Resultats i Discussió

9 ARTICLE "ANÁLISIS DE 6 MUTACIONES LEBER EN 31 INDIVIDUOS CON ATROFIA ÓPTICA. ESTUDIO DE SU TRANSMISIÓN EN 5 FAMILIAS"

ADDENDA

Gairebé el 70% de les mostres corresponents a individus amb atròfia òptica no presentaren cap de les sis mutacions LHON analitzades. No es trobaren diferències estadísticament significatives quant a freqüència de mutacions primàries entre els pacients estudiats i altres poblacions, però probablement això fou a causa de la mida de la mostra. En la **INTRODUCCIÓ** de la memòria (pàg. 34) es diu que, segons altres estudis en població europea, el 90% dels pacients LHON presenten alguna de les mutacions primàries (11778A, 3460A o 14484C). Dels 31 pacients examinats a l'Hospital de Bellvitge que foren posteriorment estudiats al laboratori de genètica, únicament nou tenien alguna de les mutacions primàries, el que representa el 29% dels casos. Des de la publicació de l'article s'han analitzat 70 mostres més, sense que aquest percentatge hagi augmentat.

A l'article s'apunten com a possibles explicacions l'existència d'algun gen nuclear alterat en aquests individus amb atròfia òptica, o la presència d'altres mutacions al mtDNA d'aquests pacients. Pel que fa a la idea d'un gen nuclear, diversos grups han investigat sobre l'existència d'un gen de susceptibilitat a pèrdua visual en el cromosoma X. Per exemple, el 1991 Bu i Rotter suggeriren la implicació en l'atròfia òptica de Leber d'algun gen al cromosoma X (Bu 1991); l'any següent, però, Sweeney *et al*. demostraren en famílies britàniques i italianes que no existia lligament entre el locus al cromosoma X proposat i la malaltia (Sweeney 1992). Més recentment s'ha tornat a descartar la participació d'algun gen al cromosoma X (Handoko 1998). En qualsevol cas no es pot excloure el paper (primari) d'altres gens nuclears en l'etiologia de la LHON, tot i que *a priori* aquesta possibilitat sembla poc probable.

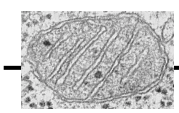
La possibilitat que altres mutacions al mtDNA siguin les responsables de l'atròfia òptica en el grup estudiat no es pot rebutjar si no es seqüencien les 16,5 kb del mtDNA dels pacients. Amb tot, pensem que és poc factible que existeixin tants pacients amb mutacions LHON "alternatives". Quant a les mutacions secundàries, tradicionalment s'havia considerat que la presència de més d'una en una persona (sense que existís cap de primària) podia provocar la LHON[33]. En el grup d'afectats analitzat en l'article tan sols es van trobar dues mutacions secundàries en un pacient, però cap la possibilitat que en algun altre pacient coincideixin mutacions diferents a les estudiades. No obstant això, novament sembla difícil que aquest sigui el cas dels 22 individus que no presenten cap mutació primària.

Una altra opció és que alguns d'aquests casos siguin conseqüència de delecions al mtDNA en zones implicades amb l'atròfia òptica de Leber. Aquesta hipòtesi és també força inversemblant ja que les delecions probablement haurien de provocar alteracions multisistèmiques, a banda del fet que no es coneixen casos LHON causat per delecions al mtDNA. En tot cas, les persones estudiades en el present treball tindrien aquestes delecions en heteroplàsmia per tal com les regions que envolten les mutacions LHON primàries es van amplificat mitjançant PCR i s'obtingueren productes de mida normal.

En qualsevol cas, sembla molt poc probable que cap d'aquestes especulacions expliqui l'atròfia òptica patida per 21 dels 31 pacients estudiats. El baix percentatge de mutacions LHON primàries observat en pacients espanyols tampoc pot ésser degut a la pertinença a haplotips de mtDNA diferents als europeus (Corte-Real 1996) ni a una base genètica nuclear substancialment diferent a la d'altres poblacions europees (Cavalli-Sforza 1993). Tanmateix, la població finlandesa amb LHON també sembla tenir unes característiques especials, ja que no s'hi ha detectat la mutació 14484 en 24 pacients i les freqüències de les mutacions 11778 o 3460 són significativament més baixes que les mundials (Lamminen 1997).

Cal plantejar-se, doncs, si la selecció dels pacients es va fer amb uns criteris de sospita de neuropatia òptica de Leber equivalents als seguits en altres estudis, o bé si l'anàlisi molecular es va plantejar per a descartar la LHON sense que les dades neurooftalmològiques fossin clarament indicatives de la mateixa. En aquest sentit seria molt interessant estudiar malalts atesos a altres centres hospitalaris, és a dir seleccionats per altres

[33] En l'actualitat hom pensa que es tracta més probablement de polimorfismes sense cap efecte patogènic.



equips mèdics. L'Hospital de Bellvitge atén una zona molt àmplia, però l'estudi de pacients d'altres àrees geogràfiques podria proporcionar una representativitat més gran de la població espanyola.

L'estudi de mutacions LHON ha generat altres dades que sí són "típiques" —freqüentment observades— de la malaltia:

- Dels 31 individus afectats, 24 són barons. Aquest predomini masculí també s'observà dins d'alguns dels pedigrís analitzats (famílies 1 i 2)
- La recuperació visual de l'individu I3 de la família 2 és un fenomen sovint descrit entre pacients que presenten la mutació 14484 (Riordan-Eva 1995)
- La família 2 també aporta un exemple de la penetració variable d'aquesta malaltia. Seria molt important, però, conèixer quina és la proporció de molècules mutants a les cèl·lules del nervi òptic. Pot ser que el percentatge en sang de molècules de mtDNA amb la mutació no sigui un reflex del percentatge a nervi òptic, i és important tenir en compte això quan es discuteix sobre la penetració de la LHON.

Si el model que proposa el Dr Howell és correcte (**FIGURA 10**, pàg. 34), el principal repte que planteja l'atròfia òptica de Leber és aturar el procés que condueix cap a la fase atròfica en els pacients amb mutacions LHON. En tots els casos seria molt interessant elucidar els factors secundaris que precipiten l'atròfia òptica. L'estudi de l'afectació del nervi òptic de malalts amb les diverses mutacions pot ajudar a definir els mecanismes patogènics precisos que la provoquen, però aquesta estratègia es veu impedida per la dificultat d'obtenir mostres adequades: nervi òptic de pacients recentment diagnosticats.

ERRATA

A l'article, a l'apartat *Métodos* dins de **Pacientes y métodos**, es diu que les tres mutacions primàries afecten la subunitat I del complex I de la CRM, però en realitat les mutacions 11778A i 14484C alteren els gens ND4 i ND6, respectivament.

10 ARTICLE "MITOCHONDRIAL DNA LHON MUTATIONS IN ALCOHOLIC PATIENTS DEVELOPING AMBLYOPIA"

ADDENDA

El percentatge d'individus amb alguna de les 10 mutacions LHON analitzades és del 53% en el grups d'alcohòlics ambliòpics (AA), 36% en el grup d'alcohòlics no ambliòpics (ANA), i del 24% dins del grup d'individus no alcohòlics (C). Malgrat aquest gradient percentual en el qual el grup considerat més sa és el que presenta un percentatge més baix de mutacions LHON, les diferències entre els AA i els ANA, o entre els ANA i els C no són estadísticament significatives. En canvi, quan es consideren només les persones que tenen una mutació primària o més d'una mutació secundària (les que *a priori* tenen més risc de patir atròfia òptica) les comparacions sí revelen diferències significatives entre AA i ANA, entre AA i C, i entre AA i el total d'individus no alcohòlics (ANA + C).

Quan s'analitzen les dades clíniques obtingudes dels individus estudiats en el present treball s'observa que l'ambliopia del grup AA no sembla causada per un estat nutricional deficient en aquestes persones, ja que no existeixen diferències en cap de les magnituds bioquímiques determinades respecte al grup alcohòlic control ANA. No es pot assegurar, però, que els AA no patissin algun dèficit alimentari important en el moment en el qual començaren a perdre agudesa visual. Si això fos cert, en el temps transcorregut aquestes persones haurien recuperat els valors normals de vitamines i altres substàncies però no la visió. El consum d'alcohol dels dos grups alcohòlics també és comparable, i per tant no explica la progressió cap a la ceguesa dels AA.

En rates el consum d'etanol provoca un augment de la concentració de 8–hidroxidesoxi-guanosina al mtDNA, és a dir, augmenta el dany oxidatiu mitocondrial (Cahill 1997; Cahill 1999). El dany és més gran a mida que la dieta amb etanol es prolonga. El consum d'etanol per part dels individus alcohòlics (AA + ANA) no ha provocat l'aparició de les mutacions LHON, ja que el percentatge detectat és comparable al de la població control (C).

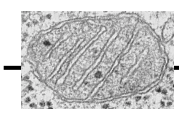
Per últim es pot assenyalar que, com ja s'havia evidenciat en els pocs treballs existents sobre ambliopia tòxica, només una petita proporció dels pacients diagnosticats amb ambliopia alcohol-tabac presenten mutacions LHON. Al marge de si aquests presenten o no manifestacions clíniques típiques de la LHON[34], encara resten per descobrir els mecanismes patogènics que s'engeguen mercè a la presència de substàncies tòxiques a l'organisme i que condueixen a la pèrdua de la visió en la majoria d'afectes de TAA.

ERRATA

En general abunden les errades tipogràfiques a tot l'article, per causa de manca de zel de la revista. Algun dels errors o mancances que poden confondre són els següents:

- A la Taula 1, el període d'alcoholisme s'indica en anys
- El colesterol del grup AA és de 203,4 ± 44,3 mg/dL
- La pre-albúmina del grup ANA és 25,8 ± 11,2 mg/dL

[34] Els exàmens neuroftalmològics dels dos individus ambliòpics amb la mutació LHON primària 3460 no suggerien l'atròfia òptica de Leber. A més, van recuperar la visió en abandonar el consum d'alcohol i tabac i en incloure a la dieta el complex vitamínic $B_1B_6B_{12}$, observacions gens típiques a la LHON. El consum de tabac i alcohol d'aquestes dues persones fou en un cas: 20 cigarretes per dia, i 3 cigalons, 1 litre de vi i 2 conyacs per dia; en l'altre: 40 cigarretes per dia, i 2 cigalons, 1 litre de vi i 2-3 copes d'aiguardent per dia.



11 ARTICLE "PRESENCE OF A MAJOR *WFS1* MUTATION IN SPANISH WOLFRAM SYNDROME PEDIGREES"

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Presence of a Major *WFS1* Mutation in Spanish Wolfram Syndrome Pedigrees

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Wolfram syndrome (WS) is an autosomal recessive neurodegenerative disease mainly characterized by familial diabetes mellitus and optic atrophy. WS patients frequently present with other clinical features such as diabetes insipidus, renal abnormalities, psychiatric disorders, and a variety of neurologic symptoms: deafness, ataxia, peripheral neuropathy. A gene responsible for Wolfram Syndrome (*WFS1*) has been recently identified on chromosome 4p16.1. Twenty-two Wolfram patients from 16 Spanish families were screened for mutations in the *WFS1* coding region by SSCP analysis and direct sequencing. Since WS has been considered a mitochondrial disorder for some time, mitochondrial DNA (mtDNA) in these families was also examined. *WFS1* mutations were detected in 75% of families (12 of 16). One of these mutations, an insertion of 16 base pairs in exon 4, turned out to be notably frequent in Spanish pedigrees. As many as 50% of pedigrees with *WFS1* mutations harbored this insertion, either in one (33% of cases) or in two chromosomes (67%). Ten other mutations were identified: 7 missense changes, 2 deletions, and 1 nonsense mutation. Only 3 of these changes had been previously described in non-Spanish pedigrees. Large mtDNA rearrangements and LHON point mutations were detected in four and six families, respectively. No correlation could be established between *WFS1* gene mutations and specific point mutations or rearrangements in mtDNA. We would suggest first screening for the 16-bp insertion in exon 4 when a new Spanish WS case is reported.

Key Words: **Wolfram syndrome; DIDMOAD; *WFS1* mutations; mtDNA deletions; mtDNA point mutations.**

Wolfram syndrome (WS, MIM 222300, also referred to as DIDMOAD) is a progressive neurodegenerative disease that primarily consists in the occurrence of juvenile-onset diabetes mellitus and optic atrophy, typically during the first decade of life (1). Some other features such as diabetes insipidus, deafness, urinary tract abnormalities, and psychiatric disorders may appear during the second decade (2). WS is transmitted in an autosomal recessive manner. The diversity in the clinical symptoms suggested deficiencies in the mitochondrial energy supply system and led several groups to consider WS as a mitochondrial disorder (3–6). Many investigators did not find mitochondrial DNA (mtDNA) mutations in WS samples, but others detected rearrangements: multiple deletions were observed in two families studied by our group (7) and at least two cases of single deletions were also described (8,9). Additionally, a WS patient harboring the primary Leber's hereditary optic neuropathy (LHON) mutation G11778A was reported (10). Polymeropoulos and colleagues (11) had demonstrated linkage of WS to some markers on the short arm of chromosome 4. In 1998 a gene responsible for the syndrome (*WFS1*) was identified in chromosome 4p16.1 (12,13), and a spectrum of *WFS1* mutations in British Wolfram patients was subsequently reported (14). Evidence for genetic heterogeneity had been provided in the past (15) and only a few months ago the second locus involved in WS was mapped in 4q22–q24 (16). The *WFS1* gene consists of eight exons coding for a putative 890-amino-acid protein named wolframin that has nine predicted helical transmembrane seg-

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ments. Thus, wolframin is thought to be a transmembrane protein. Northern blot analysis revealed *WFS1* expression in heart, placenta, pancreas, lung, and brain.

To further characterize the types of mutations in this gene, we investigated the *WFS1* coding region of Spanish WS patients. Samples from 22 patients belonging to 16 families were analyzed and the segregation of the mutations detected was studied in 58 relatives. We wanted to ascertain the spectrum of *WFS1* gene mutations present in Spanish patients, and if it was possible to establish a relationship between genotypes and observed phenotypes.

The mtDNA was also studied in these families to determine whether mitochondria are commonly affected in WS patients, and if mutations in *WFS1* correlate with specific mtDNA point mutations or rearrangements.

MATERIALS AND METHODS

Subjects and Controls

Sixteen WS families from various regions of Spain participated in the study (Fig. 1). DNA samples of 22 affected individuals (10 females and 12 males) and 58 available relatives were collected. The main clinical features of WS patients are compiled in Table 1. All families presented with one affected individual, except families WS4, WS7, and WS18, with three affected members, and family WS13, with two patients.

