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

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RETROSPECTIVE LYSOSONAL ACID LIPASE DEFICIENCY PATIENT IDENTIFICATION

2 Samples collection

PRIMARY HYPERCHOLESTEROLEMIA COLLECTION (PH)
N = 2,040

81 SUBJECTS INCLUDED.
Total Chol, c-HDL ↓ & NO MUTATIONS LDLR, APOB, PCSK9

ALTERED LIPID-LIVER PROFILE = 3

NORMAL LIPID-LIVER PROFILE
PH = 78
LSD= 66

ALTERED LSD BIOMARKERS = 20
NORMAL LSD BIOMARKERS = 124

LYSOSOMAL STORAGE DISEASE COLLECTION (LSD)
N = 1,295

67 SUBJECTS INCLUDED
Total Chol, c-HDL ↓ & NEGATIVE GAUCHER AND NIEMANN-PICK

ALTERED LIPID-LIVER PROFILE = 1

LIPA SANGER SEQUENCING
PH COLLECTION = 8
LSD COLLECTION = 16

2 LALD PATIENTS
1 LALD CARRIER
Evaluation of two approaches to lysosomal acid lipase deficiency patient identification: An observational retrospective study

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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 (C-C 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.
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, 83 LIPA mutations are listed in The Human Gene Mutation Database (www.hgmd.cf.ac.uk), with c.894A>G (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 a 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 retrospective study, we considered two approaches for screening individuals enrolled in two Spanish sample collections: primary hypercholesterolemia subjects (PH, n=2040) and LSD-suspected subjects (n=1295). 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 $\geq 60$ UI/L).

2.2.2. Plasma chitotriosidase analysis and CHIT1 gene genotyping

The ChT activity was determined using the fluorogenic substrate 4-methylumbelliferyl $\beta$-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 (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 ≥ 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 (25\(^{th}\)-75\(^{th}\) 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 660/2040 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, 67/1295 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 (20\(^{th}\) percentile). However, individuals belonging to Set 1 were overweight on average compared to those in Set 2 \((p<0.001; \text{ Table 1})\).

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 $\text{ChT} \geq \text{cut-off},$
$\text{CCL18/PARC} \geq \text{cut-off}$ or both (LSD 1).

Finally, 8 subjects were considered **HS**-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 $\text{ChT} \geq \text{cut-off}, \text{CCL18/PARC} \geq \text{cut-off}$ or both (LSD 2).

Finally, 16 subjects were considered **HS**-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 $c.-106C>A$ in the 5'-UTR in heterozygosity. Subject-2 had the known E8SJMJ mutation in
homozygosity, and Subject-3 carried the known E8SJM mutation [2] in combination with a non-previously described 25-bp deletion c.95+111_del25 (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). 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). The FATHMM-MKL score indicated an important deleterious effect (\(p=0.72; p > 0.5\) for a deleterious effect) that caused a premature stop codon in amino acid 33.

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 LAL 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.

Among the retrospective studies conducted in familial hypercholesterolemia (FH) cohorts without a definitive diagnosis, Pullinger et al. [31] analysed 1,357 FH patients and found 5 E8SJM heterozygous subjects and 1 homozygous subject (0.07%). Sjouke et al. [32], unfortunately, did not find any LALD patients in their cohort, probably due to the small study population (n=273), although 5 carriers and, interestingly, the enrichment of LIPA mutations in the FH cohort were identified. Recently, Chora et al. [33] studied two Portuguese FH cohorts (n=750), identifying 4 E8SJM homozygous subjects (0.5%, 3 index cases and 1 relative), as well as 7 novel possibly pathogenic variants in LALD. In our study, we did not find any LALD patients or carriers from the PH collection (n=81) and, just as Sjouke et al. concluded, the small study population of an ultra-rare pathology is probably the main cause [32].

Current plasma-subrogated biomarkers are not LALD specific. The ChT activity, is elevated in LSDs and atherosclerosis [9,11,13,14]. Moreover, the plasma CCL18/PARC concentration can also be elevated LSDs and atherosclerosis [16–19].
One interesting biomarker related to LALD is 7-ketocholesterol. This analyte results from the non-enzymatic oxidization of Chol and is found to be moderately increased in LALD patients [34,35]. However, presumed loss of stability of the analyte due to the long period of storage (up 2 years at -80ºC) of plasma samples in retrospective collections [36] discouraged the study 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.

Financial support

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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.

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Figure legends

Figure 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.
### Tables

**Table 1.** - Demographical data in the screened population.

<table>
<thead>
<tr>
<th></th>
<th>Female n (%)</th>
<th>Male n (%)</th>
<th>Age (y.o.)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BMI&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1 (PH)</td>
<td>81 (54.7)</td>
<td>24 (29.6)</td>
<td>49.0 (0.1-89.0)</td>
<td>26.7 (24.8-28.4)&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Set 2 (LSD)</td>
<td>67 (45.3)</td>
<td>32 (47.8)</td>
<td>52.5 (20.0-74.0)</td>
<td>22.6 (19.7-26.4)&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>TOTAL</td>
<td>148</td>
<td>56 (37.8)</td>
<td>51.0 (0.1-89.0)</td>
<td>26.2 (23.4-28.1)</td>
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y.o. = years old; BMI = Body Mass Index (kg/m<sup>2</sup>).

<sup>a</sup> Minimum-maximum.

<sup>b</sup> Median (25<sup>th</sup>-75<sup>th</sup> percentile).

<sup>c</sup>p ≤0.001.

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

<table>
<thead>
<tr>
<th>Subject</th>
<th>LALD Status</th>
<th>Gender</th>
<th>Age y.o.</th>
<th>TChol</th>
<th>c-HDL</th>
<th>c-LDL</th>
<th>TG</th>
<th>AST</th>
<th>ALT</th>
<th>ChT</th>
<th>CCL18/PARC</th>
<th>Nucleotide&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amino acid&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>S11</td>
<td>Carrier</td>
<td>F</td>
<td>1</td>
<td>115</td>
<td>18</td>
<td>75</td>
<td>111</td>
<td>89</td>
<td>10</td>
<td>47.5</td>
<td>77</td>
<td>c.-106C&gt;A</td>
<td>-</td>
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<tr>
<td>S22</td>
<td>Affected</td>
<td>F</td>
<td>18</td>
<td>200</td>
<td>35</td>
<td>146</td>
<td>96</td>
<td>28</td>
<td>47</td>
<td>140</td>
<td>140</td>
<td>c.894G&gt;A</td>
<td>p.Ser275_Gln298del</td>
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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/PARC = chemokine (C-C motif) ligand 18/pulmonary and activation-regulated chemokine (ng/mL).

<sup>a</sup> NM_000235.2 coding reference sequence (www.ncbi.nlm.nih.gov).

Highlights

1. Plasma LSD biomarkers was a good approximation to identify LALD patients.

2. Combination of LSD biomarkers and LLP enhanced the power to identify LALD patients in natural history cohorts.

3. Lysosomal storage disease suspected patients was queried for *LIPA* mutations.

4. Novel *LIPA* mutations were arisen in this study.
AUTHOR DECLARATION TEMPLATE

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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