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RESEARCH REPORT

Lysosomal Acid Lipase Deficiency in 23 Spanish Patients: High Frequency of the Novel c.966+2T>G Mutation in Wolman Disease

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Abstract Lysosomal acid lipase (LAL) is a lysosomal key enzyme involved in the intracellular hydrolysis of cholesteryl esters and triglycerides. Patients with very low residual LAL activity present with the infantile severe form Wolman disease (WD), while patients with some residual activity develop the less severe disorder known as Cholesteryl ester storage disorder (CESD). We present the clinical, biochemical, and molecular findings of 23 Spanish patients (22 families) with LAL deficiency. We identified eight different mutations, four of them not previously reported. The novel c.966+2T>G mutation accounted for 75% of the Wolman disease alleles, and the frequent CESD associated c.894G>A mutation accounted for 55% of the CESD alleles in our cohort. Haplotype analysis showed that both mutations co-segregated with a unique haplotype suggesting a common ancestor. Our study contributes to the LAL deficiency acknowledgement with novel mutations and with high frequencies of some unknown mutations for WD.

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This chapter does not contain any studies with animal subjects performed by any of the authors.

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Introduction

Lysosomal acid lipase (LAL; EC 3.1.1.13) is a lysosomal key enzyme involved in the intracellular hydrolysis of cholesteryl esters (CE) and triglycerides (TG) that have been internalized via receptor-mediated endocytosis of plasma lipoprotein particles. In this process, the released free cholesterol regulates its own endogenous synthesis, the uptake of LDL, and cholesterol esterification (Assman and Seedorf 2001). LAL is a 399-amino acid protein encoded by the *LIPA* gene (MIM *613497) located in chromosome 10 (10q23.2-q23.3) (Anderson et al. 1993; Aslanidis et al. 1994). Mutations in *LIPA* gene cause LAL deficiency, which is associated with a wide spectrum of clinical manifestations. Patients with very low residual LAL activity present with Wolman disease (WD, MIM 278000), while patients with some residual activity (i.e. 3–8% of controls in blood lymphocytes or fibroblasts) develop the less severe disorder known as cholesteryl ester storage disorder (CESD, MIM 278000) (Assman and Seedorf 2001). Wolman disease is a rare recessive disorder characterized by massive storage of CE and TG in most tissues, vomiting, diarrhea, anemia, failure to thrive, hepatosplenomegaly, adrenal calcification, and early death (usually before 1 year of age) (Assman and Seedorf 2001). Patients with CESD show a broad spectrum of severity of clinical presentation, but usually they present in childhood with hepatomegaly, hypercholesterolemia, hypertriglyceridemia and most of them are diagnosed by their second decade (Assman and Seedorf 2001).

About 50 mutations have been reported in the *LIPA* gene (see HGMD® Human Gene Mutation Database), most of them always associated with Wolman disease or with CESD, and most of them located in exon 8 (Assman and Seedorf 2001; Aslanidis et al. 1994). One of the most

frequent mutation associated with CESD is c.894G>A, that affects the last nucleotide of exon 8, does not change the amino acid (p. Q298Q), but causes the skipping of exon 8 causing an in-frame deletion of 24 amino acids (p. S275_Q298del) (Klima et al. 1993).

Here we present the clinical, biochemical, and molecular findings of 23 Spanish patients with LAL deficiency corresponding to 22 different families.

Materials and Methods

We analyzed 23 patients corresponding to 22 families originated from different regions in Spain. All patients were Caucasian and ten of them were of gipsy origin. No relation was reported between these ten families, but some of the surnames are shared, so we cannot be sure they are not related. The diagnoses of the patients were made between 1992 and 2015 in our lab. Clinical information was provided by the patient's physician and in all cases the diagnosis was confirmed biochemically by determination of the acid lipase activity in fibroblasts.

Acid lipase activity was measured in fibroblasts with the fluorogenic substrates 4-methylumbelliferyl-Palmitate (MU-palmitate) and/or 4-methylumbelliferyl-Oleate (MU-Oleate) (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Kelly and Bakhru-Kishore 1979; Koster et al. 1980). Protein concentration was determined using the Lowry method.

