

Large-Scale Screening of Lipase Acid Deficiency in at Risk Population

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Lysosomal acid lipase deficiency (LALD, OMIM#278000) is a rare lysosomal disorder with an autosomal recessive inheritance. The main clinical manifestations are related to a progressive accumulation of cholesteryl esters, triglycerides or both within the lysosome in different organs such as the liver, spleen, and cardiovascular system. A wide range of clinical severity is associated with LALD including a severe very rare antenatal/neonatal/infantile phenotype named Wolman disease and a late-onset form named cholesteryl ester storage disease (CESD). 63
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This study aimed to investigate a cohort of at-risk patients (4174) presenting with clinical or biological signs consistent with LALD using the assessment of LAL activity on dried blood spots. 70
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LAL activity was lower than 0.05 nmol/punch/L (cut-off: 0.12) in 19 patients including 13 CESD and 6 Wolman. Molecular study has been conducted in 17 patients and succeeded in identifying 34 mutated alleles. Fourteen unique variants have been characterized, 7 of which are novel. 73
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Conclusion 76

This study allowed to identify a series of patients and expanded the molecular spectrum knowledge of LALD. Besides, a new screening criteria grid based on the clinical/biological data from our study and the literature has been proposed in order to enhance the diagnosis rate in at risk populations. 77
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Keywords: LAL; Acid lipase deficiency; screening; Wolman; CESD; Cholesterol ester storage disease; Dried blood spot; DBS 80
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1. Introduction

Lysosomal acid lipase (LAL - EC.3.1.13) acts mainly in the hepatocytes following low-density lipoproteins (LDL) endocytosis to hydrolyze cholesterol esters (CE) and triglycerides (TG) into glycerol, free cholesterol (FC) and fatty acids. The reaction products are released into the cytosol and are involved in different metabolism such as energy, transport and anabolism [1]. FC is involved in several regulatory pathways. Sterol regulatory element binding proteins (SREBPs) modulate the expression of several genes involved in cholesterol and lipid synthesis. After their translation in the endoplasmic reticulum (ER), SREBPs form a complex with SREBP Cleavage Activating Proteins (SCAPs). In the presence of high FC concentrations, SCAP maintains SREBP in the ER. Conversely, in the presence of low cytosolic FC concentrations, SCAP brings SREBP to the Golgi apparatus where it becomes active through proteolytic cleavage steps. Then, SREBP translocates into the nucleus and binds to specific sequences located in the 5'UTR/enhancer regions on the target genes named SRE for Sterol Response Elements [2]. ABCA1, is an ATP-Binding-Cassette transporter that mediates the transport of cytosolic FC and phospholipids to pre-High-Density Lipoprotein (pre-HDL) particles to form HDLs [3].

LAL deficiency (LALD, OMIM#278000) is a rare autosomal recessive lysosomal disorder. The decrease or the loss of enzyme activity leads to a progressive accumulation of the enzyme substrates namely, CE, TG or both within the lysosome in different organs mainly the liver, spleen, and cardiovascular system [1, 4]. In LALD, intracellular FC depletion induces through SREBP regulation (i) an increase of cholesterol and apolipoprotein B synthesis with a subsequent increase of Very Low-Density Lipoproteins (VLDL) secretion, (ii) an increase of LDL receptor expression with a subsequent increase of LDL uptake. Besides, the low cytosolic FC concentration prevents ABCA1-mediated HDL particle formation. As a result, the lipid profile in LALD displays a high total cholesterol, LDL and TG concentrations, and a decreased HDL level [1, 4].

This lipid accumulation causes several cellular damages. In the liver, microvacuolar steatosis, elevation of transaminase activity, fibrosis and cirrhosis have been reported. Splenomegaly, the subsequent anemia and thrombocytopenia are probably related to lipid build-up within the macrophages. Lipid accumulation in the intestinal mucosa induces several gastrointestinal

disturbances such as diarrhea, abdominal and epigastric pain. Steroid hormones are synthesized in the adrenal cortex from FC. In LALD, low FC hampers the steroid synthesis and results in adrenal insufficiency. In severe antenatal forms of LALD, adrenal calcification related to extensive fetal adrenal necrosis can be detected by ultrasound [5]. The dyslipidemia associated to LALD underlies the increased cardiovascular risk.