Ascertainment criteria for WS were the occurrence of diabetes mellitus and optic atrophy. In one case (patient WS3 II3), optic atrophy was not diagnosed, but the presence of many neurological and psychiatric abnormalities induced us to examine his *WFS1* gene (Table 1).

Fifty-nine healthy unrelated individuals participated in the study as controls. Neither family history of diabetes mellitus nor optic atrophy was reported in their medical records.

DNA Extraction

DNA was extracted from peripheral blood samples obtained after informed consent, in accordance with our institution's guidelines. Brain and muscle samples of deceased members WS4 II4 (patient) and WS5 I3 (mother of WS patient) had been obtained earlier. These two families were included in previous mtDNA studies (7). Total DNA was extracted following standard procedures.

Analysis of WFS1 Mutations

Thirteen pairs of primers were used to PCR-amplify exons 2 to 8 of the *WFS1* gene as reported elsewhere (13). Standard SSCP analysis or direct sequencing with the same pair of primers was then performed. Premade 12.5% acrylamide SSCPs gels (ExcelGel DNA analysis kit, Pharmacia Biotech, Sweden) were run at 5°C on a Multiphor II System apparatus (Pharmacia Biotech). A 20-min prerun at 100 V, 23 mA, 5 W was performed, followed by a 2-h 30-min to 3-h 30-min run at 600 V, 30 mA, 18 W. Silver staining was completed with the DNA Silver Staining kit on an automatic gel stainer (Pharmacia Biotech).

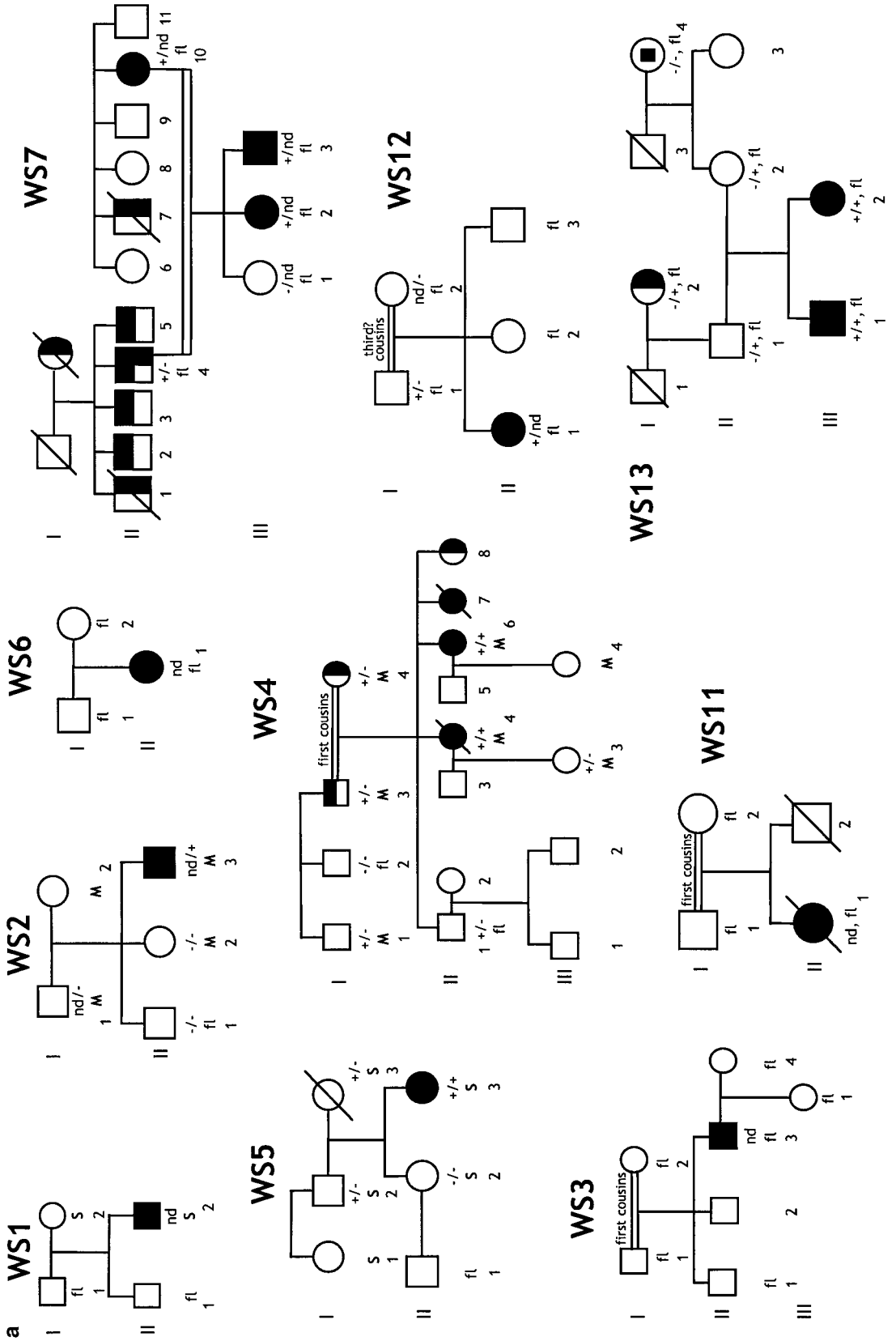
Sequencing reactions were carried out using primers at 0.3 μM and Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). Sequencing PCR consisted of an initial denaturation step at 94°C for 5 min, 30 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 15 s, and extension at 60°C for 3 min, then a 10-min final extension at 60°C. PCR products were purified through G-50 columns (Pharmacia Biotech) and loaded in a ABI Prism 377 sequencer (Applied Biosystems). Sequences were afterward analyzed using the program Sequencher v 3.0 (Gene Codes Corporation, USA).

mtDNA Analysis

Mitochondrial DNA was checked for major rearrangements by Southern blot. DNA samples were digested with *Pvu*II restriction endonuclease (Boehringer Mannheim, Germany), thus linearizing the mtDNA molecule. Digestions were then electrophoresed through an 0.8% agarose (FMC Bioproducts, USA) gel and blotted onto nylon membrane by alkali transfer according to the manufacturer's instructions (Amersham Life Sciences, UK). Hybridization with total mtDNA labeled with [α^{32}P]dCTP (Amersham Life Sciences, UK) or with fluorescence (Gene Images labeling and detection kits, Amersham Life Sciences, UK) was followed.

Additionally, several point mutations frequently identified in mitochondrial disorders that share phenotypic traits with WS were also analyzed in the mtDNA of patients, as well as some relatives. The point mutations studied were:

(i) G11778A, G3460A, T14484C, G15257A, G15812A, T3394C, A4136G, T4216C, and A4917G, associated to LHON. They affect genes coding for subunits 1, 2, 4, and 6 of NADH dehydrogenase and cytochrome b (17–19).


a
WS1
WS2
WS3
WS4
WS5
WS6
WS7
WS11
WS12
WS13
first cousins
third? cousins

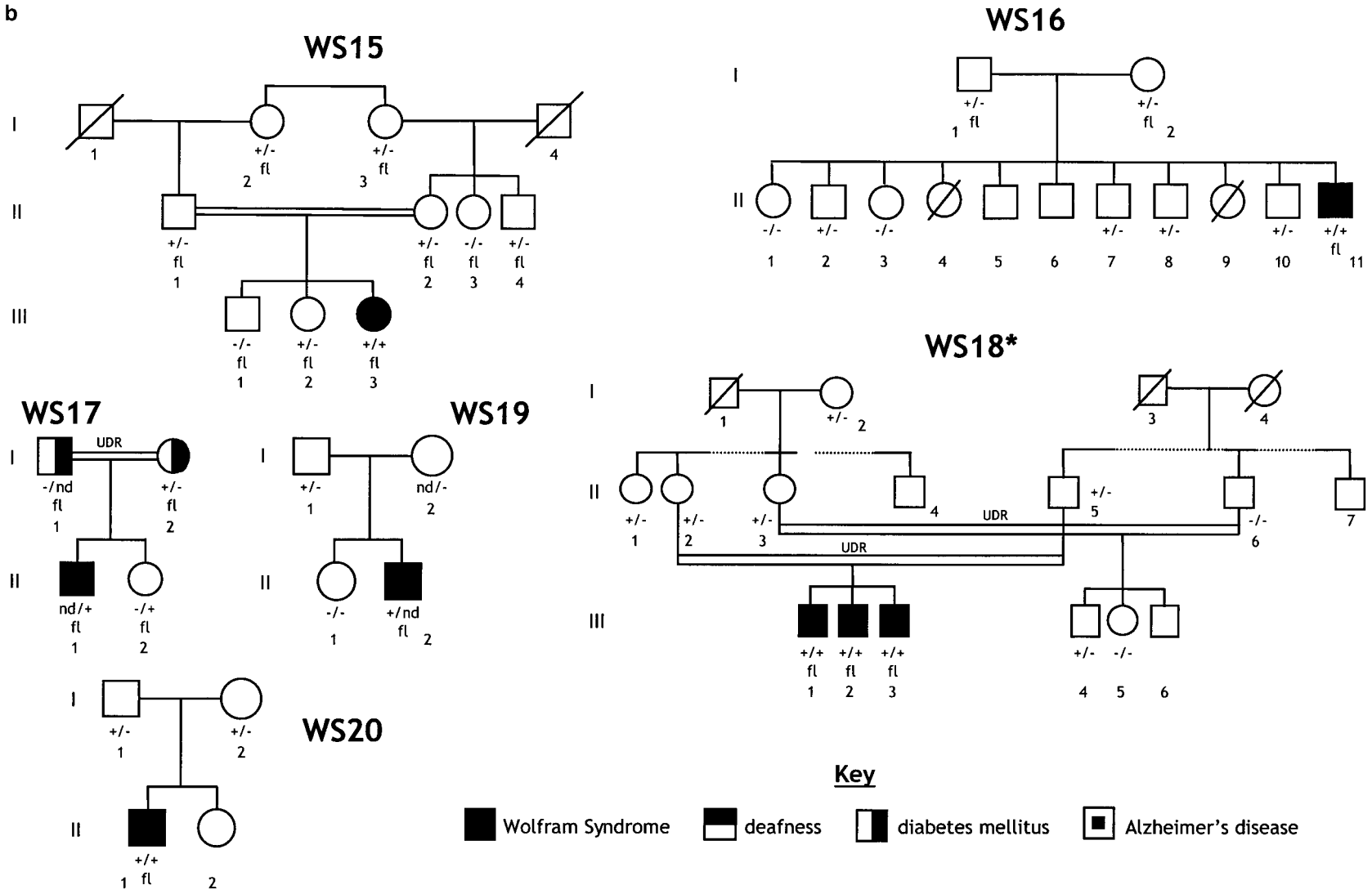


FIG. 1. WS families studied. Mitochondrial and *WFS1* gene mutation analyses were carried out for patients and also for relatives whenever the availability of DNA made it possible (for this reason, families WS8, WS9, and WS10 could not be included in the study). (−) Absence of *WFS1* mutations, (+) presence of *WFS1* mutations, (nd) no mutation detected, used for patients and obligate carriers. Slashes separate chromosomes 4. Detected *WFS1* mutations are specified in Table 2. (**S**) Single deletion, (**M**) multiple deletions, (fl), full-length (wild-type) mtDNA. UDR, unknown degree of relationship. (*) For the sake of clarity this pedigree has been greatly simplified. Dotted lines indicate the existence of other siblings.

TABLE 1
Main Characteristics of Spanish Wolfram Syndrome Patients

Family and patient number[a]	Age[b]	Gender	Consang.	DM	OA	DI	Deaf.	Renal tract abnormalities	Neurological abnormalities	Other complications	Family history
WS1 II2	34	M	−	24	+	−	+				
WS2 II3	33	M	−	8	16	23	16				
WS3 II3	33	M	+	20	−	−	−	Urethral sphincter disturbances	Cerebral atrophy, ataxia, tremor	Psychiatric disorders	
WS4 II4	42†	F	+	22	32	−	−	Urethral sphincter disturbances	Tremor, cerebral atrophy, Purkinje cell loss in cerebellum	Anxiety, abnormal behavior, anosmia, dysphagia. Death after food aspiration	DM, Deafness
WS4 II6	38	F	+	20	33	−	−	Urethral sphincter disturbances		Anxiety, abnormal behavior, anosmia, dysphagia, aspiration pneumonias	DM, Deafness
WS4 II7	35†[c]	F	+	18	30	−	−	Urethral sphincter disturbances	Tremor, cerebral atrophy, Purkinje cell loss in cerebellum	Anxiety, abnormal behavior, anosmia, dysphagia. Death after food aspiration	DM, Deafness
WS5 II3	26	F	−	8	11	13	16	Recurrent pyelonephritis, bilateral ureterohydronephrosis, weak bladder	Limb and truncal ataxia, brain stem and vermis atrophy	Dysmetria	
WS6 II1	18	F	−	3	+	−	+			Megaloblastic anemia	
WS7 II10	45	F	+	23	20	−	−		Atrophy of olfactory bulbs and tracts, atrophy of optic nerves and pons		DM, Deafness
WS7 III2	19	F	+	12	13	−	−				DM, Deafness
WS7 III3	17	M	+	12	10	−	−				DM, Deafness
WS11 II1	43†	F	+	10	15	+	−		Cerebral atrophy	Secondary amenorrhoea	
WS12 II1	36	F	+	4	7	25	18	Bilateral hydronephrosis			
WS13 III1	31	M	−	6	19	16	21	Neurogenic bladder	Cerebral and cerebellar atrophy, ataxia		DM
WS13 III2	26	F	−	5	10	16	18	Weak bladder	Cerebellar atrophy, paroxysmal vertigo		DM
WS15 III3	21	F	+	8	14	−	−		Ataxia		
WS16 II11	23	M	−	2	10	21	+	Urinary incontinence, weak bladder	Truncal and cerebellar atrophy		
WS17 II1	16	M	+	3	13	8	−		Cerebral and cerebellar atrophy		DM
WS18 III1	32	M	+	4	5	16	20	Urethral sphincter disturbances, one kidney non functional	Atrophy of pons and cerebellum	Hypogonadism, testicles atrophy, hemianosmia	
WS18 III2	30	M	+	8	9	14	+			Hypogonadism	
WS18 III3	28	M	+	10	9	12	+	Urethral sphincter disturbances		Hypogonadism	
WS19 II2	15	M	−	7	8	−	−		Ataxia, cerebellar hypoplasia	Antisocial behavior	
WS20 II1	45	M	−	3	+	−	33	Neurogenic bladder	Ataxia, vermis atrophy		

Note. M, male; F, female; Consang., consanguinity; DM, diabetes mellitus; OA, optic atrophy; DI, diabetes insipidus; Deaf., deafness. Ages of onset indicated in years, whenever known. −, absence of condition. +, presence of condition. †, Death of patient.

