

Research Article

Characteristics of the Phenotype-Genotype Correlation in Cohort of Russian and Ukrainian Patients with Lysosomal Acid Lipase Deficiency

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Abstract

Lysosomal Acid Lipase Deficiency (LAL-D) is a rare autosomal recessive disease caused by mutations in the *LIPA* gene encoding the enzyme of cholesterol metabolism Lysosomal Acid Lipase (LAL).

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The common variant of *LIPA* is c.894G>A caused the disease in more than a half of cases in Europe.

The aim of our work was to study *LIPA* gene mutations spectrum and find correlations of the genotype and secondary biochemical markers in cohort of Russian and Ukrainian LAL-D patients.

In 39 patients LAL-D diagnosis was confirmed. Molecular analysis of *LIPA* revealed the variant c.894G>A in homozygous or compound-heterozygous state in 34 of them. Fifteen other variants were defined, 11 of them were novel.

Chitotriosidase activity and the oxysterols levels of homozygous and compound-heterozygous for c.894G>A patients were compared.

Two common mutations among the Russian and the Ukrainian patients were defined. Seventeen identified variants with 11 of them novel and unique demonstrate the variety of *LIPA* mutations spectrum in Russia.

Heterozygous severe defects of *LIPA* were found to increase the chitotriosidase activity and the oxysterols levels in the patients. It indicates the higher extent of macrophages activation/liver fibrosis and dyslipidemia progression, respectively. These data may have a prognostic significance.

Keywords: Chitotriosidase; Cholesterol; Lysosomal acid lipase deficiency; *LIPA* gene; Lysosomal storage diseases; Oxysterols

Abbreviations

- 7-KC - 7-Ketocholesterol Concentration
- ALT - Alanine Aminotransferase
- AST - Aspartate Aminotransferase
- CESD - Cholesteryl Ester Storage Disease
- Cht - Chitotriosidase
- CT - Computer Tomography
- C-triol - Cholestane-3 β ,5 α ,6 β -triol
- DBS - Dried Blood Spots
- HDL - High Density Lipoprotein
- LAL - Lysosomal Acid Lipase
- LAL-D - Lysosomal Acid Lipase Deficiency
- LDL - Low Density Lipoprotein
- LOF - mutation - mutation caused total Loss of Enzyme Function
- LSD - Lysosomal Storage Disease
- TC - Total Cholesterol
- TG - Triglycerides
- US - Ultrasound
- WD - Wolman Disease

Introduction

Lysosomal Acid Lipase Deficiency (LAL-D; MIM#278000) is a rare autosomal recessive Lysosomal Storage Disease (LSD) caused by mutations in the *LIPA* (MIM 613497) gene encoding the enzyme

Acid Lipase (LAL) that plays a key role in lipid metabolism. LAL splits LDL-derived neutral lipids into free cholesterol and fatty acids. Dysfunction of the enzyme leads to progressive accumulation of Cholesteryl Esters (CE) and Triglycerides (TG), mainly in cells of the macrophage/monocyte system of the liver and spleen and hepatocytes, too [1,2]. LAL-D results in the impairment of liver function in the form of elevation of serum aminotransferases and dyslipidemia.

LAL-D is considered as a continuum of phenotypes, historically divided into two clinical forms: Wolman Disease (WD) with neonatal onset and rapidly progressing fatal course, and Cholesteryl Ester Storage Disease (CESD) with late onset, mild course and a varied severity of symptoms [2].

LIPA is located on the chromosome 10q23.31. Over 60 variants of this gene are known to be associated with LAL-D. The c.894G>A (E8SJM^{-1G>A}) variant is the most common identified in European populations and is responsible for more than half of reported cases [2-5]. For some other alleles, an ethnic background is observed [3,6]. Patients harboring the common c.894G>A allele or a missense mutation that ensures the significant residual enzyme activity in at least a heterozygous state, have CESD phenotype, regardless how severe is the other variant. WD phenotype is the result of two severe deleterious alleles in a homozygous or compound-heterozygous state [2,3,7].

The definite incidence of LAL-D is unknown. The disease is considered as pan-ethnic and its frequency in Europe is estimated from 1/300 000 to 1/40 000 depending on ethnicity and geographical location [2,7-9].

The measurement of LAL activity could be carried out in cultures of skin fibroblasts, peripheral leukocytes or liver tissue cells. The method used for the measurement in Dried Blood Spots (DBS) in the presence of specific inhibitor (Lalistat-2) introduced by Hamilton et al., in 2012 has been found to be effective and the most rapid; nowadays it is considered as the first diagnostic step for LAL-D screening [10].

