (succinato y ADP/ATP), la inhibición del complejo IV (Citocromo c Oxidasa o COX) de la cadena respiratoria mitocondrial o la disminución del contenido mitocondrial en carnitina[13-15]. Además, para éste y otros ITIAN, también se ha descrito la inhibición *in vivo*[16] o *in vitro*[17] de la transcripción mitocondrial. Por ejemplo, resultados experimentales *in vivo* realizados en células mononucleares en nuestro laboratorio revelan la existencia de mecanismos compensatorios mitocondriales de tipo transcripcional o traduccional que mantendrían la homeostasis funcional de la mitocondria aun cuando su contenido en ADNmt se encuentra deplecionado, en pacientes infectados que se hallan en tratamiento con estavudina y didanosina[18].

Además, los ITIAN se han propuesto como fármacos inductores de apoptosis a través de ejercer una lesión mitocondrial crónica que ocasiona la generación de estrés oxidativo. De esta forma, la célula queda en una situación de compromiso energético y metabólico (especialmente por déficit en ATP) que puede desencadenar la muerte celular programada a través de la vía intrínseca[19-22]. Este fenómeno es especialmente negativo cuando afecta a los linfocitos T CD4+ o a las células de tejido adiposo lipoatrófico.

Inhibidores de la transcriptasa inversa no análogos de los nucleósidos

La transcriptasa inversa del VIH también puede bloquearse mediante la administración de los antirretrovirales inhibidores de la transcriptasa inversa no análogos de nucleósido o nucleótido (ITINAN). Aunque el efecto es el mismo, su mecanismo de acción es distinto al de los ITIAN. Los ITINAN no son análogos de los nucleósidos o nucleótidos fisiológicos que bloquean la retrotranscripción del material genético viral cuando son incorporados en la cadena en síntesis,

Bloqueo de la retrotranscripción del ácido ribonucleico del VIH a ácido desoxirribonucleico proviral por parte de los inhibidores de la transciptasa inversa no análogos de nucleósido o nucleótido (ITINAN)

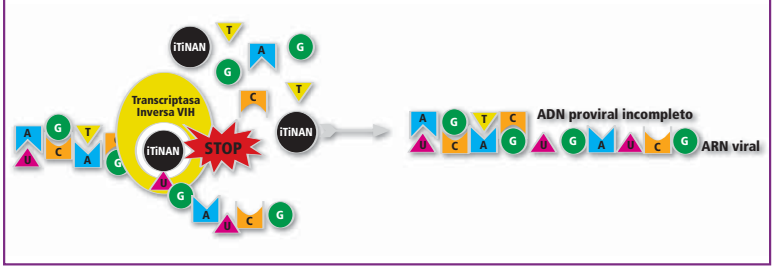

FIGURA **5.**
ADN: Ácido desoxirribonucleico; ARN: Ácido ribonucleico; ITINAN: Inhibidores de la transcriptasa inversa no análogos de nucleósido o nucleótido; Deoxinucleótidos (nucleótidos que forman parte del ADN): A (Adenina), G (Guanina), T (Timina), C (Citosina); Ribonucleótidos (nucleótidos que forman parte del ARN): A (Adenina), U (Uracilo), T (Timina), C (Citosina).

sino que interaccionan directamente con la transcriptasa inversa del virus y bloquean su actividad enzimática. (**Figura 5**). Se unen a esta enzima induciendo un cambio en su conformación que bloquea su centro catalítico y su actividad y, a diferencia de los ITIAN, no deben activarse (fosforilándose, por ejemplo) para ejercer su actividad antirretroviral.

Estos antirretrovirales, a diferencia de los ITIAN, no inhiben ninguna ADN polimerasa celular, ni siquiera la mitocondrial (γ) y su uso continuado sólo se ha asociado, quizás, a un leve aumento de los fenómenos apoptóticos. Este posible efecto inductor de apoptosis se ha documentado especialmente para el ITINAN efavirenz (EFV), que también podría alterar la proliferación de los linfocitos T[23] y la diferenciación in vitro de los adipocitos mediada por el factor de transcripción SREBP-1c[24]. Sin embargo, otro ITINAN como la nevirapina (NVP) podría tener el efecto contrario[25]. En cualquier caso, los efectos mitocondriales e incluso apoptóticos de los ITINAN están poco estudiados y se sugiere que son de baja intensidad.

Actualmente se están desarrollando ITINAN de nueva generación como el etravirine (ETR, TMC125) o el rilpivirine (TMC278), cuya toxicidad no se ha estudiado.