LALD, as all inborn errors of metabolism (IEMs), is primarily due to a monogenic alteration. But it is to be noted that a large number of genetic and environmental factors may modulate phenotypic expression. Thus, a wide range of clinical severity is associated with LALD. This continuum encompasses a severe antenatal/neonatal/infantile phenotype named Wolman disease and a late-onset form named cholesteryl ester storage disease (CESD) [1].

Wolman disease is an ultra-rare condition (incidence: 1/500,000) with a very severe outcome. Prior to enzymotherapy treatment survival expectancy was less than 2 years [6]. The symptom onset may occur in the fetal stage with 50% of affected infants presenting with antenatal adrenal calcifications. Massive hepatomegaly and failure to thrive with malabsorption contribute to the premature death.

CESD incidence is estimated at 1/40,000 [7]. Hepatomegaly is often present at diagnosis and lysosomal lipid accumulation leads to progressive organ damages including cardiovascular alteration, fibrosis and cirrhosis. Recently, the prevalence at birth of LAL deficiency has been estimated at 3.45–5.97 cases per million births in European-ancestry populations [8]

At the molecular level, LALD results from the *LIPA* gene alterations. This gene is located on chromosome 10q23.2 and includes 10 exons. To date, 111 *LIPA* variants have been reported [1, 4] with a splicing variant being frequently present (NM_000235:c.894G>A - p.(Gln298=)) [9].

LALD treatment used to be based on low-fat diet, lipid reduction and hematopoietic stem cell transplant or liver transplantation with variable outcome. An enzyme replacement therapy (ERT), Sebelipase alfa, has been recently approved considering the clinical benefit especially in the most severe cases with a significant survival increase [6].

This study reports the screening of a cohort of at-risk patients presenting with clinical or biological signs consistent with LALD.

2. Materials and Methods

2.1. Cohort description

This cohort is based on 4174 dried blood spot (DBS) samples obtained from individuals, who were suspected of having LALD after clinical screening (the presence of unexplained hepatomegaly or an increase in transaminase activity by ≥ 1.5 times the upper reference limit, or a disturbed serum lipid profile with or without splenomegaly or gastrointestinal dysfunction or hepatic microvacuolar steatosis/fibrosis/cirrhosis), and submitted to our laboratory for the assessment of LAL activity between 2015 and 2019. The cohort included 1690 females (age = 42.78 ± 22.56 years) and 2494 males (age = 44.88 ± 20.49 years). Informed consents were obtained from all subjects involved in the study. Seventeen written informed consents have been signed for the molecular analysis.

2.2. Lysosomal acid lipase

All described laboratory procedures were performed at the Metabolic Biochemistry Department, Rouen University Hospital, Rouen, France

For all analyses presented in this study, DBS were prepared by spotting EDTA venous blood on filter paper (PerkinElmer 226). Fresh DBS were analyzed within three days upon preparation and were archived at -20 °C in individual plastic bags with a desiccant.

The enzymatic assay was carried out based on the method described by Hamilton *et al* [10, 11] using 4-methylumbelliferyl palmitate (4-MU) as a substrate and the LAL inhibitor, Lalistat-2. Briefly, DBS was incubated in 200 μ L of water for 1 hour. Then, 40 μ L aliquots of DBS water extracts were preincubated with 10 μ L of 30 μ M Lalistat-2 or water for 10 minutes in a 96-well flat bottom black plate. Then, 150 μ L of reaction buffer solution containing 4-MU-Palm (0.35 mM) and cardiolipin (0.032%) in a 0.15 M acetate buffer with 1% Triton X-100, pH 4.0 was added. The plate was sealed and incubated at 37 °C for 3 hours. Following the incubation, ten 4-MU standards serially diluted in water (Range: 0.02 - 11.34 μ mol 4-MU) were added to the plate.