There are also present other secondary biochemical markers of the disease. Chitotriosidase (Cht) is a human chitinase secreted by the activated macrophages. Cht activity is a classic marker of gaucher disease and moderately elevated values are found in a variety of some other LSDs including LAL-D [11]. Due to the storage process and subsequent macrophages activation, Cht activity is increased in the course of the disease, so it could be considered as a serum marker for LAL-D. Oxysterols are oxidized derivatives of cholesterol, almost always produced by non-enzymatic processes. Two such compounds, cholestane-3 β ,5 α ,6 β -triol (C-triol) and 7-Ketocholesterol (7-KC), have been shown to be elevated in patients with Niemann-Pick type C disease, cerebrotendinous xanthomatosis and also LAL-D [12].

The aim of our work was to study LIPA gene mutations spectrum and find correlations of the genotype and secondary biochemical markers, including serum chitotriosidase activity, cholestane-3 β ,5 α ,6 β -triol and 7-ketocholesterol levels, in the cohort of Russian and Ukrainian LAL-D patients.

Materials and Methods

Patients

Between 2009 and 2015, 11 patients (5 male and 6 female) from

10 unrelated families, with age ranged from 2 month to 23 years old, suspected to have LAL-D based on clinical and biochemical features, were referred for molecular analysis of the LIPA gene.

Since 2016 the enzymatic assay for LAL activity in DBS in the presence of Lalistat-2 was added to the list of diagnostic screening tests on LAL-D carried out in Research Centre for Medical Genetics of Moscow.

The selective criteria (uncombined) of suspected LAL-D were the following:

- Hepatomegaly, feeding difficulties, frequent vomiting, diarrhea, swelling of the abdomen and anemia presented in the first 6 months of life
- Hepatomegaly observed in childhood
- ALT > 1,5-fold upper limit of normal
- Presence of dyslipidemia type II

From January 2016 till January 2019, a total number of tested DBS specimens were 1998. Patients were originated from medical centers of different regions from Russia and Ukraine. Sex distribution was approximately equal, the age varied from the first week of life to 45 years.

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 (5). Informed consent was obtained from all patients included in the study or from their legal guardians.

Patients' medical records

A retrospective chart review of patients' medical records concerning the first presented signs and symptoms, as well as biochemical (liver transaminases, platelet count, total cholesterol, HDL-C, LDL-C, TG), histological (histopathological examination of liver biopsy specimens) were collected. Some clinical and laboratory data have been published previously [13,14]. An informed consent for collecting clinical data and blood samples for biobanking was obtained from all patients included in the study.

DNA sequencing

DNA was extracted from whole blood with EDTA and DBS using the DNAprep100 Kit (IsoGene, Moscow, Russia).

Coding exons of LIPA (exons 2 - 10) were amplified by PCR and analyzed by direct sequencing on ABI PRISM 3500xL Genetic Analyzer (Applied Biosystems, USA). Primers sequences and PCR protocols are available on request. Analysis of Sanger sequencing data was performed using Chromas software and Nucleotide-BLAST software. LIPA variants are numbered according to the guidelines from the Human Genome Variation Society (<http://www.hgvs.org/mutnomen>) using NCBI Reference Sequences NM_000235.3, NG_008194.1.

Biochemical assays

LAL activity was measured by fluorimetric analysis in DBS as was reported by Hamilton et al. [10]. Cht activity was measured by fluorimetric analysis using the method described by vom Dahl et al., and adapted for DBS, according to own adaptation (unpublished) [11]. Oxysterols (CT and 7-KC) levels were measured by HPLC-MS/

MS as described by Boenzi et al., with modification [15]. Chromatographic separation was performed on column Gold C18 (2.1X100 mm, 5 microns) using HPLC-system LC20 (Shimadzu, Japan) with detection on mass-spectrometer Sciex 3200Trap (ABSciex, USA).

Determination of variant pathogenicity

Classification of novel variants by their pathogenicity was performed according to standards and guidelines for the interpretation of sequence variants [16]. For missense and intronic variants, was based on minor allele frequencies of the mutation detected by genetic sequencing (GnomAD, PMID: 27535533), parental segregation analysis (if performed) and in silico pathogenicity prediction algorithms. In silico analysis was performed using predictive algorithms/software MutationTaster, PolyPhen2, SIFT, PROVEAN (for missense variants), Human Splicing Finder and NetGene2 (for variants of alternative splicing). For novel synonymous mutation c.600G>A, determination of pathogenicity was based on cDNA analysis by direct sequencing and functional analysis by minigene splicing assay.

Statistic analysis

Statistic analysis was performed using GraphPad Prism software (version 6.01).

Results and Discussion

Thirty-nine patients with LAL-D were enrolled into the study. The ethnic distribution was as follows: 32 Russians, 6 Ukrainians, 1 Azerbaijani. The diagnosis was confirmed by molecular analysis of the LIPA gene or by the measurement of LAL activity in DBS and molecular analysis. The LAL activity in affected patients was noted as less than 0.04 nmol/punch/h.

