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Rare dyslipidemias

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Rare dyslipidemias

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Editorial: Rare dyslipidemias

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Editorial on the Research Topic
Rare dyslipidemias

Rare diseases are defined in the European Union (EU) as those that affect less than 1 person in 2000; in total, between 6,000 and 8,000 different rare diseases affect an estimated 30 million people in the EU (European Commission, 2021). Rare dyslipidemias encompass a diverse group of rare inherited metabolic disorders that are either autosomal dominant, codominant, semi-dominant or recessive (Hegele et al., 2020). At least 25 different monogenic rare dyslipidemias have been identified, caused by pathogenic variants in 23 genes with varied biochemical and clinical features (Hegele et al., 2015). Owing to their complex etiology and clinical features, these diseases pose a significant challenge in diagnosis, which is generally based on the analysis of clinical phenotypes; however, genetic testing provides a definitive diagnosis (Hegele et al., 2020). Extreme deviations in lipoprotein levels particularly at a younger age and with a positive family history of the disease raise suspicion for these rare disorders (Berberich and Hegele, 2022).

Rare dyslipidemias are characterized by abnormal levels of total and low-density lipoprotein (LDL) cholesterol, triglycerides (TG), lipoprotein (a) [Lp(a)], and high-density lipoprotein (HDL) cholesterol. These disorders can have long-term life-threatening consequences including increased risk for atherosclerotic cardiovascular disease (ASCVD), which is the leading cause of morbidity and mortality around the world. Other complications include pancreatitis, fatty liver disease, and fat-soluble vitamin deficiencies (Berberich and Hegele, 2022).

The Research Topic collection on "*Rare dyslipidemias*" presents papers on various aspects of several related disorders, including homozygous familial hypercholesterolemia, familial chylomicronemia syndrome due to different genetic causes, hypobetalipoproteinemia, hypoalphalipoproteinemia, dysbetalipoproteinemia, cerebrotendinous xanthomatosis, and lysosomal acid lipase deficiency.

Homozygous familial hypercholesterolemia (HoFH), marked by exceedingly high levels of LDL cholesterol (>400 mg/dL or >10 mmol/L) due to an impaired clearance of LDL particles, is a major rare dyslipidemia with estimated prevalence of 1 in 300,000 individuals (Berberich and Hegele, 2022; Tromp et al., 2022; Cuchel et al., 2023). It is caused by bi-allelic pathogenic variants in the *LDLR* gene encoding LDL receptor in 85%–90% of cases, in the *APOB* gene encoding apolipoprotein (apo) B in 5%–10% of cases and in the *PCSK9* gene encoding proprotein convertase subtilisin/kexin type 9 in 1%–3% of cases. Clinical features

include tendon xanthomas, corneal arcus, and very premature ASCVD and aortic disease (Cuchel et al., 2023; Hegele et al., 2020). Globally, <10% of the FH population is diagnosed and treated, due to a lack of awareness among the general public and medical community (Vallejo-Vaz et al., 2021; Wilemon et al., 2020; Sadiq et al., 2023). Effective management of HoFH remains challenging and includes lipoprotein apheresis to delay ASCVD (Cuchel et al., 2023). Kayikcioglu et al. present an interesting report on the use of low-dose lomitapide on top of standard lipid-lowering therapy resulting in decreased apheresis frequency.

Familial chylomicronemia syndrome (FCS), is a rare (1 in 1,000,000) recessive disorder, that results from the decreased clearance of large TG-rich lipoproteins (chylomicrons), even after prolonged fasting. The major life-threatening complication of FCS is acute pancreatitis, specifically at TG > 10 mmol/L (>885 mg/dL) (D'Erasmo et al., 2019). Most FCS cases are caused by biallelic pathogenic variants in the *LPL* gene encoding lipoprotein lipase (LPL), the enzyme responsible for the breakdown of TG within chylomicrons and very-low-density lipoproteins (VLDL) (Hegele et al., 2020). Causal variants in other genes such as *APOC2* encoding apo C-II, *APOA5* encoding apo A-V, *LMF1* encoding lipase maturation factor 1, *GPIHBP1* encoding glycosylphosphatidylinositol-anchored HDL binding protein 1 account for 10%–20% of the FCS cases (D'Erasmo et al., 2021). A systematic review by Sustar et al. covers the spectrum of *GPIHBP1* gene variants among the hypertriglyceridemia population. Whole genome sequencing of a Chinese proband followed by functional analyses of the genes revealed the digenic origin of hypertriglyceridemia caused by *LMF1* and *LPL* gene double heterozygosity in the patient (Guo et al.). Another study from China reported variants in the *GPD1* gene encoding glycerol-3-phopshate dehydrogenase 1 and their effect on transient infantile hypertriglyceridemia, a rare autosomal recessive disorder that may increase the risk of cardiovascular and metabolic disorders later in life (Wang et al.).

Consanguineous marriages are still prevalent in South Asia, the Middle East, and North Africa for local cultural and social reasons, and are associated with increased risk for inherited diseases with severe health consequences. Al-Waili et al. reported a novel pathogenic variant in the *LPL* gene in a family with double-cousin marriages. In addition, digenic (double heterozygous) pathogenic variants in *LPL* and *APOA5* genes were identified in another Omani family. Similarly, Ayoub et al. reported the first case with pathogenic variants of both *LPL* and *PCSK9* genes in a Syrian family that had migrated to Lebanon. So far, genomic studies have predominantly focused on populations of European ancestry (Fatumo et al., 2022). Jurado-Camacho et al. have reported several novel variants of genes involved in lipid metabolism among the Mexican population. Genetic testing of underrepresented non-European populations can help identify the actual burden of rare dyslipidemias in different genetic backgrounds, and may help guide diagnostic and therapeutic approaches (Al-Waili et al., Jurado-Camacho et al.).

Unlike FCS, multifactorial chylomicronemia syndrome is a complex, often polygenic form of severe hypertriglyceridemia that results from multiple underlying genetic factors coupled with environmental triggers, including medications (Goldberg and Chait, 2020). An example of an interaction between genetic and non-genetic determinants was seen in a normolipidemic patient with a novel apo C-III (*APOC3*) variant who experienced drug-induced hypertriglyceridemia (Iannuzzi et al.).

Lysosomal acid lipase deficiency (LAL-D, also called cholesterol ester storage disease) is an autosomal recessive disorder, caused by biallelic pathogenic variants in the *LIPA* gene encoding lysosomal acid lipase. Besler et al. comprehensively reviewed several aspects of LAL-D, including the role of *LIPA* variants in predicting ASCVD risk from genome-wide association studies and also the cell type-specific role of enhancing LAL activity as a novel treatment strategy of ischemic cardiovascular disease and fatty liver. LAL-D is globally underdiagnosed, partly due to clinical features that may resemble more common dyslipidemias and fatty liver disease, and partly due to lack of access to genetic and enzyme activity testing in clinical practice. Early diagnosis followed by timely management with intravenous enzyme replacement therapy (sebelipase) are achievable by reproducing the Slovenian Universal FH screening strategy which has helped identify patients homozygous for *LIPA* pathogenic variants. LAL-D-positive children have higher liver transaminases (AST and ALT) that clinically differentiate them from FH patients (Sustar et al.).

Several rare dyslipidemias can present with either low LDL cholesterol (hypobetalipoproteinemia) or low HDL cholesterol (hypoalphalipoproteinemia) (Hegele et al., 2020). Disorders characterized by decreased HDL cholesterol, also called familial hypoalphalipoproteinemia, include Tangier disease, an autosomal recessive disorder caused by pathogenic variants in *ABCA1* encoding the ATP binding cassette transporter A1, which is responsible for modulating the flux of cellular cholesterol and phospholipids into the reverse cholesterol transport pathway. Individuals with Tangier disease appear to have a higher incidence of coronary artery disease than normolipidemic subjects, irrespective of gender. Other forms of familial hypoalphalipoproteinemia include those caused by pathogenic variants in *APOA1* encoding apo A-I and *LCAT* encoding lecithin cholesterol acyltransferase (Oram, 2000; Hegele et al., 2020). Alves et al. presented 7 cases with rare dyslipidemias associated with either low LDL or low HDL cholesterol values, who were evaluated with next-generation DNA sequencing. The genetic basis was confirmed in 6/7 patients: one had fisheye disease due to variant *LCAT*, one had hypoalphalipoproteinemia and 5 had either abetalipoproteinemia due to *MTTP* variants or familial hypobetalipoproteinemia (FHBL) due to *APOB* variants. These results stress the important role of genetic testing in the diagnosis of rare dyslipidemias (Alves et al.). Another paper by Molk et al. reports a novel pathogenic *APOB* variant in a child with heterozygous FHBL and non-alcoholic fatty liver disease and comprehensively reviews the existing literature on *APOB* variants causing heterozygous FHBL.

Dysbetalipoproteinemia (also called hyperlipoproteinemia type III, HLP3) is characterized by elevated levels of both triglycerides and cholesterol due to abnormally elevated remnant particles resulting in part from homozygosity for the apo E2 isoform (Bea et al., 2023). This lipid profile is highly atherogenic and predisposes to premature ASCVD. To overcome technical issues in the clinical diagnosis of HLP3, Sampson et al. have described a validated equation for the indirect calculation of cholesterol content of VLDL which is abnormally increased in HLP3.

Cerebrotendinous xanthomatosis (CTX) is a rare lipid storage disease, caused by deficiency of sterol-27-hydroxylase (CYP27). Patients with CTX present with elevated cholesterol, xanthomas, and neurological deterioration that can lead to premature death. Cohen et al. reported detailed metabolic abnormalities and premature atherosclerosis among CTX patients.

The management of rare dyslipidemias is challenging and depends on accurate and timely diagnosis. Genetic testing promises greater accuracy and also insight into pathogenesis (Guo et al., Alves et al.). The discoveries of rare variants in extreme and unexplained phenotypes, with the help of advanced genetic techniques, might help us better understand pathophysiology and lead to the discovery of novel therapeutic targets. Deng et al. describe a proband with sitosterolemia and its successful treatment with ezetimibe. Several promising therapeutic approaches for dyslipidemias are in the pipeline (Merćep et al., 2022). For instance, lomitapide, an inhibitor of microsomal triglyceride transfer protein (MTP) is an important therapeutic option for difficult-to-manage HoFH patients (Kayikcioglu et al.). Interestingly, D'Erasmo et al. have demonstrated the efficacy and safety of lomitapide in autosomal recessive hypercholesterolemia (ARH), an ultra-rare autosomal recessive disorder of LDL metabolism resembling HoFH caused by pathogenic variants in *LDLRAP1* encoding the LDL receptor adaptor protein 1.

In summary, the Research Topic "*Rare dyslipidemias*" has further expanded knowledge on several aspects of these conditions, including their genetic background, phenotypic characteristics, and treatment. Many ethnic groups remain underrepresented in genomic studies. Genetic testing and applicable screening strategies should be more broadly implemented. Collaborative registries are also required to improve health policy for the care of patients with rare dyslipidemias (Hegele et al., 2020).

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BRIEF RESEARCH REPORT
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Remediation of *ABCG5*-Linked Macrothrombocytopenia With Ezetimibe Therapy

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To investigate refractory hypercholesterolemia, a female patient and relatives were subjected to whole-genome sequencing. The proband was found to have compound heterozygous substitutions p. Arg446Gln and c.1118+3G>T in *ABCG5*, one of two genes causing sitosterolemia. When tracing these variants in the full pedigree, all maternally related heterozygotes for the intronic *ABCG5* variant exhibited large platelets (over 30 fl), which segregated in an autosomal dominant manner, consistent with macrothrombocytopenia, or large platelet syndrome which may be associated with a bleeding tendency. *In vitro* cell-line and *in vivo* rat model experiments supported a pathogenic role for the variant and the macrothrombocytopenia was recapitulated in heterozygous rats and human cell lines exhibiting that single variant. Ezetimibe treatment successfully ameliorated all the symptoms of the proband with sitosterolemia and resolved the macrothrombocytopenia of the treated heterozygote relatives. Subsequently, in follow up these observations, platelet size, and size distribution were measured in 1,180 individuals; 30 were found to be clinically abnormal, three of which carried a single known pathogenic *ABCG5* variant (p.Arg446Ter) and two individuals carried novel *ABCG5* variants of uncertain significance. In this study, we discovered that identification of large platelets and therefore a possible macrothrombocytopenia diagnosis could easily be inadvertently missed in clinical practice due to variable instrument settings. These findings suggest that *ABCG5* heterozygosity may cause macrothrombocytopenia, that Ezetimibe treatment may resolve macrothrombocytopenia in such individuals, and that increased attention to platelet size on complete blood counts can aid in the identification of candidates for *ABCG5* genetic testing who might benefit from Ezetimibe treatment.

Keywords: platelet, blood, sitosterolemia, hypercholesterolemia, ezetimibe (EZE)

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Abbreviations: ABCG5, ATP-binding cassette transporters G5; ABCG8, ATP-binding cassette transporters G8; NPC1L1, Niemann–PickC1-Like1; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; WGS, whole genome sequencing; FH, familial hypercholesterolemia; GATK, Genome Analysis Tool Kit; ACMG, American College of Medical Genetics and Genomics; MPV, mean platelet volume; PLT, platelet; fL, femtoliters.

BACKGROUND

Plasma cholesterol homeostasis is maintained by the synthesis, intestinal absorption, and biliary and fecal excretion of sterols. The biosynthesis of cholesterol is a well-defined energy-consuming and feedback-regulated process (Howe et al., 2016). ATP-binding cassette transporters G5 (*ABCG5* gene) and G8 (*ABCG8* gene) usually form a heterodimer (G5G8) which inhibits the absorption of cholesterol and plant sterols by promoting the efflux of these sterols from enterocytes back into the gut lumen, and the secretions from hepatocytes into bile (Wang et al., 2015; Lee et al., 2016; Sun et al., 2021). In contrast, sterol transporter Niemann–PickC1-Like1 (NPC1L1) promotes intestinal cholesterol absorption and biliary cholesterol re-absorption (Jia et al., 2011). Together, they maintain sterol balance without direct involvement in sterol synthesis.

Homozygous or compound heterozygous variants in either *ABCG5* or *ABCG8* cause autosomal recessive sitosterolemia (OMIM 210250) (Myrie et al., 1993; Berge et al., 2000), characterized by elevated plasma levels of plant sterols (Bhattacharyya and Connor, 1974; Myrie et al., 2012; Brautbar et al., 2015). However, patients with *ABCG5/8* pathogenic variants show significant phenotypic heterogeneity (Wang et al., 2004). The other features also variably include hypercholesterolemia, xanthomas, xanthelasma, and premature atherosclerosis. Affected individuals may also suffer from diverse hematological alterations such as a decreased platelet count, an increased mean platelet volume (large platelets/macrothrombocytosis or macrothrombocytopenia if accompanied by reduced platelet count), and/or hemolytic anemia.

Management of sitosterolemia aims to reduce plasma plant sterol accumulation. Ezetimibe, a small molecule inhibitor of NPC1L1 approved for the treatment of hypercholesterolemia (Suchy et al., 2011), is considered the preferred treatment for sitosterolemia (Salen et al., 2004; Yu et al., 2005; Salen et al., 2006; Lutjohann et al., 2008; Tsubakio-Yamamoto et al., 2010; Altemus et al., 2014; Erkan, 2014; Hu and Tomlinson, 2014; Othman et al., 2015; Yoo, 2016). Studies using knock-out mouse models have successfully shown that Ezetimibe treatment can correct multiple symptoms, including reduction of large platelets and restoration of the platelet count to some extent, caused by pathogenic variants in *ABCG5/8*; similarly, clinical trials also show improvement in patients with sitosterolemia after Ezetimibe therapy (Salen et al., 2004; Lutjohann et al., 2008; Tsubakio-Yamamoto et al., 2010; Hu and Tomlinson, 2014; Othman et al., 2015). Nevertheless, the clinical improvement in these patients may be attributed at least in part to plasma cholesterol reduction, and the benefits of reduction of plant sterol storage remain unclear (Ajagbe et al., 2015).

Here we described the identification of two variants of *ABCG5* in a proband with apparent autosomal recessive hypercholesterolemia recalcitrant to statin therapy who exhibited large platelets without dysfunction of blood coagulation, neither platelet counts nor their functions. Unexpectedly, large platelets (over 30 fl) were a dominant phenotype in family members heterozygous for the c.1118+3G>T variant in *ABCG5*. Large platelet syndrome describes a group of unique disorders characterized by the presence of abnormally large platelets and is usually accompanied by thrombocytopenia. Thus, it is also termed macrothrombocytopenia. Functional studies showed that variant can cause deletion of exon 8 in human *ABCG5*, and disruptions of *ABCG5*/*ABCG8* genes recapitulated the occurrence of hypercholesterolemia and large platelets in rats. These variants were ultimately classified as pathogenic and likely pathogenic. Interestingly, the macrothrombocytosis was also successfully treated with Ezetimibe in heterozygotes in the pedigree. Large platelets can be occasionally seen in individuals without other obvious abnormal hematologic findings, and we further sequenced *ABCG5* in a large unrelated cohort of individuals with large platelets to see if *ABCG5* variant heterozygosity is a common cause; this study was carried out to determine whether a subset of individuals with large platelets might be expected to respond to Ezetimibe treatment, as an example of the potential application of pharmacogenomics that benefits from genetic analysis (Yu and Hu, 2021).

METHODS

Proband Description and Blood Sample Collection

A 6-year-old girl with refractory hypercholesterolemia was referred to our institution for the development of a suitable therapeutic regimen. Before the study, atorvastatin had been administered to the patient for more than 2 years, but her plasma total cholesterol level was decreased to only ~200 mg/dl and large platelets persisted. Additionally, the patient appeared to have atherosclerotic plaque (6 mm × 7 mm) in her left common carotid artery (**Figure 1A**, right) upon referral. The patient's initial presenting clinical and laboratory features included profoundly increased levels of total cholesterol (638 mg/dl) and low-density lipoprotein cholesterol (LDL-C, 527 mg/dl); a reduced level of high-density lipoprotein cholesterol (HDL-C, 46 mg/dl) and a slight elevation of plasma β-sitosterol (3.26 mg/dl). Although there was an elevation of β-sitosterol, criteria for the diagnosis of sitosterolemia were initially felt not to be met (Salen et al., 1985). In addition, the patient had abnormally large platelets (**Figure 1A**, left); over 41% of her platelets had a size over 30 fl. Clinically, Bernard-Soulier syndrome, of which giant platelets are a feature, was excluded as a plausible diagnosis through examination of clotting time and platelet aggregation, which were all normal in the proband (**Table 1**).

To track genetic variants in the full pedigree, we also collected blood samples from maternal and paternal relatives (I-1,2,3,4, and II-1,4). DNA was extracted from 400 μl whole blood per sample using the (Qiagen, Hilden, Germany), and all blood samples were stored at −80°C before usage. For screening for candidate *ABCG5* variants in a general population, we also enrolled individuals over 2 months in 2019, the Second Affiliated Hospital of Nanchang University, Beijing Anzhen Hospital of Capital Medical University, and Peking University

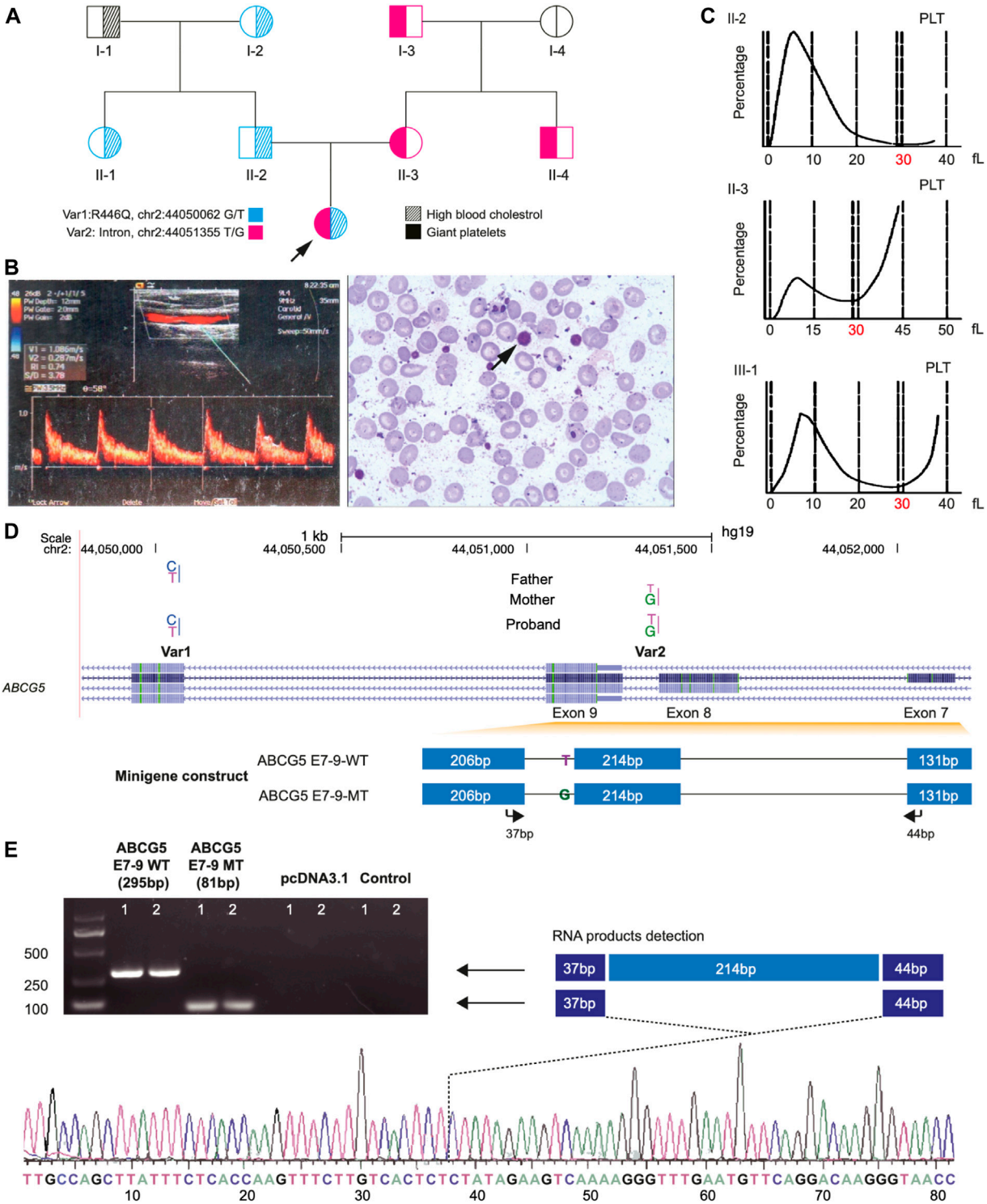

FIGURE 1 | Clinical Phenotypes and Identification of Two Novel Mutations of *ABCG5* Gene of the proband in a pedigree family. Panel **(A)** shows the large platelet in the blood smear **(left)** and the atherosclerotic plaque by B-mode ultrasound measurement from the proband. Panel **(B)**, a pedigree chart shows the phenotype distribution in all family members and the transmission of two novel variants (Var 1 and Var 2) of *ABCG5* in the family. Panel **(C)** shows the size distribution of platelets (PLT) in the proband and her parents. The dash lines represent the platelets' volume, and the double dash lines indicate 30 fl, in which 40 fl is the up limitation for the normal distribution of PLT. Panel **(D)** shows the *ABCG5* gene model from the NCBI database and the inserted fragments in the constructs for wild type and Var2 mutant in the splicing pattern assay. The arrows indicate the primer pairs used for the detection of the mRNA products which were transcribed from the constructs. Panel **(E)** shows the PCR amplification results of two products generated from wild-type and Var2 mutant constructs, respectively. The product from the wild-type construct is 295 bp, and the product for the mutant is 81 bp due to deleting exon 8 of the *ABCG5* gene. The truncated transcripts were further verified by Sanger sequencing.

TABLE 1 | Clinical characteristics of the family members.

	Id									Normal value of rang
	I-1	I-2	I-3	I-4	II-1	II-2	II-3	II-4	III-1	
Gender	Male	Female	Male	Female	Female	Male	Female	Male	Female	
Age	69	66	58	52	45	40	34	32	9	
TC	169.76	213.46	155.07	200.31	212.69	258.7	134.96	97.84	638.06	<200 mg/dl
LDL-C	99	129.93	96.29	123.74	124.9	163.19	71.54	50.66	527.46	≤120 mg/dl
HDL-C	49.88	61.49	43.7	51.43	68.83	67.29	53.75	39.06	45.63	50–60 mg/dl
β-Sitosterol	1.06	1.44	1.58	1.14	1.3	1.23	1.52	1.49	3.26	0.31~0.80 mg/dl
PLT	275	324	172	298	363	240	250	217	289	85-303 109/L
PDW	10.5	10.9	NaN	15.4	11.4	12.1	NaN	NaN	15.7	11~26.5 fl
MPV	10.2	10.3	NaN	12	10.6	10.8	NaN	NaN	12.1	7.6~13.2 fl
P-LCR	25	26.1	NaN	39.2	28.5	30.1	NaN	NaN	41	13~43%
PT			11 (9–13)				9.4 (9–13)	10 (9–13)	10 (9–13)	
APTT			29 (20–40)				26.8 (20–40)	28 (20–40)	27.3 (20–40)	
TT			17.1 (14–24)				15 (14–24)	16.7 (14–24)	15 (14–24)	
FIB			3.1 (2–4)				3.84 (2–4)	3.62 (2-4)	3.6 (2–4)	
PTA			120 (70–150)				106.8 (70–150)	117 (70–150)	116.5 (70–150)	
PAgT			61 (35–75)				65.7 (35–75)	63 (35–75)	64.3 (35–75)	

Total cholesterol, TC, Low-density lipoprotein cholesterol; LDL-C, High-density lipoprotein cholesterol; HDL-C, Platelet count; PLT, Platelet distribution width; PDW, Mean platelet volume; MPV, Platelet-large cell ratio; P-LCR, prothrombin time; PT, activated partial thromboplastin time; APTT, thrombin time; TT, fibrinogen coagulative time; FIB, prothrombin time activity; PTA, platelet agglutination test, PAgT.

Third Hospital. The samples were examined in the Second Affiliated Hospital of Nanchang University. In all, 30 patients had abnormal platelet size distribution and underwent *ABCG5* gene sequencing.

Gene Panel Test for Familial Hypercholesterolemia

We designed a custom panel of DNA oligonucleotide primers covering 216 amplicons using the Ion AmpliSeqTM platform (**Supplementary Table S1**, Life Technology, Thermo Fisher, USA). These amplicons covered all coding exons of four familial hypercholesterolemia (FH) causal genes (*APOB*, *LDLRAP1*, *LDLR*, and *PCSK9*). Sequencing Libraries were barcoded (IonXpress Barcode Kit, Life Technologies) and equalized (Ion Library Equalizer Kit) to a final concentration of approximately 100 pM. Emulsion PCR was performed using the OneTouch DL instrument, and template-positive Ion Sphere particles were enriched using the OneTouch ES instrument according to the manufacturer's instructions. Sequencing was performed on a 318 chip on the Ion Torrent PGM following the recommended protocol. Reads were aligned to hg19 and variants were called using the TorrentSuite (version 4.0.2).

Whole Genome Sequencing Analysis and Identification of Pathogenic Variants

WGS was conducted in the proband and her parents. Each sequencing library with an average insert size of 250 bp was loaded into an Illumina HiSeq X ten. Roughly 90 Gb of high-quality data in 150 bp pair-end reads was obtained for each library, reaching an average of 30-fold genome coverage for each individual. The quality evaluation was performed using FastQC software, and sequences from adapters or having low Q-score were removed with cutadapt software (Martin, 2011). The sequencing reads were mapped to the reference human genome (hg19) by the BWA algorithm (Li and Durbin, 2009). PCR duplicates were removed with Picard software. Processed bam files of humans were processed via local indel realignment and base-quality recalibration using the Genome Analysis Tool Kit (GATK) (McKenna et al., 2010). Subsequently, sorted BAM files were used for SNV calling and the UnifiedGenotyper method based on a Bayesian genotype likelihood model was applied with GATK. All detected SNVs were annotated using ANNOVAR. VarSelect in TGex platform was applied to filter for variants in WGS data of the family trio, to identify variants most likely responsible for the phenotypes of the proband (Dahary et al., 2019).

Blood Test and Analysis of Plasma Sterol Levels

The blood lipid measurements were routinely performed by the second Affiliated Hospital of Nanchang University. β-Sitosterol and other plant sterols were analyzed by liquid chromatography-mass spectrometry (AB SCIEX Triple Quad 4500) according to previous studies(Kasama et al., 1987; Hidaka et al., 1990). For each sample, a volume of 20 μl was injected into Agilent Eclipse Plus reversed-phase column (C18, 2.1 × 50 mm). The column temperature was maintained at 35°C. The mobile phase was acetonitrile-methanol (4:1, v/v) at a flow rate of 0.6 ml/min. The mass spectrometer was operated in positive ion polarity mode in the extended dynamic range (1,700 m/z, 2 GHz) with the following parameters: Curtain Gas (GUR) 40 psi; Collision Gas (CAD) 9; IonSpray Voltage (IS) 5,500.0 V; Temperature (TEM) 350°C; Ion Source Gas 1 (GS1) 50 psi; NC 3V. Blood samples from 10 healthy individuals were examined as control samples.

Platelet Size Analysis

Two ml of peripheral blood was collected using standardized tubes (INSEPACK ST serials, Beijing, China) and all tubes were stored at room temperature. Within 30 min, these samples were analyzed on SysmexXE-2100 Haematology System (Sysmex Corporation, Kobe, Japan) for lipid profile and coagulation tests following the methods previously developed protocols (**Table 1**) (Barsam et al., 2011; Depoorter et al., 2015). Large platelets are identified based on the volume (>30 fl). For each sample, we prepared two blood films dyed by Wright's dye for platelet morphology analysis using a BX53 microscope (OLYMPUS, Japan). The criteria for large platelets is a diameter greater than 4 microns. All blood films were reviewed by two independent examiners.

Splicing Pattern Assay for ABCG5 Var2

Var2 is located at a splice region of *ABCG5*, and we performed the splicing pattern assay to verify its functional effect. Genomic DNAs including reference (WT) or *ABCG5* variant were isolated from peripheral whole blood of all individuals in the study with Blood Genomic DNA Mini Kit (CWBIO, CW 2087S) and PCR was performed using PrimeSTAR® MaxDNA Polymerase (TAKARA, R045A). The 947 bp PCR fragments of *ABCG5* gene, spanning from part of exon 7 (44 bp) to part of exon 9 (37 bp) with exon8 (214 bp) and its flanking intron 7 and 8, were amplified by the primer pair, *ABCG5*-minigene_Forward (5′-GCGGTACCGCGGAAATGC TTGATTTCTT-3′) with KpnI restriction site and *ABCG5*-minigene_Reverse (5′-GCCTCGAG TTAAAGGAGGAACAAACC CATGA-3′) with XhoI restriction site. The genomic fragments (both WT and variant, MT) containing the intron of interest were cloned into the pcDNATM-3.1 (+) vector and then all cloned plasmids were verified by sequencing to confirm whether the insertion contained the WT or MT.

HepG2 cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco). After seeded in 6-well plates for 24 h, HepG2 cells were transiently transfected with 1ug prepared vectors with the allele of the WT or MT and corresponding empty vectors respectively, using Superfectin II *In Vitro* siRNA Transfection Reagent (Shanghai Pufei Biotech). After 48 h, total RNA was extracted by TRIzol reagent (Invitrogen) and 1 mg total RNA was used for reverse transcription using PrimeScript RT reagent kit with gDNA eraser (Takara) according to the manufacturer's instructions. The primer sequences of quantitative RT-PCR were: *ABCG5*-MGQ_Forward (5′-CGGTTACCCTTGTCCTGAAC-3′) located in exon 7 and *ABCG5*-MGQ_Reverse (5′-TGCCAGCTTATTTCTCACCA-3′) located in exon 9. Quantitative RT-PCR was performed with SYBR Green dye using ViiA7Real-Time PCR System (Applied Biosystems). The relative mRNA expression was calculated by the comparative Ct method using *GAPDH* as a control. PCR reactions were performed in triplicate. The RT-PCR products were separated on a 2% agarose gel and detected with Chemidoc Xrs Gel Doc Xr (Bio-Rad Universal Hood Ii 2, USA).

Generation of ABCG5/ABCG8 Double Knockout Rat

ABCG5/ABCG8 double knockout rat model was created by Beijing Biocytogen. In brief, a 19 Kb region was knocked out using a CRISPR/Cas9 system, with two sgRNAs targeting one site in the intron 4 of *ABCG5*, and the other in the intron 6 of *ABCG8*. The design was adapted from the protocol developed by Yu L, et al. The sgRNA activity was evaluated by the UCA^{TM} (Universal CRISPR Activity Assay), developed by Biocytogen. By zygote microinjection, transferred zygotes of SD rats were obtained and the founders were positively confirmed by PCR product sequencing. The genotyping primers were Forward: 5′-ctaggt ccaccaagccatgtgaaca and Reverse: 5′-attttctgggcaccctgtgttccac. The animal study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University (SYXK-20150001).

Sanger Sequencing of ABCG5

Exon amplification was performed using LA Taq (Takara, Osaka, Japan). **Supplementary Table S2** listed all the primers for amplification of 13 exons of *ABCG5* extending 50 bp towards both upstream and downstream to cover the splice regions and corresponding PCR protocols for each primer pair. All reactions were performed on a PTC-200 Peltier Thermal Cycler (MJ Research, MA, USA). PCR products were sequenced by Sangon Biotech (Shanghai, China), and the results were manually checked using SeqMan (DNAstar 5.0, WI, USA).

Statistical Analysis

The *t*-test was used to compare the mean difference of the blood lipid levels in the rat model using R 3.4(R Core Development Team, 2018).

RESULTS

Identification of Compound Heterozygous Variants in ABCG5

This proband had hypercholesterolemia with atypical changes in the β-Sitosterol level in comparison with previous reports (**Supplementary Table S3**). To identify pathogenic variants responsible for the hypercholesterolemia of the proband, a customized Gene Panel Test was initially performed to screen for variants within all coding regions of four common familial hypercholesterolemia genes: *APOB*, *LDLR*, *LDLRAP1*, and *PCSK9*. The sequencing results showed that no family member carried any rare coding variants (<1%, MAF in CHB of 1,000 genomes) in these genes. Next, we performed WGS for the proband and her parents (~30X coverage on average for each person). Two heterozygous variants of the *ABCG5* gene were found in the proband, and no other variant was observed in the known genes related to hypercholesterolemia. The proband inherited Var1 (chr2: 44050062G/T, NM_022436.3:c.1337G>A, p. Arg446Gln) from her father and Var2 (chr2:44051355T/G, splice region, c.1118+3G>T) from her mother. These two variants (Var1 and Var2) were further verified by Sanger sequencing (**Supplementary Figure S1**).

Var1 was reported to be a variant of uncertain significance for sitosterolemia in both Clinvar (Accession: VCV000289811) and gnomAD (2-44050062-C-T, allele

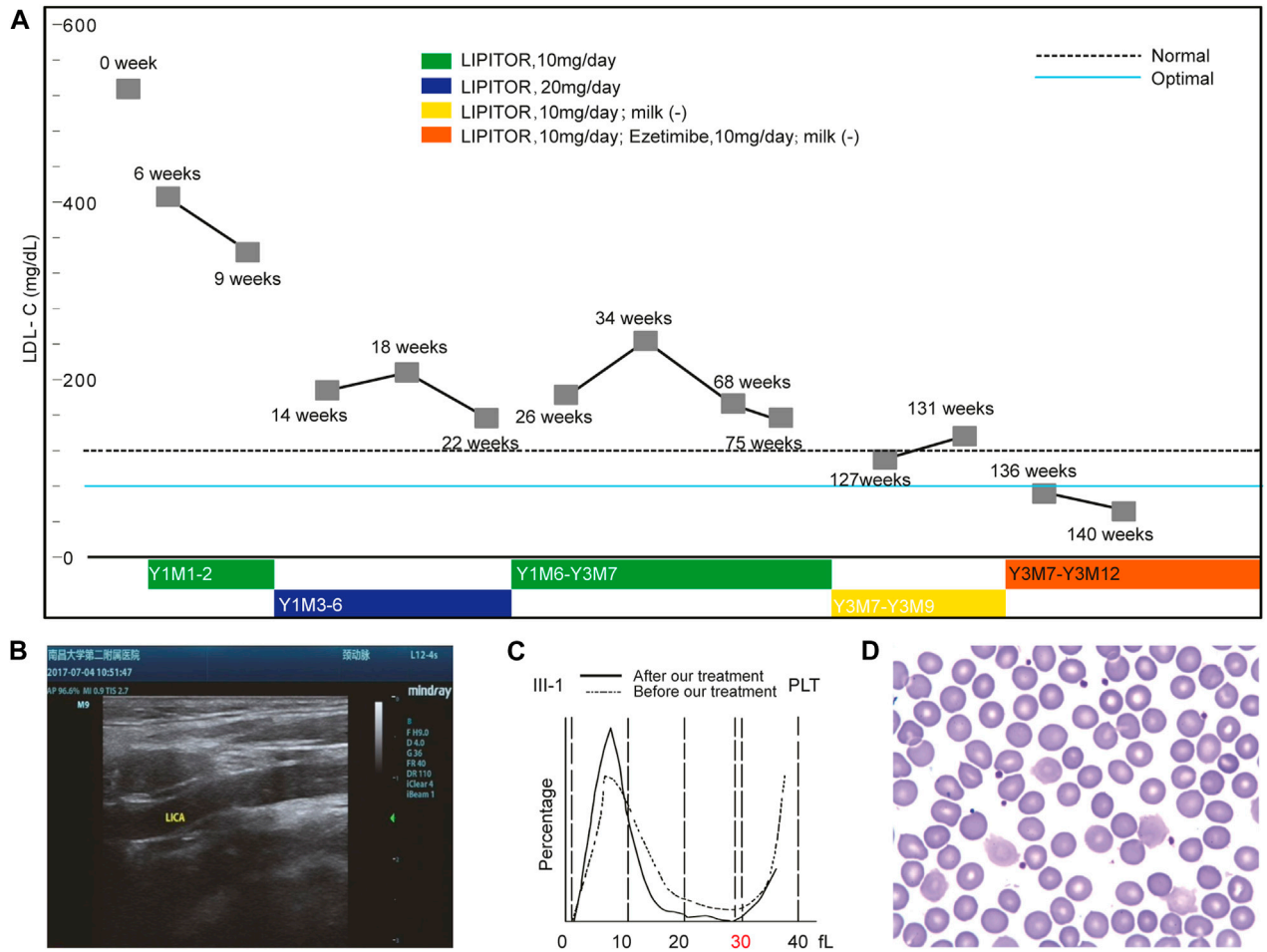

FIGURE 2 | Ezetimibe Corrected the Phenotypes of the Proband. Panel **(A)** shows the changes in the LDL-C levels under an optimized treatment regimen. Panel **(B)** shows the disappearance of the aforementioned atherosclerotic plaque revealed by the ultrasound scan after Ezetimibe treatment. Panel **(C)** shows the comparison of platelet size distributions before and after Ezetimibe treatment in the proband. Panel **(D)** shows the disappearance of the large platelet in the blood smear of the proband after treatment of Ezetimibe.

frequency: 6.74e-5 and no homozygote). While at the same position, p. Arg446Ter (Accession: VCV000030485), has been previously reported as pathogenic for sitosterolemia and hypercholesterolemia (Wang et al., 2014; Buonuomo et al., 2017; Pek et al., 2018). Var2 was not documented in either Clinvar or gnomAD; it was predicted to be a splice site variant. The blood total cholesterol level of the proband was similar to the level in previously reported individuals with biallelic *ABCG5* variants, ranging from 116 mg/dl to 870 mg/dl (**Supplementary Table S3**).

Subsequently, these two variants were examined in all family members. The results showed that these variants were transmitted across all three generations in heterozygous form (**Figure 1B**). Var1 was specific to the paternal side, and Var2 was only detected in the maternal lineage. However, individual I-1 did not carry any variants in the *ABCG5* gene. In addition, all maternal members heterozygous for Var2 exhibited the large platelet phenotype without dysfunction of blood coagulation (**Figure 1C**; **Table 1**; **Supplementary Figure S2**).

Functional Assessment of Var2 Mutation

Since Var2 was predicted to be a splice site variant, we examined if Var2 can result in novel splicing transcripts of *ABCG5* (**Figure 1D**). As shown in **Figure 1E**, the mRNA transcripts from the reference and Var2 fragments were 295 bp and less than 100 bp, respectively. Subsequent Sanger sequencing verified that this "truncated" transcript was 81 bp and excluded exon 8. Therefore, this result suggests that Var2 leads to deletion of exon 8 of *ABCG5* during transcription, and the creation of a premature termination codon triggering nonsense-mediated mRNA decay.

Treatment With Ezetimibe

Before the study, atorvastatin 10 mg/day was first administered to the proband and her plasma LDL-C level gradually decreased to around 200 mg/dl within 2 years (**Figure 2A**). Although the proband's serum LDL-C level was reduced to about 150 mg/dl when atorvastatin was increased to 20 mg/day, she experienced severe adverse effects (**Supplementary Table S4**). By reducing atorvastatin back to 10 mg/day, the proband's LDL-C level remained around 200 mg/dl.

Considering the insufficient response to atorvastatin which aims to inhibit cholesterol biosynthesis, we suspected that the proband may have an abnormality in cholesterol excretion instead. At that point, the treating physician suggested stopping cow milk intake (450 ml/day), following a similar infant case with a limited increase in β-Sitosterol where breastfeeding was arrested to ameliorate the symptoms (Rios et al., 2010).

After 1 month, the proband's LDL-C level was reduced to 130 mg/dl without further decrease. The effect caused by the reduction of cholesterol intake supported the hypothesis of a cholesterol excretion dysfunction since this strategy is not effective for individuals with abnormal cholesterol synthesis. WGS analysis identified compound heterozygous variants in *ABCG5* responsible for abnormal cholesterol excretion consistent with the known disorder sitosterolemia, which supported our hypothesis. Following this finding, the physician in our group recommended a combination of atorvastatin (10 mg/day) and Ezetimibe (10 mg/day) be administered to the proband.

Within 2 months, the patient's LDL-C level was further decreased to an optimal level (<80 mg/dl). After treatment for 39 months, atorvastatin was discontinued but Ezetimibe treatment was continued with no milk intake. Thus far, the patient's LDL-C level has been maintained at around 80 mg/dl without any obvious adverse effects. An ultrasound scan 2 months following the revised treatment showed that the proband's atherosclerotic plaque disappeared from her left carotid artery (**Figure 2B**).

Interestingly, the proband's macrothrombocytopenia nearly completely resolved within 1 month after the addition of Ezetimibe while the β-Sitosterol level was reduced to normal values (0.83 mg/dl). The size distribution of platelets normalized, and only 25% of platelets remained over 30 fl (**Figures 2C,D**). ABCG5 functional inactivation leads to relatively active NIPIC, targeting the NIPIC by Ezetimibe was shown to restore the balance of cholesterol absorption and excretion maintained by them. These results suggested that the macrothrombocytopenia of the proband was likely caused by the *ABCG5* variant. More unexpectedly, all Var2 heterozygotes in the family had large platelets, and after 1 month of Ezetimibe treatment alone, the macrothrombocytopenia of her maternal uncle (II-2, **Figure 2**) also disappeared (**Supplementary Figure S3**).

Disruptions of the ABCG5/ABCG8 genes mimic phenotypes of the proband in double knockout rat.

To further determine if heterozygotes of *ABCG5/ABCG8* genes could drive hypercholesterolemia and macrothrombocytopenia phenotypes, we created a double knockout rat (**Figure 3A**), having a growth curve similar to the WT strain without visible abnormalities (**Supplementary Figure S4A**) and succesful depletion of ABCG5 and ABCG8 expression in liver and intestine (**Supplementary Figure S4B**). As expected, the $G5G8^{-/-}$ rats had a significant elevation of blood lipid levels (**Figure 3B**, left) with large platelets (4 out of 6 animals) compared with wild-type rats. However, the alterations in blood lipids were not strictly correlated with the occurrence of large platelets (**Figure 3B**, right), further supporting the observations of phenotypic heterogeneity of *ABCG5/ABCG8* variants. In particular, one heterozygous rat ($G5G8^{+\ +/-\ -}$) also had this biased distribution of platelet size (**Figure 3C**), similar to the maternal kindred with Var2, suggesting that heterozygotes of this *ABCG5* variant might be sufficient to cause macrothrombocytopenia. Considering these functional studies, Var2 may be classified as "Likely pathogenic" according to the standards and guidelines of the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015): in this case PS3, our functional studies show a deleterious effect; PM2, absent in population databases. Addtionally, the homozygous KO rats had significantly higher β-Sitosterol level (mean, 7 mg/dl) than WT strain (mean, 2.5 mg/dl) when even being fed by normal diet. We further treated 2 KO rats with Ezetimibe 0.2mg/Kg. Their serum TC reduced from 2.3 and 2.4 mmol/L to 1.7 and 1.9 mmol/L , and β-Sitosterol level reduced from 6.5 and 7.5 mg/dl to 2.6 and 2.7 mg/dl.

Screening for ABCG5 Variant Carriers in the General Population

We decided to determine whether *ABCG5* variants are associated with large platelets in the general population. Routine automated blood cell counting systems differentiate blood cells by their size and do not recognize large platelets as platelets, these instruments may not accurately detect macrothrombocytopenia. The mean platelet volume also does not reflect actual platelet size in the case of large platelets. Platelet count should therefore be determined manually in a calculating chamber or on peripheral blood smears when suspecting the condition. On routine clinical laboratory examination, biased platelet distribution can be observed, with an abnormal "NaN" value instead of specific values. Without other indicators, large platelets may be easily overlooked.

Here, we intended to examine if platelet size could be used to identify *ABCG5* heterozygotes (Methods). We collected platelet size distribution results for all individuals having this examination within 2 months in several hospitals. Among 1,180 individuals screened, 30 had evidence of large platelets (>30 fl, **Supplementary Figure S5**). In *ABCG5* sequencing of the 30 individuals (**Figure 4**, **Supplementary Table S5**), we identified a known pathogenic variant (p.Arg446Ter, gnomad:2-44050063-G-A; allele frequency:1.70e-4 and no homozygotes) in one individual. In addition, two novel variants, Var3 (hg19-chr2:44039644, NM_022436:c.*610_*611insCCCAGTGATTTTACTGAGGATTA) and Var4 (hg19-chr2:44039650, NM_022436:c.*604_*605insTACAGAGCACCCAGTGATTTTACTGA, hg19), insertions in 3' UTR region, were also identified in three other individuals, respectively. These are classified as variants of uncertain significance (no record in gnomAD, thus only having PM2 evidence according to ACMG standards and guidelines). Hence, this population-based work along with segregation of an *ABCG5* variant in a large pedigree with sitosterolemia identifies heterozygosity for specific *ABCG5* variants as an additional genetic cause of macrothrombocytopenia. Our data suggest that 2.5% of individuals requiring complete blood counts may have large platelets, and roughly 10% of those with large platelets may have *ABCG5* variants that are causative. Therefore, screening for large platelets may be helpful to identify heterozygotes for *ABCG5* pathogenic variants; conversely, sequencing *ABCG5* in individuals with large platelets may be useful in identifying patients amenable to Ezetimibe treatment, if it were shown to be effective and beneficial in future large clinical trials.

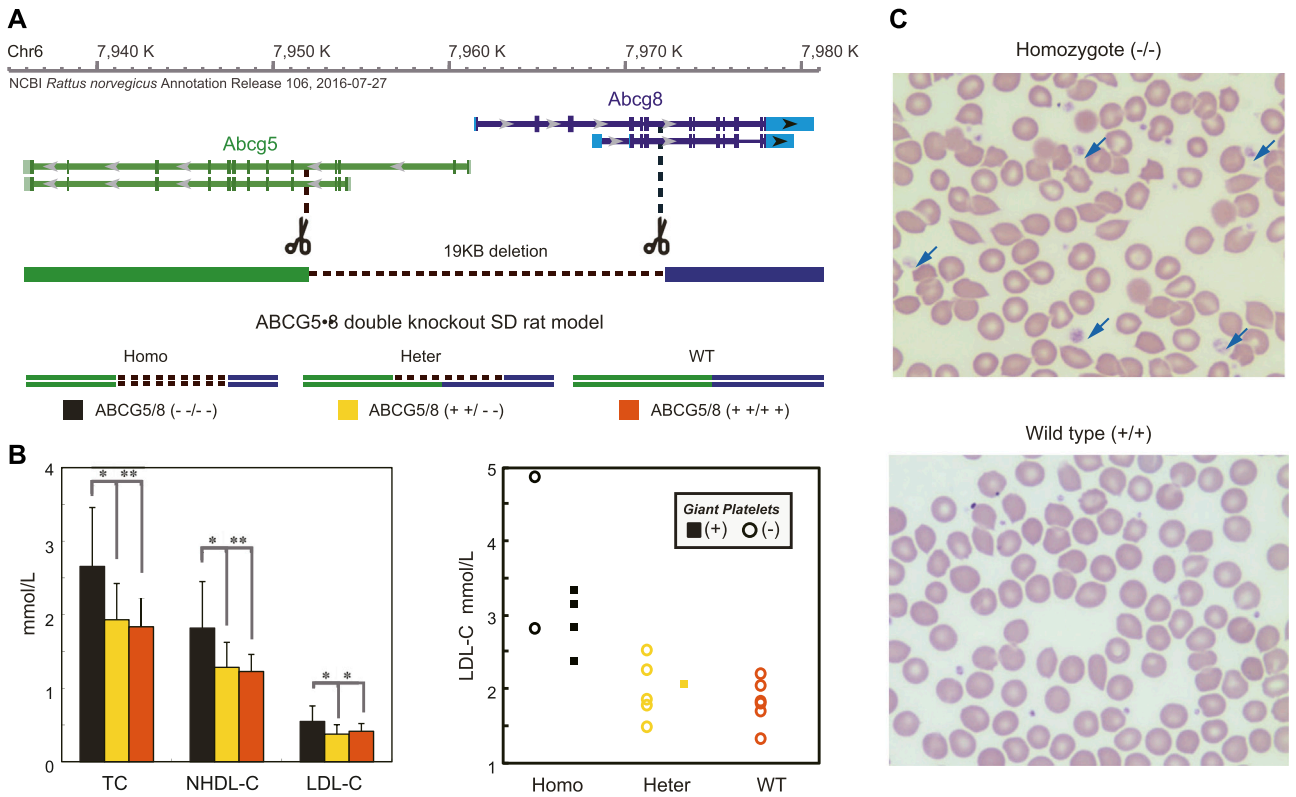

FIGURE 3 | Disruptions of *ABCG5/8* Genes Mimicked the Phenotypes of the Proband in the Double Knockout Rats. Panel **(A)** shows the strategy for the generation of *ABCG5/8* double knockout using the CAS9/sgRNA system. Panel **(B)** showed significantly increased blood lipid levels **(left)** and the large platelet **(right)** in the homozygotes compared with two other groups. For each group, the blood lipid levels and the large platelet were determined from randomly selecting rats. The values of the blood lipid levels are expressed as the mean ± SE. $n = 6$. *$p < 0.05$, **$p < 0.01$. The large platelet was detected in over 80% of homozygotes, and none was found in the wild-type strain. Panel **(C)** shows a blood smear for three groups of the rat model.

DISCUSSION

We identified a proband with profound hypercholesterolemia, recalcitrant to atorvastatin therapy. The pedigree analysis did not support dominant inheritance and gene panel testing for several genes associated with FH was non-diagnostic. *ABCG5* Var1 did not behave in a classical dominant way that most other variants responsible for FH lead to abnormal lipid levels of affected individuals when they were young. It is important to note that the increased lipid levels of paternal individuals may be confounded by lifestyles that promote hyperlipidemia. Additionally, this family appears to show an allele dosage effect where heterozygous carriers of a single putative pathogenic variant result in mild signs/symptoms, especially macrothrombocytopenia.

Hematological alterations have long been noticed in individuals with sitosterolemia, such as decreased platelet counts, increased mean platelet volume, and/or hemolytic anemia (Wang et al., 2004; Patel, 2014; Ajagbe et al., 2015). In addition to hypercholesterolemia, the proband had large platelets. Sitosterolemia can cause both phenotypes. Unexpectedly, all heterozygotes for Var2 in the maternal lineage exhibited large platelets without any changes in blood lipid profile. This is the first study to report a sign or symptom caused by heterozygosity for an *ABCG5* variant.

It has been long noticed that homozygous mutant mice with disruption of Abcg5 and Abcg8, Del (17Abcg5-Abcg8)1Hobb, have increased mean platelet volume (Yu et al., 2002). Nevertheless, this had not been previously reported in heterozygotes. We generated an ABCG5/8 double knockout rat, which illustrated that heterozygous *ABCG5* variants can lead to large platelets independent of hypercholesterolemia. Moreover, this trait was successfully corrected by Ezetimibe treatment in alone both the compound heterozygous proband and heterozygote II-4. Additionally, our preliminary experiment showed that Ezetimibe resolved both hypercholesterolemia and macrothrombocytopenia in two knockout rats. Taken together, Ezetimibe treatment can improve platelet production via unknown mechanisms.

Large platelets are usually accompanied by thrombocytopenia. Thus, it is also termed macrothrombocytopenia. Macrothrombocytopenia is usually due to acquired disorders; inherited large platelet disorders are rare. The mechanisms of large platelet formation and thrombocytopenia are poorly understood. Treatment for acquired versus inherited forms differs. Several different genes have been implicated as causes (Saito et al.). The most common clinical manifestation of inherited large platelets includes bleeding tendency (Mhawech and Saleem, 2000). Platelet count is usually decreased along with their increased size, which explains the abnormal blood

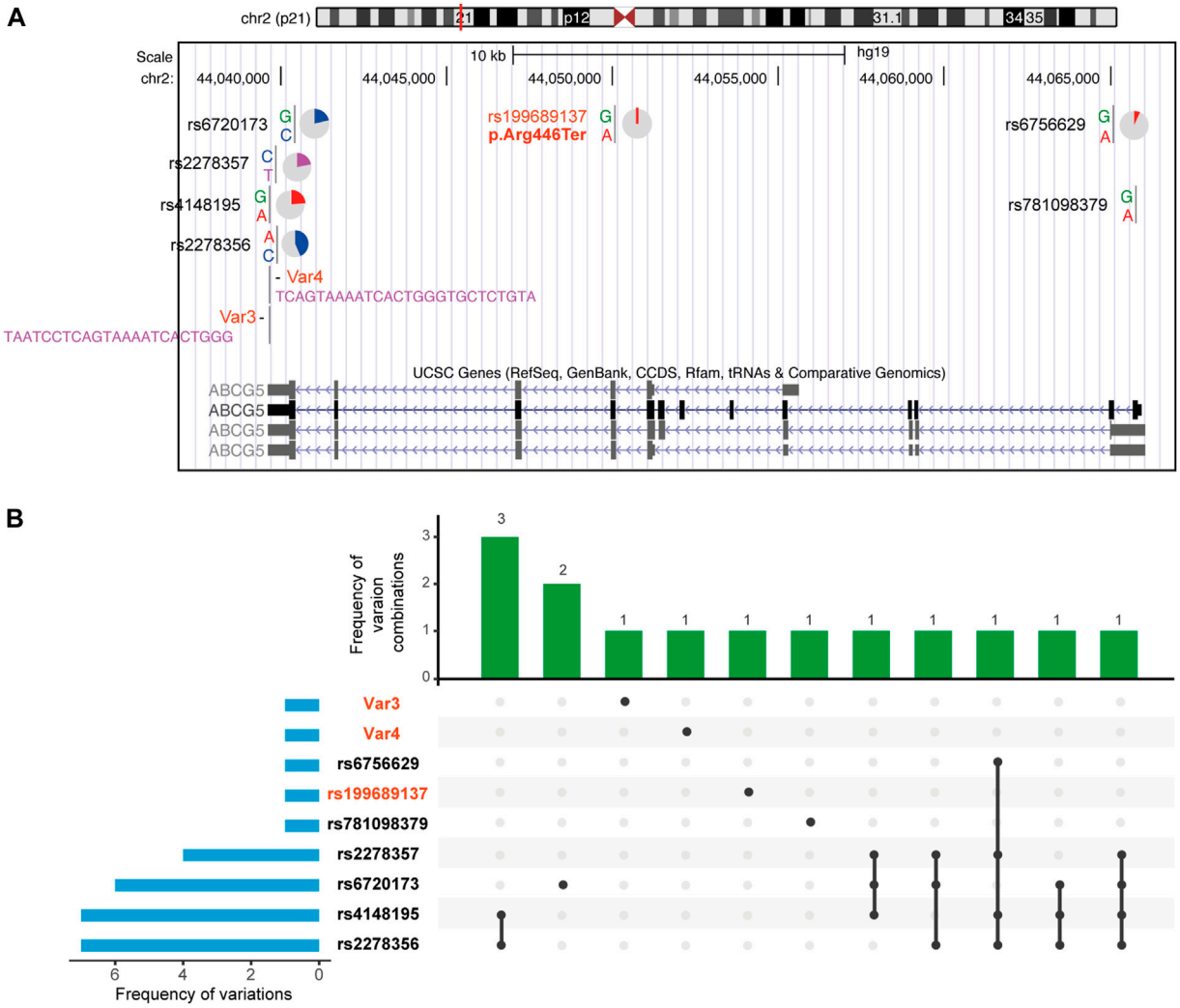

FIGURE 4 | Nine ABCG5 variants in 30 individuals with abnormal platelet size distribution. Details of each variation are in **Supplementary Table S4**. Genetic variations of them are more commonly seen in the 3′UTR of the gene model. Except for 3 pathogenic and likely pathogenic variations are heterozygotes in affected individuals, other variations are all homozygotes.

coagulation. Overall, Var2 carriers other than the proband seem to be asymptomatic, similar to a few cases exhibiting large platelet syndrome. It is noteworthy that individual I-3 exhibited cerebral ischemic stroke at the age of 58, with normal blood pressure and lipid profile. Particularly, previous studies pointed out the large platelets may increase the stroke risk (O'Malley et al., 1995; Bath et al., 2004). Recently, it has also been reported that heterozygous carriers of ABCG5 variants had a 2-fold increase in the risk of coronary artery disease (Nomura et al., 2020). Therefore, determining the disease risk of Var2 carriers may require future evaluation in cohort studies, as a potential benefit for Ezetimibe treatment in these carriers.

Frequencies of pathogenic variants of *ABCG5* should be quite low in the general population; by July 2021, pathogenic or likely pathogenic variants have been reported for 66 cases in ClinVar, and the allele frequency is about 0.04% (126/282856) in gnomAD (2.1.1). The present study, however, found a high prevalence of *ABCG5* variants in a general population with large platelets. Screening for large platelets, therefore, may be helpful to identify heterozygotes for *ABCG5* pathogenic variants; conversely, sequencing *ABCG5* in individuals with large platelets may be useful in identifying patients amenable to Ezetimibe treatment, since a heterozygous family member with large platelets responded to this treatment.

Generally, physicians may only review the average volume/size of platelets, such as mean platelet volume (MPV), examined by the automated blood cell counting systems. But they may commonly ignore an abnormal "NaN" value of MVP in the system report, which stands for a biased platelet size distribution, particularly when patients have normal functions of blood coagulation as our observation in this pedigree. Here we showed the abnormal "NaN" value of MVP stood for the occurrence of large platelets over 30 fl, which can be the only symptom for some carriers of *ABCG5* variants. In addition, this volume is equivalent to spheres 4 microns in diameter to identify large platelets, which we adopted at the

criteria in the blood film examination. Our study suggested the review of abnormal platelet size distribution may help to identify these carriers more easily. Meanwhile, they may have an increased risk of other complications such as stroke. Further larger studies could incorporate improved methods for detection of large platelets, identification of *ABCG5* variants in those with large platelets, the risk of clinical complications in such patients, and the effects of Ezetimibe treatment.

CONCLUSION

In summary, WGS and plant sterol analysis of a family trio identified sitosterolemia in a proband with recalcitrant hypercholesterolemia and macrothrombocytopenia; she was found to have compound heterozygous variants in the *ABGC5* gene. Diagnosis confirmed in this manner guided appropriate therapeutic decisions, leading to clinical improvement. Interestingly, the therapeutic regimen not only reduced the proband's blood cholesterol level to normal levels but also resolved macrothrombocytopenia. In the course of the evaluation, *ABCG5* heterozygosity was identified as a cause of autosomal dominant inherited macrothrombocytopenia; the role of *ABCG5* in macrothrombocytopenia and its therapy with Ezetimibe should be further investigated.

DATA AVAILABILITY STATEMENT

The detailed methods described above and other measurements/assays for plasma cholesterols and platelets were available in the **Supplementary Appendix** of the full text of this article. WGS and Gene panel raw data are available from the corresponding author on reasonable request with consent from the patient family. VCF files for variants in ABCG5 genes and the Gene panel are publically available at https://github.com/humangenetest/ABCG5variants.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University (IRB#2016-3). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HX, LD, and DZ designed the study, planned experiments. LD, DZ, and JX, analyzed data, and drafted the manuscript. JX, QC, BY, XL, and XW collected the clinical samples, performed the required examinations, and analyzed clinical information. LD, WC, SG, and ZC designed and performed the sequencing analysis of the pedigree. ZC, YX, and KD performed the cell-line functional analysis and analyzed the data for the animal model. HX, LD, RS, DZ, and SS interpreted data and participated in manuscript preparation. All authors reviewed the manuscript and agreed with the final version.

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SUPPLEMENTARY MATERIAL

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CASE REPORT
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Case Report: Genetic Analysis of PEG-Asparaginase Induced Severe Hypertriglyceridemia in an Adult With Acute Lymphoblastic Leukaemia

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PEG-Asparaginase (also known as Pegaspargase), along with glucocorticoids (predominantly prednisolone or dexamethasone) and other chemotherapeutic agents (such as cyclophosphamide, idarubicin, vincristine, cytarabine, methotrexate and 6-mercaptopurine) is the current standard treatment for acute lymphoblastic leukaemia in both children and adults. High doses of PEG-asparaginase are associated with side effects such as hepatotoxicity, pancreatitis, venous thrombosis, hypersensitivity reactions against the drug and severe hypertriglyceridemia. We report a case of a 28-year-old male who was normolipidemic at baseline and developed severe hypertriglyceridemia (triglycerides of 1793 mg/dl) following treatment with PEG-asparaginase for acute lymphoblastic leukaemia. Thorough genetic analysis was conducted to assess whether genetic variants could suggest a predisposition to this drug-induced metabolic condition. This genetic analysis showed the presence of a rare heterozygous missense variant c.11G > A-p.(Arg4Gln) in the APOC3 gene, classified as a variant of uncertain significance, as well as its association with four common single nucleotide polymorphisms (SNPs; c.*40C > G in APOC3 and c.*158T > C; c.162-43G > A; c.-3A > G in APOA5) related to increased plasma triglyceride levels. To our knowledge this is the first case that a rare genetic variant associated to SNPs has been related to the onset of severe drug-induced hypertriglyceridemia.

Keywords: PEG-asparaginase, acute lymphoblastic leukaemia, hypertriglyceridemia, APOC3, genetic testing

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INTRODUCTION

L-asparaginase has been used for over 40 years for the treatment of Acute Lymphoblastic Leukaemia (ALL), especially in paediatric patients, where the incidence of this disease is highest. It has been demonstrated that using L-asparaginase in adult patients improves prognosis and, when used at higher doses, its therapeutic effect is the same as in paediatric patients (Quist-Paulsen et al., 2020). It is worth remembering that L-asparaginase treatment can cause several side effects, which may require a suspension of treatment. Among the most frequently observed side effects are hepatotoxicity, pancreatitis, venous thrombosis (sometimes complicated by pulmonary emboli),

and severe hypertriglyceridemia (Hoogerbrugge et al., 1997; Grace et al., 2011; Truelove et al., 2013; Oparaji et al., 2017; Finch et al., 2020a; Burke et al., 2020; Underwood et al., 2020; Chen et al., 2021; Kumar et al., 2021). Moreover, asparaginase therapy can cause hypersensitivity reactions and development of asparaginase-antibodies that limit the action of the drug and increase the risk of leukemia relapse. Clinical pharmacogenetics studies suggest that the family of nuclear factor of activated T-cells transcription factors could play a role in increasing the risk of hypersensitivity reactions due to L-asparaginase; genetic inhibition of these factors protects against asparaginase hypersensitivity, at least in mice (Woo et al., 1998; Fernandez et al., 2015; Rathod et al., 2019a; Rathod et al., 2019b; Rathod et al., 2020). There are three formulations of asparaginase: L-asparaginase, which is endogenously produced by *Escherichia coli*, Pegylated asparaginase (PEG-ASP) which is a pegylated form of L-asparaginase and results in a prolonged half-life and decreased immunogenicity and *Erwinia* asparaginase, which is derived from the bacterium *Erwinia* chrysanthemi, and is immunologically different from the *Escherichia coli* derived asparaginase forms and which could be used in patients allergic to other formulations. At present the pegylated long-acting form of L-asparaginase (PEG-ASP) is by far the most used therapeutic option in both adult and paediatric ALL patients due to a more prolonged effect, a reduced incidence of silent antibody and more rapid clearance of lymphoblasts than native asparaginase (Avramis et al., 2002). The aim of this work is to describe a clinical case of severe hypertriglyceridemia arising following treatment with PEG-asparaginase in a patient with ALL, and to verify whether or not a genetic profile leading to the onset of this serious complication can be identified.

Case Description and Diagnostic Assessment

A 28-year-old male, with Ph negative ALL underwent chemotherapy according to the GIMEMA LAL 1913 protocol (approved by the Cardarelli Hospital Etichal Committee) and achieved complete remission with minimal residual disease (MRD), while both immunophenotyping by flow cytometry and molecular testing yielded a negative result. This protocol involves the administration of PEG-ASP during induction/consolidation (cycles 1, 2, 5, and 6), in addition to other agents, including prednisone, dexamethasone and other chemotherapeutic agents (such as cyclophosphamide, idarubicin, vincristine, cytarabine, methotrexate and 6-mercaptopurine). On admission to hospital (31 August 2020) the blood pressure was 150/90 mmHg; heart rate 124 beats per minute and the cardiologist prescribed bisoprolol fumarate. On physical examination mildly enlarged lymph nodes were palpable in latero-cervical chains and in the left axilla. Palpation of the abdomen revealed mild hepatomegaly and splenomegaly. His family history was noteworthy for breast cancer in his mother. Laboratory Data: Glucose was 93 mg/dl; triglycerides 116 mg/dl; cholesterol 220 mg/dl; HDL-cholesterol 102 mg/dl; amylase 38 IU/L; lipase 26 IU/L; lactate dehydrogenase 1169 IU/L; alanine transaminase 164 IU/L; aspartate transaminase 71 IU/L; gamma-glutamyl transpeptidase 339 IU/L; C-Reactive Protein 57 mg/L; Hematocrit 40%; Hemoglobin 14.2 g/dl; Platelets 101.000/mm^3; White-cell count 33.380/mm^3 (Differential count: Neutrophils 13.7%; Lymphocytes 75.6%; Monocytes 7.5%; Eosinophils 0.6%; Basophils 2.6%). A CT-study showed bilateral cervical and left axillary lymphadenopathy (max 1 cm), mild hepatomegaly and splenomegaly. Following the second round of treatment with PEG-ASP he was found to have: glucose 83 mg/dl; **triglycerides 1793 mg/dl**; cholesterol 390 mg/dl; HDL-cholesterol 15 mg/dl; amylase 32 IU/L; lipase 23 IU/L; lactate dehydrogenase 270 IU/L; alanine transaminase 44 IU/L; aspartate transaminase 20 IU/L; gamma-glutamyl transpeptidase 126 IU/L. There were no signs, symptoms, or investigations suggestive of acute pancreatitis. PEG-ASP was no longer administered to the patient. The nutritionist prescribed a hypocaloric, higher-protein, lower carbohydrate diet, and the internist prescribed omega-3 fatty acids and a statin. At follow-up his lipid profile was normal. **Figure 1** shows concentrations of triglycerides, total cholesterol, HDL-cholesterol at different interval times during chemotherapy.

In May 2021 the patient had a relapse of ALL and was treated for refractory acute lymphoblastic leukemia with high dose cytosine arabinoside and mitoxantrone (HAM).

In August 2021: the patient moved to another city in North Italy and was lost to follow-up.

Genetic Screening

Genomic DNA was extracted from peripheral blood and genetic screening was performed using a custom panel of 16 genes, associated to severe hypertriglyceridemia: APOA1, APOA4, APOA5, APOC1, APOC2, APOC3, APOC4, APOE, ANGPTL3, LPL, GPIHBP1, LMF1, GPD1, CREB3L3, GCKR, LRP1. For each gene the coding regions, 25 bp in each of the intronic boundaries, the 5′UTR and the 3′UTR regions, were included. Genomic libraries were obtained using the SureSelect Target Enrichment protocol (Agilent Technologies) and the high throughput sequencing was performed using Illumina technology (PE 2 × 150 bp). Data analysis was carried out using Alissa Interpret Rev.5.2.6 software (Agilent Technologies). Variants were firstly filtered on their minor allele frequency (MAF), and pathogenicity of rare prioritized variants (with a MAF <1%) was assessed according to the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015). All the prioritized variants were also validated by Sanger sequencing. The Multiplex Ligation-dependent Probe Amplification was also performed to verify the presence of rearrangements such as deletions and/or duplications in the coding region of the lipoprotein-lipase (LPL) gene (Giacobbe et al., 2020). Genetic analysis in this patient showed no evidence of variants causing severe familial hypertriglyceridemia. No common LPL gene variants (Asp9Asn, Asn291Ser, Trp86Arg, Gly188Glu, Pro207Leu, Asp250Asn) associated with the increase of plasma triglyceride levels were highlighted (Gehrisch, 1999). However, a rare missense variant, c.11G > A–p.(Arg4Gln), in the *APOC3* gene and four single nucleotide polymorphisms (SNPs) associated with increased plasma triglyceride levels at heterozygous state, were found. The APOC3 rare variant was classified as of uncertain clinical significance based on the ACMG guidelines. Genetic profile of patient is shown in **Table 1**.

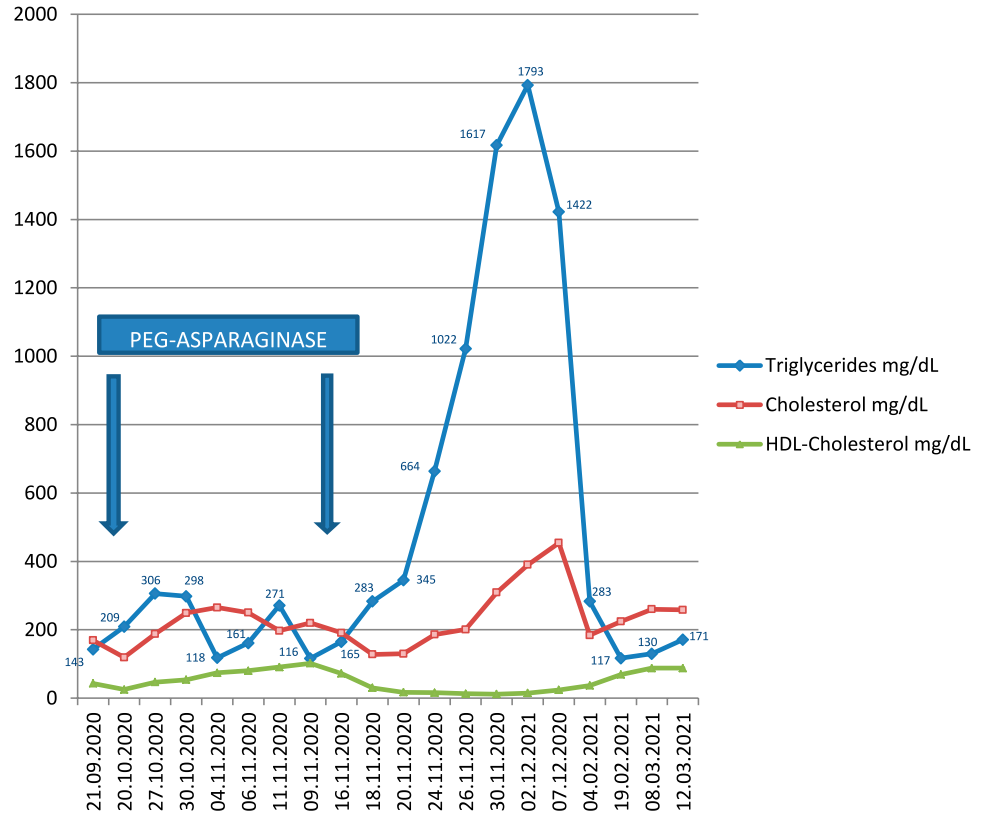

FIGURE 1 | Timeline graphic showing Triglycerides, Total Cholesterol and HDL-Cholesterol concentration during therapy for acute lymphoblastic leukemia in a 28 years man following Ginema LAL 1913 protocol (first 4 cycles). [Numbers in blue represent concentrations of triglycerides at different interval times].

TABLE 1 | Variants identified in this patient.

Nucleotide substitution	Amino acid substitution	dbSNP[1] code	MAF[2] (%)	Genotype[3]	HGMD database[4]	Phenotype[5]	Variant classification[6]	PMID[7]
APOC3 (NM_000040.3)								
c.11G > A	p.(Arg4Gln)	rs779597455	0.002	Hz	—	—	Uncertain significance	—
c.*40C > G	—	rs5128	13	Hz	CR041556	Increased plasma triglyceride levels	Benign	25928461
APOA5 (NM_052968.5)								
c.*158T > C	—	rs2266788	6.3	Hz	CR014773	Increased plasma triglyceride levels	Benign	11588264 24387992
c.162-43G > A	—	rs2072560	5.8	Hz	CS014761	Increased plasma triglyceride levels	Benign	11588264
c.-3A > G	—	rs651821	10	Hz	CR080753	Increased plasma triglyceride levels	Benign	28008009 24387992

[1]*www.ncbi.nlm.nih.gov*
[2]*MAF: minor allele frequency; gnomad.broadinstitute.org.*
[3]*Hz: Heterozygous.*
[4]*Human Gene Mutation Database (HGMD® Professional 2021.3).*
[5]*Clinical phenotype associated with the SNP.*
[6]*American College of Medical Genetics and Genomics (ACMG; Richards et al. Genet Med 2015).*
[7]*PMID: PubMed ID references.*

DISCUSSION

Glucocorticoids and PEG-ASP are fundamental treatments for ALL in both adults and children. However, these therapies, especially when combined, can result in side effects which include alteration of the lipid profile, particularly severe hypertriglyceridemia. The mechanism by which these drugs lead to the onset of hypertriglyceridemia has not yet been

elucidated. Glucocorticoids increase the synthesis of triglycerides and mobilise fatty acids, while activating lipoprotein lipase (LPL), an enzyme responsible for the hydrolysis of triglycerides (Peckett et al., 2011). PEG-ASP, on the other hand, seems to inhibit LPL activity (Hoogerbrugge et al., 1997). Therefore, when glucocorticoids are given simultaneously with PEG-ASP there is an increase in triglyceride synthesis without an increase in hydrolysis. In children the frequency of hypertriglyceridemia following treatment with corticosteroids and PEG-ASP for ALL is between 4 and 19%, depending on the protocol followed (Steinherz, 1994; Parsons et al., 1997; Cohen et al., 2010; Bhojwani et al., 2014). In young adults, as in our case, the frequency of severe hypertriglyceridemia is around 11% (Stock et al., 2019). Among the disorders of lipid metabolism, the genetic spectrum for familial hypercholesterolemia and its associated clinical implications is well established (Romano et al., 2010; Di Taranto et al., 2021). The relationship between severe hypertriglyceridemia and its clinical implications is less evident, whereas there is a well documented relationship with acute pancreatitis (Scherer et al., 2014). In patients with ALL the association between severe hypertriglyceridemia and pancreatitis is rarely seen (Tong et al., 2014). Severe hypertriglyceridemia, in the form of Familial Chylomicronaemia Syndrome (FCS), is a rare autosomal recessive disease caused by pathogenic variants especially in the LPL gene but also in APOC2, APOA5, LMF1 and GPIHBP1. Recently, it has been hypothesised that severe hypertriglyceridemia could be more frequently caused by the coexistence of rare variants in genes recognized in FCS, as well as by the presence of common or rare variants currently not recognized in the metabolism of triglycerides. This condition has been named multifactorial chylomicronaemia syndrome (MCS) (Dron and Hegele, 2020; Paquette et al., 2021). MCS represents a predisposing condition which could expose the patients to a 4 times greater risk of developing severe hypertriglyceridemia (Dron et al., 2019). Patients with the same heterozygous changes in the same genes who have normal or only slightly elevated triglyceride values, were also described, suggesting that these variants are only partially penetrating. Most variants interfering with triglyceride metabolism and causing hypertriglyceridemia are loss of function variants (LOF), and mainly found in the LPL, APOC2, APOA5, LMF1 and GPIHBP1 genes. Recently a gain of function (GOF) variant, in the APOC3 gene has been described in patients with severe hypertrigliceridemia (Sundaram et al., 2017). This APOC3 gene encodes a protein component of VLDL and chylomicrons and can inhibit lipoprotein lipase enzyme activity. Elevated plasma levels of APOC3 are also considered as a major risk factor for hypertriglyceridemia (Witztum et al., 2019). In light of the above the question we asked ourselves was: why, with equal doses of PEG-ASP, severe hypertriglyceridemia only occurs in a limited number of cases, even if these patients have a normal lipid profile at baseline? We can hypothesize that there are genetic variants which affect the onset of severe hypertriglyceridemia after certain treatments, for example with PEG-ASP. It is possible that non-genetic factors, such as the use of some drugs, could result in the development of severe hypertriglyceridemia in an individual who is predisposed to hypertriglyceridemia due to being a carrier of a gene with an increased associated risk. In a previous paper, Finch et al. found no significant genetic predisposition to hypertriglyceridemia in patients with ALL treated with PEG-ASP (Finch et al., 2020b). In our clinical case we identified a heterozygous variant, associated with triglyceride metabolism and potentially implicated in the origin of severe hypertriglyceridemia induced by PEG-ASP. To the best of our knowledge, this is the first case in which a potentially triglyceride-raising genetic variant has been detected in a patient with normal lipids at baseline and subsequent severe hypertriglyceridemia and hypercholesterolaemia following treatment with PEG-ASP. To date the APOC3 variant c.11G > A–p.(Arg4Gln) found in our patient and classified as of uncertain clinical significance according to ACMG guidelines, has never been described as associated to severe hypertriglyceridemia and if its pathogenicity resulted as GOF, the patient would be considered as carrier of a rare pathogenic variant.

