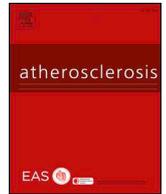




ELSEVIER

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Evaluation of two approaches to lysosomal acid lipase deficiency patient identification: An observational retrospective study



Jorge J. Cebolla^{a,b,c,d,*}, Pilar Irún^b, Pilar Mozas^d, Pilar Giraldo^{a,b,c}

^a Instituto de Investigación Sanitaria Aragón (IIS Aragón), GIIS-012, Zaragoza, 50009, Spain

^b Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), CB/06/07/1036, Instituto de Salud Carlos III (ISCIII), Hospital Universitario Miguel Servet, Zaragoza, 50009, Spain

^c Fundación Española para el Estudio y Terapéutica de la Enfermedad de Gaucher y Otras Lisosomales (FEETEG), Zaragoza, 50008, Spain

^d Departamento de Bioquímica, Biología Molecular y Celular, Facultad de Ciencias, Universidad de Zaragoza, Zaragoza, 50009, Spain

HIGHLIGHTS

- Plasma lysosomal storage disorder (LSD) biomarkers was a good approximation to identify lysosomal acid lipase deficiency (LALD) patients.
- Combination of LSD biomarkers and lipid-liver profile (LLP) enhanced the power to identify LALD patients in natural history cohorts.
- Lysosomal storage disease suspected patients was queried for *LIPA* mutations.
- Novel *LIPA* mutations were found in this study.

ABSTRACT

Background and aims: Lysosomal acid lipase deficiency (LALD) leads to the accumulation of cholesteryl esters and/or triglycerides (TG) in lysosomes due to the lack of the enzyme codified by the *LIPA* gene. The most common symptoms are dyslipidaemia and hypertransaminasemia, together with manifestations common to other lysosomal storage disorders (LSDs), including visceromegalies and elevated plasma biomarkers. Alteration of the lipid-liver profile (LLP) has been widely applied as a criterion for LALD screening, but the usefulness of biomarkers has not yet been explored. Our purpose was to explore the utility of plasma chitotriosidase activity (ChT) and CCL18/PARC concentration in addition to LLP to identify LALD patients in an observational retrospective study of two different sample collections.

Methods: Biological samples refining: Collection 1 (primary hypercholesterolemia suspected) included unrelated individuals with hyperlipidaemia and without *LDLR*, *APOB* and *PCSK9* gene mutations (Set 1), and Collection 2 (LSD suspected) included individuals without definitive LSD diagnosis (Set 2). We assessed plasma LLP (total cholesterol and its fractions, TG concentration and transaminases activities), as well as plasma ChT and CCL18/PARC. All subjects with anomalous LLP and/or biomarker levels were *LIPA* sequenced.

Results: Twenty-four subjects showed altered LLP and/or biomarkers. We identified two LALD patients (one homozygous and one compound heterozygous) and one carrier of a novel *LIPA* variant.

Conclusions: The measurement of plasma ChT and CCL18/PARC combined with LLP will be a useful approach to identifying LALD patients in retrospective LALD patient studies.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCL18/PARC, chemokine (CeC motif) ligand 18/pulmonary and activation-regulated chemokine; CESD, cholesteryl ester storage disease; c-HDL, high-density lipoproteins cholesterol; c-LDL, low-density lipoproteins cholesterol; c-VLDL, very low-density lipoproteins cholesterol; Chol, cholesterol; ChT, chitotriosidase activity; E8SJM, exon 8 splice junction mutation; FH, familial hypercholesterolemia; LALD, lysosomal acid lipase deficiency; LLP, lipid-liver profile; LSD, lysosomal storage disease; PH, primary hypercholesterolemia; SREBP, sterol regulatory element-binding protein; TChol, total cholesterol; TG, triglyceride; UTR, untranslated region

* Corresponding author. Unidad de Investigación Traslacional, Instituto de Investigación Sanitaria Aragón (IIS Aragón), GIIS-012, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), CB/06/07/1036, Hospital Universitario Miguel Servet, P° Isabel La Católica 1-3, 50009, Zaragoza, Spain.

E-mail address: jorgecebollasan@gmail.com (J.J. Cebolla).

<https://doi.org/10.1016/j.atherosclerosis.2019.03.013>

Received 23 August 2018; Received in revised form 6 March 2019; Accepted 14 March 2019

Available online 18 March 2019

0021-9150/ © 2019 Elsevier B.V. All rights reserved.

1. Introduction

Lysosomal acid lipase deficiency (LALD, MIM#278000) is an ultra-rare autosomal recessive inborn error of metabolism caused by a deficiency of lysosomal acid lipase (EC.3.1.13). This enzyme is codified by the *LIPA* gene (MIM*613947) located on chromosome 10 (10q23.2-q23.3) [1]. Currently, 96 *LIPA* mutations are listed in The Human Gene Mutation Database (www.hgmd.cf.ac.uk), with NM_000235.2:c.894A > G (NP_001121077.1:p.Ser275_Gln298del) considered the most common pathogenic allele in LALD via an “exon 8 splice junction mutation” (E8SJM) [2]. Loss of enzyme activity triggers the progressive and pathological accumulation of cholesteryl esters, TG or both, inside the lysosome. There are two main phenotypes related to LALD that can be distinguished by the level of residual enzyme activity *in vitro* [3]: Wolman disease, with the neonatal-infantile onset, and cholesteryl ester storage disease (CESD), with a later onset.