Genetic analysis

Among 39 patients, 34 had a common splice-junction c.894G>A variant of LIPA, identified in homozygous (15 pts) or compound-heterozygous (19 pts) state. Eight patients had c.796G>T (p.G266*) variant identified only in compound-heterozygous state. We also found the 4 other known mutant alleles: c.309C>A, c.347G>A, c.398delC, c.894+1G>A and 11 novel variants. The latter were presented in

Azerbaijani and some of Russian patients. Some of the novel variants were published by us earlier without any pathogenicity analysis [17].

Three out of eleven novel variants were classified as nonsense substitutions: c.348G>A (p.W116*), c.398C>A (p.S133*) and c.420G>A (p.W140*), what is “very strong” criterion of pathogenicity [16]. The other five variants were classified as leading to frame-shift alterations: 167_170dup4 (p.L61Yfs*5), c.421delG (p.A141Lfs*20), c.442delG (p.A148Qfs*13), c.817_818delAA (p.N273Yfs*3), c.911_912delinsT (p.K304Ifs*4) and the substitution c.-3A>G (p.?) that affects the canonical acceptor splicing site. In addition, there are the described mutations in codons 116, 133 and 140 leading to the same predictable changes in the primary structure of the enzyme (“strong” criterion of pathogenicity [16]). The bioinformatic analysis of the substitution c.-3A>G with Human Splicing Finder and NetGene-2 showed a high probability of alteration of the constitutive acceptor splicing site in intron 1, that resulted in skipping of exon 2 and the initiating codon of LIPA gene cDNA. The synonymous variant c.600G>A in exon 6 showed a high probability of exonic cryptic splicing site activation leading to 63 b.p. deletion corresponding to c.541_603del63 of the cDNA. The variant c.600G>A was inherited from both patient’s parents, who were heterozygous, and the variants c.-3A>G, c.398C>A and c.420G>A reside in trans-configuration with the variant c.894G>A, subsequent to the results of the parental segregation analysis (“moderate” criterion of pathogenicity [16]). For the variant c.600G>A the functional analyses by minigene assay was performed [14].

The remaining variant is the missense substitution c.956A>T (p.H319L). Predictable pathogenicity of these missense variant was valued as possible damaging.

In accordance to the gnomAD database, the allelic frequencies of all defined variants in the healthy cohort were extremely low (Table 1). A retrospective analysis of the exomic sequencing results of 50 DNA samples was performed (100 chromosomes). The samples were provided by patients who applied to the Research Centre for Medical Genetics of Moscow with different diagnoses and had LAL activity in DBS at the reference range. There were not any of revealed in LAL-D patients variants in the 100 chromosomes of patients with normal LAL activity.

№	Variant	Effect	Allelic frequency (gnomAD)	HGMD ID	References	PolyPhen-2	SIFT	PROVEAN	Variant significance
Known variants									
1	c.309C>A (p.S103R)	Missense	0.000008	CM1617783	[5,18]	Probably damaging	Damaging	Deleterious	Likelypathogenic
2	c.347G>A (p.W116*)	Nonsense	ND	CM993350	[19-21]	-	-	-	Pathogenic
3	c.398delC (p.S133*)	Nonsense	0.00002	HD971471	[3,22]	-	-	-	Likelypathogenic
4	c.796G>T (p.G266*)	Nonsense	ND	CM960945	[19,20,23]	-	-	-	Pathogenic
5	c.894G>A(p.275_298del)	Synonymous	0.000827	CS951467	[4,21]	High probability of donor splice site alteration (analyzed by Human Splicing Finder and NetGene-2)			Pathogenic
6	c.894+1G>A (p.275_298del)	Spliceregion alteration	ND	CS963024	[19,23]	High probability of donor splice site alteration (analyzed by Human Splicing Finder and NetGene-2)			Pathogenic
Novel variants									
1	c.-3A>G (p.?)	Spliceregion alteration	ND	-	-	-	-	-	Likelypathogenic
2	c.177_180dup4 (p.L61Yfs*5)	Frame-shift	ND	-	-	-	-	-	Likelypathogenic

3	c.348G>A (p.W116*)	Nonsense	ND	CM172457	[17]	-	-	-	Pathogenic
4	c.398C>A (p.S133*)	Nonsense	ND	CM1724568	[17]	-	-	-	Pathogenic
5	c.420G>A (p.W140*)	Nonsense	ND	CM1724569	[17]	-	-	-	Pathogenic
6	c.421delG (p.A141Lfs*20)	Frame-shift	ND	-	-	-	-	-	Likelypathogenic
7	c.442delG (p.A148Qfs*13)	Frame-shift	ND	-	-	-	-	-	Likelypathogenic
8	c.600G>A (p.180_200del)	Cryptogenic splice site	ND	-	-	High probability of exonic cryptic splice site activation (analyzed by Human Splicing Finder and NetGene-2); 63 b.p. deletion in mRNA			Pathogenic
9	c.817_818delAA (p.N273Yfs3*)	Frame-shift	ND	CD1724570	[17]	-	-	-	Likelypathogenic
10	c.911_912delinsT (p.K304Ifs4*)	Frame-shift	ND	-	-	-	-	-	Likelypathogenic
11	c.956A>T (p.H319L)	Missense	ND	-	-	Possibly damaging	Damaging	Deleterious	Uncertain significance

Table 1: The classification of defined LIPA variants.