Polymorphisms in the *APOC3* and *APOA5* genes, from the APOA1/APOC3/APOA4/APOA5 gene cluster on chromosome 11q23, have been associated with interindividual variation in plasma triglycerides. However, the degree to which polymorphisms in the *APOC3* and *APOA5* genes can be independently associated with triglyceride levels remains to be determined (Hallman et al., 2006). The close genomic locations of these two genes as well as their functional similarity have hindered efforts to define whether each gene independently influences human triglyceride concentrations. Several studies reported the existence of a common haplotype in the APOAV region influencing plasma triglyceride levels in some race-specific populations (Lai et al., 2003; Lai et al., 2004; Klos et al., 2005). Three APOA5 SNPs identified in our patient (rs651821, rs2072560 and rs2266788) were described by Dror et al. as associated with another SNP (rs662799) in significant linkage disequilibrium as well as with a 27–38% increase in triglyceride concentration in three ethnic groups (Ken-Dror et al., 2010). A large meta-analysis highlighted the association of the APOC3 rs5128 polymorphism with highly increased triglyceride plasma levels (Song et al., 2015).

Furthermore, Olivier et al. demonstrated that the APOA5 locus is separated from the other apolipoprotein genes by a region of increased recombination, thereby supporting the idea that APOA5 represents an independent risk gene affecting plasma triglyceride concentrations in humans (Olivier et al., 2004). The presence of the *APOC3* rare variant c.11G > A–p.(Arg4Gln) together with the common SNPs associated with high levels of triglycerides [c.*40C > G in *APOC3* (NM_000040.3) and c.*158T > C; c.162-43G > A; c.-3A > G in *APOA5* (NM_052968.5)] could lead to classify our patient as suffering from MCS.

This latter condition could be considered as a predisposing factor of developing a hypertriglyceridemic phenotype after PEG-ASP treatment. Of course, a single case cannot demonstrate with any certainty whether there is a genetic cause at the root of severe hypertriglyceridemia induced by drugs. However, this could

encourage further research into genes less frequently associated with the phenotypic manifestation of severe hypertriglyceridemia that could play a significant role as co-factors responsible for some forms of hypertriglyceridemia.

Strengths and limitations: The principal strength of this paper is the original finding (to our knowledge for the first time in the literature) of a potentially triglyceride-raising genetic variant implicated in the origin of severe hypertriglyceridemia induced by PEG-ASP in a patient with normal lipids at baseline. Moreover, this study highlights that the use of a custom capture-based target enrichment NGs panel -containing 16 genes to date known as being associated with Familial Chylomicronemia Syndrome and severe hypertriglyceridemia-allowed us to find a clear genetic pattern characterized by a rare variant in the apo-C3 gene together with four common SNPs that could be related to the onset of severe drug-induced Hypertriglyceridemia. A limitation of the present study is that no mechanistic study supports our findings and we can conclude that genetic variants could be considered as a predisposing factor of developing a hypertriglyceridemia and may not be solely responsible for severe hypertriglyceridemia after treatment with PEG-ASP.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by A. Cardarelli Hospital Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: AI, MA; Methodology: CG, DP; Validation: GF; Writing, original draft preparation: AI, EA, AB; Writing, review and editing: GF, GI.

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A Heterozygous LMF1 Gene Mutation (c.1523C>T), Combined With an LPL Gene Mutation (c.590G>A), Aggravates the Clinical Symptoms in Hypertriglyceridemia

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Hypertriglyceridemia is an important contributor to atherosclerotic cardiovascular disease (ASCVD) and acute pancreatitis. Familial hypertriglyceridemia is often caused by mutations in genes involved in triglyceride metabolism. Here, we investigated the disease-causing gene mutations in a Chinese family with hypertriglyceridemia and assessed the functional significance *in vitro*. Whole-exome sequencing (WES) was performed revealing that the severe hypertriglyceridemic proband carried a missense mutation (c.590G > A) in exon 5 of the *LPL* gene, as well as a missense mutation (c.1523C > T) in exon 10 of the *LMF1* gene. Conservation analysis by Polyphen-2 showed that the 508 locus in the LMF1 protein and 197 locus in the LPL protein were highly conserved between different species. I-TASSER analysis indicated that the *LMF1* c.1523C > T mutation and the *LPL* c.590G > A mutation changed the tertiary structure of the protein. A decrease in mRNA and protein expression was observed in 293T cells transfected with plasmids carrying the *LMF1* c.1523C > T mutation. Subcellular localization showed that both wild-type (WT) and mutant LMF1 protein were localized at the cell cytoplasm. In the cell medium and cell lysates, these *LMF1* and *LPL* gene mutations both caused a decreased LPL mass. Moreover, the combination of *LMF1* and *LPL* gene mutations significantly decreased LPL levels compared to their individual effects on the LPL concentration. Both the clinical and *in vitro* data suggest that severe hypertriglyceridemia was of digenic origin caused by *LMF1* and *LPL* mutation double heterozygosity in this patient.

Keywords: hypertriglyceridemia, lipase maturation factor 1, lipoprotein lipase, missense mutation, heterozygous mutation

Abbreviations: APOC2, apolipoprotein C2; APOA5, apolipoprotein A5; GPIHBP1, glycosylphosphatidyl-inositol-anchored high-density lipoprotein binding protein 1; HTG, hypertriglyceridemia; HEK293T cells, Human embryonic kidney 293T cells; LMF1, Lipase maturation factor 1; LPL, lipoprotein lipase; TG, triglyceride; VLDL, very-low-density lipoprotein.

INTRODUCTION

Associated with the occurrence and development of acute pancreatitis and atherosclerotic cardiovascular disease (Hegele et al., 2014), hypertriglyceridemia (HTG) is a common disease characterized by triglyceride levels higher than 150 mg/dl (1.7 mmol/L). Notably, when triglyceride levels exceed 1,000 mg/dl, the incidence of acute pancreatitis will significantly increase to 5% (Scherer et al., 2014). To the best of our knowledge, HTG is related to environmental and genetic factors, and is induced by common conditions. These include pregnancy, hypothyroidism, chronic kidney disease, and metabolic syndrome (Pearce, 2004; Goldberg and Hegele, 2012; Catapano et al., 2016). Primary severe HTG has both monogenic and polygenic determinants. Similarly, a range of monogenic and polygenic variants can lead to either the severe or mild-to-moderate primary HTG. Familial HTG is often caused by loss of function mutations in the five main genes, namely lipoprotein lipase (*LPL*), apolipoprotein C2 (*APOC2*), apolipoproteinA5 (*APOA5*), glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1 (*GPIHBP1*), or lipase maturation factor 1 (*LMF1*), that regulate TG-rich lipoprotein lipolysis.

LPL is the key enzyme in systemic lipid metabolism that mediates intravascular hydrolysis of triglycerides into chylomicrons and very-low-density lipoprotein (VLDL). As a lipase chaperone located in the endoplasmic reticulum, LMF1 is required for LPL folding and/or dimerization. Homozygous mutations in a locus called cld were found to cause severe HTG for the first time in the mouse model in previous studies, and heterozygous mice had normal TG levels (Péterfy et al., 2007). Peterfy et al. (Péterfy et al., 2007) identify the cld gene and name it *Lmf1*, and also identify a human individual with combined LPL deficiency who was homozygous a nonsense *LMF1* mutation. In humans, while a few common or rare *LMF1* variants have been reported in HTG cases, no clear evidence of functional analyses exists. Only three homozygous nonsense *LMF1* mutations (p.Tyr439*, p.Tyr460*, and p.Trp464*) have been identified as being causative of hyperchylomicronemia. The p.Ser137Leu missense variant in the *LMF1* gene was shown to be causative of severe hypertriglyceridemia through functional analysis (Péterfy et al., 2018).

In this study, we investigated a Chinese family spanning four generations that has been diagnosed with HTG. The clinical phenotypes and the functions of the pathogenic mutations were analyzed in this family so as to study the pathogenesis of HTG.

MATERIALS AND METHODS

Patients

This study was approved by the Nanfang Hospital Ethics Committee, an affiliate of the Southern Medical University (Guangdong, China). Written informed consent was obtained from all participants. The proband (II-4), a 43-year-old male, was admitted to the Nanfang Hospital's Department of Cardiology with repeated fasting serum triglyceride (TG) concentration measurements exceeding 20 mmol/L. Plasma lipid profile analysis and other routine examinations of the proband and his family members, which included his father, mother, brother, daughter and son, were performed in Nanfang Hospital.

Mutation Screening

Blood samples obtained from the proband were sent to the Nanfang Hospital Precision Medicine Center for Whole-exome sequencing (WES) of genomic DNA. Illumina HiSeq platform was used for WES. The criteria established and revised by the American College of Medical Genetics and Genomics (Richards et al., 2015) was used to classified the variants. Two hypertriglyceridemia associated genes mutations, within exon 10 of the *LMF1* gene and exon 5 of the *LPL* gene identified by WES, were verified using Sanger sequencing. Standard phenol/chloroform extraction was performed to extract genomic DNA from the peripheral blood acquired from the proband and his family members (I1, I2, II4, II2, III4, and III3). The PCR primers that were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) were as follows: *LMF1*: Exon-10 forward primer: 5′-CCGTCTCAGCCACCAGAAAA-3′, Exon-10 reverse primer: 5′-CACGGCTGGTTTGGTTTGAG-3'; and *LPL*: Exon-5 forward primer: 5′-CCAGCCATCCTGAGTGGAAA-3′, Exon-5 reverse primer: 5′-GGCTCTAAGGTGGTCATGCT-3'.The PCR products were then analyzed by agarose gel electrophoresis and submitted to Invitrogen (Shanghai, China) for Sanger sequencing.

Bioinformatics

For WES, BaseSpace BWA Enrichment App was used to perform read alignment and variant calling. The sequence was aligned with BWA Genome Alignment Software. Variant calling was performed with GATK using the human reference sequence GRCh37. Variant annotation was performed by ANNOVAR software. Available genomic databases (dbSNP, 1,000 Genomes Project, Exome Variant Server, Exome Aggregation Consortium and a local Paris Descartes Bioinformatics platform database) were used to filter exome variants and exclude variants with a frequency >1%.

The three-dimensional (3D) protein structures of wild-type and mutant LMF1 and LPL proteins were predicted using I-TASSER (Iterative Threading ASSEmbly Refinement, http://zhanglab.ccmb.med.umich.edu/I-TASSER/). Amino acid sequences were submitted to the online I-TASSER software, which then provided the predicted 3D protein structure. The conservation of LMF1 and LPL proteins was analyzed by Polyphen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) to predict the harmfulness of mutations.

Plasmid Constructs and Mutagenesis

Total RNA was extracted from peripheral blood using Trizol reagent (Invitrogen, California, United State). The HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Jiangsu, China) was used

for the synthesis of first-strand cDNA according to the manufacturer's instructions. The coding sequence (CDS) regions in *LMF1* gene were amplified using primers containing BglII and SalI restriction sites, while the CDS regions in *LPL* gene were amplified using primers containing NheI and BamHI-HF restriction sites. The primers were as follow: *LMF1*: forward primer: 5′-CGAGATCTATGCGCCCTGACAGCCCAA-3′, reverse primer: 5′- ACGCGTCGACAAGAGGGGCCCGGG CAGAG-3′; and *LPL*: forward primer: 5′-CTAGCTAGCATG GAGAGCAAAGCCCTGC-3′, reverse primer: 5′-CGGGATCCG CCTGACTTCTTATTCAGAGACTTG-3′. The WT coding region of the *LMF1* gene was then cloned into the BglII and SalI sites of the pEGFP-N1 vector (GENEWIZ, New Jersey, United States) and the WT coding region of the *LPL* gene were cloned into the NheI and BamHI-HF sites of the pcDNA3.1-cFLAG plasmid (Life Technology, Thermo Fisher Scientific, Waltham, Massachusetts, United States). Moreover, the mutation plasmid were generated by PCR-based site-directed mutagenesis. The primers for this were as follows: *LPL*: forward primer: 5′-CAGAAGCCCCGAGTCATCTTTCTCCTGATG-3′, reverse primer: 5′- CATCAGGAGAAAGATGACTCGGGG CTTCTG-3′; and *LMF1*: forward primer: 5′- TCGCGGGCA GGCCCCTGCCCAGGTGGGTCC-3′, reverse primer: 5′- GGA CCCACCTGGGCAGGGGCCTGCCCGCGA-3′. Recombinant plasmids were purified using the Plasmid Miniprep Kit (Axygen, New York, United States) and sequenced to exclude the presence of random mutations.

Cellular Localization

Human embryonic kidney 293T cells (HEK293T cells) were used for functional analysis of *LMF1* variants. HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, New York, United States) supplemented with 10% fetal bovine serum (Gibco) at 37°C and 5% CO_2.

The subcellular location of LMF1 proteins was detected by immunological fluorescence assays. The recombinant plasmids containing wild-type or mutant *LMF1* genes were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 36 h, the successfully transfected HEK293Tcell were fixed for 30 min with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, United States) after washing three times with PBS (Sigma-Aldrich). Following discarding of the paraformaldehyde, the samples were again washed three times with PBS. Cells were then incubated in 0.1% Triton X-100 (Thermo Fisher Scientific, Massachusetts, United States) to increase the permeability of the cytomembrane. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI; Sigma). The stained cells were viewed using a confocal fluorescence microscope (LSM 880; Carl Zeiss AG, Jena, Germany).

RNA Analysis

HEK293T cells were transfected with 1 μg of the recombinant plasmids containing wild-type or mutant genes using Lipofectamine™ 2000 transfection reagent (Invitrogen). After 36 h of transfection, total RNA was extracted from the cell lysates using Trizol reagent (Invitrogen) and reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (Takara, Dalian, China). Quantitative real-time PCR was performed to compare the relative mRNA levels between lysates of HEK293T cells transfected with wild-type and mutant genes using the 2 × RealStar Green Fast Mixture (GenStar, Beijing, China). The GAPDH gene was chosen as the reference gene. The primers were as follows: *LMF1*: forward primer: 5′-CGTAACAACTCCGCCCCATT-3′, reverse primer: 5′-TCCGAGTACCCA GTCTTCCG-3'; and GAPDH: forward primer: 5′-GTGAAGGTCGGAGTCAACG-3′, reverse primer: 5′-TGAGGTCAATGAAGGGGTC-3'. RNA analyses were repeated three times, and there was three groups of sample in each time (n = 9).

Western Blot Analysis

HEK293T cells transfected with the recombinant plasmids containing wild-type or mutant genes were collected and washed with cold PBS (Sigma-Aldrich). Cell lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with 1% phenylmethanesulfonyl fluoride (PMSF, Beyotime Biotechnology) was used to lyse cells and prevent protein degradation. The concentration of cell lysates was determined using the Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific) after removing cell debris by centrifugation. The obtained protein samples were separated by sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Massachusetts, United States). The membranes were blocked with 5% nonfat milk for 60 min at room temperature, and then incubated with GFP-tagged mouse monoclonal antibody (Ray Antibody Biotech, Beijing, China) or anti-GAPDH mouse monoclonal antibody (Sigma Aldrich) overnight at 4°C. After washing with Tris Buffered Saline-Tween 20 (TBST) three times, the membranes were incubated with goat anti-mouse IgG (Sigma-Aldrich) at room temperature for 2 h. Protein expression was detected by SuperSignal West Pico ECL (Thermo Fisher Scientific) and a digital chemiluminescence system (Tanon Science& Technology, Shanghai, China). ImageJ software was used to quantify the intensities of the protein bands. LMF1 expression was normalized against GAPDH levels. Western blot analyses were repeated three times, and there was three groups of sample in each time (n = 9).

ELISA Analysis

HEK293T cells were transfected with a total of 3 μg of the recombinant plasmids containing wild-type or mutant *LPL* and *LMF1* genes (*LPL*:*LMF1* of 2:1) using Lipofectamine™ 2000 transfection reagent (Invitrogen). Thirty hours post-transfection, 1 ml of complete medium with10 U heparin was added into the cells cultured in 6-well plates, and the supernatants were collected after centrifugation. The cell lysates were obtained by freeze-thaw cycles. The samples were then analyzed using the human lipoprotein lipase (LPL) ELISA KIT (LunChangShuo Biotechnology, Xiamen, China) in accordance with the manufacturer's instructions. ELISA analyses were repeated three times, and there was two groups of sample in each time (n = 6).

TABLE 1 | plasma triglyceride levels in the proband and his relatives.

	Genotype		TG (mmol/L)	TC (mmol/L)	LDL-C (mmol/L)	VLDL-C (mmol/L)	HLDL-C (mmol/L)	nHLDL-C (mmol/L)	Sd (mmol/L)
	LPL	LMF1							
I1	GA	CC	5.06	4.99	2.26	1.87	0.86	4.13	1.32
I2	GG	CT	1.70	8.94	6.74	0.46	1.74	7.20	3.10
II2	GA	CC	12.92	5.06	1.39	—	1.15	—	—
II4	GA	CT	25.19	9.20	1.10	7.62	0.48	8.72	—
III3	GA	CC	0.83	5.62	3.71	0.40	1.51	4.11	0.99
III4	GA	CC	5.78	5.48	2.87	1.94	0.67	4.81	1.35

TG, triglyceride; TC, total cholesterol; LDL-C, Low-Density Lipoprotein Cholesterol; VLDL-C, Very-Low-Density Lipoprotein Cholesterol; HLDL-C, High-density lipoprotein cholesterol; n HLDL-C, non-High-density lipoprotein cholesterol; sd, small dense Low-Density Lipoprotein.

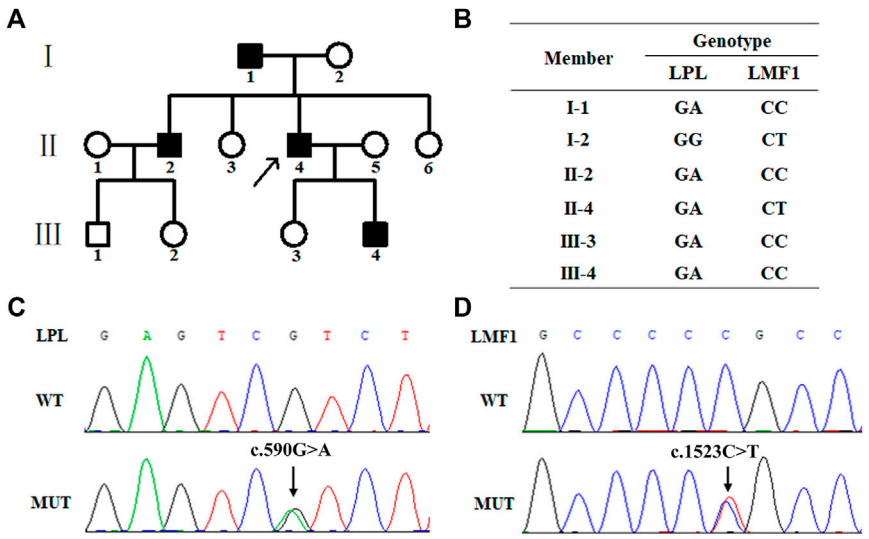

FIGURE 1 | Family pedigree and mutation screening of samples from a family with hypertriglyceridemia. **(A)**. Family pedigree. The arrow indicates the proband. **(B)**. The genotype of six family members. The proband (Ⅱ4), I1, Ⅲ4 and Ⅲ3 carry the heterozygous missense mutation (c.1523C > T) in the *LPL* gene. The proband (Ⅱ4) and his mother (I2) carry heterozygous missense mutation (c.590G > A) in the *LMF1* gene. **(C)**. Sanger sequencing results of the heterozygous mutation (c.1523C > T) in the *LPL* gene. **(D)**. Sanger sequencing results of the heterozygous mutation (c.590G > A) in the *LMF1* gene.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism software. Data were presented as the mean ± standard deviation (SD). The independent samples t-test was used for determining the statistical significance of the differences between the two groups. A p-value < 0.05 was considered to be statistically significant. Significance was further indicated using asterisks, where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

Clinical Features

The proband (II-4), a 43-year-old male, was admitted to the Nanfang hospital Department of Cardiology with repeated measurements of fasting serum TG concentration exceeding 20 mmol/L. Neither acute pancreatitis nor eruptive xanthoma occurred. The proband had no history of chronic kidney disease, diabetes mellitus, or hypothyroidism. On the occasion of his admission, the proband presented with serum TG levels of 25.19 mmol/L. After the onset of lipid lowering drug therapy (fenofibrate 0.2 g qd, and ezetimibe 10 mg qd) in the first day of admission, the serum TG levels of the proband decreased steadily to 8.15 mmol/L. The plasma lipid profile of his family members is shown in **Table 1**. The proband, I-1, II-2, and III-4 are HTG patients charactered by plasma triglyceride levels of **>** 1.7 mmol/L. The proband's mother and daughter are healthy (**Figure 1A**)

Genetic Analysis

WES was performed using genomic DNA from peripheral blood obtained from the proband to identify the possible pathogenic mutation. The proband carried a missense mutation (c.1523C > T) in the *LMF1* gene (GenBank accession no. NM_022773 exon10, p.Pro508Leu, rs372213215) and a missense mutation (c.590G > A) in the *LPL* gene (GenBank accession no. NM_000237 exon5, p.Arg197His, rs372668179). These genes

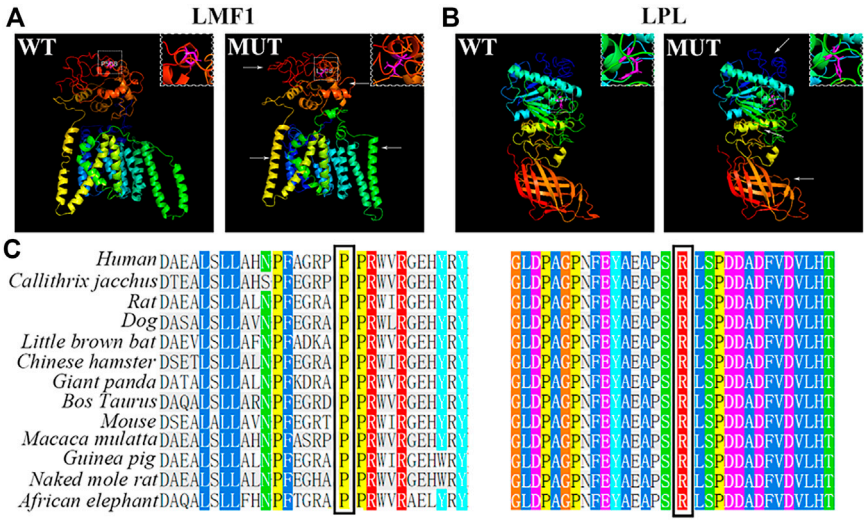

FIGURE 2 | Bioinformatics analysis of the mutation. **(A)**. Prediction of wild-type (left) and mutant (right) protein structures of LMF1 by I-TASSER. **(B)**. I-TASSER prediction of wild-type (left) and mutant (right) protein structures of LPL. **(C)**. Sequence alignment of mutant amino acids on LMF1 (P508) or LPL (R197) across different species. White box: the mutated position; Arrows: changes compared to the wild-type structure; Magenta: mutant amino acids.

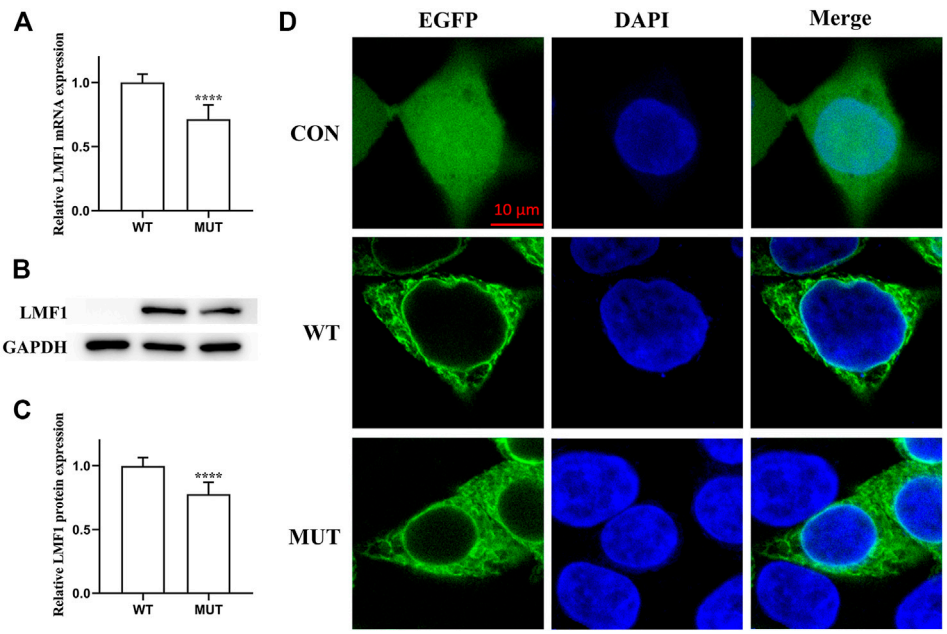

FIGURE 3 | Functional analysis of the LMF1 mutant protein. **(A)**. mRNA expression levels of wild-type and mutant LMF1 genes in HEK293T cells. There was a significant difference in the mRNA level of the wild-type and the mutant genes (****$p < 0.0001$). **(B)**. Western blotting of LMF1 expression. **(C)**. Protein expression levels of wild-type and mutant LMF1 genes in HEK293T cells. The mutant LMF1 protein expression was lower than that of the wild-type proteins in HEK293T cells (****$p < 0.0001$). **(D)**. Subcellular localization of wild-type and mutant LMF1 in HEK293T cells.

regulate TG-rich lipoprotein lipolysis. These two variants were classified as uncertain clinical significance, PM2, PP2 and PP3 for *LPL* c.590G > A, and PM2, PP3 for *LMF1* c.1523C > T. The genotype of the proband and his five family members is shown in **Figure 1B**. The proband (II4), his father (I1), his brother (II2), his son (III4), and his daughter (III3) carried the c.1523C > T missense mutation, while the proband (II4) and his mother (I2) carried the c.590G > A missense mutation (**Figures 1C,D**).

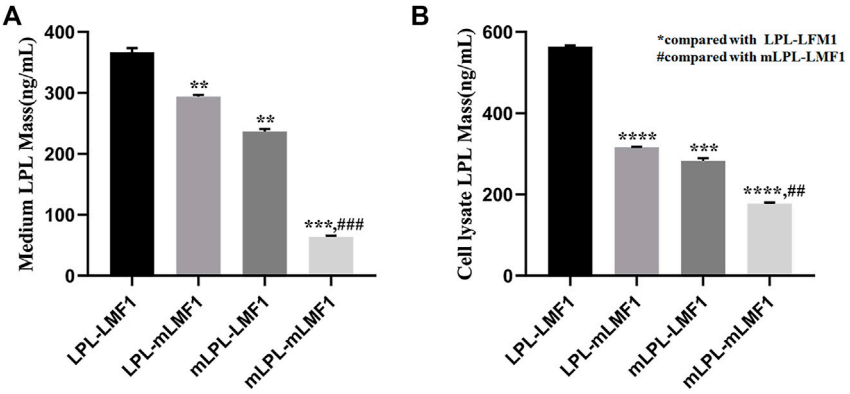

FIGURE 4 | LMF1 and LPL double mutants significantly decreased LPL levels. **(A)**. The *LMF1* and *LPL* gene mutation both caused decrease of LPL levels in the cell medium. The combination of *LMF1* and *LPL* gene mutation significantly decreased LPL mass in the cell medium (*compared to LPL-LMF1, #compared to mLPL-LMF1; ****$p < 0.0001$, **$p < 0.01$). **(B)**. The *LMF1* and *LPL* gene mutation caused decrease of LPL mass in the cell lysate. The combination of *LMF1* and *LPL* gene mutation significantly decreased LPL mass in the cell lysate (*compared to LPL-LMF1, #compared to mLPL-LMF1; ##$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Functional Analysis of LMF1 and LPL

The conservation analysis of the *LMF1* and *LPL* gene loci, P508 and R197, respectively, showed that the two mutations were located in evolutionary conserved regions. As shown in **Figure 2C**, the proline amino acid at LMF1 position 508, and arginine amino acid at LPL position 197, are consistent across different species. Additionally, I-TASSER indicated that the *LMF1* c.1523C > T mutation and the *LPL* c.590G > A mutation both altered the tertiary structure of the protein (**Figures 2A,B**). The helix changed into coil in the mutant position. Changes in other position of the structure were also found. The cellular localization of the LMF1 protein was analyzed in HEK293T cells transfected with wild-type and mutant constructs. The proteins were both localized in the cytoplasm and no differences were observed in the subcellular localization between the mutant and wild-type proteins (**Figure 3D**). Moreover, we found significant differences in the levels of LMF1 mRNA and protein expression using wild-type and mutant constructs in HEK293T cells. As shown in **Figures 3A–C**, mutant LMF1 mRNA and protein expression were both lower than that of the wild-type in HEK293T cells.

LMF1 and *LPL* Double Mutants Significantly Decreased LPL Levels

To evaluate the effect of the *LMF1* mutations, the level of LPL in HEK293T cells transfected with plasmids carrying wild-type, mutant *LPL* and *LMF1* genes were measured. As shown in **Figures 4A,B**, these *LMF1* and *LPL* mutations both caused a decreased LPL level in the cell medium and cell lysates compared to the wild-type LPL concentrations. Moreover, *LMF1* and *LPL* double mutants (mLPL-mLMF1) significantly decreased LPL level in comparison to the decrease observed for the *LMF1* (c.1523C > T) or *LPL* (c.590G > A) mutation homozygote (LPL-mLMF1 or mLPL-LMF1).

DISCUSSION

In this study, a heterozygous *LMF1* missense mutation (c.1523C > T) and a heterozygous *LPL* missense mutation (c.590G > A) were identified in the proband who presented with severe HTG using WES. The mutations were confirmed in the proband and his family members by Sanger sequencing. Within the family, there are four *LPL* c.590G > A heterozygous carriers, one *LMF1* c.1523C > T heterozygous carriers, and one double *LPL/LFM1* heterozygote. Except the proband's daughter, the *LPL* c.590G > A heterozygous carriers were diagnosed with HTG. The proband who carried double *LPL/LFM1* heterozygote had extremely high TG levels. The heterozygous mutation was first reported in a male Caucasian patient with severe HTG by Wright in 2008 (Wright et al., 2008). Surendran and Rabacchi subsequently also identified this mutation in HTG patients (Surendran et al., 2012; Rabacchi et al., 2015). These studies merely mention the *LPL*-associated c.590G > A mutation found in HTG patients. In this study, it was demonstrated that the c.590G > A mutation in the *LPL* gene could result in a decreased LPL mass in the cell medium and the cell lysates. Compared clinical characteristics of patients carried *LPL* c.590G > A mutation, age and TG level were in a big different, also the history of pancreatitis were different. The proband's daughter, who carries the c.590G > A mutation, presented with normal TG levels. Nevertheless, the regulation of triglyceride is a complex process. Since her LPL levels were not measured, there is a possibility that she carried a different mutation, which could also increase the LPL activity. It was reported that the p.Thr143Met mutation, which could increase the activity of LPL, was identified in a patient with Familial hyperchylomicronemia (Plengpanich et al., 2020).

The c. 1523G > A missense mutation in the *LMF1* gene has not previously been linked to HTG. Moreover, the allele frequency of the *LMF1* c. 1523C > T variant in the ExAC and gnomAD databases for the East Asian population was 0.0001 and 5.831e-

05, respectively. In short, the *LMF1* c. 1523C > T variant is extremely rare in populations when the allele frequency less than 0.1%.

Deficiency of LMF1 was identified in a cld mouse model characterized with a progressive increase in triglycerides that resulted in death 2–3 days after birth (Péterfy et al., 2007). Thus far, most mutations in the *LMF1* gene are heterozygous mutations found in HTG patients, but there is a lack of empirical evidence to support their pathogenicity. Only three rare nonsense variants, p.Tyr439Ter, p.Tyr460Ter, and p.Trp464Ter, have been reported on both alleles of the *LMF1* gene in HTG cases (Péterfy et al., 2007; Cefalù et al., 2009; Cefalù et al., 2017). The first two homozygous *LMF1* truncating mutations have previously been experimentally demonstrated to impact LMF1 activity. To date, p.Ser137Leu was identified and reported as the first missense mutation affecting LMF1 function (Péterfy et al., 2018). This variant severely diminishes the expression of mutant LMF1 and dramatically reduces the specific activity of LMF1. The c.1523C > T mutation in our study also decreased the expression of LMF1 mRNA and protein. *LMF1* and *LPL* double mutants also significantly decreased LPL levels. These results are consistent with the increased severity of HTG in the double heterozygous patient, but the fact that the model simulates double homozygosity and the patient is a double heterozygote may be considered a limitation. Thus, it is reasonable to infer that this mutation affects the expression of LMF1 protein through its transcription. We could not exclude the possibility that this mutation diminishes the expression of mutant *LMF1* by reducing its stability and promoting the turnover of the protein.

The human *LMF1* gene encodes a 567 amino acid protein that contains five transmembrane domains and an evolutionarily conserved domain. This domain constitutes most of its C-terminal end, which is important for the function of LMF1 protein. The c.1523C > T mutation was located in the region recognized as being a domain of unknown function 1,222 (DUF1222). This region is found in over 50 proteins and covers a wide taxonomic range from bacteria to humans. The nonsense mutations that were recognized to be pathogenic, such as p.Tyr439Ter, p.Tyr460Ter, and p.Trp464Ter, all resulted in the large fragment deletion at the C-terminal end of the *LMF1* gene. Moreover, the activity of LPL is associated with the length of the remaining C-terminal end. Compared to patients carrying the Y439X mutation in the *LMF1* gene who lacked LPL activity, the patients carrying W464X retained 40% LPL activity. The c.1523C > T mutation in our study was also located in the C-terminal end of the LMF1 protein. This was predicted to change the tertiary structure of the protein, resulting in a defect of its function in LPL maturation. At present, the specific mechanism responsible for this is still unclear. Besides the proband, this mutation was also found in his mother who had normal TG levels. The probable explanation is that additional proteins are involved in the maturation and secretion of LPL, and partially compensate for the function of LMF1. SEL1L also plays an important role in the maturation and secretion of active LPL. In the absence of SEL1L, LPL remains and aggregates in the endoplasmic reticulum, and is then degraded through autophagy (Sha et al., 2014).

Metformin increases the expression of LMF1 in the heart, suggesting that the mechanism of metformin in reducing TG may be associated with LMF1. Contrarily, fenofibrate has no effect on the expression of LMF1 protein (Forcheron et al., 2009). The proband did not respond well to the lipid-lowering fenofibrate therapy; plasma TG levels did not return to normal. This may have resulted from the deficiency in the expression of the LMF1 protein that could not have been compensated for by fenofibrate.

Besides affecting the TG levels through LPL, the LMF1 protein also influences cholesterol concentrations in plasma. The proband's mother, who carried the *LMF1* gene mutation, had TG values in the normal range but presented with hypercholesterolemia. The possible mechanism by which this happens is that LMF1 can regulate the redox homeostasis in the endoplasmic reticulum, thereby affecting not only the proper folding of LPL, but also the formation of disulfide bonds necessary for other secretory proteins such as fibronectin and LDLR (Roberts et al., 2018). Therefore, the decrease in LDLR levels leads to the deficiency in the ability to clear cholesterol in plasma, thereby increasing the plasma cholesterol level.

CONCLUSION

In summary, A missense mutation (c.590G > A) in exon 5 of the *LPL* gene and a missense mutation (c.1523C > T) in exon 10 of the *LMF1* gene were identified in a severe case of HTG. Both the mutations reduced the mass of LPL. The combination of *LMF1* and *LPL* gene mutations significantly decreased LPL mass, which contributes to severe hypertriglyceridemia. Our study underscores the complexity of hypertriglyceridemia and the need for the combination of extensive molecular genetic testing and clinical characterization; in addition, expands the spectrum of *LMF1* mutations. Finally, it helps the family members prevent and treat the disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Nanfang Hospital Ethics Committee. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

DG and HZ were responsible for the initial plan, study design, statistical analysis, and the conduct of the study.

DG, YZ, ZG, YG, and SJ were responsible for experimentation and data collection. DG.and YZ were responsible for data extraction and data interpretation. DG and YZ wrote the manuscript's draft and FX and FY were responsible for the manuscript review. FX and HZ was the guarantor for the present paper and has full responsibility for the present study.

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Exome Sequencing Data Analysis and a Case-Control Study in Mexican Population Reveals Lipid Trait Associations of New and Known Genetic Variants in Dyslipidemia-Associated Loci

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Background: Plasma lipid levels are a major risk factor for cardiovascular diseases. Although international efforts have identified a group of loci associated with the risk of dyslipidemia, Latin American populations have been underrepresented in these studies.

Objective: To know the genetic variation occurring in lipid-related loci in the Mexican population and its association with dyslipidemia.

Methods: We searched for single-nucleotide variants in 177 lipid candidate genes using previously published exome sequencing data from 2838 Mexican individuals belonging to three different cohorts. With the extracted variants, we performed a case-control study. Logistic regression and quantitative trait analyses were implemented in PLINK software. We used an LD pruning using a 50-kb sliding window size, a 5-kb window step size and a r^2 threshold of 0.1.

Results: Among the 34251 biallelic variants identified in our sample population, 33% showed low frequency. For case-control study, we selected 2521 variants based on a minor allele frequency ≥1% in all datasets. We found 19 variants in 9 genes significantly associated with at least one lipid trait, with the most significant associations found in the *APOA1/C3/A4/A5-ZPR1-BUD13* gene cluster on chromosome 11. Notably, all 11 variants associated with hypertriglyceridemia were within this cluster; whereas variants associated with hypercholesterolemia were located at chromosome 2 and 19, and for low high density lipoprotein cholesterol were in chromosomes 9, 11, and 19. No significant associated variants were found for low density lipoprotein. We found several novel variants associated with

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different lipemic traits: rs3825041 in *BUD13* with hypertriglyceridemia, rs7252453 in *CILP2* with decreased risk to hypercholesterolemia and rs11076176 in *CETP* with increased risk to low high density lipoprotein cholesterol.

Conclusions: We identified novel variants in lipid-regulation candidate genes in the Mexican population, an underrepresented population in genomic studies, demonstrating the necessity of more genomic studies on multi-ethnic populations to gain a deeper understanding of the genetic structure of the lipemic traits.

Keywords: association study, genetic variants, dyslipidemia, exome analysis, mexican population

INTRODUCTION

Currently, metabolic diseases have become one of the most challenging health concerns worldwide; they account for nearly 45% of all deaths worldwide (Forouzanfar et al., 2016). Prevention and management of these diseases are complicated, due to their long latency periods, numerous risk factors, and the presence of co-morbidities (Sevick et al., 2007). Among the known metabolic diseases, dyslipidemias comprise a well-established risk factor for cardiovascular diseases, which are the leading cause of death worldwide (Libby, 2002).

Although factors like diet and lifestyle (Wietlisbach et al., 1997) are recognized as important determinants in the clinical development of dyslipidemias, these diseases have also a strong genetic component (Gao et al., 2018). For instance, lipid disorders are highly prevalent in populations with Amerindian ancestry (up to 85.9%), compared with the prevalence among individuals from other ancestries (e.g., Caucasian 31.2% and African 41.1%) (Aguilar-Salinas et al., 2014; Noubiap et al., 2018). In addition, a large number of genome-wide association studies (GWAS) performed in populations of different ancestries have described more than 175 loci associated with dysregulated levels of plasma lipids. However, the largest body of information generated to date has relied mostly on evidence from Caucasian and Asian cohorts, with very few studies analyzing Latin American populations (Teslovich et al., 2010; Asselbergs et al., 2012; Willer et al., 2013; Wu et al., 2013; Surakka et al., 2015).

Recent findings have demonstrated that genetic factors associated with metabolic traits, including dyslipidemias, exhibited significant heterogeneity in allele frequency and in variant effects across groups with different ancestries (Klarin et al., 2018; Martagón et al., 2018). A study analyzing populations from multiple ethnicities has found important differences in the levels of association, allele frequencies, and haplotype distributions of several lipid loci (Wu et al., 2013). Several population-specific signals at these loci have been reported in non-European populations. For example, the association between the regulatory variant, rs12740374, in the *CELSR2/PSRC1/SORT1* locus, and low-density lipoprotein cholesterol (LDL-C) is higher in African-American individuals than in individuals of European descent (Buyske et al., 2012). Furthermore, the R230C variant of the *ABCA1* gene, which is associated with low levels of high-density lipoprotein cholesterol (HDL-C), is private to individuals with Amerindian ancestry (Acuña-Alonzo et al., 2010). Thus, the distribution of variants located in lipid-related genes might vary between populations of different ethnicities.

The modern Mexican population is mainly composed of Mestizo individuals, who are the result of the recent admixture of original Amerindians, Europeans (mainly Spaniards) and, to a lesser extent, sub-Saharan Africans (Moreno-Estrada et al., 2014). The recent and complex admixture in the Mexican Mestizo population might have produced a high level of genetic heterogeneity in variants in lipid-related loci. Therefore, this study aimed to determine the frequency distribution of variants located at genes related to lipid traits in Mexican individuals, followed by an association testing and the identification of potential variants for lipemic traits, using previously published exome sequencing data (Estrada et al., 2014; García-Ortíz et al., 2021).

MATERIAL AND METHODS

Study Populations

This study included 2838 Mexican individuals belonging to the Mestizo cohorts Diabetes in Mexico Study (DMS, n = 968) and Mexico City Diabetes Study (MCDS, n = 796), published previously as part of the Slim Initiative in Genomic Medicine for the Americas (SIGMA) Type 2 Diabetes Consortium (Estrada et al., 2014), as well as to the indigenous cohort Metabolic Analysis in an Indigenous Sample (MAIS, n = 1074) (García-Ortíz et al., 2021). The sample design was previously described in Estrada et al. and García-Ortiz et al. (Estrada et al., 2014; García-Ortíz et al., 2021). The MAIS sample belongs to 71 indigenous communities representing 60 ethnic groups from 10 linguistic families. All participants were unrelated volunteers and provided signed informed consent. This investigation was approved by the local ethics and research committees from the National Institute of Genomic Medicine and was conducted according to the principles of the Declaration of Helsinki. The genetic structure of DMS and MCDS population was previously described, with a mean (SD) proportion of Amerindian ancestry of 66 ± 17%, whereas the proportion of Amerindian ancestry in the MAIS cohort was of 93.2 ± 8.7% (Estrada et al., 2014; García-Ortíz et al., 2021).

Lipid Measurements in Plasma

Levels of triglycerides (TG), total cholesterol (TC), and HDL-C were measured from blood samples collected after overnight

fasting, using a Synchron CX5 Analyzer System (Beckman Coulter Fullerton, CA, United States). LDL-C values were calculated with the Friedewald formula, excluding those samples with TG > 400 mg/dl (Warnick et al., 1990).

Each lipid disorder was diagnosed according to the American Heart Association and National Heart, Lung, and Blood Institute guidelines (AHA/NHLBI; http://www.nhlbi.nih.gov). An individual was diagnosed with a lipid disorder when serum levels showed any of the following: TG ≥ 150 mg/dl (hypertriglyceridemia; HTG), TC ≥ 200 mg/dl (hypercholesterolemia; HTC), LDL-C ≥130 mg/dl (elevated LDL), or HDL-C ≤50 mg/dl in females or ≤40 mg/dl in males (low HDL-C). Individuals with desirable lipid values were assigned as controls. Data on lipid-lowering medications were available for over 80% of the participants and adjustment was done for TG and TC binary phenotypes.

Dataset Building and Single-Nucleotide Variant Annotations

First, we analyzed the Variant Call Format (VCF) files previously obtained from all participants. Quality controls for whole-exome sequencing (e.g., read depth, mean coverage, and missing rate) were described previously (Estrada et al., 2014). From these files, we extracted all the biallelic variants (mapped in the Genome Reference Consortium Human genome build 37) of 177 candidate genes for any lipid trait (**Supplementary Table S1**). These genes were selected from: 1) the Global Lipid Genetics Consortium (n = 165) (Willer et al., 2013), 2) meta-analysis studies (n = 10) (Teslovich et al., 2010; Weissglas-Volkov and Pajukanta, 2010), and 3) re-sequencing and clinical exome studies (n = 2) (Estrada et al., 2014; Williams-Amy et al., 2014). We annotated the variants with the ENSEMBL Variant Effect Predictor (https://www.ensembl.org/info/docs/tools/vep/index.html; version 87) (McLaren et al., 2016). Finally, we constructed PLINK files that comprised clinical-demographic and genotyping data for the association analyses, which employed VCF tools (v0.1.12b) (Danecek et al., 2011).

Statistical Analysis

A case-control study was conducted in each cohort to identify low-frequency SNVs [minor allele frequency (MAF) = 0.01–0.05] and common SNVs (MAF >0.05) associated with lipid traits. To analyze associations between lipid components and alleles we estimated the odds ratio (OR) using logistic regression. We performed quantitative trait analyses with linear regression (Beta value). Both methods were performed using an additive model, adjusting by age, sex and the first 10 eigenvectors from the principal component analysis as covariates. All analyses were performed using PLINK v.1.9 software (Purcell et al., 2007).

Next, we performed a fixed effects model meta-analysis for each trait using a weighted inverse variance model in the software package METAL (Willer et al., 2010). Also, genomic control was applied to each study within METAL by adjusting for the genomic inflation factor prior to meta-analysis, to correct for possible residual population stratification.

Significant threshold was determined following the approach by Kanai et al. (Kanai et al., 2016). According to this approach an LD pruning was done with PLINK v.1.9 using a 50-kb sliding window size, a 5-kb window step size and a r^2 threshold of 0.1. According with this, a total of 780 independent SNVs were identified giving a significant threshold of 6.4×10^{-5}.

Significant threshold was determined following the approach by Kanai et al. (Kanai et al., 2016). According to this approach, high LD SNVs in 177 candidate lipids genes were filtered based on LD pruning performed in PLINK v.1.9 using a 50-kb sliding window size, a 5-kb window step size and a r^2 threshold of 0.1. According with this, a total of 780 independent SNVs were identified that were used to establishing the genome-wide significant threshold *via* a straight-forward Bonferroni correction $p < (6.4 \times 10^{-5})$.

RESULTS

Study Participants

Our study population comprised 2838 individuals belonging to three different cohorts, the Diabetes in Mexico Study (DMS), the Mexico City Diabetes Study (MCDS) and the indigenous cohort Metabolic Analysis in an Indigenous Sample (MAIS) (**Table 1**). The DMS sample was composed of 968 individuals: 681 females (70.3%) and 287 males (29.7%). The mean age of the participants was 54.1 ± 9.8 years and the mean body mass index BMI was 28.4 ± 4.9 kg/m^2. According to the AHA/NHLBI cutoff points, the lipid trait with the highest prevalence in this sample was low HDL-C (70.8%), followed by HTC (65.1%), HTG (54.9%), and high LDL-C (37%). The MCDS sample consisted of 796 individuals: 482 females (60.6%) and 314 males (39.4%) with a mean age of 62.9 ± 7.6 years and a mean BMI of 29.5 ± 4.9 kg/m^2. The most frequent lipid alteration in this population was low HDL-C (82.8%), followed by HTG (58.8%), HTC (44.1%), and high LDL-C (40.0%). The MAIS sample was composed of 1074 individuals: 679 females (63.2%) and 395 males (36.8%) with a mean age of 58.6 ± 12.1 years and a mean BMI of 27.5 ± 5.0 kg/m^2. Similar to the two previous cohorts, low HDL-C (75.2%) was the most common lipid trait, followed by HTG (66.8%), HTC (29.7%) and high LDL-C (14.9%) (**Table 1**).

Analysis of the Variation in Candidate Dyslipidemia-Related Genes

After analyzing the exome data from the three analyzed cohorts, we found a total of 34251 biallelic variants with a MAF above 1% within the 177 genes previously related to dyslipidemia. Among these variants, 33% were detected at low frequency (MAF = 0.01–0.05), and 67% were common (MAF >0.05) (**Figure 1A**). According to their position in the gene, 53% of the variants were in coding regions, and the remaining 47% were found in non-coding regions (**Figure 1B**). According to the predicted annotation obtained with the Variant Effect Predictor web tool (https://grch37.ensembl.org/Tools/VEP) 61% of the variants found in the coding regions were synonymous, 37.2% were non-synonymous, 0.2% were stop-gain and 1.6% were unknown (**Figure 1C**).

TABLE 1 | Clinical and demographic characteristics of the studied cohort.

Characteristic		DMS	MCDS	MAIS
		n = 968	*n* = 796	*n* = 1074
Women/Men (%)		70.3/29.7	60.6/39.4	63.2/36.8
Age (Years)		54.1 ± 9.8	62.9 ± 7.6	58.6 ± 12.1
BMI (kg/m^2)		28.4 ± 4.9	29.5 ± 4.9	27.5 ± 5.0
FG (mg/dl)		125.2 ± 61.4	114.3 ± 54.2	114.2 ± 63.9
HTG (%)		54.9	58.8	66.8
Mean TG (mg/dl)		203.7 ± 129.3	176.3 ± 96.3	207.5 ± 119.5
HTC (%)		65.1	44.1	29.7
Mean TC (mg/dl)		201.1 ± 43.8	195.9 ± 36.5	182.8 ± 38.3
Low HDL-C (%)		70.8	82.8	75.2
Mean HDL-C (mg/dl)	Women	39.7 ± 12.1	31.5 ± 8.5	40.6 ± 12.6
	Men	37.4 ± 13.0	28.6 ± 13.4	38.7 ± 12.6
High LDL-C (%)		37	40.0	14.9
Mean [1]LDL-C (mg/dl)		120.7 ± 35.3	131.5 ± 33.6	103.7 ± 29.5

Data are presented as the mean ± SD, or the percentage, as indicated. BMI, body mass index; FG, fasting glucose; HTG, hypertriglyceridemia; TG, triglycerides; HTC, hypercholesterolemia; TC, total cholesterol; HDL-C, high density lipoprotein; LDL-C, Low density lipoprotein. [1]LDL-C values were calculated with the Friedewald equation.

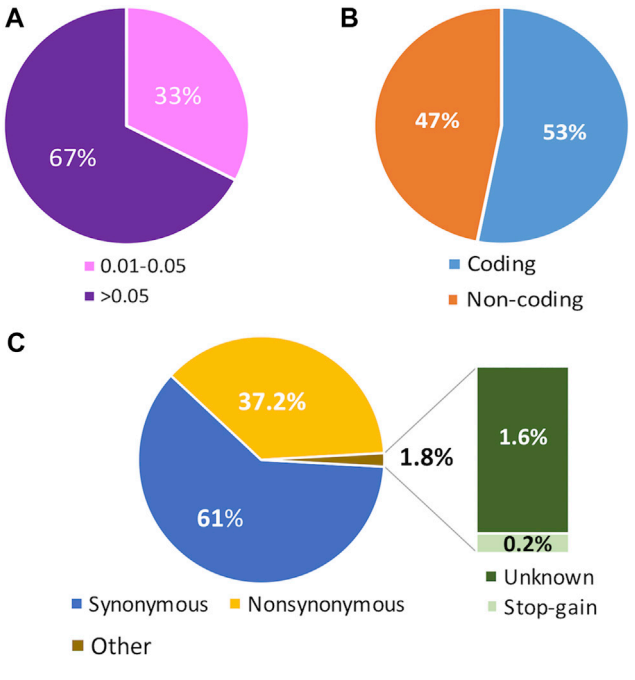

FIGURE 1 | Features of the analyzed gene variant set. **(A)** Cumulative variant distribution according to the minor allele frequency (MAF). Rare variants include Singletons, Doubletons, and variants with MAFs <0.01. Low Frequency variants include variants with MAFs = 0.01–0.05. Common variants are those with MAFs >0.05. **(B)** Distribution of coding and non-coding annotations, according to the Variant Effect Predictor tool (VEP) in the entire set of variants.

Low-Frequency and Common Variant Association Study

For the case-control study we used a meta-analysis approach to evaluate low-frequency and common variants pruned for LD (MAF >0.01; n = 780). We explored associations between individual variants and each different lipid phenotype with binary logistic regression. Quantile-quantile (QQ) plots for each analyzed phenotype showed a genomic inflation factor for HTG, HTC, low HDL-C and high LDL-C of λ = 1.0, 1.02, 1.03, and 0.91 respectively (**Supplementary Figure S1**). A total of 19 variants in 9 genes achieved a $p < 6.4 \times 10^{-5}$ or higher significant association with at least one of the analyzed lipid components (**Figure 2**). Remarkably, the 11 variants that were associated with HTG were found on chromosome 11, located in *BUD13*, *APOA5*, *APOC3*, *APOA1*, and *APOA4* genes (**Supplementary Table S2**). In the case of HTC, associated variants were found on chromosome 2 and 19, in *APOB* and *CILP2* genes; whereas in the case of low HDL-C, two of the associated variants were found on chromosome 11, in *BUD13* gene, four on chromosome 16 in *CETP* and one on chromosome 9 in *ABCA1* (**Supplementary Table S2**). We did not find any variant associated with high LDL-C.

The 11 variants associated with HTG were all located in the *APOA1/C3/A4/A5-ZPR1-BUD13* gene cluster (**Figure 2**; **Supplementary Table S2**). The strongest signal for the variants associated with increased risk to HTG was detected for rs3825041 in *BUD13* (OR = 1.53, $p = 7.55 \times 10^{-9}$; β = 22.24 mg/dl) (**Figure 3**; **Supplementary Table S2**), followed by rs651821 (OR = 1.48, $p = 5.70 \times 10^{-8}$; β = 21.40 mg/dl) and rs2072560 in *APOA5* (OR = 1.48, $p = 6.18 \times 10^{-8}$; β = 21.65 mg/dl), rs2070665 in *APOA1* (OR = 1.41, $p = 3.79 \times 10^{-7}$; β = 17.71 mg/dl), rs5128 in *APOC3* (OR = 1.40, $p = 4.22 \times 10^{-7}$; β = 18.31 mg/dl), rs5104 (OR = 1.34, $p = 6.1 \times 10^{-6}$; β = 19.61 mg/dl) and rs5092 in *APOA4* (OR = 1.33, $p = 8.07 \times 10^{-6}$; β = 18.97 mg/dl) and rs11820589 in *BUD13* (OR = 1.35, $p = 5.71 \times 10^{-5}$; β = 21.92 mg/dl). In contrast, the variants rs4520 in *APOC3* (OR = 0.74, $p = 2.96 \times 10^{-7}$; β = −19.03 mg/dl) rs5070 in *APOA1* (OR = 0.78, $p = 4.27 \times 10^{-5}$; β = −15.75 mg/dl) and rs10488698 in *BUD13* (OR = 0.73, $p = 5.80 \times 10^{-5}$; β = −20.98 mg/dl) were associated with protection against HTG. All 11 variants were also significantly associated with TG levels in the quantitative trait analysis (**Supplementary Figure S2**; **Supplementary Table S2**).

Moreover, the variant rs1367117 in *APOB* (OR = 1.36, $p = 1.88 \times 10^{-6}$; β = 4.89) was significantly associated with HTC and with a

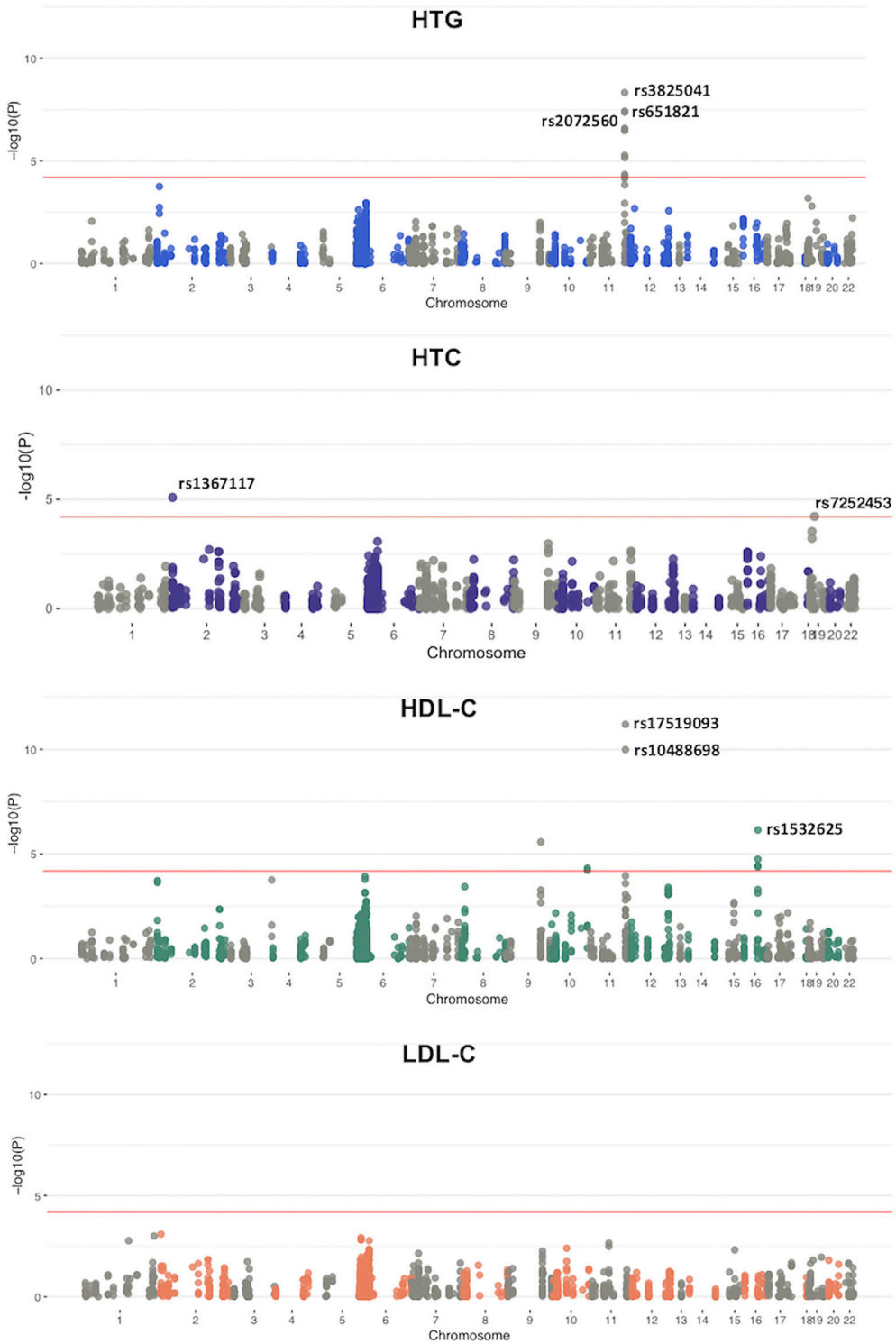

FIGURE 2 | Manhattan plots from a meta-analysis based on the genome-wide association analysis. Panels show the –log *p* value for SNVs for each lipemic traits. Top hits for each trait are indicated in the figure. Red line indicates the significant threshold line: $p = 6.4 \times 10^{-5}$.

significant increase in serum TC levels in the quantitative trait analysis (**Figure 3**; **Supplementary Table S2**). On the other hand, the variant rs7252453 in *CILP2* (OR = 0.73, $p = 4.40 \times 10^{-5}$; β = −5.17) was significantly associated with protection against HTC (**Supplementary Figure S3**; **Supplementary Table S2**). Regarding low HDL-C, the variants rs17519093 and

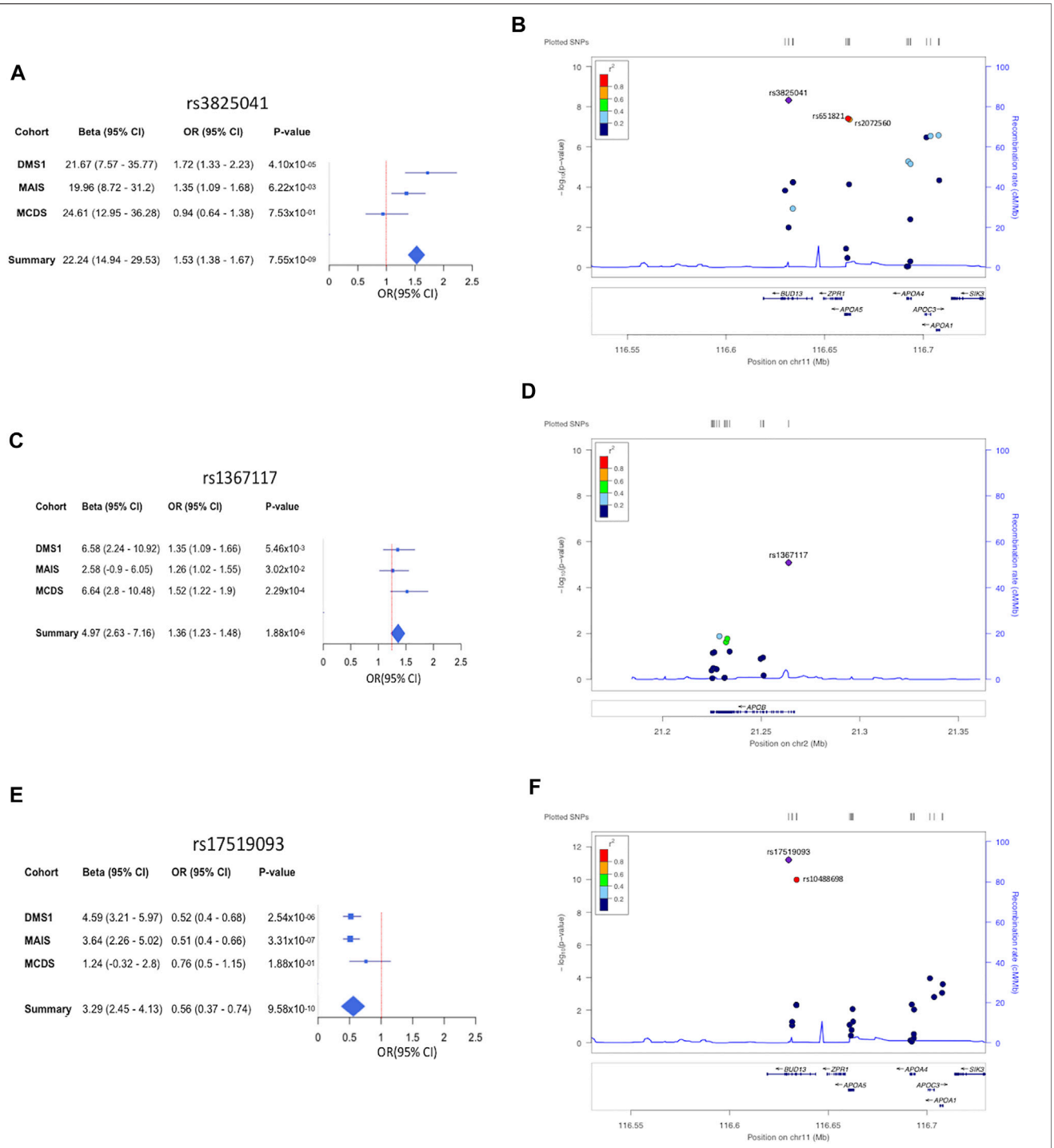

FIGURE 3 | Forest and regional plots showing the top hits associated with lipemic traits. Forest plot showing odds ratio estimates and 95% confidence intervals (squared boxes) from the 3 cohorts DMS, MCDS, and MAIS, included in the study in HTG **(A)**, HTC **(C)**, and low HDL-C **(E)**. Odds ratios for the meta-analyses are represented with a diamond. Regional plot of associated SNVs in HTG **(B)**, HTC **(D)**, and low HDL-C **(F)**.

rs10488698 in *BUD13* (OR = 0.56, $p = 9.58 \times 10^{-10}$, β = 3.29; OR = 0.58, $p = 7.34 \times 10^{-9}$; β = 3.12, respectively) and rs1532625 in *CETP* (OR = 0.72, $p = 6.56 \times 10^{-6}$; β = 2.21) were significantly associated with decreased risk in both the binary and the quantitative trait analysis (**Figure 3**; **Supplementary Figure S4**; **Supplementary Table S2**). Finally, the variants rs708272, rs11076176, and rs289714 in *CETP* (OR = 1.32, $p = 1.75 \times 10^{-5}$, β = −2.12; OR = 1.32, $p = 3.64 \times 10^{-5}$, β = −1.83; OR = 1.32, $p = 4.03$

$\times 10^{-5}$, $\beta = -1.75$, respectively) as well as rs9282541 in *ABCA1* (OR = 1.67, $p = 3.34 \times 10^{-5}$; $\beta = -2.50$) showed a significant association with increased risk to low HDL-C in both the binary and the quantitative trait analysis (**Supplementary Figure S4**; **Supplementary Table S2**).

DISCUSSION

The contribution of genetic variation to human diseases is widely recognized (Xue et al., 2012). In particular, loss-of-function or modifier variants are usually related to changes in the biological activity of the corresponding gene. Several genomic regions are currently recognized as drivers of dyslipidemias in populations from several ethnicities, although in some populations, such as those of Amerindian or African origin, genomic studies have been scarce. Furthermore, many of these loci have displayed highly different associations with these entities across different ancestries (Lek et al., 2016; Martin et al., 2017). Therefore, lipid-associated genes should be analyzed in all populations.

Here, by analyzing SNVs within 177 candidate genes, we found several associations with different types of dyslipidemias in Mexican Mestizos, an admixed population with strong Amerindian (51%) and European (46%) components (Norris et al., 2018).

In our population we were able to replicate several associations with different lipemic traits previously reported in other ethnicities. For example, variants in the gene cluster *APOA1/C3/A4/A5-ZPR1-BUD13*, such as the regulatory SNVs rs5128 in *APOC3*, and rs651821 in *APOA5*, as well as the missense SNV rs2072560 also within *APOA5*, and the intronic variant rs2070665 in *APOA1*, were all associated with HTG in our population, as they are in European, Asian, and African populations (Feng et al., 2016; Fu et al., 2015; Jasim et al., 2018; Ken-Dror et al., 2010; Song et al., 2015; Zhou et al., 2013). Likewise, the association of the missense SNV rs1367117 in *APOB* with HTC has also been reported in populations of European ancestry (Lu et al., 2010), whereas the association of the missense SNVs rs10488698 in *BUD13* and rs9282541 in *ABCA1* with low HDL-C has also been observed in Asian and Latino American populations, respectively (Zhang et al., 2017; Acuña-Alonso et al., 2010). We also observed novel lipemic trait–associated variants in *BUD13*, such as rs3825041 and rs17519093, both localized within introns, associated with HTG and high levels of HDL-C, respectively. Using public data contained in the Common Metabolic Diseases Knowledge Portal (https://t2d.hugeamp.org/), we were able to confirm the associations of rs3825041 and rs17519093 with different lipemic traits. Thus, one of the genomic regions most consistently associated with lipid traits in human populations of diverse ethnic origins is the cluster *APOA1/C3/A4/A5-ZPR1-BUD13* (Teslovich et al., 2010; Willer et al., 2013; Parra et al., 2017; Bai et al., 2019). Importantly, all 11 variants associated with HTG in our study, were in this cluster. Among them, the novel variant rs3825041 in *BUD13* showed the strongest association. Notably, this variant showed high LD with rs651821 ($r^2 = 0.84$) and rs2072560 ($r^2 = 0.89$) within *APOA5*, which have both been previously reported as associated with HTG in several populations of different ancestries, including in Mexicans (Ken-Dror et al., 2010; Kim et al., 2019). Taken together, these data provide more insights about the variants at the *APOA1/C3/A4/A5-ZPR1-BUD13* gene cluster as a relevant risk factor for dyslipidemias such as HTG and low HDL-C, and highlight the notion that these could be biomarkers for susceptibility to these traits.

Others novel association signals were observed with the synonymous SNV rs7252453 in *CILP2* and decreased risk to HTC and the intronic SNV rs11076176 in *CETP* and increased risk to low HDL-C serum levels. On *CETP* we also observed an association of the intronic variants rs708272 and rs289714 with high risk to low HDL-C, as well as the association of the intronic variant rs1532625 with protection to this dyslipidemia. These findings are in line with those reported previously in Mexican individuals (Vargas-Alarcon et al., 2018), in a meta-analysis involving six independent Hispanic cohorts (Gao et al., 2018) and in Chinese population (Guo et al., 2015).

In summary, despite our selecting candidate genes were previously associated with dyslipidemia in other populations, we were able to find additional variants showing the strongest associations with lipid traits in Mexican individuals. These differences support the notion that high allelic heterogeneity exists in lipid loci across populations. Remarkably, the new associations of variants in genes previously related to dyslipidemia, points out the importance of studying different ethnicities, since different associated variants within the same genes could be particular to one or another population ancestry. It is also worth to note that several of the risk variants previously associated with lipemic traits in different ethnic groups, including European, Asian and African populations were replicated in our study. Taken together, our results suggest that genetic architecture of dyslipidemias is partially share among different ethnic groups.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the European Variation Archive repository (EVA https://www.ebi.ac.uk/eva/), accession number PRJEB52611.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local ethics and research committees from the National Institute of Genomic Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHORS CONTRIBUTIONS

LO and EC contributed to the conception and design of the work. PJ-C, FB-O, HG-O, and MC-S performed the bioinformatic

analyses for variant calling. PB-P, AM-H, MG-V, and CG-V contributed in acquisition of data. FC-C, CC-C, YS-A, GS-M, and EM-C participated in the association studies. PJ-C, LO, and EC wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.807381/full#supplementary-material

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The Genetic Spectrum of Familial Hypertriglyceridemia in Oman

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Familial hypertriglyceridemia (F-HTG) is an autosomal disorder that causes severe elevation of serum triglyceride levels. It is caused by genetic alterations in *LPL*, *APOC2*, *APOA5*, *LMF1*, and *GPIHBP1* genes. The mutation spectrum of F-HTG in Arabic populations is limited. Here, we report the genetic spectrum of six families of F-HTG of Arab ancestry in Oman. Methods: six Omani families affected with triglyceride levels >11.2 mmol/L were included in this study. Ampli-Seq sequencing of the selected gene panels was performed. Whole-exome sequencing and copy number variant analysis were also performed in cases with negative exome results. Three novel pathogenic missense variants in the *LPL* gene were identified, p.M328T, p.H229L, and p.S286G, along with a novel splice variant c.1322+15T > G. The *LPL* p.H229L variant existed in double heterozygous mutation with the *APOA5* gene p.V153M variant. One family had a homozygous mutation in the *LMF1* gene (c.G107A; p.G36D) and a heterozygous mutation in the *LPL* gene (c.G106A; p.D36N). All affected subjects did not have a serum deficiency of LPL protein. Genetic analysis in one family did not show any pathogenic variants even after whole-exome sequencing. These novel *LPL* and *APOA5* mutations are not reported in other ethnic groups. This suggests that patients with F-HTG in Oman have a founder effect and are genetically unique. This warrants further analysis of patients of F-HTG in the Middle East for preventative and counseling purposes to limit the spread of the disease in a population of high consanguinity.

Keywords: familial hypertriglyceridemia, lipoprotein lipase, gene variant, gene mutation, LPL

INTRODUCTION

Hypertriglyceridemia (HTG) is a metabolic disorder characterized by elevated levels of fasting serum triglyceride (TG) (Lewis et al., 2015; Lahoz and Mostaza, 2021). Based on the Endocrine Society Clinical Practice Guideline, HTG is classified as mild (TG 1.7–2.2 mmol/L), moderate (TG 2.3–11.2 mmol/L), severe (TG 11.3–22.5 mmol/L), and very severe (TG ≥ 22.6 mmol/L) (Berglund et al., 2012). Familial hypertriglyceridemia (F-HTG) is a rare genetic disorder that is found in less than 5% of HTG cases. It is caused by mutations in at least five different genes, namely, lipoprotein lipase (*LPL*), apolipoprotein C-II (*APOC2*), apolipoprotein A-5 (*APOA5*),

glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein (*GPIHBP1*), and lipase maturation factor 1 (*LMF1*) genes. Secondary HTGs are more frequent and attributed to obesity-related diabetes mellitus, hypothyroidism, nephrotic syndrome, or drug-induced disorders (Clement et al., 2014; Cruz-Bautista et al., 2021).

The most severe forms of HTG are seen in both familial chylomicronemia (type I hyperlipidemia, OMIM#238600) and primary mixed hyperlipidemia (type V hyperlipidemia, OMIM#144650). Type I hyperlipidemia is characterized by defective clearance of chylomicrons and has an isolated elevation of blood chylomicrons, while type V hyperlipidemia shows elevation in both chylomicrons and very-low-density lipoprotein (VLDL) (Hegele, 2009; Brandts and Ray, 2021). The LPL enzyme is expressed on the surface of endothelial cells of capillaries, and it releases fatty acids from triglycerides from the intestine and liver into the bloodstream. A higher density of disease-causing mutation mainly exists in the middle of the *LPL* gene. The majority of loss-of-function mutations are located in exons 5 and 6 of the *LPL* gene since these exons encode important functional domains of the enzyme (Murthy et al., 1996; Shakhtshneider et al., 2021; Wu et al., 2021). Up to date, there is no study conducted on common genes associated with F-HTG in the Arabian region, with the exception of a case report from Israel on an Arab decent child with a mutation in the *LPL* gene (p.Arg270His) and p.Ser286Arg in a Moroccan patient (Bouabdellah et al., 2015; Dron and Hegele, 2017). In this study, we identified six families of F-HTG of Arab ancestry and reported their genetic causes. We further investigated the genetic causes of F-HTG and correlated the genotypes with LPL protein expression.