Inhibidores de la proteasa

Los inhibidores de la proteasa bloquean la actividad de esta enzima viral impidiendo que se generen las proteínas estructurales y los enzimas virales (transcriptasa inversa, proteasa e integrasa) derivadas de la traducción de los genes Gag y Pol del VIH, esenciales para el ensamblaje de nuevos viriones capaces de infectar nuevas células. (**Figura 6**).

Se trata de bloquear la replicación del VIH en una etapa avanzada del ciclo viral, cuando éste ya ha infectado la célula y ha empezado su ciclo lítico. Este ciclo lítico, en el caso de las células inmunitarias, a menudo empieza cuando la célula se activa. Por lo tanto, son fármacos activos contra células que ya se encuentran infectadas y son portadoras del ADN proviral en su genoma, de manera crónica, pudiendo actuar como reservorios.

Pero además de su actividad terapéutica antirretroviral, a los inhibidores de la proteasa se les ha asignado efectos secundarios de tipo metabólico, mitocondrial y apoptótico, siendo estos dos últimos motivo actual de controversia.

Los efectos adversos metabólicos de los inhibidores de la proteasa pueden explicarse por la homología estructural que existe entre la proteasa del virus y ciertas proteínas celulares, a las que podría inhibir de la misma manera que inhibe al enzima viral. Concretamente, se ha descrito homología de la proteasa del virus con CRABP-1 (proteína tipo 1 de unión citoplasmática al ácido retinoico), que interviene en la diferenciación adipocitaria, así como con LRP (proteína asociada al receptor de la lipoproteína de baja densidad), receptor hepático y del endotelio capilar que degrada los triglicéridos circulantes a ácidos grasos aptos para la acumulación adipocitaria. La alteración de la función de estas proteínas tiene un efecto directo sobre el metabolismo lipídico al inhibir la formación de células adipocitarias y favorecer la acumulación de

Bloqueo del procesamiento proteolítico de las proteínas del VIH por parte de los inhibidores de la proteasa del virus

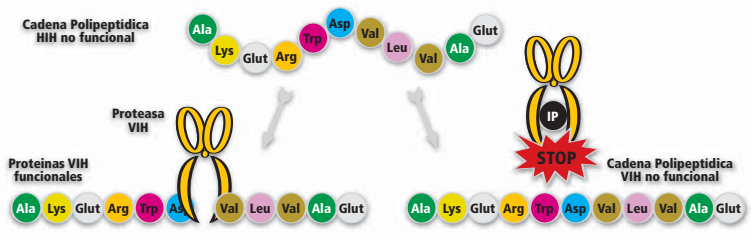

FIGURA 6.
IP: Inhibidor de la proteasa

lípidos en sangre.

Los inhibidores de la proteasa también alteran el metabolismo de la glucosa. Algunos de estos fármacos (como nelfinavir e indinavir) se ha demostrado que inhiben la translocación o activación de los transportadores de glucosa que importan este metabolito hacia el interior de la célula, como GLUT1 o GLUT4, ya sea por interacción directa[26,27], o a través de intermediarios como la proteína quinasa B (PKB), que interviene en la supervivencia celular y media los efectos de la insulina sobre el metabolismo celular, o por interacción con el factor de transcripción SREBP-1 (proteína tipo 1 de unión al elemento regulador del esterol), que interviene en la diferenciación de adipocitos e incrementa la captación de glucosa[28].

El resultado de estas interacciones comporta el aumento de la concentración plasmática de glucosa y triglicéridos y la disminución de su disponibilidad celular. Esta deprivación puede promover un déficit energético que puede ocasionar la muerte celular por apoptosis. Además, favorece el desarrollo de la resistencia a la insulina, que juntamente con la alteración del metabolismo glucídico y lipídico, constituye el denominado síndrome metabólico.

Sin embargo no existe consenso sobre los efectos mitocondriales de los inhibidores de la proteasa, aunque en caso de ser deletéreos, se conciben de menor potencia que los inducidos por los ITIAN. Algunos autores apuntan a que los inhibidores de la proteasa podrían prevenir la pérdida del potencial de membrana de este orgánulo, que además de ser necesario para su correcto funcionamiento, precede la liberación de factores apoptogénicos hacia el citoplasma[29]. Esta teoría atribuiría efectos anti-apoptóticos y de protección mitocondrial a estos fármacos, aunque otros autores afirman que a dosis superiores, su efecto sería el opuesto y actuarían promoviendo la despolarización del orgánulo y la consecuente entrada de la célula en apoptosis[30]. A favor del efecto mitotóxico de los inhibidores de la proteasa se ha descrito otro mecanismo de lesión mitocondrial indirecta, especialmente para ritonavir, que actuaría inhibiendo la actividad de la glicoproteína-p[31], encargada de expulsar los ITIAN fuera de la célula, y el citocromo p-450[32], que detoxifica algunos fármacos antirretrovirales. La combinación de inhibidores de la proteasa con ITIAN, característicos de la mayoría de regímenes TARGA, potenciaría el daño mitocondrial que ejercen estos últimos.