The estimated prevalence of Wolman is 1:350,000 births, while that of CESD ranges from 1:60,000–1:130,000, depending on the ethnic group and geographical location [1,2,4,5]. However, the real prevalence is probably underestimated since clinical symptoms are hidden by more prevalent clinical features or because mild forms remain oligosymptomatic.

The absence of enzyme activity produces a shortage of cytosolic free cholesterol (Chol), TG or both, therefore leading to activation of several metabolic pathways governing the synthesis/release of endogenous Chol and endocytosis of exogenous Chol, processes mediated by sterol regulatory element-binding proteins (SREBPs). SREBPs modulate the cellular uptake of low-density lipoproteins Chol (c-LDL) through LDL receptors, a reduction in high-density lipoproteins Chol (c-HDL) efflux and an increase in the release of very low-density lipoproteins Chol (c-VLDL) [3,6,7]. Thus, a steady trapping of lipids inside lysosomes causes engorgement in several tissues, mainly hepatocytes, which leads to microvesicular steatosis, elevation of transaminases activity (especially alanine aminotransferase [ALT] and aspartate aminotransferase [AST]), liver fibrosis, cryptogenic cirrhosis and, finally, liver failure [3,7]. Progressive lipid build-up in the monocyte-macrophage lineage elicits splenomegaly and hence anaemia and cytopenias [3,7]. Moreover, macrophage activation produces the release of pro-inflammatory molecules (e.g., cytokines, enzymes, etc.) and could promote atherosclerosis [8].

Signs and symptoms of LALD may sometimes be confused with those of other lysosomal storage disorders (LSDs) causing visceral involvement, such as Gaucher disease, Niemann-Pick disease types A/B/C [9,10] etc. In addition, plasma LSD biomarkers such as chitotriosidase (ChT) or chemokine (C–C motif) ligand 18/pulmonary and activation-regulated chemokine (CCL18/PARC) are elevated in LSDs and atherosclerosis [9,11–14]. Nevertheless, certain patients could show reduced or null plasma ChT activity due to the presence of certain polymorphic genetic variants in the gene encoding ChT (*CHIT1*; MIM*600031). Therefore, genetic characterization of *CHIT1* is highly recommended for appropriate interpretation of results [15]. Moreover, the plasma CCL18/PARC concentration is not a specific biomarker and can also be elevated in other pathologies such as in Gaucher (29-fold in symptomatic patients), Niemann-Pick and atherosclerosis [16–19].

To date, LALD patient findings studies have focused on lipid-liver profile (LLP) disturbances (high Total Chol [TChol], high c-LDL, low c-HDL), high transaminases, hepatomegaly, liver steatosis or cirrhosis [7,20]. However, ChT and CCL18/PARC have not been explored in retrospective LALD findings programs. The goal of this study was to perform an observational retrospective study of the incidence of LALD in unrelated individuals previously screened for primary hypercholesterolemia (PH) but without mutations on the most frequent genes associated with this disorder (*LDLR* [MIM*606945], *APOB* [MIM*107730], *PCSK9* [MIM*607786]) as well as in unrelated patients without a conclusive diagnosis of Gaucher and Niemann-Pick diseases.

2. Materials and methods

2.1. Patients, study design and workflow

In this observational retrospective study, we considered two approaches for screening individuals enrolled in two Spanish sample collections: PH subjects (n = 2,040) and LSD-suspected subjects (n = 1,295). The first approach consisted of analysing the plasma LLP, and the second consisted of analysing the plasma LSD biomarkers (ChT activity and CCL18/PARC concentration). All the biological fractions handled in this study were isolated from EDTA whole-blood samples and stored under appropriate conservation conditions. All individuals and controls provided written informed consent.

The flow of the study is available in [Supplemental Fig. 1](#). Briefly, both collections were refined to establish analytical sets. Subjects from the PH collection formed Set 1 according to plasma TChol (≥ 200 mg/dL) and c-HDL levels (male ≤ 40 mg/dL and female ≤ 50 mg/dL), as well as having no pathological mutation found on *LDLR*, *APOB* and *PCSK9* genes using a LIPOchip[®] DNA array-based platform [21]. Subjects from the LSD collection formed Set 2 in compliance with plasma TChol (≥ 200 mg/dL) and c-HDL levels (male ≤ 40 mg/dL and female ≤ 50 mg/dL), as well as negative studies for Gaucher and Niemann-Pick diseases. Two consecutive screening steps were applied to each set of individuals based on LLP and LSD biomarkers to identify LLP and LSD groups. Subjects included in these groups were considered “high-suspicion-LALD” subjects and completed *LIPA* test groups in which *LIPA* gene sequencing was accomplished.

Forty-two healthy volunteers were recruited to establish an intra-laboratory reference group.

The Aragon regional government's Ethics Committee for Clinical Research, Zaragoza, Spain approved the study, and research was conducted in accordance with the principles stated in the Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects, Helsinki, Finland, 1964 and as amended in Fortaleza, Brazil, 2013.