MATERIALS AND METHODS

Study Subjects

Blood samples were collected from patients with a clinical diagnosis of F-HTG at the lipid clinic in Sultan Qaboos University Hospital, Oman. Samples were also collected from their relatives. The study involved six families resulting in a cohort of 28 individuals consisting of 12 F-HTG affected patients (11 males and 1 female). Patients with mild or moderate HTG, history of uncontrolled diabetes, hypothyroidism, significant proteinuria or nephrotic syndrome, obesity, alcohol consumption, and paraproteinemia disorders were excluded from the study. Blood samples were collected after overnight fasting from all individuals for molecular and functional studies. Serum samples were collected and stored at −80°C for further analysis. The aliquoted serum samples were used for lipoprotein lipase immunoblotting. The whole blood EDTA samples were stored immediately at −80°C for DNA extraction. Family pedigrees were illustrated using cranefoot_3.2.3 software (Mäkinen et al., 2005).

Genetic Analysis and Bioinformatics

Genomic DNAs were extracted from whole blood using Qiagen mini kit (QIAamp DNA Mini). Ampli-Seq technology on the Ion Proton platform (Thermo Fisher Scientific, Inc.) was performed to sequence the F-HTG gene panel: *LPL*, *APOC2*, *APOA5*, *GPIHBP1*, and *LMF1*. The exon flanking introns and promotor regions were included in the panel. For individuals with negative exome results, DNA samples were sent to a service provider for whole-exome sequencing, Macrogen © South Korea. DNA was barcoded and enriched for the coding exons of targeted genes using hybrid capture technology (Agilent SureSelect Human All-exons-V6). Prepared DNA libraries were then sequenced using Next-Generation Sequencing (NGS) technology (NovaSeq 6000, 150) bp paired-end, at 200X coverage).

Sequencing data were processed by the Torrent Suite and reads were aligned to the hg19 reference genome. Variant call files were then generated using Torrent Variant Caller plugins. Variant annotation was performed using ANNOVAR and variants were linked to ExAC, and Greater Middle East-Arabian Peninsula (GME-AP) databases for allele frequencies. . Effect of amino acid changes was predicted by LRT, CADD, and MutationTaster. Pathogenic variants were identified from allele frequency of <1% or novel, coverage depth >30, the damaging effect from at least two of the three prediction algorithms, and segregation with the disease in the family. For CNV analysis, we used the CNVkit tool for patients with no pathogenic variant identified in the LPL gene (Talevich et al., 2016).

Immunoblotting

The serum of all affected cases including two serum samples from the control group was examined. Clear serum was collected from the bottom of the tube (milky ring avoided) after maximum centrifugation. A total of 200 µl serum was first cleared from IgG using washed magnetic beads (PureProteome™ Protein A/G Mix Magnetic Beads, Millipore). 1 µl of LPL antibody (LPL Antibody F-1, mouse monoclonal IgG, Santa Cruz Biotechnology) was added to IgG-cleared serum and incubated overnight in the cold room. Then LPL protein was immunoprecipitated using washed IgG beads under rotation for 3 h at room temperature. Then final beads were washed and mixed with reducing buffer. Proteins were separated in 12% Sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. Western blot was performed using primary LPL antibody. We used total protein loading as a normalization technique (Aldridge et al., 2008). Total lane intensity was used to measure total protein loading per sample.

RESULTS

Family Pedigrees

The lipid profiles of the affected family members are described in **Figure 1**. **Table 1** summarizes the identified mutations in the patients.

Family 1 (**Figure 1A**) is made of double cousin marriage with three affected individuals AA1 (age 11-yrs), GG1 (12-yrs), and GG4 (19yrs) with a severe elevation of triglycerides. Clinically, three patients suffered from recurrent pancreatitis and did not have lipemia retinalis, hepatosteatosis, and a history of

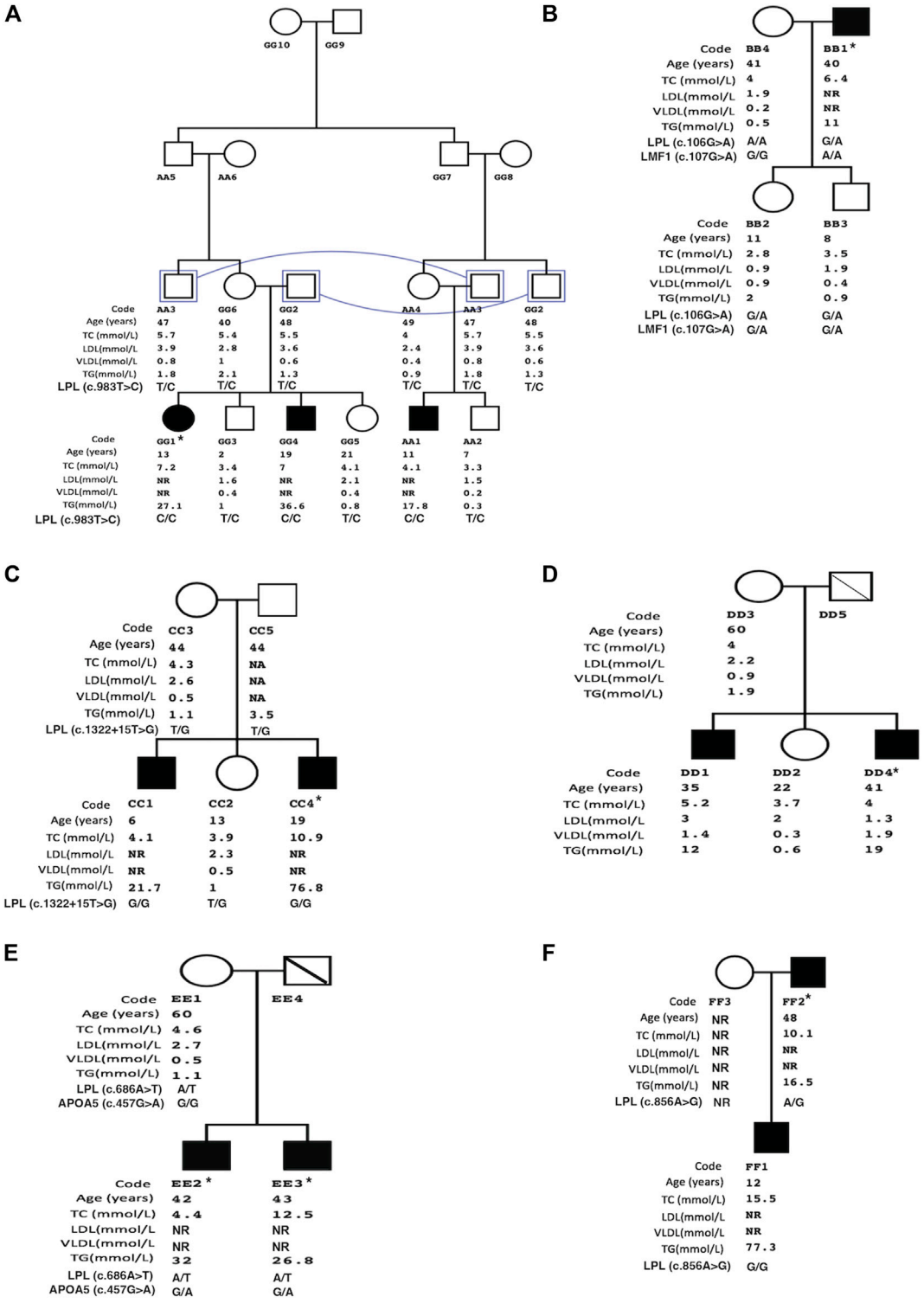

FIGURE 1 | Family pedigree structures of patients with familial hypertriglyceridemia. Illustrates family trees of study groups (A= Family1 to F= Family 6). Study group data includes age, total cholesterol (TC), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), and triglycerides (TG). Squares and circles represent male and female family members, respectively, and filled symbols represent affected subjects with familial hypertriglyceridemia. * Index case, NR: not recorded, connecting line: same individual, diagonal line: deceased subject.

TABLE 1 | List of pathogenic variants in the families.

Gene	APOA5	LMF1	LPL				
cDNA change	c.457G > A	c.107G > A	c.983T > C	c.106G > A	c.1322+15T > G	c.686A > T	c.856A > G
A.A change	p.V153M	p.G36D	p.M328T	p.D36N	-	p.H229L	p.S286G
exon No.	5	1	6	2	8	5	6
ExAC-All	0.02	0.18	Novel	0.015	Novel	Novel	Novel
GME-AP	0.06	0.06	Novel	0.018	Novel	Novel	Novel
Mutation Taster prediction	Polymorphism		Damaging	Probably harmless	-	Damaging	Damaging
LRT prediction	Neutral	Neutral	Damaging	Neutral	-	Damaging	Damaging
CADD	1.57	1.03	17.51	10.57	-	29.3	16.92

Allele frequencies were reported using ExAC-all and GME-AP databases. Exonic variant effects were assessed using the Mutation Taster, LRT, and CADD prediction tool.

cardiovascular disease, obesity, alcoholism, renal disease, or diabetes mellitus. Sequencing of the 5 candidate genes showed a novel *LPL* gene variant (c.983T > C, p.M328T) with all affected siblings being homozygous. The rest of the family members were found heterozygous carriers. The *LPL* p.M328T mutation is not reported in the *LPL*-Leiden Open Variation Database [14] and is located in exon 6 of the gene with the predicted damaging effects, **Table 1**.

In family 2, only one affected member (father, BB1) had clinical criteria for F-HTG with severe triglyceride levels. The father had obesity with no signs of lipemia retinalis. There was no history of acute pancreatitis, diabetes mellitus, renal disease, cardiovascular disease, or steatohepatitis in the family. The analysis of both targeted and whole-exome sequencing data did not identify pathogenic variants. The condition in the father could be due to double alteration in *LPL* (c.G106A; p.D36N) and *LMF1* (c.G107A; p.G36D). The *LPL* variant p.D36N is likely a benign variant (rs1801177) shown by the prediction algorithms, **Table 1**. The family's two siblings were heterozygous in two genes variants, however, they showed normal triglyceride levels. Yet, the father was homozygous for the *LMF1* (NM_001352020) p.G36D variant which may explain his severe phenotype. Furthermore, analysis of whole-exome sequencing data did not identify any CNV nor pathogenic variants in glycerol-3-phosphate dehydrogenase 1 (*GPD1*) to exclude possible Transient Infantile Hypertriglyceridemia.

In Family 3, two of the five members were affected with severe HTG. The affected sons, CC1(6 yrs) and CC4 (19 yrs) had fasting TG levels of 21.7 mmol/L and 76.8 mmol/L, respectively. Recurrent pancreatitis was present in the older son only. Both affected siblings did not have signs of lipemia retinalis and had normal blood glucose levels. Genetic analysis of family 3 showed a splice variant in the exon8-intron8 junction of the *LPL* gene. Both affected sons were homozygous, while the unaffected sibling and parents were heterozygous carriers.

In family 4, fasting TG levels of the affected males DD1 (35 yrs) and DD4 (41 yrs) were severely high at 12 mmol/L and 19 mmol/L, respectively. TG level of the mother (DD3) was normal. There was no history of acute pancreatitis and signs of lipemia retinalis in the family. Patient DD1 had obesity and steatohepatitis associated with hepatitis B infection. Patient DD4 had a history of transient ischemic heart disease and stage 2 chronic kidney disease. Using filtering strategy, no CNV nor pathogenic variants were identified in the Ampli-Seq panel or whole-exome sequencing.

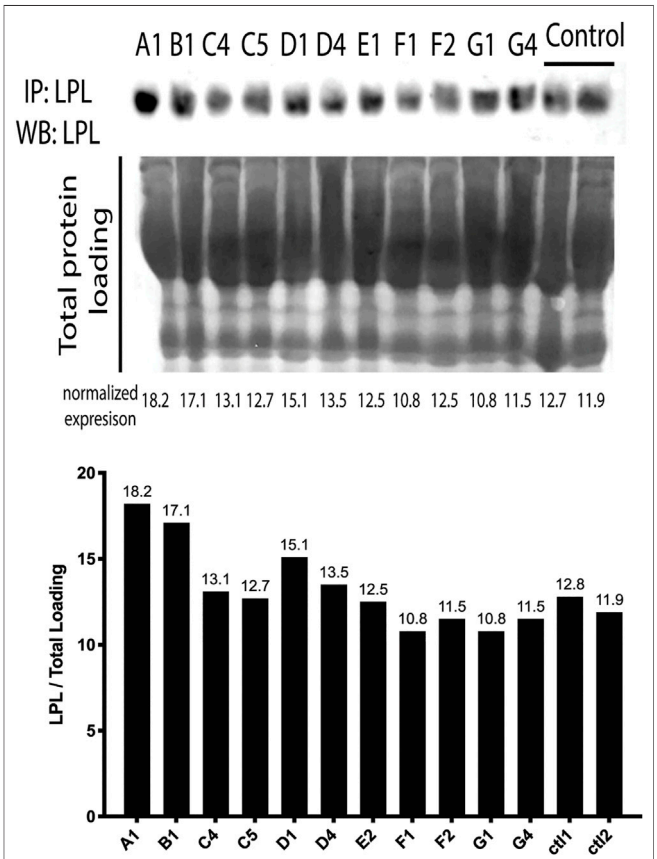

FIGURE 2 | LPL protein levels in the serum of affected individuals. The LPL densities were normalized to total protein loading. Both Ctl1 and Ctl2 are normal control samples. Individuals 'labels are as follows: A1:AA1, B1:BB1, C4:CC4, C5:CC5, D1:DD1, D4:DD4, E2: EE2, F1: FF1, F2: FF2, G1: GG1, G4: GG4.

Family 5 consisted of two affected patients with severe TG levels and a mother with normal TG levels (**Figure 1**). Recurrent acute pancreatitis was present in the affected patients but not in the mother. Genetic analysis of the two affected patients revealed a novel pathogenic *LPL* gene variant (c.686A > T, p.H229L) according to the predictions algorithm. However, the healthy

mother was also heterozygous like the affected siblings. Therefore, we analyzed the family using a double heterozygous model. We identified a polymorphic variant in the *APOA5* gene in affected patients but not in the mother. Therefore, this family had a compound heterozygous mutation in *LPL* and *APOA5* genes.

In family 6, the TG levels of an affected son (FF1) was 77.3 mmol/L which was higher than the affected father (FF2) with a TG level of 16.5 mmol/L. Both patients had recurrent attacks of acute pancreatitis and no sign of lipemia retinalis. The affected father had a history of diabetes mellitus with nephropathy. There was no history of steatohepatitis and cardiovascular disease in the family. A novel *LPL* gene variation (c.856A > G, p.S286G) was identified in the existing panel screening. The affected son with severely elevated TG was homozygous at the mutation while the father with a lower TG level was heterozygous (**Figure 1**). The mother did not attend the clinic for investigation.

Serum LPL Protein Levels

We then analyzed the LPL protein expression in serum to investigate if patients had an absolute deficiency of enzyme. To normalize the LPL expression, we used total protein loading in each Western blot lane, **Figure 2**. LPL was immunoprecipitated and expression was compared to two control cases. We found that LPL protein was expressed in all cases of F-HTG, which indicates that mutations impact the protein's functioning rather than its synthesis or stability.

DISCUSSION

Primary disorders of triglyceride metabolism result from genetic defects in triglyceride synthesis and metabolism. Here, we report the genetic spectrum of six families with severe familial hypertriglyceridemia of Arab ancestry in Oman. Three families presented with novel pathogenic variants in the *LPL* gene: two missense variants p.M328T and p.S286G and one splice variant c.1322+15T > G. One family had double heterozygous variants in *LPL* (p.H229L) and *APOA5* gene (p.V153M). Another family had double heterozygosity between *LPL* (p.G36N) and homozygous *LMF1* mutation (p.G36D). All affected subjects did not have serum deficiency of LPL protein level and most likely the variants affect the lipolytic activity of LPL. Previous studies on other disease-causing mutations also the variable effect of protein levels of LPL (Kozaki et al., 1993; Hooper et al., 2008). Genetic analysis in one family did not show any pathogenic variants even with whole-exome sequencing.

The novel pathogenic missense variant in the *LPL* gene (rs1181582051, c.983T > C, p.M328T) causes a substitution in the carboxy-terminal structural region of LPL, which spans 313–448 amino acids, on the highly conserved beta-strand (uniport: LIPL_HUMAN). The LPL-mediated lipoprotein absorption through the cell surface receptor catalyzes the first contact between LPL and the substrate in this domain (Murthy et al., 1996). Furthermore, beta-strands are crucial secondary structural elements that affect protein folding, structure, and topology, as well as the protein's functional confirmation and activity (Henderson et al., 1993; Jin et al., 2021). When compared to controls, this variant was detected in family 1 members who had normal LPL expression. This indicates that the mutation had no effect on the stability of the protein.

The novel missense mutation p.S286G in exon 6 of the *LPL* gene occurs in a less-conserved region of the gene. The 286 residues are located very close to the S-S bridge between the two cysteine residues (278–283) which plays an important role in stabilizing the heparin-binding (R279-K280 and R282) (Murthy et al., 1996). A previous case with F-HTG was reported in a Moroccan patient but with homozygous c.858T > A/p.S286R mutation (Bouabdellah et al., 2015). The heterozygous p.S286G carriers in the study family had triglycerides half levels of those of homozygote cases. suggesting that the mutation has a dose effect.

A missense mutation (c.686A > T, p.H229L) in the *LPL* gene has been detected in a family of 4 members even in the mother with normal TG levels. To the best of our knowledge, this mutation is not reported in any population. Amino acid residues from 228 to 234 of LPL make up the ß-strand in the N -terminal domain, UniProt. Studies by Dugi et al. (1992) and Henderson et al. (1993) highlighted the region from Cys218 to Cys 239 as the lid/loop domain which is stabilized by one of the four disulfide bonds of LPL protein. The lid/loop is involved in the hydrolysis of some triglycerides and phospholipids, the initial identification of the lipase substrate, and giving excess to the catalytic triad (Goldberg and Chait, 2020). At the lid proximal and distal regions, the charge and periodicity play a critical role in maintaining the catalytic activity and the apical residues of the loop contribute minimally to LPL function (Dron and Hegele, 2017). Therefore, to have a dominant effect on serum TG levels, the patient phenotype suggested the presence of a compound heterozygous model. Earlier reports show that more than two-thirds of F-HTG is caused by an autosomal recessive variant in the *LPL* gene [22, 23]. Less abundant cases are due to autosomal recessive or double heterozygous *LPL* variants with variants in *LMF1*, *APOA5*, *APOC2,* or *GPIHBP1* gene (Basel-Vanagaite et al., 2012; Goldberg and Chait, 2020). The double heterozygosity affects the function and maturity of LPL leading to elevated serum TG. LPL activity requires apoC2 and apoA5, while the maturity and transport of LPL are controlled by LMF1. GPIHBP1 is required for surface attachment of LPL at the endothelial cell surface. The compound heterozygosity in family B explains that the presence of homozygous p.G36D variant in *LMF1* along with heterozygous changes in *LPL* and *GPIHBP1* causes the severe elevation in the affected father BB1. The p.G36D of *LMF1* was earlier reported in a Thai family with F-HTG with one patient being homozygous and 4 were heterozygous (Plengpanich et al., 2020).

None of the cases presented clinically as transient infantile hypertriglyceridemia. These patients usually present with moderate hypertriglyceridemia at birth and gradually decrease with growth [24]. The condition is caused by an autosomal recessive mutation in the glycerol-3-phosphate dehydrogenase 1 gene (*GPD1*).

CONCLUSION

Hypertriglyceridemia can present as a monogenic (familial) or polygenic disease. Monogenic hypertriglyceridemia conditions are very rare and commonly cause severe elevation of serum TG. Here, we described six families of F-HTG in Oman. Four pathogenic variants in the *LPL* gene were identified: p.M328T, c.1322+15T > G. p.S286G, and p.H229L. The latter variant existed as double heterozygous with the *APOA5* variant p.V153M variant. One family with severe hypertriglyceridemia resulted in negative exome data. Further whole-genome sequencing and epigenetic studies are needed to explore the genetic basis in these families. The reported novel mutations in the study suggest that patients with F-HTG in Oman may have a founder effect and are genetically unique. This warrants further analysis of patients with F-HTG in the Middle East for the preventative and counseling purpose to limit the spread of the disease in a population of high consanguinity.

DATA AVAILABILITY STATEMENT

The datasets for novel mutation presented in this study can be found in online repositories: Global Variome shared LOVD (https://databases.lovd.nl/shared/individuals/LPL) with accession individual I.D numbers 00408363, 00408402, 00408403, 00408404.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Research Ethics Committee (SQU-EC/006/19 #1842), Sultan Qaboos University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

The article was written by FZ and MA. The revision was carried out by KA-R, RZ, and PK. Data acquisition was performed by KA-W, ABDA-M, SA-Y, AR, HAMA-B, SH, ZMA-D, HALA-B, MH, and MH. Data analysis was performed by FZ, KA-W, MH, AR, and ALMA-M. Funding was provided by FZ and KA-W.

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BRIEF RESEARCH REPORT
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Early Discovery of Children With Lysosomal Acid Lipase Deficiency With the Universal Familial Hypercholesterolemia Screening Program

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Lysosomal acid lipase deficiency (LAL-D) is an autosomal recessive lysosomal storage disorder, caused by homozygous or compound heterozygous pathogenic variants in the *LIPA* gene. Clinically, LAL-D is under- and misdiagnosed, due to similar clinical and laboratory findings with other cholesterol or liver misfunctions. As a part of the Slovenian universal familial hypercholesterolemia (FH) screening, LAL-D is screened as a secondary condition among other rare dyslipidemias manifesting with hypercholesterolemia. Out of 669 children included, three were positive for a homozygous disease-causing splicing variant NM_000235.4: c.894G > A (NP_000226.2:p. Gln298Gln) in the *LIPA* gene (NG_008194.1). The mean age by the diagnosis of LAL-D was 9.8 ± 0.9 years. Moreover, all three LAL-D-positive children had an important elevation of transaminases and decreased activity of the lysosomal acid lipase enzyme. Abdominal MRI in all children detected an enlarged liver but a normal-sized spleen. In conclusion, universal FH screening algorithms with the confirmatory genetic analysis in the pediatric population enable also rare dyslipidemia detection at an early age. An important clinical criterion for differentiation between FH and the LAL-D-positive children has elevated transaminase levels (AST and ALT). In all three LAL-D positive children, an improvement in cholesterol and transaminase levels and steatosis of the liver has been seen after early treatment initiation.

Keywords: cholesterol ester storage disease, CESD, lysosomal acid lipase deficiency, LAL-D, *LIPA* gene, hypercholesterolemia, universal screening, pediatric population

INTRODUCTION

Lysosomal acid lipase deficiency (LAL-D) is an autosomal recessive lysosomal storage disorder, caused by homozygous or compound heterozygous disease-causing variants in the *LIPA* gene located on chromosome 10q23.2 (Pisciotta et al., 2017; Wilson and Patni, 2020). Depending on the genetic variant in the *LIPA* gene and consequently residual lysosomal acid lipase (LAL) activity, LAL-D can result in the very severe, infantile-onset, and lethal form known as Wolman disease, or in the milder, late-onset phenotype, known also as cholesteryl ester storage disease (CESD) (Reiner et al., 2014; Pullinger et al., 2015; Sjouke et al., 2016; Maciejko, 2017).

In CESD, various symptoms are present and usually begin in midchildhood with an average age of 5 (Maciejko, 2017), although they can develop later in adulthood. Most of the affected children present symptoms, such as elevated low-density lipoprotein cholesterol (LDL-C) levels, low high-density lipoprotein cholesterol (HDL-C) levels, and accelerated atherosclerotic cardiovascular disease (CVD) hepatomegaly, and splenomegaly, gradually leading to liver fibrosis and cirrhosis. Moreover, some children suffer from malabsorption, vomiting, diarrhea, steatorrhea, and growth failure (Reiner et al., 2014; Pullinger et al., 2015; Sjouke et al., 2016; Maciejko, 2017).

Clinically, LAL-D is under- and misdiagnosed, due to similar clinical and laboratory findings with familial hypercholesterolemia (FH) or nonalcoholic fatty liver disease (NAFLD), leading to a delay in treatment or mistreatment. A correct diagnosis of LAL-D is critical for the appropriate clinical management of children (Pullinger et al., 2015). The standard way of the LAL-D diagnosis is a demonstration of decreased LAL enzyme activity in DBS, serum, or skin fibroblasts. Elevated liver transaminases and elevated serum TC or LDL-C could raise the suspicion of the LAL-D diagnosis (Pullinger et al., 2015; Maciejko, 2017). Most cases are diagnosed in the first two decades of life (Muntoni et al., 2007; Bernstein et al., 2013; Burton et al., 2015). Usage of a human recombinant LAL (sebelipase alpha, Kanuma®) for the LAL-D treatment was approved in 2015 resulting in significant improvements in the hepatic and lipid profiles of children with LAL-D (Sjouke et al., 2016; Camarena et al., 2017).

The prevalence of the Wolman disease is estimated at approximately 1/350,000, whereas the prevalence of the CESD is estimated between 1/40,000 and 1/300,000, depending on ethnicity and geographic location. However, due to milder phenotypes of the CESD and overlap with other more frequent pathologies, the prevalence might be underestimated (Pullinger et al., 2015; Camarena et al., 2017).

In the study, we assessed the adoption of universal FH screening for early LAL-D detection in preschool children. Furthermore, we evaluated clinical criteria for differentiation between FH and the LAL-D-positive children. Moreover, we presented clinical characteristics of the LAL-D-positive children.

METHODS

Cohort Description

Slovenia has been implementing universal FH screening in children since 1995 as a routinely part of the blood checkup at the programmed visit of all 5-year-old children to the primary care pediatrician (Sedej et al., 2014; Klancar et al., 2015; Groselj et al., 2018, 2022), lately reaching more than 90% of 5-year-old children (of approximately 20,000) each year (**Supplementary Figure S1**). As a part of the universal FH screening program total cholesterol (TC) was measured. In 2011, routine genetic diagnostic for the FH-related genes was introduced at the University Children's Hospital Ljubljana, Clinical Institute of Special Laboratory Diagnostics (Klancar et al., 2015; Groselj et al., 2018; Marusic et al., 2020). According to the clinical guidelines, additional cascade screening of family members and further clinical care are performed as required and financed by the Slovenia's national health insurance system.

As the patients with LAL-D also have elevated TC levels, they are detected by our universal FH screening and referred to the Universal Children's Hospital Ljubljana. Since 2018, in our expended NGS (next-generation sequencing) panel, other genes also associated with dyslipidemia are included. The *LIPA* gene is also one of those included on the NGS panel. Until recently, 669 children had the *LIPA* gene sequenced. For the children referred as a part of the FH screening before applying the expended NGS panel (prior 2018), who were negative for the FH-related genes and had elevated aspartate aminotransferase – AST (>0.58 μkat/L) – and/or alanine aminotransferase – ALT levels (>0.74 μkat/L), Sanger sequencing for the *LIPA* gene was additionally performed. After 2018, all FH screening positive children were with the FH-related genes simultaneously also tested for the pathogenic variants in the *LIPA* gene. A flowchart of the included children is presented in **Supplementary Figure S2**.

The principles of the Declaration of Helsinki were followed, and the Slovenian National Medical Ethics Committee approved the study (#25/12/10, #63/07/13, 0120-14/2017/5, 0120-273/2019/9 and 0120-273/2019/19). Written informed consent was obtained from all parents or legal guardians.

Genetic Analyses

Genomic DNA was isolated from the whole blood samples of 669 children using the FlexiGene isolation kit (Qiagen, Germany). Two different sequencing methods for *LIPA* gene sequencing were used over time: targeted Sanger sequencing (n = 28) and xGen® Lockdown® NGS Probes (IDT, United States) (n = 641). Targeted Sanger sequencing was applied for FH-negative children with elevated aspartate aminotransferase – AST (>0.58 μkat/L) – and/or alanine aminotransferase – ALT levels (>0.74 μkat/L), and for the cascade LAL-D screening of the positive siblings. With the usage of the NGS probes, the *LIPA* gene was included in our expanded dyslipidemia gene panel. Samples were sequenced on the MiSeq sequencer with MiSeq Reagent Kit (Illumina, United States) following the manufacturer's instructions including recommendations for quality control parameters. All variants identified with NGS sequencing were reconfirmed by targeted Sanger DNA sequencing. The pathogenicity of the identified variants was assessed using Human Gene Mutation Database Professional and ClinVar (Landrum et al., 2018) databases. For variants of unknown clinical significance *in silico* prediction tools (CADD, SIFT, MutationTaster, PolyPhen2) were utilized (Ng and Henikoff, 2003; Schwarz et al., 2010; Adzhubei et al., 2013; Rentzsch

et al., 2019). Variants were classified according to the American College for Medical Genetics and Genomics (ACMG) classification criteria (Richards et al., 2015).

Laboratory Findings, Medical Imaging and Pathology Evaluation

Lipid profiles [TC, LDL-C, HDL-C, and triglycerides (TG)] for all the included children were routinely measured with an automated analyzer Advia 1800 (Siemens Healthcare, Erlangen, Germany), using the direct enzymatic colorimetric method. Additionally, transaminase [AST, ALT, and amma-glutamyl transferase (GGT)] levels were measured. South Glasgow Hospital performed acid lipase enzyme assays for the LAL-D-positive children with a pathogenic variant in the *LIPA* gene. Liver MRI elastography and liver ultrasound were performed in the LAL-D-positive children. In two patients, liver biopsy was also performed before considering treatment with sebelipase alpha (Kanuma®).

Arterial Stiffness and Endothelial Function Evaluation

The ultrasound examination was performed using the Aloka 5500 SSD Pro-sound ultrasound machine. The UST-5539 7.5 linear probe was used for vascular examination, and the auto-IMT modality of ultrasound machine software was used. The maximum cIMT value, expressed in millimeters, was used for analysis and determined as the mean of all six measurements performed bilaterally. Using a high-definition echo-tracking system (Aloka implemented E-tracking software) the pressure–diameter curve of the artery was derived and from the time delay between the two adjacent distension waveforms, one-point local pulse wave velocity and beta stiffness was calculated.

The endothelial function measurement was performed on the peripheral arterial tone using the EndoPAT 2000 device (Itamar Medical Ltd., Caesarea, Israel); the reactive hyperemia index (RHI) was calculated using the manufacturer's algorithm based on the ratios between pulse wave amplitudes during the reactive hyperemia and baseline phases.

Prevalence Estimation of Heterozygous *LIPA* Variants in the General Population

The prevalence of LAL-D in the Slovenian population was determined based on the cohort who did not express FH phenotype (n = 1,915). The number of heterozygous carriers of the pathogenic alleles in the *LIPA* gene was determined, and the prevalence of homozygous LAL-D in our population was then calculated with the Hardy–Weinberg equation.

RESULTS

LAL-D-Positive Children Diagnosis

To date, 669 children (demographical data of the cohort is presented in **Table 1**) were included in the *LIPA* gene analysis. 664 of them were referred to our clinic because of elevated cholesterol levels found at the universal FH screening in children program in Slovenia; four children were referred as symptomatic for LAL-D and one as a part of a sibling-cascade LAL-D screening. Due to the FH screening program, the median age at the first appointment at our institution was 6.3 (IQR: 5.8–7.2) years.

Of all the 669 children analyzed for the *LIPA* and FH-related genes (*APOB*, *LDLR*, and *PCSK9*), 189 were classified as positive or with a variant of uncertain significance (VUS) for the FH-related genes. From the remaining group of 480 children, three were classified as LAL-D-positive. All of them were homozygous for NM_000235.4:c.894G > A variant in the *LIPA* gene (NG_008194.1) resulting in NP_000226.2:p. Gln298Gln (rs116928232) synonymous protein variant affecting the splice site at the end of exon 8. According to the ACMG criteria, the variant was classified as pathogenic (PP5, PP3, PP1, and PM2) (Richards et al., 2015). Two children were detected directly based on the universal FH screening program and one based on the cascade LAL-D screening of siblings. None of the children who were referred as symptomatic for LAL-D (with elevated aminotransferase levels) was positive for a pathogenic variant in the *LIPA* gene. The mean age of the diagnosis was 9.8 ± 0.9 years. Demographical and clinical characteristics of the LAL-D-positive children are presented in **Table 2**. There was no evidence of consanguineous marriage in the family.

In all three LAL-D positive children, the enzyme activity of LAL performed from a sample of dried blood spot showed a decreased activity of under 0.02 nmol/punch/hour (reference range: 0.37–2.30 nmol/punch/hour). In patient 3, a liver biopsy was performed before the genetic result was available and showed small droplet steatosis in hepatocytes and rarely captured macrophages. A mildly multiplied and condensed connective tissue in the portal field, in the sinusoids and segmentally in the bile ducts and between hepatocytes, was present. No cholesterol needles were found. Also, a liver biopsy was performed in patient 1 before consideration for the Kanuma treatment, but a milder phenotype was present, and patient 1 was not eligible for the treatment.

Clinical Follow-Up of the LAL-D-Positive Children

When comparing TC, LDL-C, HDL-C, TG, AST, and ALT levels of the FH screening positive children and the LAL-D-positive children, increase in TC and LDL-C levels can be observed in LAL-D-positive children versus the median of the FH screening positive children group (**Figure 1**) at their first visit at our clinic. Moreover, all three LAL-D-positive children had an important elevation of transaminases.

Before treatment, the mean height percentile of the children was 14.3 ± 5.7, and the mean BMI percentile was 30.4 ± 16.7. The children were not malnourished, but an improvement was seen in the height and BMI percentiles after treatment initiation. The mean percentile of height after treatment was 22.6 ± 3.2, and the mean BMI percentile was 45.9 ± 10.6. Ezetimibe (10 mg) was administered to all three children in the initial phase (**Figure 2**).

TABLE 1 | Demographical data of the children included in the study.

		M (N = 269)	F (N = 398)	Total (N = 667)
At first examination at our clinic				
Age		6.4 (5.8, 7.4)	6.3 (5.8, 7.1)	6.3 (5.8, 7.2)
Weight	kg	24.4 (21.3, 29.6)	22.8 (20.1, 27.5)	23.4 (20.5, 28.4)
Height	cm	122.0 (117.0, 130.3)	120.5 (115.5, 127.0)	121.2 (116.0, 128.5)
LDL-C	mmol/L	3.4 (2.9, 3.9)	3.5 (3.1, 4.1)	3.5 (3.0, 4.1)
	mg/dL	131.5 (112.1, 150.8)	135.3 (119.9, 158.5)	135.3 (116.0, 158.5)
TC	mmol/L	5.5 (5.0, 5.9)	5.6 (5.1, 6.1)	5.5 (5.0, 6.0)
	mg/dL	212.7 (193.4, 228.2)	216.6 (197.2, 235.9)	212.7 (193.4, 232.0)
HDL-C	mmol/L	1.5 (1.3, 1.7)	1.5 (1.3, 1.8)	1.5 (1.3, 1.8)
	mg/dL	58.0 (50.3, 65.7)	58.0 (50.3, 69.6)	58.0 (50.3, 69.6)
TG	mmol/L	0.9 (0.7, 1.4)	0.9 (0.7, 1.3)	0.9 (0.7, 1.3)
	mg/dL	79.7 (62.0, 124.0)	79.7 (62.0, 115.1)	79.7 (62.0, 115.1)
AST	μkat/L	0.5 (0.4, 0.6)	0.5 (0.4, 0.6)	0.5 (0.4, 0.6)
ALT	μkat/L	0.3 (0.3, 0.4)	0.3 (0.3, 0.4)	0.3 (0.3, 0.4)

Age, weight, height, and lipid profile of the cohort were included in the LIPA sequencing, at the first examination at our clinic. Data are presented as median (IQR). LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; AST, aspartate transaminase; ALT, alanine transaminase.

TABLE 2 | Demographical and clinical characteristics of the LAL-D positive patients.

	Patient 1	Patient 2	Patient 3
At diagnosis			
Age (years)	10.3	8.7	10.3
Screening type	Cascade	Universal	Universal
At first examination at our clinic			
Age (years)	5.8	7.0	5.8
Weight [kg and (percentile)]	19.3 (39.4)	19.2 (10.3)	17.4 (9.5)
Height [cm and (percentile)]	109.2 (15.2)	114.1 (8.3)	108.5 (9.7)
BMI [kg/m^2 and (percentile)]	16.2 (67.2)	14.8 (27.9)	14.8 (27.5)
LDL-C (mmol/L)	3.6	4.5	5.3
LDL-C (mg/dL)	139.2	174.0	204.9
TC (mmol/L)	5.3	6.4	7.1
TC (mg/dL)	204.9	247.5	274.6
HDL-C (mmol/L)	1.2	1.5	1.3
HDL-C (mg/dL)	46.4	58.0	50.3
TG (mmol/L)	1.2	0.9	1
TG (mg/dL)	106.3	79.7	88.6
AST (μkat/L)	1.8	1.3	1.4
ALT (μkat/L)	2.3	1.6	1.4

Age at diagnosis, screening type, and lipid profile is represented for the LAL-D-positive patients. BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; AST, aspartate transaminase; ALT, alanine transaminase.

At age 10.4, patient 3 started receiving combination therapy with ezetimibe (10 mg) and Kanuma® (1 mg/kg, every 2 weeks) in a clinical trial abroad. In 2017/2018, there was an interruption for 8 months with Kanuma®, because of organizing the therapy at a home institution, after discontinuing his treatment abroad. For patient 3, we noticed a decrease in TC, LDL-C, AST, ALT, and yGT levels on ezetimibe treatment alone, but a more important decrease is seen when Kanuma was added to the therapy, as seen in **Supplementary Table S1**. At age 16.8 in patient 1, no sufficient lowering of cholesterol and liver enzymes with ezetimibe was present at first and was not applicable for the Kanuma treatment, started with a combination of ezetimibe (10 mg) and rosuvastatin (10 mg). Moreover, the patient already had signs of endothelial dysfunction. We concluded that statins would be more beneficial to the patient because of persistently elevated cholesterol levels, but the patient had side effects (muscle pain and fatigue), and rosuvastatin was discontinued.

The levels of TC, LDL-C, HDL-C, TG, AST, and ALT levels in all three LAL-D children over time are presented in **Figure 2**. Vertical lines indicate therapy initiation and modification dates for all three children. Before the treatment, the mean AST and ALT levels were 1.33 ± 0.25 μkat/L and 1.61 ± 0.15 μkat/L, respectively. After treatment initiation, a decrease in the mean AST (0.73 ± 0.11 μkat/L) and ALT (1.13 ± 0.23 μkat/L) levels were observed. In the lipid profile after treatment, more favorable mean levels were present. The mean levels of TC (6.46 ± 0.95 mmol/L), LDL (4.34 ± 0.54 mmol/L), and triglycerides (1.44 ± 0.66 mmol/L) before treatment were higher than the mean TC (5.25 ± 0.40 mmol/L), LDL (3.38 ± 0.56 mmol/L), and triglycerides (1.16 ± 0.41 mmol/L) on treatment. Furthermore, HDL (1.29 ± 0.25 mmol/L) before treatment has increased on treatment (1.37 ± 0.33 mmol/L).

Chitotriosidase levels before treatment are only available in patient 2; the value was 408 nmol/mLh (reference range: 3–65 nmol/mLh). Comparing the first half (earlier period) of treatment with the second half (last period) of treatment in patients 2 and 3, we can see a decrease in chitotriosidase levels (from 426 ± 148 nmol/mLh to 300 ± 159 nmol/mLh in patient 2 and from 578 ± 197 nmol/mLh to 388 ± 57 nmol/mLh in patient three). In patient 1, the chitotriosidase levels were elevated in the second half of treatment; the values went from 654 ± 209 nmol/mLh to 682 ± 239 nmol/mLh.

Liver MRI in all children detected an enlarged liver but a normal-sized spleen. Mild fibrosis and steatosis of the liver in patients 1 and 2 were observed on MRI liver elastography, and in patient 3, high fibrosis in regions five and six has been present. The size of the liver in child 1 at 12.3 years was in the midclavicular plane in the craniocaudal line 159 mm and transverse line 185 mm. The degree of steatosis was 18%-19%. In the control after 4.5 years, the degree of steatosis was 10.2%. The size of the liver in patient 2 at 9.8 years was in the midclavicular plane in the craniocaudal line at 122 mm and transverse line at 190 mm, and the degree of steatosis

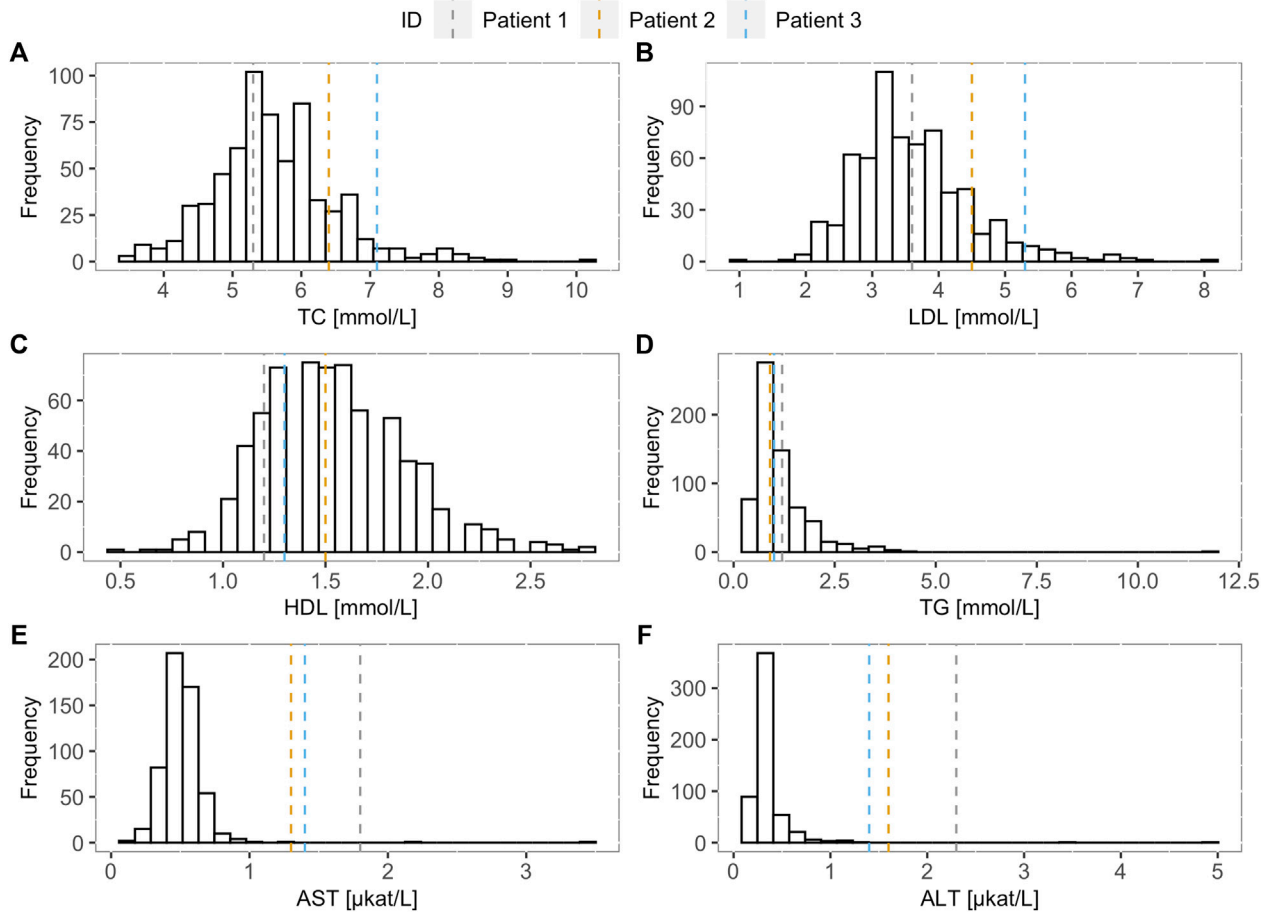

FIGURE 1 | Lipoprotein and transaminase levels of the LAL-D positive children compared to the LAL-D negative cohort. Distribution of **(A)** total cholesterol, **(B)** low-density lipoprotein cholesterol, **(C)** high-density lipoprotein cholesterol, **(D)** triglycerides, **(E)** aspartate transaminase, and **(F)** alanine transaminase for LAL-D negative population. The vertical dotted lines show the values of each parameter for the LAL-D-positive patients. LDL-C: low-density lipoprotein cholesterol; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides; AST: aspartate transaminase; ALT: alanine transaminase.

was 16%-17%. In the control after 2.5 years, the degree of steatosis was 11%. The size of the liver in patient 3 at 10.2 years was in the midclavicular plane in the craniocaudal line at 148 mm and transverse line at 184 mm, and the degree of steatosis was 10%. In the control after 6.5 years, the degree of steatosis was 7%–10%. In all children, the spleen structure was normal on MRI elastography.

The mean cIMT was 0.353 ± 0.08 mm. By the references according to Drole Torkar et al. (2020), in patient 2, cIMT was between 25. and 50. p, and in the other two patients, it was under 5. p. The mean beta stiffness was 3.5 ± 0.5. By the references according to Doyon et al. (2013), patient 1 and patient 2 had beta stiffness above 50. p, and patient 3 under 50. p. The mean RHI was 1.38 ± 0.38. Using the threshold 1.35, suggested by Bonetti et al. (2004) classified patient 1 as having already endothelial disfunction, while patients 2 and 3 had a normal endothelial function. The mean PWV was 3.94 ± 0.46 m/s. By the references according to Reusz et al. (2010), patient 1 had PWV under the 5. p, and patients 1 and 2 were classified between 10. and 50. p.

LAL-D Prevalence Estimation

The LAL-D prevalence in our population based on the data of heterozygous carriers of the pathogenic alleles in the *LIPA* gene was estimated at 1/406,193.

DISCUSSION

In the present study, we indicated the importance of the FH screening for the detection of dyslipidemia clinically expressed similarly to FH. As a result of having unspecific symptoms, LAL-D is diagnosed late in life, misdiagnosed, or overlooked. However, we aimed to distinguish between the phenotype of the children with FH and children with a *LIPA* disease-causing variant in our cohort. Furthermore, the prevalence of LAL-D in our population was estimated.

Cascade FH screening was successfully implemented in the Netherlands (Wiegman et al., 2015). Furthermore, as a great advantage of the universal hypercholesterolemia screening in

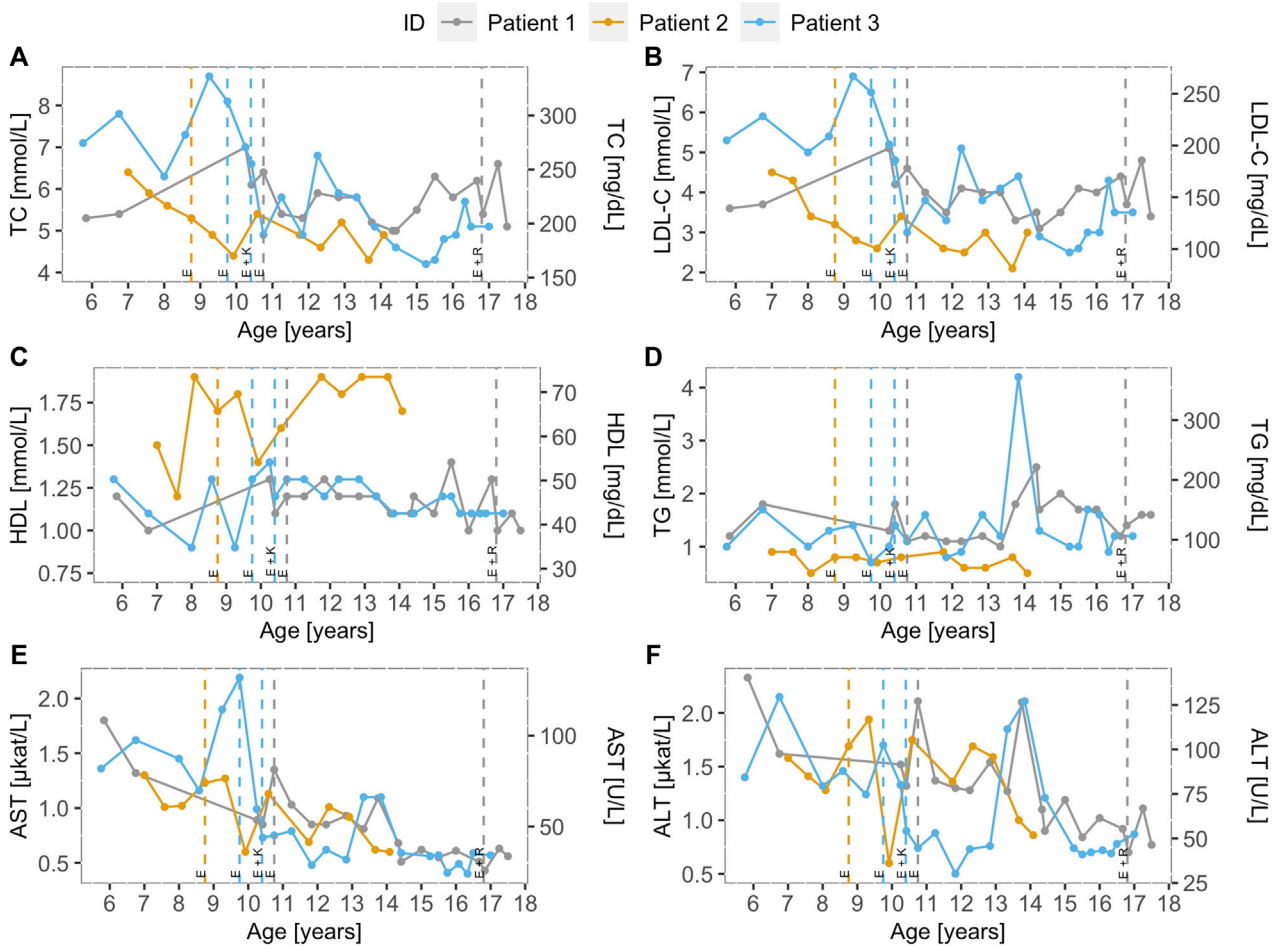

FIGURE 2 | Lipoprotein and transaminase levels of the LAL-D positive children over time. Distribution of **(A)** total cholesterol, **(B)** low-density lipoprotein cholesterol, **(C)** high-density lipoprotein cholesterol, **(D)** triglycerides, **(E)** aspartate transaminase, and **(F)** alanine transaminase for LAL-D-positive population over time. Dotted vertical lines represent the beginning of the treatment and treatment modification for each patient. All three patients were treated with ezetimibe (10 mg). Furthermore, patient 3 was treated with a combination of ezetimibe (10 mg) and Kanuma® and patient 1 was treated with a combination of ezetimibe (10 mg) and rosuvastatin (10 mg) from the age of 16, but the combination therapy was discontinued because of fatigue. LDL-C: low-density lipoprotein cholesterol; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides; AST: aspartate transaminase; ALT: alanine transaminase; E: Ezetimibe (10 mg), K: Kanuma (40 mg), R: Rosuvastatin (10 mg).

preschool children (Groselj et al., 2018), in the absence of other FH monogenic or polygenic factors, simultaneously other dyslipidaemias could be detected. Moreover, LAL-D screening should be considered for children and young adults with unexplained hepatic elevated AST/ALT levels in combination with elevated LDL-C (>160 mg/dl, 4.1 mmol/L) and low HDL-C levels (<35 mg/dl, 1.0 mmol/L). Also, by universal FH screening, the LAL-D positive children are diagnosed, and appropriate treatment at an early age before the onset of serious clinical signs and disease progression is introduced (Strebinger et al., 2019). Another aim of early treatment is also the prevention of potential later cardiovascular complications as in one of our three children endothelial dysfunction was probably already present, considering that the RHI value was below the RHI cut-off value for adults (1.35) and lower RHI seen in the children from the same age group (Kelly et al., 2014).

All three children were homozygous for a pathogenic NM_000235.4: c.894G > A variant in the *LIPA* gene (NG_008194.1), which was previously functionally characterized as affecting a 5′ splice-region causing deletion of a 72-base exon 8 (p.Ser275_Gln298del) from the mRNA for LAL causing CESD letting 5%–10% of the wildtype activity (Klima et al., 1993; Ameis et al., 1995; Fasano et al., 2012). The presence of the c.894G > A variant is associated with a milder clinical phenotype of LAL-D with slow progression of liver fibrosis and cirrhosis (Lipiński et al., 2018). Rashu et al. (2020) describe two adult *LIPA* compound heterozygous siblings for the c.894G > A and c.482del variants and Gürbüz et al. (2020) report a 14-year-old and 3-year-old siblings with homozygous c.894G > A variant with persisting gastrointestinal symptoms (hepatosplenomegaly, malabsorption, and diarrhea, combined with elevated

transaminases and dyslipidemia). A Columbian boy was confirmed with LAL-D with the c.893G > A variant in the *LIPA* gene at age 14 after isolated hepatomegaly and dyslipidemia at age 6 were detected (Botero et al., 2018).

LAL-D is an autosomal recessive disease since it has been demonstrated that heterozygous *LIPA* disease-causing carriers generally do not express an FH phenotype, while FH is a disease with an autosomal dominant inheritance pattern (Sjouke et al., 2016). The prevalence of LAL-D was previously estimated to be 1/40,000 to 1/300,000 individuals (Pullinger et al., 2015; Camarena et al., 2017). Based on the cohort of 1,915 children from our population the prevalence of homozygous LAL-D was estimated to be 1/1/406,193. Since we only considered pathogenic variants for the genetic computation of prevalence, rather than VUS, the prevalence in our population was slightly lower than expected compared to the literature.

Due to the unspecific symptoms, LAL-D is typically diagnosed late in life, misdiagnosed, or overlooked (Strebinger et al., 2019). In CESD, deaths because of liver disease progression have been reported already at the age of 7 years (Bernstein et al., 2013). Typically, children with LAL-D have elevated cholesterol levels, whereas the parents of the index case have normal lipid profiles (Sjouke et al., 2016). An important indicator for diagnosing children with LAL-D is significantly elevated transaminase levels, which is a sensitive and specific way of detecting this disorder. However, as screening for FH with cholesterol is already implemented in Slovenia, measuring TC levels at the first step and liver enzymes at the specialist clinic was more feasible. Although LAL-D should still be considered a differential diagnosis in children with increased liver enzymes or other clinical signs indicative of LAL-D. Moreover, LAL-D newborn screening (NBS) should be considered in the future (Remec et al., 2021); nevertheless, LAL-D currently is not screened in Europe and elsewhere by NBS (Loeber et al., 2021).

Moreover, statin therapy reduces LDL-C levels, but on the other hand, they promote the delivery of cholesteryl esters to hepatocytes and consequently worsens the effects on liver function (Pullinger et al., 2015; Sjouke et al., 2016). Liver transplantation has not been proven as a successful treatment option for LAL-D. Bernstein et al. (2013) reported that 11 out of 18 LAL-D children had multisystemic progression of the disease (for example, growth failure, anemia, sepsis, transplant rejection, right lower lobe collapse, atelectasis, pulmonary lipid deposition, dyslipidemia, atherosclerosis, and heart failure). Hematopoietic stem cell transplantation seems to be a more successful treatment in Wolman disease than liver transplantation, but there are also reports about disease progression and fatal transplant-related complications (Strebinger et al., 2019).

The use of a human recombinant LAL (sebelipase alpha, Kanuma®) for the LAL-D treatment was approved in 2015, not only resulting in significant improvements in the hepatic and lipid profiles of children with LAL-D but also increased survival rates in infants with Wolman disease (Sjouke et al., 2016; Camarena et al., 2017). In the results of the ARISE study, improvements in liver enzymes and lipid profile were seen with the Kanuma® treatment. At the last open-label assessment, ALT and AST normalization were achieved by 47% and 66% of children, respectively. A 25% reduction in median (IQR) percent changes in LDL-C and a 27% (19%, 44%) increase in high-density lipoprotein cholesterol in the median (IQR) percent changes were observed (Burton et al., 2022). In 13 children who experienced an infusion-associated reaction, one was categorized as serious; the others were mild to moderate. Antidrug antibodies were found in 6 children in the middle of treatment, but in the end, 5 out of 6 children tested antidrug antibody negative, and the only one that was still positive was the patient that had developed activity-neutralizing antibodies (Burton et al., 2022). Although in 2017 in the National Institute for Health and Care Excellence (NICE) guidelines Kanuma® was not recommended for long-term enzyme replacement therapy for treating LAL-D in babies with rapidly progressive disease and also not in children and adults (National Institute for Health and Care Excellence, 2017) since 2021 NICE is proceeding with a new Highly Specialised Technologies Evaluation for sebelipase (National Institute for Health and Care Excellence, 2021). In all three children from our study, an improvement in cholesterol levels, liver enzymes, and also of steatosis of the liver has been seen after early treatment initiation.

In 2020, consensus recommendations from an international collaborative working group were published on the initial assessment and ongoing monitoring of LAL-D in children and adults (Kohli et al., 2020). International guidelines on the treatment of LAL-D are lacking. In 2012 (recruiting is still in progress), an international registry was started to improve the understanding of therapeutic interventions and their long-term effectiveness (Soll et al., 2019).

One of the present study's important limitations was a delay in diagnosing LAL-D when compared to age at FH screening. As a part of the universal FH screening, TC levels at the primary-care level were measured at age 5-6, and the median age by the referral to our institution was 6.3 (IQR: 5.8–7.2) years. Before an expanded NGS panel for dyslipidemia containing the *LIPA* gene was established, genetic testing was first performed to exclude FH, and therefore, genetic testing for LAL-D was performed later. After 2018, *LIPA* sequencing is performed on all FH screening positive children. Therefore, we do not anticipate any further delays in the future detection of the pathogenic variants in the *LIPA* gene.

In conclusion, our results show that universal FH screening in children is effective also for simultaneous identification of children with other rare dyslipidemia manifesting with hypercholesterolemia (such as LAL-D), as secondary screening conditions. An important clinical criterion for differentiation between FH and the LAL-D positive children was shown to be elevated levels of transaminases (ALT and AST). Early detection and treatment of children with LAL-D are important to prevent long-term consequences.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and link to the data can be found: Mendeley; https://data.mendeley.com/datasets/zn7b85hgk6/1.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Slovenian National Medical Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

UG and TH, the corresponding author, had full access to all the data and took responsibility for the integrity of the data and the accuracy of the data analysis. US, MM, KT, TB, and TH contributed to the study concept and design, contributed data, and advised on the analysis or interpretation of the data. US, MM, TH, and UG wrote the first draft of the manuscript. KT, JK, AD, MT, AS, and TB commented on and revised the manuscript and approved the submission.

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Identification of Dysbetalipoproteinemia by an Enhanced Sampson-NIH Equation for Very Low-Density Lipoprotein-Cholesterol

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Dysbetalipoproteinemia (hyperlipoproteinemia type III, HLP3) is a genetic disorder that results in the accumulation of cholesterol on highly atherogenic remnant particles. Traditionally, the diagnosis of HLP3 depended upon lipoprotein gel electrophoresis or density gradient ultracentrifugation. Because these two methods are not performed by most clinical laboratories, we describe here two new equations for estimating the cholesterol content of VLDL (VLDL-C), which can then be used for the diagnosis of HLP3. Using results from the beta-quantification (BQ) reference method on a large cohort of dyslipidemic patients (N = 24,713), we identified 115 patients with HLP3 based on having a VLDL-C to plasma TG ratio greater than 0.3 and plasma TG between 150 and 1,000 mg/dl. Next, we developed two new methods for identifying HLP3 and compared them to BQ and a previously described dual lipid apoB ratio method. The first method uses results from the standard lipid panel and the Sampson-NIH equation 1 for estimating VLDL-C (***S***-VLDL-C), which is then divided by plasma TG to calculate the VLDL-C/TG ratio. The second method is similar, but the Sampson-NIH equation 1 is modified or enhanced (***eS***-VLDL-C) by including apoB as an independent variable for predicting VLDL-C. At a cut-point of 0.194, the first method showed a modest ability for identifying HLP3 (sensitivity = 73.9%; specificity = 82.6%; and area under the curve (AUC) = 0.8685) but was comparable to the existing dual lipid apoB ratio method. The second method based on ***eS***-VLDL-C showed much better sensitivity (96.5%) and specificity (94.5%) at a cut-point of 0.209. It also had an excellent AUC score of 0.9912 and was superior to the two other methods in test classification. In summary, we describe two new methods for the diagnosis of HLP3. The first one just utilizes the results of the standard lipid panel and the

Abbreviations: apoB, apolipoprotein B; ASCVD, atherosclerotic cardiovascular diseases; ß-VLDL, beta-migrating very low-density lipoproteins; BQ-VLDL-C, beta-quantification of VLDL-C; eS-VLDL-C, enhanced Sampson equation for VLDL-C; F-VLDL-C, Friedewald formula for VLDL-C; HLP3, hyperlipoproteinemia type III/dysbetalipoproteinemia; HTG, hypertriglyceridemia; S-VLDL-C, original Sampson equation for VLDL-C, the Sampson-NIH equation 1; VLDL-C, very low-density lipoprotein-cholesterol.

Sampson-NIH equation 1 for estimating (VLDL-C) (***S***-VLDL-C) and can potentially be used as a screening test. The second method (***eS***-VLDL-C), in which the Sampson-NIH equation 1 is modified to include apoB, is nearly as accurate as the BQ reference method. Because apoB is widely available at most clinical laboratories, the second method should improve both the accessibility and the accuracy of the HLP3 diagnosis.

Keywords: ASCVD, cholesterol, dysbetalipoproteinemia, equation, VLDL

INTRODUCTION

Dysbetalipoproteinemia, which is often also called hyperlipoproteinemia Type III (HLP3) or broad beta (β) disease, is a highly atherogenic genetic disorder of lipoprotein metabolism (Hopkins et al., 2014). HLP3 patients have a marked increased risk of both coronary artery disease (CAD) and peripheral vascular disease (Mahley et al., 1999; Yuan et al., 2007). The prevalence of HLP3 in the U.S. was calculated to be approximately 2% based on a cross-sectional analysis of U.S. adults from the National Heart and Nutrition Examination Survey and the Very Large Database of Lipids (Pallazola et al., 2019) but is likely more prevalent in patients seen at lipid clinics with mixed dyslipidemias. It is usually characterized by an almost equal increase in total plasma cholesterol (TC) and triglycerides (TG), although the elevation of these two lipids is relatively modest compared to some other types of dyslipidemias. The main metabolic abnormality in HLP3 is the accumulation of abnormal cholesterol-enriched β-migrating very low-density lipoproteins (ß-VLDL) due to impaired TG lipolysis and plasma clearance (Mahley et al., 1999). Patients with HLP3 are often first diagnosed in middle adulthood by laboratory testing or by the presence of either striate palmer xanthomas, which are considered pathognomonic for this disorder, or by the presence of tubero-eruptive xanthomas on other body surfaces, which occur in other types of dyslipidemias (Durrington, 2007).

The apolipoprotein E2 (apoE) isoform, which differs from the most common apoE3 isoform by a single amino acid substitution (Cys150→Arg), is associated with HLP3 (Mahley et al., 1999). Homozygosity of apoE2 results in reduced binding of VLDL particles to the low-density lipoprotein-receptor (LDLR) and related lipoprotein receptors, but not all homozygous patients for apoE2 have clinically manifested disease, and thus, secondary contributory factors are often also involved (Koopal et al., 2017). Dominantly inherited forms of HLP3 have also been reported for other single amino acid substitutions in apoE (Mahley et al., 1999) (Arg136→Ser/Cys; Arg142→Cys/Leu; Arg145→Cys; Lys146→Gln/Glu; Lys146→Asn; Arg147→Trp) or from the insertion of a tandem repeat of amino acids (apoE-Leiden; residues 121–127). Secondary factors that can contribute to the formation of the HLP3 phenotype in patients with predisposing apoE variants include obesity, diabetes, and hypothyroidism (Durrington, 2007). There is no specific therapy for HLP3, but making this diagnosis is prognostically useful because these patients are at a greater risk for atherosclerotic cardiovascular diseases (ASCVD) than would be estimated from their plasma lipid profile (Hopkins et al., 2005).

Results from the standard lipid panel, TC, TG, low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) cannot be used to identify HLP3 because of an overlap with other dyslipidemias (Sniderman et al., 2018). The gold standard method for identifying HLP3 is the demonstration of the presence of the ß-VLDL band by agarose gel electrophoresis. Alternatively, increased levels of cholesterol in VLDL (VLDL-C) isolated after density gradient ultracentrifugation have also long been used for identifying this disorder (Fredrickson et al., 1967). A VLDL-C/plasma TG ratio of 0.3 or more (if lipids are expressed in mg/dL) when plasma TG is between 150 and 1,000 mg/dl is the criteria most often used when the diagnosis is based on ultracentrifugation (Fredrickson et al., 1967; Fredrickson et al., 1975). Because neither agarose gel electrophoresis nor ultracentrifugation is widely used by routine clinical laboratories, the ratio of plasma TC and TGs to apolipoprotein B (apoB), the main structural protein component of VLDL, has been used to screen for HLP3 (Sniderman et al., 2007).

In 2020, we described a new and more accurate equation for estimating LDL-C (Sampson-NIH equation) based on the results of the standard lipid panel (Sampson et al., 2020). This equation consists of two components: equation 1 for estimating VLDL-C, which we call here the original Sampson VLDL-C equation (***S***-VLDL-C), and the other was Sampson-NIH equation 2 for LDL-C. The main improvement in more accurately estimating LDL-C was the more accurate estimate of VLDL-C. ***S***-VLDL-C was designed to match the level of VLDL-C found in hypertriglyceridemic (HTG) patients when measured by the reference ultracentrifugation method, beta-quantification (BQ). The ***S***-VLDL-C equation was more accurate than the Friedewald formula for VLDL-C (***F***-VLDL-C), which assumes a constant VLDL-C/TG ratio of 0.2 (Friedewald et al., 1972), or the Martin method, which uses 180 empirically derived VLDL-C/TG ratios ranging from 0.3 to 0.08 (Martin et al., 2013).

In this study, we first examined whether the improved estimate of VLDL-C by the ***S***-VLDL-C equation could be used for identifying HLP3, and we found that it can potentially be used as a screening test for HLP3. Next, we developed the new enhanced Sampson equation for VLDL-C (***eS***-VLDL-C), which is a modified ***S***-VLDL-C equation with added apoB as an independent variable. The ***eS***-VLDL-C equation had the best specificity and sensitivity for identifying HLP3 compared to ***S***-VLDL-C and ***F***-VLDL-C.

TABLE 1 | Lipid test values and demographics of the patient cohort.

	Non-HLP3 (N = 24,431)			HLP3 (N = 115)			
Test	**Mean (IQR)**	**Min**	**Max**	**Mean (IQR)**	**Min**	**Max**	***p* value**
TC	194 (157–225)	27	672	340 (243–401)	171	811	<0.0001
Non-HDL-C	147 (111–175)	12	657	301 (208–372)	132	777	<0.0001
TG	164 (83–179)	5	2,931	375 (212–512)	152	811	<0.0001
ApoB	99 (78–117)	5	377	118 (79–144)	50	401	<0.0001
HDL-C	47 (37–56)	2	201	40 (31–44)	16	90	<0.0001
HDL-TG	18 (11–23)	3	359	22 (12–28)	3	120	0.0022
LDL-C	121 (90–146)	7	593	149 (93–169)	53	628	<0.0001
LDL-TG	45 (30–51)	0	577	87 (53–103)	31	260	<0.0001
VLDL-C	26 (13–30)	0	573	152 (85–203)	46	512	<0.0001
VLDL-TG	101 (34–110)	0	2,674	267 (134–335)	35	678	<0.0001
VLDL-C/TG	0.16 (0.13–0.19)	0.00	0.69	0.4 (0.32–0.44)	0.30	0.69	<0.0001
Age	55 (45–66)	18	90	52 (44–60)	23	90	0.0244
%male	50	—	—	50	—	—	0.4428

METHODS

Deidentified lipid test results were obtained from the cardiovascular laboratory medicine program at Mayo Clinic in Rochester, MN. All results from lipoprotein metabolism profiling (which includes lipoprotein electrophoresis, apoB measurement, and the BQ reference method) ordered for clinical management between 2011 and 2021 were included (N = 24,713).

The BQ reference method was performed by a combination of ultracentrifugation and LDL precipitation as previously described (Meeusen et al., 2014; Meeusen et al., 2015). Cholesterol and nonglycerol blanked TG were measured by enzymatic methods on a Cobas 501 instrument (Roche Diagnostics, IN). Samples with detectable Lipoprotein-X on agarose gel electrophoresis (N = 126) and/or TG >3,000 mg/dl (N = 38) or TC >1,500 mg/dl (N = 3) were excluded from analysis. VLDL-C was calculated by subtracting cholesterol in the infranatant (d <1.006 g/ml), which contains LDL and HDL, from TC in plasma. ApoB was measured by the immunoturbidometric method, using a Cobas c501 analyzer (Roche Diagnostics, IN). HLP3 was defined as having a VLDL-C to plasma TG ratio greater than 0.3 and plasma TG between 150 and 1,000 mg/dl (Fredrickson et al., 1975) as determined from the BQ analysis.

The BQ dataset was randomly divided into a training dataset (N = 12,141) to first develop by a regression analysis the newly described equations, which were then validated in the other half of the data (N = 12,405). The least-square analysis for developing the new ***eS***-VLDL-C equation was done first by calculating values for the terms in the ***S***-VLDL-C equation in Excel, using the results of the standard lipid panel and then adding separate terms for apoB and an interaction term between apoB and TG. Stepwise multiple regression for predicting VLDL-C as measured by BQ was then performed with JMP software for calculating the coefficient values for each term of the ***eS***-VLDL-C equation. A similar approach was used to develop the HLP3-specific equations for predicting the cholesterol and TG content of VLDL, but only those patients identified as having HLP3 by BQ were used during the regression analysis. The receiver operating characteristic (ROC) analysis was also done with JMP software, and the optimum cut-point was calculated based on the value that gave the greatest sum of sensitivity plus specificity for identifying HLP3 when compared to the classification by the BQ analysis. Percent concordance with the BQ method for identifying HLP3 was done by calculating the percent of correctly identified patients (true positives + true negatives) out of the total number of test classifications (true positives + true negatives + false positives + false negatives). VLDL-C and LDL-C were also calculated by Sampson equations 1 and 2, respectively, as previously described (Sampson et al., 2020) using Excel. Mean lipid values were compared between groups by nonpaired *t*-test. Excel spreadsheets for performing all the calculations, including the new equations described here, can be freely downloaded at https://figshare.com/articles/software/Sampson_enhanced_VLDLC_phenotype_calculator/19666347.

RESULTS

Lipid values as determined by the BQ reference method and demographic characteristics of patients in the cohort are shown in **Table 1**. Based on these results, approximately 0.5% of patients were classified as having HLP3 by having a VLDL-C/TG ratio ≥0.3 and a plasma TG between 150 and 1,000 mg/dL. As expected, patients classified as HLP3 had modest increases in plasma TC, non-HDL-C, and TG. They also had an increase in the mean level of apoB compared to the non-HLP3 patients, but the relative increase in apoB was less than for the changes observed in plasma lipids. Although VLDL-TG was also increased in HLP3, there was a much larger relative increase in VLDL-C, leading to a mean VLDL-C/TG ratio of 0.4, which was more than two times the mean value observed in the non-HLP3 group. We did not observe any significant difference in the sex distribution and only a small difference in the mean age between the two groups.

We also observed enrichment of TG in both HDL and LDL in HLP3 patients (**Table 1**), suggesting an abnormal lipid composition for these lipoprotein particles too. This was further analyzed in **Figure 1** by examining the ratio of cholesterol and TG in the major lipoprotein fractions. A much greater fraction of plasma TC, almost 90% on average, was

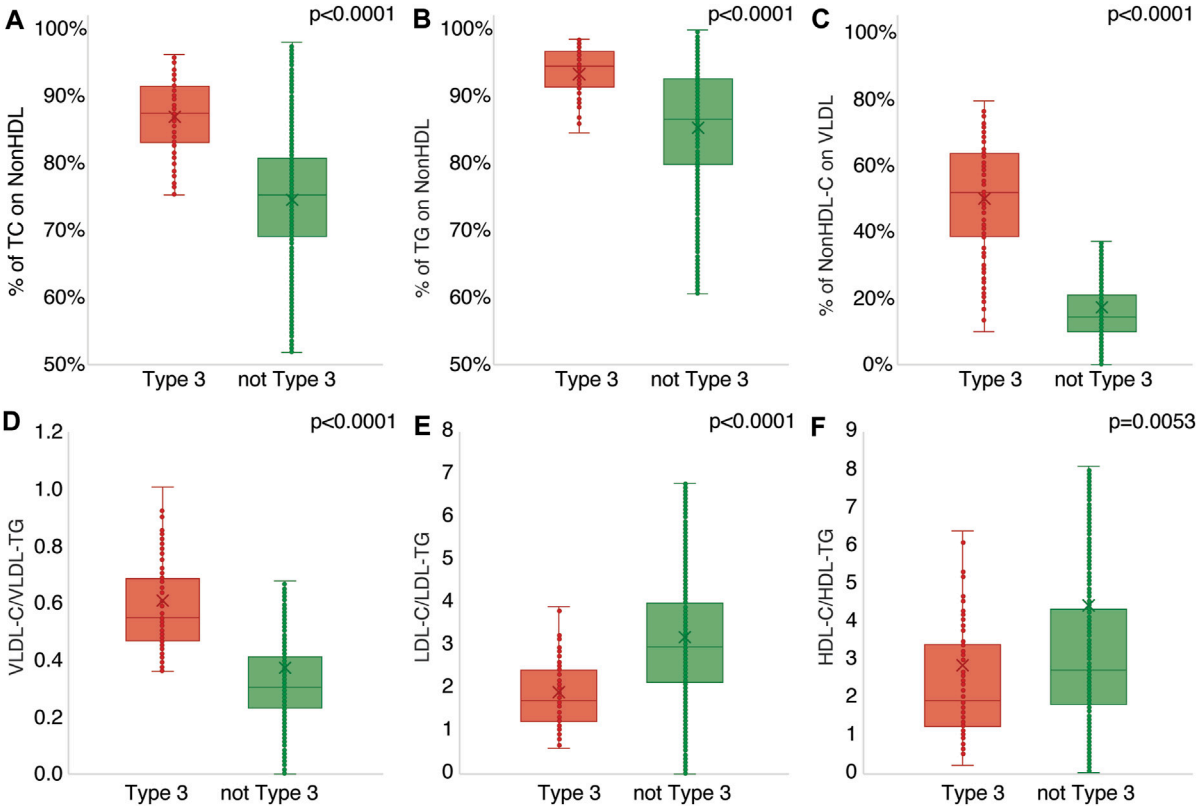

FIGURE 1 | Comparison of lipid ratios between HLP3 and non-HLP3 patients. Specimens determined to have HLP3 (N = 115, red) by BQ were compared against those without HLP3 (N = 24,431, green) for the % of TC on non-HDL **(A)**, % of TG on non-HDL **(B)**, % of non-HDL-C on BQ VLDL **(C)**, the ratios of VLDL-C/VLDL-TG **(D)**, LDL-C/LDL-TG **(E)**, and HDL-C/HDL-TG **(F)**.

present in the non-HDL fraction for HLP3 patients (**Figure 1A**). This was due to the lower level of HDL-C in these patients and the enrichment of cholesterol on VLDL (**Table 1**). The percent of TG found in non-HDL was also higher for HLP3 than non-HLP3 (**Figure 1B**), but the difference was less than what was observed for cholesterol (**Figure 1A**). Unlike the non-HLP3 group in which most of the cholesterol that makes up non-HDL-C is in LDL, we found that approximately half of the cholesterol on nonHDL particles is found in VLDL in HLP3 patients (**Figure 1C**). As previously reported, this is a consequence of delayed plasma clearance of VLDL and the replacement of TG in VLDL for cholesteryl esters (CE) by Cholesteryl Ester Transfer Protein (CETP) mediated lipid exchange (Barter et al., 2003), which results in the increased VLDL-C/VLDL-TG ratio in HLP3 (**Figure 1D**). CETP-mediated lipid exchange likely also accounts for the observed enrichment of TG over cholesterol in both LDL and HDL (**Figures 1E,F**) because when CETP removes TG from VLDL in exchange for CE, it causes the TG-enrichment of LDL and HDL (Barter et al., 2003).