Then, 100 μ L of 0.15 M acetate buffer with 1% Triton X-100 was added to all wells, and the fluorescence was measured in a plate reader (F7000, Hitachi, Japan) (excitation λ = 320 nm; emission λ = 446 nm). LAL activity was calculated by subtracting the value in the inhibited reaction (with Lalistat-2) from the value of reaction without the inhibitor (total lipase activity). The reference range was between 0.32 and 2.08 nmol/punch/1 hour with a cut-off set at 0.12 for LALD.

2.2. Molecular Analysis

DNA extraction: for NGS analysis, genomic DNAs were extracted from blood using a silica-membrane-based DNA purification method (QIAamp DNA Blood Mini Kit, QIAGEN). NGS sequencing was performed at the IRIB-Rouen University Hospital Facility (Service Commun de Génomique).

The promoter region and the flanking intronic sequences of the *LIPA* gene (NM_000235) are included in our in-house lysosomal disease NGS panel. The NGS sequencing method has been reported previously [12]. Briefly, custom primers were designed using the SureDesign software (Agilent Technologies, Santa Clara, California, USA). The library preparation protocol was set up using the QXT SureSelect enrichment kit from Agilent. Library construction was done using enzymatic fragmentation and the SureSelectQXT kit (Agilent Technologies, Santa Clara, California, USA) to capture targeted sequences. Patients' libraries were pooled after the enrichment step. Libraries were sequenced on a MiSeq or a NextSeq 500 platform (Illumina, San Diego, USA) using 2x150 bp paired-end sequencing. For the detection of SNVs, indels and CNV, a double bioinformatics pipeline was used with complementary algorithms in order to optimize the disease-causing variant detection rate.

An in-house software, CanDiD allowed for the prioritization and filtration of variants using defined criteria such as minor allele frequency in public databases or consequences of the variant (missense, synonym, nonsense, splicing) [13]. The filtered variants were compared to variant databases including dbSNP (<https://www.ncbi.nlm.nih.gov/snp/>), GnomAD (<https://gnomad.broadinstitute.org/>), HGMD (<http://www.hgmd.cf.ac.uk/>) and LOVD (<https://databases.lovd.nl/shared/genes>).

Evaluation of the pathogenicity of the variants were analyzed with in silico tools such as SIFT [14], PolyPhen2 [15] or MutationTaster [16] and M-CAP [17] to predict potential deleterious effect on protein function, and HumanSplicingFinder 2.4.1 [18], MaxEntScan [19], NNSPLICE [20], GeneSplicer [21], SpliceSiteFinder [22], and ESEFinder [23] for possible effect on splicing. Variant classification was done according to the recommendations of the American College of Medical Genetics [24].

The control of the sample identity was performed using a multiplex SNaPshot analysis comparing five SNPs located within the captured regions of 5 genes unrelated to LDs included in the panel.

3. Results

3.1. Patients

We assessed 4174 DBS samples (**Supplementary Table S1**). In females (n = 1,690, 40% of the cohort), the overall mean age was 42.78 ± 22.56 years, with a mean LAL activity of 0.98 ± 0.59 nmol/punch/1 hour. For males (n = 2,494, 60% of the cohort), mean age was 44.88 ± 20.49 years, with a mean LAL activity of 0.89 ± 0.54 nmol/punch/1 hour. One hundred and fifty-seven samples came from children below 2 years old (3.7 %) including 73 females and 84 males. Four thousand and seventeen (96.3%) samples came from patients older than 2 years old and included 1607 females and 2410 males.

Based on this cohort, 19 patients (**Figure 1 and 2**) have been detected with an LAL activity lower than 0.05 nmol/punch/1 including 13 CESD (9 females and 4 males) with an average age at diagnosis of 22.42 ± 18.23 years and 6 Wolman (4 females and 2 males) with an average age at diagnosis of 3.12 ± 4.08 months. For CESD patients, laboratory work-up showed an impaired lipid profile (**Supplementary Table S2**) with a total cholesterol = 2.72 ± 0.47 g/L (<2.3), HDL cholesterol = 0.35 ± 0.09 g/L (N>0.45), LDL cholesterol = 2.31 ± 0.5 g/L (N<1.3), triglycerides = 1.92 ± 1.26 g/L (N<1.33). For the Wolman patients, laboratory work-up showed an impaired lipid profile, cholesterol = 1.53 ± 0.87 g/L, HDL cholesterol = 0.15 ± 0.08 g/L, LDL cholesterol = 1.3 ± 0.92 g/L, triglycerides = 3.26 ± 1.42 g/L.