Genomic DNA was extracted from cultured fibroblasts using standard protocols. Exons two to ten and their flanking intronic regions of the *LIPA* gene were PCR-amplified using self-designed oligonucleotides. PCR reactions were performed according to standard protocols. Fragments were directly sequenced by the dideoxy termination method. Gene nucleotides were numbered according to the RefSeq NM_000235 sequence considering nucleotide +1 the A of the ATG start codon in exon 2. The ATG codon represents +1 for the amino acid numbering according to preprotein NP_000226 sequence.

Single nucleotide polymorphisms (SNPs) were analyzed using the same oligonucleotides as for mutation analysis, to construct haplotypes co-segregating with the changes found in WD and CESD patients: (1) c.1-65G>C, rs2250781; (2) c.46A>C (p. Thr16Pro), rs1051338; (3) c.67G>A (p. Gly23Arg), rs1051339; (4) c.676-42G>A, rs1556478; (5) c.966+46C>T, rs3802656.

Results

We studied 23 patients of 22 families with LAL deficiency. Thirteen of them were diagnosed as WD, while ten patients were diagnosed as CESD. Clinical and molecular findings of all patients are shown in Table 1.

Regarding the clinical manifestations, all WD patients were diagnosed at early infancy with a mean age at diagnosis of 48 days and all deceased before the year of life. They showed the typical intestinal malabsorption, hepatic and adrenal failure pattern. The CESD patients were diagnosed at a mean age of 13 years of life and their clinical affectation was mainly hepatomegaly and hyperlipidemia.

Biochemical analyses allowed the LAL deficiency diagnosis of the patients. WD patients showed a mean activity of 11.7% (±10.2) and 4.7% (±2.6) respect to controls using MU-Oleate and MU-palmitate respectively, while CESD patients showed a mean activity of 12.4% (±7.5) and 8.2% (±3.2) respect to controls using the same substrates respectively.

Molecular analysis of 23 LAL patients let us to identify eight different mutations, four of them not reported before. The novel changes were one missense mutation (c.256C>T, p. H86Y), one amino acid deletion (c.1055_1057delACG, p. D352del), one splicing alteration (c.966+2T>G), and a change in the same base as the common c.894G>A mutation that also changes amino acid (c.894G>C, p. Q298H). Mutation p. H86Y was predicted to be probably damaging by Polyphen-2 predictor (http://genetics.bwh.harvard.edu/pph2/) and MutPred (http://mutpred.mutdb.org/). The c.966+2T>G change affecting the invariant dinucleotide "GT" of the intron 9 donor splice site was expected to alter pre-mRNA splicing. To assess its potential effect on mRNA splicing the relative strength of the 5′ splice-site signal of intron 9 was evaluated using the BDGP Splice Site prediction (http://www.fruitfly.org/seq_tools/splice.html), NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/), and Splice Site Score Calculation (http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html). All the algorithms predicted that c.966+2T>G mutations abolished the function of the donor splice site of intron 9. Finally, the novel change c.894G>C causes a missense mutation, p. Q298H, also predicted to be probably damaging by Polyphen-2 predictor and MutPred, but also affects the same exon 8 last base that the common mutation c.894G>A. All the new changes were not found in 100 control chromosomes.

The described mutations that were also identified in our cohort were the common c.894G>A (p. S275_298del) splicing mutation, the small deletion c.398delC (p. S133X), the complex mutation c.230-33_230dup;c.232_245del (p. G77fsX82), and the missense change c.386A>G (p. H129R).

We also performed haplotype analysis to investigate the origin of the common mutations in our cohort (Table 2). We observed that both the two common mutations in our series