№	Sex	First signs and symptoms	Age of diagnosis	First consultation– laboratory data	Liver biopsy (if performed); Liver in US or CT examination (if performed)	Activity of the enzymes	Oxy-sterols levels	Genotype
Homozygous for the variant c.894G>A patients								
1	M	recurrent abdominal pain, vomiting; hepatomegaly (4y)	14y	ALT 180.0 AST 95.0 TC 8.35 HDL n.d. LDL n.d. TG 0.94	Decreased echogenicity of liver in US, hyperechogenic inclusions in the parenchyma (calcifications);	LAL 0.00 Cht752	No data	c.894G>A/ c.894G>A
2	M	enlarged abdomen; hepatosplenomegaly (3y)	5y	ALT 104.0 AST 89.4 TC 9.68 HDL 0.58 LDL 7.95 TG n.d.	Hepatosplenomegaly and lymphadenopathy in CT; Liver biopsy – lipid storage vacuoles in hepatocytes and Kupffer cells	LAL 0.00 Cht 25	C-triol 24.4 7-KC 99	c.894G>A/ c.894G>A
3	F	recurrent abdominal pain; elevated transaminases (7y)	8y	ALT 163.0 AST 131.0 TC 9.33 HDL 1.20 LDL 7.53 TG 1.69	Diffuse heterogeneity, increased echogenicity of liver in US;	LAL 0.00 Cht 61	C-triol 48.9 7-KC 223	c.894G>A/ c.894G>A
4	F	no complain; elevated transaminases (6y)	7y	ALT 124.0 AST 81.0 TC 9.55 HDL 0.92 LDL 8.40 TG 1.34	Diffuse heterogeneity and increased echogenicity of liver in US;	LAL 0.01 Cht58	C-triol33.4 7-KC 189.6	c.894G>A/ c.894G>A
5	F	recurrent abdominal pain; elevated transaminases, hyperbilirubinemia (2y)	9 y	ALT 183.2 AST 144.0 TC 6.16 HDL 1.28 LDL 4.60 TG 0.94	Diffuse heterogeneity, increased echogenicity of liver in US;	LAL 0.00 Cht 39	No data	c.894G>A/ c.894G>A
6	F	recurrent icteric sclerae, apathy; mild hepatomegaly, elevated transaminases (9y)	19y	ALT 70.0 AST 54.0 TC 7.80 HDL 1.53 LDL 5.32 TG n.d.	Diffuse changes of liver parenchyma, signs of fibrosis in US; Liver biopsy (10y) – periportal fibrosis	LAL 0.01 Cht 334	No data	c.894G>A/ c.894G>A
7	F	no complain; elevated transaminases (5y)	13y	ALT 114.0 AST 148.0 TC 8.20 HDL 1.10 LDL 6.19 TG 1.97	Diffuse changes of liver parenchyma in US; Liver biopsy – periportal fibrosis Liver fibrosis in METAVIR score F-3	LAL 0.00 Cht 15	No data	c.894G>A/ c.894G>A