De igual manera, existe una gran controversia en torno a los efectos celulares que ejercen los inhibidores de la proteasa: algunos autores aseguran que son anti-apoptóticos[33-37] y otros pro-apoptóticos[38-40], aunque la mayoría afirma que su efecto dual podría depender de la dosis[30,35,36].

Las teorías que refuerzan los efectos anti-apoptóticos de estos antirretrovirales esgrimen razones como que estos fármacos modulan la actividad de los proteasomas y la presentación de antígenos por parte del complejo mayor de histocompatibilidad de clase I (MHC-I), de manera que disminuyen la activación inmunológica y la apoptosis derivada de esta activación[34]. También hay autores que describen la disminución en la expresión de proteínas pro-apoptóticas como la caspasa-1[35-37], aunque existe mucha polémica al respecto[41,42]. A favor del carácter pro-apoptótico de los inhibidores de la proteasa está el papel que se les asigna en la pérdida de adipocitos en las regiones lipoatróficas del síndrome de lipodistrofia por alteración de la diferenciación de estas células mediante interacción con la proteína CRABP-1[38] o el factor de transcripción SREBP-1[39], anteriormente mencionados. También se han asociado *in vitro* a la reducción de la proliferación de células T[38,40], a la muerte por apoptosis de células endoteliales expuestas a concentraciones plasmáticas de estos fármacos[43] o la actividad anticancerígena por inducción de muerte celular descrita para algunos de ellos[44-46].

En resumen, los inhibidores de la proteasa se asocian a la alteración del metabolismo de los carbohidratos y de los lípidos que caracteriza el síndrome metabólico, pero sus efectos sobre el estado mitocondrial y apoptótico no están del todo esclarecidos. La mayoría de los estudios que apuntan a propiedades protectoras para la mitocondria y anti-apoptóticas por parte de los inhibidores de la proteasa están realizados *in vitro*[29,35-37] con concentraciones de estos fármacos inferiores a las plasmáticas, especialmente cuando estos fármacos se potencian con ritonavir. Por contra, estudios realizados con concentraciones próximas a las que alcanzan estos fármacos a nivel fisiológico, abogan por efectos pro-apoptóticos y dañinos para la mitocondria (por pérdida de potencial de membrana, déficit energético o potenciación de los efectos adversos de los ITIAN)[21].

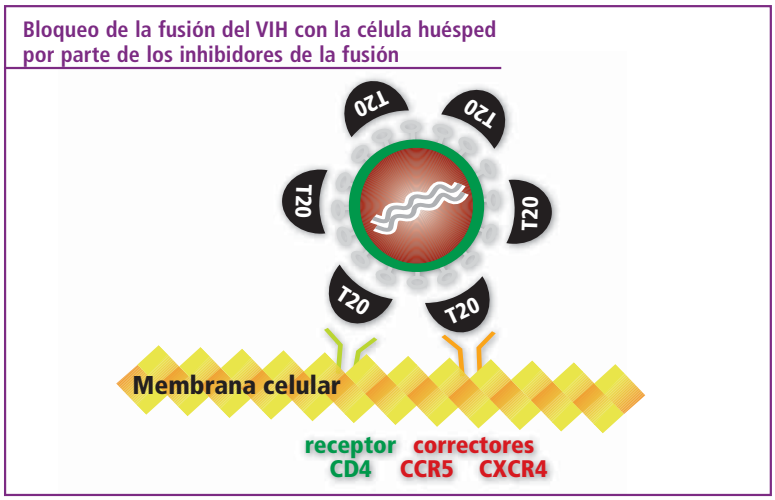

FIGURA **7.**
IF: Inhibidor de la fusión; T20: Enfuvirtide; CD4, CCR5 y CXCR4: receptor (CD4) y correceptores (CCR5 y CXCR4) de la célula huésped que reconoce el VIH.

En cualquier caso, resultados experimentales obtenidos *in vivo* en nuestro laboratorio con nelfinavir demuestran que el grado de apoptosis que pudiera inducir un TARGA que incluye un inhibidor de la proteasa es mucho menor al grado de apoptosis inducido por el propio virus, puesto que el tratamiento con nelfinavir se asocia a una tasa de apoptosis en células mononucleares muy inferior a la de aquellos sujetos infectados no sometidos a TARGA[47].