The main clinical phenotype of Wolman patients was as follows: all patients were from consanguineous parents and presented with hepatomegaly and hypertriglyceridemia, 5/5 showed adrenal calcification, 5/6 presented with anemia, 5/6 with splenomegaly and 5/5 with elevated serum transaminases, 4/6 presented failure to thrive and 4/6 died in infancy, 2/2 vomiting, 2/6 with hypercholesterolemia, 2/5 hepatic fibrosis, 2/5 hepatic steatosis, 1/3 diarrhea, 1/3 abnormality in cardiovascular system and 1/5 with cirrhosis. Regarding CESD patients, 13/13 presented with hepatomegaly, 11/11 showed elevated in serum transaminases, 9/9 presented with hypercholesterolemia, 7/11 with hepatic steatosis, 5/7 with hypertriglyceridemia, 3/9 with hepatic fibrosis and 2/10 with splenomegaly (**Supplementary Table S2 and Table S3**).

Molecular investigation has been conducted in seventeen patients. It yielded 34 mutated alleles (**Figure 4 and Supplementary Table S4**). Fourteen unique variants have been identified, 7 have been previously reported [9, 25-30]. The common variant, c.894G>A / p.(Gln298=) [9], has been identified in 12 mutated alleles. Seven variants were novel and included four in CESD form: NM_000235:c.340G>T - p.(Asp114Tyr), c.232_243delins - p.(Pro76Alafs*5), c.538+1G>A 6 p.(?), c.647T>G - p.(Leu216*) and three in Wolman disease: c.429-1G>C - p.(?), c.601G>C - p.(Gly201Arg) and c.538G>A - p.(Gly180Ser).

4. Discussion

LALD presentation covers a very wide range of clinical severity spanning from a life-threatening pediatric form to a non-specific, slowly progressive adult form.

The progressive lipid accumulation, primarily in hepatocytes and macrophages, underlies the organomegaly, gastro-intestinal dysfunction, liver disease, and dyslipidemia observed in LALD patients. First-line diagnosis relies on LAL enzyme activity assessment using peripheral leukocytes or DBS. The recent development of rapid and reliable fluorimetric method using DBS [10] enabled the screening of large at-risk populations. Molecular study of *LIPA* gene is indicated to confirm LALD diagnosis. Considering the ever-growing advances in LD therapies, an early and accurate diagnosis of LALD is critical. Unexplained liver disease and/or lipid abnormalities should prompt to assess LAL enzyme activity. Besides, hypercholesterolemic status may be misleading. Indeed, known hypercholesterolemia disorders are often evoked as first-line diagnosis while this phenotype might be consistent with LALD [31].

The results presented here are issued from the diagnostic work-up of patients presenting with clinical or biological signs consistent with LALD, namely unexplained hepatomegaly or increased transaminase activity (≥ 1.5 times the upper reference limit), or abnormal serum lipid profile with or without splenomegaly or gastrointestinal dysfunction or hepatic microvacuolar steatosis/fibrosis/cirrhosis. Nineteen patients have been diagnosed with LALD with an LAL activity below 0.05 nmol/punch/h (LALD patients' overall mean = 0.01). No difference has been observed in LAL activity values between CESD and Wolman patients. Six patients were diagnosed with Wolman disease out of 157 children below 2 years old (3.82%). Regarding CESD, 13 patients were identified out of 4017 individuals older than 2 years of age (0.32%). This very low diagnosis yield prompted us to reevaluate the screening criteria considering the clinical and biological features present in the patients characterized in this study and others [32-37]. In our series, hepatic damages were present in all patients and dyslipidemia was noted in 15/15 patients. Besides, Wolman patients presented with additional features such as gastrointestinal disturbances, anemia, failure to thrive, adrenal calcification and premature death. The screening criteria grid proposed here prioritizes liver damages and dyslipidemia and takes into account the main features reported in the literature and in our series. The suggested criteria are presented in **Table 1** with a weighted scoring for each feature. To conduct LALD screening, a total score higher than four is required with either (i) at least one liver/spleen and one dyslipidemia feature or (ii) one feature across three different categories. This stringent strategy may allow a sharper, more targeted, and therefore more efficient screening for LALD.

