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Molecular analysis of three known and one novel *LPL* variants in patients with type I hyperlipoproteinemia

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Missense and frameshift variants;
LPL

Abstract *Background and aims:* Type I hyperlipoproteinemia, also known as familial chylomicronemia syndrome (FCS), is a rare autosomal recessive disorder caused by variants in *LPL*, *APOC2*, *APOA5*, *LMF1* or *GPIHBP1* genes. The aim of this study was to identify novel variants in the *LPL* gene causing lipoprotein lipase deficiency and to understand the molecular mechanisms.
Methods and results: A total of 3 individuals with severe hypertriglyceridemia and recurrent pancreatitis were selected from the Lipid Clinic at Sahlgrenska University Hospital and *LPL* was sequenced. *In vitro* experiments were performed in human embryonic kidney 293T/17 (HEK293T/17) cells transiently transfected with wild type or mutant *LPL* plasmids. Cell lysates and media were used to analyze LPL synthesis and secretion. Media were used to measure LPL activity.

Patient 1 was compound heterozygous for three known variants: c.337T > C (W113R), c.644G > A (G215E) and c.1211T > G (M404R); patient 2 was heterozygous for the known variant c.658A > C (S220R) while patient 3 was homozygous for a novel variant in the exon 5 c.679G > T (V227F). All the *LPL* variants identified were loss-of-function variants and resulted in a substantial reduction in the secretion of LPL protein.
Conclusion: We characterized at the molecular level three known and one novel *LPL* variants causing type I hyperlipoproteinemia showing that all these variants are pathogenic.

Introduction

Type I hyperlipoproteinemia, also known as familial chylomicronemia syndrome (FCS) or familial lipoprotein lipase (LPL) deficiency, is a rare recessive disorder characterized by a severe reduction in the catabolism of triglyceride-rich lipoproteins. This reduction results in a massive accumulation of plasma chylomicrons leading to

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hypertriglyceridemia [1] with triglyceride levels typically >17 mmol/L [2–4]. Clinical signs and symptoms, often appearing in neonatal period/early infancy, include failure to thrive, eruptive xanthomas, hepatomegaly, lipaemia retinalis, severe and recurrent abdominal pains. Of these, acute pancreatitis is the most serious clinical manifestation [5–8]. The prevalence of familial LPL deficiency is estimated to be 1 to 2 per million in the general population [4,9] although it is higher in isolated ethnic groups [10,11]. Loss of function variants in 5 genes (*LPL*, *APOC2*, *APOA5*, *LMF1* and *GPIHBP1*) [12–17] cause type I hyperlipoproteinemia with *LPL* variants accounting for the majority of all reported variants.

The *LPL* gene encodes for a secreted protein of 448 amino acids with a 27-amino-acid signal peptide [18]. LPL is an enzyme with triglyceride lipase activity and it is involved in the partitioning of lipids contained in triglyceride-rich lipoprotein particles [19]. LPL is primarily synthesized in the parenchymal cells of the heart, skeletal muscle and adipose tissues, and then transported to the luminal surface of vascular endothelial cells [18]. After cell secretion the formation of dimers is a key step for the LPL activity [20].

More than 200 pathogenic variants in the *LPL* gene have been identified [21–23]. These affect LPL activity by interfering with secretion, heparin binding or enzymatic activity [23,24].

The clinical management of these patients is very burdensome and consists of a very low fat diet which usually has poor compliance. New drugs are under investigation for these patients.

In the present study, we have identified and characterized one novel variant and characterized three previously reported *LPL* variants in three patients with severe hypertriglyceridemia, consanguinity and recurrent pancreatitis. We showed, by *in vitro* experiments, that all the examined variants are loss of function variants characterized by a prominent reduction in protein secretion.

Methods

Hypertriglyceridemic patients

Three patients from the Lipid Clinic at Sahlgrenska University Hospital in Gothenburg (Sweden) were selected between June 2015 and April 2017. The selection criteria were: diagnosis of severe hypertriglyceridemia (>10 mmol/L for at least three times), recurrent pancreatitis episodes, consanguinity and the absence of secondary risk factors for hypertriglyceridemia such as alcohol abuse, type 2 diabetes mellitus and metabolic syndrome. Each individual gave informed consent to DNA extraction, analysis and publication.