8	M	enlarged abdomen; hepatomegaly (7m)	32y	ALT 188.0 AST 80.0 TC 7.01 HDL 0.80 LDL 5.02 TG 2.56	Features of liver fibrosis in US	LAL 0.00 Cht 32	C-triol 42.3 7-KC 104.4	c.894G>A/ c.894G>A
9	F	recurrent abdominal pain; hepatosplenomegaly, elevated transaminases (5y)	7y	ALT 134.0 AST 86.0 TC 8.00 HDL 1.00 LDL 7.07 TG n.d.	Diffuse heterogeneity of liver parenchyma in US	LAL 0.00	No data	c.894G>A/ c.894G>A
10	M	recurrent abdominal pain, poor appetite, vomiting; elevated transaminases (4y)	6y	ALT 88 AST 125 TC 5.90 HDL 1.05 LDL 4.58 TG 0.94	Mildly increased echogenicity of liver in US; Liver biopsy (6y) – fibrosis, lipid storage vacuoles in hepatocytes and Kupffer cells; Liver fibrosis in METAVIR score F2-F3	LAL 0.03 Cht 42	C-triol 26.7 7-KC 137.5	c.894G>A/ c.894G>A
11	F	no complain; hepatomegaly, elevated transaminases (3y)	9y	ALT 210.0 AST 146.0 TC 11.10 HDL 1.14 LDL 9.08 TG 1.51	Diffuse heterogeneity, increased echogenicity of liver in US;	LAL 0.02 Cht 220	C-triol 101.1 7-KC 181.8	c.894G>A/ c.894G>A
12	F	no complain; mild hepatomegaly, elevated transaminases (2y)	5y	ALT 160.0 AST 118.0 TC 9.07 HDL 1.45 LDL 8.24 TG n.d.	Increased echogenicity of liver in US; Liver biopsy (3y) – lipid storage vacuoles in hepatocytes and Kupffer cells	LAL 0.02	No data	c.894G>A/ c.894G>A
13	F	no complain; mild hepatomegaly (1y 6m)	2y	ALT 32.0 AST 58.0 TC 6.41 HDL 0.60 LDL 3.79 TG 1.68	Normal echogenicity of liver in US	LAL 0.03 Cht 54	C-triol 21.3 7-KC 144.3	c.894G>A/ c.894G>A
14	M	recurrent vomiting, jaundice; hepatomegaly, elevated transaminases (4y)	6y	ALT 117.0 AST 97.0 TC 7.89 HDL 0.84 LDL 6.93 TG n.d.	Diffuse heterogeneity, decreased echogenicity of liver in US	LAL 0.00 Cht 884	No data	c.894G>A/ c.894G>A
15	F	no complain; elevated transaminases, dyslipidemia (15y)	17y	ALT 114.0 AST 89.0 TC 11.2 HDL 1.55 LDL 9.63 TG n.d.	Increased echogenicity of liver in US;	LAL 0.01 Cht 79	No data	c.894G>A/ c.894G>A
Heterozygous for the variant c.894G>A patients								
1	F	no complain; hepatomegaly (9m)	8y	ALT 198.0 AST 78.7 TC 7.89 HDL 0.70 LDL 6.00 TG n.d.	Homogeneity and increased echogenicity of liver parenchyma in US;	LAL 0.00	No data	c.894G>A/c.894+1G>A
2	M	no complain; hepatomegaly (3y)	7y	ALT 147.5 AST 124.1 TC 9.70 HDL n.d. LDL 7.90 TG n.d.	Porto-portal fibrosis in US	LAL 0.02 Cht 274,3	No data	c.894G>A/c.796G>T
3	F	recurrent abdominal pain; hepatosplenomegaly, elevated transaminases (3y)	4y	ALT 150.0 AST 165.4 TC 8.05 HDL n.d. LDL 7.30 TG n.d.	Decreased echogenicity of liver in US	LAL 0.01 Cht 485	No data	c.894G>A/c.796G>T

4	F	weakness, tightness of the right hypo-chondrium; hepatosplenomegaly, elevated transaminases (7y)	18y	ALT 122.0 AST 99.0 TC 8.67 HDL 2.07 LDL 5.57 TG 2.26	Mildly diffused heterogeneity, increased echogenicity of liver in US	LAL 0.00 Cht 1229,6	C-triol143 7-KC 650	c.894G>A/c.796G>T
5	M	no complain; hepatomegaly, elevated transaminases (4y)	14y	ALT 109.0 AST 82.0 TC 6.18 HDL 0.90 LDL 5.59 TG n.d.	Diffuse changes of liver parenchyma, features of liver fibrosis in US	LAL 0.02 Cht 148	C-triol75 7-KC 150.6	c.894G>A/ c.421delG
6	F	no complain; elevated transaminases (4y)	5y	ALT 128 AST 62 TC 6.18 HDL 1.20 LDL 5.85 TG n.d.	Diffuse changes of liver parenchyma, features of liver fibrosis in US	LAL 0.04 Cht 595,5	No data	c.894G>A/ c.796G>T
7	M	no complain; hepatosplenomegaly, elevated transaminases (7y)	10y	ALT 143 AST 207 TC 7.59 HDL 0.80 LDL 4.63 TG 1.76	Homogeneity, decreased echogenicity of liver parenchyma in US;	LAL 0.01 Cht 356	C-triol 155 7-KC 405	c.894G>A/c.420G>A
8	M	no complain; mild hepatomegaly, elevated transaminases (3y)	8y	ALT 258 AST 183 TC 6.73 HDL 0.59 LDL 5.49 TG 2.20	Diffuse changes of liver parenchyma in MRT;	LAL 0.00 Cht 398	C-triol44 7-KC 370	c.894G>A/ c.796G>T
9	F	no complain; diagnosed through family screening, mild hepatomegaly, elevated transaminases (9y)	9y	ALT 88 AST 56 TC 9.05 HDL 0.80 LDL 7.77 TG 2.36	Diffuse changes of liver parenchyma in US	LAL 0.02 Cht 67	No data	c.894G>A/c.420G>A
10	M	recurrent abdominal pain; mild hepatomegaly, elevated transaminases (3y)	6y	ALT 132 AST 106 TC 7.04 HDL 0.90 LDL 5.66 TG 1.99	Diffuse changes of liver parenchyma in US;	LAL 0.01 Cht 45	C-triol53.5 7-KC 165	c.894G>A/c.420G>A
11	F	recurrent abdominal pain; hepatosplenomegaly, elevated transaminases, hyper-bilirubinemia (8y)	12y	ALT 96 AST 74 TC 9.74 HDL 1.35 LDL 7.94 TG 2.17	Small-focal heterogeneity, increased echogenicity of liver parenchyma in US;	LAL 0.00 Cht 42	C-triol59.2 7-KC 167.4	c.894G>A/ c.817_818delAA
12	M	recurrent abdominal pain; elevated transaminases, hyperbilirubinemia (3y)	6y	ALT 115 AST 111 TC 7.20 HDL 0.80 LDL 5.60 TG n.d.	Diffuse changes of liver parenchyma, features of fibrosis in MRT;	LAL 0.00 Cht 165,8	C-triol96.8 7-KC 523	c.894G>A/c.398C>A
13	M	no complain; hepatomegaly, elevated transaminases (6m)	5y	ALT 406 AST 14 TC 6.63 HDL 0.96 LDL 5.30 TG n.d.	Diffuse heterogeneity, increased echogenicity of liver in US	LAL 0.00 Cht 97,9	No data	c.894G>A/c.309C>A
14	M	protracted jaundice, pain in the right hypo-chondrium; hepatosplenomegaly, elevated transaminases, hyperbilirubinemia (18y)	21y	ALT 358 AST 47 TC 5.84 HDL 1.56 LDL 3.69 TG 1.29	Mild diffuse heterogeneity, increased echogenicity of liver in US	LAL 0.00 Cht 43	No data	c.894G>A/ c.309C>A