Table 1 Clinical and molecular findings in Spanish LAL deficiency patients

Family	DNA	Age at diagnosis	LAL form	Genotype (cDNA)	Genotype (protein)	Cons.	AD	D/MD	H	PD	HM	SM	SGS	V	D	HCH	LC	HT	HTR
F1	W1	15 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	–	–	Yes	Yes	Yes	Yes	–	Yes	Yes	–	–	–	–
F2	W2	1 month	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	–	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	–	–	–	–
F3	W5	ND	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F4	W6	15 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	Yes	–	–	–	Yes	Yes	Yes	–	Yes	–	–	Yes	–
F5	W13	2 months	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	–	–	–	–	Yes	Yes	Yes	–	–	–	–	–	–
F6	W15	41 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	–	–	–	Yes	–	–	Yes	Yes	Yes	–	–	–	–
F7	W16	2 months	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	–	Yes	Yes	Yes	Yes	–	Yes	–	Yes	–	–	–	–
F8	W18	15 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	Yes	Yes	–	–	Yes	Yes	Yes	Yes	Yes	–	–	–	–	–
	W23	4 months	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F9	W22	25 days	Wolman	**[c.966+2T>G]; [c.966+2T>G]**	**[IVS9+2T>G]; [IVS9+2T>G]**	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F10	W7	45 days	Wolman	**[c.894G>C]; [c.894G>C]**	**[p. Q298H]; [p. Q298H]**	–	Yes	–	–	Yes	Yes	Yes	–	Yes	Yes	–	–	–	–
F11	W10	1 month	Wolman	**[c.894G>C]; [c.894G>C]**	**[p. Q298H]; [p. Q298H]**	–	Yes	–	–	Yes	Yes	Yes	Yes	–	–	–	–	–	–
F12	W14	2 months	Wolman	[c.398delC]; [c.398delC]	[p. S133X]; [p. S133X]	Yes	–	–	–	–	Yes	Yes	Yes	Yes	–	–	–	–	–
F13	W3	33 years	CESD	[c.894G>A]; [c.894G>A]	[p. S275_298del]; [p. S275_298del]	–	–	–	–	–	Yes	–	–	–	–	–	Yes	Yes	Yes
F14	W8	9 years	CESD	[c.894G>A]; [c.894G>A]	[p. S275_298del]; [p. S275_298del]	–	–	–	–	–	Yes	–	–	–	–	–	–	–	–
F15	W9	7 years	CESD	[c.894G>A]; [c.894G>A]	[p. S275_298del]; [p. S275_298del]	–	–	–	–	–	Yes	–	–	–	–	Yes	–	–	Yes
F16	W17	2.5 years	CESD	[c.894G>A]; [c.894G>A]	[p. S275_298del]; [p. S275_298del]	–	–	–	–	–	Yes	–	–	–	–	Yes	–	Yes	Yes
F17	W11	3 years	CESD	[c.894G>A]; **[c.256C>T]**	[p. S275_298del]; **[p. H86Y]**	–	–	–	–	–	Yes	–	–	–	–	Yes	–	Yes	–
F18	W12	15 years	CESD	[c.894G>A]; [c.386A>G]	[p. S275_298del]; [p. H129R]	–	–	–	–	Yes	Yes	–	–	–	–	Yes	–	Yes	Yes
F19	W21	10 years	CESD	[c.894G>A]; [c.398delC]	[p. S275_298del]; [p. S133X]	–	–	–	–	Yes	Yes	–	–	–	–	Yes	–	Yes	Yes
F20	W20	11 years	CESD	[c.386A>G]; [c.386A>G]	[p. H129R]; [p. H129R]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F21	W19	11 years	CESD	[c.386A>G]; [c.230-33_230dup; c.232_245del]	[p. H129R]; [p. G77fsX82]	–	–	–	–	Yes	Yes	Yes	–	–	–	–	Yes	Yes	Yes
F22	W4	26 years	CESD	[c.386A>G]; **[c.1055_1057del]**	[p. H129R]; **[p. D352del]**	–	–	–	–	Yes	Yes	Yes	–	–	–	–	Yes	Yes	Yes

In bold novel mutations. *Cons* consanguinity, *AD* abdominal distension, *D/MD* dystonia/muscular dystrophy, *H* hypotonia, *PD* ponderal delay, *HM* hepatomegaly, *SM* splenomegaly, *SGS* suprarenal gland stones, *V* vomiting, *D* diarrhea, *HCH* hypercholesterolemia, *LC* liver cirrhosis, *HT* hypertriglyceridemia, *HTR* high transaminases, *ND* no data, (−) no data

Table 2 Haplotypes co-segregating with LIPA mutations

Mutation (cDNA level)	Mutation (protein level)	*n*	c.1-65G>T rs2250781	c.46A>C (p. Thr16Pro) rs1051338	c.67G>A (p. Gly23Arg) rs1051339	c.676-42G>A rs1556478	c.966+46C>T rs3802656
c.966+2T>G	IVS9+2T>G	20	T	C	G	G	C
c.894G>A	p. S275_298del	11	T	A	G	A	T
c.230-33_230dup; c.232_245del	p. G77fsX82	1	T	C	G	G	T
c.256C>T	p. H86Y	1	T	A	G	A	C
c.386A>G	p. H129R	5	G/T	A	G	G	C
c.398delC	p. S133X	3	G/T	A	G	G/A	C
c.894G>C	p. Q298H	4	G	A	G	G	T/C
c.1055_1057del	p. D352del	1	G	A	G	G	T

n Number of alleles

(c.966+2T>G and c.894G>A) segregated with a unique haplotype each one in all the patients. On the contrary, the other seven mutations co-segregated with different haplotypes (Table 2).