2.2. Study variables

2.2.1. Plasma lipid-liver profile determination

The concentrations of TChol, c-LDL, c-HDL and TG and ALT and AST activities (LLP panel) were quantified in plasma samples using an automatic analyser BA400 (BioSystems, Barcelona, Spain). The reference range for the concentrations of TChol, c-LDL, c-HDL and TG were chosen according to US National Cholesterol Education Program recommendations (2002) and for ALT and AST activities according to our established intra-laboratory values (activity ≥ 60 UI/L).

2.2.2. Plasma chitotriosidase analysis and *CHIT1* gene genotyping

The ChT activity was determined using the fluorogenic substrate 4-methylumbelliferyl β -D-N,N',N'-triacetylchitotrioside (Sigma Chemical Co., St Louis, MO, USA) [15]. Genotyping for the 24-bp insertion in exon 10 of the *CHIT1* gene (NM_003465.3:c.1049_1072dup24) was performed as described by Irún et al. [15]. Given that ChT activity is roughly half of normal or zero in subjects who are heterozygous or homozygous for the c.1049_1072dup24 *CHIT1* polymorphism, respectively, ChT levels were multiplied by two in heterozygous individuals, while the assessment of CCL18/PARC levels was mandatory for homozygous individuals. The cut-off was estimated according to the intra-laboratory reference group mean plus two standard deviations (ChT ≥ 150 nmol/mL/h).

2.2.3. Plasma chemokine CCL18/PARC quantification

The concentration of the chemokine CCL18/PARC was analysed by an enzyme-linked immunosorbent assay (R&D Systems Europe, Ltd, UK) according to the manufacturer's instructions. The cut-off was estimated according to the intra-laboratory reference group mean plus two

standard deviations (CCL18/PARC \geq 115 ng/mL).

2.3. LIPA sequencing and in silico analysis

The 5' and 3' untranslated regions (UTR), 10 exons and exon-intron boundaries of the *LIPA* gene were amplified by polymerase chain reaction using custom primer pairs designed on the basis of the *LIPA* genomic sequence released in the GenBank database (www.ncbi.nlm.nih.gov, accession no. NG_008194.1). All primer sequences are available upon request. Bidirectional Sanger sequencing was carried out. All genetic variations identified were described in compliance with the latest Human Genetic Variation Society recommendations (www.hgvs.org/mutnomen). Main polymorphism information was gathered from the Genome Reference Consortium Human Build 38 patch release 7 (GRCh38.p7, www.ncbi.nlm.nih.gov) using the biomaRt package from Bioconductor (www.bioconductor.org).

The *in silico* pathogenicity of non-previously described genetic variants was evaluated primarily by MutationTaster software [22]. FATHMM-MKL software [23] was used to evaluate the deleterious effects caused by nonsense mutations. PROMO software [24] was employed to estimate the potential effects of genetic variants on the binding of *trans*-acting regulatory elements and on the *LIPA* promoter.

2.4. Statistical analysis

Statistical analysis was performed using R freeware (version 3.4.0) [25]. Qualitative variables were given as frequencies and percentages and compared using Chi-squared and Fisher tests. Quantitative variables were analysed for normal distributions by the Kolmogorov-Smirnov test. Non-normally distributed variables were given as medians and interquartile ranges (25th-75th percentile) and compared using non-parametric Mann-Whitney U tests.

All statistical tests considered and took as bilateral significance a level of $\alpha = 0.05$.

3. Results

3.1. Patients

A total of 660/2,040 subjects from the PH collection satisfied inclusion criteria, and plasma and DNA samples were available from 81/660 individuals (Set 1). Regarding the LSD collection, 67/1,295 subjects accomplished inclusion criteria, and plasma and DNA samples were available from 67/67 subjects (Set 2).

No significant differences were observed between the sets regarding gender distribution, age and quantile age distribution (20th percentile). However, individuals belonging to Set 1 were overweight on average compared to those in Set 2 ($p < 0.001$; Table 1).

3.2. High-suspicion-LALD subjects in set 1

The Set 1 screening process is summarized in Fig. 1A. We found anomalous levels of TChol, c-HDL, c-LDL, TG and AST or ALT in 3 subjects (LLP 1). In the remaining 78 subjects, ChT and CCL18/PARC

levels were determined. Two subjects showed ChT null activity and were homozygous for a 24-bp duplication in *CHIT1*; therefore, CCL18/PARC was analysed, showing low levels. These subjects were not included in the LSD 1 group. Five subjects who were LLP-negative yielded ChT \geq cut-off, CCL18/PARC \geq cut-off or both (LSD 1).

Finally, 8 subjects were considered high-suspicion-LALD (3 belonging to the LLP 1 group and 5 to the LSD 1 group) and underwent a sequencing test (*LIPA* test 1).

Further information about the LLP and LSD biomarker levels of Set 1 subjects is available in Supplemental Tables 1 and 2

3.3. High-suspicion-LALD subjects in set 2

The Set 2 screening process is summarized in Fig. 1B. One subject showed elevated levels of all LLP parameters (LLP 2). In the other 66 individuals, ChT and CCL18/PARC levels were assessed. Fifteen subjects who were LLP negative yielded ChT \geq cut-off, CCL18/PARC \geq cut-off or both (LSD 2).

Finally, 16 subjects were considered high-suspicion-LALD (1 belonging to the LLP 2 group and 15 to the LSD 2 group) and underwent genetic testing (*LIPA* test 2).