15	F	no complain; mild hepatomegaly, elevated transaminases (5y)	5y	ALT 178 AST 247 TC 7.27 HDL 1.30 LDL 5.86 TG 1.21	Diffuse heterogeneity, increased echogenicity of liver in US;	LAL 0.01 Cht 89	No data	c.894G>A/ c.911_912de- linsT
16	M	enlarged abdomen; mild hepatomegaly (2y 8m)	9y	ALT 40.0 AST 57.0 TC 8.50 HDL 0.57 LDL 6.58 TG n.d.	Hepatomegaly and increased echogenicity of liver in US	LAL 0.00 Cht 884	No data	c.894G>A/ c.796G>T
17	M	recurrent abdominal pain; hepatosplenomegaly, elevated transaminases (2y)	7y	ALT 203 AST 142 TC 9.50 HDL 1.10 LDL 7.09 TG n.d.	Decreased echogenicity of liver in US	LAL 0.01 Cht 364	C-triol60.6 7-KC 400.1	c.894G>A/ c.796G>T
18	M	no complain; mild hepatomegaly (4y)	15y	ALT 78 AST 89 TC 6.25 HDL n.d. LDL 5.10 TG n.d.	Homogeneity, decreased eho- genicity of liver parenchyma in CT; Liver biopsy (5y) – periportal fibrosis, lipid storage vacuoles in hepatocytes and Kupffer cells. Liver fibrosis in METAVIR score F2	LAL 0.02 Cht 672	No data	c.894G>A/ c.347G>A
19	F	no complain; mild hepatomegaly, elevated transaminases (3y)	6y	ALT 128 AST 79 TC 7.24 HDL 0.71 LDL 5.85 TG 1.20	Diffuse heterogeneity, increased echogenicity of liver in US	LAL 0.02 Cht 46	No data	c.894G>A/ c.-3A>G
Patients without c.894G>A variant in the genotype								
1	F	no complain; hepatosplenomegaly, elevated transaminases (1y 6m)	13y	ALT 308 AST441 TC 11.80 HDL 0.80 LDL 8.98 TG 2.30	Liver chirrosin MRT (14y) Liver biopsy (16y) – periportal fibrosis liver fibrosis in METAVIR score F-4 (cirrhosis)	LAL 0.01 Cht 251	No data	c.600G>A/ c.600G>A
2	F	no complain; mild hepatomegaly (7m)	19 y	ALT 129.0 AST 120.0 TC 6.80 HDL 1.46 LDL 4.29 TG n.d.	Increased echogenicity of liver in US (3y); Liver biopsy (18y) – periportal fibrosis liver fibrosis in METAVIR score F-4 (cirrhosis)	LAL 0.00 Cht 99	No data	c.177_180dup4/c.956A>T

Table 2: Individual characteristics of cholesteryl ester storage disease patients.