In addition to the standard lipid panel, the only other routine lipid-related diagnostic test that could potentially improve VLDL-C estimation is apoB, which is present as a single copy per VLDL particle (Elovson et al., 1988). Using the same terms as in the original Sampson-NIH equation 1 for VLDL-C, called here ***S***-VLDL-C (Sampson et al., 2020), and adding apoB and an interaction term between apoB and TG, we developed by the regression analysis the following enhanced Sampson VLDL-C equation (***eS***-VLDL-C):

$$\textit{eS-VLDL-C} = \frac{\textit{non-HDL-C}}{3.81} - \frac{\textit{HDL-C}}{8.93} + \frac{\textit{TG}}{7.73} + \frac{(\textit{non-HDL-C} \times \textit{TG})}{2050} - \frac{\textit{TG}^2}{13300} - \frac{\textit{ApoB}}{2.49} - \frac{(\textit{ApoB} \times \textit{TG})}{3550} + 7.46.$$

Based on the mean absolute difference (MAD) and other quantitative metrics of accuracy (slope, intercept, correlation coefficient (R^2), and root mean square error (RMSE)), the ***eS***-VLDL-C equation appeared to yield relatively accurate results, which had a broad range of VLDL-C (0–514 mg/dl) and TG values (5–2,853 mg/dl) in the training dataset (**Figure 2**).

Next, we evaluated the accuracy of the new ***eS***-VLDL-C equation, the ***S***-VLDL-C equation, and the Friedewald equation (TG/5) for VLDL-C (***F***-VLDL-C) in the validation dataset when compared to the BQ reference method (**Figure 3**). Based on MAD and other metrics of accuracy shown in the three plots, the ***eS***-VLDL-C equation

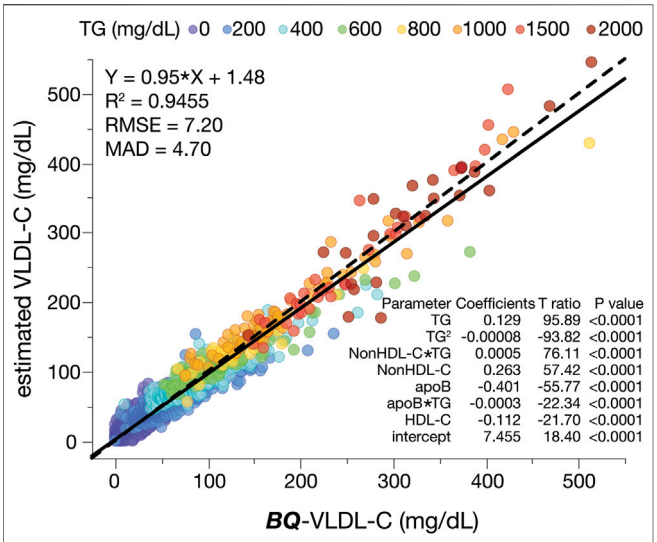

FIGURE 2 | Development of the enhanced VLDL-C equation (*eS*-VLDL-C). The training dataset (N = 12,141) was used in multiple regression analysis with the indicated input variables. The solid line is the linear fit for indicated regression equation. A dashed line is the line of identity. Results are color-coded by TG levels with the value in the legend indicating the start of each interval.

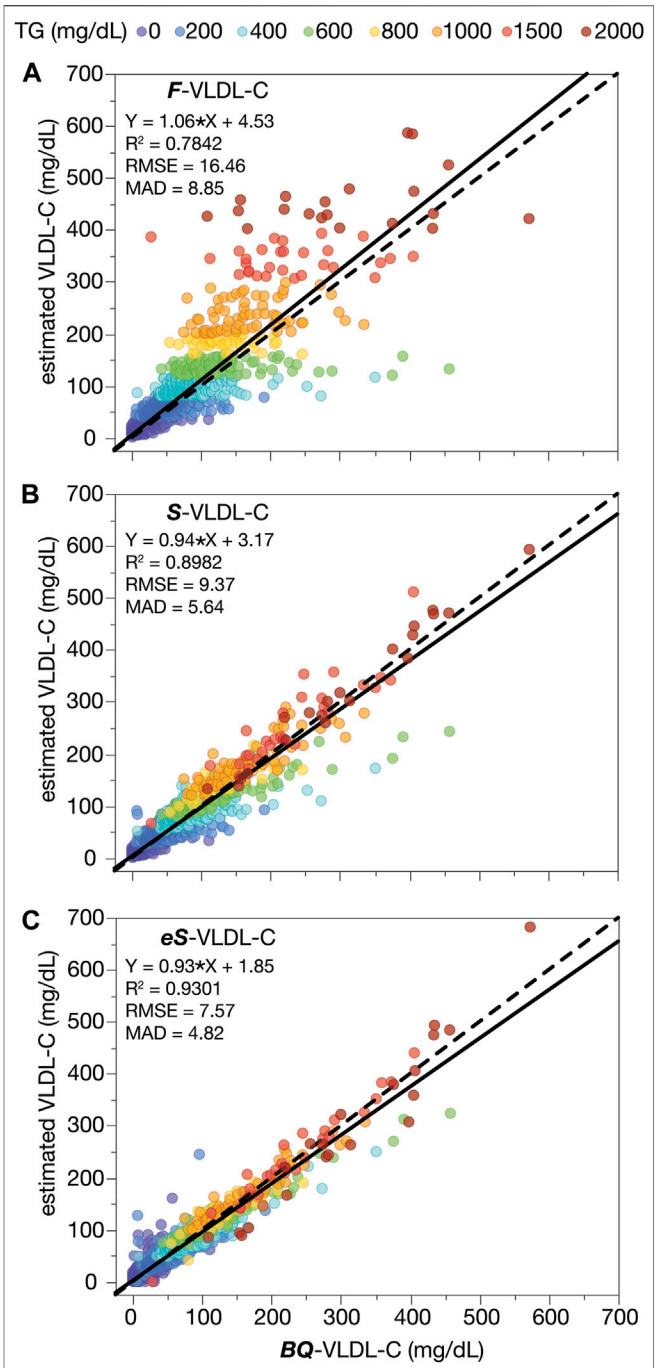

FIGURE 3 | VLDL-C by calculation versus β-quantification. VLDL-C was calculated from the results of a standard lipid panel in the validation dataset (N = 12,405) by the Friedewald VLDL-C formula (*F*-VLDL-C) **(A)**, the Sampson VLDL-C equation (*S*-VLDL-C) **(B)**, and the enhanced Sampson VLDL-C equation (*eS*-VLDL-C) **(C)** and plotted against VLDL-C as measured by BQ. Solid lines are the linear fit for indicated regression equations. Dashed lines are the line of identity. Results are color-coded by TG levels with the value in the legend indicating the start of each interval.

(**Figure 3C**) showed the greatest accuracy followed by the original ***S***-VLDL-C equation (**Figure 3B**) and then the ***F***-VLDL-C equation (**Figure 3A**). As has been well described (Sampson et al., 2020), the Friedewald equation shows a positive bias for VLDL-C with increasing TG. In addition, the Friedewald equation for VLDL-C shows a marked increase in its variance for high TG samples, making it unreliable when TG is greater than 400 mg/dl. This problem occurs to a lesser degree with the ***S***-VLDL-C equation and an even lesser degree with the ***eS***-VLDL-C equation that includes apoB for estimating VLDL-C.

In **Figure 4**, the VLDL-C to plasma TG ratio (VLDL-C/TG) was calculated by the ***S***-VLDL-C equation and the new ***eS***-VLDL-C equation and compared to results obtained by the BQ reference method. Only a small percent of patients (11%) identified as having HLP3 by BQ had a VLDL-C/TG ratio greater than 0.3 when VLDL-C was calculated by the ***S***-VLDL-C equation (**Figure 4A**). Those patients classified as non-HLP3 by the BQ reference method did, however, have a much lower VLDL-C/TG ratio than the HLP3 group. In contrast, approximately 41% of HLP3 patients as determined by BQ did have a VLDL-C/TG ratio greater than 0.3 when calculated by the ***eS***-VLDL-C equation (**Figure 4B**).

Because HLP3 patients were only a small minority of the total number of patients that we used to develop our regression equations for estimating VLDL-C, it is not unexpected that the regression equations would underestimate VLDL-C given that the mean VLDL-C level for the HLP3 group is almost 5 times higher than the non-HLP3 group (**Table 1**). We, therefore, investigated by the ROC analysis whether a different cut-point for the VLDL-C/TG ratio could better discriminate between the two groups. At a cut-point of 0.194, the ***S***-VLDL-C equation did show a modest ability to identify HLP3, with a sensitivity of 73.9% and a specificity of 82.6% (**Figure 4C**). It also showed a moderately good AUC score, an overall metric of test

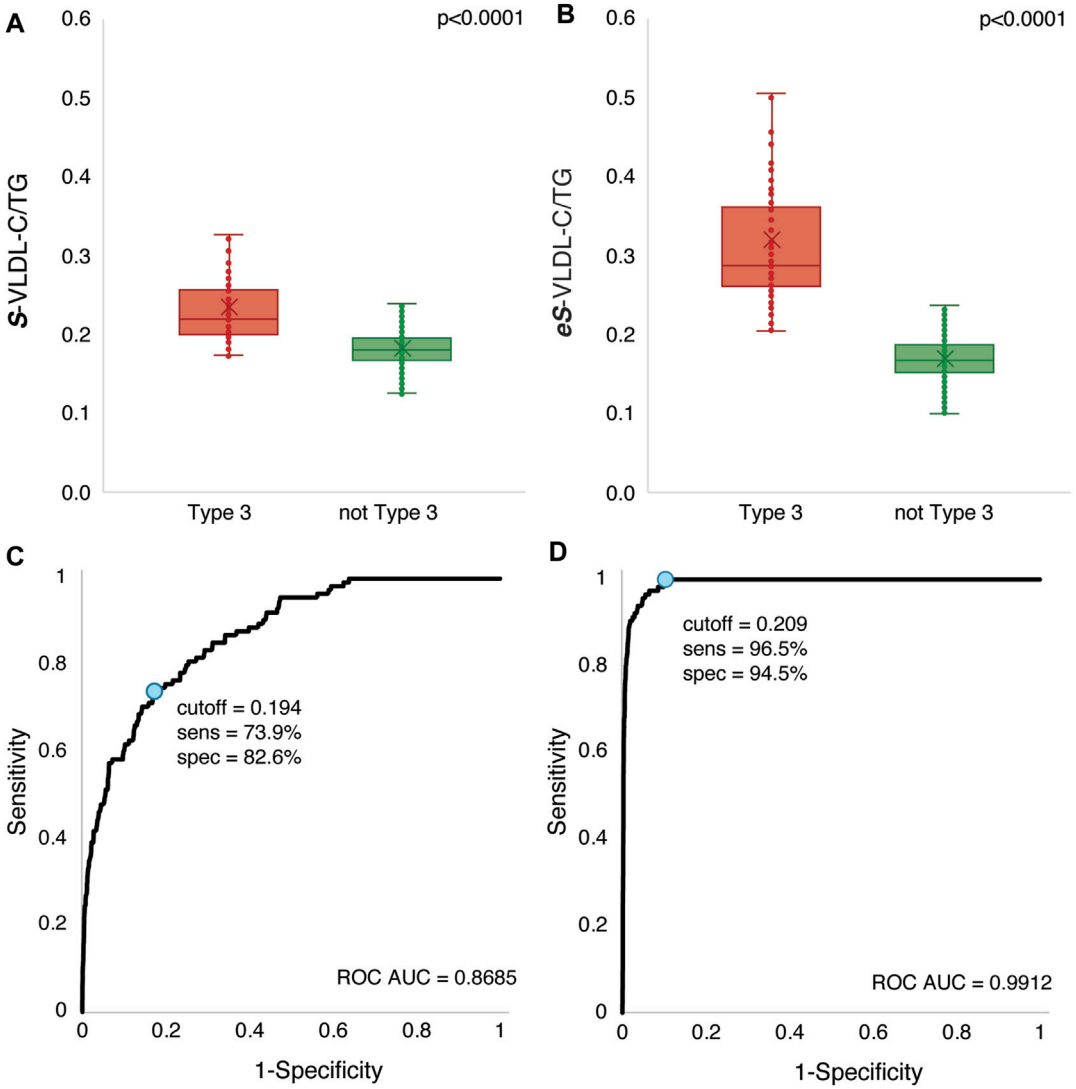

FIGURE 4 | VLDL-C/TG ratio comparison in HLP3 and non-HLP3 patients. VLDL-C was calculated by the Sampson VLDL-C/TG (*S*-VLDL-C/T) **(A)** and enhanced Sampson VLDL-C/TG (*eS*-VLDL-C/T) **(B)** equations in the validation dataset (N = 12,405) from the results of a standard lipid panel. HLP3 was defined as VLDL-C/TG > 0.3 mg/dl and TG between 150–1,000 mg/dl using the BQ results. The ROC curves are plotted for the Sampson VLDL-C/TG (*S*-VLDL-C/T) **(C)** and enhanced Sampson VLDL-C/TG (*eS*-VLDL-C/T) **(D)** equations. The optimal cut-point (blue circle) and its corresponding sensitivity and specificity are indicated for each equation.

classification, of 0.8685. At a cut-point of 0.209, the ***eS***-VLDL-C, however, showed much better sensitivity (96.5%) and specificity (94.5%) and an excellent AUC score of 0.9912 (**Figure 4D**).

Next, we compared the ***S***-VLDL-C and ***eS***-VLDL-C equations to the previously described dual lipid apoB ratio method (Sniderman et al., 2007; Sniderman et al., 2018), which requires both TC/apoB≥6.2 and TG/apoB≤10 ratios (when units are in mmol/L) for identifying HLP3 (**Figure 5**). When used at the near-optimal cut-point shown in **Figure 4**, the ***S***-VLDL-C equation was much more sensitive than the dual lipid apoB ratio test but much less specific (**Figure 5A**). The ***eS***-VLDL-C equation at its optimal cut-point was more sensitive than the dual apoB ratio method but had a similar specificity. The ***eS***-VLDL-C equation was both more sensitive and specific than the ***S***-VLDL-C equation (**Figure 5A**) and had an overall concordance with the BQ method of 94.6%. Next, we changed the cut-point of the ***S***-VLDL-C and ***eS***-VLDL-C equations to match either the sensitivity (**Figure 5B**) or specificity (**Figure 5C**) of the dual lipid apoB ratio method. Based on this analysis, the ***S***-VLDL-C equation was similar in diagnostic test performance to the dual lipid apoB ratio method for identifying HLP3, and the ***eS***-VLDL-C equation appeared to show overall the best sensitivity and specificity compared to the two other methods. Similar conclusions were found on the relative diagnostic value of the three methods by giving equal value to sensitivity and specificity (Sen + Spec/2) and by using the normalized Matthews correlation coefficient (MCC) index (**Figure 5D**), which adjusts for the prevalence of disease (Chicco et al., 2021).

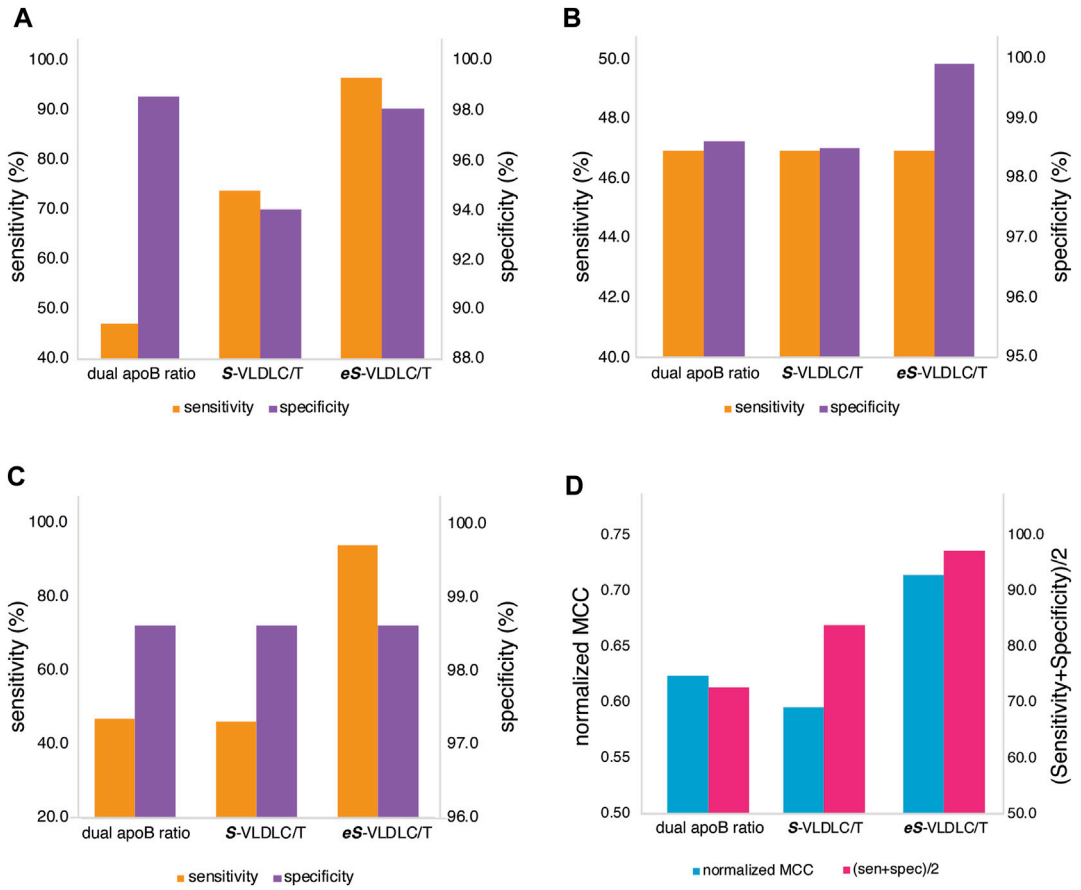

FIGURE 5 | Comparison of test accuracy for HLP3 classification. Classification accuracy in the validation dataset (N = 12,405) by sensitivity (orange) and specificity (purple) for the dual apoB ratio method, the Sampson VLDL-C/TG (*S*-VLDL-C/T), and enhanced Sampson VLDL-C/TG (*eS*-VLDL-C/T) methods are shown for the optimal cut-points **(A)**, after adjustment to match the dual apoB ratio sensitivity **(B)**, and after adjustment to match the dual apoB ratio specificity **(C)**. The normalized MCC score (blue) and the average of sensitivity and specificity (sen + spec/2; pink) are shown for the optimal cut-points **(D)**.

Finally, given the residual bias observed in the ***eS***-VLDL-C equation (**Figure 3**), we also developed specific equations that utilize apoB for estimating both VLDL-C (**Figure 6A**) and VLDL-TG (**Figure 6B**), once a patient is identified as having HLP3. As will be discussed in the following, these equations may potentially be used for monitoring response to lipid-lowering therapy for HLP3 patients.

DISCUSSION

Identification of HLP3 is useful in the management of these patients because their risk for cardiovascular diseases (CVD) is greater than what would be expected based on their standard lipid profile. In particular, LDL-C is a poor risk marker in this type of dyslipidemia not only because of their elevated TG but also because of a much greater fraction of cholesterol carried on remnant particles, which are known to be particularly proatherogenic (Hopkins et al., 2014). In addition, although these patients frequently show a good response to statins and lifestyle changes, which diminishes the impact of secondary contributory factors for this disorder, they can sometimes still have residual risk from remnant lipoproteins after statin treatment and may require a second lipid-lowering agent for lowering plasma TG.

We describe here two new methods for identifying HLP3. The first simply involves using results from the standard lipid profile for estimating VLDL-C, which is the original Sampson-NIH equation 1 (***S***-VLDL-C) (Sampson et al., 2020) that is currently used by clinical laboratories together with the Sampson-NIH equation 2 for LDL-C calculation. This approach has not been previously used in the case of the ***F***-VLDL-C equation because it always assumes a fixed ratio of VLDL-C/TG of 0.2. Although our new method based on the standard lipid panel shows a reasonably good sensitivity of over 70% for detecting HLP3, its specificity of about 80% is not ideal for a relatively rare disorder like HLP3. It may, however, have some utility as a screening test, because a positive presumptive score by this method could trigger the performance of a physical exam to look for signs and symptoms of HLP3. Results from this test could also be used by clinical laboratory information systems to automatically reflex to measuring apoB in order to make a more definitive diagnosis. We recently reported on a simple

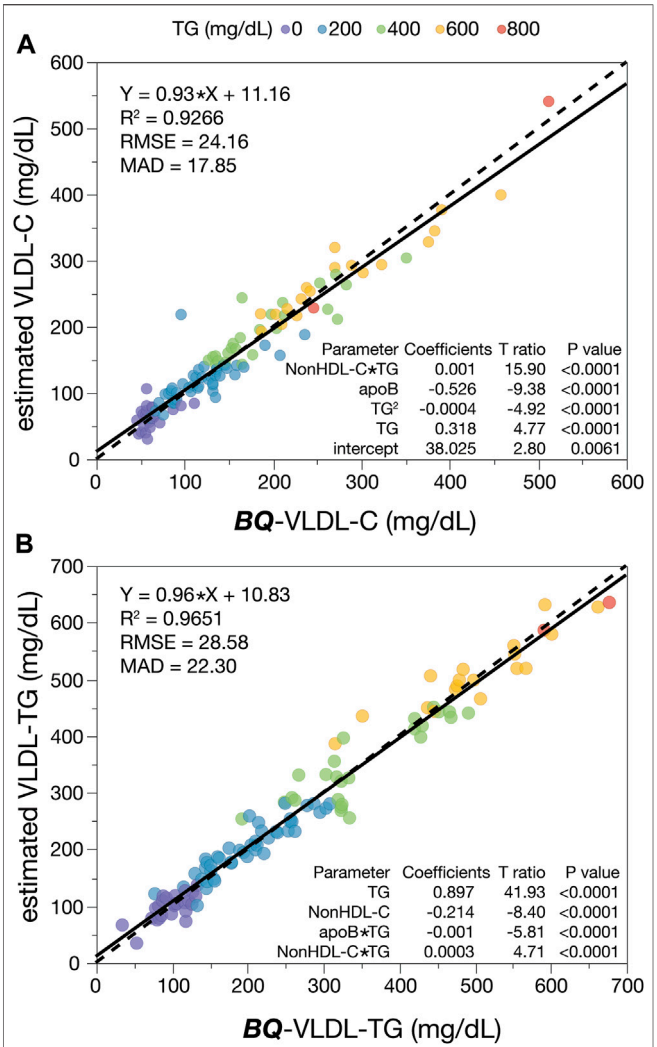

FIGURE 6 | HLP3 specific equations for VLDL-C and VLDL-TG. HLP3 patients (N = 115) were used to develop a regression analysis for VLDL-C **(A)** and VLDL-TG **(B)** with the indicated input variables that include apoB. Solid lines are the linear fit for the indicated regression equation. Dashed lines are the line of identity. Results are color-coded by TG levels with the value in the legend indicating the start of each interval.

algorithm for using plasma non-HDL-C and TG for classifying all the common lipoprotein dyslipidemic phenotypes except for HLP3 and described how it can be used as a practical aid in the clinical management of patients (Sampson et al., 2021). With the use of the new method described here for HLP3, one can now identify all the common lipoprotein phenotype disorders from the standard lipid panel and the phenotypes could potentially be automatically reported along with the lipid test results.

The second method we developed for identifying HLP3 is both more sensitive and specific than the ***S***-VLDL-C equation, the ***F***-VLDL-C formula, or the dual lipid apoB ratio test. By using apoB, we were able to modify the ***S***-VLDL-C equation for improving its accuracy for VLDL-C. ApoB is present as a single copy per LDL (Knott et al., 1986), VLDL (Elovson et al., 1988), and chylomicron (Phillips et al., 1997) particle and thus provides a measure of the particle number for these lipoproteins. All of these lipoprotein particles are highly diverse in their size, which affects their TG and cholesterol carrying capacity. This likely accounts for why the inclusion of apoB in the ***eS***-VLDL-C equation improves its accuracy, particularly for HLP3, which is characterized by having a large number of small VLDL particles (Hopkins et al., 2014). Both terms in the ***eS***-VLDL-C equation that includes apoB have negative coefficients, which indicates that the use of apoB helps compensate for the overestimation of VLDL-C by the other variables in the equation. It is also worth noting that although apoB was not markedly elevated in HLP3 like it has been described for familial combined hyperlipidemia (Sniderman et al., 2018), it was modestly increased in at least some subjects. It is likely given the known metabolic defect in HLP3 that some of the excess apoB in HLP3 would reside in small dense VLDL rather than LDL, but this was not directly tested in this study and would be a valuable future research direction.

We also describe two other equations, which are specific for calculating the cholesterol and TG content of VLDL for already diagnosed HLP3 patients. These equations cannot be used for initially identifying HLP3 and should also not be used for normolipidemic patients because it will lead to an overestimation of VLDL-C and VLDL-TG. Nevertheless, these two equations may be potentially useful for monitoring response to lipid-lowering therapy for HLP3, but this will require additional future studies to determine if this is valuable or not.

A limitation of our approach is that an additional test, namely, apoB, will be required to more definitively make the diagnosis of HLP3. It has recently been shown, however, in several large studies that apoB is the most accurate lipid-related measure of CVD risk (Sniderman et al., 2019; Marston et al., 2022), and thus it will likely be used more often in the future even for primary screening. The 2019-European Society of Cardiology/European Atherosclerosis Society guidelines for the management of dyslipidemias (Mach et al., 2019), recommended that physicians consider measuring apoB for CVD risk assessment, particularly in patients with HTG, diabetes, obesity, metabolic syndrome, or low levels of LDL-C. These guidelines also state that when available, apoB can also be used as an alternative to LDL-C as the primary means to screen, diagnose, and manage patients.

Another limitation of our study is that we only compared the ***S***-VLDL-C equation (the original Samspon-NIH equation 1) and the ***eS***-VLDL-C equation to the ***F***-VLDL-C formula and did not examine other potential LDL-C equations. Because the original Sampson-NIH equation was previously shown to be more accurate than all other LDL-C equations, particularly for high TG samples (Sampson et al., 2020), it is unlikely that any alternative equation would be superior for identifying HLP3. In addition, none of the previously developed LDL-C equations, including the original Sampson-NIH equation, contained apoB as an independent variable like the ***eS***-VLDL-C equation. We also did not directly assess the clinical utility of the new ***eS***-VLDL-C equation in the routine clinical laboratory setting, which will have to await a future study. It will also be important to test the new ***eS***-VLDL-C equation in a wide variety of different ethnic/racial groups to determine its generalizability.

In summary, the two new equations, ***S***-VLDL-C and ***eS***-VLDL-C, that we describe for identifying HLP3 potentially have great practical value for the clinical management of this disorder because they only involve the use of the standard lipid panel and apoB, which are both relatively inexpensive and widely available. Future studies, however, will be needed in different types of patient cohorts to assess the clinical impact of early detection and monitoring of HLP3 patients by these equations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study of human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/ participants or patients/participants legal guardian/ next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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A homozygous variant in the *GPIHBP1* gene in a child with severe hypertriglyceridemia and a systematic literature review

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Background: Due to nonspecific symptoms, rare dyslipidaemias are frequently misdiagnosed, overlooked, and undertreated, leading to increased risk for severe cardiovascular disease, pancreatitis and/or multiple organ failures before diagnosis. Better guidelines for the recognition and early diagnosis of rare dyslipidaemias are urgently required.

Methods: Genomic DNA was isolated from blood samples of a Pakistani paediatric patient with hypertriglyceridemia, and from his parents and siblings. Next-generation sequencing (NGS) was performed, and an expanded dyslipidaemia panel was employed for genetic analysis.

Results: The NGS revealed the presence of a homozygous missense pathogenic variant c.230G>A (NM_178172.6) in exon 3 of the *GPIHBP1* (glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1) gene resulting in amino acid change p.Cys77Tyr (NP_835466.2). The patient was 5.5 years old at the time of genetic diagnosis. The maximal total cholesterol and triglyceride levels were measured at the age of 10 months (850.7 mg/dl, 22.0 mmol/L and 5,137 mg/dl, 58.0 mmol/L, respectively). The patient had cholesterol deposits at the hard palate, eruptive xanthomas, lethargy, poor appetite, and mild splenomegaly. Both parents and sister were heterozygous for the familial variant in the *GPIHBP1* gene. Moreover, in the systematic review, we present 62 patients with pathogenic variants in the *GPIHBP1* gene and clinical findings, associated with hyperlipoproteinemia.

Conclusion: In a child with severe hypertriglyceridemia, we identified a pathogenic variant in the *GPIHBP1* gene causing hyperlipoproteinemia (type 1D). In cases of severe elevations of plasma cholesterol and/or triglycerides genetic testing for rare

dyslipidaemias should be performed as soon as possible for optimal therapy and patient management.

KEYWORDS

hypertriglyceridemia, triglycerides, glycosylphosphatiliylinositol-anchored high-density lipoprotein-binding protein 1, hyperlipidemia, GPIHBP1

Introduction

Affecting 15–20% of the population (Parhofer and Laufs, 2019; Basit et al., 2020), hypertriglyceridemia has been associated with an increased risk for pancreatitis (Carrasquilla et al.; Simha, 2020; Álvarez-López et al., 2021). In adults with severe hypertriglyceridemia only up to 2% of the cases could be explained by a monogenic variant in genes involved in triglyceride (TG) metabolism (Hegele et al., 2020). Polygenic variants of smaller effects combined with environmental factors are considered a primary cause of hypertriglyceridemia. Secondary causes include diabetes mellitus, metabolic syndrome, alcohol and commonly used drugs (Simha, 2020).

Hypertriglyceridemia is defined as fasting triglyceride (TG) levels over 2 mmol/L (180 mg/dl), whereas in severe hypertriglyceridemia fasting TG levels exceed 10 mmol/L (885 mg/dl) (Hegele et al., 2020). Patients with the most severe phenotypes start expressing clinical symptoms at a younger age, usually have serum TG levels above 11.3 mmol/L (1000 mg/dl), and in some cases also have abdominal pain related to acute pancreatitis, hepatosplenomegaly, lipemia retinalis, and eruptive xanthomata already in childhood (Gonzaga-Jauregui et al., 2014; Brown et al., 2020; Hegele et al., 2020).

Lipoprotein lipase (LPL) mediates the hydrolysis of triglycerides packed in lipoproteins such as chylomicrons and very-low-density lipoprotein (VLDL) (Wu et al., 2021). Many factors interact with LPL affecting TG metabolism. Dysfunction of LPL and other factors interacting with LPL may lead to hypertriglyceridemia (Liu et al., 2018). Besides *LPL* there are other genes involved in the LPL-mediated lipolysis of chylomicrons and VLDL: *ANGPTL4* (angiopoietin-like 4), *APOC2* (apoprotein C-II), *APOA5* (apolipoprotein A-V), *LMF1* (lipase maturation factor 1), and *GPIHBP1* (glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1) (Hegele et al., 2020; Kersten, 2021).

GPIHBP1 binds and transports LPL to the capillary lumen from interstitial space, where it hydrolyses TG and triglyceride-rich lipoproteins (TRLs) (Supplementary Figure S1). In patients with GPIHBP1 deficiency, LPL is mislocalized and intravascular hydrolysis of triglycerides is impaired (Beigneux et al., 2017; Hegele et al., 2020; Wu et al., 2021). The consequence is low plasma levels of LPL, and severe hypertriglyceridemia (Beigneux et al., 2017). The prevalence of type I hyperlipoproteinemia because of a pathogenic variant in the *GPIHBP1* is estimated between 1:500,000 to 1:1,000,000 (Gonzaga-Jauregui et al., 2014).

Methods

Study design and family description

The National Medical Ethics Committee approved the study in Slovenia (0120-14/2017/5, and 0120-273/2019/9), and the Ethics Committee approved the study in Pakistan (033-523-2019). The principles of the Declaration of Helsinki were followed. Written consent of the patient's parents was obtained before inclusion.

Lipid profile analysis

Serum samples were analyzed for lipids including TC, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and TG. TC, TG and LDL-C were measured by a homogenous enzymatic method (Cobas 8000 c502 module, Roche, United States).

Lipid levels were considered as normal if: TC < 4.4 mmoL/L (170 mg/dl), LDL-C <2.8 mmoL/L, (110 mg/dl) HDL-C >1.2 mmoL/L (45 mg/dl), TG < 0.8 mmoL/L (75 mg/dl) for children <9 years of age and <1 mmoL/L (90 mg/dl) if > 9 years of age. Lipid levels were considered as elevated/lowered if: TC > 5.2 mmoL/L (200 mg/dl), LDL-C >3.4 mmoL/L (130 mg/dl), HDL-C <1 mmoL/L (40 mg/dl), and TG > 1.1 mmol/L (100 mg/dl) for children <9 years of age and >1.5 mmoL/L (130 mg/dl) if > 9 years of age. The lipid levels in-between the cut-offs were considered borderline (De Jesus, 2011).

Genetic analyses

All genetic analyses were performed at the University Children's Hospital Ljubljana in Slovenia in the same way as Slovenian national genetic testing for the universal familial hypercholesterolemia (FH) screening program in preschool children (Groselj et al., 2018, 2022; Sustar et al., 2022). Genomic DNA was isolated from the patient's and his family members (mother, father and sister) peripheral blood using a Flexigene kit (Qiagen). xGen® Lockdown® NGS Probes (IDT,

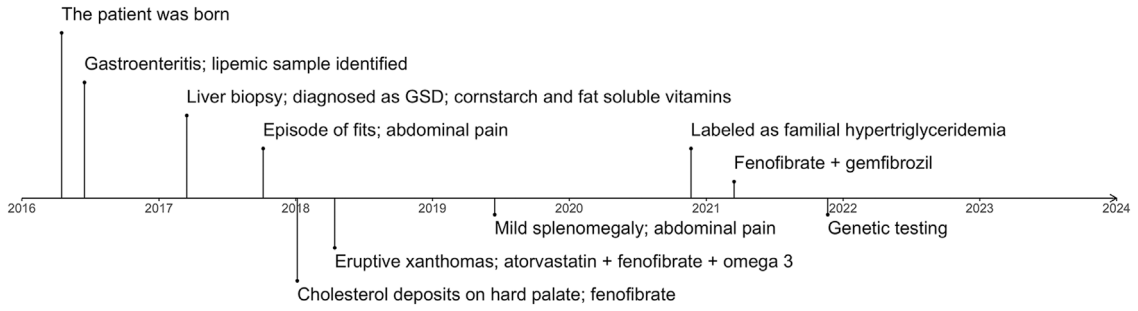

FIGURE 1
The timeline represents important complications and milestones in diagnosing and managing the patient. GSD, glycogen storage disease.

United States) for detection of coding and promoter regions for the genes associated with dyslipidemia were used (*ABCA1, ABCG5, ABCG8, ALMS1, APOA1, APOA5, APOB, APOC2, APOC3, APOE, CREB3L3, GPIHBP1, LDLR, LDLRAP1, LIPA, LMF1, LPL,* and *PCSK9*). Samples were sequenced on MiSeq sequencer with MiSeq Reagent Kit (Illumina, United States) following the manufacturer's protocol including recommendations for quality control parameters. For variant annotation and filtration, the VafAFT tool was applied (Desvignes et al., 2018). The detected variants were classified by the American College of Medical Genetics and Genetics and the Association for Molecular Pathology (ACMG-AMP) (Richards et al., 2015) classification criteria as (likely) benign, variants of uncertain significance (VUS) and (likely) pathogenic. The pathogenic variant in the *GPIHBP1* gene was reconfirmed by targeted Sanger DNA sequencing.

Systematic literature review

We gathered all accessible scientific case report publications for the systematic review of pathogenic variants in the *GPIHBP1* gene. The systematic review was registered at PROSPERO (CRD42022336232). An electronic search was performed using the keyword "GPIHBP1" in the PubMed database on 11 June 2022. Moreover, we searched for the articles related to pathogenic variants also through the search in the Human Gene Mutation Database (Stenson et al., 2003) and the Franklin by Genoox tool based on the pathogenic variant, confirmed in our patient "NM 178172.6:c.230G>A" (scope: "Gene"). By going through all of the abstracts and titles found, we included all articles meeting the following requirements: 1) articles in English published after 2002, 2) articles containing human data, 3) only articles or data from articles describing pathogenic variants in the *GPIHBP*1 gene, 4) reported patients were homozygous or compound heterozygous for pathogenic variants, and 5) clinical data on patients was provided.

Results

The proband was a 5.5-year-old boy. He was born at a full-term birth weight of 3.7 kg with no known antenatal issues. The timeline of the diagnosing, treatment and management of the patient is represented in Figure 1. The parents of the patient are in a consanguineous marriage and are first-degree relatives. The patient's mother has had an early abortion and has a 2-year-old healthy daughter. The maternal grandfather of the patient had a heart attack at 28 years of age. No family history of pancreatitis is known. The lipid profile of the proband and his family members is represented in Supplementary Figure S2.

At 2 months of age, the patient had an episode of gastroenteritis needing intravenous hydration. At that time mother was first notified of "pink blood" (lipemic sample). Repeated sampling confirmed the same finding. The child was later taken to a local hospital where he was seen by a paediatric gastroenterologist who advised liver biopsy. The child had an initial liver biopsy done at 10 months of age, which showed a severely autolytic sample with Periodic acid–Schiff (PAS) stain positive in preserved areas suggestive of glycogen storage disease (GSD). At the same time, other laboratory measurements were elevated: total serum cholesterol of 850.7 mg/dl (22.0 mmol/L) and serum triglycerides of 5,137 mg/dl (58.0 mmol/L). However, fasting glucose was raised significantly (29.5 mmol/L) along with total bilirubin levels (110.0 umol/L) and ALT 550 U/L. The TC and TG levels of the patient over time are represented in Figure 2. On examination, he had pallor and a soft abdomen with no hepatomegaly. The patient was labelled as a case of GSD. Based on that, the patient was advised to start taking cornstarch, fenofibrate, allopurinol, and vitamins A, D and E.

Aged 1 year and 5 months the patient presented to the Paediatric outpatient department in Shifa Hospital for the first time with complaints of one episode of vague seizure-like activity and abdominal pain for 3 months. At age 1 year and 8 months, the patient developed hoarseness of voice. He was noted to have 2–3 yellow plaques on the hard palate. At this visit, his TC level

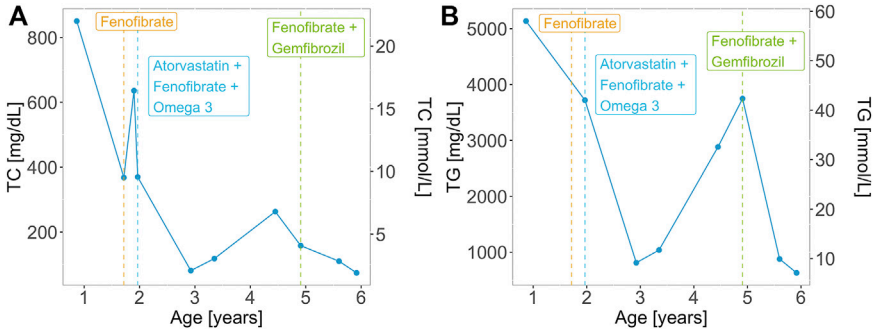

FIGURE 2
(A) Total cholesterol (TC) and **(B)** triglyceride (TG) levels over time for the patient with the homozygous GPIHBP1 pathogenic variant. Vertical lines represent the initiation and/or modification of the treatment.

was 368.0 mg/dl (9.5 mmol/L). Two months later he had eruptive xanthomas, a TC levels of 636.0 mg/dl (16.4 mmol/L). Aged 2 his TC levels were 370 mg/dl (9.6 mmol/L) and his TG levels were 3,720 mg/dl (42.0 mmol/L). Along with fenofibrate and omega 3 the patient was prescribed atorvastatin (5 mg) with increasing dosage (10 mg) upon a follow-up visit. Treatment of the patient over time is represented in Figure 2. The patient presented for follow-ups with lab results that repeatedly showed TG and total serum cholesterol above normal limits. He never had any documented hypoglycemia, drowsiness or seizure activity. He didn't have hepatomegaly.

Two years following the initial presentation, with regular follow-ups in between, the boy presented to the outpatient department with pain in the abdomen, lethargy and poor appetite. There was mild splenomegaly and upon repetition of laboratory tests, ALT (29 U/L) and serum lipase levels were normal, TC level was 81.0 mg/dl (2.1 mmol/L), LDL-C 18.0 mg/dl (0.5 mmol/L), HDL 8 mg/dl (0.2 mmol/L), triglycerides were 1,068 mg/dl (12.1 mmol/L) and 2,883 mg/dl (32.6 mmol/L) subsequently, despite receiving the treatment with atorvastatin, fenofibrate 50 mg once daily and omega 3 capsules twice a day.

The diagnosis was reviewed at age 4.5 years due to persistently high serum TG and relabeled as a case of primary/familial hypertriglyceridemia based on the lipid profile. The patient was advised to stop using cornstarch, allopurinol and atorvastatin and continue fenofibrate. On follow-up, the patient, weighing 14 kg at 4 years and 11 months of age, had his TC within normal range, however, TGs were still deranged (3749 mg/dl, 42.3 mmol/L). Response to fenofibrate (67 mg twice a day) was inadequate with persistent high TGs (>1000 mg/dl; 11.3 mmol/L), therefore gemfibrozil (300 mg twice a day) was added to the treatment regime. Aged 5 years and 7 months the triglycerides were 878.0 mg/dl (9.9 mmol/L), TC 110.0 mg/dl (2.8 mmol/L), LDL-C 10 mg/dl (0.3 mmol/L), HDL-C 0.2 mmol/L (8.0 mg/dl). His weight and height were at the 5th centile for his age and gender. At age 5 years and 11 months his lipid profile levels were: TC: 74 mg/dl (1.9 mmol/L), LDL-C: 10 mg/dl (0.3 mmol/L), HDL-C: 8 mg/dl (0.2 mmol/L) and TG: 630 mg/dl (7.1 mmol/L). At that point, he had no xanthomas.

At age 5.5 genetic testing was performed and we identified a homozygous variant c.230G>A (NM_178172.6) in exon 3 of the *GPIHBP1* gene (NG_034256.1) leading to a protein change p.Cys77Tyr (NP_835466.2). The patient's family members (father, mother and sister) were heterozygous for the c.230G>A variant (Supplementary Figures S2, S3). The variant has already been reported in the ClinVar (VCV000917845.1) (Landrum et al., 2018) as likely pathogenic in association with hyperlipoproteinemia (type ID) phenotype. The variant was classified as pathogenic by the *in silico* prediction tools (Revel, MetaLR, MetaSVM) (Ioannidis et al., 2016). The frequency of the variant in the gnomAD population databases (Karczewski et al., 2020) is extremely low. Following the American College of Medical Genetics and Genetics and the Association for Molecular Pathology (ACMG-AMP) criteria (Richards et al., 2015) the variant was classified as likely pathogenic.

In Table 1 we reviewed the literature on the pathogenic variants in the *GPIHBP1* gene containing additional clinical information about the patients. Fifty-four patients were homozygous while seven were compound heterozygous for a pathogenic variant in the *GPIHBP1* gene. One patient was heterozygous for variants in *GPIHBP1* and *APOC2* genes. We presented 32 unique variants in the *GPIHBP1* gene. 5 pathogenic variants are located in exon 1, 3 in exon 2, 10 in exon 3 and 10 in exon 4. 4 variants represent major deletion of a whole exon/multiple exons/whole *GPIHBP1* gene.

TABLE 1 Review of pathogenic variants in *GPIHBP1* gene from the literature.

Reference	Nationality	Gender	Age	HGVS Transcript and Protein Change	Zygosity	TG (mg/dL)	TG (mmol/L)	EX	HSM	AP	C
(Ariza et al., 2016)	Ecuadorean	F	25	NM_178172.6:c.3G>T, NP_835466.2:p.(Met1?)	HOM	3,82	43.1	No	No	Yes	Yes
(Hegele et al., 2018)				NM_178172.6:c.17C>A, NP_835466.2:p.(Ala6Asp)	HOM						
(Paquette et al., 2018)	Vietnamese	F	33	NM_178172.6:c.40_41insGCGG, NP_835466.2:p.(Phe14CysfsTer25)	HOM	5,973	67.4	No	No	Yes	No
(Yamamoto et al., 2013)	Japanese	F	54	NM_178172.6:c.202T>C, NP_835466.2:p.(Cys68Arg)	HOM	2,64		No	No	Yes	Yes
(Liu et al., 2022)	Chinese	F	29 days	NM_178172.6:c.45_48dupGCGG, NP_835466.2:p.(Pro17AlafsTer22)	HOM	2,255	25.46		No		No
(Lin et al., 2020)	Chinese	F	35	NM_178172.6:c.48_49insGCGG, NP_835466.2:p.(Pro17AlafsTer22)	HOM	1,514	17.09	No	Yes	Yes	
(Ahmad and Wilson, 2014)	Caucasian	F	2 mth	NM_178172.6:c.85_88GAGGdel, NP_835466.2:p.(Glu29ThrfsTer50)	CHET	2,663	30.1	Yes	No	Yes	No
				NM_178172.6:c.267C>A, NP_835466.2:p.(Cys89Ter)							
(Buonuomo et al., 2015)	Italian		3 days	NM_178172.6:c.154_162AACAGGCTCdelTCTTins, NP_835466.2:p.(Asn52SerfsTer253)	CHET	1,667	18.8			No	
				NM_178172.6:c.319T>C, NP_835466.2:p.(Ser107Pro)							
(Wang and Hegele, 2007)		F	47	NM_178172.6:c.166G>C, NP_835466.2:p.(Gly56Arg)	HOM	7,094	80.1			Yes	No
(Wang and Hegele, 2007)		M	52	NM_178172.6:c.166G>C, NP_835466.2:p.(Gly56Arg)	HOM		48.0			Yes	
(Lima et al., 2021)	Brazilian	F	30	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	2,498	28.2	No	No	Yes	No
(Lima et al., 2021)	Brazilian	F	11	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	1,248	14.1	No	No	No	No
(Lima et al., 2021)	Brazilian	M	15	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	2,204	24.9	Yes	No	No	No
(Lima et al., 2021)	Brazilian	F	48	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	2,84	32.1	Yes	No	Yes	Yes
(Lima et al., 2021)	Brazilian	F	42	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	6,381	72.0	Yes	No	Yes	Yes
(Lima et al., 2021)	Brazilian	F	37	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	1,975	22.3	No	No	Yes	No
(Lima et al., 2021)	Brazilian	F	1	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	20,6	232.6	No	No	No	No
(Lima et al., 2021)	Brazilian	F	1	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	4,142	46.8	Yes	No	No	Yes
(Lima et al., 2021)	Brazilian	F	30	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	885	10.0	No	No	No	Yes
(Lima et al., 2021)	Brazilian	M	27	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	924	10.4	No	No	No	Yes
(Lima et al., 2021)	Brazilian	F	0.5	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	18	203.2	No	No	No	No
(Lima et al., 2021)	Brazilian	M	0.6	NM_178172.6:c.182-1G>T, NP_835466.2:p.?	HOM	40,141	453.2	No	No	No	Yes
(Surendran et al., 2012)	Dutch			NM_178172.6:c.194G>A, NP_835466.2:p.(Cys65Tyr)	HOM						
(Franssen et al., 2010)	UAE	M	3	NM_178172.6:c.194G>A, NP_835466.2:p.(Cys65Tyr)	HOM	4,005	45.2	No	No	Yes	
(Olivecrona et al., 2010)	Swedish	M	10	NM_178172.6:c.194G>C, NP_835466.2:p.(Cys65Ser)	CHET	1,727	19.5	No	Yes		No
				NM_178172.6:c.202T>G, NP_835466.2:p.(Cys68Gly)							
(Iacocca et al., 2019)	Swedish	F	9 mth	NM_178172.6:c.194G>C, NP_835466.2:p.(Cys65Ser)	CHET	5,049	57.0	No	Yes	Yes	No
				NM_178172.6:c.202T>G, NP_835466.2:p.(Cys68Gly)							
(Iacocca et al., 2019)	Swedish	F	16 mth	NM_178172.6:c.194G>C, NP_835466.2:p.(Cys65Ser)	CHET	4,296	48.5	No	Yes	Yes	No
				NM_178172.6:c.202T>G, NP_835466.2:p.(Cys68Gly)							

(Continued on following page)

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TABLE 1 (*Continued*) Review of pathogenic variants in *GPIHBP1* gene from the literature.

Reference	Nationality	Gender	Age	HGVS Transcript and Protein Change	Zygosity	TG (mg/dL)	TG (mmol/L)	EX	HSM	AP	C
(Coca-Prieto et al., 2011)	Spanish	F	30	NM_178172.6:c.203G>A, NP_835466.2:p.(Cys68Tyr)	HOM	>1,000	>11.3	No	No	Yes	
(Chokshi et al., 2014)	Salvadorean	F	24	NM_178172.6:c.203G>A, NP_835466.2:p.(Cys68Tyr)	HOM			Yes	Yes	Yes	No
(Rios et al., 2012)	Salvadorean	F	36	NM_178172.6:c.203G>A, NP_835466.2:p.(Cys68Tyr)	HOM	6,48	73.2	Yes	No	Yes	No
(Ariza et al., 2016)	Pakistani	M	39	NM_178172.6:c.239C>A, NP_835466.2:p.(Thr80Lys)	HOM	4,489	50.7	No	No		
(Rabacchi et al., 2016)			42	NM_178172.6:c.247T>C, NP_835466.2:p.(Cys83Arg)	HOM					Yes	
(Rabacchi et al., 2016)		M	40	NM_178172.6:c.247T>C, NP_835466.2:p.(Cys83Arg)	HOM					No	
(Charrière et al., 2011)		M	6 mth	NM_178172.6:c.266G>T, NP_835466.2:p.(Cys89Phe)	CHET	1,736	19.6			Yes	
				ex1_3 del							
(Rabacchi et al., 2016)		F	55	NM_178172.6:c.267C>A, NP_835466.2:p.(Cys89Ter)	HOM				Yes	Yes	
(Plengpanich et al., 2014)	Thai	F	46	NM_178172.6:c.320C>G, NP_835466.2:p.(Ser107Cys)	HOM	3,164	35.7	No		No	
(Plengpanich et al., 2014)	Thai	M	64	NM_178172.6:c.320C>G, NP_835466.2:p.(Ser107Cys)	HOM	842	9.5			No	
(Plengpanich et al., 2014)	Thai	M	43	NM_178172.6:c.320C>G, NP_835466.2:p.(Ser107Cys)	HOM	673	7.6			No	
(Chyzhyk et al., 2019)	Middle East	F	6 weeks	NM_178172.6:c.323C>G, NP_835466.2:p.(Thr108Arg)	HOM	3,15	35.6			No	No
(Chyzhyk et al., 2019)	Middle East	F	2	NM_178172.6:c.323C>G, NP_835466.2:p.(Thr108Arg)	HOM	1,838	20.8			Yes	No
(Surendran et al., 2012)	Caucasian	M	1	NM_178172.6:c.323C>G, NP_835466.2:p.(Thr108Arg)	HOM					Yes	
(Gonzaga-Jauregui et al., 2014)	Spanish	F	5 weeks	NM_178172.6:c.331A>C, NP_835466.2:p.(Thr111Pro)	CHET	12,046	136.0	No		Yes	No
				NM_178172.6:c.413_429del, NP_835466.2:p.(Pro140SerfsTer161)							
(Beigneux et al., 2009a)	Colombian	M	33	NM_178172.6:c.344A>C, NP_835466.2:p.(Gln115Pro)	HOM	3,366	38.0	No	Yes	No	
(Surendran et al., 2012)	Dutch			NM_178172.6:c.344A>C, NP_835466.2:p.(Gln115Pro)	HOM						
(Hegele et al., 2018)				NM_178172.6:c.394C>T, NP_835466.2:p.(Gln132Ter)	HOM						
(Jung et al., 2017)	Algerian	M	1 mth	NM_178172.6:c.476delG, NP_835466.2:p.(Gly159AlafsTer94)	HOM	>5,000		No	No	No	
(Ariza et al., 2018)	Spanish			NM_178172.6:c.502delC, NP_835466.2:p.(Leu168SerfsTer85)	HOM						No
(Charrière et al., 2011)		M	35	NM_178172.6:c.523G>C, NP_835466.2:p.(Gly175Arg)	HOM	2,303	26.0			Yes	
(Rios et al., 2012)	Algerian	M	26	NM_178172.6:c.523G>C, NP_835466.2:p.(Gly175Arg)	HOM	2,303					No
(Chyzhyk et al., 2019)		M	43	NM_178172.6:c.523G>C, NP_835466.2:p.(Gly175Arg)	HET	>5,000	>56.4			Yes	
				NM_000483.5:c.2-4G>C, NP_000474.2:p.?							
(Berge et al., 2014)	Pakistani	F	22	ex3 and 4 deletion	HOM	5,314	60.0			Yes	Yes
(Berge et al., 2014)	Pakistani	M	37	ex3 and 4 deletion	HOM	8,857	100.0			Yes	Yes
(Berge et al., 2014)	Pakistani	M	40	ex3 and 4 deletion	HOM	2,073	23.4			No	Yes
(Berge et al., 2014)	Pakistani	F	37	ex3 and 4 deletion	HOM	2,028	22.9			Yes	Yes
(Iacocca et al., 2019)		M	22	ex3 and 4 deletion	HOM	2,737	30.9			No	
(Iacocca et al., 2019)		M	39	ex3 and 4 deletion	HOM	1,329	15.0			No	
(Iacocca et al., 2019)		M	50	ex3 and 4 deletion	HOM	957	10.8			Yes	

(Continued on following page)

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TABLE 1 (*Continued*) Review of pathogenic variants in *GPIHBP1* gene from the literature.

Reference	Nationality	Gender	Age	HGVS Transcript and Protein Change	Zygosity	TG (mg/dL)	TG (mmol/L)	EX	HSM	AP	C
(Rios et al., 2012)	Indian	M	2 mth	17.5 kbp deletion (included GPIHBP1)	HOM	37,284	421.0	No	No	No	No
(Iacocca et al., 2019)	Indian	F	44	17.5 kbp deletion (included GPIHBP1)	HOM					Yes	No
(Patni et al., 2016)	Indian	M	10	Entire gene deletion (54,623 bp)	HOM	2,274	25.7	No	Yes	No	No
(Chokshi et al., 2014)	Indian	M	2 mth	Entire gene deletion	HOM	>20,000	>225.8	No	No	Yes	No
(Iacocca et al., 2019)		M	37	Entire gene deletion	HOM	3,082	34.8			Yes	

HO, homozygous; CHE, compound heterozygous; HE, heterozygous; EX, eruptive xanthoma; HSM, hepatosplenomegaly; AP, acute pancreatitis; C, consanguinity.

Discussion

We presented a patient with severe hypertriglyceridemia as a consequence of a homozygous pathogenic variant in the *GPIHPB1* gene. Additionally, we reviewed the literature on described cases with pathogenic variants in the *GPIHBP1* gene.

Elevated TG levels have been linked to cardiovascular disease (CVD) and pancreatitis (Carrasquilla et al., 2021). For severe hypertriglyceridemia, pathogenic variants in genes *LPL*, *APOA5*, *APOC2*, *GPIHBP1,* and *LMF1*, associated with hyperlipidemia should be considered (Goldberg and Chait, 2020). *GPIHBP1* is located on chromosome 8q24.3 and is composed of 4 exons (Liu et al., 2018). *GPIHBP1* is expressed mostly in the capillary endothelial cells of the heart, brown adipose tissue and skeletal muscle, involved in energy and lipid metabolism (Cruz-Bautista et al., 2021). GPIHBP1 acts as an important partner of the LPL in plasma triglyceride metabolism (Supplementary Figure S1). The loss-of-function pathogenic variant in *GPIHBP1* impairs LPL activity and its lipolytic processing of chylomicrons and very-low-density lipoproteins (Voss et al., 2011; Gin et al., 2012). LPL unfolds spontaneously and remains within the interstitial spaces. Due to the loss of its catalytic activity, triglycerides are not hydrolysed resulting into severe hypertriglyceridemia (Arora et al., 2019; Miyashita et al., 2020) (Supplementary Figure S1).

GPIHBP1 is a protein composed of 184 amino acids from the lymphocyte antigen (Ly6) family and consists of a N-terminal signal peptide region, an amino-terminal acidic domain, a Ly6 domain of ten disulfide-bonded cysteines and a highly hydrophobic carboxyl-terminal motif that is replaced within the endoplasmic reticulum by a glycosylphospatidylinostiol (GPI) anchor for tethering to the cell membrane. GPIHBP1 is distinguishable from the other Ly6 family members by the presence of a high acidic domain composed of 21 aspartates or glutamates located 12 amino acids prior to the Ly6 motif (Beigneux A. R. et al., 2009; Liu et al., 2018). Both, a high acidic and Ly6 domain are important for the GPIHBP1 and LPL interaction. Genetic modifications encoding for one of these two domains result in chylomicronemia. Most missense variants break down the disulfide bond of the Ly6 domain, resulting in the production of GPIHBP1 dimers and multimers, which are incapable of binding LPL (Beigneux et al., 2015; Fong et al., 2016).

Amino acids at positions 24-50 (exon 2) compose the acidic domain and amino acids at positions 63-148 (exons 3 and 4) compose the Ly6 domain of the GPIHBP1 protein (Figure 3). Amino acids at positions 27-50 (exon 2) are important for LPL transport to the lumenal surface of endothelial cells. Amino acids at positions 103-109 (exon 4) are important for interaction with LPL (UniProt, Q8IV16). The majority of pathogenic variants disrupt the Ly6 domain's folding, resulting in multimerized and defective GPIHBP1 molecules on the cell surface (Holmes and Cox, 2012; Mysling et al., 2016).

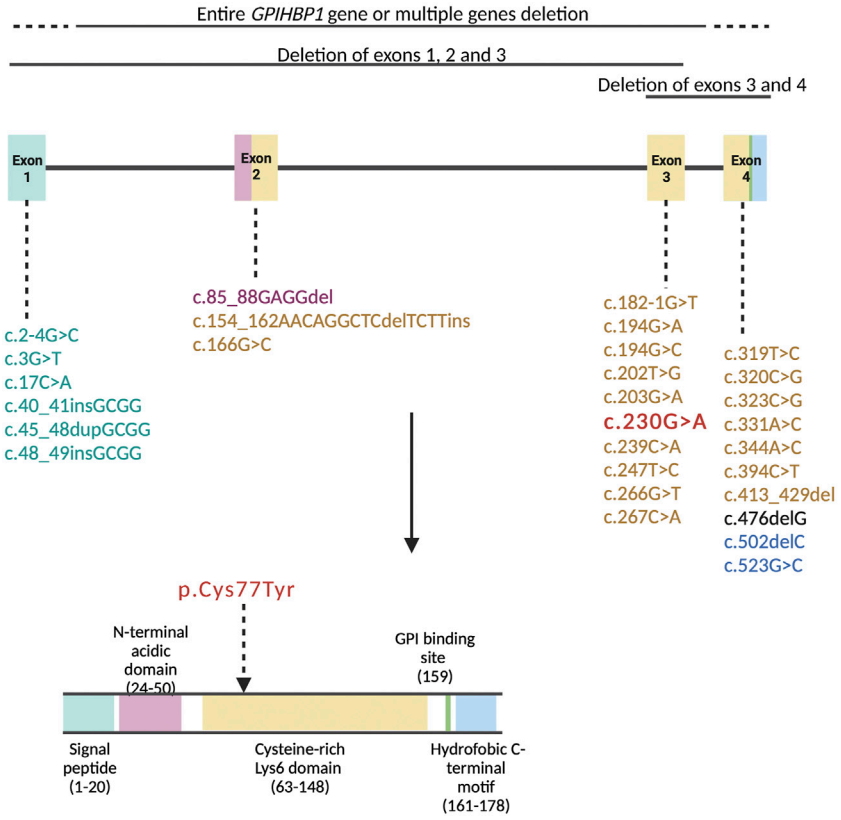

FIGURE 3
Pathogenic variants in the *GPIHBP1* gene based on the literature review represented in Table 1 are organized by exon numbers. The colour of the exon denotes the protein domain affected by the pathogenic variation. The variant of our patient (c.230G > A) is in bold red colour.

Patients with GPIHBP1 deficiency express a similar phenotype as patients with LPL deficiency, such as severe chylomicronemia (plasma TG levels above 1,500 mg/dl (16.9 mmol/L) presented already in childhood and high risk of pancreatitis (Miyashita et al., 2020). Highest reported TG and TC levels for the patient at age 10 months were 5,137 mg/dl (58.0 mmol/L) and 850.7 mg/dl (22.0 mmol/L) respectively. Because hyperbilirubinemia was detected during this examination, extreme TC levels could be the consequence of biliary congestion. Hyperbilirubinemia was not detected on further examinations, yet increased TC and TG levels remained.

Moreover, as the recessive manner of inheritance of the disorder, the parents and the younger sister of the patient did not express the extreme phenotype associated with the disorder, although the father has elevated TC and TG levels (Supplementary Figure S1), similarly, as reported by Wang and Hegele, 2007 for heterozygous family members of a proband with a homozygous variant in the *GPIHBP1* gene. Likewise, our patient's father may have an extra genetic variant that contributes to his phenotype but is not present in the patient's mother or sister.

The variant p.Cys77Tyr of our patient is located in exon 3, encoding for the Ly6 domain of the GPIHBP1 protein (Figure 3). Cysteine residues of Ly6 domain are essential for the 3-fingered structural motif formation. Interfering with any of the disulfide links is expected to cause significant structural changes in the protein (Rabacchi et al., 2016). Beigneux et al. (2009b) report that cells expressing the cysteine mutants in *GPIHBP1* are unable to bind and transport LPL from the subendothelial space to the endoluminal surface of the endothelial cells. Furthermore, several genetic variants involving cysteine alterations to another amino acid have previously been documented. Eight patients with homozygous and four with compound heterozygous variants p.Cys65Tyr, p.Cys68Gly, p.Cys83Arg, p.Cys83Arg, p.Cys89Phe and p.Cys89Ter (Olivecrona et al., 2010; Charrière et al., 2011; Iacocca et al., 2019) with severe elevation in TG (1,000–6,480 mg/dl, 11.3–73.2 mmol/L) and episodes of acute pancreatitis have previously been described. Moreover, Lima et al. (2021) present a series of twelve cases with an intronic variant c.182-1G>C, resulting in the skipping of cysteine-rich exon 3. These patients likewise

had extremely elevated TG levels (885–40,141 mg/dl, 10–453.2 mmol/L), but interestingly only four suffered from acute pancreatitis, none of them had hepatosplenomegaly and four of them had eruptive xanthomas. Additionally, twelve patients with deletion of exon 3 and 4 or entire gene deletion had TG values between 957 and 37,284 mg/dl (10.8 and 421.0 mmol/L) and seven of them were affected by acute pancreatitis (Rios et al., 2012; Berge et al., 2014; Chokshi et al., 2014; Patni et al., 2016; Iacocca et al., 2019). Table 1 contains additional information about these patients.

European Atherosclerosis Society provided practical clinical guidelines for rare dyslipidaemia management for patients with extreme LDL-C, TG and HDL-C levels (Hegele et al., 2020). Early detection of rare dyslipidemias in a pre-clinical stage is possible with an effective FH screening program capable of detecting other dyslipidemia than FH (Groselj et al., 2018, 2022; Marusic et al., 2020; Sustar et al., 2022) or with a gene panel applied as a part of a newborn screening program (Remec et al., 2021). It is important to implement a worldwide registry for rare dyslipidemias, comparable to what already exists for FH/homozygous FH (Tromp et al., 2022; Vallejo-Vaz et al., 2018, 2021).

Statin therapy substantially reduced the CVD risk in patients with high LDL-C levels (Silverman et al., 2016). Nevertheless, other factors, such as triglycerides or triglyceride-rich lipoproteins (TRLs), contribute to the CVD risk with low-grade inflammation, as a part of atherosclerosis (Nordestgaard, 2016). The goal of the treatment is to reduce plasma TG levels to less than 500–1,000 mg/dl to prevent acute pancreatitis (Okazaki et al., 2021). To prevent abdominal pain and acute pancreatitis, patients should be on a low-fat diet of total dietary fat intake of <10–15% of daily calories (<15–20 g per day) and treated with common lipid-lowering drugs (fibrates, omega-3 fatty acids, statins) (Esan and Wierzbicki, 2020; Navarro Hermoso and Valdivielso, 2021). Volanesorsen, an antisense oligonucleotide inhibitor of apoC3 is a promising medicine for the reduction of TG levels by 70–80% (Esan and Wierzbicki, 2020). Furthermore, gemfibrozil is a useful medication for the reduction of TGs in patients with very high TG serum levels. The mechanism of action of gemfibrozil is based on the activation of nuclear transcription factors for up-regulation of LPL transcription and down-regulation of the LPL inhibitor apo C-III, resulting in a decrease in triglyceride levels and an increase in HDL. Moreover, gemfibrozil reduces hepatic triglyceride synthesis by inhibiting peripheral lipolysis and decreasing hepatic removal of free fatty acids. It inhibits the synthesis and increases the clearance of very low-density lipoprotein (VLDL) (Goldberg and Hegele, 2012). Our patient was managed with statins (atorvastatin) and later with a combination of fibrates (fenofibrate and gemfibrozil). Combination therapy with fenofibrate and gemfibrozil has helped in lowering TG levels (<1000 mg/dl) which was not achieved by monotherapy with fenofibrate. Statins were stopped when the diagnosis of GSD was dismissed, and the patient was treated as a case of primary HTG.

In conclusion, genetic testing for rare dyslipidaemias should be considered early in cases of severe elevations of plasma cholesterol and/or triglycerides, to enable adequate and precise management of the patient. Besides *LPL*, there are other genes involved in primary hypertriglyceridemia phenotypes (most notably, *APOC2, APOA5, LMF1,* and *GPIHBP1*). Our paediatric patient had a homozygous pathogenic *GPIHBP1* variant, causing severe hypertriglyceridemia, cholesterol deposits at the hard palate, eruptive xanthomas, lethargy, poor appetite, and mild splenomegaly. Combination treatment with fenofibrate and gemfibrozil was shown to help reduce TG levels.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee Pakistan (033-523-2019). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

UG and FS designed this study. FS, SS, SK, and US collected and analysed the data. US drafted the manuscript. All authors contributed to the manuscript and approved the final version for submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.983283/full#supplementary-material

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Clinical characteristics and variant analyses of transient infantile hypertriglyceridemia related to GPD1 gene

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Objective: Our study aims to summarize and analyze the clinical characteristics of transient infantile hypertriglyceridemia (HTGTI) and variants in the glycerol-3-phosphate dehydrogenase 1 (*GPD1*) gene and the effect of HTGTI on the protein structure of *GPD1*.

Methods: Retrospective analysis, using the general data, symptoms, signs, and auxiliary examinations, was performed on patients with HTGTI, which were confirmed by genetic testing in our hospital and reported cases online. The clinical data were analyzed using statistical and bioinformatic approaches.

Results: A total of 31 genetically confirmed HTGTI patients were collected from our hospital and cases reported in the literature. The clinical manifestations showed the median age of onset was 6.0 (1.9, 12.0) months. All the patients had normal psychiatric status, but 22.6% of them presented growth retardation and short stature, 93.5% had hepatomegaly, and 16.1% had splenomegaly. Just a few children were reported with jaundice, cholestasis, and obesity (3.2–6.5%). The laboratory investigations showed that 96.8% of them had hypertriglyceridemia (HTG) with a median level of 3.1 (2.1, 5.5) mmol/L, but only 30.0% had returned to normal during follow-up. In addition, 93.5% of patients had elevated alanine aminotransferase (ALT) with an average level of 92.1 $\pm$ 43.5 U/L, while 38.7% had hypercholesterolemia. Upon abdominal imaging, all patients presented fatty liver and liver steatosis, with 66.7% of patients showing hepatic fibrosis. Statistical differences in triglyceride (TG) level were observed in the $\leq$6 months group compared with the older groups and in the 13 months to 6 years group with >6 years group (H = 22.02, P < 0.05). The restricted cubic spline model showed that severe HTG decreased in the early stage of infants to the normal level; however, it rebounded again to a mild or moderate level after the following days. The genetic test revealed that the main variant types of the *GPD1* gene were missense variants (51.6%), followed by splicing variants (35.5%) and nonsense variants (12.9%). Of patients, 87.1% had homozygous variants, with the most frequent loci being c.361-1G > C and c.895G > A.

Conclusion: The common manifestations of HTGTI were HTG, hepatomegaly, elevated liver transaminases, and hepatic steatosis in early infancy. However, the recurrence of aberrant HTG may pose long-term detrimental effects on HTGTI patients.

KEYWORDS

glycerol-3-phosphate dehydrogenase 1 (GPD1), transient infantile hypertriglyceridemia (HTGTI), hypertriglyceridemia, hepatic steatosis, hepatomegaly

Introduction

Hypertriglyceridemia (HTG), which refers to elevated fasting triglyceride (TG) that exceeds 1.7 mmol/L, is a relatively prevalent condition in clinical practice (Berglund et al., 2012). It affects 10–20% of the adult population with considerable interregional variation in the incidence rate (Parhofer and Laufs 2019; Laufs et al., 2020). In addition, HTG is a significant risk factor for obesity, atherosclerotic vascular diseases, type 2 diabetes, acute pancreatitis, and other related metabolic syndromes. Based on the genetic and nongenetic etiologies of HTG, the current categorization of HTG segregates primary and secondary causes for each category (Brunzell 2007; Hegele et al., 2014). HTG is considered a complicated phenotype resulting from the complex regulatory networks of multiple susceptibility genes (common and rare genetic variants) and environmental factors (Johansen et al., 2010; Hegele et al., 2014). The single-gene variants of primary HTG identified by gene sequencing include *LPL*, *APOC2*, *APOA5*, *LMF1*, *GPIHBP1*, and *GPD1*. Severe HTG (TG > 10.0 mmol/L) is more likely to be associated with single-gene variation, especially in infants and adolescents (Laufs et al., 2020; Shah and Wilson, 2020).

Transient infantile hypertriglyceridemia (HTGTI) is a sporadic autosomal recessive hereditary disease with lipid metabolic disorder. It is caused by the inactivation and variant of glycerol-3-phosphate dehydrogenase 1 (GPD1, MIM138420) located on chromosome 12q12-q13, which is also a crucial molecular cause of congenital HTG in infants. The *GPD1* gene encodes intracytoplasmic NAD-dependent GPD1, which plays an essential role in lipid and carbohydrate metabolism by catalyzing the reversible redox reaction of dihydroxyacetone phosphate (DHAP) and reduced nicotine adenine dinucleotide (NADH) to glycerol-3-phosphate (G3P) and NAD^+ (Ou et al., 2006). To date, 31 genetically confirmed patients have been reported worldwide in 10 case reports. The most common clinical manifestations were transient hypertriglyceridemia, hepatomegaly, elevated liver transaminases, and hepatic steatosis in early infancy (Basel-Vanagaite et al., 2012; Joshi et al., 2014; Dionisi-Vici et al., 2016; Li et al., 2017; Li et al., 2018; Matarazzo et al., 2020; Xie et al., 2020; Kumar and Sharma 2021; Tesarova et al., 2021; Wang et al., 2021). In some patients, HTG is normalized with age. However, during follow-up, other patients may exhibit mild HTG accompanied by abnormal liver transaminases, fatty liver, and even cirrhotic manifestations. However, there is inadequate understanding of the multiple clinical phenotypes and long-term prognosis of HTG, and it requires detailed systematic and comprehensive studies. This article aims to summarize and analyze the clinical characteristics of HTGTI and variants in the *GPD1* gene, which will serve as a reference for further clinical diagnosis, therapy, and research.

Materials and methods

Source of cases and search strategy

One confirmed case of HTGTI was from our hospital. Meanwhile, to retrieve the other subjects from previously published reports, we searched the relevant literature about HTGTI caused by the *GPD1* gene variant in databases. The search was conducted up to September 2021 using the following electronic databases: Medline, Cochrane Library, EMBASE, PubMed, and Web of Science. The search terms used to retrieve data were "Glycerol-3-Phosphate Dehydrogenase (NAD^+)," "Glycerol-3-phosphate dehydrogenase 1," "*GPD1*," "HTGTI," or "transient infantile hypertriglyceridemia," in association with a list of sensitive terms to search for experimental studies or case reports about humans. In addition, the reference lists provided in the selected articles were hand-searched to investigate further relevant studies.

Data extraction

The general characteristics of studies were extracted, including the age, gender, nationality, age of onset, clinical manifestations, laboratory and genetic examination results, treatments, and follow-up of patients. The collected data were collated and summarized in tables and figures.

Sequencing study and analysis

The genetic testing was performed after obtaining written informed consent from our patient's parents, and the peripheral venous blood of the proband and her parents were collected for genetic testing using next generation of whole genomic

DNA(Wang et al., 2021). The captured libraries were sequenced using Illumina HiSeq, analyzed using clinic sequence analyzer from WuXiNextCODE (China), and tested using the SureSelect Human All Exon V5 kit (Agilent Technologies, Inc.), Illumina Cluster kit, and SBS kit according to the manufacturer's standard procedure. The average sequencing depth of exome target sequencing is greater or equal than 90×, in which 95% of the target sequencing is over 20×. Base calling was carried out for all sequences.

In the second round, sequencing data were mainly analyzed using Sentieon. The reads were mapped to the reference of Sentieon BWA and UCSC hg19. The sequencing depth and variants prediction of each base were extracted from all genomic sequencing data. Variants were noted by VEP (Variant Effect Predictor, Ensembl73). Three databases including ClinVar, OMM, and HGMD were employed to filter known and possible pathogenic variants, and other tools were adopted to predict the function of missense mutation and to note non-coding sequence. Large-scale sequencing databases including 1000 Genome Project, Exome Sequencing Project, Exome Aggregation Consortium, and Genome Aggregation Database of Tokyo and the Netherlands, were used to filter frequent variants. Every variant was assessed with clinic sequence analyzer, named in accordance with the guidelines of Human Genome Variation Society, and classified based on the standards and guidelines for the interpretation of sequence variants by American College of Medical Genetics and Genomics. Variants were verified in the patient and her parents by Sanger sequencing. The other children with HTGTI also underwent similar tests with gene sequencing, which were described in the above case reports.

Structural diagram of glycerol-3-phosphate dehydrogenase 1 gene and protein

The nucleotide reference sequence of the *GPD1* gene was derived from NCBI (transcript: NM_005276.4). The crystal structure of human GPD1 was retrieved from the Protein Data Bank; its primary citation of related structures is 1X0V. The picture of the gene structure was prepared using illustrator for biological sequences (IBS) (Liu et al., 2015), and the protein structures of GPD1 were drawn using PyMOL software (The PyMOL Molecular Graphics System, http://www.pymol.org).

Statistical analyses

Excel 2019 software and SPSS 18.0 statistical software were used for statistical analysis. The normally distributed data were expressed as mean ± SD, while the nonnormally distributed variables were expressed as median M (Q1, Q3); however, the count data were expressed as percentages (%). Normal distributed continuous variables were analyzed using either one-way analysis of variance or the LSD test. Differences in the characteristics between groups were analyzed using the Kruskal–Wallis test with the Dunn *post hoc* tests (for continuous variables, R package FSA) or the χ^2 tests with *post hoc* tests (for categorical variables, R package fifer). We also used restricted cubic spline models fitted for the hazards models of TG with age using R with ggplot2 (Xu et al., 2021). Values of p less than 0.05 were considered statistically significant.