Further information about the LLP and LSD biomarker levels of Set 2 subjects is available in Supplemental Tables 1 and 2

3.4. LIPA mutations

High-suspicion-LALD individuals ($n = 24$) underwent *Sanger* sequencing, which revealed that in *LIPA* test 2, there were three subjects with genetic variants in *LIPA*: one carrier of a novel variant (Subject-1), one homozygous (Subject-2) and one compound heterozygous (Subject-3).

The genetic and biochemical features of these subjects with positive genetic analyses, are given in Table 2. Subject-1 with the novel genetic variant had the mutation NM_000235.2:c.-106C > A in the 5'-UTR in heterozygosity, Subject-2 had the known E8SJM mutation in homozygosity, and Subject-3 carried the known E8SJM mutation [2] in combination with a non-previously described 25-bp deletion NM_000235.2:c.95 + 111_del25 (NP_001121077.1:p.Thr33*) located on exon 1-intron 1.

MutationTaster analysis showed probabilities close to 1 for c.-106C > A and p.Thr33* mutations, indicating a "high probability" of being disease-causing; hence, computed different bioinformatics tools were used for further analysis, depending on the genetic nature of the mutation (Supplemental Table 4).

The *in silico* analysis of the novel variant c.-106C > A suggested pathogenic significance when it was segregated in homozygosity (MutationTaster probability score = 0.9990, maximum = 1.0000). PROMO results suggested that one of two consensus binding sites of transcription factor Sp1 in the *LIPA* promoter was abolished.

The *in silico* analysis of the p.Thr33* proposed a pathogenic effect when it was segregated in homozygosity (MutationTaster probability score = 0.9998, maximum = 1.0000). The FATHMM-MKL score indicated an important deleterious effect ($p = 0.72$; $p > 0.50$ for a deleterious effect) that caused a premature stop codon in amino acid 33.

Table 1
Demographical data in the screened population.

	n (%)	n Female (%)	n Male (%)	Age (y.o.) ^a	BMI ^b
Set 1 (PH)	81 (54.7)	57 (70.4)	24 (29.6)	49.0(0.1–89.0)	26.7(24.8–28.4) ^c
Set 2 (LSD)	67 (45.3)	35 (52.2)	32 (47.8)	52.5(20.0–74.0)	22.6(19.7–26.4) ^c
Total	148	92 (62.2)	56 (37.8)	51.0(0.1–89.0)	26.2(23.4–28.1)

y.o. = years old; BMI = Body Mass Index (kg/m²).

^a (Minimum-Maximum).

^b Median(25th–75th Percentile).

^c $p \leq 0.001$.

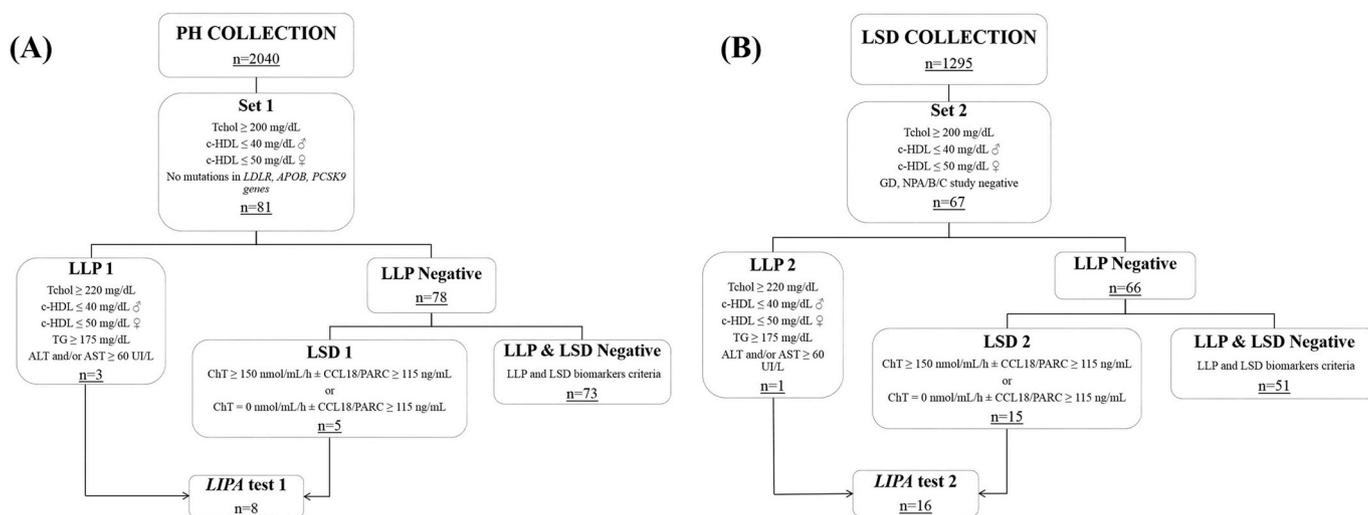


Fig. 1. High-suspicion-LALD subjects screening.

(A) After applying inclusion criteria from the PH collection, 81 subjects were screened by lipid-liver profiles (LLP, LLP 1 subset n = 3), and the rest of the 78 LLP-negative individuals were screened by LSD biomarkers levels (LSD 1 subset n = 5). The *LIPA* test 1 subset was composed of 8 high-suspicion-LALD individuals who underwent sequencing of the *LIPA* gene. (B) After applying inclusion criteria from the LSD collection, 67 subjects were screened by lipid-liver profiles (LLP, LLP 2 subset n = 1), and the other 66 LLP-negative individuals were screened by LSD biomarker levels (LSD 2 subset n = 15). The *LIPA* test 2 subset was composed of 16 high-suspicion-LALD individuals who underwent sequencing of the *LIPA* gene.