ALT - Alanine Aminotransferase Concentration, U/L (<37)
 AST - Aspartate Aminotransferase Concentration, U/L (<47)
 TC - Serum total cholesterol concentration, mmol/L (3,20-5,20)
 HDL - serum High Density Lipoprotein cholesterol concentration, mmol/L (0,90-2,10)
 LDL - serum Low Density Lipoprotein cholesterol concentration, mmol/L (1,55-3,80)
 TG - serum Triglycerides concentration, mmol/L (0,10-1,70)
 LAL - Lysosomal Acid Lipase activity, nmol/punch/h (0,16-1,80)
 Cht - Chitotriosidase activity, nmol/punch/h (<100)
 C-triol - Cholestane-3β,5α,6β-triol concentration, ng/mL (<50,0)
 7-KC - 7-Ketocholesterol Concentration, ng/mL (<75,0)
 US - Ultrasound
 CT - Computer Tomography
 METAVIR F0 - F4 - the stages of fibrosis according to METAVIR scoring system from no fibrosis to cirrhosis respectively, estimated based on histology data

Patients with cholesteryl ester storage disease

The remaining 36 patients demonstrated symptoms of CESD and 34 of them had at least one copy of c.894G>A variant. From the rest

two patients, one had a pathogenic variant c.177_180dup4 and an un-described substitution c.956A>T with doubtful pathogenic features; the second one was homozygous for the variant c.600G>A. The genotypes of patients are presented in table 2.

The age at which the first clinical signs were reported by a physician varied from 4 months to 18 years (median 3 years). Hepatomegaly was the most common first abnormality observed in 28 patients (71.8%), while 9 (23.1 %) patients were noted to have mild splenomegaly. Twenty-seven patients (69.2 %) had also mildly to moderately elevated liver transaminases.

In all patients dyslipidemia type II was presented: serum total cholesterol and LDL-cholesterol were found to be elevated in all cases. In addition, cholesterol levels tended to increase with age. The maximum concentration of total cholesterol amounted 11.8 mmol/L (at the age of 13y), the maximum concentration of LDL - 9.63 mmol/L (17y) (for reference ranges see Table 2). At the background of hypercholesterolemia HDL-cholesterol level was normal in 20 patients and decreased in 12 patients (in 4 cases was not performed). Serum triglycerides concentration was performed for 19 patients and noted to be normal in 11 of them, in 8 patient's triglycerides level was elevated.

Most of the patients demonstrated increased echogenicity of liver and signs of fibrosis in US, CT and MRT studies. Histological data performed for 6 patients, shows periportal fibrosis and microvesicular steatosis of liver. In 5 of them fibrosis with septa was observed (METAVIR score higher than F-0). Three patients had cirrhosis at the age of 10, 14 and 18 years.

Secondary biomarkers in the CESD patients

It is known, that the presence of the splice-junction mutation c.894G>A alleviates the clinical presentation of LAL-D [5]. On the other hand, the presence of two LOF-mutations in a genotype dramatically worsens the disease course leading to WD phenotype. The majority of variants identified in a compound-heterozygous state with the allele c.894G>A found to be LOF-mutations: nonsense variants, frame-shift deletions and insertions. We hypothesized about a significant impact of LOF-alleles presented in a compound-heterozygous state with the allele c.894G>A on the severity of symptoms in comparison with patients homozygous for the "mild" splice-junction variant.

The clinical data presented in table 2 shows that such features as liver transaminases or US findings varied widely in patients with the same genotype, even in one family. That is why we compared the secondary biomarkers of LAL-D, chitotriosidase activity and oxysterols levels in the groups of homozygotes and compound-heterozygotes for c.894G>A patients, respectively. Increase Cht activity reflects the involvement of liver and spleen macrophages [24]. Increased oxysterols could be considered as secondary to lipid profile alterations.

Cht activity was measured in 15 homozygous and 19 compound-heterozygous patients. The enzyme activity in compound-heterozygous patients was found to be significantly higher than in homozygotes ($P < 0.05$). Therefore, statistic data argues for correlation of the marker of liver injure with a LIPA genotype. The degree of macrophages activation and fibrogenesis depends on the age of the clinical presentation and the time from first symptoms (due to accumulation of the substrate). Regarding that we could assume that all these characteristics of the disease course directly correlate with a patient genotype.

The oxysterols levels were measured in 7 homozygous and 8 compound-heterozygous patients. The significant difference among 7-KC

and C-triol levels between the groups was observed ($P < 0.05$). However, it should be remarked that there is no any difference among standard characteristics of lipid profile as total cholesterol and LDL-cholesterol. So, the markers of dyslipidemia do not demonstrate one-valued association with one LOF-mutation of LIPA in patients' genotypes.

The intervals of the significant different characteristics in the two groups are presented on figure 1.