Inhibidores de la fusión

Los inhibidores de la fusión del virus con la célula huésped (**Figura 1**), familia a la que pertenece enfuvirtide (T-20), se unen a las proteínas víricas que forman parte de la envuelta lipídica del virus e impiden su interacción con los receptores y correceptores de la célula huésped, bloqueando así la entrada del virus y la infección de la célula huésped (**Figura 7**).

Como la interacción del fármaco con la célula huésped es exclusivamente extracelular, se espera una nula toxicidad mitocondrial para este tipo de fármacos, aunque existen pocos estudios al respecto. Estudios realizados *in vivo* en nuestro laboratorio con células mononucleares de individuos infectados sometidos al cambio de una pauta antirretroviral que contenía fármacos de contrastada toxicidad mitocondrial por otro régimen que contenía enfuvirtide se asoció a la mejora de las actividades enzimáticas y oxidativas de la cadena respiratoria mitocondrial y al aumento en la tasa de expresión de proteínas mitocondriales [48]. Por otra parte, en células mononucleares de individuos no infectados a los que se les ha administrado durante una semana enfuvirtide se ha observado un efecto nulo sobre el contenido en ADNmt (datos no publicados). Finalmente, tampoco existen evidencias clínicas que indiquen toxicidad mitocondrial para este fármaco [49, 50]. Por todo ello, no es esperable el desarrollo de efectos mitocondriales adversos durante los próximos años al tratamiento con enfuvirtide.

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9.2. COMUNICACIONS PRESENTADES A CONGRESSOS

A continuació es descriuen les **comunicacions** escrites i orals que la doctoranda ha presentat en diversos congressos d'àmbit internacional fruit de la labor experimental dels diferents treballs d'investigació en els que ha participat al llarg del doctorat:

Autors: Negredo E, Miró Ò, **Garrabou G**, Morén C, Cardellach F, Estany C, Rodríguez-Santiago B, Masabeu A, Force L, Barrufet P, Cucurull J, Domingo P, Alonso C, Pérez-Álvarez N, Bonaventura C.
Títol: 'Lopinavir-ritonavir plus nevirapine as a strategy to improve nucleoside-related mitochondrial toxicity in chronically treated HIV-infected patients: 48 week follow-up of MULTINEKA study'.
Tipus de participació: Pòster.
Congrés: 'XVII International AIDS Conference'
Lloc de celebració: Ciutat de Mèxic (MÈXIC) Dates: 3-8 Agost 2008.

Autors: **Garrabou G**, Morén C, Rodríguez V, Inoriza JM, Martí MJ, Olíu G, Miró Ò, Casademont J, Cardellach F.
Títol: 'Mitochondrial lesion in acute carbon monoxide poisoning: effects of normobaric or hyperbaric oxygen treatment'.
Tipus de participació: Pòster.
Congrés: 'The 7th European Meeting on Mitochondrial Pathology (Euromit): From basic mechanisms to disease and ageing'
Lloc de celebració: Stockholm (SUÈCIA) Dates: 11-14 Juny 2008.

Autors: **Garrabou G**, Morén C, Rodríguez V, Trullàs JC, Mondón S, Navarro V, Jou J, Nogué S, Miró Ò, Casademont J, Cardellach F.
Títol: 'Mitochondrial effects of methylene chloride substance abuse'.
Tipus de participació: Pòster.
Congrés: 'The 7th European Meeting on Mitochondrial Pathology (Euromit): From basic mechanisms to disease and ageing'
Lloc de celebració: Stockholm (SUÈCIA) Dates: 11-14 Juny 2008.

Autors: **Garrabou G**, Morén C, Rodríguez V, Coll O, Durban M, López S, Vidal R, Suy A, Hernandez S, Casademont J, Cardellach F, Mataro D, Miro Ò.
Títol: 'Oocyte mitochondrial DNA content implication on HIV-infected antiretroviral-treated women fertility outcome'.
Tipus de participació: Pòster.
Congrés: 'The 7th European Meeting on Mitochondrial Pathology (Euromit): From basic mechanisms to disease and ageing'
Lloc de celebració: Stockholm (SUÈCIA) Dates: 11-14 Juny 2008.

Autors: **Garrabou G**, Morén C, López S, Rodríguez V, Cardellach F, Miró Ò, Casademont J.
Títol: 'Mitochondrial effects of sepsis'.
Tipus de participació: Pòster.

Congrés: 'The 7th European Meeting on Mitochondrial Pathology (Euromit): From basic mechanisms to disease and ageing'
Lloc de celebració: Stockholm (SUÈCIA) Dates: 11-14 Juny 2008.