Further, 15 common *LIPA* variants or polymorphisms were also observed, as shown in Supplemental Table 3.

4. Discussion

Since 2012, a non-laborious methodology for prospective screening of lysosomal acid lipase activity in dried blood spot samples has been available, showing high precision and accuracy to detect potential LALD patients [26]. Recently, a consensus update of the technique has been published, highlighting improvements and interpretations of the results of routine screening processes [27]. In 2015, the first human lysosomal acid lipase recombinant enzyme therapy (Sebelipase alfa; Alexion Pharmaceuticals) was approved as a LALD treatment by the European Medicines Agency, since its efficacy was proven in different clinical trials [28–30]. Major clinical achievements include the normalization of transaminases and lipid levels, reduction in fat content and liver volume, improvement in growth parameters and, most importantly, increased survival in children.

Considering this state-of-the-art, the retrospective identification of undiagnosed subjects from stored sample collections constitutes an important task for many physicians. Most hospitals have identified individuals with clinical suspicion of dyslipidaemia, liver disease, cardiovascular disease, or even LSD, without a definitive diagnosis.

Table 2
Biochemical and genetic features of LALD cases and LALD carriers.

Subject	LALD Status	Gender	Age y.o.	TChol	c-HDL	c-LDL	TG	AST	ALT	ChT	CCL18	Nucleotide ^a	Amino acid ^b
1	Carrier	F	1	115	18	75	111	89	10	47.5	77	c.-106C > A	-
2	Affected	F	18	200	35	146	96	28	47	140	140	c.894G > A	p.Ser275_Gln298del
												+	+
												c.894G > A	p.Ser275_Gln298del
3	Affected	M	2	458	40	363	273	106	77	824	402	c.894G > A	p.Ser275_Gln298del
												+	+
												c.95 + 111_del25	p.Thr33*

F = Female; M = Male; y.o. = years old; TChol = total cholesterol (mg/dL); c-HDL = high-density lipoproteins cholesterol (mg/dL); c-LDL = low-density lipoproteins cholesterol (mg/dL); TG = triglycerides (mg/dL); AST = aspartate aminotransferase (UI/L); ALT = alanine aminotransferase (UI/L); ChT = chitotriosidase activity (nmol/mL/h); CCL18 = chemokine (C-C motif) ligand 18/pulmonary and activation-regulated chemokine (ng/mL).

^a NM_000235.2 coding reference sequence (www.ncbi.nlm.nih.gov).

^b NP_000226.2 protein reference sequence (www.ncbi.nlm.nih.gov).

[36] discouraged the assessment of this biomarker in our study.

Finally, the *LIPA* characterization of 24 individuals considered high-suspicion-LALD subjects allowed us to identify one homozygous mutation for E8SJM, one compound heterozygous mutation for E8SJM and the novel nonsense mutation p.Thr33*, whose biochemical phenotype matched that of enzyme deficiency. Otherwise, we were able to detect one heterozygous novel mutation in 5'-UTR with probable pathogenic significance when segregated in homozygous (c.-106C > A) individuals due to abolishing one of the consensus binding sites of the *trans*-regulatory element Sp1, widely involved in activation of gene expression [37]. Unfortunately, no familial study was available, and we could not deepen the c.-106C > A characterization. Further studies could determine the implication of this novel mutation in LALD (e.g., electrophoresis mobility shift assay, etc.). Despite the negative identification of LALD patients in Set 1 (PH collection), we advocate for the suitability of LALD patient identification in FH suspected subjects without a definitive diagnosis because effective treatment is available for LALD [31,33,38,39].

In the two approaches mentioned in our study design, if the LLP, biomarkers or both were elevated versus the control population, we considered this enough evidence to analyse the *LIPA* gene. A limitation is the lack of DNA sequence analysis of all subjects reported here. Direct sequencing of the *LIPA* gene would be a powerful tool to detect LALD patients in the cohort of LSD. In this work, it was outside the scope of the study that aims to evaluate the weight of biomarkers to identify suspicions without conducting genetic studies indiscriminately and for which we lacked funding.

Our findings strongly support the value of LSD biomarkers, ChT and CCL18/PARC in retrospective LALD patient identification from stored sample collections.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

JJC and PG conceived and designed the experiments. JJC and PI performed the experiments. JJC carried out the statistical analysis. PM Contributed reagents/materials/analysis tools, JJC drafted the manuscript. All the authors read and approved the final manuscript.

Financial support

This research has been partially supported by a grant from the Fundación Española para el Estudio y Terapéutica de la Enfermedad de Gaucher y Otras Lisosomales (FEETEG).

Acknowledgements

The authors gratefully thank Sofía Aznárez, María Pueyo, Mark Strunk and Dr Isabel de Castro for technical support; Dr Laura López de Frutos and Dr Ralf Köhler for critical revision of the manuscript.

The authors would like to acknowledge the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atherosclerosis.2019.03.013>.