Results

General information of patients

Our search strategy identified 152 potentially relevant articles from the online databases. After removing duplicates, 140 studies remained. Reading the titles and abstracts of these references led us to exclude a further 128 identified articles that did not meet the inclusion criteria. After reading the full text of the remaining 12 articles, 2 were excluded because of the lack of relevant information. In the end, a total of 10 case reports were retrieved related to HTGTI with GPD1 variants from the above-mentioned medical research databases, including 31 genetically confirmed patients. The selection process is shown in Figure 1. Among these patients, 13 (61.9%) and 8 (38.1%) were male and female, respectively, with a male-to-female ratio of 1.6:1; however, 10 cases lack the related information. Meanwhile, their onset age was widely variable, ranging from 0.0 to 7.0 years, with a median age of 6.0 (1.9, 12.0) months. Ethnic distribution shows that 12 patients were Arabs; 9 patients were Roma; 4 patients were Chinese; 2 patients were Italian; 1 patient each from Russia, Caucasus, and India; and only 1 patient was unknown. In addition, 12 patients come from families with a high degree of consanguinity, 5 patients were from nonconsanguineous families, and 14 patients were unknown. Just one patient had a family history of fatty liver in parents, one had a morbidly obese father, and others were unknown.

Clinical manifestation

We analyzed the clinical characteristics of these 31 patients with HTGTI. According to the data from the beginning of the visit to the last follow-up, the median follow-up time was 4.1 (0.9, 12.6) years. The age of the original diagnosis of HTGTI was 0.0–24.0 months, and the median age was 6.0 (1.8–10.0) months. All the patients had normal psychiatric status, but 22.6% of them presented with growth retardation and short stature. HTG was identified in 96.8% (30/31) of patients, with elevation ranging from 1.9 to 70.6 mmol/L, and the median level was 3.1 (2.1, 5.5) mmol/L. However, the HTG level of only 30.0% (6/20) of

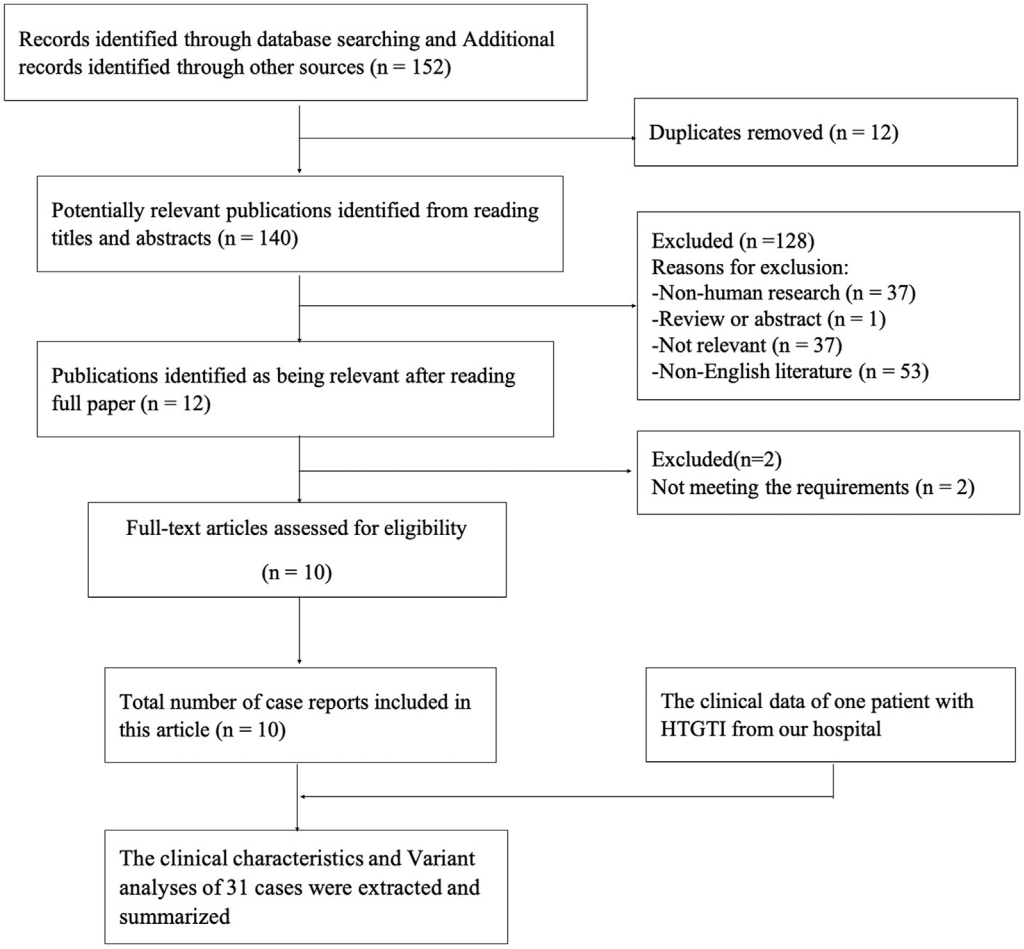

FIGURE 1
The flow diagram of the research process.

children returned to normal during follow-up. Hepatomegaly, which was palpated less than 10 cm below the costal margin, was observed in 93.5% (29/31) of patients. Splenomegaly, which was palpated less than 5 cm below the costal margin, was reported in 16.1% (5/31) of patients. A few patients presented with jaundice (6.5%), elevated cholestasis (3.2%), hypoglycemia (9.7%), and obesity (3.2%). Imaging analysis showed that 100.0% of patients had fatty liver (Figure 2). The liver biopsy specimens from 15 patients presented steatosis, of which 66.7% (10/15) were accompanied by hepatic fibrosis. Blood screenings were normal, but 6.7% (2/30) of urine screenings reported urine dicarboxylic acids. Of patients, 87.1% had homozygous variants, with the most frequent loci being c.361-1G > C and c.895G > A (Table 1).

Furthermore, to analyze the average levels of serum TG, total cholesterol (TC), and alanine aminotransferase (ALT) of 31 patients, we divided them into four groups: younger than 6 months (≤6 months group), 7–12 months (7–12 months group), 13 months to 6 years (13 months to 6 years group), and older than 6 years (>6 years group) (Table 2; Figure 3). The median levels of TG, TCHO, and ALT were 5.85 (4.16, 9.33) mmol/L, 4.90 (3.13, 6.50) mmol/L, and 90.00 (57.25, 112.00) U/L, respectively. Next, we drew boxplots based on the laboratory data from the reported cases. As shown in Figure 3, the median level of TG was highest in the ≤6 months group. In contrast, the level of TG tended to decrease with age but rebounded again in the >6 years group. Furthermore, statistical differences (using the Kruskal–Wallis test with Dunn's test) in TG levels were observed among four groups. Results indicated that the ≤6 months group showed a significant increase in TG levels compared to the other three groups, and the 13 months to 6 years group was comparable with the >6 years group (H = 22.02, $P < 0.05$). The level of TCHO was lower in the 7–12 month age group than in the ≤6 month age group, but it was higher in the 13 months to 6 years group and the >6 years group. Significant differences were also observed in the ≤6 months group compared with that of the >6 years

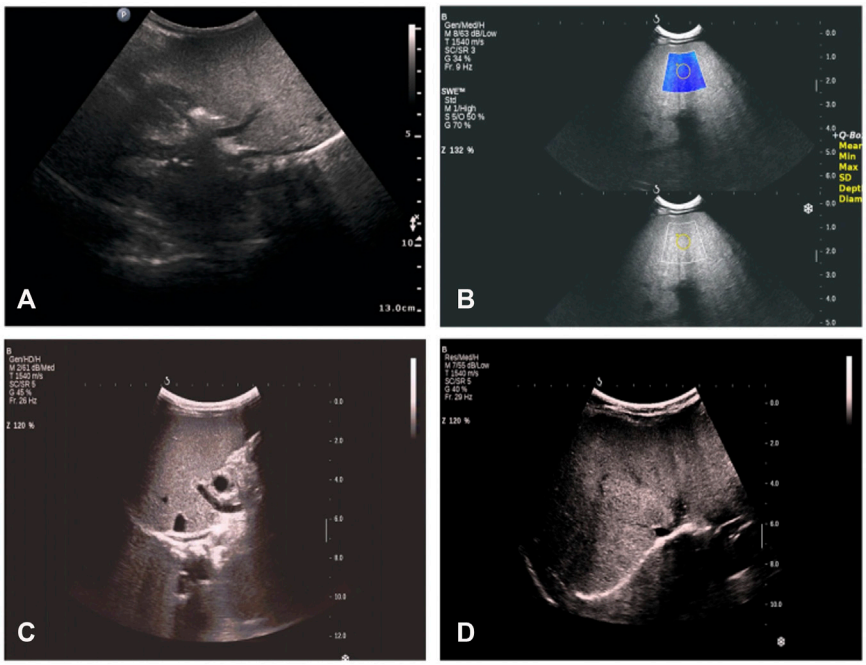

FIGURE 2
Radio findings in proband with glycerol-3-phosphate dehydrogenase 1 (GPD1) mutation from our hospital. **(A)** at the age of 1 month 26 days: hepatosplenomegaly, thicker parenchyma echo spots, slightly coarse gallbladder wall, and gallbladder contraction rate was less than 50% after 1 h of breastfeeding. **(B)** at the age of 2 months 4 days: hepatosplenomegaly, thinner parenchyma echo spots, strengthened echo, thicker and coarse gallbladder wall, and liver tissue elastic modulus was approximately 15 kPa. **(C)** at the age of 7 months 10 days: hepatosplenomegaly, stronger and diffused echo, and liver tissue elastic modulus was approximately 17 kPa. **(D)** at the age of 1 year and 4 months: fatty liver change; no obvious abnormality of gallbladder, pancreas, liver, and spleen; liver tissue elastic modulus was approximately 14.3 kPa.

group, and the 7–12 months group showed significant difference compared with the >6 years group ($H = 17.77$, all $P < 0.05$). No significant differences were observed in the levels of serum ALT between or within the four age groups ($H = 5.35$, all $P > 0.05$).

Correlation between blood triglyceride and age using restricted cubic spline

To further analyze the TG trends with the age of HTGTI patients, we used restricted cubic spline (RCS) with four knots at 3, 12, 60, and 150 months to flexibly model the association of serum TG with age (Figure 4). The association between the levels of TG on a continuous scale and the age of these patients was U-shaped. This curve shows a good simulation of the correlation between these binary variables (P for nonlinearity <0.001). It predicted that the high level of TG in the early stage of infants decreased sharply with age and became normal (1.7 mmol/L) at the age of 23 months. Still, TG level rebounded again after infants reached 50 months old and maintained at relatively stable levels. Considering that all patients with HTGTI exhibited HTG, steatosis, and even hepatic fibrosis during the onset and follow-up period, HTG does not mean a transient phenomenon only in infancy. Meanwhile, we also believe that rebounded or sustained elevation of TG was associated with an increased risk of disease. Although the severe HTG may be transient in infancy in HTGTI, mild to moderate dyslipidemia is likely to be persistent in many of these patients. Even though there is still a lack of abundant and longer-term follow-up data, this situation may be associated with future adverse health outcomes.

Analysis of glycerol-3-phosphate dehydrogenase 1 genetic test

The *GPD1* gene (NG_032168.1, NC_000012.12, including 7306 bases, gene ID: 2819) found in the GenBank gene database shows that the *GPD1* gene has a total length of 7,306 bp and is located on the long arm of human chromosome 12 (12q13.12). *GPD1* includes eight exons and seven introns, while coding sequence (CDS) encodes 349 amino acid residues (Figure 5). Analysis of *GPD1* gene variant loci and clinical phenotype (Table 1) shows that the variant sites of the *GPD1* gene were mainly missense variants (16/31), followed by splicing variants (11/31) and nonsense variants (4/31). These reported variants resulted in the dysfunction of GPD1.

TABLE 1 GPD1 gene variant and phenotype of 31 patients with HTGTI.

References	Descent	Number of patients	c.DNA variant		Hom/Het	Amino acid change	Variant region	Variant type	Main phenotype of pathogenic variant
			Maternal	Paternal					
Basel-Vanagaite et al. (2012)	Israeli-Arab	10	c.361-1G > C	c.361-1G > C	Homozygous	Ile119fsX94	Intro 3	Splicing variant	Hepatomegaly, fatty liver, HTG, 4/10 had short stature, vomit, slow weight gain, elevated ALT + GGT
Joshi et al. (2014)	Caucasian	1	c.686G > A	a small deletion	Compound heterozygous	R229Q	Exon 6	Missense variant	Hepatomegaly, fatty liver, HTG, maldevelopment, vomit, elevated transaminase + GGT + TC [11]
Dionisi-Vici et al. (2016)	Arab-Muslim	1	c.806G > A	c.806G > A	Homozygous	Arg269Gln	Exon 6	Missense variant	Hepatomegaly, fatty liver, HTG, elevated transaminase, recurrent fasting hypoglycemia
	NA	1	c.361-1G > C	c.361-1G > C	Homozygous	Ile119fsX94	Intro 3	Splicing variant	Hepatomegaly, fatty liver, HTG, elevated transaminase + GGT + ALP
	Italian	1	c.640T > C	c.640T > C	Homozygous	Cys214Arg	Exon 6	Missense variant	Hepatosplenomegaly, fatty liver, HTG, elevated transaminase + total bile acid (TBA), urine dicarboxylic acids, minimal lesion nephropathy
	Italian	1	c.640T > C	c.640T > C	Homozygous	Cys214Arg	Exon 6	Missense variant	Hepatomegaly, fatty liver, HTG, elevated transaminase + TC + TBA
Li et al. (2017)	Chinese	1	c.820G > A	c.220–2A > G	Compound heterozygous	Ala274Thr	Exon 6 and 3	Missense variant	Obesity, fatty liver, short stature, insulin resistance, dermal abnormalities, elevated dehydroepiandrosterone sulfate and lipoprotein-α(LP-α), normal lipid
Li et al. (2018)	Chinese	1	c.523C > T	c.523C > T	Homozygous	Q175*	Exon 5	Nonsense variant	Hepatomegaly, fatty liver, HTG, elevated transaminase + GGT + TBA
Matarazzo et al. (2020)	Russian	1	c.895G > A	c.895G > A	Homozygous	G299R	Exon 7	Missense variant	Hepatomegaly, fatty liver, cirrhosis, HTG, elevated transaminase + TBA
Wang et al. (2021)	Chinese	1	c.901G > T	a short deficiency	Compound heterozygous	E301X	Exon 7	Nonsense variant	Hepatomegaly, fatty liver, jaundice, cirrhosis, elevated transaminase + GGT + TBA, urine dicarboxylic acids
Xie et al. (2021)	Chinese	1	c.901G > T	c.931C > T	Heterozygous	E301X + Q311X	Exon 7	Nonsense variant	Hepatomegaly, HTG, elevated transaminase
Kumar and Sharma (2021)	Indian	1	c.500G > A	c.500G > A	Homozygous	Gly167Asp	Exon 5	Missense variant	abdominal distention, Hepatomegaly, HTG, slight Splenomegaly, elevated ALP
Tesarova et al. (2021)	Romani	9	c.895G > A	c.895G > A	Homozygous	Gly299Arg	Exon 7	Missense variant	9 of 10 had hepatomegaly, elevated transaminase, low-normal growth; 2 had mild repeated hypoglycemia
	Palestinian Arab	1	c.116G > A	c.116G > A	Homozygous	Trp39*	Exon 2	Nonsense variant	

Items: HTG, hypertriglyceridemia; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; TC, total cholesterol; ALP, alkaline phosphatase; TBA, total bile acid.

TABLE 2 Differential analysis of TC, TCHO, and ALT in different age groups. Significant differences existed in TG and TCHO in different age groups ($P < 0.05$), but there were no statistical differences in ALT between four groups ($P > 0.05$) (a, b, c, and d are used to represent the statistical difference between groups with the same superscript letter).

Items	subgroup	Median/mean	H-value	*p*-value
TG	≤6 months	5.85 (4.16, 9.33)[a]	22.023	0.000
	7–12 months	2.92 (2.05, 3.73)[a]		
	13 months to 6 years	2.28 (1.64, 2.93) [ab]		
	>6 years	3.27 (2.07, 4.86) [ab]		
TCHO	≤6 months	3.30 (2.82, 4.46)[c]	17.767	0.001
	7–12 months	2.75 (2.56, 4.01)[d]		
	13 months to 6 years	4.95 (3.84, 5.47)		
	>6 years	6.28 (4.89, 6.90) [cd]		
ALT	≤6 months	107.50 (80.5, 156.25)	5.35	0.148
	7–12 months	92.50 (54.00, 143.75)		
	13 months to 6 years	71.50 (48.25, 91.25)		
	>6 years	87.5 (54.00, 108.00)		

Figure 6 and Figure 7 show the schematic diagrams of the protein structure (dimer) of GPD1 missense variant loci, which mainly exists in the dimer binding surface (R229Q), active site (R269Q), helical bundle (C214R, A274T), near active site (Q299R), and β-folding (G167D), in patients with HTGTI. Arg269, as a conserved active site of the ternary complex of GPD1/DHAP/NAD^+, is in direct contact with the substrate and provides 3 of 11 hydrogen bonds in the dimer interface. Gly299 constitutes the other fixed point of the three-dimensional structure of the binary complex of GPD1 with NAD^+ (Ou et al., 2006). Furthermore, we can see the simulation diagram of amino acid/protein three-dimensional structural changes caused by a missense variant of the *GPD1* gene. These six missense variants of the *GPD1* gene result in the substitution of one amino acid for another. We can see these tertiary structural changes in the spatial structure of amino acid disability before and after missense variants at these sites. The defective protein assembly and connective enzyme structure may lead to loss of GPD1 enzyme activity.

At present, 10 types of GPD1 variants in HTGTI patients have been reported. As this disease is a very rare genetic metabolic disease in children, the specific carrying frequencies of many mutation sites of the *GPD1* gene in the population was not available in the public databases such as Genecards and dbSNP. The most frequent variant types of GPD1 are c.361-1G > C (35.5%) and c.895G > A (32.3%). The odds ratio (OR) of c.361-1G > C to c.895G > A (32.3%) is 1.15 (95% confidence interval (CI) 0.40–3.31, $P = 0.39$), and the OR of c.361-1G > C to c.640T > C and c.901G > T was 7.98 (95% CI 1.59–39.9, $P = 0.006$); moreover, the OR of c.361-1G > C to c.686G > A, c.806G > A, c.820G > A, c.220–2A > G, c.523C > T, c.931C > T, c.500G > A, and c.116G > A was 16.5 (95% CI 1.97–138.0, $P = 0.005$).

Discussion

Transient infantile hypertriglyceridemia (HTGTI) was first reported by Basel-Vanagaite et al. (2012). They explicitly showed primary dyslipidemia, a lipid metabolism disorder, in early infancy is caused by a monogenic variant in the *GPD1* gene. Among the reported 31 patients all over the world, the typical clinical manifestations are hypertriglyceridemia, hepatomegaly, elevated transaminase, hepatic steatosis, and early-stage hepatic fibrosis. Additional heterogeneous phenotypes include prolonged jaundice, cholestasis, fasting ketosis, hypoglycemia, insulin resistance, obesity, kidney disease, and growth retardation. The cases of HTGTI have been reported in different regions of the world and among various ethnic groups. It suggests that there may be no regional and ethnic differences in prevalence. Although the ratio of males to females is 1.6:1, we still have no evidence for the characteristic of sex-controlled inheritance. The onset age of the disease is very early with a median age of 6 months, and the morbidity rate was 75% before 1 year of age. As an autosomal recessive disease, the parents of HTGTI patients showed no related symptoms, but consanguineous marriage may be a high-risk factor for the disease. Therefore, we should be alert to the possibility of HTGTI when some infants and children are accompanied by unexplained HTG, hepatosplenomegaly, and abnormal liver function.

It is generally considered that HTG caused by the *GPD1* variant is temporary, which will gradually become regular with age. However, the analysis of the laboratory data of TG provided by these patients showed that only 30% of the patients recovered to normal from HTG during follow-up. The remaining patients still had different degrees of HTG during the follow-up period. This abnormal situation even lasted until the age of 31 years. Our fitting curve also shows that HTG in early infants decreases

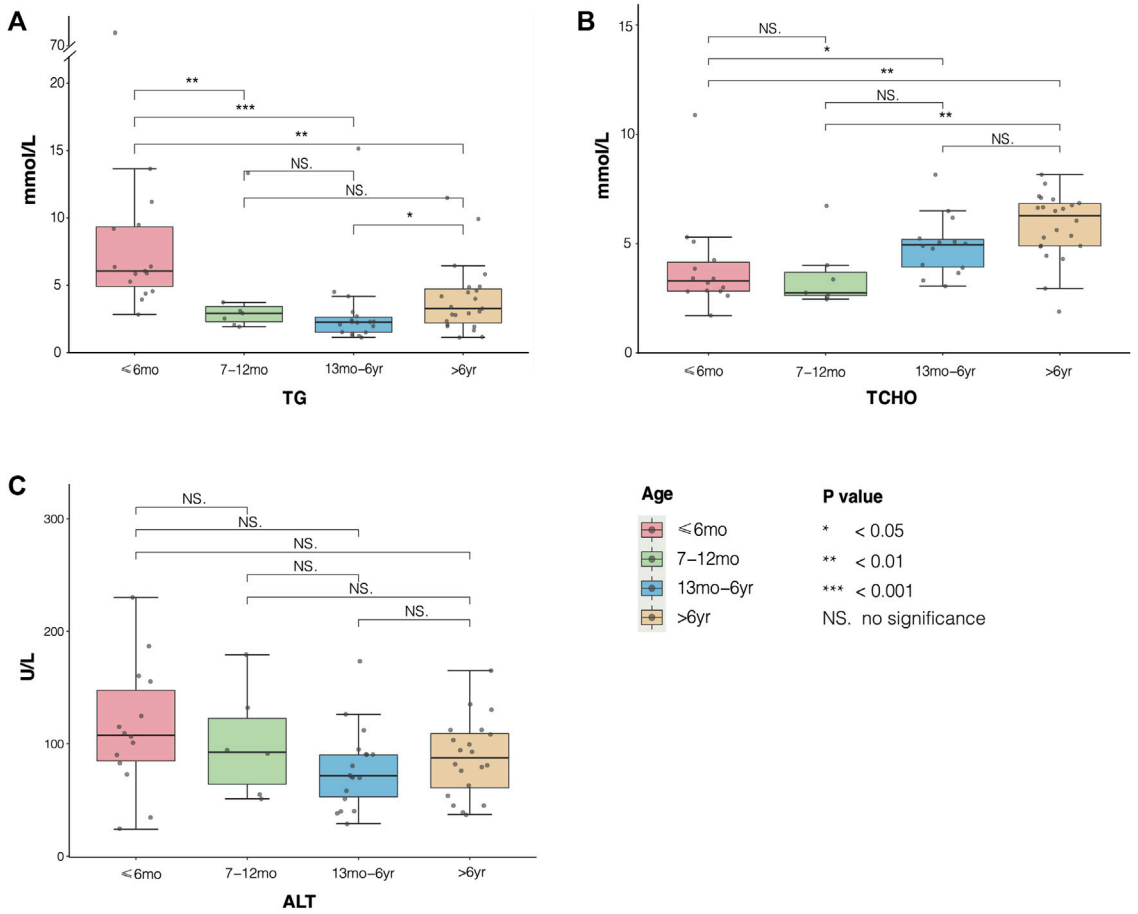

FIGURE 3
Distribution of serum triglyceride (TG), TCHO, and alanine aminotransferase (ALT) in different age groups of patients with transient infantile hypertriglyceridemia (HTGTI). The boxplots showed median and interquartile level values of TC, TCHO, and ALT in the four age groups. **(A)** statistical differences in TG were shown in group ≤ 6months compared with the other three groups, and group 13 months to 6 years with group >6 years ($P < 0.05$). **(B)** significant differences in TCHO were observed in group ≤6 months compared with group 13 months to 6 years and group >6 years, and group 7–12 months with group 6 years (all $P < 0.05$). **(C)** there were no significant differences in serum ALT among the four groups (all $P > 0.05$).

rapidly with age, especially within 1 year of age. However, its average level still exceeded the normal upper limit during the later period. Meanwhile, some children were still accompanied by continuous mild to moderate fatty liver, elevated transaminase, and liver cirrhosis. Therefore, the characteristic of mild or moderate persisting HTG in some patients indicates that it is a prolonged process rather than temporary. This also reflects the persistent disorder of lipid metabolism, which may have a long-term adverse effect on health. Epidemiological survey data show that the morbidity of HTG in adults was 10–20% (Parhofer and Laufs 2019; Laufs et al., 2020), but there are no relevant investigation data of infants for use. At the same time, the overall prevalence of NAFLD in children was approximately 3–10% (Anderson et al., 2015). In particular, many studies indicate that HTG is an independent or increased risk factor for atherosclerosis, coronary heart disease, diabetes, acute pancreatitis, and even tumors (Cullen 2000; Huang et al., 2016; Packard et al., 2020). These findings may help us better understand the disease and maintain more sustained attention to dyslipidemia in childhood.

Dyslipidemia is the most common lipid metabolism disorder. However, its exact etiology and pathogenic mechanisms remain elusive. The occurrence of HTG results from either increased synthesis in the liver or decreased TG degradation and uptake. The HTG phenotype is regulated by the complex networks of multiple gene variation and secondary factors (Hegele et al., 2014; Lewis et al., 2015; Liu et al., 2019). Previous studies have demonstrated that monogenic HTG in patients with severe HTG (TG > 10 mmol/L) displays classic autosomal recessive hereditary disorders. Affected individuals are often

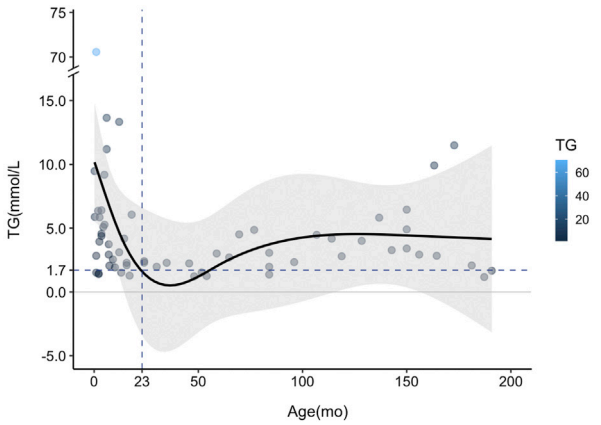

FIGURE 4
Curve fitting of serum triglyceride (TG) changing trend with age in patients with transient infantile hypertriglyceridemia (HTGTI). Restricted cubic spline (RCS) with four knots was used to model the association of serum TG with age. The curve shows a good simulation of the correlation between this binary ($p < 0.001$). The TG level decreased sharply from the peak point in the early age of infants to the normal value (1.7 mmol/L) but rebounded during follow-up, especially after approximately 50 months old.

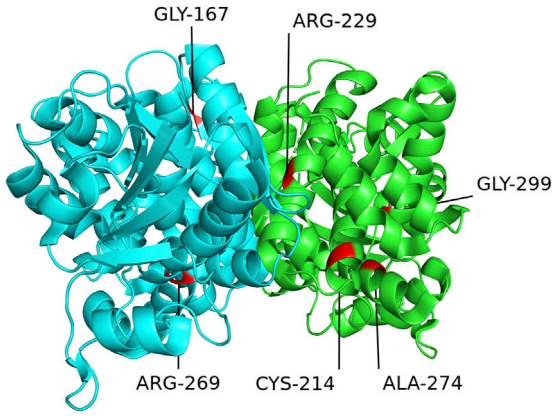

FIGURE 6
Schematic diagram of protein structure of mutation loci of the glycerol-3-phosphate dehydrogenase 1 (*GPD1*) gene. This diagram shows the location of the GPD1 missense mutation sites in the protein structure (dimer) in patients with transient infantile hypertriglyceridemia (HTGTI): it mainly exists in the dimer binding surface (R229Q), active site (R269Q), helical bundle (C214R, A274T), near active site (Q299R), and β-folding (G167D). These protein structures of GPD1 were drawn using PyMOL software.

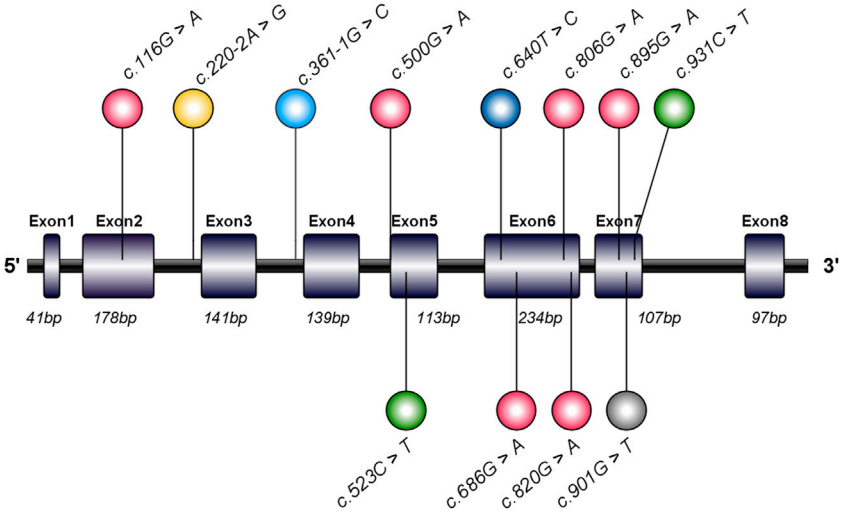

FIGURE 5
Schematic diagram and mutation sites of glycerol-3-phosphate dehydrogenase 1 (*GPD1*) gene in transient infantile hypertriglyceridemia (HTGTI) patients. The human GPD1 contains eight exons and encodes a protein consisting of 349 amino acid residues. This image illustrates the spectrum of GPD1 mutation sites associated with HTGTI from the reported cases, marked in the lollipop style, including 10 mutation sites on exons and 2 on introns. This spectrum was generated using illustrator for biological sequences (IBS).

homozygous or compound heterozygous for large-effect loss-of-function variants in genes that regulate catabolism of TG-rich lipoproteins, such as *LPL* (Henderson et al., 1991), *APOC2* (Baggio et al., 1986), *APOA5* (Kao et al., 2003), *LMF1* (Peterfy et al., 2007), *GPIHBP1* (Wang and Hegele 2007), and *GPD1* (Hegele et al., 2014; Liu et al., 2019).

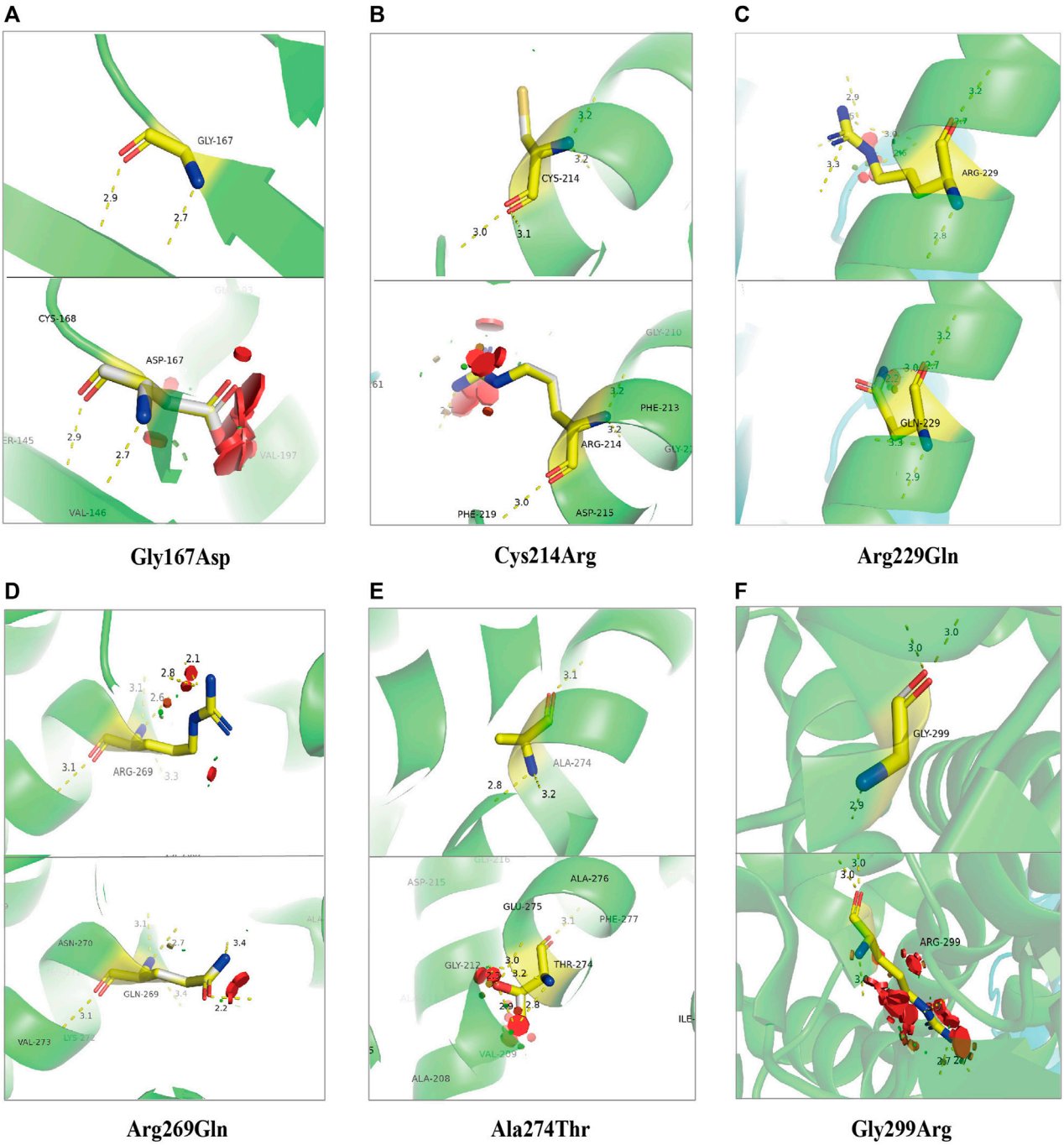

FIGURE 7
Stimulation diagram of amino acid/protein three-dimensional structure changes caused by missense variants of *GPD1* gene. They exist in the β-folding [**(A)**. Gly167Asp], helical bundle [**(B)**. Cys214Arg and **(E)**. Ala274Thr], the dimer binding surface [**(C)**. Arg229Gln], active site [**(D)**. Arg269Gln and **(F)**. Gly299Arg]. We can see these tertiary structural changes in the spatial strcture of amino acid disability before and after missense variants at these sites. This picture was drawn using PyMOL software.

The human *GPD1* gene is located on chromosomal region 12q12-q13 (RefSeq: NM_005276.2) and encodes cytoplasmic NAD-dependent GPD1. The open reading frame of mRNA, including eight coding exons, encodes a protein of 349 amino acid residues, which catalyzes the reversible biological conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate

(G3P). The variants in the *GPD1* gene have been reported to cause HTGTI in these 31 patients. The primary variant sites of the *GPD1* gene were missense variants (51.6%), followed by splicing variants (35.5%) and nonsense variants (12.9%). Research has shown that the splice-site homozygous variant in intro 3 (c.361-1G > C) creates a truncated protein of 213 residues and missing some major secondary structures and active sites (Basel-Vanagaite et al., 2012). The compound heterozygous missense variant in exon 6 (c.686G > A) is associated with a lack of GPD1 protein and a reduction in CPT1 and CPT2 activity (Joshi et al., 2014). Another compound heterozygous missense variant in exon 6 (c.820G > A) and splicing variant in exon 3 (c.220–2A > G) generated a decreased expression of the protein and the loss of bases (Li et al., 2017). The crystal structure of GPD1 in Rao's study showed that Arg229 provides 3 of 11 hydrogen bonds in the dimer interface. Arg269, as a conserved active site of the ternary complex of GPD1/DHAP/NAD^+, is in direct contact with the substrate, while Gly299 constitutes the other fixed point of the three-dimensional structure of the binary complex of GPD1 with NAD^+(Ou et al., 2006). Therefore, variants at these loci resulted in the loss or low activity of the GPD1 enzyme, which further led to the disorder of glucose and lipid metabolism.

The deep investigation of monogenic dyslipidemias has the potential to reveal previously unrecognized key pathways in lipid metabolism. HTGTI is caused by the inactivation and variant of *GPD1*, which plays a critical role in carbohydrate and lipid metabolism. The *GPD1* gene variant is one of the significant molecular etiologies of primary HTG with the onset of disease in infancy (Basel-Vanagaite et al., 2012). Basel-Vanagaite et al. (2012) confirmed that the transience of HTG might follow the pattern of hepatocyte triglyceridemia, that is, a higher secretion rate in the newborn than in adults. However, the exact mechanism behind the multiple clinical characteristics and pathogenesis of the disease remains largely unknown, which needs to be further clarified. At present, there is no satisfactory therapeutic approach available for this inherited disorder. The Summary Report of the Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents advises that people aged 10–21 years with lipid abnormalities (LDL cholesterol level of ≥250 mg/dl and/or TG level of >500 mg/dl) should be handled for 3–6 months with diet adjustments (Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents; National Heart, Lung, and Blood Institute, 2011). A diet rich in low-fat medium-chain TG or antihyperlipidemic drugs may be effective in some people for lowering TG levels. Sometimes, pharmacological therapy should be considered for children and adolescents (Fiorentino and Chiarelli 2021), while efficacy and safety data for triglyceride-lowering drugs are still prudent under 18 years old (Valaiyapathi et al., 2017; Fiorentino and Chiarelli 2021).

Owing to the absence of specific biochemical markers, the underdiagnosis of HTGTI is quite possible in children or adolescents with hepatomegaly or hepatopathy of unknown origin or nonalcoholic steatohepatitis (NASH) (Tesarova et al., 2021). In recent years, with the application of gene sequencing in the diagnosis of rare diseases, more and more hereditary metabolic diseases have been confirmed and gained effective treatment and prognostic guidance. Meanwhile, it has also increased the awareness of the features and mechanisms of these sorts of diseases, for example, the diagnosis and treatment of HTGTI have also benefited a lot from it, and new cases are being discovered constantly (Lin et al., 2021). We believe that our understanding about HTGTI will gradually grow in the future. However, it is necessary to update the knowledge that dyslipidemia may not be transient in infancy, and HTG may rebound and present long-lasting abnormality with mild to moderate levels for the majority of these patients. Furthermore, cirrhosis developing at such a young age in some children opposes the prevailing perception of GPD1 being a benign or transient disease. Therefore, some specialists advise that the original name of the disease, "transient infantile hypertriglyceridemia," should be abandoned because it cannot precisely reflect the condition of the patient (Tesarova et al., 2021).

Conclusion

Although severe HTG is transient at the early age of infants for some patients, it may not maintain normal with age spontaneously for most of these patients. Meanwhile, persistent mild HTG and liver damage indicate long-lasting detrimental effects on the health of patients rather than transience. However, as the specific signs and symptoms for diagnosis and the long-term follow-up data are still unavailable, the clinical manifestations and molecular pathogenesis of HTGTI are not entirely understood. Therefore, a lipid-clinic evaluation and a close follow-up are recommended for the patients throughout their life.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, and further inquiries can be directed to the corresponding author.

Ethics statement

Since the patients and data of this study were extracted from published case reports, and have no any experiments in

this study, ethical review and written consent are not required.

Author contributions

JW and DL: conceptualization and original manuscript drafting; XS and ML: data curation; XS, LJ, and ZX: formal analysis and editing; JW, YZ, and PX: investigation and Figures 1–7; TT and RL: investigation and Tables 1–2. RF: writing-review and editing. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Low circulating PCSK9 levels in *LPL* homozygous children with chylomicronemia syndrome in a syrian refugee family in Lebanon

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Familial chylomicronemia syndrome is a rare autosomal recessive disorder of lipoprotein metabolism characterized by the presence of chylomicrons in fasting plasma and an important increase in plasma triglycerides (TG) levels that can exceed 22.58 mmol/l. The disease is associated with recurrent episodes of abdominal pain and pancreatitis, eruptive cutaneous xanthomatosis, lipemia retinalis, and hepatosplenomegaly. A consanguineous Syrian family who migrated to Lebanon was referred to our laboratory after perceiving familial chylomicronemia syndrome in two children. The *LPL* and *PCSK9* genes were sequenced and plasma PCSK9 levels were measured. Sanger sequencing of the *LPL* gene revealed the presence of the p.(Val227Phe) pathogenic variant in exon 5 at the homozygous state in the two affected children, and at the heterozygous state in the other recruited family members. Interestingly, PCSK9 levels in homozygous carriers of the p.(Val227Phe) were ≈50% lower than those in heterozygous carriers of the variant (p-value = 0.13) and ranged between the 5th and the 7.5th percentile of PCSK9 levels in a sample of Lebanese children of approximately the same age group. Moreover, this is the first reported case of individuals carrying simultaneously an *LPL* pathogenic variant and *PCSK9* variants, the L10 and L11 leucine insertion, which can lower and raise low-density lipoprotein cholesterol (LDL-C) levels respectively. TG levels fluctuated concomitantly between the two children, were especially high following the migration from a country to another, and were reduced under a low-fat diet. This case is crucial to raise public awareness on the risks of consanguineous marriages to decrease the emergence of inherited autosomal recessive diseases. It also highlights the importance of the early diagnosis and management of these diseases to prevent serious complications, such as recurrent pancreatitis in the case of familial hyperchylomicronemia.

KEYWORDS

chylomicronemia syndrome, type 1 hyperlipoproteinemia, LPL gene, triglycerides, Lebanon, Syria, PCSK9 levels

Introduction

Genetic rare diseases usually require special care that could be challenging and hardly achievable, especially in war crises or migration situations. This is the case of type I hyperlipoproteinemia (T1HLP).

T1HLP (OMIM 238600), also known as familial hyperchylomicronemia, familial lipoprotein lipase deficiency (Pingitore et al., 2016), or familial chylomicronemia syndrome (FCS) (Caddeo et al., 2018), is a rare autosomal recessive disorder of lipoprotein metabolism characterized by the presence of chylomicrons in fasting plasma and an important increase in plasma triglycerides (TG) levels that can exceed 2,000 mg/dl (22.58 mmol/l) (Burnett et al., 1993). This condition is associated with recurrent episodes of abdominal pain and pancreatitis, eruptive cutaneous xanthomatosis, lipemia retinalis, and hepatosplenomegaly (Brunzell and Deeb, 2019). Recurrent episodes of pancreatitis in these patients can affect the functions of the pancreas and can, in severe cases, lead to a multi-organ failure and an increase in morbidity and mortality (Regmi and Rehman, 2021). The prevalence of the disease in the general population is estimated to be one to two per million (Abifadel et al., 2004; Rodrigues et al., 2016), however, it is higher in some isolated ethnic groups (i.e., French Canadians, Afrikaner) (Gagné et al., 1989; Henderson et al., 1992; Foubert et al., 1996; Pingitore et al., 2016), and in populations with a high incidence of consanguinity. T1HLP is caused in most cases by loss-of-function variants in the lipoprotein lipase (*LPL*) gene (Langlois et al., 1989; Caddeo et al., 2018). The latter encodes a secreted protein that plays a crucial role in lipid metabolism and homeostasis through the hydrolysis of TG transported by TG-rich lipoproteins [very low-density lipoproteins (VLDL) and chylomicrons] to decrease plasma TG and generate free fatty acids that are either stored in the adipose tissue or oxidized by the muscles (Wion et al., 1987; Pingitore et al., 2016; Rodrigues et al., 2016). In rarer cases, T1HLP is caused by variants in the apolipoprotein C2 (*APOC2*) (Catapano and Capurso, 1986; Cox et al., 1988; Zanelli et al., 1994) and apolipoprotein A5 (*APOA5*) (Pennacchio and Rubin, 2003; Priore Oliva et al., 2005) genes encoding respectively ApoC2 and ApoA5 which are LPL activators, the glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (*GPIHBP1*) gene which encodes a protein that plays a role in the transport and binding of LPL to the endothelial cell wall and its entry into the capillaries (Beigneux et al., 2009; Davies et al., 2010; Song et al., 2022), and in the lipase maturation factor 1 (*LMF1*) gene encoding an endoplasmic reticulum membrane protein, that plays a role in the posttranslational folding, assembly and stabilization of active homodimerized LPL (Péterfy et al., 2007; Péterfy, 2012).

An early diagnosis of the disease is important in order to instore as early as possible a very low-fat diet consisting of reducing the dietary fat to ≤20 g/day or 15% of the total daily energy intake to prevent abdominal pain and recurrent pancreatitis. The goal is to maintain plasma TG levels below 1,000 mg/dl (11.29 mmol/l). It is noted that recurrent abdominal pain is prevented when TG levels are maintained below 2,000 mg/dl (22.58 mmol/l) (Burnett et al., 1993). However, the compliance to the diet is usually poor (Caddeo et al., 2018), and its control is difficult, especially among migrants or low-income populations.

Interestingly, patients with chylomicronemia syndrome generally present low low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) levels besides high TG levels (Fojo and Brewer, 1992; Hegele, 2013; Blom et al., 2018; O'Dea et al., 2019). More recently, *PCSK9* has been identified as a major protagonist in lipid metabolism and familial hypercholesterolemia (FH) (Abifadel et al., 2003). Many studies have reported a potential correlation between plasma PCSK9 levels and LDL-C, but also TG, VLDL-C, and intermediate-density lipoprotein cholesterol (IDL-C). Investigations linking these actors of the lipid pathway are being conducted (Lakoski et al., 2009; Druce et al., 2015; Norata et al., 2016; Baragetti et al., 2018; Warden et al., 2020).

In this article, we report the case of a consanguineous family of Syrian refugees in Lebanon with two children suffering from familial hyperchylomicronemia. To our knowledge, this is the first study to measure circulating PCSK9 levels in familial hyperchylomicronemia and to identify individuals carrying simultaneously variants in the *LPL* and the *PCSK9* genes.

Materials and methods

Study participants

A Syrian family who migrated to Lebanon was referred to our laboratory upon perceiving familial hyperchylomicronemia in two children. The parents and four of their children were recruited. We collected the clinical history and anthropometric data for the recruited members. The parents signed the informed consent to participate with their children in our study. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Hôtel Dieu de France Hospital and the Saint Joseph University of Beirut.

Laboratory and biochemical tests

Blood samples were obtained after overnight fasting, plasma, and serum were prepared and stocked at −80°C. Lipid measurements were determined on a COBAS INTEGRA®

analyzer (Roche Diagnostics, Basel, Switzerland). Non-HDL-C was calculated by subtracting HDL-C from total cholesterol (TC).

DNA analysis and variant detection

Genomic DNAs of all the participants were extracted from peripheral blood leukocytes using the illustra™ blood genomicPrep Mini Spin Kit according to the manufacturer's instructions. The exons and the flanking exon-intron boundaries of the *LPL* and *PCSK9* genes were amplified by polymerase chain reaction (PCR) and sequenced using the Sanger method. PCR conditions and primers' sequences are available upon request. For DNA sequence assembly and variant detection, the CodonCode Aligner® Software was used.

In silico analysis of the variant

The Genome Aggregation Database (gnomAD; http://gnomad.broadinstitute.org/) was used for frequency determination of the variant. The Polymorphism Phenotyping version 2 (PolyPhen-2; http://genetics. bwh.harvard.edu/pph2/), Protein Variation Effect Analyzer (PROVEAN; http://provean.jcvi.org/index.php), Mutation Taster (http://www.mutationtaster.org/), and the Combined Annotation Dependent Depletion score (CADD score; https://cadd.gs.washington.edu/snv) were used to predict the pathogenicity of the variant.

PCSK9 measurements

We measured PCSK9 levels in the plasma of the recruited members using a commercial ELISA kit (Human Proprotein Convertase 9/PCSK9 Duoset catalog no. DY3888; R&D Systems, Minneapolis, MN, United States) and the Bio-Plex Pro assay technology (Luminex Corporation, Austin, TX, United States) as previously described (El Khoury et al., 2018).

Statistical analysis

The variables were analyzed using the GraphPad Prism version 9. Results for quantitative variables were expressed as median with its interquartile ranges (first quartile and third quartile). Spearman correlation was performed to measure the strength and direction of the linear relationship between PCSK9 and other quantitative variables. The Mann–Whitney U test was used to compare PCSK9 values between homozygous and heterozygous carriers of the p.(Val227Phe) variant in the *LPL* gene.

Results

Clinical characteristics and biochemical analysis

In the recruited family, the parents were first-degree cousins and two of their six children suffered from familial hyperchylomicronemia confirmed by lipoprotein electrophoresis and later by genetic sequencing (Figure 1).

The proband (III.6) was a girl aged 6 years at the time of recruitment. She presented high TG levels (7.51 mmol/l) and low LDL-C and HDL-C levels (0.38 and 0.33 mmol/l respectively). At the age of 9 years, she was underweight with a BMI of 16.5 kg/m^2. She suffered from recurrent abdominal pain, vomiting, and diarrhea almost every week, and the echography showed that she presented splenomegaly. Infectious gastrointestinal causes were ruled out. She suffered as well from chronic anemia. Thalassemia was excluded by the normal results of hemoglobin electrophoresis. Mediterranean fever was suspected and the *MEFV* gene (NM_000243), responsible for the disease was studied by Sanger sequencing. Analysis of the exons and the flanking intronic regions did not reveal the presence of any pathogenic variant. However, this study does not preclude large deletions. Recently, she had an outbreak of the disease with TG levels reaching 54.33 mmol/l and they were rapidly reduced to 4.29 mmol/l following a very strict low-fat diet.

Her brother (III.5), aged 7 years at the time of recruitment, had TG levels fluctuating between 33.22 mmol/l for the highest value and 3.175 mmol/l for the lowest. At recruitment, he presented high TG levels (4.99 mmol/l) and low LDL-C and HDL-C levels (0.51 and 0.36 mmol/l respectively). At the age of 10 years, he was also underweight with a BMI of 14.6 kg/m^2. Clinically, he presented cutaneous xanthomatosis on his elbows and asthenia. He was diagnosed with splenomegaly consecutive to echography. He presented hemoglobin levels that fluctuated between normal and low. The normal results of hemoglobin electrophoresis allowed to rule out thalassemia. Recently, he presented moderately high levels of TG.

Curiously, TG levels fluctuated concomitantly between the two children and were especially high following the migration from a country to another. For example, the TG levels were the highest upon the arrival of the family to Lebanon, and then they were gradually reduced.

Both children were under fenofibrate but in a discontinuous way, omega 3, vitamin D, folic acid, iron, and a low-fat diet.

Two of their siblings (III.1 and III.4) presented normal TG and TC levels (Table 1). Their parents (II.3 and II.4) presented moderate hypertriglyceridemia according to the 2018 ACC/AHA classification (Table 1) (Grundy et al., 2019). The father aged 45 years was obese.

PCSK9 measurements

Patients with T1HLP generally present low LDL-C levels and HDL-C levels besides high TG levels (Fojo and Brewer,

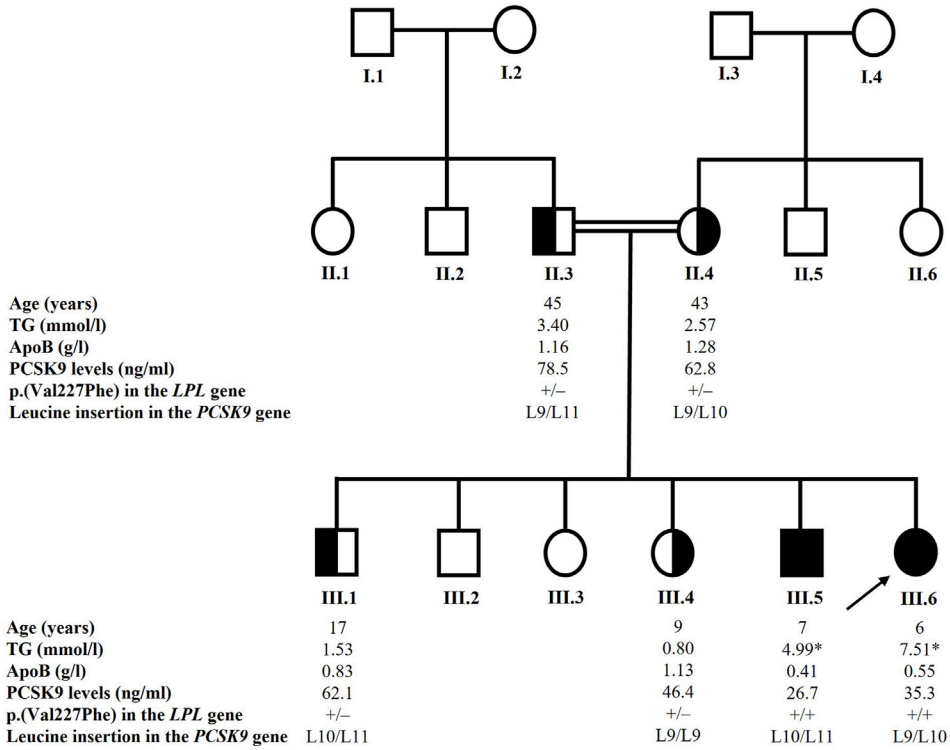

FIGURE 1
Pedigree of the family. The arrow indicates the proband. Blackened symbols indicate affected homozygous carriers of the p.(Val227Phe) variant in the *LPL* gene. Half-blackened symbols indicate heterozygous carriers of the p.(Val227Phe) variant. Only individuals with available and reported data were included in the study. The * sign indicates TG value while the patient was under a very strict low-fat diet. The +/+ sign indicates that the individual is homozygous for the p.(Val227Phe) variant in the *LPL* gene and the +/– sign indicates that the individual is heterozygous for the variant. The L9 designates the normal allele in exon 1 of the *PCSK9* gene, the L10 designates the p.Leu21dup or p.L15_L16insL and the L11 variant designates the p.Leu21tri or p.L15_L16ins2L. Age at recruitment is given in years. TG levels are given in mmol/l, ApoB levels are given in g/l and PCSK9 levels are given in ng/ml. ApoB: apolipoprotein B; LPL: lipoprotein lipase; PCSK9: proprotein convertase subtilisin/kexin type 9; TG: triglycerides.

1992; Hegele, 2013; Blom et al., 2018; O'Dea et al., 2019). Moreover, many studies have reported a potential correlation between plasma PCSK9 levels and LDL-C, but also triglycerides-rich lipoproteins and investigations linking these actors are being conducted (Lakoski et al., 2009; Druce et al., 2015; Norata et al., 2016; Baragetti et al., 2018; Warden et al., 2020). For these reasons, we measured plasma PCSK9 levels in the recruited members of the family. The results are presented in Table 1 and Figure 2. Homozygous carriers of the p.(Val227Phe) variant (III.5 and III.6) presented a ≈50% decrease in PCSK9 levels compared to heterozygous carriers (II.3, II.4, III.1, and III.4) [median with its interquartile ranges (first quartile–third quartile) of 31.00 (26.70–35.30) ng/ml versus 62.45 (50.33–74.58) ng/ml, respectively] without being significant (p-value = 0.13). Moreover, using Spearman correlation PCSK9 levels were positively correlated with age, ApoB levels and with BMI in all family members (r = 0.94, p-value = 0.017; r = 0.89, p-value = 0.033 and r = 0.94, p-value = 0.017, respectively).

Genetic analysis

We sequenced all the exons and the flanking exon-intron boundaries of the *LPL* gene knowing that most cases of T1HLP are caused by loss-of-function variations in it. Sequencing of this gene revealed the presence of the c.679G > T variation in exon 5 (NM_000237.3) at the homozygous state in both affected children (III.5 and III.6) (Figure 1). This nucleotide change causes a valine to phenylalanine substitution at position 227 of the amino acid chain [p.(Val227Phe)]. The parents (II.3 and II.4) and the two recruited siblings (III.1 and III.4) were heterozygous for the p.(Val227Phe) variation (Figure 1).

In silico analysis revealed that the amino acid valine at position 227 is well conserved among species. The variation is not present in the gnomAD database and was predicted to be disease-causing on Mutation Taster (Grantham Matrix score of 50), deleterious on PROVEAN (with a score of -4.549), with probably damaging consequences on the functionality of the protein according to Polyphen-2 (score of 0.960, sensitivity: 0.63;

TABLE 1 Lipid measurements and characteristics of the recruited members.

Subject	Age	Gender	BMI at recruitment	TC	TG	LDL-C	HDL-C	Non-HDL-C	ApoB	Plasma PCSK9 levels	p.(Val227Phe) variant in the *LPL* gene	Leucine insertion in the *PCSK9* gene
			(kg/m²)			*(mmol/l)*			*(g/l)*	*(ng/ml)*		
II.3	45	M	33.4	4.42	3.40	2.07	0.67	3.75	1.16	78.5	+/–	L9/L11
II.4	43	F	25.4	4.92	2.57	2.69	0.93	3.99	1.28	62.8	+/–	L9/L10
III.1	17	M	24.5	3.00	1.53	1.57	0.62	2.38	0.83	62.1	+/–	L10/L11
III.4	9	F	14.8	4.32	0.80	2.69	0.93	3.39	1.13	46.4	+/–	L9/L9
III.5	7	M	13.9	5.46	4.99*	0.51	0.36	5.10	0.41	26.7	+/+	L10/L11
III.6¥	6	F	15.7	5.54	7.51*	0.38	0.33	5.21	0.55	35.3	+/+	L9/L10

¥sign indicates the proband.

*sign indicates TG value while the patient was under a very strict low-fat diet.

The +/+ sign indicates that the individual is homozygous for the p.(Val227Phe) variant in the *LPL* gene and the +/– sign indicates that the individual is heterozygous for the variant. The L10 designates the p.Leu21dup or p.L15_L16insL in the *PCSK9* gene and the L11 variant designates the p.Leu21tri or p.L15_L16ins2L.

Age at recruitment is given in years. BMI, is given in kg/m^2. TC, TG, LDL-C, HDL-C, and non-HDL-C levels are given in mmol/l, ApoB levels are given in g/l and PCSK9 levels are given in ng/ml.

ApoB, apolipoprotein B; F, female; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LPL, lipoprotein lipase; M, male; PCSK9, proprotein convertase subtilisin/kexin type 9; Non-HDL-C, non high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

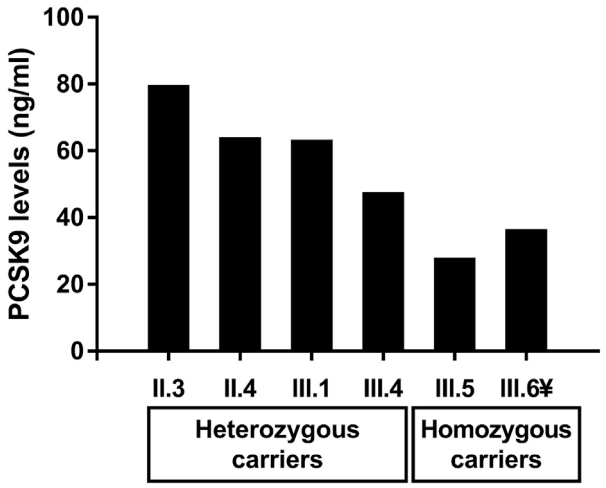

FIGURE 2
Levels of circulating PCSK9 in heterozygous and homozygous carriers of the p.(Val227Phe) variant in the *LPL* gene in the recruited family. The ¥ sign indicates the proband.

specificity: 0.92), and presented a CADD score of 25.8 suggesting that this variant is predicted to be among the top 1% of the most deleterious variants in the human genome.

Interestingly, another *LPL* variant in the same codon (p.Val227Ala) was also reported to be responsible for T1HLP (Maruyama et al., 2004; Caddeo et al., 2018).

We also sequenced the *PCSK9* gene to investigate the presence of an eventual variant that might explain the low levels of PCSK9 observed in some individuals of the family. The sequencing revealed the presence of two types of variations in exon 1 of the *PCSK9* gene: the leucine insertion L10 also designated p.Leu21dup or p.L15_L16insL and the leucine insertion L11 also designated p.Leu21tri or p.L15_L16ins2L (Abifadel et al., 2008). The father (II.3) was heterozygous for the L11 variation while the mother (II.4) and one of the affected children (III.6) were heterozygous for the L10 variation. One child (III.4) carried the normal L9 alleles, and two children (III.1 and III.5) were compound heterozygotes for the L10 and L11 alleles. The results are presented in Figure 1.

Discussion

The p.(Val227Phe) pathogenic variant has been recently reported by Caddeo et al. (2018) in a University Hospital in Gothenburg (Sweden) at the homozygous state in a proband aged 29 years who presented a mean TG level of 18.7 ± 3.2 mmol/l and recurrent pancreatitis episodes. His brother also carried the pathogenic variant. They have likely migrated from the Middle East to Sweden where the center received migrants from Syria and Iran (Caddeo et al., 2018). Functional studies demonstrated the pathogenicity of the p.(Val227Phe) loss-of-function variant. These studies suggested that this variant affects protein production and secretion, but not its degradation. Results showed that the transfection of the HEK 293T/17 cells with the p.(Val227Phe) variant caused a reduction in protein synthesis by 35%–40% and a decrease by at least 80% in the secretion of the LPL in the media of these cells compared to the wild type LPL. Moreover, LPL enzymatic assay showed that LPL activity was absent in the media of the cells transfected with the

variant. It is noteworthy that there was no difference in the intracellular degradation rate between the wild type and the mutant *LPL* transfected cells (Caddeo et al., 2018).

We identified the p.(Val227Phe) variant in the *LPL* gene causing T1HLP in a consanguineous Syrian family who migrated to Lebanon. Indeed, this is the first study to measure PCSK9 levels in T1HLP. In this family, homozygous carriers of the p.(Val227Phe) variant presented lower levels of PCSK9 than heterozygous carriers [median (first quartile–third quartile) of 31.00 (26.70–35.30) ng/ml versus 62.45 (50.33–74.58) ng/ml, respectively]. PCSK9 levels were positively correlated with age, ApoB levels and with BMI in all family members (*p*-values = 0.017, 0.033, and 0.017 respectively).Compared to a cohort of 279 Lebanese children of approximately the same age group (8–11 years) in whom PCSK9 levels were measured using the same method [median (first-third quartile) of 64.30 (46.95–93.38) ng/ml] (Azar et al., 2022), the two homozygous children (III.5 and III.6) presented PCSK9 levels that were approximately between the fifth (30.40 ng/ml) and the 7.5th percentile (33.25 ng/ml) of PCSK9 levels in the group, while their heterozygous sibling (III.4) presented PCSK9 levels (46.40 ng/ml) that were approximately within the 25th percentile (46.95 ng/ml). The sibling (III.1) presented PCSK9 levels of 62.1 ng/ml, which is normal compared to a group of 159 Lebanese adolescents aged 15–18 years [median (first-third quartile) of 60.55 (44.51–93.96) ng/ml] (Azar et al., 2022). The presence of loss-of-function variants in the *PCSK9* gene has been studied by sequencing the whole gene. Interestingly, this is the first report of a family with an *LPL* pathogenic variant also carrying variants in *PCSK9*, more precisely two types of leucine insertion variants in the exon 1 of the *PCSK9* gene (Table 1). These polymorphisms occur in the signal peptide region of the PCSK9 protein and are characterized by the insertion of one or two leucines into a stretch of nine leucines (Chen et al., 2005; Yue et al., 2006; Abifadel et al., 2008). They might lead to a structural change in the signal peptide causing an impairment in its cleavage and processing in the endoplasmic reticulum (Yue et al., 2006; Pisciotta et al., 2012; Benito-Vicente et al., 2022). In a study conducted on 1,745 apparently healthy individuals, plasma PCSK9 levels were significantly lower in individuals carrying a leucine insertion in exon 1 of the *PCSK9* gene (Awan et al., 2013). Moreover, the insertion of two leucines in the signal peptide has been reported in a family with familial combined hyperlipidemia and two patients with FH (Abifadel et al., 2008) and *in vitro* studies have shown that it causes a reduction in the secretion of the mature form of PCSK9 compared to the wild type PCSK9 (Benito-Vicente et al., 2022). However, these variations might not alone explain the very low levels of PCSK9 observed in homozygous carriers of the *LPL* variant in our study.

Moreover, homozygous carriers of the p.(Val227Phe) variant in this family presented low levels of LDL-C and HDL-C. This was also observed in the study conducted by Caddeo et al. (2018), as well as in patients suffering from T1HLP described in the literature (O'Dea et al., 2019; Susheela et al., 2021). The low levels of LDL-C in our studied family cannot be explained by the presence of the leucine insertions in the signal peptide of PCSK9. On one hand, the leucine insertion L10 also designated p.Leu21dup or p.L15_L16insL is a common variation associated with lower levels of LDL-C in populations with normal to low LDL-C levels, but also in patients suffering from FH carrying the same p.(Cys681X) mutation in the LDL receptor (*LDLr*) gene (Abifadel et al., 2009). On the other hand, the leucine insertion L11 also designated p.Leu21tri or p.L15_L16ins2L is a rare variant that has been associated with familial combined hyperlipidemia and was found at a low frequency in subjects presenting LDL-C levels of 2.96–4.90 mmol/L and coronary lesions in the American population (Chen et al., 2005; Abifadel et al., 2008; Benito-Vicente et al., 2022). In fact, low levels of LDL-C and HDL-C in T1HLP may be explained by the disruption in the activity of the LPL (Hegele et al., 2018). Variations in LPL activity in humans result in changes in lipoproteins metabolism (Goldberg et al., 1990). Indeed, low or absent activity of LPL results in an impairment in the conversion of TG-rich particles to their remnant lipoproteins, including chylomicron remnants, VLDL, VLDL remnants, intermediate density lipoprotein and LDL. The subsequent decrease in the available cholesterol from VLDL, LDL, and peripheral tissues causes a decrease in HDL-C levels (Hegele et al., 2018). Other studies attribute the low levels of LDL-C and HDL-C to an increase in their catabolism, besides the decrease in their synthesis (Fojo and Brewer, 1992). Interestingly, the low levels of LDL-C might explain the observed low levels of plasma PCSK9. In fact, many studies have shown a positive correlation between circulating plasma PCSK9 and LDL-C (Shapiro et al., 2019). It is suggested that this correlation is due to the fact that PCSK9 acts as an important regulator of LDL metabolism through targeting the LDLR for lysosomal degradation, but also due to a direct interaction between PCSK9 and LDL (Abifadel et al., 2010; Tavori et al., 2015). Further studies of PCSK9 levels in patients with hyperchylomicronemia would be interesting in order to verify if all homozygous patients with T1HLP have low PCSK9 levels, or if it is specific to the patients in the studied family, and to decipher the causes of this decrease, as well as the mechanism, the role of PCSK9, and its correlation with ApoB levels (Ayoub et al., 2021).

It is noteworthy that anemia has been described as a clinical sign of T1HLP that occurs in some cases (Rahalkar and Hegele, 2008; Brahm and Hegele, 2013). In a case series of infants with T1HLP, 7 out of 16 infants presented normocytic anemia which cause was unknown, and both males and females were affected (Feoli-Fonseca et al., 1998). In another study conducted to determine the phenotype-genotype relationships between different subgroups of T1HLP, all the female participants (n = 7) suffered from anemia (Chokshi et al., 2014).

TABLE 2 Summary of the described variations in the *LPL* gene in the Middle East and the Mediterranean regions.

Region	Amino acid variation in the *LPL* gene according to the article	Case presentation	References
Lebanon, 2004	Homozygous for the p.(Asp174Val)/p.(Asp201Val) variant (according to the original/present nomenclature)	A 34-year-old male with TG levels of 34.3 mmol/l lowered to 6.37 mmol/l under medication and a low-fat diet. A 7-year-old boy with a TG peak of 30.45 mmol/l during an episode of pancreatitis and recurrent abdominal pain since 3 years old	Abifadel et al. (2004)
Greece, 2004	Compound heterozygous for the p.(Gly188Glu) and p.(Met301Arg) variants (according to the original nomenclature)	A 32-day-old girl with TG levels of 169.3 mmol/l at the time of admission to the hospital and rapidly lowered to 11.2 mmol/l 10 days after the administration of medium-chain triglycerides enriched milk	Kavazarakis et al. (2004)
Middle East, 2013	Homozygous for the p.(Arg270His) variant (according to the present nomenclature)	A 2-month-old Arab infant with a TG peak of 276.6 mmol/l rapidly lowered to 4.93 mmol/l at the time of discharge from the hospital after the administration of a medium-chain triglycerides-rich diet.	Hegele et al. (2018)
Morocco, 2015	Homozygous for the p.(Ser286Arg) variant (according to the present nomenclature)	A 19-year-old girl with TG levels of 199 mmol/l lowered to 14.15 mmol/l 2 months after the administration of an appropriate diet, a maximal dose of fenofibrate and simvastatin along with heparin and insulin	Bouabdellah et al. (2015)
Middle East, 2016	(p.Gly256Thrfs*26) (according to the present nomenclature) (p.Met404Arg) (according to the present nomenclature)	A 19-year-old female with TG levels of 60.7 ± 7 mmol/l and a 32-year-old male with TG levels of 31.7 ± 7.8 mmol/l A 28-year-old male with TG levels of 45 ± 26.9 mmol/l	Pingitore et al. (2016)
Some of them from the Middle East, 2017	Compound heterozygous for the p.(Trp113Arg), the p.(Gly215Glu), and the p.(Met404Arg) variants (according to the present nomenclature)	A 49-year-old female with TG levels of 39.7 ± 13.6 mmol/l	Caddeo et al. (2018)
	Heterozygous for the p.(Ser220Arg) variant (according to the present nomenclature)	A 69-year-old male with TG levels of 19.6 ± 9.4 mmol/l	Caddeo et al. (2018)
	Homozygous for the p.(Val227Phe) variant (according to the present nomenclature)	A 29-year-old male with TG levels of 18.7 ± 3.2 mmol/l	Caddeo et al. (2018)

The original nomenclature considers the amino acid numbering of the mature protein [without the signal peptide of 27 amino acids (Deeb and Peng, 1989)], while the present international nomenclature considers the initiator methionine as the first amino acid of the LPL (NP_000228.1).

Although the parents (II.3 and II.4) were heterozygous carriers of the p.(Val227Phe) variant, they presented moderately high levels of TG. The phenotypic expression of heterozygous LPL deficiency is not clinically and biochemically well described yet. It has been reported that heterozygous carriers of one defective allele in the *LPL* gene do not present chylomicronemia nor other manifestations of the disease. They may present normal or moderately increased fasting TG levels, especially when fed a high-calorie, high-fat diet (Miesenböck et al., 1993; Hölzl et al., 2000; Abifadel et al., 2004). The presence of precipitating factors such as age, obesity, pregnancy, hyperinsulinemia, and lipid-raising drugs would contribute to the phenotypic expression of heterozygous LPL deficiency (Wilson et al., 1990; Abifadel et al., 2004).

The identification of heterozygous carriers of an *LPL* mutation is of major importance, especially in countries with a high frequency of consanguineous marriages (Abifadel et al., 2004). It is also necessary for the prevention of precipitating factors knowing that the lipoprotein phenotype in heterozygous carriers of a defective *LPL* allele has been considered atherogenic (Reymer et al., 1995; Bijvoet et al., 1996; Wittrup et al., 1997; Hölzl et al., 2000).

The high prevalence of consanguineous marriages in Middle Eastern countries increases the risk of autosomal recessive genetic diseases (Al-Herz and Al-Mousa, 2013) such as hyperchylomicronemia.

A study conducted to determine the prevalence of consanguineous marriages in Syria showed that the overall frequency was 30.3% in urban areas and 39.8% in rural ones, with an overall rate of 35.4%. In some provinces, the frequency could reach 67.5%. Among this type of marriage, first cousins' marriages were the most common with a rate of 20.9% (Othman and Saadat, 2009). It is noteworthy that the natality rate increases in refugees' camps or migrant populations, as well as the risk of consanguineous marriages.

To date, more than 200 pathogenic variants in the *LPL* gene have been reported to cause T1HLP (Caddeo et al., 2018). However, rare cases have been described in the Middle East and the Mediterranean regions. A summary of the described mutations in this region is presented in Table 2.

Conclusion

This is the first study to measure plasma PCSK9 levels in T1HLP. We found that homozygous affected patients presented low levels of PCSK9 compared to heterozygous non-affected members of the family and to children from the same age group. Further studies of PCSK9 levels in patients with hyperchylomicronemia would be interesting in order to verify if all homozygous patients with T1HLP have low PCSK9 levels. This would help decipher the causes of this decrease, as well as the mechanism and the role of PCSK9 in this disease. It might also be interesting to elucidate pathways linking PCSK9 to apolipoprotein B or eventually to triglycerides-rich lipoproteins metabolism.

The identification of the same mutation in the *LPL* gene in two distinct families with T1HLP originated most probably from the same region in Syria and that migrated to either Lebanon or Sweden, should lead us to search for this mutation as the first cause of hyperchylomicronemia in patients originated from Syria. A founder effect could be hypothesized but needs further investigation and cases to be confirmed.

Public awareness and education concerning the medical risks of consanguineous marriages are important and should be included in the international effort and politics that provide care, birth control, and genetic counseling when needed. They might help in decreasing the emergence of inherited autosomal recessive diseases.

Finally, handling a metabolic disease that needs specific care and compliance to a drastic regimen or treatment to prevent some fatal complications is not easy, especially in a crisis. The main solution is to prevent the emergence of these diseases by raising awareness of the risks of consanguineous marriages.

It is more and more urgent to address genetic lipid disorders and more generally genetic diseases that need special treatment, diagnosis, management, and prevention especially in refugee populations or in war or economic crisis countries.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of the Hôtel Dieu de France Hospital and the Saint Joseph University of Beirut. Written informed consent to participate in this study was provided by the participants or by the participants' legal guardian/next of kin for children.

Author contributions

Conceptualization: MA, PE, and SJ; methodology: CA, YA, YA-K, YG, SE, PE, and MA; investigation: DM; writing—original draft preparation: CA, YA, YA-K, YG, SE, PE, and MA; writing—review and editing: CA, PE, SE, YG, YA, YA-K, MV, CB, and MA; supervision: MA and PE; funding acquisition: MA, MV, and CB. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Efficacy of Long-Term Treatment of Autosomal Recessive Hypercholesterolemia With Lomitapide: A Subanalysis of the Pan-European Lomitapide Study

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Background and aim: Autosomal recessive hypercholesterolemia (ARH) is a rare autosomal recessive disorder of low-density lipoprotein (LDL) metabolism caused by pathogenic variants in the *LDLRAP1* gene. Like homozygous familial hypercholesterolemia, ARH is resistant to conventional LDL-lowering medications and causes a high risk of atherosclerotic cardiovascular diseases (ASCVDs) and aortic valve stenosis. Lomitapide is emerging as an efficacious therapy in classical HoFH, but few data are available for ARH.

Results: This is a subanalysis carried out on nine ARH patients included in the Pan-European Lomitapide Study. The age at starting lomitapide was 46 (interquartile range (IQR), 39.0–65.5) years, with a median treatment duration of 31.0 (IQR 14.0–40.5) months. At baseline, four (44.4%) patients had hypertension, one (11.1%) had diabetes mellitus, two (22.2%) were active smokers, and five (55.5%) reported ASCVD. The baseline LDL-C was 257.0 (IQR, 165.3–309.2) mg/dL. All patients were on statins plus ezetimibe, three were receiving Lipoprotein apheresis (LA), and one was also receiving proprotein convertase subtilisin/kexin type 9 inhibitors (PCSK9i). The addition of lomitapide (mean dose, 10 mg) resulted in the achievement of a median on-treatment LDL-C of 101.7 mg/dL (IQR, 71.3–138.3; 60.4% reduction from baseline), with a best LDL-C value of 68.0 mg/dL (IQR, 43.7–86.7; 73.5% reduction from baseline). During follow-up, one patient stopped both PCSK9i and LA. Recurrence of ASCVD events was reported in one patient. The median on-treatment aspartate transaminase and alanine transaminase values were 31.1 (IQR, 22.6–48.3) U/L and 31.1 (IQR, 27.2–53.8) U/L, respectively. Among six ARH patients

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with available fibroscan examination, liver stiffness values recorded at the last visit were within the normal range (median, 4.7 KPa; IQR, 3.6–5.3 KPa).

Conclusion: Lomitapide is effective and safe in ARH therapy as well as in classical HoFH.