Autors: Morén C, Rovira N, Noguera A, **Garrabou G**, Rodríguez V, Cardellach F, Miró Ò, Fortuny C.
Títol: 'Mitochondrial DNA amount restoration after planned antiretroviral interruption in HIV-infected children'.
Tipus de participació: Pòster.
Congrés: 'The 7th European Meeting on Mitochondrial Pathology (Euromit): From basic mechanisms to disease and ageing'
Lloc de celebració: Stockholm (SUÈCIA) Dates: 11-14 Juny 2008.

Autors: Morén C, Molina E, Noguera A, **Garrabou G**, Rodríguez V, Casademont J, Cardellach F, Fortuny C, Miró Ò.
Títol: 'Mitochondrial damage in peripheral blood mononuclear cells in HIV-infected pediatric patients'.
Tipus de participació: Pòster.
Congrés: 'The 7th European Meeting on Mitochondrial Pathology (Euromit): From basic mechanisms to disease and ageing'
Lloc de celebració: Stockholm (SUÈCIA) Dates: 11-14 Juny 2008.

Autors: Cardellach F, **Garrabou G**, Inoriza JM, Martí MJ, Olíu G, Miró Ò.
Títol: 'Mitochondrial oxidative and enzymatic damage in acute carbon monoxide poisoning: membrane lipid peroxidation and complex IV activity from peripheral lood lymphocytes and its modification after oxygen therapy'.
Tipus de participació: Pòster.
Congrés: '47th Society of Toxicology (SOT) Annual Meeting'.
Lloc de celebració: Seattle (EEUU) Dates: 16-20 Març 2008.

Autors: Fortuny C, Noguera A, Morén C, Simó M, Sànchez E, **Garrabou G**, Rodriguez V, Miró Ò.
Títol: 'Aumento significativo en los niveles de ADN mitocondrial en células mononucleares de sangre periférica tras la interrupción del TARGA en pacientes pediátricos infectados por el VIH'.
Tipus de participació: Pòster.
Congrés: 'IV Congreso de la Sociedad Española de Infectología Pediátrica'.
Lloc de celebració: Marbella (ESPANYA) Dates: 6-8 Març 2008.

Autors: Noguera A, Morén C, Corrales E, Sanchez E, **Garrabou G**, Rodriguez V, Miró Ò, Fortuny C.
Títol: 'Partial restoration of mitochondrial DNA content in perinatally HIV-infected pediatric patients after planned interruption of HAART'.
Tipus de participació: Pòster.
Congrés: '15th Conference on Retrovirus and Oportunistic Infections (CROI)'.
Lloc de celebració: Boston (EEUU). Dates: 3-6 Febrer 2008.

Autors: **Garrabou G**, López S, Morén C, Rodriguez V, Milinkovic A, Martinez E, Riba J, Casademont J, Cardellach F, Gatell JM, Miró Ò.
Títol: 'Mitochondrial impairment in mononuclear cells of hyperlactatemic patients on HAART'.
Tipus de participació: Pòster.
Congrés: '4th IAS Conference on HIV Pathogenesis, Treatment and Prevention'.
Lloc de celebració: Sydney (AUSTRÀLIA) Dates: 22-25 Juliol 2007.

Autors: **Garrabou G**, López S, Morén C, Rodriguez V, Milinkovic A, Martinez E, Riba J, Casademont J, Cardellach F, Gatell JM, Miró Ò.
Títol: 'Mitochondrial impairment in mononuclear cells of hyperlactatemic patients on HAART'.
Tipus de participació: Pòster.
Congrés: '9th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'.
Lloc de celebració: Sydney (AUSTRÀLIA) Dates: 19-22 Juliol 2007.

Autors: Cardellach F, **Garrabou G**, Inoriza JM, Martí MJ, Oliu G, López S, Casademont J, Miró Ò.
Títol: 'Acute carbon monoxide poisoning: treatment with normobaric or hyperbaric oxygen and its correlation with mitochondrial complex IV activity from peripheral blood lymphocytes'.
Tipus de participació: Pòster.
Congrés: '46th Society of Toxicology (SOT) Annual Meeting'.
Lloc de celebració: Charlotte (EEUU) Dates: 25-29 Març 2007.

Autors: **Garrabou G.**
Títol: 'Fertilidad y mitocondria en mujeres VIH-positivas'.
Tipus de participació: Oral.
Congrés: 'Sessió Informativa-Clínica Eugin'.
Lloc de celebració: Barcelona, Catalunya (ESPANYA) Dates: 7 Febrer 2007.

Autors: **Garrabou G.**
Títol: 'ADN mitocondrial, ovocitos e infertilidad'.
Tipus de participació: Oral.
Congrés: 'Reunión de Investigación VIH'.
Lloc de celebració: Barcelona, Catalunya (ESPANYA) Dates: 15 Gener 2007.