[a] As depicted in Fig. 1.

[b] Age in years.

[c] This patient died before the study was initiated.

(ii) A3243G, in the $tRNA^{Leu}$ gene. It is associated to MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) and is also described in several cases of maternally inherited diabetes mellitus and hearing loss (20).

(iii) A1555G mutation in the 12S rRNA gene, associated to nonsyndromic deafness (21), and largely detected in Spanish families with late-onset sensorineural deafness (22). This test was performed to elucidate whether deafness observed in some patients was due to WS itself or if it actually was nonsyndromic deafness.

Procedures for the screening of these point mutations were based on PCR amplification, digestion with restriction enzymes and subsequent separation of RFLPs on a 6–8% polyacrylamide

TABLE 2
Mutations in *WFS1* Gene and mtDNA of Spanish WS Families

Family	Nucleotide changes[a,b]	Amino acid changes	mtDNA point mutations	mtDNA deletions
WS1	nd	—	nd	Single
WS2	425ins16, 376G > A // nd	Multiple,[c] A126T	T14484C, T4216C	Multiple
WS3	nd	—	G15257A	nd
WS4	(2209G > A), 2452C > T // (2209G > A), 2452C > T	(E737K), R818C	A4136G	Multiple*
WS5	1046delTCT // 1046delTCT	350delF	nd	Single*
WS6	nd	—	nd	nd
WS7	1511C > T // (2020G > A)	P504L (G674R)	nd	nd
WS11	nd	—	T4216C, A4917G	nd
WS12	425ins16 // nd	Multiple[c]	T4216C	nd
WS13	425ins16 // 425ins16	Multiple[c]	nd	nd
WS15	1558C > T, 173 C > T // 1558C > T, 173C > T	Q520X, A58V	nd	nd
WS16	425ins16 // 425ins16	Multiple[c]	T4216C	nd
WS17	1060delTTC // nd	354delF	nd	nd
WS18	425ins16 // 425ins16	Multiple[c]	nd	nd
WS19	2338G > C // nd	G760R	nd	nd
WS20	425ins16 // 425ins16	Multiple[c]	nd	nd

Note. ( ), Probable polymorphism (see text for details). ins, insertion; del, deletion; nd, none detected; //, separate the two patient's chromosomes. * Detected in brain samples.

[a] The nucleotide changes in the *WFS1* gene shown in this table are those detected in patients' samples.

[b] Nucleotide position is counted from the first base of the start codon.

[c] As a result of this out-of-frame insertion, an aberrant amino acid sequence follows.

(Gibco BRL, UK) gel. Gels were stained in ethidium bromide and visualized under UV light. Oligonucleotides and enzymes are described elsewhere (22–24).

RESULTS

Clinical Data

We compiled the more relevant data of 23 WS patients (12 males and 11 females) from 16 families (Table 1). Consanguinity was reported in eight families (50%). Diabetes mellitus occurred at a median age of 8 years (range 2–24 years). Optic atrophy appeared (subsequently to diabetes mellitus in all but three cases) at a median age of 13 years (range 5–33). Twelve patients (52%) developed sensorineural deafness, and 11 suffered from diabetes insipidus (48%). These percentages might in fact be higher, because in some cases it was not possible to ascertain the occurrence of deafness or diabetes insipidus. Renal tract abnormalities (52%), neurological irregularities (61%), and other complications (52%) could also have been underestimated.

Mutations in the WFS1 Gene

Samples showing an abnormal pattern in SSCP analysis were sequenced and compared to the wolframin cDNA sequence (GenBank Access No. Y18064). Table 2 shows the mutations found in the Spanish pedigrees. No mutation was detected in families WS1, WS3, WS6, and WS11. Patients in the other 12 families harbored *WFS1* mutations, in either one or both alleles, as indicated in Table 2. Eleven allelic variants were identified: one in exon 2, two in exon 4, and eight in exon 8.

- Mutation 425ins16 in exon 4 (Fig. 2) was present in six families, that is 50% of pedigrees with *WFS1* mutations. Patients in families WS13, WS16, WS18, and WS20 were homozygotes for the insertion, whereas those in families WS2 and WS12 were heterozygotes. 425ins16 is predicted to produce an aberrant protein: assuming that no splicing alterations occur, translation will follow until residue 251, where a stop codon is created. The resultant protein is then more than two-thirds shorter than the wild type wolframin. The other identified changes are missense mutations in seven cases, in-frame deletions in two cases, and a nonsense mutation in one instance.
- In patient WS2 II3, mutations in exon 4 A126T and 425ins16 were detected in one chromosome. These mutations were not found in his father (Fig. 1) and therefore the patient must have inherited them

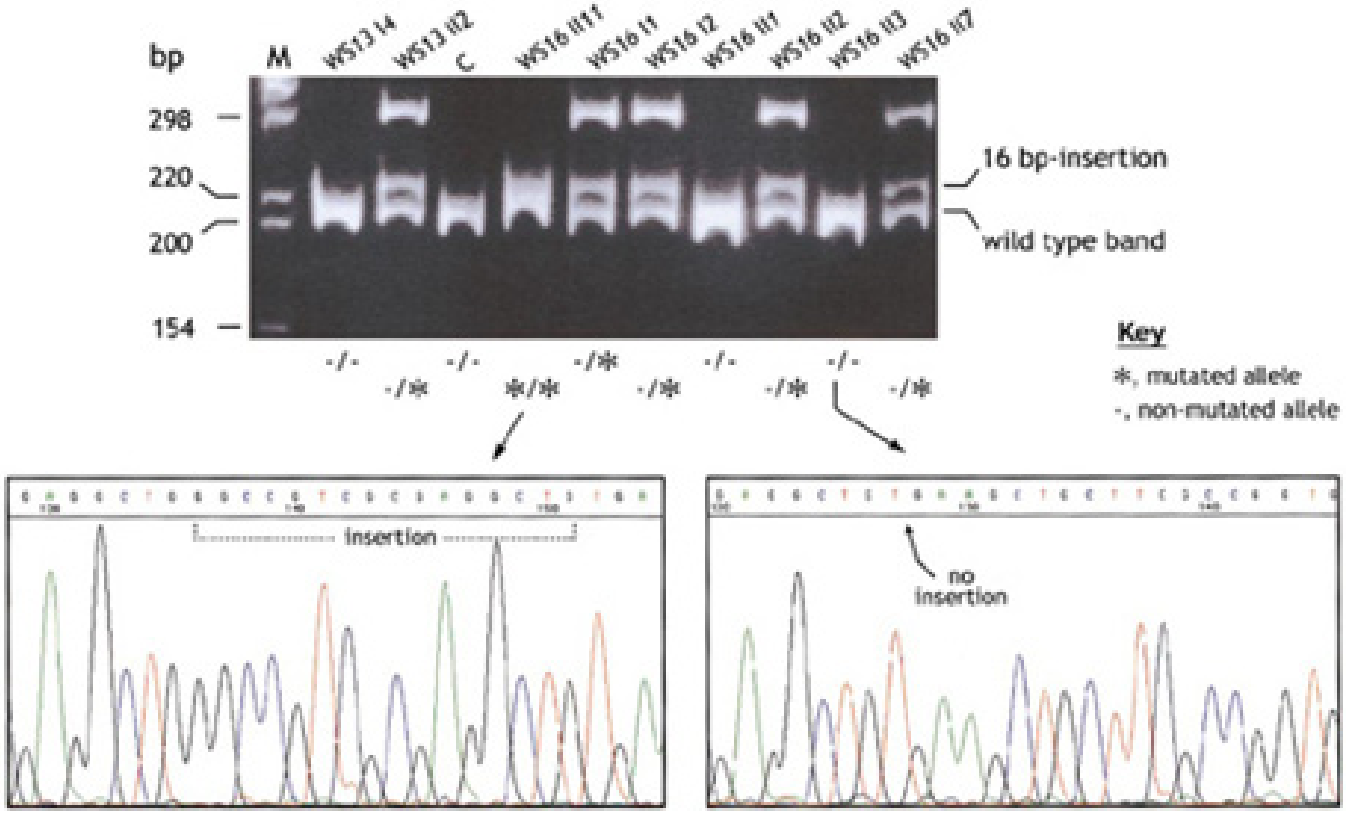


FIG. 2. Detection of mutation 425ins16. (Top) 10 exon 4 PCR products, loaded onto a 1% agarose gel. M corresponds to a DNA size standard; exact sizes in base pairs (bp) are indicated on the left. The rest of the lanes correspond to WS patients and relatives, as in Fig. 1. C, normal control. (Bottom) Exon 4 forward sequences of a homozygote for the insertion (left), and a normal individual (right).

from his mother. This could not be confirmed because no DNA sample from her was available at the time of the screening of *WFS1* gene. The paternal allele carrying a different pathogenic mutation is yet to be identified.

• DNA samples from two affected individuals in consanguineous family WS4 were homozygotes for substitutions E737K and R818C. The parents are first cousins and are heterozygotes for these mutations, as is the unaffected son WS4 II1. E737K results in a Glu → Lys exchange and R818C represents an Arg → Cys change (basic to polar residue), which is likely to cause a more important alteration in wolframin structure. In spite of not having detected it in control chromosomes, the variant 2209 G > A (E737K) could be a polymorphism in linkage disequilibrium with the mutation rather than a pathogenic mutation itself.

• In family WS7 two different variants were identified: P504L and G674R. The unaffected daughter (WS7 III1) is homozygous for G674R and therefore this change might in fact be a polymorphism. Both parents and the affected son and daughter are heterozygous for P504L. A different, as yet unidentified mutation carried by the mother (since she is a WS patient too) is needed to cause the syndrome in this pedigree.

• Patient WS15 III3 harbored in homozygosity the only nonsense mutation detected in this study. 1558C > T truncates the protein at residue 520 when a glutamine codon (CAG) is changed to a stop codon (UAG). He also carried the variant 173C > T (A58V) in both chromosomes, which probably represents a minor alteration in the wolframin. WS15 is also a consanguineous family and both grandmothers of individual III3 harbor the nonsense mutation Q520X.

• The WS patient in family WS19 was heterozygous for mutation 2338G > C, which transforms a glycine into an arginine at position 760. Because her father carries the G760R mutation, the mother is an obligate carrier of an unidentified second mutation.

• Patients in families WS5 and WS17 harbored different in-frame deletions in exon 8 (Table 2) that result in the deletion of a phenylalanine residue. According to the predicted structure of the wolframin protein (14) residues 350 and 354 are likely to be located in the first transmembrane domain. However, the ultimate effect of these deletions cannot be anticipated yet. Patient WS5 II3 was homozygous for 350delF while patient WS17 II1 was heterozygote for 354delF. He is a compound heterozygous, but the second mutation has not been identified yet. His mother also transmitted the chromosome harboring the TTC deletion to his sister (WS17 II2), who apparently did not inherit the affected chromosome from the father.

None of these *WFS1* variants were detected in 118 control chromosomes.

mtDNA Variations

• Several LHON mutations were identified in WS pedigrees (Table 2). Primary mutation T14484C and secondary mutation T4216C were detected in family WS2. Patients in families WS3 and WS4 harbored mutations G15257A and A4136G, respectively. T4216C and A4917G were found in family WS11. The same substitutions were identified in individual WS12 I1, father of a WS patient who consequently did not transmit them to his affected daughter. Likewise, in family WS16 the mutation T4216C was identified only in the father. All these point mutations except G15257A were detected in heteroplasmy. Various known mitochondrial polymorphisms were found in some families as well (data not shown).

• None of the Wolfram patients harbored mutations A3243G or A1555G, associated to maternally inherited diabetes mellitus and hearing loss, and nonsyndromic deafness, respectively.

• Previous studies demonstrated large mtDNA rearrangements in WS patients and relatives from families WS2 and WS4 (multiple deletions) (7), and WS1 and WS5 (single deletion) (9). The other family groups did not present rearrangements in their blood mtDNA (Table 2).

DISCUSSION

In view of the data that we have been able to collect, the intrafamilial variability regarding clinical manifestations is minor. Only two cases of significant differences can be noted. One is observed in family WS7, where the age of onset for diabetes mellitus in individual II10 was 10 years later than in her affected children. In addition, she suffered from optic atrophy prior to being diagnosed with diabetes mellitus (trait shared with her son III3), which is infrequent in WS. The other case of phenotypic differences within a family is seen in pedigree WS13, where patient III2 developed optic atrophy nine years earlier than her sibling.

When all the patients are considered, we can see that some present with diabetes insipidus and/or deafness, and some do not as yet. Eight families have renal tract abnormalities but not the other. The three affected brothers in family WS18 have hypogonadism, which has not been observed in the other Spanish patients. On the whole, we can consider interfamiliar variability to be quite significant among Spanish WS families.

In this study we have analyzed the presence of *WFS1* gene mutations in 22 WS patients and 58 relatives. In four families (WS1, WS3, WS6, and WS11) no mutations were detected. Clinical observations of patients from these families did not indicate atypical phenotypes except in patient WS3 II3, to whom we will refer later. Chromosome 4*p*16 haplotypes for families WS1 and WS3 do not exclude linkage to this chromosome (data not shown). Moreover, the possibility that mutations in the *WFS1* gene in these patients occur in regions other than exons 2 to 8 (promoter, intronic regions) cannot be ruled out. The genetic heterogeneity of WS has been demonstrated recently and a new locus in 4q22–q24 has been reported (16). Further studies will determine if families WS1, 3, 6, and 11 are linked to this region. Interestingly enough, likewise to those patients reported by El-Shanti and colleagues, WS patients in families 1, 3, and 6 did not suffer from diabetes insipidus. In contrast, none of these pedigrees presented with peptic-ulcer disease. Eleven *WFS1* allelic variants were detected in 18 patients, 8 being described for the first time (Table 2): seven missense changes, two in-frame deletions, one insertion, and one nonsense mutation. The three mutations previously reported are P504L, Q520X, and del354F, in Australian, Turkish, and British pedigrees, respectively (12–14).

The most frequent mutation in the Spanish pedigrees was the 16-bp insertion in exon 4, found in six pedigrees. A total of nine Wolfram patients harbored the insertion, which represents nearly 41% of cases. The high incidence of this mutation in the Spanish population could be explained by a founder effect.

Our results to date suggest first screening for this insertion when a new WS sample becomes available to the genetic laboratory.