Keywords: Real-world study, rare disease, autosomal recessive hypercholesterolaemia, LDL-C, lomitapide, long-term, efficacy, safety

INTRODUCTION

Autosomal recessive hypercholesterolemia (ARH) is an ultrarare genetic disorder of lipid metabolism caused by disruptive variants in both alleles of the gene coding for the low-density lipoprotein receptor (LDLR) adaptor protein-1 (*LDLRAP1*) (D'Erasmo et al., 2020; Bertolini et al., 2020; Tromp et al., 2022; D'Erasmo et al., 2018). LDLRAP1 is a cytosolic protein required for the LDLR-mediated internalization of low-density lipoproteins (LDL) in polarized cells, such as hepatocytes (D'Erasmo et al., 2020; Bertolini et al., 2020; Tromp et al., 2022; D'Erasmo et al., 2018). This leads to a defective clearance of LDL particles from circulation, thereby causing a severe elevation of LDL cholesterol (LDL-C). ARH patients have an increased risk of premature atherosclerotic cardiovascular disease (ASCVD), which is comparable to that observed in homozygous familial hypercholesterolemia (HoFH) with defective pathogenic variants in the *LDLR* gene (D'Erasmo et al., 2020; Bertolini et al., 2020; Tromp et al., 2022; D'Erasmo et al., 2018). It is well known that in HoFH, the magnitude and duration of exposure to elevated values of LDL cholesterol (also called as LDL-C burden) largely determine the cardiovascular prognosis (D'Erasmo et al., 2020; Tromp et al., 2022; D'Erasmo et al., 2018). A recent survey on the natural history of ARH showed considerable heterogeneity in response to treatments but also highlighted that LDL-C in ARH patients stays far from the ideal target despite intensive treatment with conventional lipid-lowering therapies (including a combination of statins and/or ezetimibe and/or lipoprotein apheresis). As a consequence, their cardiovascular risk remains very high (D'Erasmo et al., 2020; D'Erasmo et al., 2018), thereby urging the identification of more effective treatments.

Monoclonal antibodies against proprotein convertase subtilisin/kexin type 9 inhibitors (PCSK9i) and lomitapide are the newly available therapies for severe forms of genetic hypercholesterolemia (D'Erasmo et al., 2021a). However, the efficacy of PCSK9i in ARH is currently disputed because the mechanism of their actions is largely dependent on the extent of residual LDLR functionality (D'Erasmo et al., 2021a; Cesaro et al., 2022). In contrast, lomitapide inhibits the activity of microsomal triglyceride transfer protein (MTP), which decreases the secretion of very-low-density lipoprotein (VLDL) by the liver, and, consequently, the production of LDL is derived from VLDL. Thus, lomitapide acts using an LDLR-independent mechanism, therefore potentially extending its efficacy in patients with an absent LDLR function, like those with ARH. Previous findings have suggested the existence of an inverse correlation between residual LDLR activity and MTP expression and activity, thereby probably causing an inverse correlation between LDLR activity and the effectiveness of a pharmacological MTP inhibition (Sirtori et al., 2014). Although preliminary observations have indicated the efficacy of lomitapide in ARH, the size of this benefit and, more importantly, its long-term safety has been poorly investigated. We have recently completed a real-world survey, including hypercholesterolemic patients with biallelic pathogenic variants in *LDLR* and *LDLRAP1* receiving lomitapide, in Europe (D'Erasmo et al., 2021b; D'Erasmo et al., 2021c). This unique cohort provides the opportunity to perform a subanalysis of the carriers of homozygous mutations in *LDLRAP1* to specifically evaluate the long-term LDL-lowering potential and safety of lomitapide in ARH.

MATERIAL AND METHODS

Study Design and Study Patients

This is a post hoc analysis of the Pan-European Lomitapide Study (D'Erasmo et al., 2021b) performed on a subgroup of patients diagnosed with ARH due to homozygous mutation in *LDLRAP1*. The Pan-European Lomitapide Study was a multicenter, observational, and retrospective survey collecting data from all patients known to be receiving lomitapide in Europe in the context of usual clinical practice and without protocol-mandated procedures (D'Erasmo et al., 2021b). The protocol of the Pan-European Lomitapide Study has been reported in detail elsewhere (D'Erasmo et al., 2021b). For a brief period, physicians were asked to retrospectively retrieve demographic and clinical information from medical records. Baseline data were defined as those at the date of initiation of lomitapide, whereas last follow-up data were those at the time of the last clinic visit up to December 31, 2019, respectively (D'Erasmo et al., 2021b). Details of concomitant lipid-lowering therapies, dosages of lomitapide, and side effects at each visit were requested to assess the efficacy and safety of this treatment (D'Erasmo et al., 2021b).

Statistical Analysis

For descriptive statistics, continuous traits were presented as mean and standard deviation or as the median and interquartile range (IQR), as appropriate (D'Erasmo et al., 2021c). Categorical traits were shown as numbers and proportions. Comparisons were carried out using Mann–Whitney test for non-normally distributed variables and Student's t-test for normally distributed variables (D'Erasmo et al., 2021c). For differences between categorical traits, the p value was calculated using the Chi-squared or Fisher's exact test, as appropriate. Paired t-test was used to evaluate the difference between untreated, lowest, and last visit total and LDL-C, as well as LDL-C burden pre- vs. on-treatment.

Linear regression with the enter method was used to evaluate associations (D'Erasmo et al., 2021c). Values that were not normally distributed were *log*-transformed before entering the model (D'Erasmo et al., 2021c).

Statistical analyses were performed using the IBM Statistical Package for Social Sciences (IBM SPSS, version 25.0, Inc. Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

RESULTS

Baseline Characteristics

The baseline characteristics of the nine ARH patients included in the Pan-European study are summarized in **Table 1**. Patients were equally distributed between sex, and they were middle-aged (median age, 52.1 years old; IQR, 43.9–67.4 years old). Three of the four women had been pregnant before the start of lomitapide. Most of them (N = 7) were from Italy and two were West Asian–European Turkish. Approximately 90% presented with xanthomata. They were slightly overweight, with a prevalence of current smoking (22.2%) and diabetes mellitus (11.1%). Moreover, approximately half of the patients had well-controlled hypertension. The majority (55.6%) had already experienced at least one ASCVD event before starting lomitapide and, among these patients, the mean age at the first event was 45.2 ± 7.9. **Table 1** shows that the untreated mean LDL-C level was very similar to that observed in typical HoFH carrying defective *LDLR* pathogenic variants (Bertolini et al., 2020). Their median baseline LDL-C level was 257.0 mg/dL (IQR, 165.3–309.2) despite receiving a combination of lipid-lowering therapies that included statins and ezetimibe in all patients, evolocumab in one patient, and lipoprotein apheresis (LA) in three patients (one weekly, one biweekly, and one monthly). HDL-C and triglyceride levels were within normal ranges.

TABLE 1 | Baseline characteristics of ARH patients

	Lomitapide cohort (N = 9)	
Demographic Variables		
Age, years (IQR)	52.1 (43.9–67.4)	
Male, *n* (%)	5 (55.6)	
Geographic Origin, *n* (%)		
European	7 (77.7)	
West Asian-European Turkish	2 (22.2)	
Xanthomas, *n* (%)	8 (88.9)	
ARH genotypes, *n* (%)		
c.430_431insA	5 (55.5)	
c.406C > T	1 (11.1)	
c.89–1 G > C	1 (11.1)	
Not provided	2 (22.2)	
Previous Pregnancy, *n* (%)	3 (42.9)	
Risk factors		
BMI, kg/m^2 (IQR)	28.7 (22.2–35.9)	
Current Smoking, *n* (%)	2 (22.2)	
T2DM, *n* (%)	1 (11.1)	
Hypertension, *n* (%)	4 (44.4)	
PAS (mmHg)	120.0 (111.7–122)	
PAD (mmHg)	71.0 (67.7–80.0)	
Previous MACE, *n* (%)	5 (55.6)	
Age at first MACE, yrs	45.2 ± 7.9	
Plasma Lipids(mg/dl)	mg/dL	mmol/dL
Untreated		
Total cholesterol	569.5 (476.5–666.5)	14.72
LDL-C	464.0 (455.4–608.0)	11.99
Baseline		
Total cholesterol	337.0 (229.5–389.0)	8.71
LDL-C	257.0 (165.3–309.2)	6.64
HDL-C	51.0 (43.0–57.5)	1.32
Total triglycerides	123.0 (81.8–153.5)	1.39
Lipid lowering therapies, *n* (%)		
None	0	
Statin	9 (100.0)	
Ezetimibe	9 (100.0)	
PCKS9i	1 (11.1)	
Fibrates	0	
LA	3 (33.3)	
weekly	1 (11.1)	
bi-weekly	1 (11.1)	
Monthly	1 (11.1)	

Data are represented median (interquartile range) and number (percentage) as appropriate.
The worst lipid profile without any cholesterol lowering medication is reported as naïve values. Percentage associated with genotypes are reported on the whole cohort. ARH, autosomal recessive hypercholesterolemia; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; BMI, body mass index; T2DM, type 2 diabetes; LA, lipoprotein apheresis; PCKS9i, Proprotein convertase subtilisin/kexin type 9 inhibitors; MACE, major atherosclerotic cardiovascular events.

Efficacy Analysis

The median duration of follow-up of lomitapide treatment was 31 months (IQR, 14.0–50.5; range, 13–59 months). The addition of a mean dose of 10 ± 11.4 (IQR, 5–10) mg/day of lomitapide allowed the achievement of the best median on-treatment LDL-C value of 68.0 mg/dL (IQR, 43.7–86.7; $p < 0.001$) corresponding to a percent reduction from baseline of 72.0% (IQR, 59.7–79.2; **Figure 1**). At the last visit, a slight rise in the median value of LDL-C to 84.0 mg/dL (IQR, 66.1–115.4) was observed, thereby bringing the percentage reduction of LDL-C at the end of the follow-up to 64.2% from the baseline (IQR, 50.2–68.7; $p < 0.001$; **Figure 1**). Overall, the median on-treatment LDL-C was 101.7 mg/dL (IQR, 71.3–138.3), with wide intraindividual variability, ranging from a minimum to of 46.7 mg/dL to a maximum of 168.5 mg/dL (**Figure 2A**). When percentage change was considered from the baseline (**Figure 2B**), ARH patients displayed individual reductions ranging from a minimum of 30.6% to a maximum of 77.8%, but differences in the effects were independent of the lomitapide dose at the last visit (P = NS). Comparable results were obtained if the absolute LDL-C reduction was considered (data not shown). Notably, this lipid-lowering effect was obtained despite six patients receiving only 5 mg/day of lomitapide (**Figure 2A**). In the ARH patient receiving the quadruple combination of atorvastatin 40 mg, ezetimibe 10 mg, monthly LA, and evolocumab (baseline LDL-C of 461 mg/dL), the addition of lomitapide allowed the interruption of evolocumab and LA after 3 months due to a drop of 83% in LDL-C from baseline (**Figure 2**).

To identify possible explanations for variability in the individual lipid response, we performed univariate and

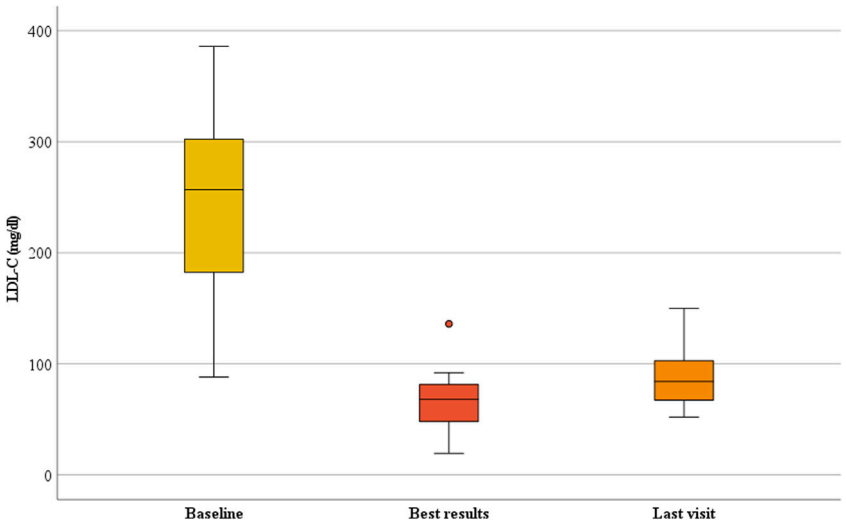

FIGURE 1 | Change in LDL-C during lomitapide therapy in ARH patients. Box plot graphs represent the median LDL-C levels in the ARH population receiving lomitapide. The yellow, red, and orange boxes represent the median LDL-C, nadir on-treatment, and last visit results, respectively. LDL-C, low-density lipoprotein cholesterol.

multivariate regression analysis to show that the achieved on-treatment LDL-C levels were independent of the duration of follow-up, sex, age at lomitapide start, untreated LDL-C, background therapy, or lomitapide dosage (data not shown).

Safety Analysis

Data on gastrointestinal side effects were not available for all patients at each timepoints. However, the most common side effect was diarrhea followed by nausea, which was reported in two patients. One patient complained of diarrhea with a mild-to-moderate intensity at each visit and she was prescribed nutritional counseling from the referring physician with benefit. She had to stop treatment because she had moved to another country, where lomitapide is not allowed. Another patient referred to the same gastrointestinal side effect and was prescribed with diet; however, he showed very poor adherence to the dietary regimen and lomitapide treatment. Indeed, he stopped lomitapide after 18 months of treatment due to gastrointestinal side effects and lack of compliance.

Changes in liver function tests during lomitapide treatment are shown in **Figure 3**. Aspartate aminotransferase (AST) levels always remained <40 U/L (**Figure 3A**) and the median on-treatment value was 31.1 U/L (IQR, 22.6–48.3 U/L). Among the four ARH patients with the longest follow-up (4 years), the median AST was 22 U/L (IQR, 16.2–36.0; range, 16–39 U/L). A similar trend was observed for alanine aminotransferase (ALT) values, whose median on-treatment value was 31.1 U/L (IQR, 27.2–53.8; **Figure 3B**). In patients with a longer follow-up, the median ALT value was 17.5 U/L (IQR, 13.5–35.7 U/L; range, 13–41 U/L).

Liver ultrasound was available in seven and eight patients at baseline and last visit, respectively (**Table 2**). Two ARH patients did not show changes in the severity of liver fat, while four experienced worsening of the hepatic fat content. Even though only one patient reported a fibroscan evaluation at baseline, this information was available in six ARH patients at the last visit. In this subgroup of patients exposed to lomitapide for a median period of 35 months (IQR, 14.0–40.2), the individual values of hepatic stiffness remained well within the normal range of <7.0 KPa (median, 4.6 KPa; range, 3.5–6.3 kPa).

In the entire group, only one patient experienced an elevation in liver function test at approximately thrice the upper limits of the normal after 15 months of treatment. AST/ALT levels returned to normal range at the next laboratory check without any change in lomitapide dosage. This patient also experienced a change in hepatic fat, from absent to moderate steatosis, at the last follow-up visit. However, the patient stopped lomitapide a few months after the liver function test elevation due to gastrointestinal side effects and poor treatment compliance.

ASCVD Outcomes

Six patients had internal carotid plaques at baseline ultrasound examination and none had progression during follow-up. Data on common carotid intima media thickness (CCA-IMT) were available in four of nine patients showing no progression of subclinical atherosclerosis. In this subgroup, the median CCA-IMT was 1.4 (0.9–2.4) mm on the right and 1.4 (1.0–1.6) mm on the left at the first lomitapide prescription. After more than 2 years of treatment, the median CCA-IMT was 1.4 (1.0–2.4) mm on the right and 1.2 (0.7–1.7) mm on the left (paired *t*-test, P = NS). One patient experienced a recurrence of ASCVD events during follow-up due to the worsening size of the abdominal aorta aneurism, coronary revascularization, and one episode of acute heart failure.

DISCUSSION

The results from the present analysis confirmed over approximately 3 years of treatment the efficacy and the

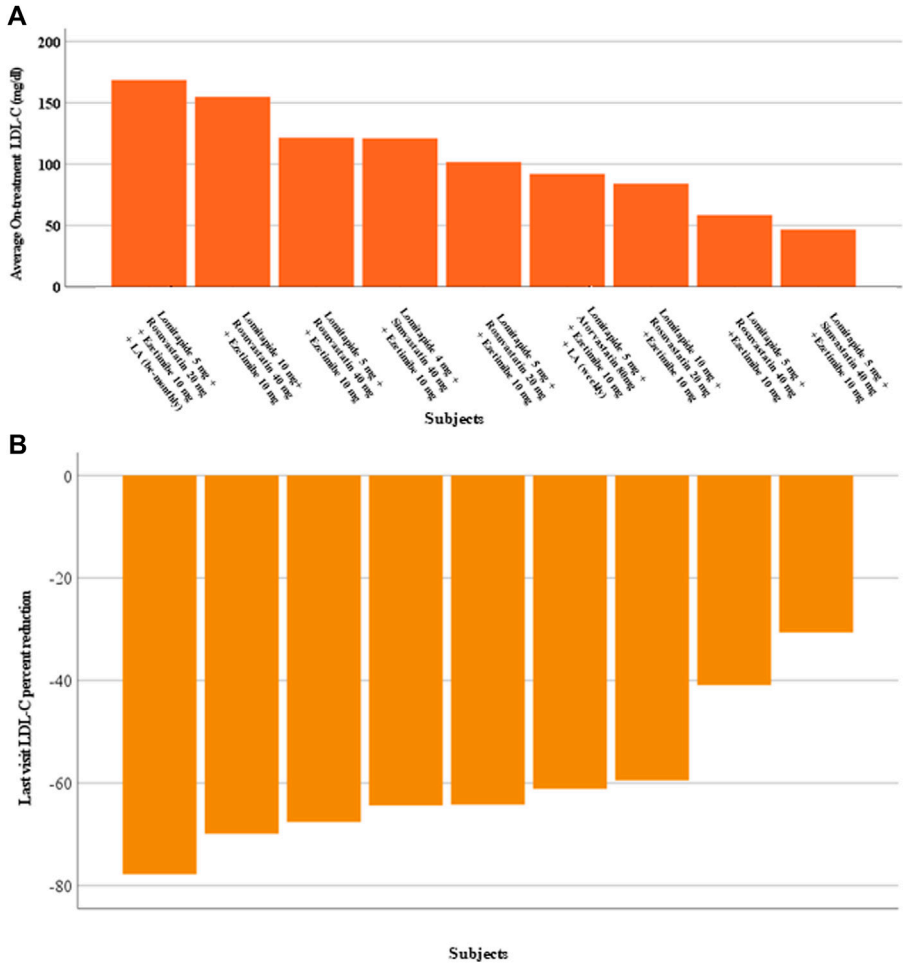

FIGURE 2 | Individual changes in LDL-C levels in ARH patients during lomitapide therapy. **(A)** This figure represents the individual achieved average on-treatment LDL-C. The therapies prescribed to the patients in the last visit are reported in the *x*-axis. **(B)** This figure represents the individual percent reduction in LDL-C from the baseline to the last visit. LDL-C, low-density lipoprotein cholesterol.

reassuring safety profile of lomitapide in ARH patients. Indeed, the adjunct of lomitapide to background lipid-lowering therapies allowed up to a 70% reduction in LDL-C from baseline, thereby resulting in an on-treatment LDL-C of 101.7 mg/dL. Moreover, neither liver function tests nor indicators of liver fibrosis showed appreciable changes in these patients during the exposure to lomitapide.

ARH patients enrolled in the present survey showed some differences compared with those previously reported by D'Erasmo *et al.* (2018). To this regard, it is interesting to note that in the Pan-European cohort (D'Erasmo et al., 2021b), we observed that ARH showed higher body mass index (BMI) compared to null/null homozygous FH patients (28.9 ± 6.6 units vs. 23.6 ± 4.2 units, respectively; $p = 0.047$). Interestingly, this difference was independent of age and gender. A recently published paper by Leigh *et al.* (2022) has demonstrated that *LDLRAP1*$^{-/-}$ mice on a chow diet gained significantly more weight and were more insulin resistant compared with control mice. Further studies are needed to confirm this observation in humans carrying bi-allelic mutations in the *LDLRAP*1 gene.

The present findings confirm those previously reported on the efficacy of lomitapide in six patients included in the paper by D'Erasmo et al. (2018), thereby showing that lomitapide reduced LDL-C from baseline up to approximately 80% with a mean dosage of approximately 20 mg/day. In the present study, longer data on the efficacy and safety of the use of lomitapide in ARH patients compared with the previously published data were included, thereby adding more granular information on the hepatic safety that was lacking in the previous observation. In addition, all the patients in the previous paper were from Italy. However, in this collection, we also retrieved information on two ARH patients treated outside Italy: one from Germany and another from the Netherlands. It must be noted that the average dosage of 10 mg/day of lomitapide recorded in the present survey was different from that of 20 mg/day reported

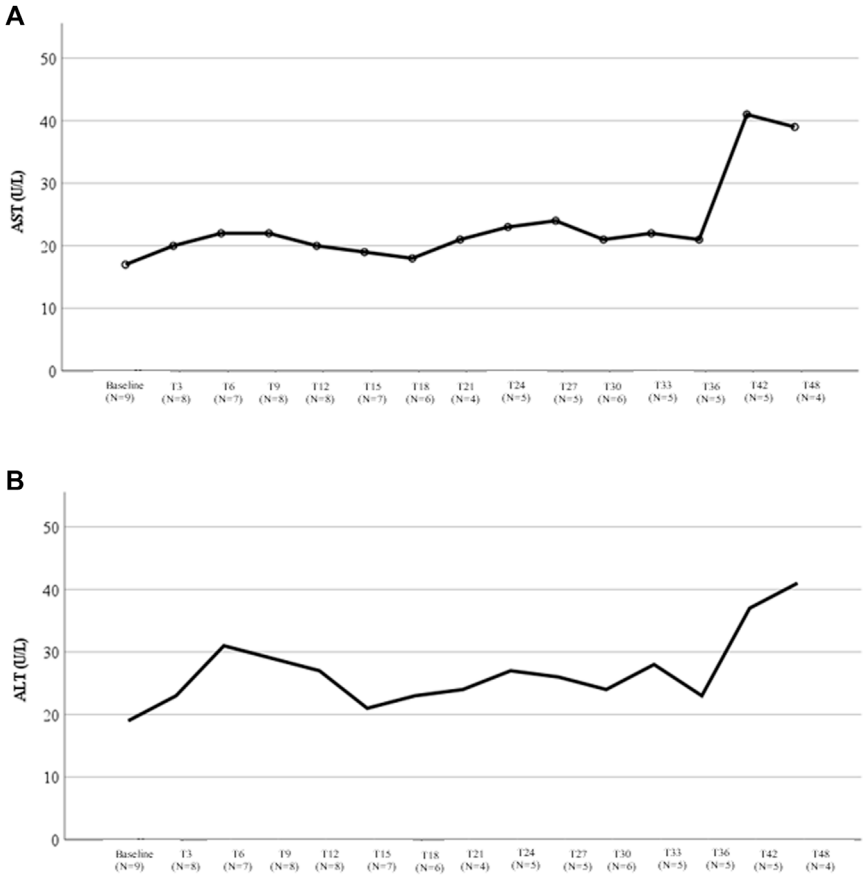

FIGURE 3 | Liver transaminases variations during therapy with lomitapide in ARH patients. **(A)** This figure represents the variation in the median AST value during treatment with lomitapide according to the months of treatment. For each timepoint, the number of patients with available data was reported. **(B)** This figure represents the variation in the median ALT value during treatment with lomitapide according to months of treatment. For each timepoint, the number of patients with available data was reported. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

in a previous study (D'Erasmo et al., 2018). This discrepancy could be explained, at least partially, with the fact that the two group of ARH patients were different. In fact, only four participated in the two surveys and two of these were enrolled in the phase 3 trial, where an escalating protocol from 5 to 60 mg/day was scheduled.

According to what observed in HoFH (D'Erasmo et al., 2021b; D'Erasmo et al., 2021c), the present study showed wide variability in the lipid-lowering response, which is difficult to explain. In the Pan-European cohort (D'Erasmo et al., 2021b), changes in LDL-C levels were obtained with a mean lomitapide dose at the last visit of 21.2 ± 14.4 mg/day in the HoFH defective/defective group, 6.7 ± 2.9 mg/day in the null/defective group, 19.1 ± 9.8 mg/day in the null/null and 10 ± 11.4 mg/day in the ARH group (data not shown). Therefore, as already shown (D'Erasmo et al., 2021b), the LDL-C lowering effect of lomitapide was independent of the residual LDLR activity and heterogeneity in the results may be explained by other factors. We can only speculate that ancestry (null/null and null/defective are mainly from abroad Italy, whereas most defective/defective and ARH are Italian), dietary habits (Perry, 2013), adherence to medications, polymorphism in the *MTP* gene (Ledmyr et al., 2002), gut microbiota characteristics, and bile metabolism (Patel et al., 2022) influence the lipid-lowering effect of lomitapide. Unfortunately, these factors were not investigated in the present work, which prevented the making of any further consideration.

Even if a direct comparison is not feasible and is out of the scope of the present study, discussing the differences in the response to PCKS9 inhibitors and lomitapide in ARH is important. The TESLA part B trial, in which 49 patients were treated with evolocumab 420 mg or placebo Q4W for 4 weeks on top of the ongoing lipid lowering therapy, reported a 30.9% reduction in LDL-C compared to baseline (Raal et al., 2015). When analyzed according to *LDLR* pathogenic variants status, the patients with *LDLR* null variants in both alleles or those with autosomal recessive HoFH did not respond to evolocumab treatment (Raal et al., 2015). A heterogeneous reduction in LDL-C levels' response to PCSK9i was also experienced in the treatment of ARH in Spain: three patients received triple and combined treatment with PCSK9i (evolocumab) as well

TABLE 2 | Baseline and last visit measurements of hepatic steatosis and stiffness in individual ARH patients treated with lomitapide

Subjects	Baseline		Last Visit	
	Ultrasound	Fibroscan	Ultrasound	Fibroscan
1	—	—	moderate	4.5
2	—	—	moderate	3.5
3	absent	—	moderate	4.8
4	Mild	—	mild	
5	absent	—	mild	3.6
6	moderate	—	Moderate	4.9
7	absent	5.7	Moderate	6.3
8	absent	—	—	—
9	absent	—	Moderate	—

Data are represented as value per each subject by reporting ultrasound and fibroscan data as previously described (D'Erasmo et al., 2021b).
For comparison data are reported at baseline and last visit. The severity of hepatic steatosis was estimated by an ultrasonographical semi-quantitative measurement of liver fat content and categorized as absent, mild, moderate, or severe (D'Erasmo et al., 2021b).

as obtained heterogeneous responses ranging from 19% to 59% reduction in LDL-C (Sánchez-Hernández et al., 2018). In contrast, with the adjunct of lomitapide, a median percent reduction of 64.2% (IQR, 50.2–68.7) at the last visit was observed with the lowest percentage of LDL-C reduction value of approximately 30%. Although both PCSK9i and lomitapide are associated with heterogeneity in the LDL-C lowering efficacy and a face-to-face comparison has never been done so far, the data of the current study suggest that discussing the possibility to tailor lipid-lowering treatments to a patient's genetic background within the scientific community can be timely, thereby potentially improving the cost–benefit balance. In the case of ARH, treatment based on the LDLR activity will not allow the achievement of LDL-C targets and, therefore, this may not be prescribed.

The analysis of the safety outcomes highlights that lomitapide is a manageable drug in ARH. According to other studies (D'Erasmo et al., 2021a; D'Erasmo et al., 2021b), gastrointestinal side effects were managed with a dietary regimen prescription. Only one patient stopped lomitapide due to diarrhea, but the responsible physicians referred poor adherence to a low-fat diet. Nutritional support may be useful for handling patients treated with lomitapide because gastrointestinal side effects in most cases are preventable and manageable with a dietary plan (D'Erasmo et al., 2021a; D'Erasmo et al., 2021b).

Even though an overall increase in liver fat, as estimated by ultrasounds, was observed in most patients, the hepatic steatosis was always described as moderate and never severe at the last follow-up visits. In the group of ARH patients who received fibroscan evaluation during follow-up, liver stiffness remained within the normal range, thereby suggesting that the increase in fat liver content was not necessarily associated with fibrosis. Moreover, the results observed in the present ARH cohort were obtained despite most patients receiving only 5 mg/day of lomitapide at the last visit. The low dose of lomitapide (mean dose, 10 mg/day) used in this population could have minimized the risk of long-term liver-associated side effects. However, the small sample size and the relatively short period of observation prevent giving any definite conclusion.

An interesting aspect to be considered is whether lomitapide may cause different liver side effects in ARH vs. HoFH. The present report was not designed to answer this question, and the findings of the Pan-European study should be referred to (D'Erasmo et al., 2018). Although no difference between HoFH and ARH in the occurrence of liver function test elevation after lomitapide was detected, an increase in liver steatosis was particularly evident among ARH patients (data not shown). Thus, ARH patients had a 2.6 (95% CI, 1.0–6.8) higher risk of having moderate steatosis compared with other genotypes, independent of age, gender, and lomitapide dose. The dysregulation in lipid storage and energy homeostasis highlighted in mice in the study by Leight *et al.* (2022) could explain this observation. However, the present data prevent us from making any definite conclusion. Moreover, whether the increase in hepatic fat content in ARH may translate into liver damage is unknown. However, the lack of change in liver stiffness up to 3 years of follow-up report in the present study argues against the signal of liver fibrosis in ARH patients exposed to lomitapide.

As an exploratory analysis, the occurrence of ASCVD during lomitapide exposure was evaluated. No progression of carotid artery disease was observed. Only one of the nine patients suffered from ASCVD recurrence. It must be noted that this patient was at really high risk as she was in her sixties and already had cardiovascular disease at the time of initiation of lomitapide. In addition, she had other cardiovascular risk factors beyond LDL-C elevation as smoking habits, hypertension, and type 2 diabetes. Although anectodical, we believe that these observations are suggestive of a potential benefit of lomitapide in halting the progression of atherosclerotic damage. Thus, additional studies focused on assessing the cardiovascular benefit of lomitapide in ARH/HoFH patients are needed to give a definite answer to this question.

LIMITATIONS

This study has several limitations that must be acknowledged. The retrospective nature of the study is the most important. Moreover, patients did not receive follow-up according to a prespecified protocol, and the management of each patient was entirely based upon the judgment of the treating physicians. This analysis included only a relatively small cohort of ARH patients. Therefore, the expansion of similar analyses to larger cohorts of ARH patients is strongly recommended. However, it should be considered that ARH is an ultrarare disease and the largest worldwide cohort described concerned 52 patients (D'Erasmo et al., 2018).

CONCLUSION

Treating ARH is an unmet clinical need because these patients are far from the recommended LDL-C goals with the conventional lipid-lowering therapies. The findings of the current study

strongly indicate that lomitapide should be considered an effective and safe LDL-lowering therapy for ARH patients, which is similar to that reported in classical HoFH (Wilemon et al., 2020).

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DATA AVAILABILITY STATEMENT

The raw data of this work will be shared upon reasonable request by the corresponding author. Requests to access the datasets should be directed to Laura D'Erasmo, laura.derasmo@uniroma1.it.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sapienza Unviersity of Rome (approval code #4928) and University Medical Centre Rotterdam (approval code #2017–1199). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: MA and LD'E; Data curation, LD'E, AG, and PS; Formal analysis, LD'E, PS, and AG; Data collection and interpretation: LD'E, ABC, ADC, SB, IM, DT, AG, MAv, GI, MDDT, GF, MG, AP, TM, CP, LC, GBV, MB, KB, FN, TS, FS, PS, CS, FF, AC, PC, FV, SDA, LP, SB, GK, EL, ED, JR, EB, KS, AV, JC, SW, MK, JR, SM-H, AE, and KL; Data Supervision: MA and LD'E; Data Validation: MA, JRvL, LD'E, AG, PS, GI, CP, AV, SB, and KS; and Writing original draft, LD'E, DT, ADC, IM SB, MA, JRvL, SB, GI, CP, and AV. All the authors have revised and approved the manuscript.

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Lysosomal acid lipase deficiency: A rare inherited dyslipidemia but potential ubiquitous factor in the development of atherosclerosis and fatty liver disease

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Lysosomal acid lipase (LAL), encoded by the gene *LIPA*, is the sole neutral lipid hydrolase in lysosomes, responsible for cleavage of cholesteryl esters and triglycerides into their component parts. Inherited forms of complete (Wolman Disease, WD) or partial LAL deficiency (cholesteryl ester storage disease, CESD) are fortunately rare. Recently, LAL has been identified as a cardiovascular risk gene in genome-wide association studies, though the directionality of risk conferred remains controversial. It has also been proposed that the low expression and activity of LAL in arterial smooth muscle cells (SMCs) that occurs inherently in nature is a likely determinant of the propensity of SMCs to form the majority of foam cells in atherosclerotic plaque. LAL also likely plays a potential role in fatty liver disease. This review highlights the nature of LAL gene mutations in WD and CESD, the association of LAL with prediction of cardiovascular risk from genome-wide association studies, the importance of relative LAL deficiency in SMC foam cells, and the need to further interrogate the pathophysiological impact and cell type-specific role of enhancing LAL activity as a novel treatment strategy to reduce the development and induce the regression of ischemic cardiovascular disease and fatty liver.

KEYWORDS

lysosomal acid lipase, LIPA, Wolman Disease, Cholesteryl Ester Storage Disease, GWAS, smooth muscle cells, atherosclerosis, nonalcoholic fatty liver disease

Introduction

Lysosomal acid lipase (LAL), encoded by the gene *LIPA*, is the sole lysosomal lipase, responsible for the critical functions of hydrolysis of cholesteryl esters (CE) contained in endocytosed lipoproteins to component free cholesterol and fatty acids, and of triglycerides to their component fatty acids. LAL also cleaves stored CE delivered to lysosomes for breakdown in the lipophagy component of autophagy (Ouimet et al., 2011), enhancement of which may be protective against atherosclerosis (Sergin et al., 2017). Complete deficiency of LAL, Wolman Disease (WD), is fatal in early life due to

malabsorption and liver disease (Grabowski et al., 2019). Near total LAL deficiency or cholesteryl ester storage disease (CESD) can have a variable phenotype but is frequently asymptomatic and difficult to identify clinically despite affected individuals having only as little as 1–12% of residual LAL activity (Bernstein et al., 2013). Recombinant LAL is now available and lifesaving for individuals with WD and useful to prevent and reverse liver fat accumulation and fibrosis and correct the dyslipidemia in CESD. In recent years *LIPA* has been identified as a cardiovascular risk allele, though the directionality of this association is not yet clear. More recently, expression of *LIPA*/LAL has been found to be low in arterial smooth muscle cells of both humans and mice, as a natural occurrence rather than due to mutations, and is the apparent cause of CE overload in lysosomes of smooth muscle cell foam cells in atherosclerosis. Low blood LAL activity has also been found to be associated with nonalcoholic fatty liver disease. In this review we summarize recent knowledge about the nature of LAL, mutations in *LIPA* leading to WD and CESD, the value of *LIPA* as a predictor of risk for atherosclerosis, and recent findings regarding its role in atherosclerosis and fatty liver disease.

LIPA/LAL regulation and function

The *LIPA* gene is located on chromosome 10q23.2–23.3 (Anderson et al., 1993) and encodes a 372 amino acid polypeptide with a 27 amino acid signal sequence necessary for both secretion and lysosomal targeting of LAL (Ameis et al., 1994). Purification of human LAL from liver tissue yields two glycoproteins of 56 kDa and 41 kDa, thought to represent a proprotein and a mature, active protein respectively (Ameis et al., 1994). The propeptide sequence, cleavage site, and location and identity of the putative responsible protease have not been identified. It has recently been suggested that LAL is not in fact a proprotein and that the 41 kDa form observed is reflective of protease cleavage during purification (Strøm et al., 2020). Mature LAL is glycosylated in the endoplasmic reticulum (ER), and mannose-6-phosphate is added in the Golgi, a critical step for lysosomal targeting and for cellular uptake of secreted or exogenous LAL by receptor-mediated endocytosis (Sando and Henke, 1982; Du et al., 1998; Zschenker et al., 2005). The pathway of trafficking and secretion of LAL, and molecular characterization of key sequences and modifications of LAL remain to be further elucidated; discussion of these remaining questions is further described in a recent review (Li and Zhang, 2019).

Structurally, LAL is similar to gastric and lingual lipase, notably containing a core domain with a catalytic triad of Ser153, Asp324, and His355 (Lohse et al., 1997), an oxyanion hole and a cap domain containing a lid which regulates substrate entry (Roussel et al., 1999; Rajamohan et al., 2020). This family of lipases is active at acidic pH, with optimal pH for LAL being 3.5–4.5 (Dubland and Francis, 2015), and is not homologous with neutral lipases, such as hormone sensitive lipase and neutral cholesteryl ester hydrolase (Anderson and Sando, 1991). Recent elucidation of the crystal structure of LAL suggests that this is mediated by protonation of Asp-361 at acidic pH, allowing opening of the lid (Rajamohan et al., 2020).

CE and triglycerides are the major substrates of LAL, and upon hydrolysis, free cholesterol and free fatty acid products are released from lysosomes. Free cholesterol and free fatty acids subsequently inhibit SREBP activation in the endoplasmic reticulum, thereby reducing new cholesterol synthesis and low density lipoprotein receptor expression, and free cholesterol can be converted to oxysterol metabolites that activate LXRα to promote expression of genes including ATP-binding cassette transporter AI (ABCA1) and ABCG1 that promote cholesterol efflux from cells (Venkateswaran et al., 2000; Horton et al., 2002). LAL therefore plays a critical role in regulation of cellular lipid metabolism in response to cellular lipid accumulation by inhibiting *de novo* cholesterol synthesis and LDL uptake, and by promoting removal of excess cholesterol from cells (Goldstein et al., 1975; Brown et al., 1976; Bowden et al., 2011). LAL is the sole neutral lipid hydrolase in the lysosome and thus is critical for hydrolysis of CE as well as triglycerides contained in endocytosed lipoproteins, and subsequent cholesterol efflux (Goldstein et al., 1975). Due to promoting ABCA1 expression and mediating release of free cholesterol from endocytosed lipoproteins (Bowden et al., 2011), LAL is a key driver of total body reverse cholesterol transport (Bowden et al., 2018). Injection of $LAL^{-/-}$ mouse macrophages containing LDL radiolabeled with ^{3}H-CE into $LAL^{+/+}$ mice resulted in significantly higher appearance of ^{3}H-cholesterol in feces compared to the same injection of lipoproteins into $LAL^{-/-}$ mice. This also demonstrated the ability of supplemental LAL to be taken up by cells and correct reverse cholesterol transport *in vivo* (Bowden et al., 2018).

LAL is similarly necessary for metabolism of lipids contained in apoptotic cells endocytosed by macrophages during efferocytosis (Viaud et al., 2018). In lipoprotein-loaded macrophages, LAL is also essential for metabolism of CE contained in cytosolic lipid droplets via lipophagy, a selective process of lipid degradation where lipid droplets are fused with lysosomes (Ouimet et al., 2011). LAL expression is also activated in conditions of nutrient deprivation by transcription factors forkhead homeobox type protein O1 (FOXO1) in adipocytes (Lettieri Barbato et al., 2013) and transcription factor EB (TFEB) in hepatocytes and other cell types (Settembre et al., 2013), as part of an autophagic and specifically lipophagic response, to mobilize fatty acids and free cholesterol. LAL also plays critical roles in lipid metabolism beyond autophagy, such as supplying free fatty acids by catabolism of triglycerides that drives alternative activation of M2 macrophages (Huang et al., 2014). During monocyte to macrophage differentiation, LAL is upregulated by Sp1 and AP-2, possibly to accommodate increased lysosomal degradation processes in macrophages (Ries et al.,

1998a). Plaque macrophages may therefore contribute to increased LAL activity in the atherosclerotic relative to normal artery wall (Dubland and Francis, 2015).

Epigenetic regulation of LIPA has been reported in a number of recent studies. Preconception maternal exposure to saturated fatty acids is associated with increased *LIPA* DNA methylation in infants (Robinson et al., 2020), and similarly, children of mothers with Type 1 Diabetes Mellitus exhibit hypermethylation and increased expression of *LIPA* (Knorr et al., 2021. Preprint.). The *LIPA* promoter is hypomethylated in obese patients with elevated LDL (Płatek et al., 2020), and *LIPA* is hypermethylated in patients with alcohol dependence (Brückmann, 2019. Dissertation.). Correlation of DNA methylation data with RNA and protein expression, and specific investigation of *LIPA* regulation and contributions to these phenotypes, will be necessary to determine the significance of these findings. DNA methylation master regulator UHRF1 and DNA methyltransferase inhibitor zebularine may upregulate *LIPA* in mesothelioma cells and mouse melanoma, respectively (Fang et al., 2021; Reardon et al., 2021), and histone deacetylase inhibitor voronistat increases LAL protein and activity in fibroblasts (Subramanian et al., 2017). Further characterization of the epigenetic regulation of *LIPA* and possible epigenetic modulators have potential for developing novel regulators of LAL expression.

LAL is present in all cell types with the exception of erythrocytes (Grabowski et al., 2019), and participates in multiple physiological processes in different tissues. Additional roles for LAL have been elucidated in thermogenesis (Duta-Mare et al., 2018; Fischer et al., 2021; Fischer et al., 2022), bile acid metabolism with effects on the gut microbiome (Sachdev et al., 2021), insulin sensitivity (Radović et al., 2016), and retinoic acid metabolism (Grumet et al., 2016).

LAL deficiency states—Wolman Disease and CESD

Mutations

Mutations in *LIPA* leading to reduced LAL activity present as two rare disorders, Wolman Disease (WD) and Cholesteryl Ester Storage Disease (CESD). They are referred to collectively as LAL deficiency (LALD). WD involves complete loss or less than 1% preservation of LAL activity (Aslanidis et al., 1996; Pagani et al., 1998; Porto, 2014), and was first described in 1956 by Wolman *et al.* who found cholesterol-laden foam cells throughout the liver, spleen, intestine, and adrenal glands of an infant female presenting with vomiting and severe hepatosplenomegaly (Abramov et al., 1956). Infants with WD typically present before 4 months of age with gastrointestinal symptoms and a reduced growth rate, and death follows in the first 6 months to 1 year of life from liver failure and malnutrition without treatment (Marshall et al., 1969; Grabowski et al., 2019). Patients with CESD, conversely, typically retain between 1–12% of LAL activity (Bernstein et al., 2013; Porto, 2014) and exhibit a range of less severe phenotypes which often remain unrecognized until later childhood or into adulthood (Bernstein et al., 2013; Aguisanda et al., 2017; Rashu et al., 2020) (Figure 1A). CESD patients characteristically will exhibit hepatomegaly, elevated serum transaminases, and dyslipidemia including reduced high density lipoprotein cholesterol (HDL-C), due to impaired upregulation of ABCA1 in response to CE sequestration in lysosomes (Bowden et al., 2011), and elevated low density lipoprotein cholesterol (LDL-C) (Bernstein et al., 2013). The progressive liver pathology caused by CESD, which involves hepatocyte accumulation of LAL substrates in the lysosome as well as lipid-overloaded liver macrophages and fibrosis (Hůlková and Elleder, 2012; Grabowski et al., 2019), may be diagnosed as non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, or cryptogenic liver disease (Bernstein et al., 2013). Often CESD patients are also at risk for accelerated atherosclerosis and premature cardiovascular disease, thought to be due to persistent dyslipidemia (Bernstein et al., 2013; Burton et al., 2015b). Additional reduction of naturally lower LAL activity in arterial smooth muscle cells (SMCs) compared to macrophages may also further promote SMC foam cell formation in atherosclerosis (see below) (Dubland et al., 2021). The most common cause of premature death in CESD patients is liver failure, which may occur in childhood, younger or older adulthood depending on the severity of LAL deficiency (Bernstein et al., 2013).

At the cellular level, LALD causes the accumulation of lipoprotein-derived CE particularly, and triglycerides, in the lysosome. This leads to a perceived cellular deficiency of both free fatty acids and free cholesterol, which cannot or are only minimally released from the lysosome due to the reduced activity of LAL. Free fatty acids and free cholesterol normally interact with SREBP (1c and 2) causing feedback inhibition of HMG-CoA reductase which reduces cholesterol synthesis, LDL receptor downregulation to reduce LDL uptake, acyl-cholesterol acyltransferase activation to esterify free cholesterol, and downregulation of fatty acid synthesis (Horton et al., 2002; Reiner et al., 2014). In LALD, the reverse occurs: in addition to increased apolipoprotein B and very low density lipoprotein (VLDL) production (Reiner et al., 2014), lack of free cholesterol released from the lysosome also impairs ABCA1 upregulation and cholesterol efflux to apolipoprotein A-I, leading to low HDL production and impaired whole body reverse cholesterol transport (Bowden et al., 2011; Bowden et al., 2018). This interruption of intracellular cholesterol flux results in excessive lipid accumulation and dysfunction in multiple cell types, manifesting most prominently in the liver in CESD and in multiple tissues in WD.

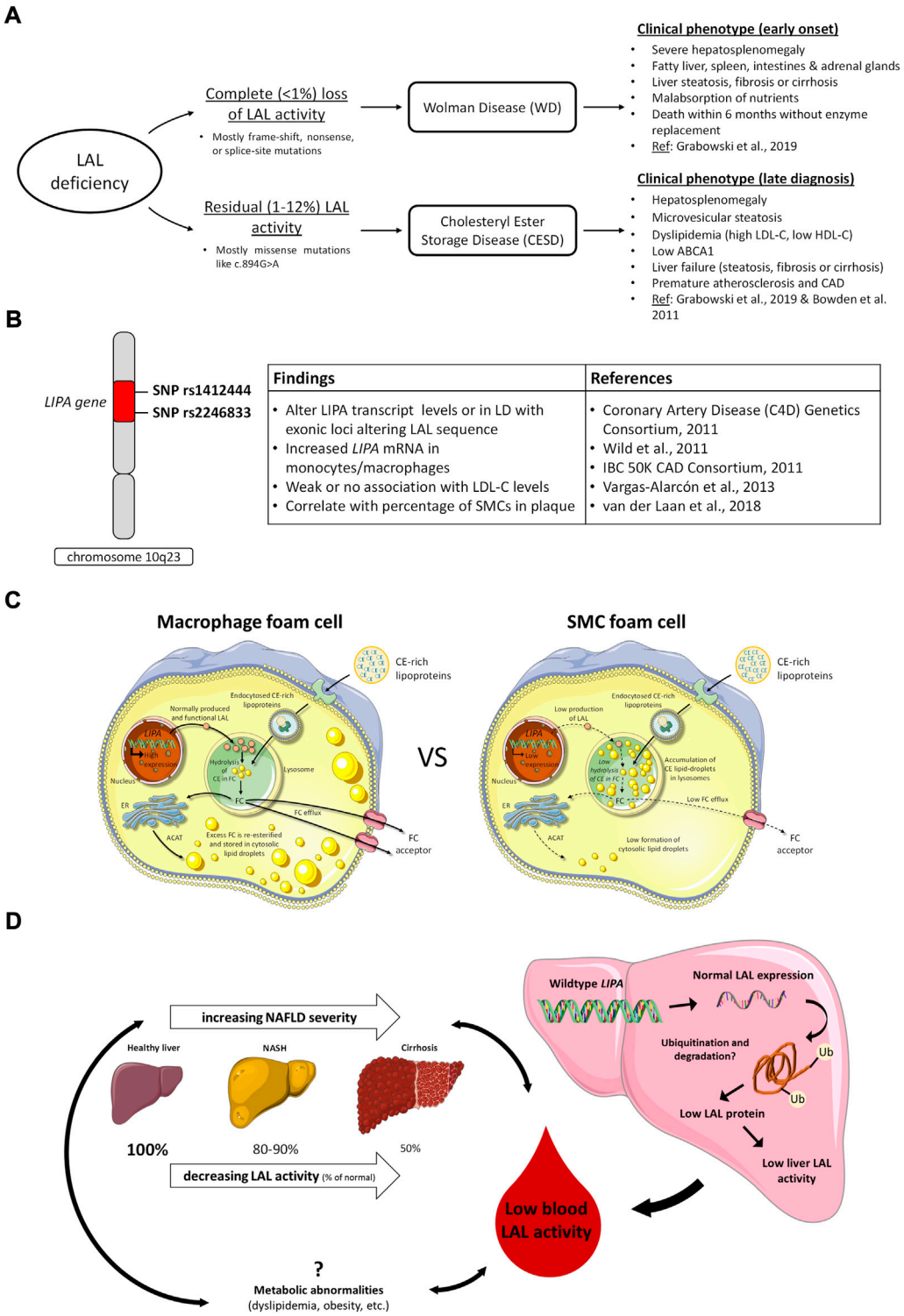

FIGURE 1
LAL in deficiency states, GWAS studies of cardiovascular risk variants, and potentially common roles of relative LAL deficiency in the development of atherosclerosis and nonalcoholic fatty liver disease. **(A)**, clinical consequences of complete and partial LAL deficiency; **(B)**, *LIPA* variants identified in GWAS studies of CVD risk and related effects in cells and tissues. LD, linkage disequilibrium; **(C)**, variable expression of *LIPA*/LAL in macrophages and smooth muscle cells (SMCs) and consequences for cell cholesterol handling. In macrophages, high levels of *LIPA*/LAL expression result in efficient hydrolysis of endocytosed lipoprotein cholesteryl esters (CE) to free cholesterol (FC), and trafficking of lysosomally-released FC for re-esterification in the endoplasmic reticulum (ER) or removal from the cell by cholesterol efflux mechanisms. In SMCs, low levels of
(*Continued*)

FIGURE 1 (Continued)
LIPA/LAL expression result in retention of lipoprotein CE in lysosomes, reducing FC available for re-esterification in the ER or efflux from cells. Adapted from (Dubland et al., 2021); **(D)**, Potential role of LAL in nonalcoholic fatty liver disease (NAFLD). LAL activity in the blood is reduced in NAFLD and lower LAL activity is associated with increased NAFLD severity. Both NAFLD and low LAL are associated with metabolic abnormalities, but no causal relationships between these factors have been determined. No variants in *LIPA* have been associated with NAFLD, and expression of LAL in NAFLD livers is normal, but activity is low (Gomaraschi et al., 2019), possibly due to accumulation of dysfunctional LAL (Carotti et al., 2021).

WD and CESD are both autosomal recessive disorders, but in most cases are caused by distinct mutations in the *LIPA* gene, and can be distinguished biochemically by the level of residual LAL activity. CESD is therefore *not* the heterozygous form of WD, but is less severe due to the nature of the *LIPA* mutations present in both alleles. WD mutations are more often frame-shift, nonsense, or splice-site mutations rather than missense mutations, which are more likely to result in CESD (Vinje et al., 2018). The most common CESD-causing variant is a splice site mutation, c.894G>A, which allows production of a small percentage of functional *LIPA* transcripts, whereas WD splice-site mutations result in a total lack of functional LAL transcripts (Aslanidis et al., 1996; Pagani et al., 1998). Similarly, degradation of LAL protein secondary to misfolding due to deletions or other mutations results in zero functional protein with WD mutations and some functional protein with CESD mutations (Fasano et al., 2012). Large structural changes in LAL or changes in the critical amino acids of the catalytic triad or other key regions also typically manifest as WD, whereas smaller structural changes are more likely to manifest as CESD (Saito et al., 2012). It must be considered that compound heterozygosity, where a patient has two different mutations in their two copies of *LIPA*, is common (Ries et al., 1998b; Santillán-Hernández et al., 2015; Cappuccio et al., 2019). This can manifest as patients having one copy of a WD-type mutation but produce some functional LAL from the other copy with a CESD mutation, and therefore present with CESD rather than WD (Lohse et al., 2000).

Prevalence

In 2007, about half of previously reported cases of CESD were c.894G>A (also known as E8SJM) carriers (Muntoni et al., 2007); the frequency of c.894G>A is therefore often used to estimate prevalence of CESD. The same 2007 study showed a 1 in 200 carrier frequency of c.894G>A in a German population, and estimated a 1 in 40,000 prevalence of CESD, whereas a recent meta-analysis by Carter *et al.* estimates a carrier frequency of 1 in 336 and CESD prevalence of 1 in 160,000 (Carter et al., 2019). It is evident that LALD frequency differs depending on ethnicity and geographical location: a New York multiethnic cohort had c.894G>A carrier frequencies of 1 in 1,000 in Asian populations, 1 in 333 for Caucasian and Hispanic, and no African American carriers (Scott et al., 2013). There are limitations to using c.894G>A for estimates of prevalence: this mutation is not present in WD patients (Grabowski et al., 2019), and the proportion of LALD cases carrying this mutation may vary in different populations. Carter *et al.*, by interrogating an international genomic database for multiple *LIPA* mutations, estimated an alternate general population LALD prevalence of 1 in 177,452 (Carter et al., 2019), while a newer study combining functional data and 165 published LALD genotypes with multiple public genomic databases estimated a prevalence between 1 in 170,000 and 290,000 (del Angel et al., 2019). It is clear that LALD is a very rare disease, with differing prevalence in diverse populations, but high carrier frequencies and presence in multiple ethnic groups suggesting potential benefit for routine screening.

Screening and diagnosis

Several recent studies screening patients with hypercholesterolemia or elevated transaminases using the dried blood spot assay (DBS) for blood LAL activity (Hamilton et al., 2012) have failed to identify cases of LALD (Pullinger et al., 2015; Sjouke et al., 2016; Ashfield-Watt et al., 2019): one Norwegian study screened 3,000 hypercholesterolemic patients without an autosomal dominant cause of hypercholesterolemia (Vinje et al., 2018), while a United Kingdom study screened 1825 patients with both low HDL-C and elevated transaminases (Reynolds et al., 2018), with no cases of LALD discovered. The majority of patients in these studies were adults. One study screening for LALD based on clinical suspicion (hepatomegaly, a 1.5-fold increase in transaminases compared to reference limits, or dyslipidemia with or without splenomegaly, gastrointestinal dysfunction, or liver steatosis, fibrosis, or cirrhosis) successfully identified 19 cases in 4,174 using DBS (Tebani et al., 2021). Another study screening 810 children in Turkey with either elevated transaminases for 3 months, hepatomegaly, or liver steatosis, fibrosis, or cirrhosis not explained by obesity or other causes by DBS found two LALD patients (Kuloglu et al., 2019). A recent study published in this series of Frontiers in Genetics screened 669 Slovenian children with familial hypercholesterolemia and detected 3 cases of LALD homozygous for c.894G>A (Sustar et al., 2022). Despite the rarity of LALD, with many cases undiagnosed until later in

life, and the availability of effective treatment, screening especially in children with hypercholesterolemia or liver disease after eliminating common causes may be beneficial (Vinje et al., 2018; Carter et al., 2019).

Diagnosis of LALD relies on measurement of LAL activity via the DBS assay. Patients with dyslipidemia, including elevated LDL and low HDL, who on taking a family history do not exhibit an autosomal dominant pattern of inheritance that would otherwise be consistent with familial hypercholesterolemia, can be examined for possibly hepatomegaly and/or splenomegaly and tested for elevated ALT. If this combination of abnormalities is present, increased liver echogenicity on ultrasound, cirrhosis or steatosis of the liver, and early cardiovascular disease, this is suggestive of LALD and LAL DBS testing should be performed. Deficient or slight residual LAL activity is diagnostic for LALD. Patients with marginal LAL activity can then be diagnosed if *LIPA* gene sequencing reveals a pathogenic variant. The reader may refer to diagnostic flowcharts published by Reiner et al. and Lipiński et al. (Reiner et al., 2014; Lipiński et al., 2018).

Although LALD is considered an autosomal recessive disease, indicating a lack of disease phenotype in heterozygous carriers, it is possible that mild phenotypes or increased risk may result from reduced LAL activity in these individuals. There is one report of carriers of c.894G>A showing elevated total serum cholesterol (Muntoni et al., 2013), and a higher prevalence of this mutation was found in familial hypercholesterolemia cohorts than in the general population in two studies (Sjouke et al., 2016; Ashfield-Watt et al., 2019); another study showed no association of c.894G>A with serum cholesterol (Stitziel et al., 2013). Heterozygous carriers of LALD mutations do not always have LAL activity falling below reference levels by DBS (Lukacs et al., 2017); it is possible that these individuals would have no phenotype. Carriers of multiple different CESD and WD mutations with no disease but between 30–70% of normal LAL activity by DBS were also shown to have altered macrophage differentiation (Rothe et al., 1997). Broader screening approaches for LALD may identify more carriers and allow further study of possible heterozygous phenotypes. We discuss below associations of cardiovascular disease risk with more common *LIPA* variants and functional correlates; note that these differ from known disease-causing mutations in *LIPA*.

Treatment

Prior to 2015, the only treatment available for LALD was supportive. Dietary restriction of fats with nutrient supplementation to prevent malabsorption in WD cannot extend survival beyond approximately 1 year of age, and lipid-lowering medications such as statins do not correct liver disease in CESD (Erwin, 2017). Liver transplantation for WD or CESD is limited by availability and by allograft rejection, and often ends in disease recurrence, possibly due to failure to correct LAL deficiency in bone-marrow derived macrophage-monocytes which infiltrate multiple tissues (Bernstein et al., 2018). Hematopoietic stem cell transplant (HSCT) for WD is limited by complications and comorbidities and rarely extends survival beyond 1 year (Jones et al., 2016; Erwin, 2017). In 2015, an enzyme replacement therapy for LAL was approved. Sebelipase alfa is a recombinant human LAL protein, administered intravenously every one to 2 weeks (CADTH Common Drug Review, 2018). It is typically dosed at 1 mg/kg for CESD and 3–5 mg/kg for WD patients (Jones et al., 2017; Demaret et al., 2021; Burton et al., 2022). Cellular uptake of sebelipase alfa occurs via the mannose receptor, which then facilitates delivery to the lysosome (Balwani et al., 2013). At treatment initiation, sebelipase alfa in CESD patients can lead to transient increases in blood triglycerides, total cholesterol, and LDL-C (Balwani et al., 2013; Burton et al., 2015a), thought to be caused by mobilization of free cholesterol and free fatty acids with increased LAL activity. After 7–20 weeks of treatment, and continuing up to 5 years after treatment initiation, triglycerides, total cholesterol, and LDL-C are lowered and HDL-C is increased. Both short term and long term reduction of serum transaminases, liver fat content, and liver volume also occur with sebelipase alfa treatment (Balwani et al., 2013; Valayannopoulos et al., 2014; Burton et al., 2015a; Malinová et al., 2020; Burton et al., 2022). Key characteristics and major findings from each sebelipase alfa trial are outlined in Supplementary Table S1.

In WD, similar improvements in lipid and liver function parameters were observed with sebelipase alfa treatment in two studies, with trends in another study compared to baseline (Jones et al., 2017; Demaret et al., 2021; Vijay et al., 2021); the smaller difference between pre and post-treatment values in the latter study may have occurred due to very early treatment initiation (median 7 weeks of age). Relief of symptoms including nausea and diarrhea, resolution of hepatosplenomegaly, and increased weight for age were also shown with treatment, as well as reduced requirement for nutritional support. Survival in the first 12 months increased from 11% in a historical untreated control population to 67–100% with sebelipase alfa, with 68% surviving to 5 years of age and one patient surviving 10 years (Demaret et al., 2021; Vijay et al., 2021) (Supplementary Table S1). Earlier initiation of treatment when patients are in more stable condition appears to improve outcome. Follow-up with surviving patients continues, and long-term effects of treatment remain to be studied.

Sebelipase alfa has been shown to be safe in both short and long-term studies, for both CESD and WD patients. Mild infusion reactions occur in most patients, with infrequent severe hypersensitivity-like responses that can be resolved with diphenhydramine or epinephrine (Balwani et al., 2013; Burton et al., 2015a; Demaret et al., 2021; Vijay et al., 2021; Burton et al., 2022). In almost all cases, reducing the dose then slowly returning to the initial dose is effective and patients are

able to continue treatment even after severe reactions. Anti-drug antibody (ADA) formation occurs in some CESD patients and most WD patients (Balwani et al., 2013; Jones et al., 2017; Demaret et al., 2021; Vijay et al., 2021; Burton et al., 2022) (Supplementary Table S1). This is likely due to the lack of endogenous LAL present in WD compared to CESD. ADA do not appear to correlate with adverse reactions in either WD or CESD, however in WD, patients with ADA may show reduced response to treatment (Vijay et al., 2021), whereas this was not observed in CESD (Burton et al., 2015a; Burton et al., 2022). The issue of reduced response to treatment due to ADA in WD patients may be addressed by combining enzyme replacement therapy with hematopoietic stem cell transplant (HSCT). A small study treating WD patients with sebelipase alfa conducted subsequent HSCT in patients who exhibited reduced response or ADA, and reported good outcomes (Potter et al., 2021). This approach may improve outcomes of HSCT, since patients are more stable upon initiation, and circumvent problems with continuous central venous access and ADA in treating WD with sebelipase alfa, since successful HSCT could provide longer-term correction of LAL deficiency (Stein et al., 2007; Potter et al., 2021).

Parents of children with LALD report feelings of uncertainty and powerlessness upon wrestling with a new diagnosis of LALD (Hassall et al., 2022). Sebelipase alfa has provided incredible increases in survival, reduction of disease in terms of lipid and liver parameters, and a good safety profile. Limitations remain however, such as infusion reactions and ADA, problems with continuous central venous access in WD patients (Demaret et al., 2021), the frequency of dosing, and the cost of treatment. Effects of treatment begin to reverse after a few weeks without sebelipase alfa such that biweekly doses are required (Balwani et al., 2013), and sebelipase alfa can cost between $892 000 and $4.9 million CDN annually per patient (CADTH Common Drug Review, 2018). Gene therapies using mRNA to promote LAL expression or gene-editing techniques to correct monogenic defects for sustained effect may be an approach to reduce costs, frequency of dosing, and ADA. Continued follow-up will also be necessary to determine longer term response to treatment.

LALD and atherosclerosis

Premature atherosclerosis has often been reported in CESD. This has not been observed in WD, perhaps due to the short lifespan of these patients (Grabowski et al., 2019). Sebelipase alfa reduces LDL-C, LDL particle number, and apolipoprotein B, and increases HDL-C and apolipoprotein A1, suggesting an anti-atherogenic effect (Wilson et al., 2018). It is unknown whether sebelipase alfa has additional effects on atherosclerosis risk outside of its modulation of lipid parameters, and larger and longer term studies would be necessary to determine cardiovascular event rates. Interestingly though, it appears that sebelipase alfa improves lipid parameters with or without previous treatment with lipid-lowering medication (LLM), and that LLMs have an additive effect with sebelipase alfa in patients who started LLMs during the trial period (Valayannopoulos et al., 2014; Wilson et al., 2018; Burton et al., 2022). These effects require confirmation in more patients with appropriate study designs. Whether enzyme replacement therapy in individuals who do not have a genetic deficiency in LAL might modulate atherosclerosis risk is a separate question, which we address below.

Identification of *LIPA* as a cardiovascular risk allele in GWAS studies

Genome-wide association studies (GWAS) of large European, South Asian and Mexican cohorts have identified *LIPA* single nucleotide polymorphisms (SNPs) rs1412444 and rs2246833 on chromosome 10q23 as common variants associated with coronary artery disease (CAD) risk (Coronary Artery Disease (C4D) Genetics Consortium, 2011; IBC 50K CAD Consortium, 2011; Wild et al., 2011; Vargas-Alarcón et al., 2013). While these SNPs are intronic and therefore not directly coding LAL sequence, the risk SNPs are proposed to alter *LIPA* transcript levels by affecting its rate of transcription, nuclear export and transcript stability, or to be in linkage disequilibrium with exonic loci altering LAL sequence. Several studies have linked these risk alleles to increased expression of *LIPA* mRNA by circulating monocytes (Coronary Artery Disease (C4D) Genetics Consortium, 2011; Wild et al., 2011; PLOS Genet 2011) but not to changes in LDL-C level (Figure 1B). No clear association has been found with *LIPA* expression in the liver. These findings suggest enhanced *LIPA* expression may not affect the CE hydrolytic activity of LAL but have some other effect on CAD risk, possibly related to endothelial dysfunction as measured by reduced flow-mediated dilatation (Wild et al., 2011). In contrast to CESD, where very low LAL activity results in elevated LDL-C, low HDL-C levels and increased risk of atherosclerosis (Dubland and Francis, 2015), CAD risk variants have generally not been found to be associated with alterations in plasma lipid levels. Potential hypotheses for increased CAD risk with increased *LIPA* transcript levels could be increased extracellular release of LAL-generated free fatty acids having a pro-inflammatory effect in the plaque intima, increased hydrolysis of CE by exocytosed LAL on LDL retained in the interstitium leading to increased interstitial free cholesterol promoting plaque inflammation, and increased LAL-modified LDL uptake and foam cell formation by intimal macrophages and SMCs.

In vitro studies to determine the effect of *LIPA* risk variants on LAL function and activity and cell cholesterol metabolism have reached differing conclusions. Morris et al.

investigated a coding variant rs1051338, which is in high linkage disequilibrium (r^2 = 0.89) with the GWAS variant rs2246833 and causes a nonsynonymous threonine to proline change within the signal peptide of LAL (Morris et al., 2017). COS7 cells transfected with the risk allele exhibited significantly less LAL protein and activity, felt to be due to an increased rate of LAL protein degradation. These results were confirmed in lysosomal extracts of macrophages from 4 individuals homozygous for either the nonrisk or risk allele of rs1051338, in which inhibition of the proteasome resulted in equal amounts of lysosomal LAL protein in risk and nonrisk macrophages (Morris et al., 2017). While this effect of reduced LAL activity would be consistent with the pro-atherogenic effect of LAL deficiency in CESD, potential weaknesses of this study are that rs1051338 is not yet proven as the causal variant at the *LIPA* GWAS locus, the small sample size, and that other aspects of LAL activity such as CE hydrolysis and autophagy were not examined (Zhang and Reilly, 2017). Evans et al. also studied the effect of rs1051338 variant in human monocytes from a larger patient cohort, n = 114, and found that it conferred increased *LIPA* expression and LAL activity, but had no effect on *LIPA* mRNA or LAL activity or secretion when transfected into HEK-293T or NIH-3T3 cells (Evans et al., 2019). Their conclusion was that common *LIPA* exonic variants in the signal peptide are of minimal functional significance and that CAD risk is instead associated somehow with increased *LIPA* function linked to intronic variants (Evans et al., 2019). The effect of *LIPA* variants identified by GWAS studies therefore remains controversial, and will require further studies to identify the role of variant expression in other relevant cell types such as SMCs, and in targeted mouse models with knock in of human *LIPA* risk and non-risk alleles to assess the effect on atherosclerosis (Zhang and Reilly, 2017). Interestingly, a study by van der Laan *et al.* using carotid plaque specimens from two independent biobanks identified expression of *LIPA* variant rs1412444 correlated most strongly with percentage of SMCs in plaque, and predicted reduced CAD risk with the GWAS SNP (van der Laan et al., 2018). If SMCs, like macrophages, have higher LAL activity with this SNP, that would imply a protective effect of increasing LAL in plaque SMCs.

Low expression of LAL by arterial SMCs and formation of SMC foam cells in atherosclerosis

Expression of *LIPA*/LAL varies considerably among tissues (Du et al., 1996). As early as 1974 de Duve proposed that low LAL might be contributing to accumulation of CE in aortic cells in atherosclerosis (de Duve, 1974). Subsequent reports indicated low levels of *LIPA* expression in mouse (Du et al., 1996) and human (Zhang et al., 2017) SMCs relative to macrophages and other tissues, but the functional significance of that in atherosclerosis was not explored. We had previously reported that low LAL-mediated hydrolysis of lipoprotein CE is the reason for low ABCA1 expression in human CESD fibroblasts, is the likely reason for low plasma HDL-C in CESD, and that supplementation of human CESD fibroblasts with exogenous LAL corrects ABCA1 expression and activity (Bowden et al., 2011). We had also reported that ABCA1 expression is low in intimal compared to medial arterial SMCs (Choi et al., 2009), and in coronary artery intima SMCs compared to intima macrophages (Allahverdian et al., 2014). We subsequently quantitated the contribution of SMCs to total atheroma foam cells, and by immunohistochemical analysis determined that, at minimum, SMCs contribute >50% of total foam cells in human coronary atheromas (Allahverdian et al., 2014). We subsequently found that, using gentle digestion of atheromas and flow cytometry, that SMCs contribute approximately 70% of foam cells in apoE-deficient mice fed a Western diet for 6 weeks, in both SMC nonlineage-tracing and lineage-tracing mice, and that SMC foam cells in mice also exhibit low ABCA1 expression relative to macrophage foam cells (Wang et al., 2019). Defective cholesterol handling by arterial SMCs is therefore a likely major cause of cholesterol accumulation in atherosclerotic plaque.

In an attempt to determine the mechanism of reduced ABCA1 expression by SMCs, we determined that SMCs exhibit reduced production of 27-hydoxycholesterol and fail to activate cholesterol esterification and inhibit *de novo* cholesterol synthesis when compared to human macrophages in response to loading with aggregated LDL (Dubland et al., 2021). These findings suggested SMCs are deficient in trafficking of lysosomally-derived cholesterol following lipoprotein loading. Strikingly, we observed sequestration of lipoprotein-derived CE within the lysosomes of SMCs even after a 24 h equilibration period following aggregated LDL loading; macrophages on the other hand sequester cholesterol within cytosolic CE droplets following hydrolysis of lipoprotein CE and trafficking of lysosomally-derived free cholesterol to the endoplasmic reticulum for re-esterification (Dubland et al., 2021). This suggested either that SMCs have impaired lysosomal function or that deficiency of *LIPA*/LAL may explain this striking phenotype. Similar to previous results from Jerome and others (Griffin et al., 2005; Cox et al., 2007), we determined that loading of human monocyte-derived macrophages with aggregated LDL led to free cholesterol accumulation and reduced acidification in lysosomes of macrophages, but we found no similar defects, nor any defect in proteolysis, in SMC lysosomes (Dubland et al., 2021). We then determined that SMCs have markedly low *LIPA* mRNA, LAL protein and LAL activity both before and after loading the cells with aggregated LDL (Dubland et al., 2021) (Figure 1C). These *in vitro* studies were confirmed in human coronary atheromas,

showing high LAL expression in macrophages but low LAL in arterial SMCs, particularly intimal SMCs (Dubland et al., 2021); these results were also corroborated in apoE-deficient mouse atheroma where macrophages showed high and arterial SMCs showed low *LIPA* expression (Dubland et al., 2021). While additional reasons for low ABCA1 in human SMCs were determined, including lower expression of sterol-27-hydroxylase and LXRα, necessary components of enhanced ABCA1 expression, incubation of human SMCs with exogenous LAL significantly increased lysosomal CE hydrolysis and cholesterol efflux to apolipoprotein A-I using the cells' existing level of ABCA1 expression (Dubland et al., 2021). This suggests that increasing *LIPA* expression and/or LAL activity in arterial SMCs may be effective in promoting removal of excess cholesterol from SMC foam cells in plaque atheroma, assuming sufficient apolipoprotein A-I and HDL acceptor particles are available for both ABCA1-dependent and–independent cholesterol efflux, respectively. Precedent for this possibility is provided by a pre-clinical study of Hong Du and others where weekly injection of yeast-derived LAL for 6 weeks both prevented appearance and induced regression of atheromas in LDL receptor-deficient mice; however, the cellular target of the exogenous LAL in those studies was unknown (Du et al., 2004). Based on plaque macrophages having abundant LAL expression (Ries et al., 1998a; Dubland et al., 2021) and not showing increased cholesterol efflux in response to LAL supplementation (Dubland et al., 2021), the results suggest plaque SMC foam cells were the target of the exogenous LAL. It also provided highly suggestive evidence that increased circulating LAL can have a major therapeutic effect upon diffusing into the artery wall.

Mechanism of reduced *LIPA* expression in arterial SMCs

The reason for low *LIPA*/LAL expression by arterial SMCs relative to macrophages is not yet known. SMCs are not designed or equipped to carry out the functions of professional macrophages. Even though plaque SMCs can express multiple macrophage markers (Allahverdian et al., 2014; Feil et al., 2014; Shankman et al., 2015), induction of macrophage protein expression by cholesterol loading SMCs in culture was accompanied by only ~25% of the ability of those SMCs to carry out phagocytosis and efferocytosis when compared to cultured macrophages (Vengrenyuk et al., 2015). The concept of a "SMC-derived macrophage" is therefore likely incorrect: even when expressing macrophage markers, dedifferentiated SMCs retain a distinct SMC-specific gene expression pattern and do not assume the capabilities of professional macrophages (Vengrenyuk et al., 2015). SMCs are however critical for the initial deposition of lipoproteins in the human artery wall, through secretion of negatively-charged proteoglycans that bind and retain positively-charged apolipoprotein B-containing lipoproteins like LDL in the intima (Hurt-Camejo and Camejo, 2018; Allahverdian et al., 2022). These retained lipoproteins surround primarily SMCs rather than macrophages in the deep intima in human atherosclerosis-prone arteries (Nakashima et al., 2007), and through expression of scavenger receptors (Allahverdian et al., 2012), though of less abundance than in macrophages, over time can be taken up to generate SMC foam cells. This capacity of SMCs to take up retained lipoproteins to become foam cells, but not have the same capacity as macrophages to catabolize lipoprotein CE due to low *LIPA* expression, may be a consequence of human evolution not progressing as rapidly as the increase in atherosclerosis as a cause of death in the last 100 years. When humans didn't live as long due to death from famine, infection or war, *i.e.*, did not live long enough to die from atherosclerosis, the inability of SMCs to catabolize lipoproteins as well as macrophages had no major consequences. Now with lifespans averaging over 80 years in many developed countries, this lack of adequate SMC LAL activity has a functional consequence. This strongly indicates the need to further investigate the pathophysiologic impact of enhancing LAL activity in SMC foam cells in the artery wall.

The possibility also exists that low *LIPA*/LAL expression represents a defect in the regulation of this gene in arterial SMCs. A generalized defect in lysosomal function in SMCs seems unlikely based on their ability to carry out lysosomal proteolysis normally (Dubland et al., 2021), and to apparently carry out autophagy normally (Salabei and Hill, 2013). A recent publication suggested SMC foam cells isolated from mouse atheromas have a higher level of dysfunctional autophagy during atherogenesis relative to macrophage foam cells (Robichaud et al., 2022). Whether upregulating known inducers of *LIPA* expression such as FOXO1 or TFEB can rescue *LIPA* expression and LAL function in SMC foam cells remains to be determined. TFEB overexpression has been shown to increase *LIPA* expression and lysosomal biogenesis in mouse peritoneal macrophages (Emanuel et al., 2014), and to reduce atherosclerosis in a mouse model (Sergin et al., 2017); whether the significantly lower *LIPA* expression in SMCs could be similarly enhanced requires further investigation.

The role of LAL in fatty liver disease

It is established that genetic LALD causes a microvesicular steatosis and fatty liver disease, but a role for LAL in the more common cases of non-alcoholic fatty liver disease (NAFLD) found in the general population is now being investigated. NAFLD is characterized by fat accumulation or steatosis in the liver, and has a worldwide prevalence of approximately 25% (Friedman et al., 2018). The beginning stages of steatosis

do not carry a high morbidity, but insidious progression to non-alcoholic steatohepatitis (NASH), fibrosis, and finally liver cirrhosis leads to liver failure and other adverse outcomes, including hepatocellular carcinoma (Friedman et al., 2018). The most common cause of death in patients with NAFLD is cardiovascular disease, probably related to a combination of both the metabolic abnormalities (diabetes, obesity, dyslipidemia, etc.) associated with both NAFLD and cardiovascular disease, and hepatic inflammation independently promoting systemic vascular damage and coagulation (Tana et al., 2019). The progression of NAFLD is often asymptomatic and may not be reflected in laboratory values. Diagnosis of NAFLD via non-invasive imaging modalities is reliable, but NASH diagnosis requires liver biopsy (Friedman et al., 2018). Blood LAL activity by DBS has been shown to be lower in NAFLD than in healthy subjects in multiple studies (Baratta et al., 2015a; Thoen et al., 2021): one key study by Baratta *et al.* shows LAL activity is inversely correlated to severity of NAFLD, with lower activity in NAFLD non-NASH than healthy patients (~10% reduction), even lower in NASH patients (~20%), and further reduced in patients with cirrhosis (~50%) (Baratta et al., 2019a). Lower LAL activity in NASH is associated with increased necroinflammation and NASH severity (Vespasiani-Gentilucci et al., 2017). Some studies did not detect differences in earlier stages of NAFLD, but LAL by DBS was found to be reduced in cirrhosis of both cryptogenic (Vespasiani-Gentilucci et al., 2016; Gravito-Soares et al., 2019) and known etiologies (Vespasiani-Gentilucci et al., 2016; Ferri et al., 2020). Gravito-Soares *et al.* showed that LAL activity can be used to predict cryptogenic cirrhosis and fibrosis more effectively than existing markers (Gravito-Soares et al., 2019). LAL is therefore a very attractive biomarker for NAFLD, because the DBS assay is non-invasive, can be incorporated as part of regular bloodwork, and appears to predict progression of NAFLD with reasonable sensitivity and specificity (Gravito-Soares et al., 2019). Continued study will be necessary to verify the utility of LAL as a biomarker of NAFLD, and it remains to be seen whether LAL activity could be used to identify patients earlier in the NAFLD progression than existing methods.