Autors: **Garrabou G.**
Títol: 'Fertilidad y mitocondia en mujeres VIH-positivas'.
Tipus de participació: Oral.
Congrés: 'V Jornades VIH y mitocondria'.
Lloc de celebració: Barcelona, Catalunya (ESPANYA) Dates: 30 Novembre 2006.

Autors: **Garrabou G**, López S, Rodriguez V, Riba N, Martinez E, Villarroel C, Manriquez M, Casademont J, Cardellach F, Miró Ò.

Títol: 'Mitochondrial DNA (mtDNA) content in enfuvirtide-treated healthy volunteers'.
Tipus de participació: Pòster.
Congrés: '8th International Congress on Drug Therapy in HIV infection'.
Lloc de celebració: Glasgow (ANGLATERRA) Dates: 12-16 Novembre 2006.

Autors: **Garrabou G**, López S, Negredo E, Cardellach F, Casademont J, Clotet B, Miró Ò.
Títol: 'Mitochondrial effects of didanosine dosage reduction when administred together with tenofovir'.
Tipus de participació: Pòster.
Congrés: '8th International Congress on Drug Therapy in HIV infection'.
Lloc de celebració: Glasgow (ANGLATERRA) Dates: 12-16 Novembre 2006.

Autors: **Garrabou G**.
Títol: 'Mitochondrial DNA (mtDNA) depletion is present in oocytes of HIV-infected antiretroviral-treated infertile women'.
Tipus de participació: Oral.
Congrés: '8th International Congress on Drug Therapy in HIV infection'.
Lloc de celebració: Glasgow (ANGLATERRA) Dates: 12-16 Novembre 2006.

Autors: **Garrabou G**, Coll O, Durban M, López S, Vidal R, Suy A, Hernández S, Cardellach F, Mataró D, Miró Ò.
Títol: 'Mitochondrial DNA (mtDNA) depletion is present in oocytes of HIV-infected antiretroviral-treated infertile women'.
Tipus de participació: Pòster.
Congrés: '8th International Congress on Drug Therapy in HIV infection'.
Lloc de celebració: Glasgow (ANGLATERRA) Dates: 12-16 Novembre 2006.

Autors: Casademont J, López S, **Garrabou G**, Infante A, Miró Ò, Pons A, Bernardo M, Cardellach F.
Títol: 'Effect of neuroleptics on OXPHOS system: peripheral blood mononuclear cells analysis in psychotic patients'.
Tipus de participació: Pòster.
Congrés: 'XIth International Congress on Neuromuscular Diseases'.
Lloc de celebració: Istanbul (TURQUIA) Dates: 2-7 Juliol 2006.
Publicat revista: Neuromuscular disorders 16: S186-187 Suppl. 1 Jul 2006.

Autors: Pedrol E, Deig E, Miró Ò, **Garrabou G**, López S, Vidal I, Infante A. B, Ruiz M, Cardellach F.
Títol: 'Efectos metabólicos y mitocondriales con el cambio de tratamiento antiretroviral a Enfuvirtide, Tenofovir y Saquinavir/Ritonavir en pacientes VIH+ pretratados'.
Tipus de participació: Comunicació oral – Pòster.
Congrés: 'XII Congreso SEIMC' (Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica).
Lloc de celebració: València (ESPANYA) Dates: 10-13 Maig 2006.

Autors: **Garrabou G.**
Títol: 'Efectos mitocondriales del cambio a una pauta de baja toxicidad mitocondrial (T20+SQV/rit+TDF)'.
Tipus de participació: Oral.
Congrés: 'IV Jornades VIH y mitocondria'.
Lloc de celebració: Barcelona, Catalunya (ESPANYA) Dates: 30 Novembre 2005.

Autors: Miró Ò, **Garrabou G**, López S, Infante AB, Casademont J, Villarroya F, Giralt M, Escayola R, Martínez E, Gatell JM, Cardellach F.
Títol: 'HAART regimens containing nelfinavir reduce peripheral blood mononuclear cells apoptosis'.
Tipus de participació: Pòster.
Congrés: '10th European AIDS Conference'.
Lloc de celebració: Dublín (IRLANDA) Dates: 17-20 Novembre 2005.

Autors: **Garrabou G**, López S, Sanjurjo E, Infante AB, Larrouse M, Milinkovic A, Martínez E, Riba J, Casademont J, Cardellach F, Miró Ò.
Títol: 'Mitochondrial function in HAART-related hyperlactatemia'.
Tipus de participació: Pòster (P-70).
Congrés: '7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'.
Lloc de celebració: Dublín (IRLANDA) Dates: 13-16 Novembre 2005.
Publicat revista: Antiviral Therapy 10(8) 2005: L43-L43.