The number of different mutations detected in this population is smaller than among the British population: 11 mutations in 16 families versus 24 mutations in 19 families. In the British study, only one individual of a group of 30 WS patients failed to present *WFS1* mutations. This observation would suggest once more genetic heterogeneity in Spanish WS patients.

Another feature of Spanish WS patients is that the types of mutations detected in our patients were different from those detected in other pedigrees (13,14). For instance, there was just one nonsense mutation among the 11 variants detected in Spanish patients (9% of detected mutations), as opposed to eight nonsense mutations among the 24 changes identified in British patients (33%).

Pathogenic LHON point mutation T14484C was identified in family WS2, along with secondary mutation T4216C. T14484C is a primary LHON mutation very frequent among LHON patients, and its presence in the mtDNA of patient WS2 II3 could explain the optic atrophy phenotype. We have not detected other primary LHON mutations in our patients and, to our knowledge, there is only one report of a WS patient harboring a primary LHON mutation (10). Since individual WS2 II3 is also a compound heterozygote for two *WFS1* mutations (the 16-bp insertion and the missense change A126T in one chromosome, and an unknown mutation in the other chromosome 4), his phenotype probably results from the combination of the mitochondrial and the nuclear mutations.

Mutation G15257A was found in homoplasmy in individual WS3 II3. The pathogenic effect of this mutation in Leber's neuropathy is quite controversial (19,25–27). WS3 II3 is a 33-year-old man who suffers from diabetes mellitus but not optic atrophy and who was included in the study because his other clinical manifestations are frequent in WS (Table 1). Since we did not detect *WFS1* gene mutations in this patient, and it is very unlikely that LHON mutation G15257A could be responsible for all his health problems, at present we should consider him not to be WS. Other mitochondrial or nuclear genetic defects not yet determined (perhaps in 4q22–q24) must account for the patient's clinical features.

The other LHON mutations identified in WS families are secondary mutations (A4136G, T4216C, and A4917G) whose clinical relevance in these families if any is probably minor.

Multiple mtDNA deletions had been found in families WS2 and WS4 (7), and single deletions in families WS1 and WS5 (9) (Fig. 1). As with *WFS1* mutations, we have not been able to find a relationship between these mtDNA deletions and patients' phenotypes. If mtDNA from target tissues (not from blood) were available for the studies, then perhaps a significant number of WS patients would show mitochondrial abnormalities. This point is illustrated by family WS4, in which patient II4 showed an average of 88% deleted mtDNA in brain regions, but only 5% in liver, skeletal muscle, or lymphocytes (7). We think that patients with no deletions in blood mtDNA could be harboring them in target tissues such as brain, pancreas or optic nerve. The role of mitochondrion in WS is yet to be discovered.

Now that a gene responsible for WS has been identified (and that researchers are currently pinpointing the second *WFS* locus in 4q), it is essential to know the cellular function of wolframin for a good understanding of the pathogenesis of the syndrome. Then we will definitively be able to assess if it is somehow implicated in mitochondrial metabolism and whether the model proposing that mitochondrial genetic defects or nuclear genetic defects would independently lead to the syndrome is correct (5). According to this model, a nuclear gene defect would interfere with the normal function of the mitochondrion, and would originate this autosomal recessive syndrome. When wolframin's function is known, we will also be able to understand the exact mechanism that underlies the presence of mtDNA deletions in some WS families, as it was proved that these deletions were linked to the disease locus (7). One hypothesis speculates that wolframin could participate in a mechanism that would protect the cells of the main tissues affected (neurons and endocrinium) from programmed cell death (28). If wolframin were in fact a member of the anti-apoptotic machinery, then a direct interaction with mitochondrion would not be too surprising.

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ADDENDA

En general les manifestacions clíniques dels pacients estudiats amb la síndrome de Wolfram són força coincidents amb les observades en d'altres poblacions europees tant quant a tipus com a edat d'aparició (Barrett 1995). És molt probable, però, que alguna de les entitats clíniques que acostumen a acompanyar la WS es trobi sub-representada a la Taula 1, potser per problemes en l'intercanvi d'informes clínics entre els diversos serveis hospitalaris que acostumen a atendre un cas WS (Endocrinologia, Neurologia, Oftalmologia, Medicina Interna, ...).

Quatre pacients pertanyents a famílies WS diferents no presentaren mutacions en cap dels exons del gen *WFS1* analitzats. Aquest fet pot tenir dues explicacions principalment: 1) que les mutacions es trobin en altres zones del gen, com ara l'exó 1 (**FIGURA 12**, pàg. 41), el promotor o les regions intròniques; 2) que aquests malalts tinguin algun altre gen mutant. En aquest sentit cabria la possibilitat que aquest gen fos *WFS2* (vegeu l'apartat **Genètica de la WS**, pàg. 39); de fet, els afectes de les famílies WS1, WS3 i WS6 no havien patit diabetis insípida fins el moment del treball, la qual cosa aniria a favor de la participació de *WFS2*. Nogensmenys en cap d'aquests malalts no consta que s'hagin donat hemorràgies gastrointestinals, detectades a 11 de 16 pacients amb mutacions en el segon locus per a la WS[35]. Un cop s'hagi situat aquest gen de forma precisa, caldrà examinar-lo sobretot en les famílies sense mutacions a *WFS1*.

El resultat més sorprenent recollit en aquest article és la presència d'una mutació prevalent en el 41% dels pacients estudiats i el 50% de les famílies amb mutacions. Això contrasta amb tots els treballs publicats fins el moment, en especial amb l'estudi de mutacions a *WFS1* en 19 famílies britàniques, en el qual en 30 malalts no es detectà cap mutació comuna (Hardy 1999). La mutació 425ins16 en l'exó 4 no ha estat per ara identificada a pedigrís WS d'altres orígens i hom podria pensar que la seva elevada freqüència és resultat d'un efecte fundador. Les sis famílies que presenten aquesta mutació provenen de Gipuzkoa (2), Andalucía (Granada i Córdoba), Valladolid i Barcelona. A la meitat de les famílies estudiades (8) s'ha identificat consanguinitat, però aquesta no consta en totes les famílies amb la inserció a l'exó 4. Les famílies WS2, WS13, WS16 i WS20 no són conscients d'unions entre parents en les generacions prèvies més immediates, malgrat a les tres darreres la inserció s'ha detectat en homozigosi.

Per tal de determinar si va ocórrer en el passat un efecte fundador que impliqués famílies amb la inserció 425ins16, o bé si l'alta proporció d'aquesta mutació es deu a múltiples esdeveniments mutacionals independents, caldria un estudi extensiu que caracteritzés genèticament el major nombre de generacions possibles de cadascuna de les genealogies implicades. Pero aquesta tasca sembla veritablement difícil, i més considerant l'esforç que ha suposat no tan sols aconseguir mostres de pacients amb la síndrome de Wolfram sinó també informació clínica d'ells i dels seus familiars. En qualsevol cas, l'existència d'una mutació tan comuna en població espanyola facilita el cribatge del gen *WFS1* en nous malalts WS, amb l'avantatge afegit que aquesta inserció es pot detectar mitjançant PCR i separació en un simple gel d'agarosa o acrilamida. La identificació d'aquesta mutació no ha d'esperar la separació i tinció d'un gel de SSCP ni la seqüenciació de l'exó.

Respecte a mutacions al mtDNA, la dada més destacable és que en un dels malalts amb la síndrome de Wolfram es va detectar una mutació primària LHON (pacient WS2 II3). Només coneixem un cas similar reportat el 1994 (Pilz 1994). A altres pacients i familiars es van identificar diverses mutacions secundàries; cap d'ells no tenia la mutació 1555G, la MELAS 3243G o les MERRF 8344G i 8356C (dada no inclosa a l'article). A banda de les delecions al mtDNA descrites prèviament (Barrientos 1996b; Barrientos 1996c) no es van detectar altres delecions a la resta de famílies analitzades. La presència de delecions es va estudiar tant per *Southern blot* com per PCR llarga, que és capaç

[35] No sembla que l'anèmia megaloblàstica de l'afecte WS6 II1 tingui relació amb les hemorràgies del tracte intestinal descrites a (al-Sheyyab 2001).

d'amplificar un percentatge molt més reduït de molècules delecionades.

Tret dels afectes de les famílies WS4 i WS5, en cap altre grup no va ser possible estudiar algun dels teixits diana de la síndrome, per això pensem que no es pot descartar l'existència de delecions al mtDNA dels pacients WS. El cas de la pacient WS4 II4 (a qui es detectà un 88% de delecions en diverses regions del cervell però tan sols un 5% en limfoblasts, fetge i múscul esquelètic), podria ser freqüent entre els pacients WS. Malauradament aquest dubte té una solució difícil, puix que la disponibilitat de teixit cerebral o pancreàtic és mínima.

Gràcies a la investigació de Takeda *et al*. se sap que *WFS1* s'expressa en molts teixits i òrgans (Takeda 2001); això no obstant, en la majoria de malalts amb la síndrome de Wolfram són principalment les cèl·lules endocrines i les regions neuronals les que es troben afectades. Aquests dos teixits tenen una necessitat d'ATP superior a la d'altres, la qual cosa fa pensar si en el mecanisme patogènic de la WS no estarà involucrada una alteració en la producció d'ATP mitocondrial. Altres òrgans amb el gen *WFS1* mutant potser no es veuen tan limitats funcionalment com el sistema nerviós central i pàncrees.

La proteïna WFS1 s'ha detectat a fraccions cel·lulars que contenen el reticle endoplasmàtic (RE), malgrat no presenta els senyals típics de reconeixement per RE[36] (Takeda 2001). La localització de WFS1 a RE suggereix tres possibles funcions per aquesta proteïna:

- moviment de membranes
- processament de proteïnes i/o
- regulació de l'homeòstasi del calci (Ca^{2+}) a RE

Takeda *et al*. suggereixen que la proteïna WFS1 podria funcionar fisiològicament en un procés que protegís certes poblacions de cèl·lules neuronals i endocrines d'una via apoptòtica exclusiva de RE. La seva localització a algunes regions cerebrals determinades indica que podria influir també en el control emocional i de comportament. En els darrers anys s'estan multiplicant els estudis sobre RE i com regula aquest les concentracions de Ca^{2+} ($[Ca^{2+}]$) citoplasmàtic en teixits del sistema nerviós. També s'està descobrint la seva participació en vies apoptòtiques descrites en malalties neurodegeneratives (Mattson 2000).

El grup de Takeda descarta la participació mitocondrial en la WS pel fet que la proteïna WFS1 es localitza a RE i no en aquests orgànuls.

Nosaltres pensem que el paper del mitocondri en la WS no es pot excloure encara. El RE és una estructura que es perllonga de la membrana nuclear externa i amb freqüència es troba íntimament associada a la membrana plasmàtica i al mitocondri. L'estreta relació espacial entre RE i mitocondri s'ha posat de manifest en cèl·lules HeLa amb la utilització de dues proteïnes fluorescents que contenen dianes per aquestes dues estructures (Rizzuto 1998). Entre un 5 i un 20% de la superfície mitocondrial es troba en

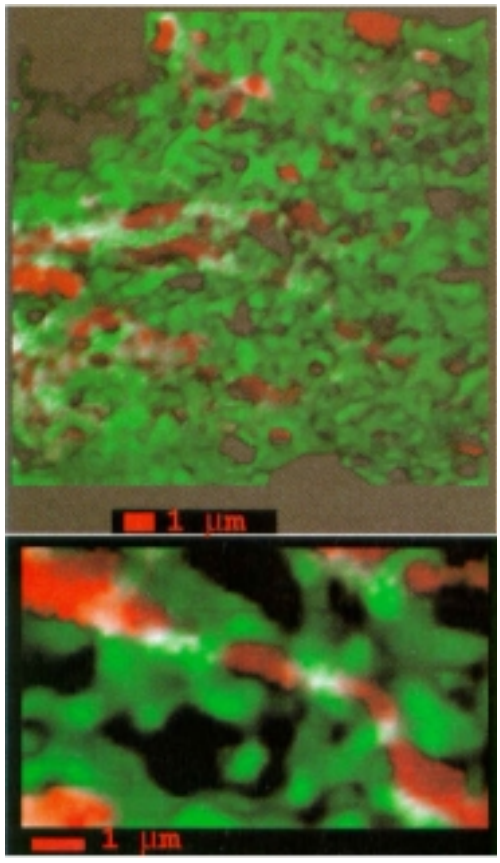


FIGURA 14. MITOCONDRIS I RE.

Reproduïda de Rizzuto *et al*. (Rizzuto 1998). Imatges combinades en 3D de mitocondris i RE en cèl·lules HeLa. Els mitocondris es veuen en color vermell i el RE en color verd. Les zones de superposició són blanques.

[36] Però no totes les proteïnes que tenen com a destí el RE porten aquests senyals.

contacte íntim amb el RE (**FIGURA 14**). En aquestes zones es poden formar microdominis de [Ca^{2+}] elevades generats en obrir-se els canals d'inositol(1,4,5)trifosfat al RE. Es pensa que aquests microdominis fan que una gran quantitat de Ca^{2+} s'incorpori a la matriu mitocondrial en un temps molt concret i curt, de forma que el potencial de membrana interna generat en la CRM no es dissipa en un flux continu i innecessari de Ca^{2+}. La coordinació entre l'alliberament del catió des del RE i la captació per part del mitocondri podria estar alterada en certes situacions patofisiològiques (Rizzuto 2000).

L'energia necessària per a la síntesi d'ATP i l'entrada de Ca^{2+} la proporciona el gradient electroquímic de protons de la CRM. Això ha estat demostrat pel grup de la doctora Rizzuto en un treball amb cíbrids preparats a partir de cèl·lules MERRF i cèl·lules NARP (Brini 1999). Mentre que les cèl·lules NARP (que tenen una mutació al gen mitocondrial de l'ATP sintasa que impedeix la síntesi d'ATP però no trastorna substancialment la CRM) presenten una resposta del Ca^{2+} mitocondrial a agonistes completament normal, en les cèl·lules MERRF aquesta està dràsticament reduïda. Les mutacions MERRF alteren un tRNA, el que provoca un dèficit en la síntesi proteica mitocondrial general que afecta la CRM i la fosforilació oxidativa.

L'alteració de l'homeòstasi del Ca^{2+} en el mitocondri pot impedir l'activació de les funcions mitocondrials dependents de calci. Els enzims de la matriu isocitrat, oxoglutarat i piruvat deshidrogenases són regulats per la [Ca^{2+}]; d'altra banda, l'augment de la [Ca^{2+}] en la matriu estimula el metabolisme mitocondrial de manera que es poden satisfer les demandes energètiques en cèl·lules estimulades (Pozzan 2000).