It should be noted that there are limitations to the DBS assay for LAL activity. DBS assays testing for other lysosomal storage diseases mostly reflect leukocyte enzyme activity, and since activity is normalized not to protein content or cell counts but to blood spot area, blood leukocyte count could importantly influence results (Civallero et al., 2006; Ceci et al., 2011). Platelets also have lysosomes and thus contribute part of the activity measured by DBS (Bentfeld-Barker and Bainton, 1982). WBC and platelet counts have both been reported to be increased in NAFLD steatosis and decreased in cirrhosis, so these factors are key to consider in interpreting DBS results (Qamar and Grace, 2009; Chao et al., 2022). A study investigating platelet and leukocyte influence on DBS results in healthy subjects showed a greater correlation of total DBS LAL activity with LAL activity in platelets than in leukocytes, and observed an association of total activity with platelet but not leukocyte count (Vespasiani-Gentilucci et al., 2017). Several of the studies showing low LAL activity in NAFLD also found that LAL activity was associated with leukocyte count (Baratta et al., 2019b) or both leukocyte and platelet counts (Vespasiani-Gentilucci et al., 2016; Tovoli et al., 2017), but in all of these LAL activity was independently associated with NAFLD after adjusting for leukocytes and platelets.

The pathogenesis of the lowered LAL activity in NAFLD is as yet unknown. Multiple studies screening for LAL mutations in NAFLD patients with low LAL activity did not find any (Vespasiani-Gentilucci et al., 2016; Gravito-Soares et al., 2019), and there are currently no GWAS reporting associations of NAFLD or other hepatic phenotypes with *LIPA* variants in the NHGRI-EBI GWAS catalog (Buniello et al., 2019), so it does not appear to be a gene polymorphism effect. An interesting study by Carotti *et al.* suggests a post-translational mechanism, demonstrated using *in vitro* and mouse models, and confirmed in NAFLD patient biopsies, that NAFLD is associated with low functional levels of LAL protein and accumulation of dysfunctional ubiquitinated LAL (Carotti et al., 2021). In agreement with this, Gomaraschi *et al.* report that low LAL activity in liver biopsies of NAFLD patients was shown to be independent of LAL expression (Gomaraschi et al., 2019). Other etiologies of fatty liver disease also exhibit lowered LAL activity, including alcoholic and HCV-related liver disease, though NAFLD shows a greater reduction of LAL (Angelico et al., 2017; Tovoli et al., 2017; Ferri et al., 2020). This suggests that lowered LAL activity is partly secondary to liver disease, since various etiologies share the same characteristic of low LAL, but the even lower LAL in NAFLD specifically suggests that the metabolic abnormalities associated with NAFLD may also be related to LAL activity directly (Baratta et al., 2015b; Angelico et al., 2017). We have discussed above the association of low LAL with dyslipidemia; Baratta *et al.* show an association of elevated LDL with low LAL activity in NAFLD patients (Baratta et al., 2015a). Thoen *et al.* similarly associate low LAL with high BMI in NAFLD patients, and *LIPA* variants have previously been associated with metabolic syndrome, though this association requires further investigation (Guénard et al., 2012; Thoen et al., 2021). Thoen *et al.* also demonstrate increased necroinflammation in NASH patients with lower LAL activity, which may speak to the immune roles of LAL (Thoen et al., 2021). It is unclear from these associations what the relationship between low LAL, metabolic or inflammatory factors associated with NAFLD, and NAFLD liver pathology is: low LAL may be secondary to both NAFLD and the identified factors, causative of both, or somewhere in between (Figure 1D).

Baratta *et al.* speculate that determining the epigenetic or metabolic factors leading to low LAL may be instrumental in treating NAFLD, and suggest that sebelipase alfa enzyme-replacement therapy may be useful in these patients (Baratta et al., 2015b). Sebelipase alfa has not yet been tested for NAFLD, CAD, or any indication other than LALD. Hepatic overexpression of LAL using lentivirus in a mouse model of NAFLD led to reduced steatosis and fibrosis, decreased liver inflammation, and decreased serum lipid levels (Li et al., 2021); however, overexpression with an adeno-associated virus in a different mouse model of NAFLD did not attenuate steatosis, and increased liver inflammation (Lopresti et al., 2021). Both studies showed increased autophagic activity in hepatocytes. *In vitro,* a novel GATA3-binding molecule morroniside increases *LIPA* expression and reduces the fibrosis response in hepatic stellate cells (An et al., 2022). Further testing in alternate animal models for NAFLD, or using different strategies to increase hepatic LAL, will be necessary to elucidate the utility of increasing LAL as a treatment for NAFLD. It is possible that some models, and some patients, with lower endogenous LAL activity may respond better to such treatment than those with normal LAL activity.

Conclusion

LAL is critical to life as the sole neutral lipid hydrolase in lysosomes, as a director of downstream cholesterol metabolism in cells, and as a driver of total body reverse cholesterol transport. Total deficiency of LAL is fatal, whereas even very low levels of residual LAL activity are compatible with life though predisposing to liver disease and potentially atherosclerosis. In addition to utilizing recombinant LAL to treat complete and partial LAL deficiency, we are now learning much more about the role of *LIPA* expression as a predictor of cardiovascular risk, and the likelihood that naturally occurring low levels of *LIPA* expression predict the formation of SMC foam cells, the proposed primary source of foam cells in atherosclerosis. It is likely that in the next decade the activity and functions of LAL will assume an ever increasing role in our understanding of the pathogenesis and potential treatment of both atherosclerosis and fatty liver disease.

Author contributions

KB and GF designed and wrote the manuscript, VB contributed the figure, and all authors contributed to review of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.1013266/full#supplementary-material

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Features of the metabolic syndrome and subclinical atherosclerosis in patients with cerebrotendinous xanthomatosis: An augmented risk for premature cardiovascular disease

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Background: Cerebrotendinous xanthomatosis (CTX) is a rare lipid storage disease, caused by deficiency of sterol-27-hydroxylase. Xanthomatous lesions in numerous tissues, and an elevation of cholestanol levels, characterize the disease. Its natural course is progressive neurologic deterioration, leading to premature death. Chronic treatment with oral chenodeoxycholic acid (CDCA) reduces cholestanol levels. Occurrence of premature atherosclerosis has been described in CTX in an unknown mechanism.

Aim: The aim of the current work was to evaluate the potential metabolic abnormalities and preclinical vascular changes in Israeli CTX patients.

Methods: Ten subjects with CTX were studied. Features of the metabolic syndrome were evaluated, and carotid intima media thickness (cIMT) was measured in the common carotid arteries.

Results: All patients were diagnosed with CTX, and all received treatment with CDCA, which resulted in normalization of their plasma cholestanol levels. At the conclusion of the follow up, risk factors for CVD and features of MS were present in all the patients and in three patients, cIMT was higher compared to control subjects.

Conclusion: Cardiovascular risk factors and premature vascular changes exist in young CTX patients and proper assessment should be implemented with

Abbreviations: CDCA, chenodeoxycholic acid; CHD, coronary artery disease; cIMT, carotid intima-media thickness; CTX, cerebrotendinous xanthomatosis; CVD, cardiovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglycerides; GGT, gamma-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

preventive measures to reduce the risk of atherosclerotic cardiovascular disease in CTX patients.

KEYWORDS

cerebrotendinous xanthomatosis, low-density lipoprotein cholesterol, coronary artery disease, cholestanol, atherosclerosis, carotid intima-media thickness, metabolic syndrome

Introduction

Cerebrotendinous xanthomatosis (CTX) is a rare autosomal recessive lipid storage disease, caused by mutations of the CYP27A1 gene, resulting in deficiency of sterol-27-hydroxylase (CYP27). CYP27 is a mitochondrial cytochrome P450 enzyme, present in most cells in the body, with a key role in gap between hepatic bile acid synthesis (Nie et al., 2014), as it catalyzes steps in the oxidation of sterol intermediates that form bile acids. Normally, the ideal substrates for mitochondrial 27-hydroxylation are 7α-hydroxylated intermediates in bile acid synthesis (Björkhem 2013).

The biochemical hallmark of CTX is impaired synthesis of chenodeoxycholic acid (CDCA) from cholesterol, that results in elevated levels of plasma and bile cholestanol (Nie et al., 2014). Xanthomatous lesions in numerous tissues, including tendons, lens, brain, and additional tissues characterize the disease (Moghadasian et al., 2002). There is considerable variability in disease onset, developmental manifestations milestones, presence of intellectual deficiency or learning disabilities, later neurological deterioration, and systemic involvement, even among patients within the same family and genotype. The natural course of CTX is progressive neurologic deterioration from childhood through adulthood, leading to diffuse damage of the central, and peripheral nervous systems and eventually to premature death. Neurological deterioration due to cerebellar, pyramidal, and extrapyramidal system involvement, as well as additional cognitive decline, psychiatric symptoms, epileptic seizures, and peripheral neuropathy usually become evident in the second or third decades of life. Common non-neurologic manifestations of CTX include infantile-onset diarrhea, childhood-onset cataracts, osteoporosis, repeated bone fractures and respiratory insufficiency (Nie et al., 2014; Moghadasian et al., 2002; Moghadasian 2004; Kuriyama et al., 1991; Patni and Wilson, 2020). Chronic treatment with oral CDCA reduces cholestanol synthesis and lowers its plasma levels. Timely treatment, started early in life, may halt, or even prevent the neurological progression of CTX, and has the potential to reverse some neurological deficits (Nakamura et al., 1991; Verrips et al., 2020; Yahalom et al., 2013; Tao et al., 2019). Nevertheless, delayed diagnosis and therapy as well as decreased compliance and availability of the drug, remains a major problem in CTX (Yahalom et al., 2013; Yunisova et al., 2019). Primary neurological involvement is the principal concern in patients with CTX; nonetheless, substantial occurrence of premature vascular involvement has also been described, with different clinical manifestations of cardiovascular disease (CVD) in more than 10% of patients with CTX (Tada et al., 2018; Souto et al., 2020; Valdivielso et al., 2004).

In a publication from 1991, describing several patients with CTX, nearly half of them presented with atherosclerotic lesions on the coronary angiogram, suggesting a high prevalence of vascular changes in CTX (Kuriyama et al., 1991). The exact mechanism leading to early onset arteriosclerosis in this disease is unknown (Björkhem and Boberg 1998). Specific abnormalities in the lipoprotein profile were not identified in CTX, and cholesterol levels usually are within normal limits (Fujiyama et al., 1991). In a case report from 2004, a young patient was described with a myocardial infarction, lacking the classic risk factors for premature arteriosclerosis, except for mild mixed dyslipidemia and elevated apolipoprotein B levels (Valdivielso et al., 2004). Furthermore, the enzyme 27-hydroxylase, which is the malfunctioning enzyme in CTX, has additional roles in cells such as macrophages, and endothelial cells and is additionally involved in the process of the transport of cholesterol from the peripheral tissues to the liver (Babiker et al., 1997). Metabolic changes in high-density lipoprotein (HDL) contribute to the premature atherosclerosis as defects in HDL functionality and cholesterol efflux capacity are associated with of subclinical atherosclerosis in young and healthy subjects and in older and CVD patients (Hunjadi et al., 2020). Hence, decreased levels or dysfunction of the HDL particle in CTX may lead to alternation in the process of reverse cholesterol transport and is a potential mechanism for the accelerated atherosclerosis in CTX (Babiker et al., 1997; Von Bahr et al., 2002).

Carotid artery intima media thickness (cIMT) is non-invasive ultrasound guided technique of cardiovascular risk stratification and it is utilized for preclinical assessment of early atherosclerotic changes (Stein et al., 2008; Liu et al., 2020). Routine use of cIMT in CTX is not practiced, though in a publication describing a young CTX patient, carotid ultrasound facilitated the recognition of preclinical atherosclerosis (Burnett et al., 2001).

The metabolic syndrome (MS) is a cluster of metabolic manifestations and risk factors for diabetes and CVD (DeFronzo and Ferrannini, 1991) and the most used definition is the one of the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) (Alberti et al., 2009). Within the general Israeli population, in a recent large cohort of 230,639 participants, the prevalence of

obesity, abnormal blood pressure, and Type 2 diabetes mellitus has increased dramatically throughout the years (Twig et al., 2019).

Additionally, studies have demonstrated a substantial relationship between fatty liver disease and CVD mortality (Ghouri et al., 2010). Elevated levels of liver function markers, gamma-glutamyltransferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), have been associated with an augmented risk of CVD (Li et al., 2016; Pahwa et al., 2021; Rahmani et al., 2019).

There are two major clusters of CTX in Israel, one is in Jews of North African decent and the second is in the Druze, a small Middle Eastern religious sect from the north of Israel and each patient group has distinct mutations. In Jews from Moroccan origin, a deletion of thymidine in exon 4 and guanosine to adenosine substitution at the 3′ splice acceptor site of intron 4 of the gene have been found. There is an additional mutation in Jews of Algerian origin, which is a cytosine to thymidine transition at cDNA position 1037 leading to a threonine to methionine substitution at residue 306. In the Druze a CYP27 a deletion of cytosine in exon two results in a frameshift leading to a premature termination signal (Yahalom et al., 2013; Leitersdorf et al.,1993; Falik-Zaccai et al., 2008; Reshef et al., 1994).

As the assessment for features of the metabolic syndrome and cIMT are not used in the routine management of CTX patients, the aim of the current work was to evaluate the prevalence of MS and vascular changes in CTX in an attempt to elucidate the mechanism leading to the phenomena of premature CVD.

Materials and methods

Subjects

Ten CTX subjects were recruited at the outpatient clinic of the Movement Disorders Institute and at the Bert W. Strassburger Metabolic Center Outpatient Clinic at the Chaim Sheba Medical Center, Israel from January 2008 to January 2022. All patients had exhibited elevated plasma levels of cholestanol before starting therapy with CDCA, several years ago.

Molecular genetics

All patients were of North African Jewish descent and were either homozygous or compound heterozygous for either one or two of the known mutations described in Jewish families of North African descent.

Metabolic syndrome

Diagnosis of the MS in adults was done according to the National Cholesterol Education Program (NCEP), Adult Treatment Panel III (ATP III) (Alberti et al., 2009). Presence of any three out of the five criteria qualifies the definition of MS: abdominal obesity, waist circumference ≥102 cm in men and ≥88 cm in females, serum triglycerides (TG) ≥150 mg/dl or drug treatment for elevated TG, serum HDL cholesterol <40 mg/dl in males and <50 mg/dl in females or drug treatment for low HDL cholesterol. Blood pressure ≥130/85 mm Hg or drug treatment for elevated blood pressure or fasting plasma glucose (FPG) ≥100 mg/dl (or drug treatment for elevated blood glucose).

Liver function markers

Following an overnight fast alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were evaluated. Presence of hepatic steatosis was evaluated by liver ultrasound scan.

Carotid artery intima-media thickness

Patients underwent a color-coded duplex examination of neck vessels using a 10 MHz linear array ultrasound (Hitachi medical corporation, Tokyo, Japan). IMT was evaluated on the common carotid arteries (CCAs) over approximately 1.5 cm proximal to the flow divider, according to standardized guidelines. IMT was measured at the thickest plaque-free point on the near and far walls with a specially designed computer program. Mean CIMT values from the far walls of the right and left common carotid arteries are reported (Liu et al., 2020). The control group for the IMT measurements, in patients less than 30 years old consisted of fifty-five (33 females) healthy normo-cholesterolemic patients aged 18–30 years, who underwent carotid IMT measurements at the Bert W. Strassburger Metabolic Center, between the years 2007–2014. Mean bilateral carotid IMT measurements in the healthy controls was 0.49 ± 0.059 mm (mean ± SD). The normal reference for patients aged 30–40 years was less than 0.65 mm and less than 0.75 mm for the age range of 40–60 years (Stein et al., 2008).

Ethics committee

Ethical approval for the study was obtained from the ethics board of the Chaim Sheba Medical Center, Tel-Hashomer, Israel number 8210-21 SMC.

TABLE 1 Baseline Biochemical and clinical characteristics of CTX patients (Features of the metabolic syndrome in bold).

No.	Gender	Follow up (years)	Age at DX[a] (years)	Age at 1st visit	Blood pressure (mmHg)	BMI (kg/m$_2$)	AST/ ALT IU/L	Fasting glucose (mg/dl)	TC (mg/dl)	HDL (mg/dl)	TG (mg/dl)	Cal LDL cholesterol (mg/dl)	Non HDL-cholesterol (mg/dl)	[b]Number of metabolic aberrations
1	M	14	13	19	126/71	**29**	23/31	90	219	47	**168**	127	172	3
2	F	14	16	40	118/79	24.5	24/28	96	188	50	72	117	138	2
3	F	11	14	38	125/70	20	20/34	87	170	53	58	109	117	1
4	F	8	21	34	**135**/80	23	**46/60**	92	256	**43**	**171**	179	213	3
5	F	3	10	33	125/80	22	31/17	97	198	57	83	124	141	2
6	M	13	6	14	**132**/82	**29**	25/30	84	164	48	113	93	116	4
7	F	2	30	40	**140**//73	**28**	30/22	83	266	47	**234**	172	219	3
8	M	10	10	29	**143**/84	23	17/25	83	118	**37**	59	70	81	2
9	M	7	16	34	121/81	22.8	18/24	89	242	44	100	178	198	2
10	M	7	13	42	126/75	**25.5**	23/34	98	113	42	**169**	138	71	3
Mean ± SD		8.9 ± 4.3	14.9 ± 6.7	32.7 ± 6.7	S-128.1 ± 7.2 D-77.5 ± 4.9	24.6 ± 3.1	25.7 ± 8.5 29.4 ± 11.8	89.9 ± 5.7	193.4 ± 53.6	46.8 ± 5.7	122.7 ± 59.6	130.7 ± 36.7	146.6 ± 352.6	

[a]Age at diagnosis and start of CDCA, treatment.

[b]End of follow up, Normal range AST-0-37 IU/L, ALT 0-37 IU/L.

M, Male; F, Female; DX, diagnosis; m, meter; BMI, Body mass index; Kgs, Kilograms; ALT, Alanine aminotransferase; AST, aspartate aminotransferase; IU, international units; MS, metabolic syndrome; SD, standard deviation; S, systolic; D, diastolic; Cal, calculated; L, liter

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TABLE 2 Imaging characteristics of the patients- Carotid intima-media thickness measurements and carotid Doppler tests.

Patient	Age at cIMT (years)	Mean cIMT (mm)[a]
1	19	1.0 mm
2	45	1.1 mm
	53	1.4 mm
3	38	0.4 mm
	47	Carotid plaque LICA-25%

[a]Mean bilateral carotid IMT, measurements healthy controls −0.49 ± 0.059 mm.
Abbreviations: cIMT, Carotid intima-media thickness; LICA, left internal carotid

Results

The baseline clinical, metabolic and biochemical characteristics of the ten patients included in the study are summarized in Table 1. None were smokers and none reported additional clinical ASCVD risk factors, symptoms, or imaging findings at the initial visit. Family history of MS or CVD was present in two of the patients at baseline.

All patients were diagnosed with CTX as children or adolescents, and one patient was diagnosed at the age of 21 years (14.9 ± 6.7 years, mean ± SD). All patients received treatment with CDCA (250 mg three times per day) that resulted in normalization of their plasma cholestanol levels (Data not shown). While neurological stabilization and steadiness was obtained in patients number 1–7, gradual deterioration was observed in patients 8–10, as described in our previous publication presenting the neurological consequences (Yahalom et al., 2013).

At the initial assessment, one patient presented with full criteria of the MS and six patients had features of the partial MS and obesity. During the mean follow up of 8.9 ± 4.3 years (mean ± SD) four additional patients fulfilled the required number of abnormalities for MS. Liver function markers of alanine aminotransferase (ALT), aspartate aminotransferase (AST) were above the normal range in one patient at presentation, and five patients developed altered liver function during follow up and ultrasonic features of fatty liver in the liver ultrasound scan. The mean time for the appearance of the metabolic abnormalities was 3.5 ± 2.2 (mean ± SD) years from diagnosis.

Hypercholesterolemia and elevated plasma LDL-cholesterol are not part of the five features of the metabolic syndrome, though they are as a well-known risk factor for CVD. Elevated levels of fasting plasma LDL-cholesterol >100 mg/dl were present in eight patients through the follow up period. At the conclusion of the follow-up, risk factors for CVD and features of MS were present in all the patients.

In two patients, the initial cIMT was higher in comparison to the normal range of their age group, with accelerated increment within 7 years in the patient number 2. In the third patient, though the initial IMT was within the normal range, atherosclerotic changes developed within 9 years (Table 2; Figure 1). To date there are no clinical events of atherosclerotic cardiovascular disease.

Discussion

CTX is a rare inherited metabolic disorder; with a relatively high estimated prevalence in Israel with a carrier frequency of 1: 80 in Jews of North African decent (Leitersdorf et al., 1993; Falik-Zaccai et al., 2008). We report here, a series of young patients with CTX, with features of the MS, hypercholesterolemia and

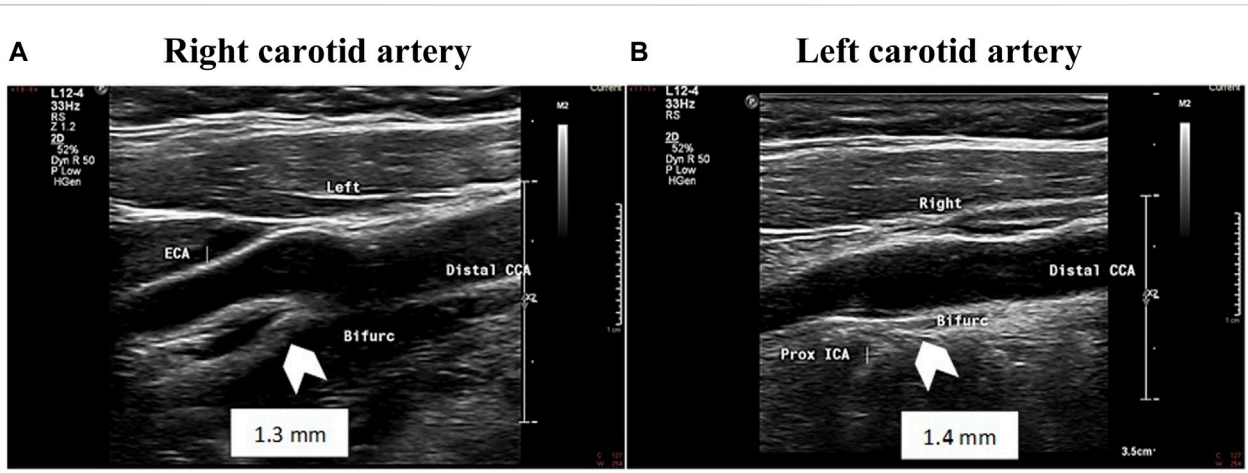

FIGURE 1
Carotid Intima media thickness. Ultrasound image showing measurement of near and far wall IMT in the distal 1 cm of the common carotid artery and increased carotid IMT of the common carotid artery (1.3 mm; 1.4 mm) of patient 2. White arrowhead -indicating increased carotid IMT. CCA, common carotid artery, ICA-internal carotid artery, Bifurc-bifurcation, mm-millimeter. **(A)** Right carotid artery, **(B)** B .Left carotid artery.

pre-clinical atherosclerotic vascular changes. Remarkable disturbances of glucose metabolism were not demonstrated. Even though, the incidence of obesity and its metabolic consequences has increased in the Israeli population in the last two decades (Twig et al., 2019), the elevated proportion of CTX patients with dys-metabolism exceeds this tendency and appeared in all of the patients in the study.

Premature vascular involvement and clinical CVD manifestations have been described globally in CTX and may be attributed to the fact that CVD is the leading cause of death in most developed countries (Minelli et al., 2020). We propose that MS and its features developed as a related phenomenon to the CTX, resulting from lifestyle modifications of an early onset chronic illness, or due to the alternations in bile acid metabolites subsequent to the inborn error of metabolism (Duell et al., 2018; Sekijima et al., 2018; Lazarević et al. .2019).

While further investigation is desired to clarify the exact mechanism of atherosclerosis in CTX, accurate CVD risk assessment should be implemented to conduct preventive measures for risk reduction of atherosclerotic cardiovascular disease in these patients, as an opportunity to reduce the burden of CVD. Performing noninvasive evaluation of preclinical atherosclerosis may serve as an additional tool of risk stratification in CTX and, may assist in detecting the patients that will benefit from intensive lifestyle changes and appropriate pharmacotherapy.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Sheba medical center. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

HC: Conception of the work, design of the work, acquisition of data, analysis of data, interpretation of data, drafting the work and revising the manuscript. SH-B: acquisition of data, interpretation of data, drafting the work and revising the manuscript. AS: Interpretation of data and revising the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Case report: Therapy adherence, *MTTP* variants, and course of atheroma in two patients with HoFH on low-dose, long-term lomitapide therapy

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Background: Homozygous familial hypercholesterolemia (HoFH) is a rare and devastating genetic condition characterized by extremely elevated levels of low-density lipoprotein cholesterol (LDL-C) leading to an increased risk of premature atherosclerosis. Patients with Homozygous familial hypercholesterolemia mostly present with mutations in *LDLR*; however, herein, we present two cases with concomitant *microsomal triglyceride transfer protein* mutations, who showed different clinical courses and treatment adherence on long-term therapy with the new MTTP inhibitor lomitapide.

Objectives: We aimed to present the possibility of preventing the progression of atherosclerotic burden with effective and safe LDL-C reduction in patients with Homozygous familial hypercholesterolemia on low-dose lomitapide therapy and emphasize the role of treatment adherence in therapy success.

Methods: We present two patients with phenotypically Homozygous familial hypercholesterolemia, a compound heterozygous woman and a simple homozygous man, both with *LDLR* and additional *MTTP* mutations, who were treated with the MTTP-inhibiting agent lomitapide, with different treatment compliances. The role of impulsivity was investigated through *Barratt Impulsivity Scale 11*, and the extent of the atherosclerotic burden was followed up using coronary artery calcium scoring, echocardiographic and sonographic findings, and, eventually, through a strict follow-up of laboratory parameters. The patients were on lomitapide for 8 and 5 years, respectively, with no adverse effects.

Conclusion: When accompanied by good adherence to therapy, low-dose lomitapide on top of standard lipid-lowering therapy with decreased frequency of lipid apheresis prevented the progression of atherosclerotic burden. Non-compliance might occur due to patient impulsivity and non-adherence to a low-fat diet.

KEYWORDS

MTTP, lomitapide, homozygous familial hypercholesterolemia, lipoprotein apheresis, low-density lipoprotein receptor, genetics

1 Introduction

Homozygous familial hypercholesterolemia (HoFH) is a rare metabolic disorder of mainly autosomal-dominant inheritance that causes extremely high low-density lipoprotein (LDL) cholesterol levels (Rosenson, 2021). Affected individuals show elevated LDL cholesterol levels from birth, which leads to premature atherosclerotic cardiovascular disease (ASCVD). Therefore, the early detection of this condition is critical to prevent early morbidity and mortality (Kayikcioglu et al., 2014; Zhang et al., 2020; Tokgozoglu and Kayikcioglu, 2021). At least four genes are associated with familial hypercholesterolemia (FH), including *LDLR*, *APOB*, *PCSK9*, and *LDLRAP1*, with phenotypic variations (Prince et al., 2013; Chemello et al., 2021; Tokgozoglu and Kayikcioglu, 2021). In most cases, the underlying mutation is in *LDLR*, which is associated with typical LDL cholesterol levels >500 mg/dl in patients with HoFH. Due to absent or defective LDL-receptor activity, individuals with HoFH are resistant to conventional lipid-lowering therapy (LLT) targeting LDL-cholesterol clearance by upregulating LDL receptors. Therefore, LDL apheresis is the most effective treatment for these patients (Kayikcioglu et al., 2018). However, the semi-invasive and time-consuming nature of apheresis is the major obstacle leading to decreased quality of life, increased risk of depression, and deterioration in mental status, leading to high treatment refusal and low adherence (Kayikcioglu et al., 2019; Tunçel et al., 2020). Therefore, effective pharmacotherapies with long-term safety are needed for patients with HoFH.

Lomitapide is a microsomal triglyceride transfer protein (MTTP) inhibitor that reduces LDL cholesterol levels through LDL receptor-independent pathways by directly decreasing apoprotein (Apo)-B levels (Cuchel et al., 2013). Herein, we present two patients with HoFH on long-term, low-dose lomitapide without drug adverse effects but different atheroma courses despite similar baseline LDL cholesterol levels.

2 Case descriptions

Both patients were undergoing long-term follow-up at the Ege University Medical School, Department of Cardiology, Lipid Clinic. They were selected by the means of need and after receiving consent for the compassionate use of lomitapide as an add-on therapy. The clinical characteristics of the patients before and after lomitapide therapy are shown in Table 1. Figure 1A,B depicts the timeline of the case symptoms, diagnosis, and management.

2.1 Genetic analysis

Blood specimens were collected from the cases into tubes containing ethylenediaminetetraacetic acid. Genomic DNA was extracted from peripheral blood samples on a MagNA Pure LC instrument using the MagNA Pure LC DNA Isolation Kit I (Roche Applied Science, Germany). To amplify *LDLR*, *APOB*, *PCSK9, LDLRAP1,* and *MTTP* regions, the Ion AmpliSeq™ Designer tool was used to design specific assay primers to generate a custom FH-DNA panel based on the human (Hg19) reference genome (Ion AmpliSeq™ Target Technology, ThermoFisher Scientific, Waltham, MA). *LDLR*, *APOB*, *PCSK9, LDLRAP1,* and *MTTP* regions of the cases were sequenced on an Ion-Torrent PGM instrument (Life Technologies, Rockville, MD) (314-chip) according to the manufacturer's recommended protocol. The Ion-Torrent suite 4.0 0.2 plugin was used to align the reads to the Hg19 reference genome. Integrative Genomics Viewer (IGV) v2.3 software http://www.broadinstitute.org/software/igv/download) was used to remove false-positive variations and possible PCR errors from the obtained variants. The Human Genetic Mutation Database (HGMD; http://www.hgmd.org/), the Variant Database (FHVD; http://www.ucl.ac.uk/ugi/fh), and https://varsome.com/databases were used for the annotation and prediction of the significance of the detected variants.

2.2 Case reports

Case 1 is a 28-year-old woman with HoFH receiving low-dose lomitapide combined with apheresis for 8 years. Her initial complaints started with chest pain at 8 years of age. She had undergone coronary artery bypass grafting (CABG) due to severe three-vessel coronary artery disease (CAD) at 10 years of age, when she was diagnosed with HoFH with an untreated LDL-cholesterol level ranging between 490 and 549 mg/dl. Her family history was remarkable for early-onset CAD and high cholesterol levels. She had received weekly or biweekly LDL-apheresis therapy since 10 years of age. As her LDL-cholesterol levels were far from the treatment goals, oral lomitapide 5 mg/dl was introduced on top of atorvastatin (80 mg/day) and ezetimibe (10 mg/day) at 20 years of age. The dose was titrated up to 20 mg/dl and has been maintained without adverse events. An additional 31% LDL-cholesterol reduction was observed in the first month of low-dose lomitapide (5 mg/day) therapy. At a dose of 20 mg/day, the LDL-cholesterol reduction reached 49% at the end of 6 months. As she declined to be weaned off apheresis, we reduced the apheresis frequency and she received low-dose lomitapide (20 mg/day)

TABLE 1 Clinical characteristics of the cases.

	Case-1	Case-2
Gender/age (years)	Female/28	Male/33
Age at initial symptom (years)	8, angina	3, xanthomas
Age at diagnosis (years)	10	20
Age of onset of CV disease (years)	8	20
Age at first LDL-apheresis (years)	10	22
Age at the introduction of lomitapide therapy (years)	20	24
CV disease	CABG in 2004	CABG in 2009
	(at age 10 years)	(at age 20 years)
	No history of MI	No history of MI
Weight (kg)	55	88
Height (cm)	160	172
Body mass index (kg/m^2)	21.5	29.7
Blood pressure (mmHg)	98/62	118/78
Hypertension	None	None
Smoking sfttatus (pack-years)	None	None
Diabetes	None	None
Consanguinity of parents (degree)	Second-degree	Second-degree
Family history of CV disease	Remarkable for premature MI both in maternal and paternal sides	Remarkable for premature MI both in maternal and paternal sides
	Father had a mortal CV event at age 38	
Xanthomas	At diagnosis she had on her knees but none now	At diagnosis, he has extensive xanthomas on his knees, elbows, fingers, buttocks, *etc.* Now, most of them vanished but still some on his knees
Corneal arcus	None	Yes
Genetic analysis		
- *LDL-R*	Heterozygous	Homozygous
	c.664T>C, (p.Cys222Arg)	c.1760dupG, (p.Ser587ArgfsTer16)
- *Apo-B*	-	-
- *PCSK9*	-	-
- *LDLRP1*	-	-
- *MTTP*	rs3816873, rs1061271, rs11944749, rs17029189, rs11944752, rs17029213, rs17029215, rs2306985, rs2718684 rs34734558, rs41275719, rs7667001, rs881981, rs982424, and rs991811	rs3816873 and rs1061271
Imaging characteristics		
A. Carotid ultrasonography		
- At baseline before lomitapide	IMT Right 1.3 mm and center 1.5 mm	IMT Right 2.3 mm
	Right ICA minor soft plaque	Left 1.4 mm
	(November 2013)	Right ICA is occluded
		Left ICA 50–70% stenotic calcific plaques
		(2017)

(Continued on following page)

TABLE 1 (*Continued*) Clinical characteristics of the cases.

	Case-1	Case-2
- On lomitapide	IMT Right 1.0 mm and Left 1.0 mm	-
	Right ICA minor calcific plaque	
	(December 2014)	
	IMT Right 0.8 mm and center 0.7 mm	-
	Right ICA minor calcific plaque	
	(November 2015)	
	IMT Right 0.8 mm and center 0.7 mm	Right ICA is occluded
	Right ICA minor calcific plaque	Left ICA 60–70% stenotic calcific plaques
	(November 2019)-	(January 2020)
B. Achilles thickness (mm)- Ultrasound		
- At baseline before lomitapide	9.4 mm	9.8 mm
	(November 2013)	
- On lomitapide	7 mm	NA
	(December 2014)	
	4.8 mm	NA
	(November 2019)	
C. Coronary artery calcium score (Agatston units)	Zero (in all CT evaluations)	Zero (in all CT evaluations)
D. Coronary computerized tomography		
- Before lomitapide	Date: 2013	Date 2014
	Both the bypass grafts of LIMA to LAD and saphenous obtuse marginal graft are patent. The saphenous to RCA graft is occluded. A soft plaque of 25% stenosis at RCA orifice	There are soft plaques and ostial stenosis at the level
		of proximal RCA and LMCA. Atherosclerotic calcifications are present in the aortic root and ascending aorta
- On lomitapide	Date: 2022	Date 2019
		There are soft plaques and ostial stenosis at the level
	Both the LIMA graft to LAD and saphenous obtuse marginal graft are patent. The saphenous to RCA bypass graft is occluded. A soft plaque of 25% stenosis at RCA orifice	of proximal RCA and LMCA
		There are focal stenosis and wall thickening at the level of the saphenous vein graft placed to
		obtuse marginal branch. Atherosclerotic calcifications are present in the aortic root and ascending aorta (progressed compared to previous CT)
E. Echocardiographic features		
-Before lomitapide	Date: 2013	Date: 2015
	Degenerated aortic cusps	Septal-lateral hypokinesia
	Mild aortic regurgitation	Sclerotic aortic valves
	Mild-to-moderate aortic stenosis	Mild aortic regurgitation
	Mean gradient: 23.00 mmHg	Mean gradient: 11.00 mmHg
	Peak gradient: 39 mmHg	Max gradient: 25.00 mmHg
		Minimal mitral regurgitation
		Minimal tricuspid regurgitation

(Continued on following page)

TABLE 1 (*Continued*) Clinical characteristics of the cases.

	Case-1	Case-2
- On lomitapide	Date: 2021	Date: 2018
	Degenerated aortic cusps	Degenerated calcific aortic valve
	Aortic regurgitation: Mild	AVA: 1.12 cm^2
	Mild-to-moderate aortic stenosis	Mild aortic regurgitation
	Mean gradient: 26 mmHg	Moderate aortic stenosis
	Peak gradient: 41 mmHg	Mean gradient: 29 mmHg
		Peak gradient: 47 mmHg
		Date: 2019
		Moderate aortic stenosis
		Mean gradient: 35 mmHg
		Peak gradient: 60 mmHg
		Date: 2020
		Moderate aortic stenosis
		Mean gradient: 36 mmHg
		Peak gradient: 68 mmHg
		AVA: 1.00 cm^2
Lipid and lipoproteins		
1. LDL-cholesterol (mg/dl)		
At diagnosis	519	582
On apheresis		
At the end of the first year of apheresis pre- and post-session (mean)	146–77	261–147
	Weekly and biweekly apheresis	Weekly and biweekly apheresis
At the end of the fifth year of apheresis pre- and post-session (mean)	249–90	466–276
	Every 2 weeks apheresis	Monthly apheresis
At the last three sessions on before lomitapide pre-post session (mean)	330–175	479–322
On lomitapide		
- Baseline	248	562
- 1st month–lomitapide 5 mg/day	170 (no apheresis)	413 (no apheresis)
- 6th month- lomitapide 20 mg/day	129 (no apheresis)	287
- 1st year pre- and post-apheresis session (3 sessions mean)	132–54	256–133
	Apheresis every 2 months	Apheresis every 2 months
2. Lipoprotein (a)		
- At diagnosis (mg/dl)	34.0	<7
- Before lomitapide (mean) (nmol/L)	28	<7
- After lomitapide (mean) (nmol/L)	21	<6
3. Lipid profile (mean, mg/dL) Before lomitapide		
- Total cholesterol	445	585

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TABLE 1 (*Continued*) Clinical characteristics of the cases.

	Case-1	Case-2
- Triglycerides	111	98
- HDL-C	32	31
- Apo A1	81	88
- Apo B	141	320
4. Lipid profile (mean, mg/dL) After lomitapide		
- Total cholesterol	198	352
- Triglycerides	78	70
- HDL-C	42	38
- Apo A1	96	99
- Apo B	79	224
Laboratory analysis (mean)		
AST/ALT (U/L) before lomitapide	24.50/27.40	16.17/15.6
AST/ALT (U/L) after lomitapide	17.92/17.14	13.00/10.00
Creatinine (mg/dl) before lomitapide	0.62	0.86
Creatinine (mg/dl) after lomitapide	0.73	0.87
Treatment		
LLT at baseline before the start of lomitapide	Atorvastatin 80 mg/day	Rosuvastatin 40 mg/day
	Ezetimibe 10 mg/day	Ezetimibe 10 mg/day
	LDL apheresis–every 15 days	LDL apheresis–monthly
LLT (current)	Atorvastatin 80 mg/day	Rosuvastatin 40 mg/day
	Ezetimibe 10 mg/day	Ezetimibe 10 mg/day
	Lomitapide 20 mg/day	Lomitapide 40 mg/day
	LDL apheresis (every 2 Months)	LDL apheresis (every 2 Months)
Treatment duration on lomitapide	8 years 1 month	5 years 9 months

CABG, coronary artery bypass grafting; LAD, left anterior descending; RCA, right coronary artery; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; Apo, apolipoprotein; LIMA, left internal mammary artery; CV, cardiovascular; IMT, intima-media thickness; LLT, lipid-lowering therapy; AST/ALT, aspartate transaminase/alanine transaminase; CT, computed tomography; ICA, internal carotid artery.

plus concomitant bimonthly apheresis. However, during the pandemic for 1 year, she could not access apheresis. Throughout the 8-year lomitapide treatment, she did not experience any increase in serum liver enzyme levels, weight loss, or liver steatosis. Moreover, no change was observed in hepatic fibro scans. She followed a fat-restricted diet compatible with lomitapide therapy to prevent steatorrhea, with concomitant supplementation of fat-soluble vitamins and essential fatty acids. Since the commencement of lomitapide therapy, the patient has remained stable at functional class (NYHA-I) without any adverse cardiovascular events.

Imaging work-up at diagnosis revealed mild aortic stenosis [aortic peak gradient (AoPG) of 30 mmHg-mean (AoMG) 23–18 mmHg] and mild aortic regurgitation with a normal ejection fraction (EF). During the 8-year follow-up, the AoMG was stable at around 25–30 mmHg. In 2022, cardiac catheterization revealed an AoPG of 30 mmHg with normal EF. The computed tomography (CT) angiography showed almost identical coronary and aortal involvement between the baseline (2013) and follow-up (2022) images (Figure 2A). These findings were confirmed by coronary angiography in 2022. Similarly, both the Achilles tendon thickness (ATT) on ultrasonography and carotid intima-media thickness (IMT) significantly decreased with lomitapide treatment. Moreover, the minor soft plaque at the orifice of the right carotid artery became calcified with no progression with lomitapide therapy.

Case 2 is a 33-year-old man with a remarkable history related to HoFH. His first symptoms were xanthomas on his ankles and xanthelasma which appeared at 3 years of age. However, he was not diagnosed with HoFH until 20 years of age. He has presented

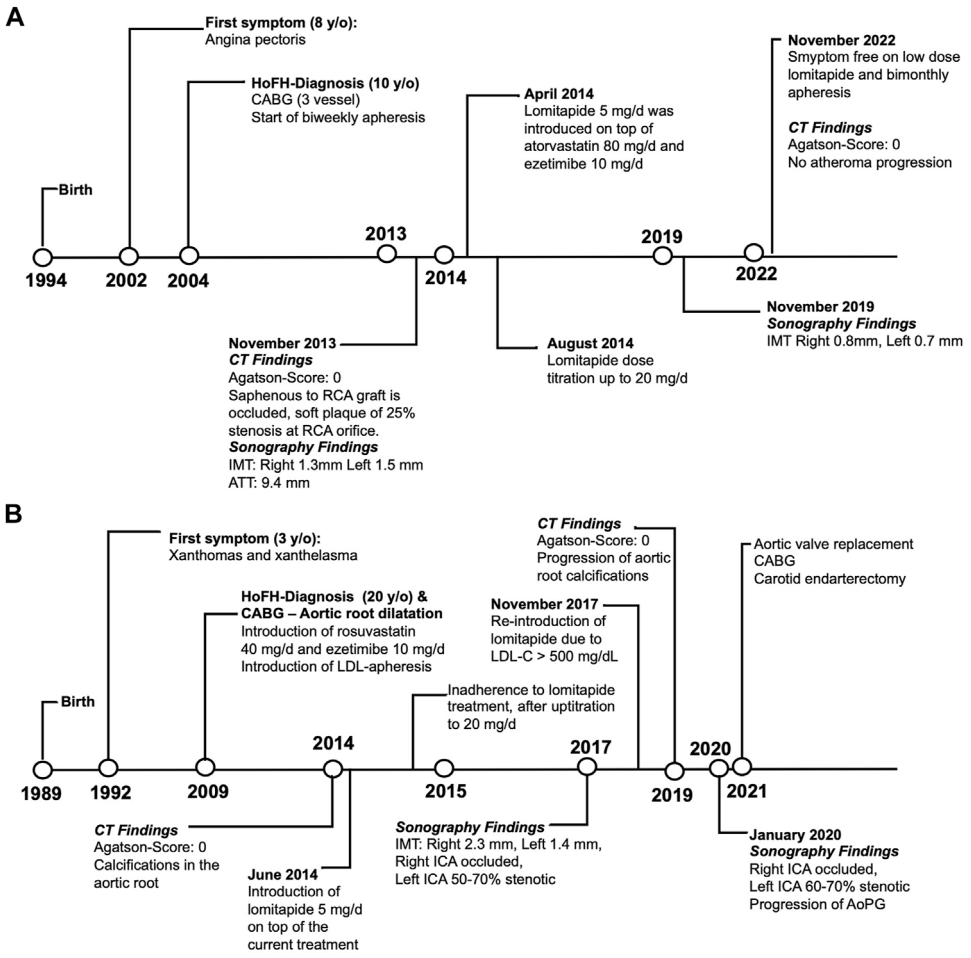

FIGURE 1
Timeline schema representing the clinical evolutions of Cases 1 **(A)** and 2 **(B)**. Abbreviations: CABG, coronary artery bypass graft; CT, computed tomography; ICA, internal carotid artery; RCA, right coronary artery; IMT, intima-media thickness; ATT, Achilles tendon thickness; HoFH, homozygous familial hypercholesterolemia; AoPG, aortic peak gradient; LDL-C, low-density lipoprotein cholesterol.

with exertional angina and angiographically extensive ostial coronary stenotic lesions and had undergone CABG accompanied by aortic root dilation at 20 years of age. One year later, he was referred to our lipid clinic for extremely high LDL-cholesterol levels ranging between 420 and 540 mg/dl. Xanthomas were apparent on his elbows, knees, and ankles. He was already prescribed rosuvastatin (40 mg/day) and ezetimibe treatment, and we introduced LDL-apheresis. He lived a 14-h drive from our center; therefore, he receive monthly apheresis. As his LDL-cholesterol levels were still >200 mg/dl post-apheresis (Mach et al., 2020), we introduced lomitapide in June 2014, when he was 25 years of age. At the initial dose of oral 5 mg/day, mild nausea was observed. With the up-titrated dose of 20 mg/day, his nausea did not increase; however, after 6 months, he declined to take his pills as he experienced an 8 kg weight loss. For the next 3 years, he continued monthly apheresis but no lomitapide. We re-introduced lomitapide in November 2017 due to persistent LDL-cholesterol levels >500 mg/dl. However, this time he was compliant with the low-fat diet and lomitapide. He did not experience any adverse symptoms or weight loss with an up-titrated dose to 40 mg/day. No alteration was observed in transaminase levels, and a recent hepatic fibroscan was normal. We observed an additional 27% LDL-cholesterol reduction for a 5 mg daily dose of lomitapide at the end of the first month, which increased to 49% at the end of 6 months at a dose of 20 mg daily. The patient has been taking the current dose of lomitapide (40 mg/day) for almost 5 years and concomitant apheresis (every 2–3 months). During the pandemic, his access to apheresis was disturbed for 1 year; however, he maintained his lomitapide therapy. He also received supplementation with omega 3-6-9 and vitamin E. However, he reported several times reasons other than adverse effects for not being adherent to lomitapide therapy. During the

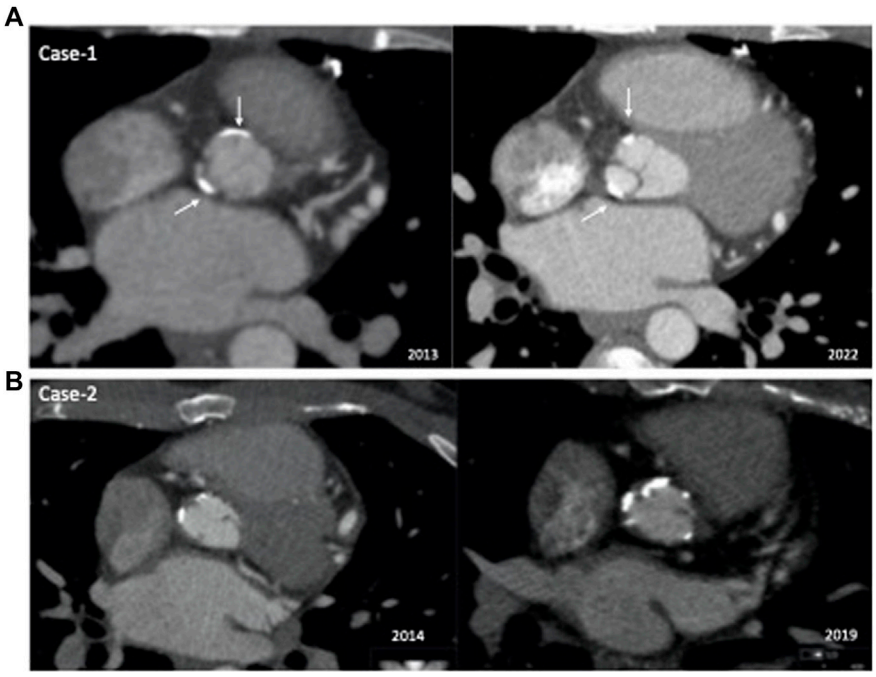

FIGURE 2
Comparison of baseline and follow-up computed tomography angiography images of the patients. **(A)** Case 1: Aortal involvement was almost identical between the baseline (2013) and follow-up (2022) images. **(B)** Case 2: Comparison of 2014 and $2019^{-\Delta\Delta CT}$ angiograms showed significant progression of calcifications in the aortic root.

follow-up, his coronary lesions and aortic stenosis progressed, and he underwent aortic valve replacement (Benthal operation), carotid endarterectomy, and repeated CABG in 2021. Figure 2B depicts the comparison of 2014 and $2019^{-\Delta\Delta CT}$ angiograms showing significant progression of calcifications in the aortic root.

2.3 Genetic results

According to the sequencing results, Case 1 was heterozygous for *LDLR* [c.664T>C, (p.Cys222Arg)], while Case 2 was homozygous for *LDLR* [c.1760dupG, (p.Ser587ArgfsTer16)]. No clinically significant mutation was found in the *APOB, PCSK9*, and *LDLRAP1* regions in both patients. Additionally, 16 different *MTTP* variants were detected in the two patients. Cases 1 and 2 shared 2 *MTTP* variants (rs3816873 and rs1061271). Case 2 also showed one mutation (c.*450A>G) with uncertain clinical significance in public databases, while Case 1 showed 13 mutations (rs11944749, rs17029189, rs11944752, rs17029213, rs17029215, rs2306985, rs2718684 rs34734558, rs41275719, rs7667001, rs881981, rs982424, and rs991811) in *MTTP*. Twelve of these 13 mutations were categorized as benign/likely benign in public databases; only *MTTP* c.3G>A (rs11944752) was associated with elevated plasma glucose, insulin (MAGIC Consortium HGVM 4589669), and cholesterol levels (Teslovich et al., 2010).

3 Discussion

We present two real-life cases of HoFH with completely different disease courses after the commencement of lomitapide therapy. Case 1 demonstrated that long-term effective LLT with low-dose lomitapide combined with apheresis safely prevented the progression of atheroma both in coronary and vascular territories and retarded the progression of aortic stenosis. However, Case 2 showed a completely different course, with significant progression of his coronary lesions and aortic involvement. The patient had to undergo re-operation despite similar baseline LDL-cholesterol levels to those in Case 1. The difference between these patients' disease courses suggests the importance of adherence to therapy for the clinical success of therapy.

Lomitapide is a new-generation potent lipid-lowering agent that acts independently of LDL receptors. Lomitapide inhibits MTTP, a cellular protein that transports neutral lipids between membrane vesicles and acts as a chaperone for the synthesis of apoB-containing triglyceride-rich lipoproteins. Lomitapide has a

critical role in the assembly and secretion of apoB-containing lipoproteins in the liver and intestines (Hussain et al., 2012). Thus, lomitapide, besides triglycerides, effectively reduces LDL-cholesterol levels in patients who are lacking or have mutant LDLR. Lomitapide has been approved by the Food and Drug Administration as a lipid-lowering agent for patients with HoFH as an adjunct to standard LLT and by the European Medicines Agency for patients treated with standard LLT with or without apheresis.

Clinical evidence indicates lomitapide as a potent LDL-receptor-independent agent that has made it possible for patients with HoFH to reach their LDL-cholesterol goals. The phase 3 open-label dose-escalation trial demonstrated that lomitapide at maximal-tolerated doses (5–60 mg/day) reduced LDL-cholesterol by 50% at 26 weeks when added to the standard of care including lipid apheresis in 29 patients with HoFH (Cuchel et al., 2013). Moreover, long-term lomitapide treatment (range 2.1–5.7 years) with a median daily dose of 40 mg reduced LDL-cholesterol levels by an average of 45.5% in 19 patients with HoFH (Blom et al., 2017). During the 246-week therapy, 14 (74%) patients achieved the LDL-cholesterol target of 100 mg/dl and 11 (58%) patients reached the target of 70 mg/dl on at least 1 occasion. We observed a 31% additional reduction in LDL-cholesterol after 1 month of very low-dose (5 mg/day) lomitapide and 49% at the end of 6 months of therapy for a dose of only 20 mg/day in Case 1. In Case 2, the LDL-cholesterol levels were reduced by an additional 27% and 49% for 5 and 20 mg daily doses of lomitapide at the end of 1 and 6 months, respectively. Due to the effective reductions in LDL-cholesterol levels, we decreased the frequency of the apheresis sessions to every 2 months. The LDL-cholesterol reductions achieved in our cases are consistent with those in an HoFH case series reporting >50% reductions for doses of 10–40 mg/day in real-life settings (Stefanutti et al., 2016), which likely led to clinically significant reductions in ASCVD. Consequently, Case 1, who showed a consistent reduction of LDL-cholesterol levels, showed no ASCVD events and no atheroma progression. However, Case 2 showed a different course, with atherosclerosis progression during the 5-year therapy. The difference in therapy adherence was likely the major explanation for the diverse disease courses after lomitapide therapy in our patients. Adherence to LLT favors the survival of patients with ASCVD (Dopheide et al., 2021). Case 1 was adherent since the first dose of therapy. However, in Case 2, the first attempt to use lomitapide was not successful as the patient declined its use due to weight loss; another major obstacle was non-compliance to the low-fat diet, which is a requirement for lomitapide use. After 3 years, we re-introduced the drug. While he was adherent to the low-fat diet, the patient had many excuses for his lack of therapy adherence, likely due to his impulsive behaviors. Barratt impulsivity Scale 11 (Tamam et al., 2013) demonstrated the high impulsivity of Case 2 and the low impulsivity of Case 1. Treatment adherence is inversely related to impulsive and compulsive behaviors (López-Torrecillas et al., 2021).

Hepato-steatosis and liver-enzyme elevation are well-known phenomena associated with lomitapide treatment. However, none of these were observed in either of our patients with the long-term use of lomitapide at doses lower than those used in clinical trials. The only adverse effect was the weight loss reported by Case 2. These findings suggest that most of the adverse events can be avoided by lowering the dosing of lomitapide, as assessed by the LOWER study, in which the long-term (5-year) follow-up of patients on lomitapide (10–20 mg/dl) showed significantly lower adverse event rates compared to the results of the Phase-3 study conducted with doses of 40–60 mg/dl (Underberg et al., 2020). Furthermore, the 20 mg daily dose of lomitapide effectively reduced LDL-cholesterol levels almost to the targets in Case 1 in the present study. Increasing the dose to 40 mg likely would have allowed us to stop apheresis; however, as the patient insisted on apheresis therapy, we continued with 20 mg lomitapide combined with bi-monthly apheresis for >7 years without adverse effects. Low-dose lomitapide plus conventional therapy (statin and ezetimibe) with a decreased apheresis frequency probably increased the adherence to long-term therapy besides efficient LDL-lowering. Similarly, the use of combination therapy was associated with a higher proportion and greater odds of achieving the therapeutic LDL goals of <70 mg/dl and <55 mg/dl,, particularly with the combination of five drugs in the HICC registry (Vallejo-Vaz et al., 2021).

An important aspect of Case 1 in this study is the stabilized course of the aortic atheroma. In patients with late apheresis initiation after 8–10 years of age, the progression of aortic atheroma to stenosis reportedly cannot be prevented even if the LDL goals are attained (Cuchel et al., 2014; Kayikcioglu et al., 2018; Kayikcioglu, 2021). However, in Case 1, after commencing lomitapide use, the aortic involvement of the valvular stenosis and aortic plaques remained stable. This is also contrary to our clinical experience that despite meeting the LDL goals, children with late-onset apheresis therapy (after 8 years of age) show a gradual progression of aortic valve sclerosis or mild stenosis to moderate and severe stenosis.

In addition, our experiences with both of these patients call attention to lipid apheresis as a treatment difficult to access during the pandemic. Thus, novel effective LDL-lowering agents such as oral lomitapide may be useful for patients with HoFH during lockdowns due to pandemics or any other disaster that could disrupt access to apheresis as a hospital-based therapy (Kayikcioglu et al., 2020). Moreover, apheresis cessation or decreased frequency with an effective LDL-lowering agent will improve the psychosocial wellbeing of patients. We previously documented that apheresis as a semi-invasive, time-consuming therapy leads to a decreased quality of life, increased risk of depression, and deterioration in mental status, which lead to high refusal and low adherence rates (Kayikcioglu et al., 2019;

Tunçel et al., 2020) noticeably leading to undertreatment of patients with HoFH, consequently affecting ASCVD outcomes.

Although both patients were phenotypically HoFH with untreated LDL-cholesterol levels >500 mg/dl, xanthomas appearing at early ages, and parental clinical features of heterozygous FH, Case 1 was heterozygous and Case 2 was homozygous for *LDLR* mutations. In addition to standard genetic analysis of the four associated genes, this study is the first to study *MTTP* in patients with HoFH. Loss-of-function mutations within *MTTP* result in severe autosomal recessive diseases, causing ataxia, failure to thrive, steatorrhea, and muscle weakness known as abetalipoproteinemia (Hussain et al., 2012). However, gain-of-function mutations in *MTTP* are not yet well understood. We identified several variants in both patients that may alter lipid levels or affect the clinical efficacy and adverse reactions to lomitapide.

The cases presented have some limitations and strengths. The evaluation of only five genes affecting LDL-cholesterol levels might be a limitation as other genes may explain the severe FH phenotype of the patients, especially in Case 1. However, the evaluation of *MTTP* variants is an important strength as these are the first case presentations of HoFH with *MTTP* variants. Detailed long-term follow-up of the cases and evaluation of impulsivity are additional strengths that allow the evaluation of adherence, clinical course, and the associated factors. Moreover, Case 1 is the first reported case to show the cessation of aortic atheroma progression despite the late onset of apheresis therapy, suggesting the importance of effective LDL-cholesterol lowering in combination with lomitapide and apheresis. Moreover, the comparison of two HoFH patients with similar lipid profiles at baseline and who received the same therapy allowed the assessment of the impact of adherence to therapy.

In conclusion, long-term, low-dose lomitapide was an effective and well-tolerated adjunct to conventional LLT and lipid apheresis in real life. Adherence to lomitapide and a low-fat diet in addition to standard LLT with decreased apheresis frequency appeared to be associated with more effective and safer management of HoFH and prevented the progression of atheroma burden in both the coronaries and aorta and regression in CIMT and ATT. Further studies are needed to illuminate the role of gain-of-function mutations in *MTTP* on lipid levels and response to lomitapide in patients with HoFH.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the patients for the publication of any potentially identifiable images or data included in this article.

Author contributions

MK and HO designed this study. MK, HO, BY, and SB collected data. MK and HO drafted the manuscript. AV performed the genetic analysis. AV searched for genetic literature. MK, HO, and BY conducted the literature search. All authors contributed to the manuscript and approved the final version for submission.

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Conflict of interest

MK has received honoraria (for lectures and consultancy) from Abbott, Jansen, AstraZeneca, Abdi Ibrahim, Nova Nordisk, Novartis, and Sanovel; has received research funding from AstraZeneca, Amryt Pharma; and has participated in clinical trials with Amgen, Bayer Schering, LIB Therapeutics, Lilly, Novartis, and Medpace for the last 3 years.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Rare primary dyslipidaemias associated with low LDL and HDL cholesterol values in Portugal

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Background: Dyslipidaemia represents a group of disorders of lipid metabolism, characterized by either an increase or decrease in lipid particles, usually associated with triglycerides, LDL cholesterol (LDL-C) and/or HDL cholesterol (HDL-C). Most hyperlipidaemias and HDL deficiencies confer an increased cardiovascular risk, while hypolipidaemia, such as abeta or hypobetalipoproteinemia, may present different manifestations ranging from poor weight progression to neurological manifestations. The aim of this study is to present 7 cases with rare dyslipidaemias associated with low LDL or low HDL cholesterol values, referred to our laboratory for the genetic identification of the cause of the dyslipidaemia.

Methods: Lipid profile was determined for each individual in an automated equipment Integra Cobas (Roche). Molecular analysis was performed by NGS with a target panel of 57 genes involved in lipid metabolism (Sure select QXT, Agilent) and samples were run in a NextSEQ Sequencer (Illumina). Only genes associated to rare forms of low HDL-c or LDL-c were analysed for this work, namely: *ABCA1, APOA1, LCAT, SCARB1, APOB, PCSK9, MTTP, SAR1B, and ANGPTL3*. All rare variants (MAF<5%) found in these genes were confirmed by Sanger sequencing.

Results and discussion: This study includes 7 index cases (IC), with the following clinical diagnoses: Fish Eye Disease (1), Hypoalphalipoproteinemia (1) and Abetalipoproteinemia (ABL) / Familial Hypobetalipoproteinemia (FHBL) (5). We have identified one IC with a compound heterozygosity in *LCAT* causing Fish Eye Disease and one IC with a variant in *ABCA1* in homozygosity causing Tangier disease. We found variants causing homozygous FHBL in 2 IC, one of whom has an undescribed pathogenic variant in homozygosity in *APOB* (c.12087+1G>A) and the other is a possible compound heterozygous for *APOB* variants c.2604+1G>A and c.4651C>T/p.(Gln1551*). In two patients

only a variant in heterozygosity (c.3365delG/p.(Gly1122Vfs*62) and c.11095A>T/p.(Arg3699*)). In the remaining patient, no variants were identified. NGS proved to be a fundamental key for genetic testing of rare lipid disorders, allowing us to find the genetic cause of disease in 6/7 patients with low HDL-c and LDL-c. Patients with these rare conditions should be identified as early as possible in order to minimize or prevent clinical manifestations. The unsolved case is still under investigation.

KEYWORDS

rare dyslipidaemias, LDL cholesterol, HDL cholesterol, hypobetalipoproteinemia, hypoalphalipoproteinemia

Introduction

Dyslipidemia is a commonly encountered clinical condition defined as an abnormal concentration of lipids in blood and ranging from raised to low plasma concentrations of total cholesterol (TC), LDL cholesterol (LDL-c), HDL cholesterol (HDL-c) or triglycerides (TGs) (Ramasamy 2016; Hegele et al., 2020). Primary dyslipidemias can be classified as hyperlipidemias or hypolipidemias, depending on whether there is an increase or decrease in plasma lipid levels. The type depends on the metabolic pathway which is affected. It is particularly important to investigate the molecular base of primary dyslipidemias since the etiology determines management and treatment for each subject affected (Hegele et al., 2020).

Primary hypobetalipoproteinemia is an inherited trait characterized by extremely low (or absent plasma) LDL-c and apolipoprotein B (apoB) concentrations. This group of diseases include Familial Hypobetalipoproteinemia (FHBL), Abetalipoproteinemia (ABL), Chylomicron Retention Disease and PCSK9 deficiency (Hegele 2013). FHBL is a rare autosomal codominant disorder characterized by low concentrations of apoB and apoB containing lipoproteins (lower than the fifth percentile for age and sex) (Moutzouri et al., 2011; Hegele et al., 2020). FHBL is caused by nonsense or frameshift variants in apolipoprotein B gene (*APOB)* leading to the formation of a truncated apoB protein of different sizes, resulting in a loss of capacity to form lipoproteins in the liver and intestine and consequently to export lipids from these organs (Ramasamy 2016). Missense variants in *APOB* gene that do not originate truncated proteins can also cause FHBL (Ramasamy 2016), but these are rare causes. Frequently, homozygous FHBL individuals have clinical manifestations such as malabsorption, deficiency of fat-soluble vitamins and acanthocytosis, atypical retina pigmentosa, neuromuscular abnormalities and hepatic steatosis. Although heterozygous FHBL also exhibit some of these manifestations, these individuals usually present a less severe phenotype (Huang et al., 1991). ABL is a rare autosomal recessive disease characterized by markedly low levels of LDL-c, triglycerides and apoB, caused by loss-of-function (LOF) alterations in the Microsomal Triglyceride Transfer Protein (*MTTP)* gene that result in truncated forms of the coded protein (Wetterau et al., 1992; Hooper et al., 2005). MTP is indispensable for the production of apoB containing lipoproteins by the liver and intestine. MTP forms a heterodimer with protein disulfide isomerase that supports the lipid transfer function. This step is important for correct folding of apoB. Due to absence of functional MTP, apoB is not correctly processed, and so the hepatic secretion of triglyceride-rich lipoproteins and fat-soluble vitamins that are transported into lipoprotein particles is impaired (Lee and Hegele 2014).

ABL patients exhibit a phenotype similar to FHBL patients being almost impossible to distinguished clinically. However, since ABL is an autosomal recessive disease, heterozygous subjects frequently have normal lipid profiles, while heterozygous FHBL patients present a characteristic phenotype with lower levels of LDL-c than the general population, consistent with a codominant inheritance mode (Lee and Hegele 2014).

Individuals with ABL or homozygous FHBL may have hepatic complications, which are however more common in individuals with FHBL. Heterozygous FHBL, can also present hepatic steatosis (Lee and Hegele 2014). The exact mechanism on the liver lipid accumulation is not clear, one idea being that the impaired lipid efflux from the liver caused by failure to assemble VLDL particles, results in chronic lipid retention (Lee and Hegele 2014).

Chylomicron retention disease, or Anderson's disease, is a rare autosomal recessive disorder caused by biallelic loss-of-function alterations in the *SAR1B* gene that leads to failure of chylomicron secretion from enterocytes (Hooper et al., 2005; Levy et al., 2019). Often, failure to thrive is observed in childhood, along with severe malabsorption, steatorrhea, and fat-soluble vitamin deficiency (Levy et al., 2019). Homozygous patients have absence of apo B-48 and chylomicrons, and heterozygous subjects presents normal lipid profiles (Hegele et al., 2020). This *SAR1B* gene codes for a small GTPase that regulates the formation and assembly of ER-derived COPII vesicles during protein export from the endoplasmatic reticulum to the Golgi. SAR1B, as the GDP-to-GTP exchanger, is a critical element in the final step of assembling this vesicular transport complex. Consequently, alterations in this gene affect pre-chylomicron

trafficking from the ER to the Golgi apparatus, leading to the absence of chylomicrons and a marked accumulation of lipids in enterocytes (Georges et al., 2011; Levy et al., 2019).

Loss-of-function variants in *PCSK9* have also been described and associated with hypocholesterolemia. The large majority are missense and nonsense alterations that prevent PCSK9-mediated LDLR degradation, and thus increase LDL uptake by the liver, resulting in a 40% reduction in plasma LDL-c levels (Lopez 2008). LOF *PCSK9* variants are not associated, until the moment, with specific clinical symptoms, and on contrary, have been associated to a lower risk of cardiovascular disease (Cohen et al., 2006).

Hypoalphalipoproteinemia is also an inherited dyslipidemia characterized by extremely low HDL-c values (<fifth percentile or <35 mg/dl). Hypoalphalipoproteinemia includes the following diseases: Tangier disease, Apolipoprotein A-I (APOA1) deficiency, and Lecithin cholesterol acyltransferase (LCAT) deficiency (including Familial LCAT deficiency (FLD) and Fish eye disease (FED)) (Hegele 2020 review). Tangier disease is inherited as an autosomal recessive trait and is caused by pathogenic variants in the *ABCA1*, which mediates the secretion of cholesterol excess from cells into the HDL metabolic pathway (Burnett et al., 2019). This results in a severe HDL deficiency and accumulation of cholesteryl esters throughout the body (Burnett et al., 2019). The major clinical signs of Tangier disease include hyperplastic yellow-orange tonsils, hepatosplenomegaly and peripheral neuropathy, which may be either relapsing-remitting or chronic progressive in nature. Rarer complications may include corneal opacities that typically do not affect vision, premature atherosclerotic coronary artery disease, occurring in the sixth and seventh decades of life (not usually before age 40 years), and mild hematologic manifestations, such as mild thrombocytopenia, reticulocytosis, stomatocytosis, or hemolytic anemia. All these clinical signs combine differently in each patient (Alshaikhli and Vaqar 2012).

APOA1 deficiency, a codominant disorder, is characterized by almost absence of HDL-c and apolipoprotein A-I (APOA-I), as well as premature coronary heart disease (Schaefer et al., 2016). To date, about 20 homozygous or compound heterozygous patients have been described worldwide with APOA1 deficiency (Hegele et al., 2020). Homozygous patients with missense variants present a very low plasma concentration of a structurally abnormal APOA-I, and can have corneal clouding, similar to Fish Eye disease (Santos et al., 2008; Schaefer et al., 2016). The homozygous patients with null alleles are clinically characterized by xanthomas, either limited to the eyelids or covering the body (Santos et al., 2008; Schaefer et al., 2016). Heterozygous patients are usually asymptomatic despite low values of HDL-c (Hegele et al., 2020).

Biallelic variants in the *LCAT* gene decrease LCAT secretion or function resulting in LCAT deficiency. LCAT is an enzyme that catalyzes the esterification of free cholesterol in HDL, thus deficiency results in accumulation of free cholesterol, but in a variability of tissues, including cornea and kidney. LCAT deficiency manifests two distinct phenotypes, FLD and FED. In FLD, alpha and beta LCAT activity is absent, leading to extremely low plasma HDL-C (below the fifth percentile). In FED, only the alpha LCAT activity is lost, the beta activity is preserved, permitting cholesterol esterification on VLDL and LDL but not on HDL (Ingle et al., 2016; Santamarina-Fojo et al., 1998). The difference between FED and FLD phenotypes seems to be on whether the variants prejudice the catalytic triad, impair the availability to the catalytic residues or affect the folding of the enzyme.

Clinical presentation of LCAT deficiency include corneal opacities, haemolytic anaemia, proteinuria, progressive chronic kidney disease (CKD), high plasma triglyceride and low HDL-cholesterol concentrations. FLD patients develop premature and progressive CKD leading to end-stage renal disease, the main cause of morbidity and mortality in these patients (Pavanello et al., 2020). Curiously, CKD is not a feature of FED patients.

Despite the profound HDL-C deficiency, LCAT deficiency does not appear to increase the risk for CVD (Zipes 2018).

The aim of this work is to present the 7 cases with low LDL and HDL cholesterol values referred to our laboratory, highlighting the most relevant clinical and molecular data.

Material and methods

The Rare Familial Dyslipidaemia Study is a research project coordinated by the National Institute of Health (INSA) supported mainly by external funds and free of charge for all patients and health institutions. INSA Ethical Committee and INSA Data Protection Officer approved the study protocol and database. Written informed consent was obtained from all participants before their inclusion in the study.

Biochemical characterisation of lipids and lipoproteins

Fasting blood samples were collected at the time of referral to the study. Total cholesterol, direct LDL-c, HDL-c, triglycerides TG, apoA1, apoB and lipoprotein(a) (Lp(a)) were determined for all individuals in a Cobas Integra 400 plus (Roche) by enzymatic colorimetric and immunoturbidimetric methods.

Molecular analysis

Genomic DNA was isolated from peripheral blood EDTA samples, using an adaptation of the protocol described in D.K.Lahiri et al. (1991) (Lahiri and Nurnberger 1991).

Samples for Next-Generation Sequencing (NGS) were prepared according to SureSelect QXT Target Enrichment kit (Agilent Technologies, United States). Targeted sequencing capture probes were custom designed using the specific online tool (SureDesign)

TABLE 1 Clinical, biochemical and molecular characterization of the index cases in this study.

ID	Age	Gender	TC	LDL-C	HDL-C	TGs	Lp(a)	ApoA1	ApoB	Clinical diagnosis	Molecular diagnosis
1	42	F	73	46	3	158	8.3	10	91	Tangier Disease	*ABCA1* gene: c.[3460A>T];[3460A>T], p.[(Lys1154*);(Lys1154*)]
2	47	M	108	31	12	98	8.3	41	61	Fish eye Disease	*LCAT* gene: c.[619G>A];[682G>A]. p.[(Gly207Ser)];[(Asp228Asn)]
3	14	F	24	<10	22	5	9	49	10	ABL/FHBL	–
4	28	F	69	18	52	13	*	115	15	ABL/FHBL	*APOB* gene: c.3365delG:p.(Gly1122Valfs*62)
5	55	F	75#	27#	46#	16#	*	*	*	ABL/FHBL	*APOB* gene: c.11095A>T, p.(Arg3699*)
6	16	F	20	<4	29	9	8.3	82.5	<20	ABL/FHBL	*APOB* gene: c.[12,087 + 1G>A];[12,087 + 1G>A], p.(?); p.(?)
7	53	M	62	9	49	15	38	110	<20	ABL/FHBL	*APOB* gene: c.[2604 + 1G>A];[4651C>T], p.[(?)];[(Gln1551*)]

Age (years); TC–total cholesterol (mg/dl); LDL-C–low density lipoprotein cholesterol (mg/dl); HDL-C, high density lipoprotein cholesterol (mg/dl); TGs, triglycerides (mg/dl); Lp(a)—lipoprotein a) (mg/dl); ApoA1—apolipoprotein A1 (mg/dl); ApoB–apolipoprotein B (mg/dl); ABL, abetalipoproteinemia; FHBL, familial hypobetalipoproteinemia; * Not determined; # on treatment.

provided by Agilent. Only genes associated to rare forms of low HDL or LDL values were analyzed for this work, namely: *ABCA1*, *APOA1*, *LCAT, SCARB1, APOB, PCSK9, MTTP, SAR1B, and ANGPTL3*. The references used for analysis were: NM_ NM_005502, NM_000039, NM_000229, NM_005505, NM_000384, NM_174936, NM_000253, NM_001033503 and NM_014495 respectively.

The manufacturer's protocols were followed during library preparation (Agilent Technologies, United States). In resume, for each sample, 25 ng of high-quality genomic DNA was fragmented and adaptors were added in a single enzymatic step with QXT reagents. The adaptor-tagged DNA library was purified and amplified. The libraries were recovered using streptavidin magnetic beads, and a post-capture PCR amplification was carried out. Post-enrichment pooling allowed sequencing of a high variety of sample amounts as well as different sample target sizes. Samples were pooled prior to sequencing to a final concentration of 4 nM and run in a NextSeq platform (Illumina, United States) available at INSA using a NextSeq 550 System generating 130M reads per run (75 base reads). The FASTQ files were analyzed using SureCall Software (Agilent Technologies, United States). Output VCF files were analyzed using wANNOVAR Software for SNVs identification (Yang and Wang 2015). Variants with a minor allele frequency (MAF) below 5% in gnomAD (Karczewski et al., 2019) in one of the genes studied in this work, were confirmed by Sanger sequencing.

Complementary DNA numbering was considered according to the Human Genome Variation Society (HGVS) nomenclature (Dunnen and Antonarakis 2000) with nucleotide c.1 being A of the ATG initiation codon p.1.

Results

This study includes seven index cases, 2 with low HDL-c values and 5 with low LDL-c values. The clinical and biochemical characterization of the individuals in this study are present in Table 1.

Four genes associated to hypoalphalipoproteinemia phenotype were analyzed (*ABCA1*, *APOA1*, *LCAT* and *SCARB1*). A total of three variants were detected, one in *ABCA1* gene (c.3460A>T, p.(Lys1154*) and two in *LCAT gene* [c.619G>A, p.(Gly207Ser) and c.682G>A, p.(Asp228Asn)] (Table 1). No rare variants were identified in *APOA1* or *SCARB1* genes.

For the cases with hypocholesterolemia phenotype, we studied five genes (*APOB*, *PCSK9*, *MTTP*, *SAR1B*, and *ANGPTL3*). Rare variants were only detected in the *APOB* gene in four index cases (Table 1). No rare variants were identified in *PCSK9*, *MTTP*, *SAR1B*, or *ANGPTL3* genes.