Analysis of the LPL gene

DNA of patients was extracted from EDTA-containing blood or saliva using DNeasy Blood kit (Qiagen, Hilden, Germany) and Puregene Buccal Cell Core kit (Qiagen) respectively, according to the manufacturer's instructions.

DNA sequencing of the exons with flanking intron sequences of the *LPL* gene was performed. The primers and conditions for thermal cycling are available upon request.

The PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) according to the manufacturer's instructions. Version 3.1 of the BigDye terminator cycle-sequencing kit (Applied Biosystems, Foster City, CA) was used for the sequencing reactions according to the manufacturer's instructions. The sequencing products were run on a Genetic Analyzer 3730 (Applied Biosystems) and analyzed using Secscape version 2.5 software (Applied Biosystems). The sequence annotation for the full length LPL protein includes the 27 residue signal peptide.

Site-direct mutagenesis and transient transfection of HEK 293T/17 cells

Human wild type *LPL* cDNA was synthesized and cloned in pcDNA3.1 containing a V5 epitope tag at the C-terminus by GeneArt Gene Synthesis (Thermo Fisher Scientific, Rockford, IL, USA) as previously described [25]. *LPL* variants were generated by site-directed mutagenesis introducing a single base-pair change in the wild type *LPL* gene sequence. To obtain the LPL W113R amino acidic substitution, a single base-pair change from thymine to cytosine at nucleotide 337 was introduced by using the following primers: primer forward TGTGGTGGACCGGCTGTCACG, primer reverse CGTGACAGCCGGTCCACCACA.

To obtain the LPL G215E amino acidic substitution (from guanine to adenine at nucleotide 644) we used the following primers: primer forward TTCACCAGAGAGTCCCCTGGT, primer reverse ACCAGGGGACTCTCTGGTGAA.

For the LPL S220R substitution (from adenine to cytosine at nucleotide 658) the following primers were used: primer forward CCCTGGTCGACGCATTGGAAT, primer reverse ATTCCAATGCGTCGACCAGGG.

To obtain the LPL V227F substitution (from guanine to thymine at nucleotide 679) the following primers were used: primer forward CCAGAAACCATTTGGGCATGT, primer reverse ACATGCCCAAATGGTTTCTGG. All the primers were purchased from Sigma–Aldrich (St. Louis, Missouri). The protocol used for mutagenesis is available upon request.

The presence of the *LPL* variants and the fidelity of each construct were confirmed by DNA sequencing (Eurofins Genomics, Germany).

Human embryonic kidney 293T/17 (HEK 293T/17) cells were purchased from American Tissue Culture Collection (Manassas, VA) and cultured in Dulbecco's Modified Eagle's Medium (high glucose from Lonza) containing 10% Fetal Bovine Serum (FBS), 5% penicillin-streptomycin and 2 mM L-glutamine.

HEK 293T/17 cells were transiently transfected with plasmids containing the human wild type *LPL* cDNA or carrying the other variants (3 μg/mL) using TurboFect transfection reagent (Thermo Fisher Scientific), according to the manufacturer's instructions.

48 h after transfection, cells and media were collected. Cells were lysed using mammalian protein extraction reagent (M-PER, Thermo Fisher Scientific) containing

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complete protease inhibitor cocktail (Sigma–Aldrich). Media were concentrated 10 times by centrifuging using VIVASPIN tubes (Sartorius Stedim Biotech, Göttingen, Germany). Cell lysates and media were used to analyze protein synthesis and secretion by western blot. Media fractions were additionally used to measure LPL activity.

Cycloheximide chase assay

HEK 293T/17 cells were seeded in six well plates and transiently transfected with plasmids containing the human wild type *LPL* cDNA or carrying the other variants (3 μg/mL). Then, cells were treated with cycloheximide (200 μg/mL) for 2, 4, 8, and 12 h. Cells and media were collected and subjected to western blot analysis. The intensity of the western blotting bands was measured by Image Lab Software (Bio-Rad) and expressed as arbitrary unit (AU).