A més, els mitocondris regulen la [Ca^{2+}] local en microdominis cel·lulars i per tant modulen el senyals de calci en molts tipus cel·lulars (Duchen 2000).

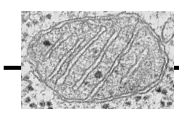
En definitiva, sembla prematur excloure la implicació mitocondrial en la WS, ja que les mutacions a *WFS1* podrien tenir com efecte l'alteració de l'homeòstasi del calci en el RE, que repercutirien en la [Ca^{2+}] mitocondrial i, conseqüentment, en la [Ca^{2+}] cel·lular. Aleshores totes les vies dependents de senyals de Ca^{2+} es veurien alterades. El mateix funcionament tissular acabaria impedit per l'augment d'espècies d'oxigen reactiu dins del mitocondri i de l'engegada de les vies apoptòtiques de RE i de mitocondri. Se sap que alteracions de l'homeòstasi del Ca^{2+} en RE poden induir apoptosi. A més, Bcl-2 s'associa a RE i a membranes mitocondrials i aquesta associació pot estabilitzar l'homeòstasi del Ca^{2+} i eliminar l'estrès oxidatiu.

És d'esperar que en poc temps s'esbrinarà completament la funció de la proteïna WFS1 i s'aclarirà aquesta qüestió. Potser llavors s'obriran noves perspectives en el tractament de malalts amb la WS.

Mentre això no passa, caldrà seguir estudiant noves famílies amb la síndrome de Wolfram, determinar el gen nuclear mutant, les alteracions al mtDNA, examinar el comportament de la CRM i de la fosforilació oxidativa. Si és possible, generar també híbrids transmitocondrials i estudiar els tipus i proporcions de RNA als teixits diana.

12 ARTICLE "RESPIRATORY CHAIN DYSFUNCTION ASSOCIATED WITH MULTIPLE MITOCHONDRIAL DNA DELETIONS IN ANTIRETROVIRAL THERAPY-RELATED LIPODYSTROPHY"

13 ARTICLE "MITOCHONDRIAL INVOLVEMENT IN ANTIRETROVIRAL THERAPY-RELATED LIPODYSTROPHY"



represent important candidate pathways for involvment in HAART-induced lipodystrophy. For example, nuclear lamins contribute to the maintenance and integrity of the nuclear envelope in various cell types. The alteration of lamin-dependent nuclear functions because of genetically determined or pharmacologically induced inhibition in dividing cells may result in cell death. However, we found no homology of the amino acid sequences between lamin A/C and HIV-1 protease, providing no evidence for the direct inhibition of lamins by HIV-1 protease inhibitors. Extending our knowledge on the interaction of intermediate filaments such as lamins and other nuclear factors in the pathogenesis of complex metabolic and tissue-related disorders will provide new strategies to elucidate HAART-associated lipodystrophy and drug-related adverse events.

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Respiratory chain dysfunction associated with multiple mitochondrial DNA deletions in antiretroviral therapy-related lipodystrophy

Highly-active antiretroviral therapy (HAART) can induce a characteristic lipodystrophy syndrome characterized by peripheral fat wasting and central adiposity, usually associated with hyperlipidaemia and insulin resistance [1,2]. Indirect data have led some authors to propose that mitochondrial dysfunction could play a role in this syndrome [3,4].To date, as recently outlined by Kakuda *et al*. [5]in this journal, HIV-infected patients developing lipodystrophy have not been studied for mitochondrial changes or respiratory chain capacity.

We studied a 67-year-old woman with an unremarkable past history except for HIV infection diagnosed *5* years earlier, without previous opportunistic infections. During the 4 months before the current evaluation, she developed central and peripheral lipodystrophy associated with raised serum triglyceride levels (from 130 to 543 mg/dl) and no changes in cholesterolaemia or glycaemia. The total CD4 lymphocyte count was 401/mm^3, and viral load was 790 copies/mm^3. She had previously received zidovudine (total cumulative dose;

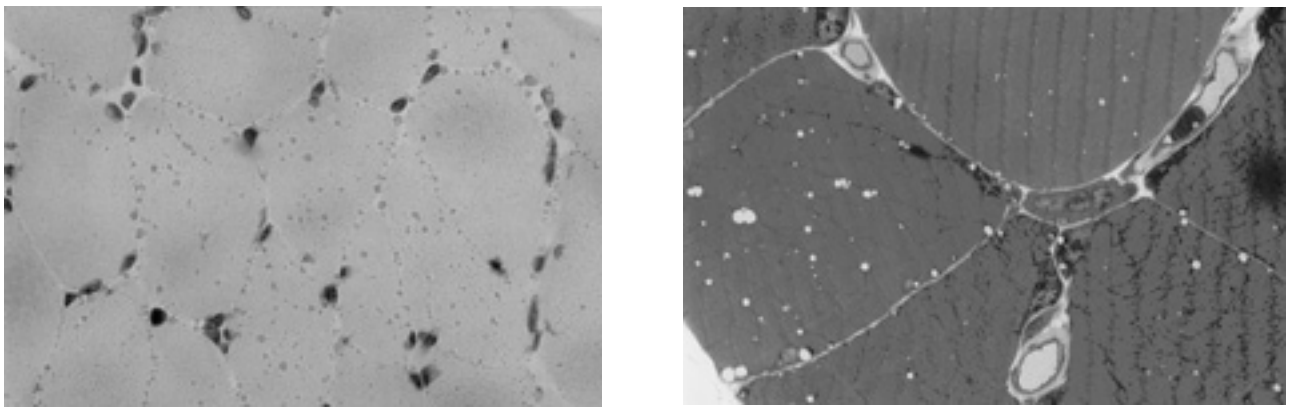


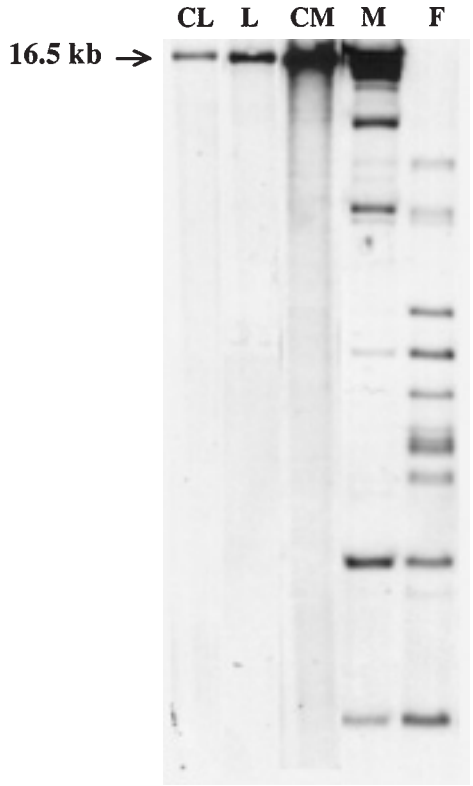
Fig. 1. Skeletal muscle from HIV patient with highly-active antiretroviral therapy-related lipodystrophy. Oil-red 0 staining shows abundant neutral lipids (up; original magnification: 200×). Lipid droplets are also evident in semithin sections (down, original magnification: 800×).

TCD 200 g) and didenosine (TCD 216 g) and, when lipodystrophy appeared, she was receiving saquinavir (TCD 1512 g), lamivudine (TCD 288 g) and stavudine (TCD 58 g). After obtaining informed consent, a muscle biopsy was performed. Some subcutaneous fat lobules were removed during the same surgical procedure. Thirty mL of venous blood was extracted for the isolation of lymphocytes.

A fragment of skeletal muscle was used for histological and ultrastructural studies. The biochemical function of the mitochondrial respiratory chain (MRC) was analysed on fresh skeletal muscle mitochondria and lymphocyte suspensions using a double method. First, state III respiratory rates were pollarographically determined using pyruvate-malate, succinate, glycerol-3-phosphate and ascorbate as substrates that transfer electrons at complexes I, II, III and IV of MRC, respectively. Intact cell respiration was also assessed for lymphocytes. Second, the individual enzyme activity for each complex of the MRC was spectrophotometrically quantified. For molecular studies, whole DNA of skeletal muscle, lymphocytes and adipocytes were extracted and analysed by Southern blotting and polymerase chain reaction (PCR), to look for rearrangements and to quantify mitochondrial DNA (mtDNA) abundance. The methodology has been reported elsewhere [6–8].

We found abundant lipid storage inside both type I and II myocytes on a histological study of skeletal muscle (Fig. 1); the rest of the morphological examination was otherwise irrelevant. With respect to biochemical analyses, clearly decreased complex III and IV activities were found in both skeletal muscle mitochondria and lymphocytes, which in turn caused a decay in state III respiratory rates for nearly all substrates. When mtDNA was analysed, multiple deletions were found in skeletal muscle and adipocytes, but not in lymphocytes (Fig. 2), whereas the total amount of mtDNA was preserved in all tissues.

We believe this is the first case of an HIV-infected patient with HAART-related lipodystrophy in whom



Enzyme activity (nmol·min^{-1}·mg protein^{-1})	L	M
Complex I	-	128 (*29-143*)
Complex II	30 (*12-72*)	67 (*42-239*)
Complex III	**16** (*70-290*)	**136** (*815-3131*)
Complex IV	**27** (*50-280*)	**182** (*301-2556*)
Oxidative activity (nmol oxygen·min^{-1}·mg protein^{-1})	L	M
Intact cell respiration	**5.6** (*6-26*)	-
Pyruvate-malate	**2.0** (*4-22*)	30.5 (*25-91*)
Succinate	**3.5** (*4-24*)	**27.2** (*43-148*)
Glycerol-3-phosphate	**2.2** (*4-20*)	**5.3** (*8-38*)
Ascorbate	**-**	**18.7** (*160-497*)

Fig. 2. Left: Southern blot. Samples were digested with *Pvu* II, electrophoresed in a 0.8% agarose gel, blotted onto nylon membrane, and hybridized with total mitochondrial DNA (mtDNA). The arrow indicates the normal 16.5 kb mtDNA bands. CL, Control lymphocytes; CM, control muscle. L, M, and F correspond to lymphocytes, muscle and fat from the patient. Muscle and fat from the patient presented abundant multiple deletions. Right: Enzyme and oxidative activities from lymphocytes and muscle were decreased (bold numbers) for most of the parameters and were evaluated with respect to control values (italic numbers between brackets).

mitochondrial dysfunction was demonstrated. Protease inhibitors have been invoked as the main cause of the syndrome through disturbing adipocyte metabolism and causing apoptosis by means of altered retinoid signalling [1,9]. In addition, it has also been proposed that the well-known mitochondrial toxicity of nucleoside analogue reverse transcriptase inhibitors [8]could contribute to the development of HAART-related lipodystrophy, on the basis of its occasional appearance in patients on protease-sparing regimens, and on its similarity with multiple symmetrical lipomatosis, a disorder sometimes associated with single and multiple mtDNA deletions and decreased complex IV activity [10]. Why the effects of adding protease inhibitors to nucleoside analogue reverse transcriptase would produce a lipodystrophy syndrome instead of the more classical phenotype resembling an inherited mitochondrial disease is not known. We hypothesized that protease inhibitors could interfere with certain patient proteases in a selective way in different tissues. In this sense, some proteases are essential for mitochondrial biogenesis and function, such as mitochondrial processing of peptidases involved in the import and activation of mitochondrial protein precursors (including diverse DNA-encoded MRC subunits) synthesized in cytoplasmic ribosomes.

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Lack of interference between ribavirin and nucleosidic analogues in HIV/HCV co-infected individuals undergoing concomitant antiretroviral and anti-HCV combination therapy

HIV and hepatitis C virus (HCV) share parenteral routes of transmission through exposure to contaminated blood. The prevalence of HCV seropositivity among HIV-infected homosexual/bisexual men and intravenous drug users has been reported to be 10 and 80%, respectively [1]. Co-infection with HIV alters the clinical course of hepatitis C by inducing a more rapid progression to fibrosis and cirrhosis [2]. The morbidity and mortality rate from liver disease is thus higher in co-infected patients than in immunocompetent patients infected with HCV alone [3], emphasizing the need for active therapeutic intervention to treat HCV infection in co-infected patients [4]. On the basis of the promising results of combination therapy with IFN-α and ribavirin for chronic hepatitis C in immunocompetent individuals [5], we have recently engaged in an open prospective trial of this combination therapy in patients co-infected with HCV and HIV [6].

As ribavirin, zidovudine (ZDV) and stavudine (D4T) are metabolized to their triphosphate active forms by thymidine kinase, ribavirin has been suggested to reduce the intracellular phosphorylation of nucleosidic reverse transcriptase inhibitors [7]. No data are, however, available at this time on the clinical relevance of this putative antagonistic interaction. We have investigated the changes in plasma HIV-RNA levels during and after combination therapy with IFN and ribavirin in the first 38 HIV/HCV co-infected patients of our cohort undergoing stable antiretroviral therapy to reach 6 months of follow-up after discontinuation of a 6 or 12 month course of IFN and ribavirin. Nineteen of the 38 patients had been treated with a combination of two nucleosidic analogues, D4T and lamivudine (3TC) ($n = 15$) or ZDV and 3TC ($n = 4$) for a mean duration of 20 ± 10 months before the initiation of IFN and ribavirin. The remaining 19 patients had been treated with a triple combination antiretroviral regimen includ-

Mitochondrial involvement in antiretroviral therapy-related lipodystrophy

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Objectives: The management of HIV infection has greatly improved during recent years essentially because of the appearance of new antiretroviral drugs. Highly active antiretroviral therapy (HAART) has achieved important reductions of viraemia and significant recoveries of $CD4^+$ cell counts in HIV-infected patients. Nonetheless, cases of HIV-infected individuals experiencing lipodystrophy (LD) are being increasingly reported. The purpose of this work was to analyse whether the presence of mitochondrial abnormalities is a frequent feature in LD, since we previously detected mitochondrial abnormalities in an HIV-patient. The second main objective was to study whether LD could be associated with a specific drug.

Design: Seven HIV patients presenting LD and five HIV non-LD controls participated in the study. LD patients met the following criteria: (1) LD was their only clinical abnormality, (2) LD was clinically relevant, (3) compliance with antiretroviral treatment was higher than 90% and (4) patients did not have personal or familial history suggestive of mitochondrial disease or neuromuscular disorder.

Methods: Histological stainings, histo-enzymatic reactions, enzymatic and respiratory activities of mitochondrial respiratory chain complexes, and mitochondrial DNA (mtDNA) depletion and rearrangements were examined on muscle mitochondria.