Clinical cases

Index case 1

A 48 years-old woman was referred to the genetic department after several myocardial infarctions (MI).

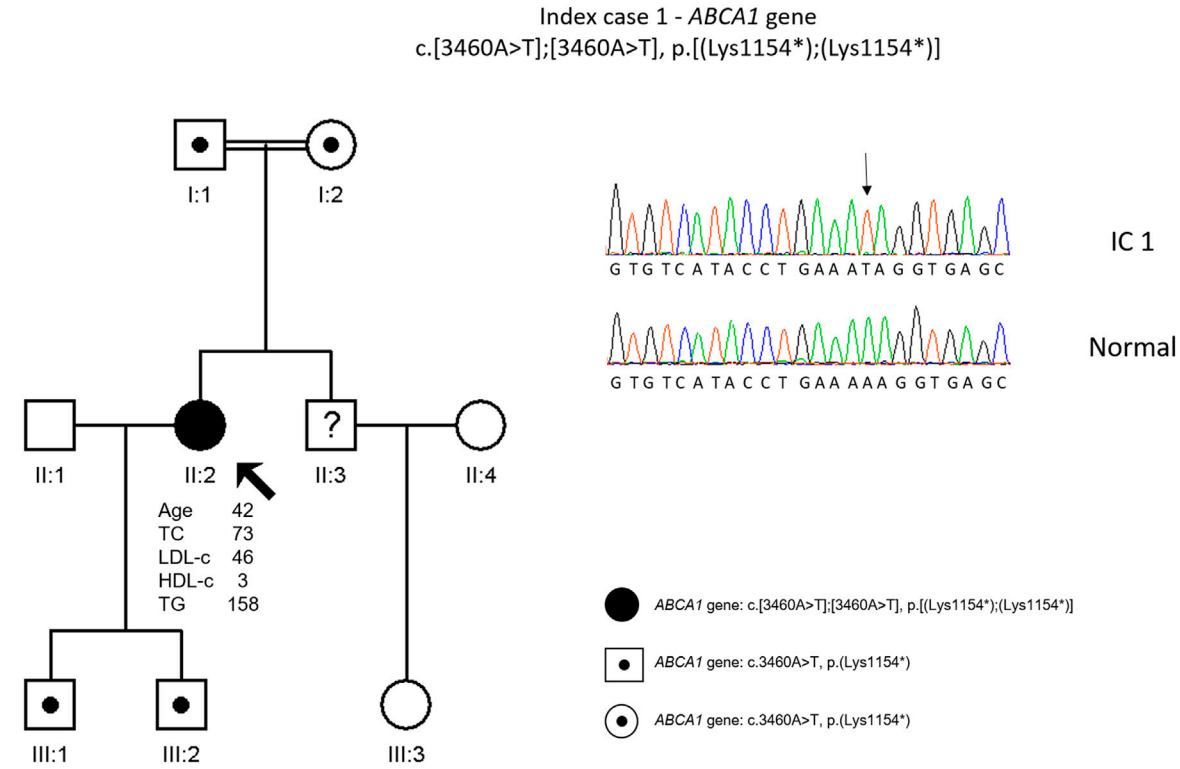

FIGURE 1
Arrow indicates index case 1 (IC1). Age (in years) and lipid profile in mg/dL are under each symbol. Point symbols represent heterozygous individuals for the variant in *ABCA1* gene, c.3460A>T, p.(Lys1154*). Black filled symbol represents homozygosity for this variant (c.3460A>T, p.(Lys1154*)). Partial sequence of *ABCA1* gene of IC1 and a normal sequence.

She is the first daughter of a consanguineous couple, and she has a healthy younger brother and two healthy sons. She had tonsillectomy at 4 years of age. She also has fatty liver disease and laparoscopic cholecystectomy was performed for chronic calculous cholecystitis.

She had the first MI at 42 years. At that time, the laboratory tests show TC 73 mg/dl, ApoA1 10 mg/dl and HDL-c values lower than 5 mg/dl. After cardiac exams, an atrophic right coronary artery was diagnosed, with atherosclerotic plaque burden superior than expected for age and gender.

At the age of 48 years, she had another MI and underwent angioplasty of the anterior descending coronary. Five months later, she had a third MI and a stent was implanted overlapping with the previous one.

Despite having a normal ophthalmological and neurological exam, severe deficiency of HDL and premature atherosclerotic coronary artery disease suggested Tangier disease.

The genetic study revealed a homozygous variant in *ABCA1* gene, c. 3460A>T, p. (Lys1154*) (Figure 1). This variant has not been previously described but is classified as pathogenic according to the American College of Medical Genetics (ACMG) guidelines (Richards et al., 2015). No relatives were available for cascade screening, but the sons are obligatory heterozygous for the mother's variant.

Index case 2

A 47-years-old male, referred from primary health center to genetic department for ocular findings combined with analytical changes, with suspected Fish-eye-disease since the age of 45. From personal medical history, there is a report of a road accident in 2008 with subsequent abdominal trauma and non-functioning right kidney.

In addition to follow-up at nephrology department, he was observed in ophthalmology, and ring opacification of the corneal periphery was revealed. Analytically, he evolved with low HDL values (6–16 mg/dl) combined with hypertriglyceridemia (158–263 mg/dl), maintained despite the fibrates therapy and diet restrictions.

The genetic testing found two missense variants in *LCAT* gene: c.619G>A, p. (Gly207Ser) and c.682G>A, p. (Asp228Asn). These variants are not described previously and are classified as variants of uncertain significance (VUS) according to the ACMG guidelines. The co-segregation study was carried out on the parents, and it was observed that the index patient inherited a

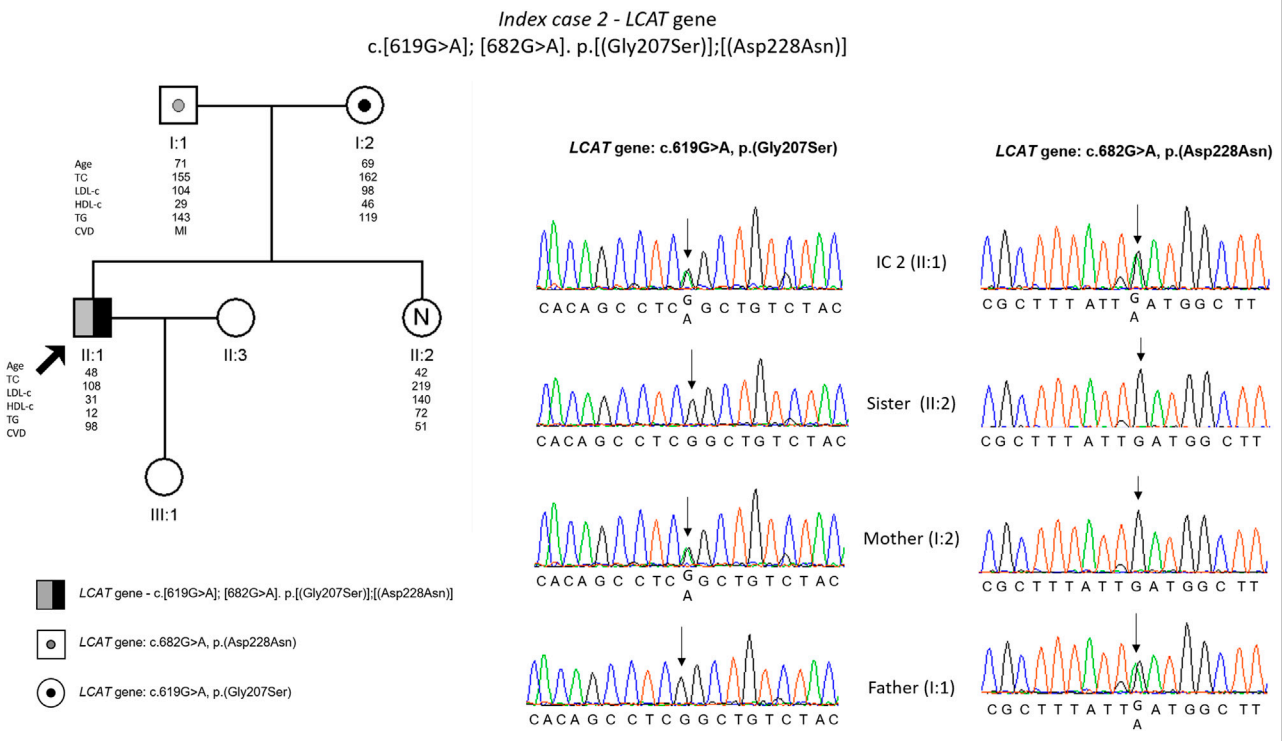

FIGURE 2
Arrow indicates index case 2 (IC2). Age (in years) and lipid profile in mg/dL are under each symbol. Black point symbol represent heterozygous individual for the variant in *LCAT* gene, c.619G>A, p.(Gly207ser); grey point symbol represent heterozygous individual for the variant in *LCAT* gene, c.682G>A, p.(Asp228Asn); the compound heterozygote is represented with half-filled in black and half-filled in grey; Partial sequence *LCAT* gene of IC2 and family members.

different variant from each parent; the sister did not carry any of the mutated alleles (Figure 2).

Index case 3

A 14 years-old female of Indian ancestry was referred to a pediatric consultation for poor weight and height progression. She also presented mild hepatomegaly and hyperechogenic liver suggesting liver steatosis. There was no history of chronic diarrhoea. Laboratory tests were performed showing normochromic anaemia, mild increase of transaminases and the following lipid profile: TC 24 mg/dl, LDL-c <10 mg/dl, HDL-c 22 mg/dl, TGs 5 mg/dl and apoprotein B 10 mg/dl.

Further investigations centered mainly on the diagnosis of a possible abetalipoproteinemia showed acanthocythosis (red blood cells with thorny spikes) on peripheral blood film, mild iron deficiency, low Vitamin D_{25}, extremely low Vitamin E and A levels. Although ophthalmological examination of fundus was initially normal, later on she developed pigmentary retinopathy, in electroretinography she presented severe and progressive macular and retinal dysfunction in internal layer. Duodenal and jejunal endoscopy showed white duodenal mucosa and the Intestinal biopsy vacuolization of enterocytes (fat-filled). Follow up showed difficulty in night vision, episodes of diarrhea (initially denied) with steatorrhea and renal lithiasis. Despite very low levels of fat-soluble vitamins she presented no ataxia, hyporeflexia or muscle weakness.

Treatment included low lipid diet, high doses of vitamin A and E, as well as vitamin D and K, calcium and iron according to blood tests, showing some improvement in growth.

Her parents have very different lipid profiles. While the father presents a typical lipid profile of heterozygous FHBL (TC 114 mg/dl, LDL-c 59 mg/dl and TGs 50 mg/dl), the mother presents a moderately high lipid profile (TC 214 mg/dl, LDL-c 150 mg/dl and TGs 100 mg/dl), but she is obese. Her brother has normal to low lipid values (TC 154 mg/dl, LDL-c 72 mg/dl and TGs 68 mg/dl) (Figure 3).

The genetic study of *APOB*, *PCSK9*, *MTTP*, *SAR1B*, and *ANGPTL3* genes has not detected any variant causing disease in these genes.

Index case 4

A 27 years-old female, was referred by an endocrinologist with hypocholesterolemia since childhood. The lipid profile was, TC 71 mg/dl, LDL-c 14 mg/dl, HDL-c 55 mg/dl, TGs 11 mg/dl and

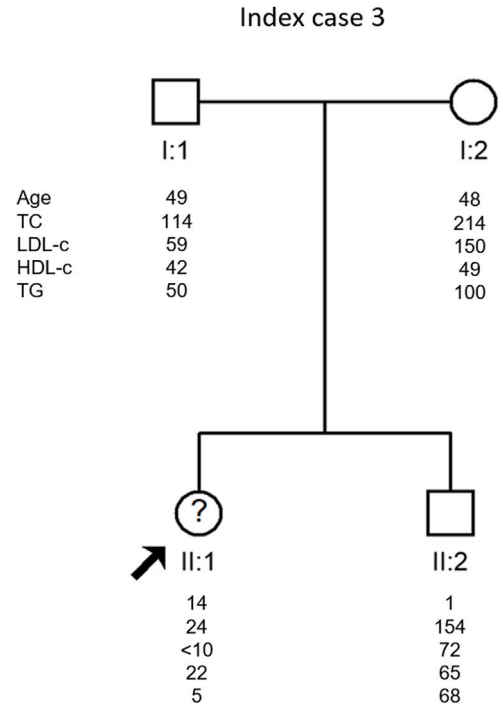

FIGURE 3
Arrow indicates index case 3 (IC3). Age (in years) and lipid profile in mg/dL are under each symbol.

APOB <26 mg/dl. The father, paternal aunts and one of the brothers were also reported to have low total and LDL cholesterol values.

The genetic study detected a heterozygous frameshift variant (c.3365delG, p. (Gly1122Valfs*62) in the *APOB* gene. This variant is classified as pathogenic according to the ACMG guidelines and was previously described in an individual with FHBL (Marmontel et al., 2018). It was not possible to carry out the co-segregation study in this family (Figure 4).

Index case 5

A 52 years-old woman with arterial hypertension and intermittent left bundle branch block in medical history, suddenly collapsed when she was working in housekeeping activities. In her family, her mother died suddenly at 48 years old, her maternal uncle also died at around 40 years and the maternal grandfather suffered sudden cardiac death at 70 years. Physical examination at the local showed no abnormalities. ECG showed a normal sinus rhythm with left bundle branch block. She was admitted to the emergency room with cranioencephalic traumatism from the fall.

An echocardiogram was performed and revealed left ventricular end-diastolic cavity at upper limits of normal and reduced left ventricular ejection fraction (LVEF) at 30–35%, with overall wall hypokinesia. She was discharged with a favourable evolution, and a cardioverter defibrillator (ICD) was implanted.

Lipid values are not available before the sudden death event. After the cardiac event she was given a statin and her LDL values reached 27 mg/dl.

The etiological investigation with a Sudden Death Gene Panel requested by the medical geneticist to an external laboratory showed a likely pathogenic variant in *RYR2* gene (c.14579C>T). Additionally, as an incidental finding a heterozygous variant was found in the *APOB* gene, c.11095A>T, p. (Arg3699*). The presence of this variant was confirmed in our laboratory. This variant is classified as pathogenic according to the ACMG guidelines and was previously described in a heterozygous FHBL patient (Martín-Morales et al., 2013). The cascade screening performed on the index daughters revealed that one of them was carrying the *APOB* gene variant (Figure 5).

Index case 6

A 16-years-old female with a history of a functional specific learning disorder and family history of remote consanguinity, with no known heredofamilial diseases was admitted to Pediatric Consultation due to nephrolithiasis. On physical examination, thinness was noted (BMI 18.02 kg/m2 (P15)). "Routine" investigation disclosed TC 30 mg/dl, LDL-c 1.7 mg/dl, HDL-c 25.3 mg/dl, TGs 14.9 mg/dl (<P5 for sex and age) and mild increase of transaminases. The hypothesis of hypobetalipoproteinemia was supported by apolipoprotein B100 < 12 mg/dl. Neurological and ophthalmological exams were normal. Liver ultrasound was also normal (no steatosis).

The dosage of vitamins A, D and E showed deficits in vitamin D (22 ng/ml; r.v.>29) and E 4.25 mg/L (r.v. 5–20). The coagulation study showed an INR of 1.28 (r.v. ≤1.2) with factor VII 41% (r.v. 50–150). At this stage she started vitamin E, D and K supplementation.

The genetic study detected a homozygous variant [c.12087 + 1G>A, p. (?)] in the *APOB* gene. This variant is novel and is classified as pathogenic according to the ACMG guidelines. The cascade screening was carried out on the parents, and it was found that the parents are both carriers of the variant in the *APOB* gene (Figure 6).

Index case 7

A 53 years-old male with a previous history of follicular variant papillary carcinoma that was removed, was referred to our lab by an internal medicine physician and presented with hypocholesterolemia, hepatomegaly, steatosis hepatic and arterial hypertension. The lipid profile was TC 62 mg/dl, LDL-c 9 mg/dl, HDL-c 49 mg/dl, TGs 15 mg/dl and mild increase of transaminases. Neurological and ophthalmological exams were normal.

The genetic study detected two variants in *APOB* gene, c.2604 + 1G>A, p. (?) and c.4651C>T, p. (Gln1551*). Both variants were described previously, the splicing variant by Pelusi and others

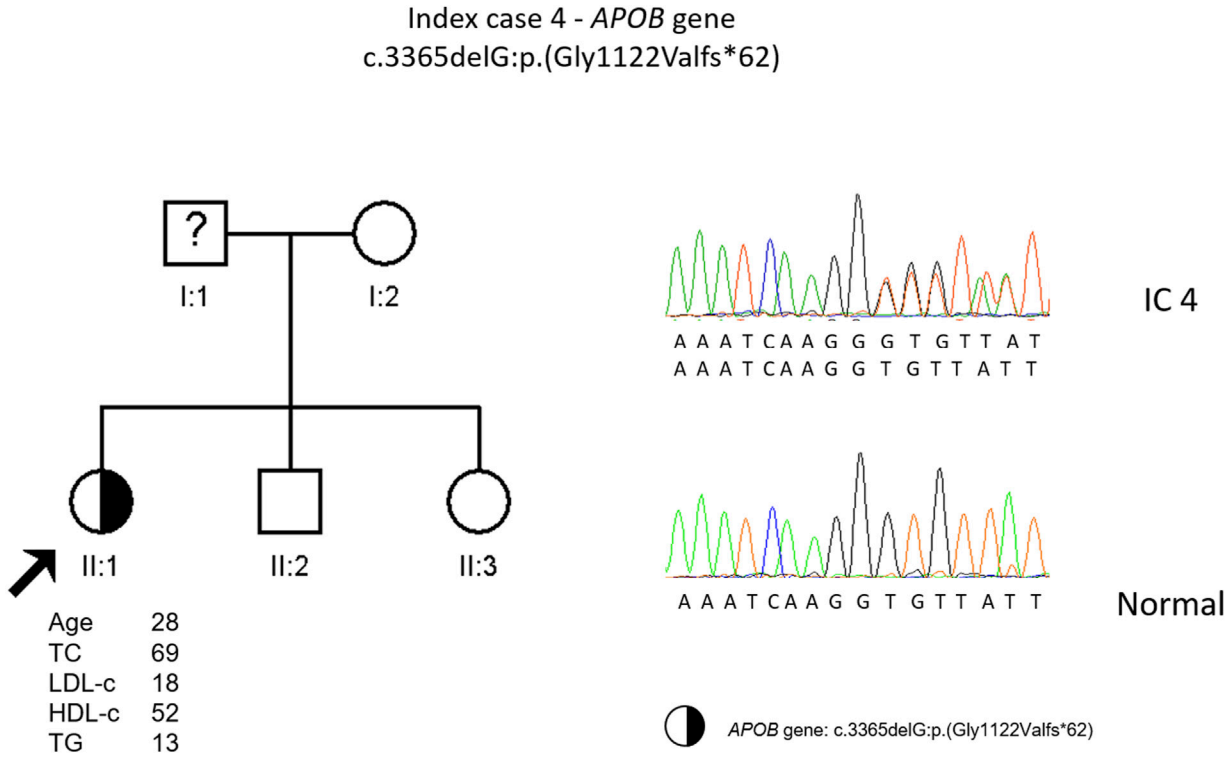

FIGURE 4
Arrow indicates index case 4 (IC4). Age (in years) and lipid profile in mg/dL are under each symbol. Half-filled symbol represents heterozygous individual for the variant in *APOB* gene, c.3365delG, p.(Gly1122Valfs*62); Partial sequence *APOB* gene of IC4 and a normal sequence.

(Pelusi et al., 2019) and the nonsense variant by Lange and others (Lange et al., 2014). These variants are classified as pathogenic according to the ACMG guidelines. The cascade screening could not be performed until now due to parents old age and reduced mobility (Figure 7).

Discussion

Serious clinical complications resulting from genetic dyslipidemias awakened the scientific community for the need to investigate these disorders.

With the advent of NGS technology, massive parallel DNA sequencing became possible, as well as the rapid and cost-effective sequencing of the entire genome hence altering the traditional laboratory approach to genetic testing and research (Sanger Sequencing) (Shendure et al., 2017). As a research tool, NGS customized panels allow a focused investigation of rare familial dyslipidemias by combining genes associated with familial dyslipidemias and genes that are candidates for an important role in lipid metabolism, allowing for a quick analysis.

In this work we present the clinical and molecular data of seven index cases (ICs), clinically diagnosed with rare dyslipidaemias: hypoalphalipoproteinemia (2 ICs) and hypocholesterolemia (5 ICs).

Hypoalphalipoproteinemia

Patient 1 with a nonsense variant in homozygosity in *ABCA1* gene [c.3460A>T, p. (Lys1154*)] presented an unusual phenotype having had several MI around 40 years old. Although the development of CHD is a characteristic of the disease, this patient presented it at an early age (Hegele et al., 2020). Furthermore, she has fatty liver disease and laparoscopic cholecystectomy was performed for chronic calculous cholecystitis. Although at the moment, there is no specific treatment for this disease, early identification could have prevented the development of CHD at such an early age. The therapeutic options are to increase HDL levels through lifestyle alterations such as physical exercise, a healthy weight, smoking cessation, and replacement of monounsaturated for saturated fatty acids in diet, in order to raise HDL cholesterol. In some cases, a combination of lipid-lowering therapies such as statins, niacin, and fibrates can be given either alone or in combinations to optimize LDL levels (Hegele et al., 2020; Alshaikhli and Vaqar 2012). However, there is no robust evidence of the efficacy of this therapeutic approach (Alshaikhli and Vaqar 2012). In this patient, LDL levels were already low and this approach was not chosen.

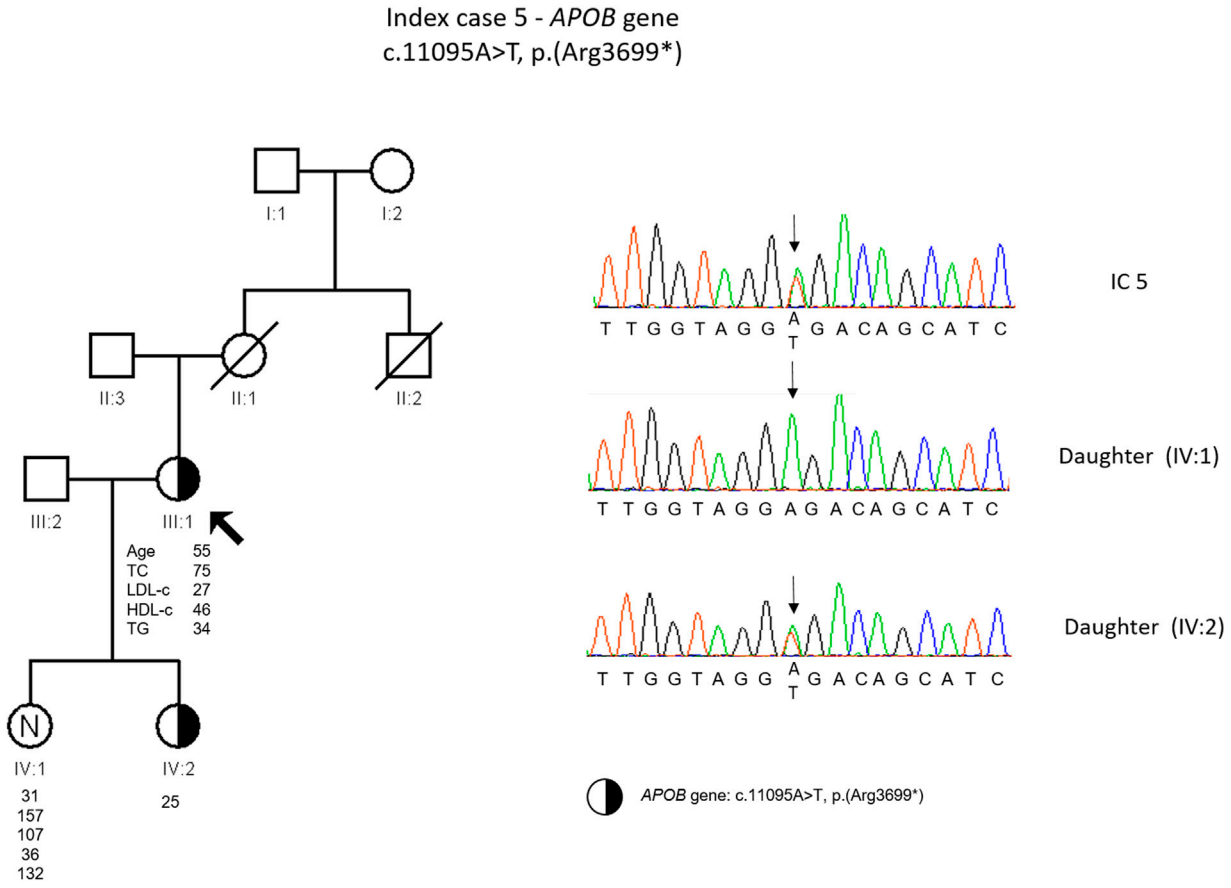

FIGURE 5
Arrow indicates index case 5 (IC5). Age (in years) and lipid profile in mg/dL are under each symbol. Half-filled symbol represent heterozygous individual for the variant in *APOB* gene, c.11095A>T, p.(Arg3699*); Partial sequence *APOB* gene of IC5 and family members

Cascade screening was not carried out but, since this patient is a true homozygous patient, her sons and parents are obligatory carriers of this variant. No health complications are predicted since they will present this variant in heterozygosity, however, for future reproductive issues the index case offspring should be offered genetic counseling since they are carriers of a pathogenic variant that in homozygosity is associated with a high cardiovascular risk.

The second patient with hypoalphalipoproteinemia presented ring opacification of the corneal periphery since childhood, characteristic of Fish Eye Disease (Hegele et al., 2020). However, the diagnosis was only made later in life. The molecular study confirmed the presence of two different variants in the *LCAT* gene, both not previously described. Parents are unrelated and so the compound heterozygosity was expected in this patient. It is described that heterozygous have HDL-cholesterol levels about half of reference values (Hegele et al., 2020), and in fact the IC parents have low HDL values, most clearly the father (29 mg/dl). As expected, the non-carrier sister has a normal HDL value (74 mg/dl).

Most patients with FED have moderately high triglyceride levels (Ramasamy 2016), since they have high levels of VLDL, TG enrichment of LDL and reduction of HDL (Schaefer et al., 2016). Our patient presented mild hypertriglyceridaemia and is receiving appropriate pharmacological treatment with fibrates.

At 35 years old he had a road accident, resulting in only one functioning kidney. As kidney disease is not associated with FED patients (Santamarina-Fojo et al., 1998), this issue will not affect his prognosis.

Hypocholesterolemia

We identified a rare variant in the *APOB* gene in four out of five index cases with a clinically diagnosis of hypocholesterolemia.

IC three was the only case where the cause of hypocholesterolemia has not yet been identified. All genes associated to date with hypocholesterolemia have been studied and no disease-causing variant has been identified. This is one of the most enigmatic cases in our laboratory since she presents a

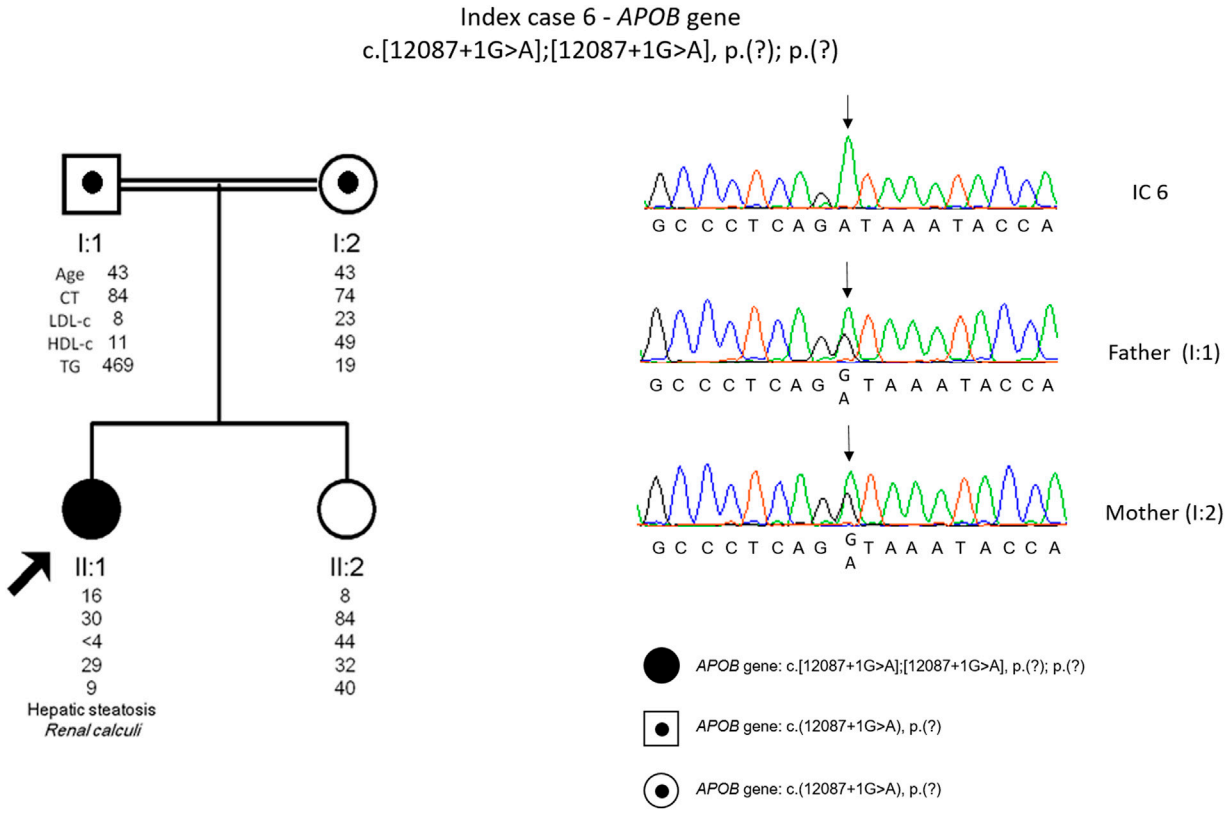

FIGURE 6
Arrow indicates index case 6 (IC6). Age (in years) and lipid profile in mg/dL are under each symbol. Black point symbol represents heterozygous individual for the variant in *APOB* gene, c.12087+1G>A, p.(?); Black symbol represent homozygous individual for the variant in *APOB* gene, c.12087+1G>A, p.(?); Partial sequence *APOB* gene of IC6 and family members.

severe hypocholesterolemia phenotype but no variant explaining the phenotype was found. Moreover, her mother seems to have a mild dyslipidemia which is not common in relatives of patients with hypocholesterolemia. In this family, a whole exome sequencing will be performed in an attempt to find the cause of hypocholesterolemia.

Patient four is also an interesting case. After the genetic study to investigate the cause of an episode compatible with the sudden death syndrome, a frameshift variant in the *APOB* gene was found as an incidental finding. This variant, c.3365delG, p. (Gly1122Vfs*62), causes an alteration in the reading frame resulting in a premature termination codon in *APOB*, not described previously. The predicted translation product with this alteration is a truncated protein, smaller than apoB-48. Due to a family history of sudden death and the finding of an alteration in *RYR2* gene variant, this was considered the cause of the disease and a statin was prescribed. In this case, being the patient heterozygous for FHBL, lipid lowering therapeutics should not be necessary since the patient naturally has low LDL values. One of the daughters is also heterozygous for a frameshift variant in the *APOB*. We do not have information about plasma levels, but we assume they will be low. Both mother and daughter should be evaluated for fatty liver disease due to lack of apoB protein produced from one allele.

IC 5 has the diagnosis of heterozygous FHBL, since a nonsense variant in the *APOB* gene in heterozygosity resulting in a premature stop codon consequently generating a truncated protein [c.11095A>T, p. (Arg3699*)] was identified. This variant has been described previously in heterozygosis, in a Spanish patient with a clinical diagnosis of FHBL (Martín-Morales et al., 2013). However, the plasma levels in our patient are lower compared to that described by Martim-Morales and others (Martín-Morales et al., 2013). In both families, there are relatives with low and normal LDL levels and there is no report of neurological disease or fatty liver. Nevertheless, the carriers of this variant should be followed in a specialized center to prevent the long-term complications.

IC 6, a definite diagnosis of homozygous FHBL was possible to achieve since the patient presents a novel splicing variant in

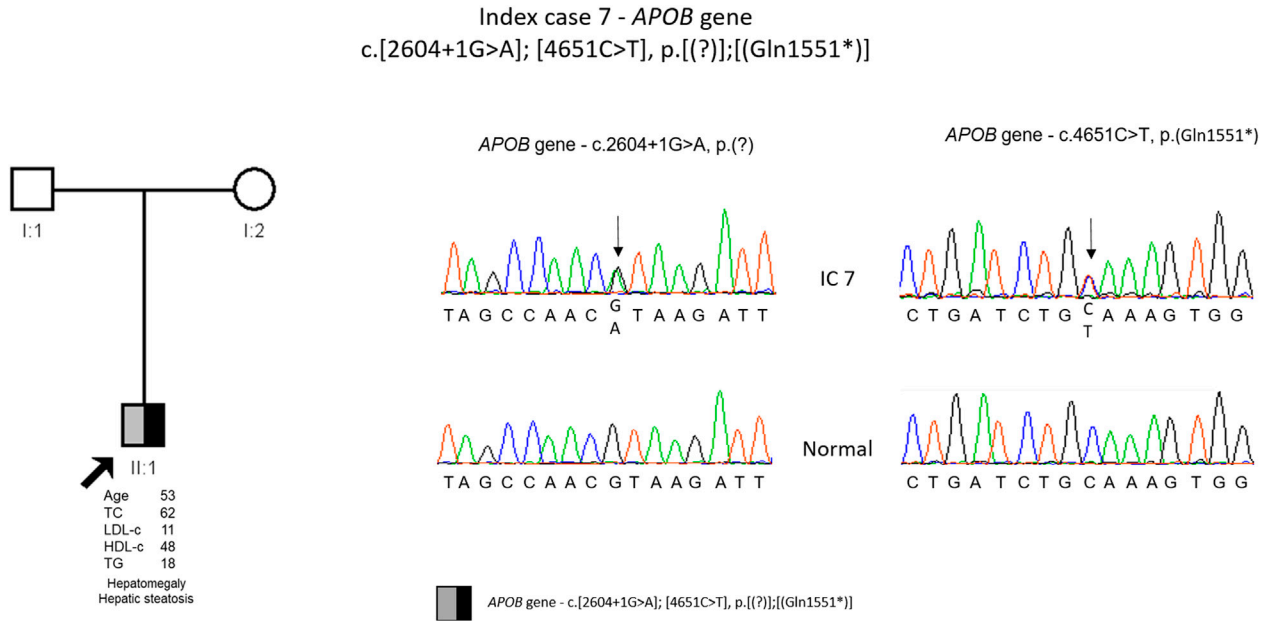

FIGURE 7
Arrow indicates index case 7 (IC7). Age (in years) and lipid profile in mg/dL are under each symbol. Black half-filled symbol represent heterozygous individual for the variant in *APOB* gene, c.2604G>A, p.(?); Grey half-filled symbol represent heterozygous individual for the variant in *APOB* gene, c.4651C>T, p.(Gln1551*); Partial sequence *APOB* gene of IC7 and normal sequence.

homozygosity that justifies the phenotype. Following the co-segregation study, it was found that the *APOB* gene variant was indeed inherited from each parent. The parents have remote consanguinity, justifying the presence of the variant in true homozygosity. To date it was not possible to perform the co-segregation study on the sister, but according to the lipid profile (LDL 44 mg/dl) she seems to be a carrier of the variant in the *APOB* (Figure 6). Although the patient has the most severe form of the disorder (homozygous form), she is in a good clinical condition without neurological symptoms, although with low levels of fat-soluble vitamins. Supplementation should prevent long-term complications, such as ataxia and peripheral neuropathy. Fatty liver, not found in the patient, should be investigated in the heterozygous parents. Considering the rarity and heterogeneity of the clinical severity of familial hypobetalipoproteinaemia, this diagnosis is a challenge in terms of therapeutic and clinical surveillance.

In IC 7, two *APOB* variants were identified, one splicing variant [c.2604 + 1G>A. p. (?)] and other non-sense [c.4651C>T, p. (Gln1551*)]. Unfortunately, the cascade screening could not be carried out in this family due to the old age of the parents who live in the islands and therefore it was not possible to confirm that the IC is a compound heterozygous. However, due to the patient phenotype there is little doubt that each variant is in a different allele. The non-sense variant creates a premature codon and consequently a truncated protein and the splicing variant, considering that is at position +1, it is in a critical location for the correct RNA splicing and most probably will disrupt RNA splicing. This will result in exon skipping or intron retention that in turn will lead to frameshift originating a truncated protein. Furthermore, both variants have been described previously in individuals with a clinical diagnosis of FHBL (Lange et al., 2014; Marmontel et al., 2018). So, both variants are considered disease causing. This patient presents hepatic steatosis, and interestingly the splicing variant was previously described in an Italian patient with non-alcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (NAFLD-HCC) (Pelusi et al., 2019). Pelusi and others observed that individuals with NAFLD-HCC and *APOB* variants had a circulating lipid profile consistent with hypobetalipoproteinemia (Pelusi et al., 2019). It was also described that variants in *APOB* also frequently occur during hepatic carcinogenesis since there is a causal role of hepatocellular lipid retention in promoting NAFLD-HCC (Ally et al., 2017). The mechanism that relates *APOB* variants with carcinogenesis is not well understood. Some of the hypotheses are induction of hepatocellular lipid accumulation, oxidative stress, and the loss of a possible tumor suppressive activity of *APOB* (G. Lee et al., 2018; Valenti and Romeo 2017).

Functional assessment of NGS detected variants in these cases is relevant to understand the effect of these specific truncations since the length of the truncated protein may have clinical significance (Richards et al., 2015). All variants found in the *APOB* gene are predicted to prevent the complete

translation of apoB mRNA, resulting in the production of truncated dysfunctional apoB proteins that are most probably degraded by the cell. Without apoB, new lipoproteins are not formed in the liver and/or intestine and exported, resulting in low or absent LDL levels in plasma (Sankatsing et al., 2005; Rabacchi et al., 2019). Liver steatosis in FHBL individuals might be explained by chronic lipid retention due to decreased production of LDL apo B-100, increased catabolism of VLDL, and extremely low secretion of the truncated apo-B (Parhofer et al., 1992). The screening of liver steatosis in these patients is seldom done in the clinical set, especially in heterozygous cases, preventing early diagnosis and treatment.

In addition, cascade screening is essential to study these inherited disorders in order to access variant pathogenicity and to early identify affected relatives who might benefit from implementation of therapeutic measures. Particularly in FHBL, due to its co-dominant inheritance pattern, heterozygous relatives are at risk and should be offered genetic assessment and counselling (Hegele et al., 2020).

Studies focused on FHBL subjects demonstrated by correlation that they are relatively protected from CVD by the (life-long) reduced levels of exposure to apo B-containing lipoproteins, suggesting that apo B-containing particles may constitute a central factor in atherogenesis (Sankatsing et al., 2005). Thus, FHBL patients might constitute a unique cohort to evaluate the impact of life-long exposure to unusually low levels of apoB-containing atherogenic lipoproteins. Nonetheless, despite low cardiovascular risk of FHBL patients, other serious conditions are associated with this disorder, such as neurological complications and steatohepatitis (J. Lee and Hegele 2014).

Conclusion

We report here the clinical and molecular characteristics of seven patients with rare lipid disorders associated with low LDL or low HDL. Two cases presented with hypoalphalipoproteinemia, and the genetic workup allowed the diagnosis of Tangier disease in one and of Fish Eye Disease in the other. These are ultrarare disorders, with 200 and 30, cases respectively reported worldwide (www.orphanet.com).

Five out of seven patients had a clinical diagnosis of hypocholesterolaemia and variants in *APOB* were identified in 4. Half have the milder form of FHBL (heterozygous form), but also need to be counselled since they are at risk for liver steatosis. In the only patient without molecular diagnosis in our cohort, extended molecular studies (eg exome) will be performed to find the cause of the severe hypocholesterolaemia.

This work shows that NGS custom panels allow for a rapid and accurate identification of patients with rare dyslipidaemia. When the cause is not found, novel genes can be investigated by exome/genome sequencing. (Santamarina-Fojo et al., 1998).

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession numbers are: OQ692142 and OQ692158. The data can be accessed here: https://www.ncbi.nlm.nih.gov/nuccore/OQ692142 and https://www.ncbi.nlm.nih.gov/nuccore/OQ692158

Ethics statement

The studies involving human participants were reviewed and approved by Comissão de Ética do INSA. Written informed consent to participate in this study was provided by the participantsandapos'legal guardian/next of kin.

Author contributions

AA and BM perform molecular analysis, including RS. AA and MB analysis and interpretation of results. OM, RG, MS, RS, LD, SS, referred the patients, provided clinical history and follow up. AA, draft manuscript preparation. MB conceived and supervised the whole study and reviewed the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Non-alcoholic fatty liver disease in a pediatric patient with heterozygous familial hypobetalipoproteinemia due to a novel *APOB* variant: a case report and systematic literature review

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Background: Familial hypobetalipoproteinemia (FHBL) is an autosomal semi-dominant disorder usually caused by variants in the *APOB* gene that frequently interferes with protein length. Clinical manifestations include malabsorption, non-alcoholic fatty liver disease, low levels of lipid-soluble vitamins, and neurological, endocrine, and hematological dysfunction.

Methods: Genomic DNA was isolated from the blood samples of the pediatric patient with hypocholesterolemia and his parents and brother. Next-generation sequencing (NGS) was performed, and an expanded dyslipidemia panel was employed for genetic analysis. In addition, a systematic review of the literature on FHBL heterozygous patients was performed.

Case report: Genetic investigation revealed the presence of a heterozygous variant in the *APOB* (NM_000384.3) gene c.6624dup[=], which changes the open reading frame and leads to early termination of translation into the p.Leu2209IlefsTer5 protein (NP_000375.3). The identified variant was not previously reported. Familial segregation analysis confirmed the variant in the mother of the subject, who also has a low level of low-density lipoprotein and non-alcoholic fatty liver disease. We have introduced therapy that includes limiting fats in the diet and adding lipid-soluble vitamins E, A, K, and D and calcium carbonate. We reported 35 individuals with *APOB* gene variations linked to FHBL in the systematic review.

Conclusion: We have identified a novel pathogenic variant in the *APOB* gene causing FHBL in pediatric patients with hypocholesterolemia and fatty liver disease. This case illustrates the importance of genetic testing for dyslipidemias in patients with significant decreases in plasma cholesterol as we can avoid damaging neurological and ophthalmological effects by sufficient vitamin supplementation and regular follow-ups.

KEYWORDS

***APOB*, *APOB* gene, familial hypobetalipoproteinemia, nonalcoholic steatohepatitis, hypocholesterolemia, pediatric, fatty liver, systematic review**

1. Introduction

Hypobetalipoproteinemias (HBLs) encompass a diverse range of disorders that are characterized by reduced levels of total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), and apolipoprotein B (apoB) in the blood, which fall below the 5th percentile of the population distribution (1).

It may develop due to primary and secondary causes (1). Primary hypobetalipoproteinemia comprises various genetic conditions, such as abetalipoproteinemia (ABL) (genetic defect in *MTP* gene), chylomicron retention disease (genetic defect in *SAR1B* gene), and combined familial hypolipidemia (genetic defect in *ANGPTL3* gene). These disorders share an autosomal recessive mode of inheritance. Another condition falling under primary hypobetalipoproteinemias is familial hypobetalipoproteinemia (FHBL) (2). The main secondary causes are malnutrition, intestinal fat malabsorption, severe liver disease, and hyperthyroidism (3).

Familial hypobetalipoproteinemia has an autosomal semi-dominant pattern of inheritance (4). Variants in the *APOB* gene are the main cause of FHBL. Additionally, less frequent variants in the *PCSK9* gene have also been associated with the disease (5, 6). Most variants in the *APOB* gene cause the formation of truncated forms of apoB, which depending on the length may or may not be secreted into the plasma (7, 8). LDL-C levels in heterozygous FHBL are low but not absent (9). Due to the lower production rate, predicted apoB levels in FHBL heterozygotes would be 50% of normal; however, they are ∼24% (10). Homozygous FHBL individuals commonly exhibit significantly low levels of total cholesterol (TC) in their bloodstream, with the presence of plasma apoB either completely absent or found only in minimal quantities (2).

We report a new heterozygous variant in the *APOB* gene identified in a patient whose symptoms were characteristic of FHBL. This case prompted us to carry out a systematic review to better define the genetic causes, already identified variants, and the clinical course in heterozygous FHBL.

2. Methods

2.1. Study design and family description

The patient has been followed regularly at the University Medical Centre Ljubljana. The medical records were used to acquire his clinical information. This report is a part of the research project approved by the National Medical Ethics Committee of Slovenia (#0120-273/2019/9). The principles of the Declaration of Helsinki were followed. Written informed consent using local consent forms was obtained from the parents of the patient for the publication of any data included in this article.

2.2. Liver enzymes, lipid profile, and fat-soluble vitamins analysis

Laboratory measurements were analyzed for lipids including TC, LDL-C, high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) using the direct enzymatic colorimetric method. Additionally, liver enzymes, including aspartate transaminase (AST) and alanine transaminase (ALT), and fat-soluble vitamins E, A, and D were measured.

2.3. Genetic analysis

Genetic analysis was performed at the University Children's Hospital Ljubljana in Slovenia. Using the FlexiGene DNA Kit 250, genomic DNA was extracted from peripheral blood samples (Qiagen, Hilden, Germany). Next-generation sequencing (NGS) was performed. Using TruSight One, Illumina, FC-14111006 (Illumina, San Diego, CA, United States), the regions of interest were enriched. Sequencing was performed on the MiSeq desktop sequencer together with MiSwq reagent kit v3 (Illumina, San Diego, CA, United States). Coding regions of 4,813 genes were analyzed. A panel of seven genes associated with hypocholesterolemia, including the *APOB* gene, was used for filtering variants. VarAFT tool was applied for annotation and filtration (11). The detected variants were classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG AMP) (12) classification criteria as (likely) benign, variants of uncertain significance (VUS), and (likely) pathogenic. The variant identified in the *APOB* gene was subsequently confirmed by Sanger sequencing.

2.4. Systematic literature review

We have collected accessible scientific report publications for the systemic review. A systematic literature review was performed on 20 June 2022, following PRISMA reporting guidelines. We searched the PubMed database for available case report articles on heterozygous patients with pathogenic and likely pathogenic variants in the *APOB* gene related to FHBL. The following search terms were used: "hypocholesterolemia" and "APOB". In addition to that, we also searched Human Gene Variant Database Professional (13) and the Franklin by Genoox tool (14) based on the variant confirmed in our patient and using scope "Gene." We found 63 articles. We read the abstracts and included all articles that (1) were published in English, (2) were fully accessible, (3) contained human data and clinical data on patients, and (4) described heterozygous cases. We excluded all articles that did not meet the criteria. All articles that met the criteria were read and analyzed in full-text form. In the end, 19 articles describing 35 cases were included in our systematic literature review. All data were collected in Microsoft Office 365 (Microsoft Corporation, Redmond, WA, USA). Systematic literature workflow is presented in Figure 4.

3. Case report

The proband was a male in his early twenties who was referred to our department for HBL at the age of 10 years. He was hospitalized in another hospital for gastroenterocolitis at the age of 7 years, where laboratory tests revealed severe hypobetalipoproteinemia. The family history revealed that his two siblings as well as his mother and his maternal grandmother all

have low serum levels of LDL-C. His mother and sister have been diagnosed with celiac disease.

The patient was born after a fourth pregnancy, the second pregnancy ended with a miscarriage. He has had atopic dermatitis since birth and in the last years, allergic rhinitis has developed. The patient also reported getting tired very quickly, with unpleasant breath in the morning. On physical examination, he was quite high for his age with a body mass index of 17.5 kg/m^2. Respiratory and cardiovascular examinations were normal. His abdomen was a little bit tender with a palpable liver of 2 cm under the rib cage and impalpable other organs or masses. Other physical findings included an asymmetric chest and irregular growth of teeth. After examination, several blood tests were performed, revealing mildly elevated levels of AST 54 IU/L (0.9 μkat/L) (<36.6 IU/L, <0.61 μkat/L) and ALT 100.8 IU/L (1.68 μkat/L) (<28.8 IU/L, <0.48 μkat/L) with normal gamma-glutamyl transferase (gGT) and bilirubin concentrations. Low levels of LDL-C of 0.4 mmol/L (2.0–3.5 mmol/L) and apoB of 0.2 (0.55–1.40 mmol/L) were found. Hormones and antibodies regarding any thyroid gland disorder were in a normal range. Coeliac disease was excluded. The proband was afterward regularly followed by our endocrinology department and by a gastroenterologist. Laboratory tests are chronologically presented in Figures 1–4.

The mildly elevated ALT and AST persisted until the end of the follow-up at our tertiary center. The concentration of ALT varied from 63.5 to 163.5 IU/L (1.08–2.78 μkat/L) and AST from 33.5 to 55.9 IU/L (0.57 to 0.95 μkat/L) (Figure 2). The concentrations of gGT and bilirubin were within normal limits. Abdominal ultrasound revealed a marginally enlarged liver and spleen (at the upper limit for age and gender). The gallbladder and bile ducts appeared normal. Other abdominal organs were without evident pathology. Magnetic resonance of the upper abdomen showed changes characteristic of hepatic steatosis. A liver biopsy was performed at the age of 16 years, which confirmed liver steatosis (steatosis was present in 50% of hepatocytes). There were no signs of liver cell injury (e.g., ballooning degeneration) or inflammation; however, there was evidence of mild fibrosis along the central veins and mild sinusoidal-pericellular fibrosis. Other causes of chronic liver diseases were excluded (e.g., viral liver disease, Wilson's disease, alpha-1-antitrypsin deficiency, autoimmune liver disease, hemochromatosis, drug-induced liver steatosis, and others). During the follow-up, the synthetic liver function was normal. There were no signs of portal hypertension or hypersplenism. The patient was without gastrointestinal symptoms throughout the follow-up, and he passed normally formed stools once or twice a day. Serological tests for coeliac disease were repeated several times and were always negative. He was not nourished, and his body mass index (BMI) ranged between 17.5 kg/m^2 at the diagnosis of hypobetalipoproteinemia and 24.2 kg/m^2 at the last follow-up examination at the age of 18 years. At that time, the values of ALT and AST were 74.7 IU/L (1.27 μkat/L) and 38.8 IU/L (0.66 μkat/L) respectively, with normal gGT and bilirubin concentrations, and there were no signs of portal hypertension on Doppler ultrasound of the portal veins.

Low levels of vitamin E were one of the major concerns regarding our proband. The blood test of proband aged 10 years revealed vitamin E levels of 13.6 μmol/L (14–23 μmol/L). He was prescribed a treatment of 10 mg of vitamin E per day and 1,000 IE of vitamin D per day. He was also treated with lecithin, complex B, and omega-3 fatty acids. As the measured vitamin E level did not improve, the dose was initially increased to 100 mg per day and later gradually to 1,000 mg per day to achieve adequate blood concentrations.

The first bone densitometry performed in 2011 was unremarkable (L1–L4 Z-score +0.3; whole body Z-score −0.3). He was prescribed calcium carbonate 1,000 mg per day and 1,000 IE of vitamin D per day due to osteopenia that was revealed on the second densitometry (L1–L4: Z-score −0.5; whole body Z-score −1.6).

During the follow-up, he was regularly followed by a clinical dietician. At the start of the follow-up, an analysis of the dietary diary revealed that the proband's intake was 2,000 kcal/day (recommended 3,000 kcal/day), fat intake was as recommended (<30% of total calories), but with long-chain fatty acids around 30 g/day, whereas 15 g/day are recommended for the patients with the HBL.

According to the following US of the abdomen in 2017, improvement was noticed. A liver biopsy revealed histologic changes in line with metabolic liver defects. It showed microvesicular steatosis in 50% of hepatocytes without steatohepatitis, fibrosis along the central veins, and mild sinusoidal-pericellular fibrosis. The first genetic analysis was performed in 2012. Exon 26 of the *APOB* gene was amplified by a polymerase chain reaction and sequenced directly to analyze the presence of variants NM_000384.3:c.10707C>T and NM_000384.3:c.10708G>A. Analyzed variants were not confirmed, thus FHBL could not be confirmed as the cause for the clinical manifestation of the disease. The diagnosis was confirmed after a second genetic analysis was performed using NGS. In the *APOB* (OMIM: +107730) gene, heterozygous variant c.6624dup has been identified, which we then confirmed with Sanger sequencing. The variant is not found in dbSNP, HGMD, or gnomAD databases. The *in silico* prediction tools report variants as pathogenic (CADD (https://cadd.gs.washington.edu/), Variant Taster). The variant is classified as pathogenic (PVS1 very strong, PM2 moderate, and PP1 supporting) according to the ACMG-AMP criteria (12). Thus, this variant was considered causal for the clinical manifestations. Later, Sanger sequencing-based segregation analysis confirmed the presence of this variant in proband's mother, but it was not confirmed in proband's brother and father. A family peedigree with c.6624dup p.Leu2209IlefsTer5 variant in the APOB gene is presented in Figure 5. We were unable to perform a genetic analysis on his sister who has normal cholesterol levels. In 2019, we expended genetic testing which revealed the variant in the *COL2A1* gene (NM_001844.5) c.375+1G>A[=], confirming the Stickler syndrome.

In the next follow-ups in 2018 and 2019, treatment and diet remained the same. The US of the abdomen in 2019 showed regression of the steatohepatitis compared with the previous US of the abdomen at the beginning of the same year, which showed moderate hepatosplenomegaly and hepatopathy. Vitamin E level was still at the lower limit of the normal range, and the supplementation dose was increased to 2,000 mg/day. The last follow-up visit was in 2022. Apart from elevated liver

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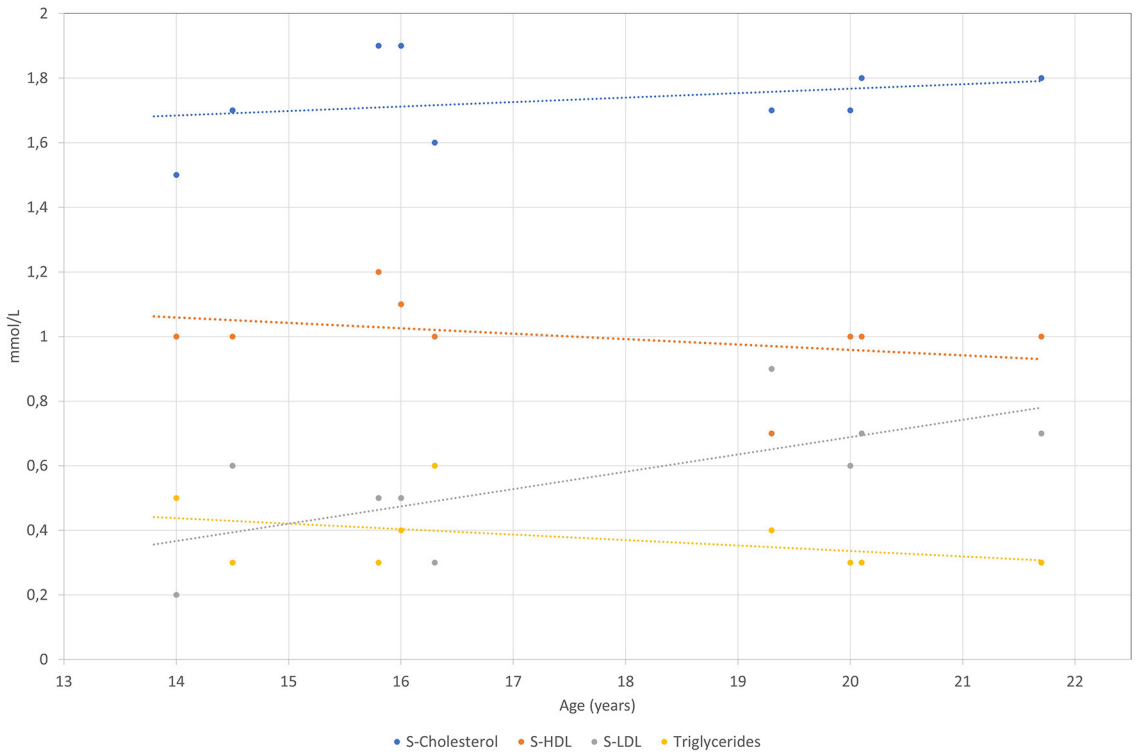

FIGURE 1
S-Cholesterol, S-HDL-C, S-LDL-C and S-TAG levels are presented over time for the patient measured in mmol/L.

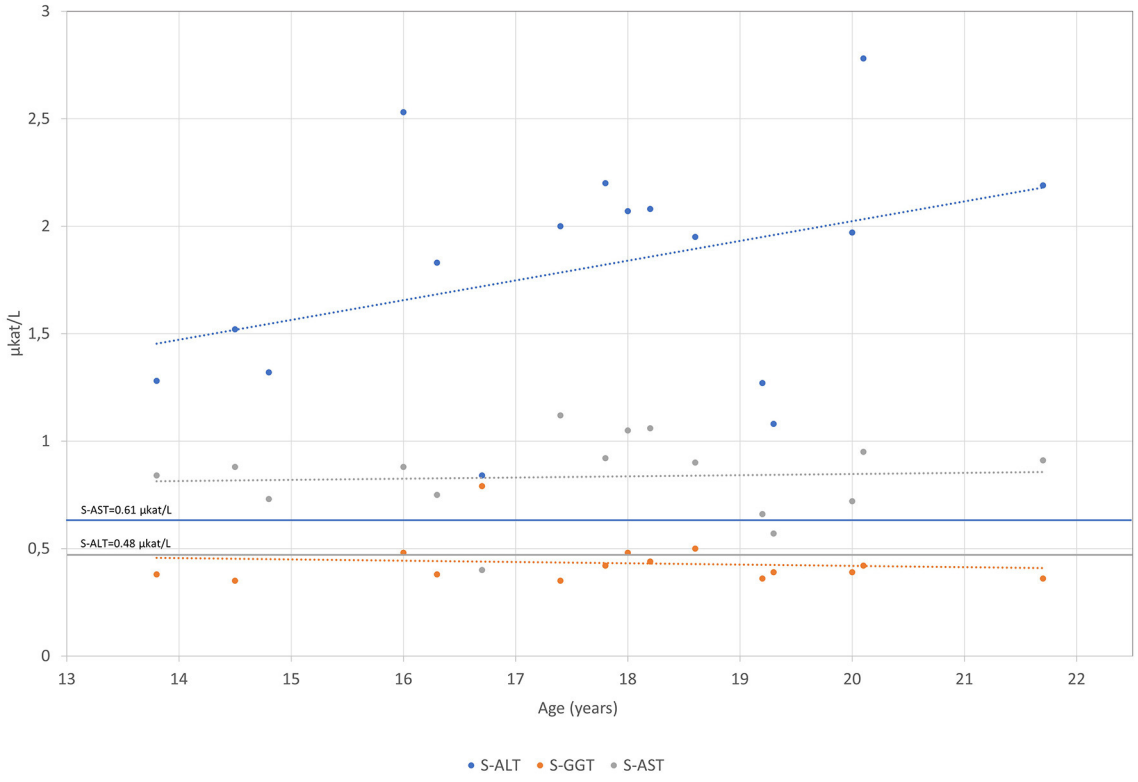

FIGURE 2
S-AST, S-ALT and S-GGT levels are presented over time for the patient measured in µkat/L.

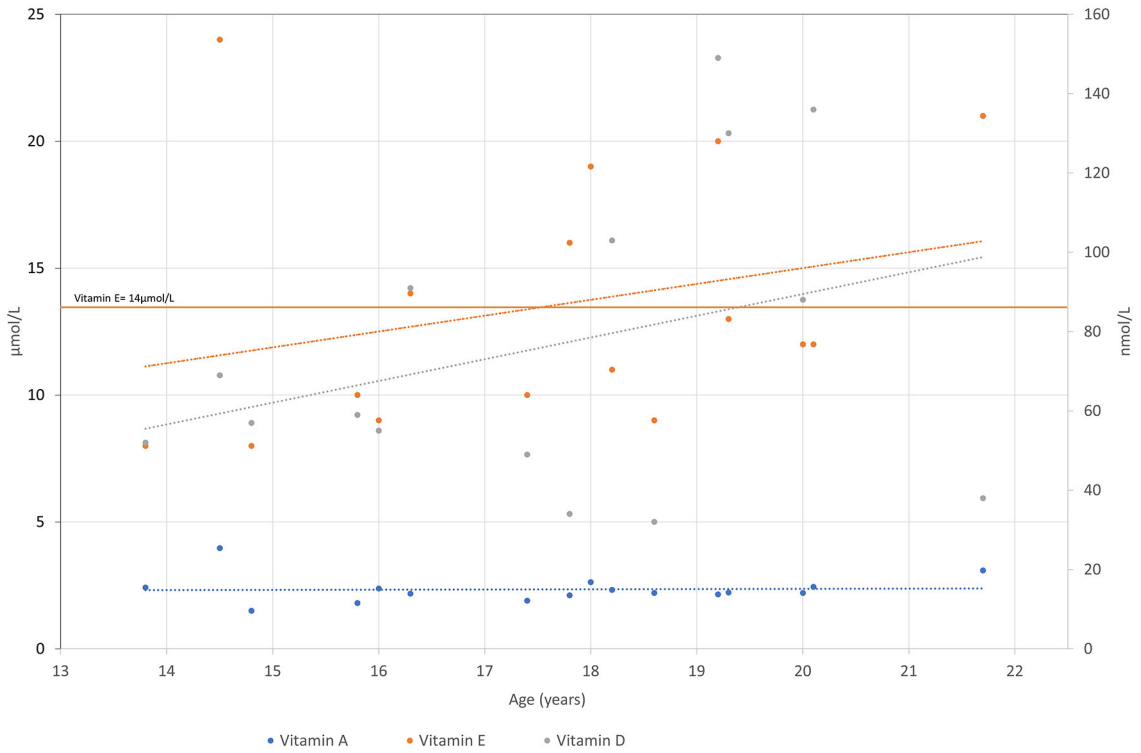

FIGURE 3
Vitamin A, E and D levels are presented over time for the patient. Vitamin A and E are measured in µkat/L and vitamin D in nmol/L.

enzymes with AST 53.5 IU/L (0.91 µkat/L) (<35.9 IU/L, <0.61 µkat/L) and ALT 128.8 IU/L (2.19 µkat/L) (<28.2 IU/L, <0.48 µkat/L), everything else remained stable and treatment remained unchanged. All biochemical measurements are collected and presented in Supplementary Table 1 and on the Mendeley Data Repository https://doi.org/10.17632/sm84p6vp26.1.

4. Discussion

Our study presented the results of a follow-up conducted over an extended period for a proband carrying a new variant in the *APOB* gene, which is associated with FHBL.

FHBL type 1 (OMIM: #615558) is a semi-dominant disorder mainly caused by protein-truncating variants (PTVs) in the *APOB* gene (15). In 1979, Steinberg and his colleagues were the first to identify a distinct condition characterized by hypobetalipoproteinemia and normal triglyceridemia. Young et al. described the first kindred with two distinct abnormal *APOB* alleles associated with FHBL (16). Multiple following kindreds have been documented since then, which has allowed the identification of numerous gene deletions, single nucleotide substitutions, and splicing variants.

The *APOB* gene, positioned on chromosome 2p24.1, is composed of 29 exons. The same gene produces two forms of protein, apoB-48 and apoB-100. ApoB-48 is produced by tissue-specific mRNA C→ U RNA editing at nucleotide position 6666 which causes premature stop codon. The modification results in the production of apoB-48, representing 48% of the amino terminus of apoB-100 (17). ApoB-48 is produced by the small intestine and lacks the LDL-receptor binding domain, but it plays a crucial role in chylomicron synthesis (18, 19). Full-length apoB-100 is produced in the liver. It plays a role as a structural component of very low-density lipoprotein (VLDL) and a ligand for receptor-mediated endocytosis of low-density lipoprotein (LDL).

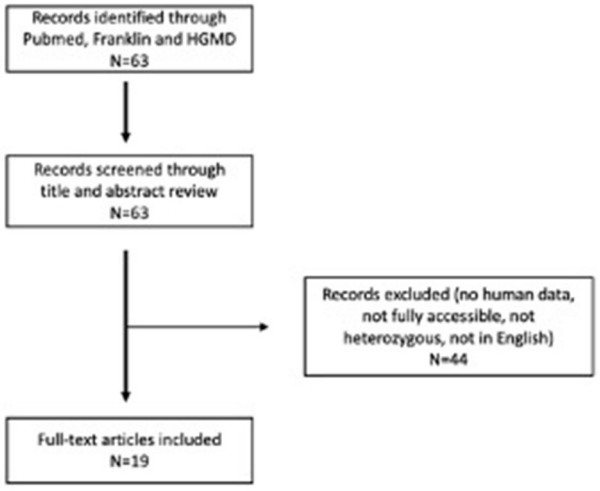

FIGURE 4
PRISMA flow diagram for systematic literature review.

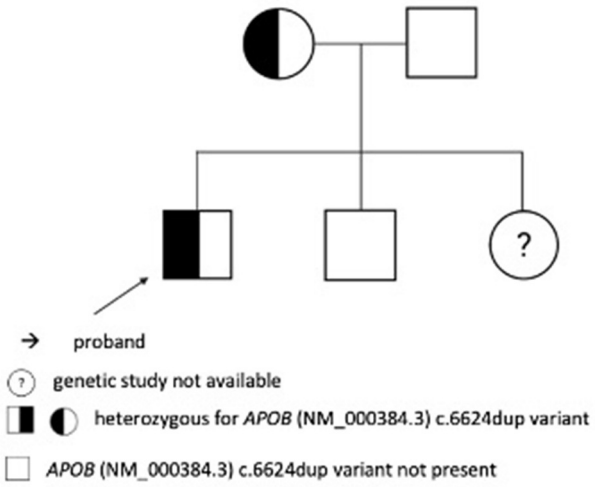

FIGURE 5
A family pedigree with c.6624dup p.Leu2209IlefsTer5 variant in the APOB gene.

Due to their enormous size and hydrophobicity, apoB-100 and apoB-48's 3D structures have not yet been fully characterized at the anatomic level. However, attempts have been made to determine the structure of the apoB-100 domains using different algorithms (20). The modeled apoB-100 protein is composed of five domains NH3-βα1-β1-α2-β2-α3-COOH. The initial assembly of triglyceride-rich lipoproteins in enterocytes and hepatocytes depends on the N-terminal βα1 superdomain (N-terminal amino acids 1–827), which has both α-helical and β-sheet secondary structures. β1 domain, which is located roughly between amino residues 827 and 2000, is involved in irreversible lipid-binding, while the β2 provides LDL receptor-binding abilities (21). The α2 domain, located roughly between amino residues 2,075 and 2,575, and the α3 domain, located roughly between amino residues 4,100 and 4,550, form amphipathic α -helices with both hydrophilic and hydrophobic characteristics. They provide a flexible area that gives the molecule elasticity and enables the recruitment of varying amounts of core lipids (22). ApoB-48 is composed of the domains βα1, β1, and a portion of the domain α2 (20).

The majority of the FHBL-causing variants identified interfere with the development of a complete apoB-100 molecule (19). The size and density of lipoprotein particles depend on the length of apoB truncation. Patients with variants causing truncated proteins have lower plasma levels of apoB possibly because of lower apoB production and higher clearance rate (23). The ability of apoB to create plasma lipoproteins in the liver or intestine and export lipids from these organs is lost when apoB-48 is in an inadequate form (24). The severity of symptoms is related to truncation lengths; long-truncated apoBs continue to have some lipid-binding ability, whereas truncations that are shorter than apoB-29/30 are primarily degraded within cells and are not released as part of lipoprotein particles. Truncations shorter than apoB-29/30 lead to a phenotype similar to ABL, characterized by neurologic dysfunctions, fatty liver, acanthocytosis, a lack of fat-soluble vitamins, and fat malabsorption (1, 25). Carriers with longer truncations are less affected (e.g., longer than apoB-75) as they still have some capacity to bind lipids and form lipoprotein particles that are then released into the bloodstream. However, in the presence of certain factors, such as a high-fat diet, alcohol consumption, or obesity, individuals with longer truncated apoBs may still develop fatty liver. Most people with heterozygous FHBL do not experience any symptoms but may have mild liver dysfunction and hepatic steatosis. However, around 5–10% of them may develop more severe non-alcoholic steatohepatitis that requires medical treatment and, in rare cases, may progress to cirrhosis (26). Other symptoms observed in heterozygous patients include increased stool frequency, chronic steatorrhea, mild vitamin deficiencies, and malabsorption, particularly after consuming high-fat meals (27).

The clinical severity of FHBL in individuals who are homozygous or compound heterozygous for apoB truncations is determined by the ability of truncated apoBs to bind lipids and form lipoprotein particles. FHBL homozygotes or compound heterozygotes carrying apoB truncations, that result in both alleles encoding truncated apoBs shorter than ApoB-29/30, do not have detectable apoB in their bloodstream. For individuals who are homozygous or compound heterozygous for truncations longer than apoB-50, the clinical phenotype can vary widely. In severe cases, it may lead to neurological complications due to the malabsorption of vitamin E (28). The malabsorption of fat can be managed through dietary modifications, such as limiting the consumption of long-chain fatty acids, and supplementing fat-soluble vitamins (1).

In Supplementary Table 2, we conducted a literature review on the variants in the *APOB* gene that have been observed in heterozygous FHBL patients so far. Our review includes 19 articles describing 35 cases. The most common type of variants observed are substitutions in exons (43%). Truncation lengths vary from 6.46–83% of the full apoB-100 length. Among the reported symptoms, fatty liver was described in 57% of cases, and it was also exhibited in our proband.

We report a novel single nucleotide duplication [NM_000384.3:c.6624dup[=]] in exon 26 of the *APOB* gene, which was found in a family with HBL. This variant causes a frameshift in translation p.Leu2209IlefsTer5 protein (NP_000375.3), early termination, and results in a truncated protein. In our case, frameshift causes termination codon TGA at the 2213th amino acid. This represents 48.5% of full apoB length or intermediate size truncation which is longer than the wild-type counterpart. This variant is located in α2 based on protein structure. Previous studies have shown that individuals who are FHBL heterozygotes and carry truncated apoBs have lower-than-expected levels of plasma LDL-C and apoB, which are approximately one-third of normal levels. This is due to a combination of reduced hepatic secretion of apoB-100, increased catabolism of VLDL, and decreased secretion of the truncated apoBs (29). Martín-Morales et al. presented a heterozygous case with the Ser2184fsVal2193X variant that produces a protein apoB-48.32 (a truncated apoB comparable in size to that found in our kindred) (2013). Like our proband, their case also showed symptoms of steatorrhea, fatty liver, and low concentrations of TC, LDL-C, and apoB. In addition, we also discovered the variant in the *COL2A1* gene (NM_001844.5) c.375+1G>A[=], confirming the Stickler syndrome. Stickler syndrome affects connective tissue and can involve multiple

systems in the body, including the inner ear, eyes, joints, and skeleton. The disorder is characterized by a range of symptoms, including myopia (nearsightedness), vitreoretinal degeneration, cataracts, and retinal detachment at a young age (30). These eye manifestations were also observed in the case being discussed. Since some of FHBL and Stickler syndrome symptoms overlap, we cannot solely attribute all the characteristics observed in our proband to the variant discovered in the *APOB* gene.

According to previous studies, patients with FHBL have been shown to have lower levels of lipid-soluble vitamins E, A, K, and D in their cellular membranes. Vitamin A plays a crucial role in phototransduction and vitamin E is important for normal retinal function. Vitamin E transport is hindered in FHBL due to the fact that it heavily relies on transport through chylomicrons and other particles that contain apo B, which are impaired in FHBL (8). Supplementation of vitamin E can prevent the onset of retinopathy, or if it has already developed, it can halt its progression (31). Homozygous FHBL patients may present neurological and ophthalmological abnormalities, which include progressive spinocerebellar degeneration, areflexia, ataxia, and retinitis pigmentosa due to lipid-soluble vitamins deficiency (4). They usually experience neurological symptoms in their first or second decade of life. Early identification of these conditions through an effective screening program is crucial, as proper treatment can prevent adverse neurological and ophthalmological outcomes (2).

The current standard treatment involves dietary restrictions and vitamin supplementation. It is recommended to consider supplementation when low levels of lipid-soluble vitamins are detected. Vitamin E doses should be close to the recommended daily intake (15 mg/day), with tolerable upper intake levels of 1,000 mg/day. In our case, our patient was prescribed up to 2,000 mg/day due to irregular intake. To prevent ophthalmologic complications, high doses of vitamin A (100–400 IU/kg/day) should be given if a deficiency is detected. Additionally, it is recommended to supplement vitamin D (800–1,200 IU/day) in all patients. Due to malabsorption, it is recommended to reduce long-chain fatty acids in the diet. Regular clinical judgment is recommended for all homozygous patients and heterozygous patients with vitamin deficiencies and fatty liver disease. Periodic transaminase tests and abdominal ultrasound can be used to monitor hepatic function. Follow-up evaluations for older patients include ophthalmologic assessments to detect atypical retinitis pigmentosa and tests to measure bone mineral density to check for osteopenia (2). Our patient was managed with vitamin E, vitamin D3, calcium carbonate, lecithin, complex B, and omega-3 fatty acids. Doses were adjusted based on the deficiency.

In conclusion, early detection and treatment of patients with FHBL are of great importance since damaging neurological and ophthalmological effects may be avoided by following up with sufficient vitamin supplementation. Early detection is possible through genetic testing of subjects showing symptoms of malabsorption and a decrease in plasma cholesterol or, ideally, through a screening program for familial hypercholesterolemia (32–35). Our proband had a novel heterozygous *APOB* variant causing significant liver steatosis, malabsorption of fat-soluble vitamins, and osteopenia. Treatment with sufficient vitamins and an adequate diet helped at limiting disease symptoms progression (36).

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the National Medical Ethics Committee (#0120-273/2019/9). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

NM and MB collected the data and prepared the first draft of the manuscript. MZ, DU, and UG followed and treated the patient, performed follow-up tests, and collected the data. MD, SB, US, and JK performed the molecular genetic analysis and data analysis with interpretation. UG supervised the work and helped write the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1106441/full#supplementary-material

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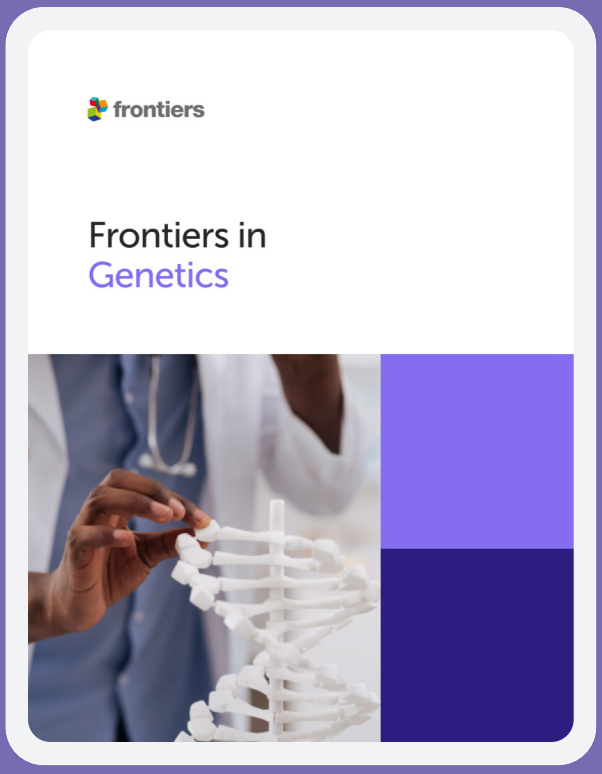

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