References

[1] G. Grabowski, L. Charnas, H. Du, Lysosomal acid lipase deficiencies: the wolman disease/cholesteryl ester storage disease spectrum, in: D. Scriver Valle, A. Beaudet,

- B. Vogelstein, K. Kinzler, S. Antonarakis, A. Ballabio (Eds.), *Metab. Mol. Bases Inherit. Dis.*, eighth ed, McGraw Hill, New York, 2012, <https://doi.org/10.1036/ommbid.172>.
- [2] S.A. Scott, B. Liu, I. Nazarenko, S. Martis, J. Kozlitina, Y. Yang, C. Ramirez, Y. Kasai, T. Hyatt, I. Peter, R.J. Desnick, Frequency of the cholesteryl ester storage disease common *LIPA* E8SJM mutation (c.894G > A) in various racial and ethnic groups, *Hepatology* 58 (2013) 958–965, <https://doi.org/10.1002/hep.26327>.
- [3] D.L. Bernstein, H. Hülkova, M.G. Bialer, R.J. Desnick, Cholesteryl ester storage disease: review of the findings in 135 reported patients with an underdiagnosed disease, *J. Hepatol.* 58 (2013) 1230–1243, <https://doi.org/10.1016/j.jhep.2013.02.014>.
- [4] S. Muntoni, H. Wiebusch, M. Jansen-Rust, S. Rust, U. Seedorf, H. Schulte, K. Berger, H. Funke, G. Assmann, Prevalence of cholesteryl ester storage disease, *Arterioscler. Thromb. Vasc. Biol.* 27 (2007) 1866–1868, <https://doi.org/10.1161/ATVBAHA.107.146639>.
- [5] N.O. Stitzel, S.W. Fouchier, B. Sjouke, G.M. Peloso, A.M. Moscoso, P.L. Auer, A. Goel, B. Gigante, T.A. Barnes, O. Melander, M. Orho-melander, S. Duga, S. Sivapalaratnam, M. Nikpay, N. Martinelli, D. Girelli, R.D. Jackson, C. Kooperberg, L.A. Lange, D. Ardisino, R. McPherson, M. Farrall, H. Watkins, M.P. Reilly, D.J. Rader, U. De Faire, H. Schunkert, J. Erdmann, N.J. Samani, L. Charnas, D. Altshuler, S. Gabriel, J.J.P. Kastelein, J.C. Defesche, A.J. Nederveen, S. Kathiresan, G.K. Hovingh, Exome sequencing and directed clinical phenotyping diagnose cholesteryl ester storage disease presenting as autosomal recessive hypercholesterolemia, *Arterioscler. Thromb. Vasc. Biol.* 33 (2013) 2909–2914, <https://doi.org/10.1161/ATVBAHA.113.302426>.
- [6] T. Reynolds, Cholesteryl ester storage disease: a rare and possibly treatable cause of premature vascular disease and cirrhosis, *J. Clin. Pathol.* 66 (2013) 918–923, <https://doi.org/10.1136/jclinpath-2012-201302>.
- [7] Z. Reiner, O. Guardamagna, D. Nair, H. Soran, K. Hovingh, S. Bertolini, S. Jones, M. Čorić, S. Calandra, J. Hamilton, T. Eagleton, E. Ros, Lysosomal acid lipase deficiency - an under-recognized cause of dyslipidaemia and liver dysfunction, *Atherosclerosis* 235 (2014) 21–30, <https://doi.org/10.1016/j.atherosclerosis.2014.04.003>.
- [8] T.J. Reape, K. Rayner, C.D. Manning, A.N. Gee, M.S. Barnette, K.G. Burnand, P.H. Groot, Expression and cellular localization of the CC chemokines PARC and ELC in human atherosclerotic plaques, *Am. J. Pathol.* 154 (1999) 365–374, [https://doi.org/10.1016/S0002-9440\(10\)65283-2](https://doi.org/10.1016/S0002-9440(10)65283-2).
- [9] S. vom Dahl, E. Mengel, Lysosomal storage diseases as differential diagnosis of hepatosplenomegaly, *Best Pract. Res. Clin. Gastroenterol.* 24 (2010) 619–628, <https://doi.org/10.1016/j.bpg.2010.09.001>.
- [10] V. Valayannopoulos, E. Mengel, A. Brassier, G. Grabowski, Lysosomal acid lipase deficiency: expanding differential diagnosis, *Mol. Genet. Metabol.* 120 (2017) 62–66, <https://doi.org/10.1016/j.ymgme.2016.11.002>.