Autors: **Garrabou G**, López S, Vidal F, Miró Ò, Domingo P, Pedrol E, Villarroya F, Saumoy M, Infante AB, Martínez E, López-Dupla M, Sambeat MA, Deig E, Villarroya J, Rodríguez-Chacón M, Fontanet A, Richart C, Giralt M, Gatell JM.
Títol: 'Peripheral blood mononuclear cells of HIV-1-infected patients long-term non-progressors show mild mitochondrial impairment and low mitochondrially-driven apoptosis'.
Tipus de participació: Pòster (P-71).
Congrés: '7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'.
Lloc de celebració: Dublín (IRLANDA) Dates: 13-16 Novembre 2005.
Publicat revista: Antiviral Therapy 10(8) 2005: L43-L44.

Autors: López S, **Garrabou G**, Negredo E, Infante AB, Puig J, Grau E, Gatell JM, Casademont J, Cardellach F, Clotet B, Miró Ò.
Títol: 'Effects of different dosis of didanosine combined with tenofovir on peripheral blood mononuclear cell (PBMC) mitochondrial parameters'.
Tipus de participació: Pòster (P-75).
Congrés: '7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'.
Lloc de celebració: Dublín (IRLANDA) Dates: 13-16 Novembre 2005.
Publicat revista: Antiviral Therapy 10(8) 2005: L46-L46.

Autors: López S, **Garrabou G**, Villaroya J, Infante AB, Rodríguez de la Concepción M, Escayola R, Giralt M, Gatell JM, Cardellach F, Casademont J, Villaroya F, Miró Ò.
Títol: 'Protease inhibitors (PI) and apoptosis: studies on peripheral blood mononuclear cells (PBMCs)'.
Tipus de participació: Pòster (P-68).
Congrés: '7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'.
Lloc de celebració: Dublín (IRLANDA) Dates: 13-16 Novembre 2005.
Publicat revista: Antiviral Therapy 10(8) 2005: L42-L42.

Autors: Miró Ò, **Garrabou G**, López S, Deig E, Vidal I, Infante AB, Cardellach F, Casademont J, Pedrol E.
Títol: 'Metabolic and mitochondrial changes after 6 months of switching of antiretroviral-experienced patients to enfuvirtide, tenofovir and saquinavir/ritonavir'.
Tipus de participació: Pòster (P-81).
Congrés: '7th International Workshop on Adverse Drugs Reactions and Lipodystrophy in HIV'.
Lloc de celebració: Dublín (IRLANDA) Dates: 13-16 Novembre 2005.
Publicat revista: Antiviral Therapy 10(8) 2005: L49-L49.

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

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

Autors: Saumoy M, Miró Ò, Domingo P, Pedrol E, Martínez E, Villarroya F, López S, Rodríguez de la Concepción M, Sambeat M, Deig E, Giralt M, López M, Fontanet A, **Garrabou G**, Villarroya J, Gatell JM, Vidal F.
Títol: 'MtDNA content and function and its relation with PBMC apoptosis of origin in HIV-1-Infected patients Long-Time Non-Progressors'.
Tipus de participació: Pòster.
Congrés: '47th Interscience Conference on Antimicrobial Agents and Chemotherapy' (ICAAC).
Lloc de celebració: New Orleans (EEUU.) Dates: 21-24 Setembre 2005.

Autors: Miró Ò, Villarroya J, **Garrabou G**, López S, Rodríguez de la Concepción M, Martínez E, Escayola R, Giralt M, Gatell JM, Cardellach F, Casademont J, Villarroya F.
Títol: 'Effects of Highly Active Antiretroviral Therapy (HAART) regimens containing the protease inhibitor (PI) nelfinavir on apoptosis of peripheral blood mononuclear cells'.
Tipus de participació: Pòster.
Congrés: '3rd IAS Conference on HIV Pathogenesis, Treatment and Prevention'.
Lloc de celebració: Rio de Janeiro (BRASIL) Dates: 24-27 Juliol 2005.

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

Autors: Ballesteros AL, Miró Ò, López S, Fuster D, Videla S, Martínez E, **Garrabou G**, Salas A, Coté H, Tor J, Rey-Joly C, Planas R, Clotet B, Tural C.
Títol: 'Mitochondrial effects of a 24-week course of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) in asymptomatic HCV/HIV co-infected patients on long-term treatment with didanosine, stavudine or both'.
Tipus de participació: Pòster (547).
Congrés: '40th Annual Meeting of the European Association for the Study of the Liver'.
Lloc de celebració: París (FRANÇA) Dates: 13-17 Abril 2005.
Publicat revista: Journal of Hepatology 42 (Suppl.2); 199.