Molecular studies were conducted in 17 patients and succeeded in confirming the molecular basis of LALD in all tested individuals. Fourteen unique variants have been identified; 7 variants have been previously reported [9, 25-30] while 7 were novel (3 missense, 1 nonsense, 2 splicing, 1 frameshift variants). The common variant, c.894G>A - p.(Gln298=), accounted for 12 out of 34 mutated alleles (Figure 4) and was exclusively identified in CESD patients. Importantly, severe truncating variants including nonsense, frameshift and splicing variants were associated with both Wolman disease and CESD phenotypes, but the variants identified in Wolman patients were distinct from those associated with CESD phenotype.

5. Conclusions

LALD is a very rare and underdiagnosed condition. This study allowed to identify a series of patients and better tune the screening criteria for LALD in at-risk populations. Besides, our study expands the molecular spectrum knowledge of lipase acid deficiency. The effect of ERT on survival rate and clinical course urges to define efficient diagnosis strategies.

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Data Availability Statement: All the data that support the findings are presented in the manuscript and the supplementary material.

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Legends

Figure 1. Cohort description.

Figure 2. LAL enzymatic activity distribution between Non-LALD, CESD and Wolman patients. The panel to the left is a zoom on low activity values.

Figure 3. Clinical phenotype overview of the CESD and Wolman patients. Blank box means that the status of clinical feature has not been reported.

*Hypercholesterolemia is defined with either increased total cholesterol or LDL cholesterol concentrations.