- [11] M. Artieda, A. Cenarro, A. Gañán, I. Jericó, C. Gonzalvo, J.M. Casado, I. Vitoria, J. Puzo, M. Pocióvi, F. Civeira, Serum chitotriosidase activity is increased in subjects with atherosclerosis disease, *Arterioscler. Thromb. Vasc. Biol.* 23 (2003) 1645–1652, <https://doi.org/10.1161/01.ATV.0000089329.09061.07>.
- [12] H. Michelakakis, E. Dimitriou, I. Labadaridis, The expanding spectrum of disorders with elevated plasma chitotriosidase activity: an update, *J. Inherit. Metab. Dis.* 27 (2004) 705–706.
- [13] F. Isman, J.A. Hobert, J.N. Thompson, M.R. Natowicz, Plasma chitotriosidase in lysosomal storage diseases, *Clin. Chim. Acta* 387 (2008) 165–167, <https://doi.org/10.1016/j.cca.2007.07.019>.
- [14] T. Kologlu, S.K. Ucar, E. Levent, Y.D. Akcay, M. Coker, E.Y. Sozmen, Chitotriosidase as a possible marker of clinically evidenced atherosclerosis in dyslipidemic children, *J. Pediatr. Endocrinol. Metab.* 27 (2014) 701–708, <https://doi.org/10.1515/jpem-2013-0365>.
- [15] P. Irún, P. Alfonso, S. Aznarez, P. Giraldo, M. Pocióvi, Chitotriosidase variants in patients with Gaucher disease. Implications for diagnosis and therapeutic monitoring, *Clin. Biochem.* 46 (2013) 1804–1807, <https://doi.org/10.1016/j.clinbiochem.2013.09.006>.
- [16] R.G. Boot, M. Verhoek, M. De Fost, C.E.M. Hollak, M. Maas, B. Bleijlevens, M.J. Van Breemen, M. Van Meurs, L.A. Boven, J.D. Laman, M.T. Moran, T.M. Cox, J.M.F.G. Aerts, Marked elevation of the chemokine CCL18/PARC in Gaucher disease: a novel surrogate marker for assessing therapeutic intervention, *Blood* 103 (2004) 33–40, <https://doi.org/10.1182/blood-2003-05-1612.Reprints>.
- [17] J. Brinkman, F.A. Wijburg, C.E. Hollak, J.E. Groener, M. Verhoek, S. Scheij, J. Aten, R.G. Boot, J.M. Aerts, Plasma chitotriosidase and CCL18: early biochemical surrogate markers in type B Niemann-Pick disease, *J. Inherit. Metab. Dis.* 28 (2005) 13–20, <https://doi.org/10.1007/s10545-005-4416-9>.
- [18] D.A. Hägg, F.J. Olson, J. Kjell Dahl, M. Jernås, D.S. Thelle, L.M.S. Carlsson, B. Fagerberg, P.A. Svensson, Expression of chemokine (C-C motif) ligand 18 in human macrophages and atherosclerotic plaques, *Atherosclerosis* 204 (2009) 15–20, <https://doi.org/10.1016/j.atherosclerosis.2008.10.010>.
- [19] I. De Castro-Orós, P. Irún, J.J. Cebolla, V. Rodríguez-Sureda, M. Mallén, M.J. Pueyo, P. Mozas, C. Dominguez, M. Pocióvi/Spanish NP-C Group, Assessment of plasma chitotriosidase activity, CCL18/PARC concentration and NP-C suspicion index in the diagnosis of Niemann-Pick disease type C: a prospective observational study, *J. Transl. Med.* 15 (2017) 43, <https://doi.org/10.1186/s12967-017-1146-3>.
- [20] C. Camarena, L.J. Aldamiz-Echevarría, B. Polo, M.A. Barba Romero, I. García, J.J. Cebolla, E. Ros, Actualización en deficiencia de lipasa ácida lisosomal: diagnóstico, tratamiento y seguimiento de los pacientes, *Med. Clin. (Barc.)* 148 (2017) 1–10, <https://doi.org/10.1016/j.medcli.2016.12.044>.
- [21] D. Tejedor, S. Castillo, P. Mozas, E. Jiménez, M. López, M.T. Tejedor, M. Artieda, R. Alonso, P. Mata, L. Simón, A. Martínez, M. Pocióvi, Reliable low-density DNA array based on allele-specific probes for detection of 118 mutations causing familial