Autors: **Garrabou G**, López S, Infante A, Negredo E, Puig J, Ruiz L, Sanjurjo E, Casademont J, Cardellach F, Clotet B, Miró Ò.
Títol: 'Addition of tenofovir to a didanosine-based HAART does not increase mitochondrial DNA depletion but decreases citochrome c oxidase function and mitochondrial mass'.
Tipus de participació: Pòster (P184).
Congrés: '7th International Congress on Drug Therapy in HIV infection'.
Lloc de celebració: Glasgow (ANGLATERRA) Dates: 14-18 Novembre 2004.
Publicat revista: Medscape General Medicine (MedGenMed). 2004;6(4) [Electronic Journal of the International AIDS Society (eJIAS). 2004; 1(2)].

Autors: López S, **Garrabou G**, Fernández-Solá J, Pedrol E, Badia E, Infante A, Martínez E, Cardellach F, Gatell J.M, Casademont J, Miró Ò.
Títol: 'HIV infection is associated with increased skeletal apoptosis assessed by TUNEL'.
Tipus de participació: Pòster (P188).
Congrés: '7th International Congress on Drug Therapy in HIV infection'.
Lloc de celebració: Glasgow (ANGLATERRA) Dates: 14-18 Novembre 2004.
Publicat revista: MedGenMed. 2004;6(4) [eJIAS. 2004; 1(2)].

Autors: López S, **Garrabou G**, Rodríguez de la concepción M, Martínez E, Pedrol E, Giralt M, Cardellach F, Gatell J.M, Vilarroya F, Casademont J, Miró Ò.

Títol: 'Compensatory mechanisms preserve mitochondrial function in HIV patients on didanosine plus stavudine exhibiting mitochondrial DNA depletion'.
Tipus de participació: Pòster (P183).
Congrés: '7th International Congress on Drug Therapy in HIV infection'.
Lloc de celebració: Glasgow (ANGLATERRA) Dates: 14-18 Novembre 2004.
Publicat revista: MedGenMed. 2004;6(4) [eJIAS. 2004; 1(2)].

Autors: López S, **Garrabou G**, Martínez E, Domingo P, Fontdevila J, Gatell J.M, Infante A, Gallart X, Milinkovic A, Cardellach F, Casademont J, Miró Ò.
Títol: 'Mitochondrial studies in adipose tissue of HIV-infected patients without fat redistribution'.
Tipus de participació: Pòster (PL 7.3)-Comunicació Oral.
Congrés: '7th International Congress on Drug Therapy in HIV infection'.
Lloc de celebració: Glasgow (ANGLATERRA) Dates: 14-18 Novembre 2004.
Publicat revista: MedGenMed. 2004;6(4) [eJIAS. 2004; 1(2)].

Autors: **Garrabou G**, López S, Infante A, Negredo E, Puig J, Ruiz L, Sanjurjo E, Casademont J, Cardellach F, Clotet B, Miró Ò.
Títol: 'Addition of tenofovir to a didanosine-based HAART does not increase mitochondrial DNA depletion but decreases cytochrome c oxidase function and mitochondrial mass'.
Tipus de participació: Pòster (Abstract 26).
Congrés: '6th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'.
Lloc de celebració: Washington DC (EEUU) Dates: 25-28 Octubre 2004.
Publicat revista: Antiviral Therapy 2004; 9(6): L19-L20.
Publicat revista: Nutrition and Metabolic Disorders in HIV infection. 2004 Oct-Dec; 3(4).

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

Autors: López S, **Garrabou G**, Martínez E, Domingo P, Fontdevila J, Gatell J.M, Infante A, Gallart X, Milinkovic A, Cardellach F, Casademont J, Miró Ò.
Títol: 'Mitochondrial studies in adipose tissue of HIV-infected patients without fat redistribution'.
Tipus de participació: Pòster (Abstract 27).
Congrés: '6th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'.
Lloc de celebració: Washington DC (EEUU) Dates: 25-28 Octubre 2004.
Publicat revista: Antiviral Therapy 2004; 9(6): L20.
Publicat revista: Nutrition and Metabolic Disorders in HIV infection. 2004 Oct-Dec; 3(4).

Autors: López S, **Garrabou G**, Rodríguez de la Concepción M, Martínez E, Pedrol E, Infante A, Giralt M, Cardellach F, Gatell J.M, Vilarroya F, Casademont J, Miró Ò.
Títol: 'Mitochondrial DNA depletion in asymptomatic HIV-infected patients receiving didanosine plus stavudine-based HAART regimen seems to be compensated by up-regulatory mechanisms'.
Tipus de participació: Pòster (Abstract 28).
Congrés: '6th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV'.
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