Results: Structural muscle abnormalities, mitochondrial respiratory chain dysfunction or mtDNA deletions were detected in all HIV lipodystrophic patients.

Conclusions: The mitochondrial abnormalities found suggest that mitochondrial dysfunction could play a role in the development of antiretroviral therapy-related lipodystrophy.

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Keywords: Lipodystrophy, HIV, antiretroviral therapy, reverse transcriptase inhibitors, protease inhibitors, mitochondrial respiratory chain, mitochondrial DNA deletions

Introduction

Lipodystrophy (LD) syndrome is characterized by atrophy of adipose tissue (mainly in subcutaneous fat), fat redistribution (limb and facial fat decreases whereas abdominal and trunk fat increases) and diverse metabolic disturbances (insulin resistance, hypercholesterolaemia and hypertriglyceridaemia) [1–4]. During the last few years incomplete variants of this syndrome have also been recognized.

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Most lipodystrophic cases have been linked to the administration of protease inhibitors (PI), although nucleoside reverse transcriptase inhibitors (NTRI) could also have a role in LD development [1]. Nowadays it is currently accepted that LD is closely associated with the introduction of highly active antiretroviral therapy (HAART) [5–7].

The pathogenesis of LD is unknown, but some hypotheses have been suggested, such as the possible interaction between the PI activity and the hepatic lipoprotein receptor binding [4]. Carr *et al.* pointed out the fact that there is a 63% homology between HIV-1 protease and the low-density lipoprotein-receptor-related protein, and 58% between this protease and the cytoplasmic retinoic-acid binding protein type 1 [8]. These molecules would be inhibited by PI and, consequently, increased apoptosis of peripheral adipocytes and hyperlipidaemia would follow. Another hypothesis considers an imbalance between peripheral lipolysis and lipogenesis, both of which are regulated by cortisol and dehydro-epiandrosterone [9]. The PI could also modulate adipocyte differentiation. In fact, subcutaneous adipocyte apoptosis occurs in lipoatrophic areas of HIV-seropositive PI-treated patients [10].

The direct involvement of mitochondria in LD is under discussion. It is well known that the mitochondrion may be damaged by NRTI, especially by zidovudine. Cellular contents of mitochondrial DNA (mtDNA) in muscle fibres are lowered by the prolonged use of zidovudine [11,12], eventually developing muscle complaints, which has been termed zidovudine-myopathy. The contents of mtDNA raises again when zidovudine is tapered [13]. It is also acknowledged that mitochondrial cardiolipins, by means of their interaction with added L-carnitine, act by preventing the myopathy that may follow a sustained treatment with NRTI [14]. Persistent mitochondrial dysfunction after perinatal exposure to NRTI has been demonstrated in some cases [15]. Some authors have postulated that, in addition to the physiopathological role of PI, the mitochondrial toxicity induced by NRTI could play an essential part in the development of LD [5,16]. Brinkman noted that many side effects attributed to the administration of NRTI (myopathy, lactic acidosis, polyneuropathy, etc.) match with clinical manifestations that are characteristic of mitochondrial diseases. Nonetheless, to date, evidence of such mitochondrial damage is essentially lacking.

Very recently, our group detected respiratory chain dysfunction and multiple mtDNA deletions in fat and skeletal muscle samples of an HIV-infected patient developing LD [17]. This case prompted us to further investigate whether the presence of mitochondrial abnormalities is a frequent feature in LD.

Materials and methods

Participants

The patients invited to participate in the present study came from a cohort of 344 HIV-positive outpatients controlled at the Hospital of Granollers. The criteria of inclusion were the following: (1) LD was the only clinical abnormality at the time of initiating the study, (2) LD was clinically relevant, (3) compliance with antiretroviral treatment was higher than 90% and (4) patients did not have a personal or familial history suggestive of mitochondrial disease or neuromuscular disorder. Patients were also investigated for previous symptoms or signs attributable to zidovudine-myopathy through a scrupulous neuromuscular examination to detect any muscular dysfunction, and they were accordingly excluded if positive. Eleven patients fulfilling the inclusion criteria were identified as potential participants in the study. Seven patients (six men and one woman) finally accepted (LD-HIV). The woman has been reported separately [17].

Five individuals with advanced HIV disease who did not present any sign or symptom of LD were included as an HIV control group (non-lipodystrophic HIV patients, NLD-HIV). Three of these patients had not received treatment (NLD-HIV no. 1, 2 and 3), and the other two had been under HAART for 18 and 6 months (NLD-HIV individuals no. 4 and 5, respectively). All these individuals were in the C_3 stage [18], and presented a mean CD4+ cell count of $86 \pm 83 \times 10^6$ cells/l. Viral loads were greater than 3000 copies/ml in all cases except individual NDL-HIV no. 4, who had < 50 copies/ml.

The muscle studies of twelve healthy non-HIV male volunteers, matched by age, who had been recruited in other previous unrelated protocols were also used as healthy controls (HC). Histological studies were available in twelve cases, biochemical studies in eleven cases, and molecular studies in six cases.

The Ethical Committee of the Hospital Clínic and Hospital Fundació-Asil de Granollers approved the protocol, and patients and controls gave informed consent.

Methods

Obtaining the samples

Skeletal muscle biopsies from quadriceps were obtained by an open surgical procedure under local anaesthesia, and were divided in fragments to perform histological, biochemical and molecular studies. When possible, subcutaneous fat lobules were removed during the same surgical procedure for molecular studies. Additionally,

30 ml of venous blood were also extracted from LD-HIV to perform molecular analysis.

Histological studies

Skeletal muscle fragments were routinely frozen in cooled isopenthane and processed for conventional staining and histo-enzymatic reactions on cryostat sections (6 μm-thick) as described elsewhere [19]. Morphologic mitochondrial irregularities were specifically analysed by Gomori's trichrome staining, which detects the presence of ragged-red fibres (RRF), and also by cytochrome *c* oxidase and succinate dehydrogenase reactions, which identify RRF-equivalents.

Biochemical studies

All biochemical analyses on skeletal muscle were carried out on enriched mitochondrial suspensions, which were prepared following standard procedures [20].

Respiratory activity

State III respiratory rate (oxygen consumption) was polarographically determined at 37°C in a water-jacketed cell (Hansatech Instruments Limited, Norfolk, UK) containing 250 μl of adequate respiratory medium using glutamate-malate, succinate and ascorbate-TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) as substrates, which respectively transfer electrons to complex I, II and IV of the mitochondrial respiratory chain (MRC). We used 10 μg of mitochondrial protein to perform each experiment.

Enzyme activity

Absolute enzyme activity (AEA) for each MRC complex was spectrophotometrically quantified (UVIKON 922, Kontron AS, Switzerland). Between 10 and 40 μg of mitochondrial protein were utilized to determine the activity of complexes, except for complex IV, for which 2–4 μg were used. Measurements of complex I (rotenone-sensitive NADH-decylubiquinone oxidoreductase), complex II (succinate decylubiquinone DCPIP reductase), complex III (ubiquinol-cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase) were performed at 37°C in a total volume of 1 ml as previously described [21].

Derivative ratios

All ratios between respiratory and enzyme activities were estimated, as they are more sensitive in detecting partial MRC defects than AEA [22,23]. To assess the coupling state of mitochondria, respiratory control ratios (RCR) for glutamate and succinate were also estimated by calculating the ratio between the oxygen consumption in the presence of ADP (state III) and the oxygen consumption in absence of ADP (state IV).

Molecular studies

Total DNA was extracted from muscle biopsies, subcutaneous fat lobules and peripheral blood samples. Isolation of DNA was based on proteinase K digestion followed by a standard phenol–chloroform extraction.

The mtDNA depletion and rearrangements were examined by Southern blot analysis: samples were digested with *Pvu* II restriction endonuclease (Boehringer Mannheim, Mannheim, Germany), electrophoresed through a 0.8% agarose gel, and blotted onto nylon membrane by alkali transfer according to the manufacturer's instructions (Amersham Pharmacia Biotech., Freiburg, Germany). Hybridization with complete mtDNA labelled with fluorescein or α–^{32}P–dCTP followed. After several exposures, the blots were stripped to allow for a second hybridization. Subsequently, to investigate mtDNA depletion two probes consisting in total mtDNA and a fragment of nuclear 18S gene were used for hybridizing together the stripped blots [11].

Additionally, two mitochondrial point mutations described in patients presenting lipomas were also studied in LD-HIV patients. MERRF (myoclonic epilepsy and ragged-red fibres) mutation A8344G (tRNA^{Lys} gene) was analysed by polymerase chain reaction (PCR) amplification using an oligonucleotide spanning positions 8201-8220 of the mtDNA light chain (according to the Cambridge sequence [24]), and the mutagenesis primer GTATTTAGTTGGGGCATTTCACTCTA at position 8375-8345 of the heavy chain. Twenty microlitres of each PCR product were digested with *Bgl* I restriction endonuclease (New England Biolabs, Hitchin, Hertfordshire, UK) and electrophoresed through a 7% acrylamide gel. Mutated samples would produce two fragments of 143 and 32 bp, in contrast to the 175 bp wild-type fragment.

The mtDNA mutation A12217G in tRNA$^{Ser(AGY)}$ gene [25] was studied by standard single-stranded conformation polymorphism analysis in 12.5% acrylamide gels (Amersham Pharmacia Biotech.) and direct sequencing.

Statistical analysis

Results are expressed as mean ± SD. Comparisons among groups were performed by means of one-way analysis of variance.

Results

The patients included in the study had developed LD after a variable period under HAART, and the time elapsed between LD diagnosis and the inclusion in the study ranged from 2.5 to 22 months (average 11.6 months). Table 1 shows clinical and anthropometric data of LD-HIV patients at the time of inclusion in the

Table 1. Main clinical, immunological and analytical data of LD-HIV patients.

Patient	Age/gender[a]	Weight (kg)	Height (cm)	BMI[b]	HIV infection (months)	LD (months)	Distribution of LD	CDC stage [18]
1	33/M	65	177	20.7	26	9	peripheral	B_3
2	36/M	60	169	21.0	56	12	peripheral	C_3
3	31/M	73	178	23.0	72	19	central, peripheral	C_3
4	43/M	81	170	28.0	75	2.5	peripheral	C_3
5	67/F	50	150	22.2	54	4	central, peripheral	B_3
6	35/M	80	172	27.0	88	13	peripheral	C_3
7	42/M	70	178	22.1	72	22	central, peripheral	A_2

Patient	CD4+ count (cells $\times 10^6$/l)	Viral load (copies/ml)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	c-HDL (mg/dl)	c-LDL (mg/dl)	Glucose (mg/dl)	C-peptide (ng/ml)
1	336	508	210/177	209/336	–/24	–/103	79/89	–/3.7
2	170	< 200	159/224	114/205	–/51	–/135	100/93	–/2.3
3	313	46494	172/176	242/203	22/24	–/70	78/77	–/1.0
4	133	1711	261/234	194/110	35/34	204/178	109/117	–/3.9
5	401	790	204/211	130/543	–/23	–/–	99/102	–/5.5
6	276	4024	150/207	139/182	–/39	–/132	78/94	–/2.2
7	571	525	271/278	175/376	41/37	125/–	98/94	–/2.8

[a]Age is indicated in years; M, male; F, female. [b]Body mass index (kg/m^2). LD-HIV, HIV-positive patients with lipodystrophy (LD): selection criteria are described in 'Participants' paragraph of 'Materials and methods'; CDC, Centers for Disease Control; c-HDL, high-density lipoprotein cholesterol; c-LDL, low-density lipoprotein cholesterol; / , separate values before and after LD diagnosis. –, not determined.

study, and the immunological and analytical parameters determined before and after the development of LD. Amylase and lipase values were normal for all of them. The LD-HIV patients no. 1, 5 and 6 showed a two- to three-fold increase in concentrations of transaminases. None of the patients exhibited clinical, radiologic or electrocardiographic evidence of heart dysfunction.

Figure 1 shows the treatment of LD-HIV patients in detail, including duration and total cumulated doses.

Histological changes in skeletal muscle of LD-HIV patients were varied and abundant. Six individuals exhibited structural abnormalities: two presented mitochondrial abnormalities (LD-HIV patients no. 1 and 3 showed 10 and 2% of RRF, respectively), two showed non-specific lipid storage, one had microvasculitis, and one presented non-specific changes. These results are summarized in Table 2. The NLD-HIV controls presented diverse structural abnormalities not involving mitochondria (Table 2), whereas the HC showed a normal skeletal muscle appearance in all cases.

Mitochondrial contents in skeletal muscle did not differ between LD-HIV, NLD-HIV and HC: 0.57 ± 0.17, 0.46 ± 0.08 and 0.47 ± 0.13 μg of mitochondrial protein per milligram of skeletal muscle, respectively (*P*, NS).

The biochemical analyses yielded the results illustrated in Figure 2a. State III oxygen utilization was diminished in LD-HIV patients in comparison with both NLD-HIV and HC for most of the substrates tested, the greatest impairment being observed with the use of complex IV substrate ascorbate. The ratios between the respiratory activities of the different substrates disclosed wide disarrays for those relationships involving ascorbate in LD patients when compared with NLD-HIV and HC (Fig. 2b).

No significant differences in RCR were found between LD-HIV patients and NLD-HIV or HC groups either for glutamate (5.02 ± 3.26, 4.25 ± 2.60 and 5.04 ± 3.88, respectively; *P*, NS) or for succinate (3.07 ± 1.28, 2.48 ± 0.55 and 3.96 ± 2.06, respectively; *P*, NS).

With respect to AEA, we found that complexes I and II were normal in nearly all LD patients, whereas complexes III and IV were clearly reduced in all LD patients, with the only exception being LD-HIV patient no. 2, who presented complex III deficiency and normal (although in the lower range) complex IV activity (Fig. 2a).