- hypercholesterolemia, *Clin. Chem.* 51 (2005), <http://clinchem.aaccjnl.org/content/51/7/1137.long>, Accessed date: 23 June 2017.
- [22] J.M. Schwarz, D.N. Cooper, M. Schuelke, D. Seelow, MutationTaster2: mutation prediction for the deep-sequencing age, *Nat. Methods* 11 (2014) 361–362, <https://doi.org/10.1038/nmeth.2890>.
- [23] H.A. Shihab, J. Gough, D.N. Cooper, P.D. Stenson, G.L.A. Barker, K.J. Edwards, I.N.M. Day, T.R. Gaunt, Predicting the functional, molecular, and phenotypic consequences of amino acid substitutions using hidden Markov models, *Hum. Mutat.* 34 (2013) 57–65, <https://doi.org/10.1002/humu.22225>.
- [24] X. Messeguer, R. Escudero, D. Farré, O. Núñez, J. Martínez, M.M. Albà, PROMO: detection of known transcription regulatory elements using species-tailored searches, *Bioinformatics* 18 (2002) 333–334 <http://www.ncbi.nlm.nih.gov/pubmed/11847087>, Accessed date: 3 May 2017.
- [25] R Core Team, R: a language and environment for statistical computing, <https://www.r-project.org/>, (2017).
- [26] J. Hamilton, I. Jones, R. Srivastava, P. Galloway, *Clinica Chimica Acta* A new method for the measurement of lysosomal acid lipase in dried blood spots using the inhibitor Lalistat 2, *Clin. Chim. Acta* 413 (2012) 1207–1210, <https://doi.org/10.1016/j.cca.2012.03.019>.
- [27] Z. Lukacs, M. Barr, J. Hamilton, Best practice in the measurement and interpretation of lysosomal acid lipase in dried blood spots using the inhibitor Lalistat 2, *Clin. Chim. Acta* (2017), <https://doi.org/10.1016/j.cca.2017.05.027>.
- [28] M. Balwani, C. Breen, G.M. Enns, P.B. Deegan, T. Honzik, S. Jones, J.P. Kane, V. Malinova, R. Sharma, E.O. Stock, V. Valayannopoulos, J.E. Wraith, J. Burg, S. Eckert, E. Schneider, A.G. Quinn, Clinical effect and safety profile of recombinant human lysosomal acid lipase in patients with cholesteryl ester storage disease, *Hepatology* 58 (2013) 950–957, <https://doi.org/10.1002/hep.26289>.
- [29] B. Burton, M. Balwani, F. Feillet, I. Baric, T. Burrow, C. Camarena Grande, C. M. A. Consuelo-Sánchez, P. Deegan, M. Di Rocco, G.M. Enns, R. Erbe, F. Ezgu, C. Ficiocioglu, K.N. Furuya, K. J. C. Laukaitis, E. Mengel, E. Neilan, S. Nightingale, H. Peters, M. Scarpa, K.O. Schwab, V. Smolka, V. Valayannopoulos, M. Wood, Z. Goodman, Y. Yang, S. Eckert, S. Rojas-Caro, A.G. Quinn, A phase 3 trial of sebelipase alfa in lysosomal acid lipase deficiency, *N. Engl. J. Med.* 373 (2015) 1010–1020, <https://doi.org/10.1056/NEJMoa1501365>.
- [30] S.A. Jones, D. Plantaz, R. Vara, S. Eckert, K.-H. Le Quan Sang, A. Brassier, J.-B. Arnoux, C. Breen, J. Jay Gargus, A.G. Quinn, S. Rojas-Caro, V. Valayannopoulos, Effect of sebelipase alfa on survival and liver function in infants with rapidly progressive lysosomal acid lipase deficiency, *Mol. Genet. Metabol.* 114 (2015) S59, <https://doi.org/10.1016/j.ymgme.2014.12.123>.
- [31] C.R. Pullinger, E.O. Stock, I. Movsesyan, M.J. Malloy, P.H. Frost, R. Tripuraneni, A.G. Quinn, B.Y. Ishida, E.J. Schaefer, B.F. Asztalos, J.P. Kane, Identification and metabolic profiling of patients with lysosomal acid lipase deficiency, *J. Clin. Lipidol.* 9 (2015) 716–726, <https://doi.org/10.1016/j.jacl.2015.07.008> e1.
- [32] B. Sjouke, J.C. Defesche, J.S.E. de Randamie, A. Wiegman, S.W. Fouchier, G.K. Hovingh, Sequencing for LIPA mutations in patients with a clinical diagnosis of familial hypercholesterolemia, *Atherosclerosis* 251 (2016) 263–265, <https://doi.org/10.1016/j.atherosclerosis.2016.07.008>.
- [33] J.R. Chora, A.C. Alves, A.M. Medeiros, C. Mariano, G. Lobarinhas, A. Guerra, H. Mansilha, H. Cortez-Pinto, M. Bourbon, Lysosomal acid lipase deficiency: a hidden disease among cohorts of familial hypercholesterolemia? *J. Clin. Lipidol.* 11 (2017) 477–484, <https://doi.org/10.1016/j.jacl.2016.11.002> e2.
- [34] S. Pajares, A. Arias, J. García-Villoria, J. Macías-Vidal, E. Ros, J. de las Heras, M. Girós, M.J. Coll, A. Ribes, Cholestane-3 β ,5 α ,6 β -triol in lipidosis: high levels in Niemann pick type C, cerebrotendinous xanthomatosis and lysosomal acid lipase deficiency, *J. Lipid Res.* 56 (2015) 1926–1935.
- [35] S. Boenzi, F. Deodato, R. Taurisano, B.M. Goffredo, C. Rizzo, C. Dionisi-Vici, Evaluation of plasma cholestane-3 β ,5 α ,6 β -triol and 7-ketocholesterol in inherited disorders related to cholesterol metabolism, *J. Lipid Res.* 57 (2016) 361–367.
- [36] C. Helmschrodt, S. Becker, J. Thiery, U. Ceglarek, Preanalytical standardization for reactive oxygen species derived oxysterol analysis in human plasma by liquid chromatography-tandem mass spectrometry, *Biochem. Biophys. Res. Commun.* 446 (2014) 726–730, <https://doi.org/10.1016/j.bbrc.2013.12.087>.
- [37] G. Suske, The Sp-family of transcription factors, *Gene* 238 (1999) 291–300, [https://doi.org/10.1016/S0378-1119\(99\)00357-1](https://doi.org/10.1016/S0378-1119(99)00357-1).
- [38] S.W. Fouchier, J.C. Defesche, Lysosomal acid lipase A and the hypercholesterolaemic phenotype, *Curr. Opin. Lipidol.* 24 (2013) 332–338, <https://doi.org/10.1097/MOL.0b013e328361f6c6>.
- [39] M.A. Iacocca, R.A. Hegele, Recent advances in genetic testing for familial hypercholesterolemia, *Expert Rev. Mol. Diagn.* (2017) 1–11, <https://doi.org/10.1080/14737159.2017.1332997>.