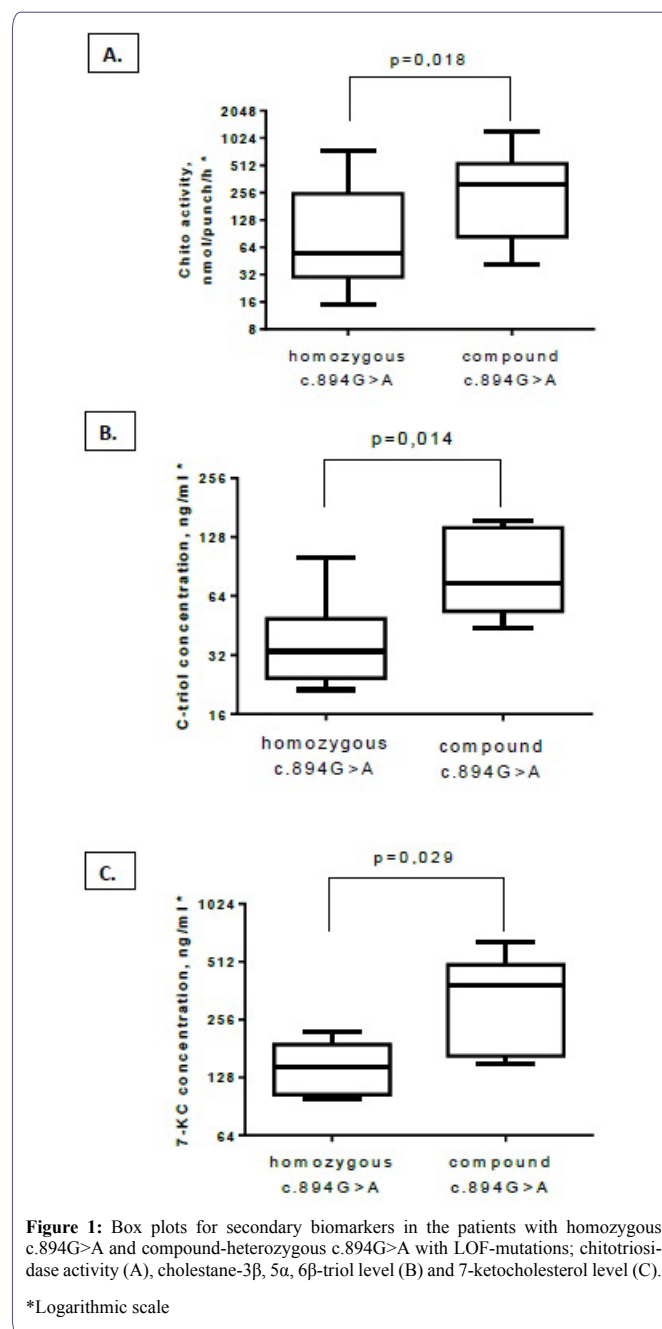


Figure 1: Box plots for secondary biomarkers in the patients with homozygous c.894G>A and compound-heterozygous c.894G>A with LOF-mutations; chitotriosidase activity (A), cholestane-3 β , 5 α , 6 β -triol level (B) and 7-ketocholesterol level (C).

Regarding a huge progress in the target of LSDs treatment observed in the last years, the clinical significance of their early and correct diagnosis is constantly growing. The problem of LAL-D is non-specific symptoms. The challenging of CESD is the lack of

specific symptoms or signs (as we see in WD where there is observed a pathognomonic adrenal gland calcification) and absence of complaints in more than half of all reported cases (this ratio is observed in our cohorts too, see table 2) [2].

Measurement of LAL activity in DBS should be considered as one of the mandatory first diagnostic steps in both listed categories. In the case of positive or doubtful results the complex study is needed [5].

The supporting biochemical data are high levels of oxysterols and Cht activity. A quantitative analysis of these biomarkers seems to be useful. The confirmation of the statistic correlations with heterozygous severe LIPA defects we defined is requiring more wide sampling of CESD patients and detailed clinical data. It seems over time measurement to be informative, especially on the background of different treatment courses. If confirmed this data should have diagnostic significance for further detection of c.894G>A and other LIPA variants. It may also have some prognostic meaning, primarily for progression of pathology in the macrophage system of liver related to the Cht activity.

Conclusion

The mutation spectrum of LIPA gene in Russian population is varied. We found 16 different pathogenic variants in LAL-D patients with 11 of them undescribed so far.

There were identified two common mutations among the Russian and the Ukrainian patients: c.894G>A variant as the most frequent like in other European countries and c.796G>T variant accounting for 10% of our cohort but comparatively rare in Europe.

Heterozygous LOF-defects of LIPA were found to increase the Cht activity and the levels of the two oxysterols in CESD patients. In comparison with the homozygotes for the “mild” variant c.894G>A, it indicates the higher extent of macrophages activation/liver fibrosis and dyslipidemia progression, respectively. Those data may have a prognostic significance but more detailed studies are required.

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Conflict of Interest

The authors EA Kamenets, SV Mikhaylova, A Tylki-Szymańska, VS Kakaulina, NL Pechatnikova, N Pichkur, P Lipiński, GV Baydakova, TY Proshlyakova, IO Nagornov, EY Zakharova declare that they have no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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