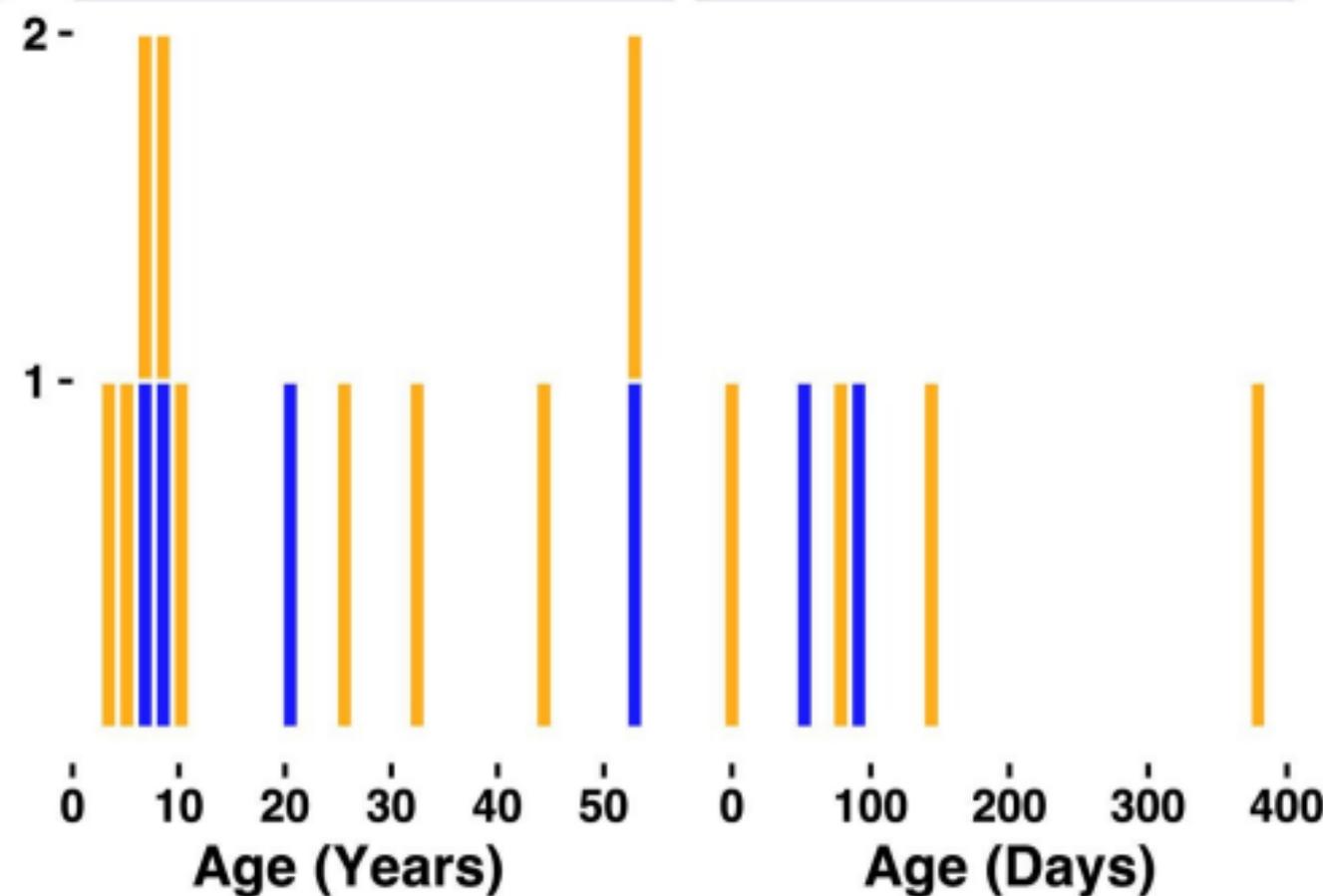
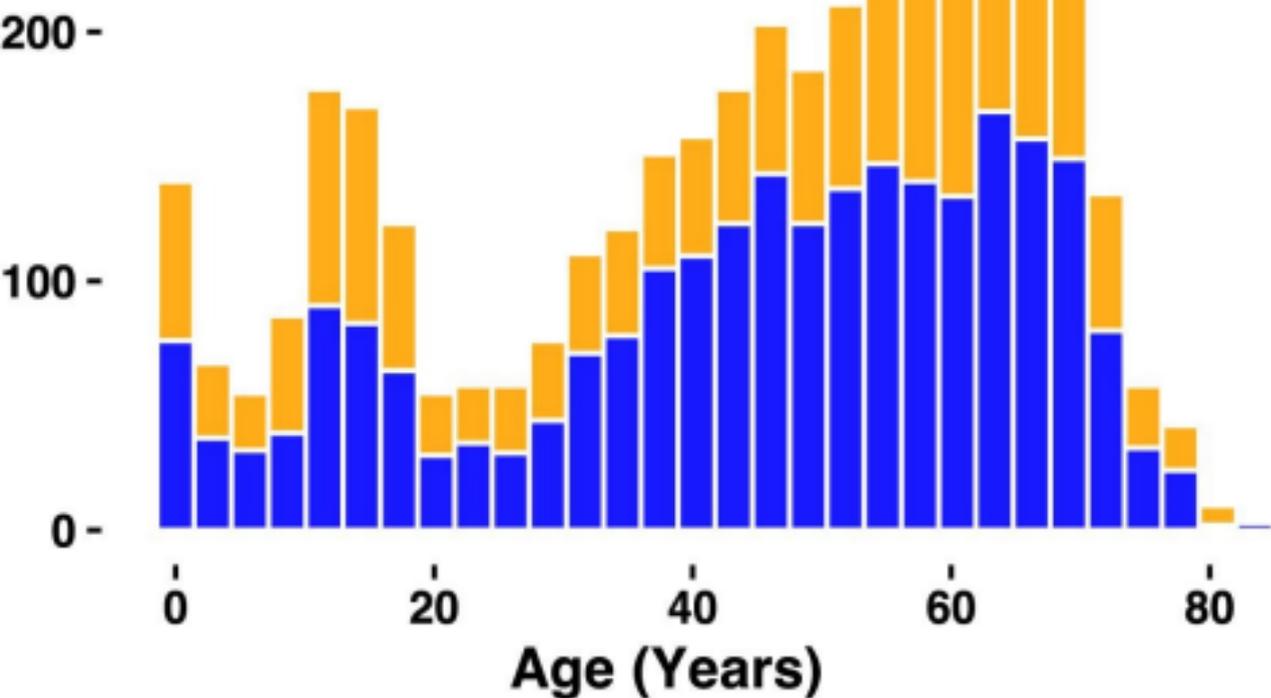
Figure 4. Molecular variations of the seventeen investigated cases describing seven novel and seven previously reported variants. The number of mutated alleles is indicated in the circle. The size of the bubble is proportional to the number of mutated alleles.