When mtDNA was analysed, samples derived from blood showed no rearrangement in any of the patients (data not shown). No deletions were observed in the muscle mtDNAs from HC (Fig. 3a) or NLD-HIV controls (Fig. 3b). Only NLD-HIV no. 1 exhibited two faint deleted bands in extremely low proportion when compared with the 16.5 kb wild-type band. This sample corresponds to a 71-year-old man. In contrast, deletions were detected in skeletal muscle samples of all LD-HIV patients (Fig. 3). Single deletions in muscle mtDNA of patients no. 1 and 2 were evident in longer exposures of the fluorescent blot (in particular, after a 60 min exposure); therefore the proportion of deleted molecules in these patients was lower than in the others. The mtDNA from adipocytes of LD-HIV no. 1

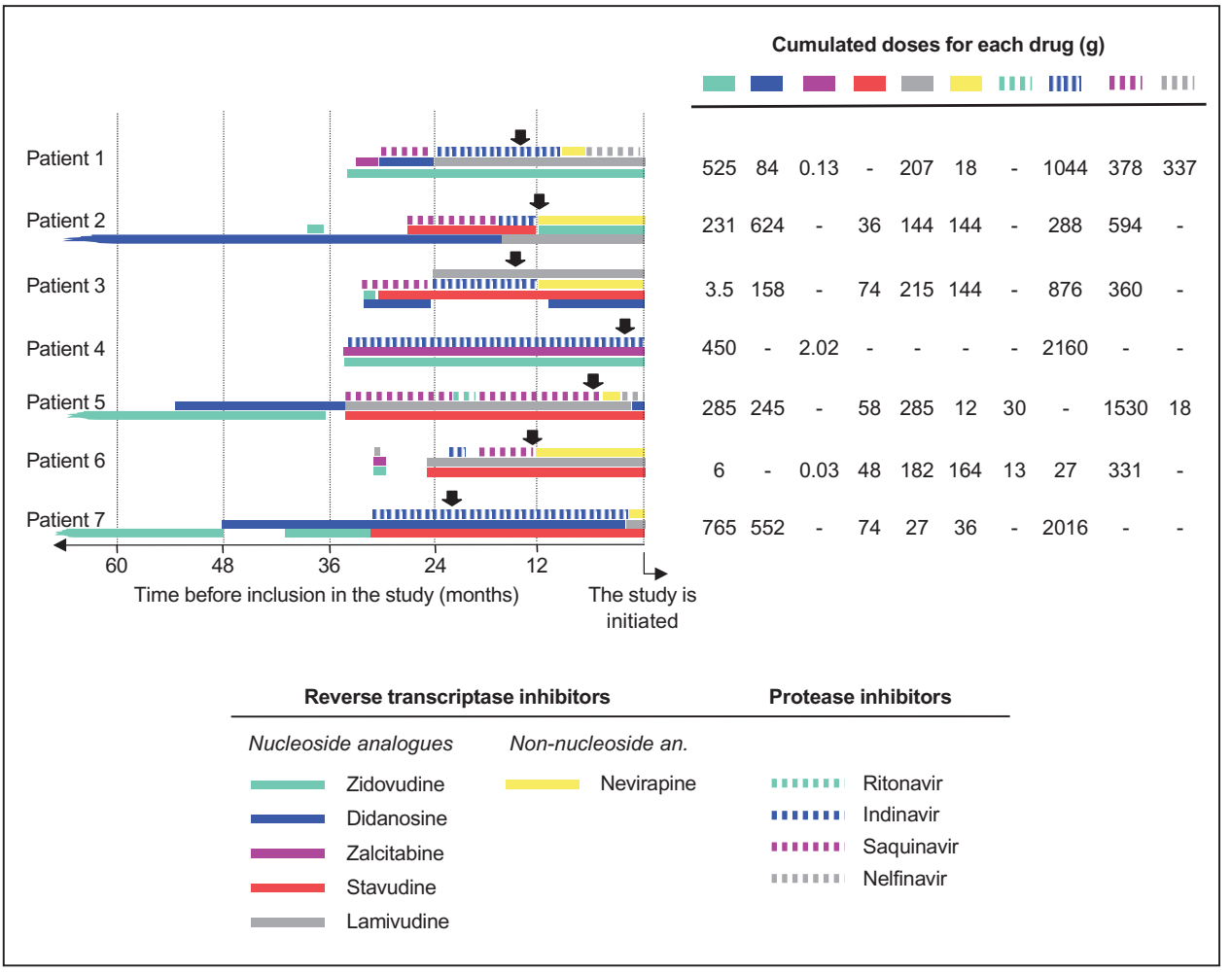

Fig. 1. Drug intake history of HIV-positive patients with lipodystrophy (LD-HIV) included in the study (selection criteria are described in 'Participants' paragraph of 'Materials and methods'). Thick arrows indicate the time at which the diagnosis of lipodystrophy was made.

was normal. The other LD-HIV patients presented multiple deletions, and participants no. 4, 5, 6 and 7 harboured some similar deleted bands (Fig. 3). Certain bands present in the muscle of LD-HIV no. 5 were also observed in the fat sample. Remarkably, the wild-type 16.5 kb band corresponding to full length mtDNA was not detected in this adipocyte sample, not even after much longer exposures.

The simultaneous hybridization of the blot including LD-HIV samples with nuclear and mitochondrial DNA did not reveal mtDNA depletion (data not shown).

Mitochondrial point mutations A12217G and A8344G were not detected in LD-HIV patients.

Table 2. Structural findings in muscle biopsies.

	LD-HIV no.	NLD-HIV no.
Size variability	1, 3	1, 2, 3, 5
Type II fibre atrophy	3	1, 2, 5
Minimal inflammatory infiltrates	3, 6	2, 3
Ragged-red fibres	1, 3	–
COX disturbances	1, 3, 6	–
SDH disturbances	1, 6	–
Lipid deposition	4, 5	–

LD-HIV, HIV-positive patients with lipodystrophy (LD): selection criteria are described in 'Participants' paragraph of 'Materials and methods'; NLD-HIV, non-lipodystrophic HIV patients; COX, cytochrome *c* oxidase; SDH, succinate dehydrogenase.

Discussion

We investigated the presence of mitochondrial abnormalities in HIV-infected patients who had developed LD under a HAART schedule. We examined the mitochondria of patients at three levels: structure, function of the respiratory chain and integrity of mtDNA molecules. The major finding of this work is the

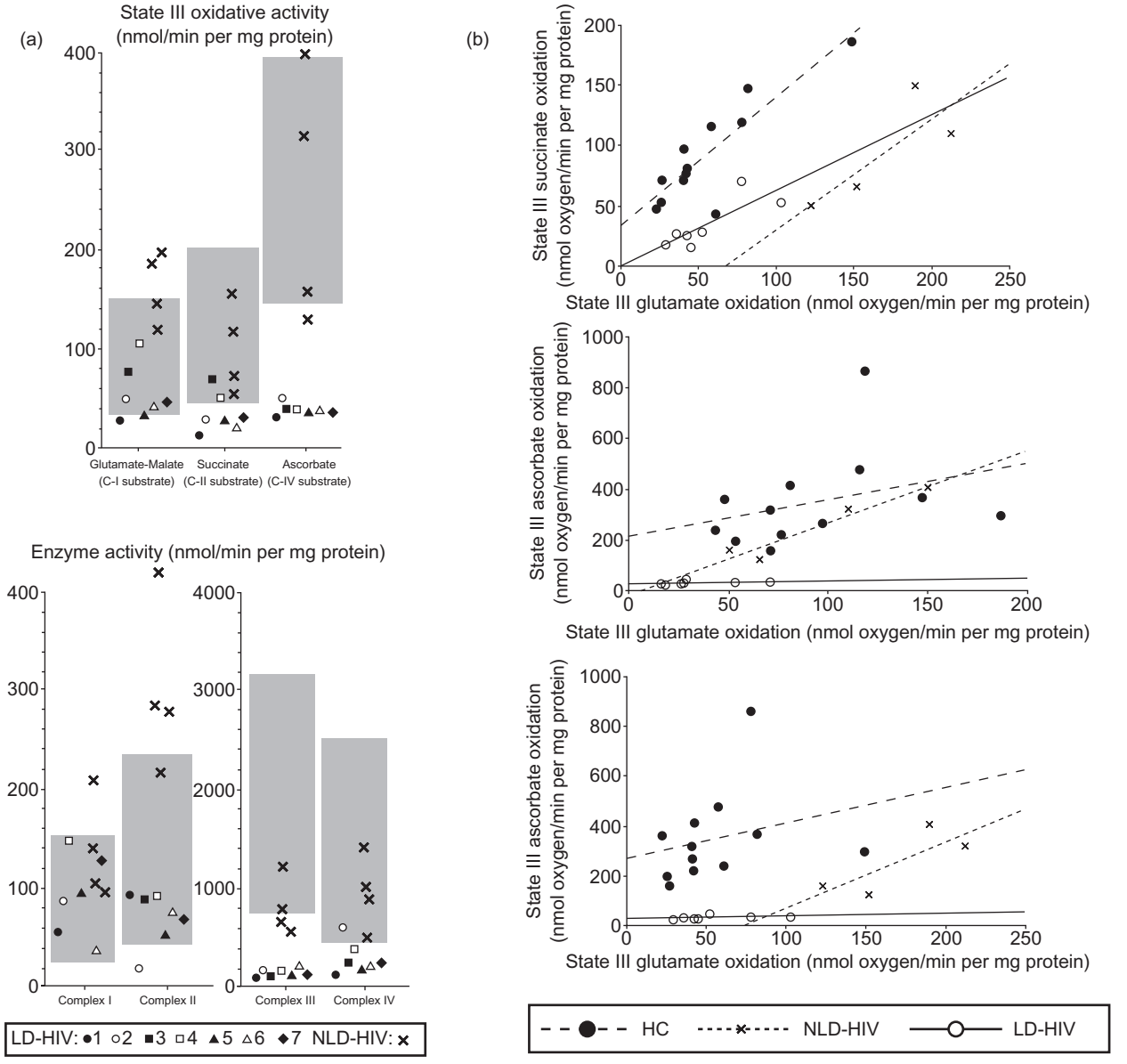

Fig. 2. (a) Respiratory chain biochemical results in skeletal muscle mitochondria from HIV-positive patients with lipodystrophy (LD-HIV). Grey boxes represent the values of healthy controls (HC). (b) Comparisons of state III oxidative activities in LD patients and controls [HIV-positive patients without LD (NLD-HIV) and HC]. Balanced ratios between respiratory chain-dependent oxidative activities are essential for an efficient electron-transfer activity. Note that there is a good correlation between activities in controls but not in LD-HIV patients for ratios involving ascorbate as substrate.

presence of mitochondrial abnormalities in all patients studied.

Although none of the HIV-infected individuals (either LD-HIV or NLD-HIV) complained of muscular symptoms and neuromuscular examinations were entirely normal, only one individual in each group showed a strictly normal skeletal muscle histology. Muscle abnormalities in HIV-patients, either related to zidovudine treatment or not, have been previously described [26–28]. LD-HIV patients no. 1 and 3 presented histological abnormalities suggesting mitochondrial dysfunction. Interestingly, whereas LD-HIV no. 1 had been treated with large amounts of zidovudine (consequently he could be presenting a zidovudine-related myopathy), only minimal doses of zidovudine had been used in LD-HIV no. 3, who also showed biochemical and molecular abnormalities in their mitochondria. Furthermore, none of these patients exhibited mtDNA depletion, the molecular hallmark of zidovudine myopathy. In our opinion, the histological anomalies detected in both patients are more likely related to the

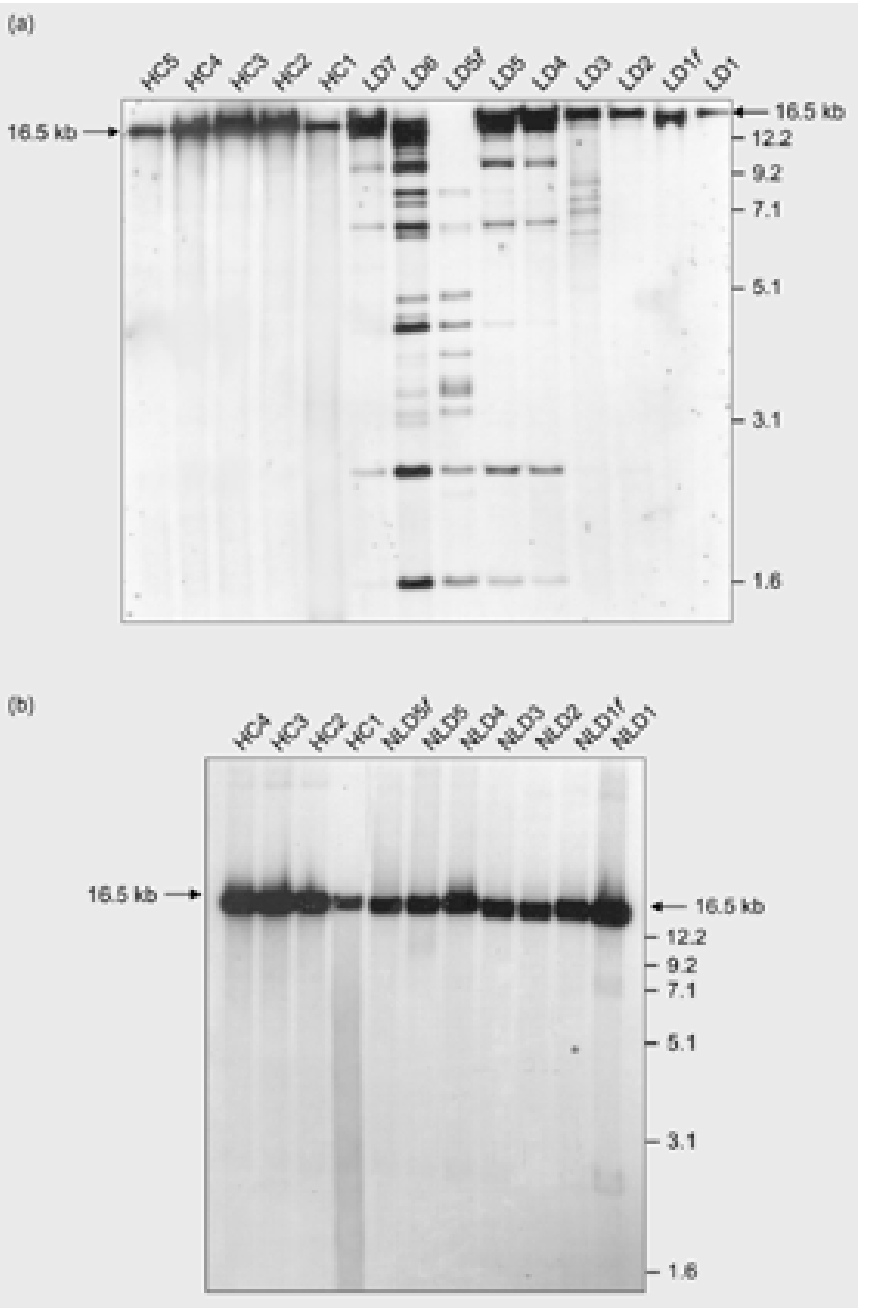

Fig. 3. (a) Fluorescent Southern blot analysis of mtDNA of HIV-positive patients with lipodystrophy (LD-HIV) and healthy controls (HC). The image corresponds to a 35 min exposure. (b) Radioactive Southern blot analysis of HIV-positive patients without LD (NLD-HIV) and HC, after 1 h exposure. Samples were *Pvu* II digested and probed with total mtDNA. All lanes correspond to mtDNA extracted from skeletal muscle except *f* lanes (fat samples). NLD, NLD-HIV control samples. Arrows indicate the wild-type mtDNA molecule.

different circumstances associated with HIV infection (opportunistic infections, neuropathies, malnutrition. . .), rather than with classical zidovudine -related toxicity.