Discussion

The thirteen patients with WD were diagnosed between 15 days and 4 months of life, with a mean age at diagnosis of 48 days. The majority of them showed a severe systemic affectation with abdominal distension, hypotonia, ponderal delay, hepatosplenomegaly, suprarenal gland stones, diarrhea, and vomiting. Only one patient showed Hypertriglyceridemia. On the contrary, the ten patients showing CESD form of the disease were diagnosed between 2,5 and 33 years of life, being 13 years the mean age at diagnosis. Nearly all of them showed hepatomegaly as the main affectation trait and some of them showed ponderal delay, hypercholesterolemia, and high transaminases (Table 1). Regarding the clinical point of view, the WD patients in our cohort showed the typical clinical WD presentation, while CESD patients also showed the typical milder form with hepatomegaly and hyperlipidemia.

Biochemical analyses showed that using MU-palmitate as substrate gave lower percentage of activity respect to controls in both WD and CESD patients (4.7 and 8.2%), than using MU-Oleate, which resulted in higher residual activity in both series of patients (11.7 and 12.4%). Both substrates allowed the diagnosis of LAL deficient patients but, to our point of view, using both of them in the diagnosis protocol gives more reliable results when analyzing patients.

After molecular analysis, we detected eight different mutations in our cohort of patients, four of them novel. The novel mutations were c.256C>T (p.H86Y), c.1055_1057delACG (p.D352del), c.966+2T>G, and c.894G>C (p.Q298H). All of them were predicted presumably damaging and were not detected in 100 control chromosomes.

The described mutations that were identified in our cohort were the common c.894G>A (p.S275_298del) splicing mutation (Klima et al. 1993), the small deletion c.398delC (p.S133X) (Sadhukhan et al. 2014), the complex mutation c.230-33_230dup;c.232_245del (p.G77fsX82) (Pisciotta et al. 2009), and the missense change c.386A>G (p.H129R) (Ries et al. 1998).

Regarding genotype-phenotype correlation, we detected a clear differentiation between Wolman and CESD patients. The novel splicing mutation c.966+2T>G in intron 9 was detected in 75% (20/26) of the Wolman patients alleles, always in homozygosity. The novel c.894G>C change was the second most frequent being detected in 17% (4/26) of the alleles also in homozygosity. In CESD patients, the most frequent mutation was the common CESD associated c.894G>A which accounts for 55% (11/20) of the alleles, followed by the already reported missense change p.R129H, which represents 25% (5/20) of the alleles. Four out of ten CESD patients were homozygous for the c.894G>A change.

All mutations detected in WD patients were not detected in CESD patients and any mutation detected in CESD patients was detected in WD patients, except for the described deletion c.398delC that was identified in homozygosity in one WD patient and in heterozygosity with the common c.894G>A in one CESD patient.

The novel highly prevalent c.966+2T>G change affects the second base of the canonic donor splicing site in intron 9. It is predicted to disrupt the correct splicing process and presumably it would cause an insertion or a

deletion of some amino acids in the protein, but studies on cDNA must be performed to know the exact effect it causes. Anyway, as when present in homozygosity it is always associated with WD, it is presumed that the protein codified by this mRNA would show very low activity. Regarding the clinical point of view, patients presenting this mutation in homozygosity did not show any differential trait compared with the other WD patients with other mutations.