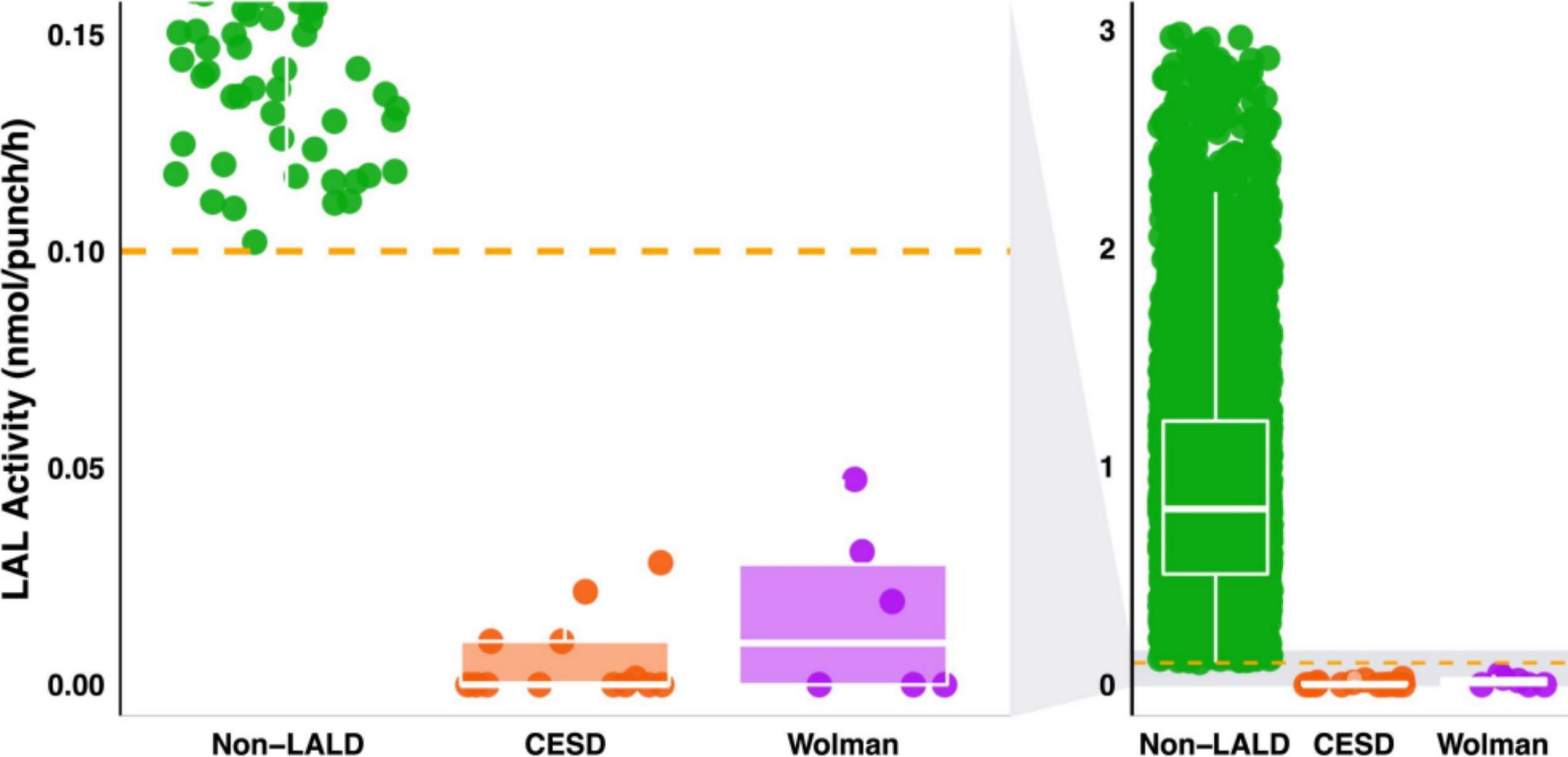
Non-LALD (n = 4155)
Female = 1677 : Male = 2488