Immunoblotting

HEK 293T/17 lysates and media fractions concentrated 10 times were mixed with Laemmli buffer containing 2-mercaptoethanol and boiled at 95 °C for 5 min. Proteins were size-separated by SDS-PAGE (10% acrylamide gel) and transferred onto nitrocellulose membranes (0.4 A, 1 h). Membranes were incubated for 1 h with primary antibodies, washed 2 times for 10 min with 0.2% tris-buffered saline (TBST) containing 0.2% tween, incubated 1 h with HRP-conjugated secondary antibodies, then washed 3 times for 10 min with 0.2% TBST. Membranes were incubated for 5 min with chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA). Bands were visualized by Chemidoc XRS System (Biorad, Hercules, CA) and quantified using Image Lab Software (Biorad).

The following antibodies were used: mouse anti-V5 (Invitrogen, P/N46-0705), rabbit anti-Calnexin (Sigma–Aldrich, C4731), mouse anti-Albumin (Sigma–Aldrich, A6684).

Lipoprotein lipase activity

LPL activity was measured in media fractions of HEK 293T/17 cells transiently transfected and overexpressing wild type LPL or mutant LPL as previously described [25,26].

Briefly, 50 μL of each concentrated medium fraction was incubated for 40 min at 37 °C with a mixture containing phosphatidylcholine (Sigma–Aldrich), cold triolein, radiolabeled [9,10-^{3}H(N)]-triolein (Perkin Elmer, Waltman, MA), heat-inactivated fetal bovine serum (FBS) and bovine serum albumin. The reaction was blocked and lipids were extracted by the addition of methanol/chloroform/heptane (10:9:7). Samples were centrifuged at 3000 g for 15 min and the upper aqueous phase of each sample was saved. The amount of free [^{3}H]-oleic acid in the upper phase was measured by scintillation counting. Mouse post-heparin plasma was used as positive control.

Results

Clinical features of the patients

A total of three individuals underwent genetic screening for the presence of variants in the *LPL* gene. The clinical characteristics of the study participants are shown in Table 1. Briefly, they were adults (mean age 49) with a mean BMI of 22.8 and a mean fasting triglyceride level of 26 mmol/L.

Genetic screening

The *LPL* gene was successfully sequenced in all the study participants. Patient 1 was compound heterozygous for three different variants: 1) c.337T > C in exon 3, resulting in a tryptophan to arginine substitution at position 113 of the LPL protein (W113R); 2) c.644G > A in exon 5, resulting in a glycine to glutamic acid substitution at position 215 (G215E); 3) c.1211T > G in exon 8, resulting in a methionine to arginine substitution at position 404 (M404R) (Fig. 1A). The first two variants (W113R and G215E) are well known causes of lipoprotein lipase deficiency [23,27]; the third variant (M404R) has already been described by our group [25].

Patient 2 was heterozygous for the nucleotide change c.658A > C in exon 5 (Fig. 1B), resulting in a serine to arginine substitution at position 220 (S220R). This variant has been described by Mailly et al. [1]. No other variants in candidate genes (*APOC2*, *APOA5*, *GPIHBP1* and *LMF1*) were found.

Patient 3 was homozygous for the nucleotide change c.679G > T in exon 5 in the *LPL* gene (Fig. 1C), resulting in a valine to phenylalanine substitution at position 227 (V227F). His brother was homozygous for the same variant.

LPL synthesis and secretion in HEK 293T/17 cells

To examine the effect of the variants in the *LPL* gene at a protein level, the wild type *LPL* cDNA was cloned into

Table 1 Clinical, anthropometric and lipoprotein profile of the individuals screened for *LPL* variants.