The second frequent WD associated c.894G>C mutation affects the same base as the common described CESD associated c.894G>A mutation. This mutation is known to result not only in a major non-functional transcript with the skipping of exon 8, causing the deletion of 24 amino acids (p.S275_Q298del), but also in a minor normally spliced transcript producing 5–10% residual LAL activity (Klima et al. 1993). The Change c.894G>C not only would destroy the same canonic splicing donor site as c.894G>A, presumably causing the same splicing effect, but also would introduce a missense mutation (p.Q298H) in the 5–10% of protein correctly synthesized. As the mutation p.Q298H is predicted to be damaging by the mutation effect predictors, its presence would cause the loss of the activity of the protein translated from the correctly spliced mRNA. So c.894G>C would generate 90–95% of inactive protein with a 24 amino acid deletion and 5–10% of inactive protein with the missense mutation p.Q298H. The presumably lack of activity correlates with the fact that we identified the c.894G>C mutation in homozygosity only in WD patients, the severe form, unlike c.894G>A, that has only been identified in CESD patients.

The other mutation detected in a WD patient of our cohort was c.398delC. It was detected in homozygosity in one WD patient but also in compound heterozygosity with the common c.894G>A in one CESD patient. These findings agree with the literature, as this mutation was previously described in homozygosity in one WD case (Sadhukhan et al. 2014) and in heterozygosity in CESD patients (Benlian et al. 2014), pointing out that the protein carrying this deletion presents very low activity, correlating with the WD genotype in homozygosity, but may be present in milder CESD patients when it is in heterozygosity with a mild mutation as c.894G>A.

Regarding CESD patients, the majority showed the reported frequent CESD associated c.894G>A mutation in homozygosity or in heterozygosity with another mutation, confirming its association with the mild forms of the disease (Klima et al. 1993) also in our cohort.

One CESD patient showed the reported p.H129R mutation in homozygosity and three patients carried the same mutation in heterozygosity, giving the idea that this change is associated with some residual activity and being associated with a milder phenotype (Ries et al. 1998).

Regarding the other mutations detected in heterozygosity in CESD patients of our cohort (p.H86Y, p.D352del and c.230-33_230dup;c.232_245del), expression studies should be performed to elucidate to what extent they allow some residual LIPA activity to explain this phenotype or if the milder form of the patient is due to the percentage of active protein caused by the mutation in the other allele.

We also performed haplotype analysis to investigate the origin of the common mutations in our cohort (Table 2). We observed that both the two common mutations in our series (c.966+2T>G and c.894G>A) segregated with a unique haplotype each one in all the patients. On the contrary, for the other six mutations, three of them were identified in only one allele, so we cannot conclude anything consistent about their co-segregation, and the other three mutations co-segregated with different haplotypes (Table 2). c.894G>A mutation co-segregates with the same haplotype previously described by Fasano et al. (2012), except for the rs2071509 polymorphism that we didn't analyze in our cohort and we do not have data. This observation strongly supports the hypothesis of a common ancestor for this mutation suggested by Fasano et al. (2012). Regarding the novel c.966+2T>G mutation that also co-segregates with a unique haplotype, it is also presumed to be transmitted by a common ancestor. For the other mutations identified in more than one allele, as they co-segregates with different haplotypes, it is to be assumed that they are recurrent mutations affecting hot spots in *LIPA* gene.

In conclusion, we identified four novel and four previously described mutations in a cohort of 23 Spanish LAL deficiency patients. The majority of the CESD patients carried the common mutant allele c.894G>A and the great majority of the WD patients carried the novel mutation c.966+2T>G. These two mutations co-segregated with a unique haplotype suggesting a presence of a common ancestor. The other mutations in WD or CESD patients co-segregated with different haplotypes.

Our study contributes to the LAL deficiency acknowledgement with novel mutations and with high frequencies of some unknown mutations for WD.

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Take-Home Message

In Spanish acid lipase deficient patients, the novel mutation c.966+2T>G is highly frequent (75% of the alleles) in Wolman disease patients, while the common CESD associated c.894G>A is also the most frequent among these patients in our cohort.

Compliance with Ethics Guidelines

Author Contributions

Carla Ruiz-Andrés collected the clinical data and performed the molecular analyses, analyzed and interpreted the data; Elena Sellés performed the enzymatic analyses and interpreted the data; Angela Arias cultured the fibroblasts and Laura Gort contributed to conception and design, analysis and interpretation of data and writing the chapter. All the authors revised the manuscript critically.

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Competing Interest Statement

All the authors declare that they have no conflict of interest.

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All the procedures were approved by the ethics committee of the Hospital Clínic, Barcelona. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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