CESD (n = 13)
Female = 9 : Male = 4

Wolman (n = 6)
Female = 4 : Male = 2

Male
Female



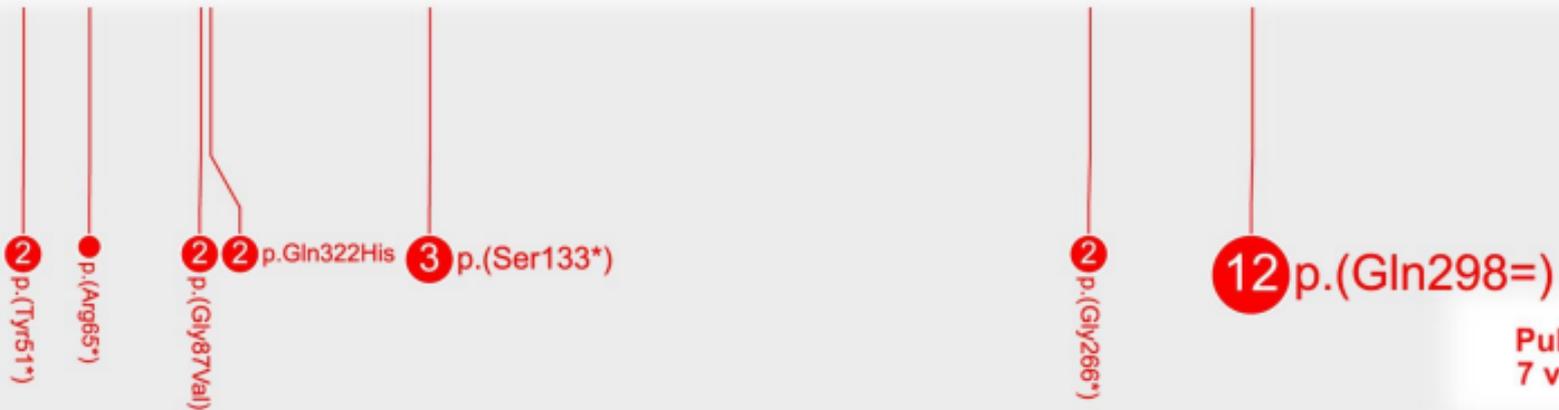
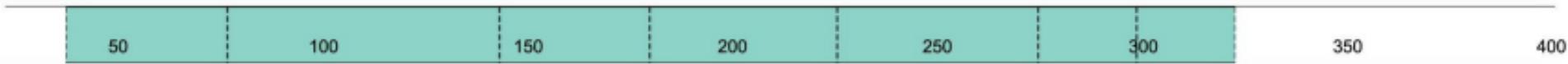




This study
7 variants



LIPA
NM_001127605
Protein length



Published
7 variants

Table 1. Screening criteria.

Categories	Features	Score
Liver/Spleen	Hepatomegaly	3
	Elevated transaminases (>1.5 N)	3
	Microvacuolar Steatosis	3
	Fibrosis/Cirrhosis	2
	Splenomegaly	1
Dyslipidemia	LDL > 1.3 g/L (under lipid lowering treatment)	3
	HDL < 0.45 g/L	3
Gastrointestinal	Diarrhea/Vomiting	3
	Ascitis	1
Other	Failure to thrive	3
	Adrenal calcification	3
	Abdominal distension/pain	1
	Thrombopenia	1
	Anemia	1

Highlights

- A screening of 4174 at-risk LALD patients is reported
- Nineteen patients including thirteen CESD and six Wolman have been identified
- Thirty-three mutated alleles have been identified including fourteen unique variants have been characterized, 7 of which are novel.
- A new LALD screening criteria grid based on the clinical/biological data is proposed.

Journal Pre-proofs