From a functional point of view we found that mitochondrial respiration was notably altered in all LD-HIV patients studied and, in some cases, for more than one substrate. This indicates that the defect could be located outside MRC (e.g. the coupling of electron transport of MRC with oxidative phosphorylation by ATPase, the dinucleotide translocase, or the mitochondrial membranes) or, alternatively, in the later steps of MRC. The lack of differences in RCR between LD-

HIV, NLD-HIV and HC makes a deficiency in the coupling of oxidative phosphorylation unlikely. On the other hand, when we tested the enzyme function of isolated MRC complexes, we found that it was clearly diminished for complexes III and IV, whereas complexes I and II remained unaffected. Consequently, these enzyme abnormalities seem to be the main cause of the reduced respiratory activities observed in the skeletal muscle of the LD-HIV patients, which were especially evident with ascorbate, a substrate donating electrons directly to complex IV of MRC. That these disarrays are result of the treatment with zidovudine is a possibility that should be considered, since this drug was used for long periods in five out of seven LD-HIV patients. Both *in vitro* and *in vivo* studies have demonstrated the ability of zidovudine to partially impair MRC complexes [29–31]. However, the fact that LD-HIV no. 3 and 6 used very low zidovudine doses (Fig. 1) indicates that zidovudine cannot be the only cause responsible for mitochondrial respiratory chain dysfunction in LD-HIV patients.

The molecular analysis revealed numerous deletions in LD-HIV patients: they were detected in every muscle mtDNA sample and in one of the two adipocyte samples. The existence of such deletions is particularly striking when compared with NLD-HIV or HC controls, which showed a unique 16.5 kb mtDNA molecule (Fig. 3). Only individual NLD-HIV no. 1 showed two deleted bands in very low proportion. It is widely accepted that mtDNA deletions may appear in old tissue, which has been attributed to the effect of the oxidative stress on the mtDNA molecule. The fact that this man was 71 years old and in a very poor physical state led us to believe that these deletions are related to these circumstances rather than to the HIV infection.

Other mtDNA deletions have been found in other groups of HIV patients [12], although in a much lower proportion than that observed in our LD patients. In that study deletions were detected by PCR and not by Southern blot, indicating that the percentage of deleted molecules was lower than 5% of total mtDNA. Furthermore, there were no significant differences in the percentage of individuals with mtDNA deletions between zidovudine-treated and non-zidovudine-treated HIV groups. Therefore, we regard it as very unlikely that the presence of deletions in LD-HIV patients is an effect of AZT. We rather believe that the whole antiretroviral treatment is implicated in the origin of the mtDNA deletions observed in LD-HIV patients. NLD-HIV controls no. 4 and 5 do not harbour mtDNA deletions at present despite being on HAART. However, what we do not know now is whether in the future, when the cumulated drug intake in these individuals is greater, LD and mtDNA deletions will appear.

As opposed to zidovudine-treated non-lipodystrophic HIV patients examined in the past [11,12], the LD-HIV patients in our study did not present depletion of their mtDNA, despite all having used zidovudine for some period of time, and that patients no. 1, 2 and 4 were still being treated with zidovudine when the biopsies were performed. Daily doses of zidovudine are nowadays substantially lower than those used earlier, therefore this could explain why our patients did not show depletion of the mitochondrial genome.

Due to the variety in treatments of our patients (Fig. 1) it is not possible to assign the development of LD to any particular HAART regimen. However, it should be noted that in two of the three LD-HIV patients in whom a history of long-term utilization of antiretroviral therapy was present, LD did not appear during utilization of NRTI alone but became evident shortly after the introduction of indinavir. Actually, five out of the seven patients studied were receiving indinavir when LD was detected, as it was the most effective and best-tolerated PI by that time. At that moment, the NRTI predominantly used was lamivudine because it was well tolerated and it did not cause side effects. Following the introduction of PI the first LD cases appeared and, consequently, PI were considered directly responsible of LD syndrome. Today we know that even though LD is more frequent in those protocols that include PI, several therapeutic schedules using other antiretroviral drugs are also involved in LD. Presumably then, more than one drug group can cause the fat redistribution phenomenon that is characteristic of LD. Individual susceptibility might be an important factor too, because many patients under HAART do not develop LD despite being maintained on those schedules over long periods of time.

Although the results of this study led us to think that mitochondria might be involved in LD, the small sample size does not allow us to present it as a general hypothesis. Should such a relationship be confirmed in the future, the role of mitochondria in LD, either primary or secondary, will still have to be ascertained.

It could be speculated that HAART would cause mitochondrial abnormalities by a mechanism that initially would lead to MRC dysfunction, and later to mtDNA deletions and fat redistribution. An interference between the PI that was used and a patient's protease could trigger the development of LD. Some proteases are essential for mitochondrial biogenesis and function, for instance, the mitochondrial processing of peptidases involved in the import and activation of mitochondrial protein precursors synthesized in cytoplasmic ribosomes (including diverse nuclear DNA-encoded MRC subunits). The characteristic tissue distribution of LD could be due to the selective involvement of tissue-specific isoenzymes.

The analysis of different subsets of LD-HIV patients should provide further information on whether any specific HAART regimen induces the extensive mitochondrial damage found in our patients. This should also help to elucidate the mechanisms involved in LD.

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ADDENDA

Els dos treballs presentats són el resultat d'un projecte que principalment pretenia determinar si existia algun tipus d'alteració mitocondrial en pacients HIV-positius que patien de lipodistròfia (LD). Un segon propòsit era estudiar si es podia atribuir l'aparició de la LD a algun antivíric o pauta de tractament concrets.

La selecció de pacients es va fer segons els criteris exposats a l'apartat **Participants** dins dels Materials i mètodes de l'article; dels 344 pacients que es visiten periòdicament a l'Hospital de Granollers només a 11 se'ls va proposar la participació, i d'aquests, set accediren a col·laborar. La inclusió en l'estudi implicà una nova extracció de sang[37] i la realització d'una biòpsia muscular.

Els individus HIV lipodistròfics (LD-HIV), sis homes i una dona, patien la LD des de temps diversos que anaven des de 2,5 mesos a gairebé dos anys (mediana: 12 mesos). La durada de la infecció amb l'HIV també diferia entre els pacients, tot i que en sis dels set pacients superava els quatre anys (mediana: sis anys).

Un cop realitzats els estudis histològics, els de funcionament de la respiració mitocondrial i dels complexos de la CRM, i analitzats els mtDNA extrets de sang i de múscul esquelètic (i de dues mostres de greix subcutani), es va observar que els mitocondris de mostres LD-HIV presentaven anomalies generalitzades. Aquestes anomalies no s'observaven en mostres d'individus no infectats amb HIV.

Per tal d'assegurar que les possibles troballes en aquests pacients estaven relacionades amb la LD i no pas amb la infecció per HIV, es va intentar seleccionar persones HIV-positives no lipodistròfiques (tractades i no tractades amb antivírics) que constituïssin un grup control amb la infecció (NLD-HIV). Aquest objectiu es preveia relativament complicat perquè requeria una biòpsia muscular de persones prou sanes malgrat la infecció vírica (tractades amb HAART), biòpsia que no havia de repercutir en cap diagnòstic ni en la millora immediata de la qualitat de vida del donant. *A priori* encara semblava més difícil aconseguir que persones infectades amb el virus de la SIDA que havien rebutjat rebre tractament acceptessin participar en l'estudi.

Després de tres mesos es va aconseguir la participació de cinc persones HIV-positives, tres de les quals no rebien ni havien rebut tractament contra la infecció que patien. L'estudi d'aquestes mostres descartà la presència de les anomalies mitocondrials detectades als pacients LD-HIV. S'hi identificaren algunes alteracions estructurals però aquestes no estaven relacionades amb els mitocondris. La respiració mitocondrial amb succinat i ascorbat i les activitats dels complexos III i IV —que estaven clarament disminuïdes en els pacients LD-HIV— eren normals en aquest grup control. L'anàlisi del mtDNA no va revelar la multiplicitat de bandes delecionades reconegudes en els músculs esquelètics dels pacients lipodistròfics (figura 3 de "Mitochondrial damage..."). Tan sols l'individu NLD-HIV 1 mostrà dues bandes delecionades que podrien ser el reflex de l'acumulació de delecions amb l'edat, aspecte ja tractat a l'apartat **L'Envelliment i...** (pàg. 26). Aquesta persona no rebia tractament per a combatre la infecció per HIV i es trobava en un estat de salut molt precari. Conseqüentment, amb les dades disponibles en aquest cas no es pogué determinar si les delecions presents al mtDNA eren resultat de l'estrès oxidatiu acumulat o bé si s'havien generat durant algun procés patogènic relacionat o no amb la infecció vírica patida.

L'estudi d'altres músculs (i si és possible, teixit adipós) de pacients LD-HIV pot demostrar que el funcionament del mitocondri i el seu DNA està majoritàriament alterat en aquests pacients. Els estudis realitzats en aquest tema posen de manifest un cop més la ineficàcia d'examinar mostres de sang en moltes malalties en les quals existeixen defectes de la funció mitocondrial, el que força a la pràctica de biòpsies musculars[38].

[37] A aquestes persones se'ls extrau sang periòdicament per a controlar, entre d'altres magnituds, les concentracions de cèl·lules $CD4^+$ i de còpies víriques.

[38] Els mètodes alternatius d'obtenció de mostres no satisfan els requisits d'un estudi d'aquest tipus. Per exemple, un raspat bucal no proporciona prou mtDNA per a un *Southern blot* i a

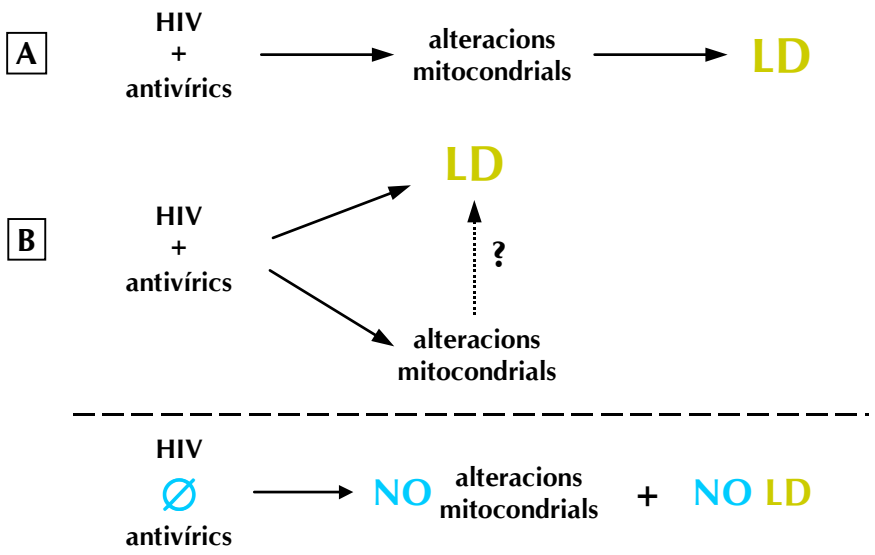

FIGURA 15. LD ASSOCIADA A TERÀPIES AMB MOLÈCULES ANTIVÍRIQUES.

Les opcions A i B s'expliquen en el text. Els individus HIV-positius que no reben tractament amb substàncies antivíriques, no pateixen lipodistròfia (LD) i tampoc presenten una funció mitocondrial alterada.

Un cop determinat que el mitocondri presenta alteracions principalment en els complexos bioquímics i en el mtDNA quan existeix LD, i no les presenta quan no hi ha LD, cal plantejar-se si el seu paper en aquesta complicació és primari o secundari.

La **FIGURA 15** esquematitza aquestes dues possibilitats. L'alternativa A proposa que la teràpia antivírica altament activa (HAART) causa alteracions mitocondrials l'efecte de les quals provocarà la síndrome de LD en moltes persones infectades amb l'HIV. Aquesta opció s'ajusta a les hipòtesis de Brinkman i de Kakuda (Brinkman 1999; Kakuda 1999); vegeu l'apartat **Implicació mitocondrial...** de la **INTRODUCCIÓ**, pàg. 43). Així doncs, si aquesta hipòtesi és correcta el mitocondri tindria un paper primari en el desenvolupament de la LD, en el sentit que el funcionament anormal del mateix seria necessari per a l'aparició d'aquest síndrome.

L'opció B presenta la LD i les anomalies mitocondrials com a dos fenòmens independents causats per la teràpia antivírica. La identificació de pacients lipodistròfics que no presentin aberracions mitocondrials aniria en favor d'aquesta hipòtesi, ja que es pot suposar que en algunes persones l'alteració mitocondrial apareixerà abans que la LD, i en d'altres, l'ordre d'esdeveniments serà l'invers. De moment, però, els resultats obtinguts no apunten en aquest sentit.

La fletxa discontínua indica que un cop provocades alteracions mitocondrials, aquestes podrien incidir en el procés de LD ja iniciat. El contrari, que la LD (que consisteix en la disminució del greix corporal en unes zones i l'acumulació en d'altres) provoqui disfunció als mitocondris de teixit adipós però també de múscul esquelètic, sembla poc versemblant.

Evidentment l'estudi d'altres pacients amb LD aclarirà la validesa d'aquestes dues alternatives, i ajudarà a elucidar els factors que influeixen en el desenvolupament de la LD. Aquests factors determinen una susceptibilitat particular de cada individu a patir la LD. En el context del present treball, és particularment interessant seguir estudiant els dos individus NLD-HIV que reben tractament contra l'HIV i veure si en el futur desenvolupen LD. El seu seguiment, però, seria molt més factible si hi hagués una manera no agressiva d'obtenir mostres d'aquestes persones en diferents moments futurs; aleshores probablement es podria esbrinar si la disfunció mitocondrial ha de precedir la LD o si aquesta pot

més no és adequat ni per a les tincions histològiques ni per a les determinacions bioquímiques.

aparèixer sense que existeixin defectes en els mitocondris.

El nombre de pacients lipodistròfics analitzats i la variabilitat en els seus tractaments no permet d'associar la LD amb cap substància antivírica o cap HAART concreta. Per això seran necessaris estudis molt més amplis i, si és possible, de caire prospectiu.