Variable	Patient 1	Patient 2	Patient 3
Age (years)	49	69	29
Gender (male/female)	F	M	M
BMI (Kg/m^2)	21.4	27.8	19.2
Alcohol intake (yes/no)	NO	NO	NO
Diabetes (yes/no)	NO	YES[a]	YES[a]
Triglycerides (mmol/L)	39.7 ± 13.6	19.6 ± 9.4	18.7 ± 3.2
Pancreatitis (yes/no)	YES	YES	YES
Number of pancreatitis	>3	>3	>3
Cholesterol (mmol/L)	8.7	8.5	5.1
HDL-cholesterol (mmol/L)	0.4	0.3	0.4
LDL-cholesterol (mmol/L)	0.6	4	<0.1

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

[a] Patient 2 and 3 had diabetes mellitus due to a reduction in the insulin secretion due to multiple pancreatitis. Triglycerides are shown as mean ± SD of 3 measurements.

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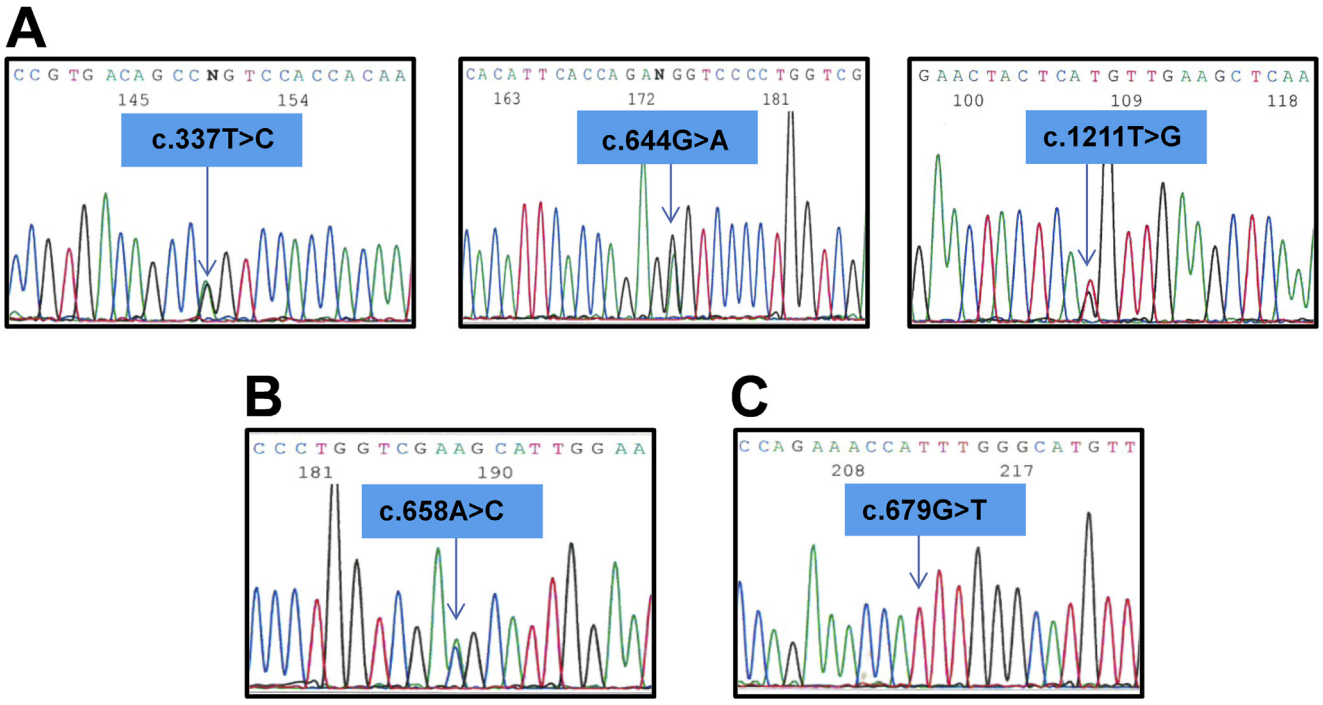

Figure 1 ***LPL*** **gene sequencing of three individuals affected by hypertriglyceridemia.** (A) DNA sequence of patient 1 shows mutated nucleotides c.337 T > C (right panel), c.644 G > A (central panel), c.1211 T > G (left panel). (B) DNA sequence of patient 2 shows mutated nucleotide c.658 A > C. (C) DNA sequence of patient 3 and 4 (brothers) shows mutated nucleotide c.679 G > T. Nucleotide changes are indicated by the arrow.

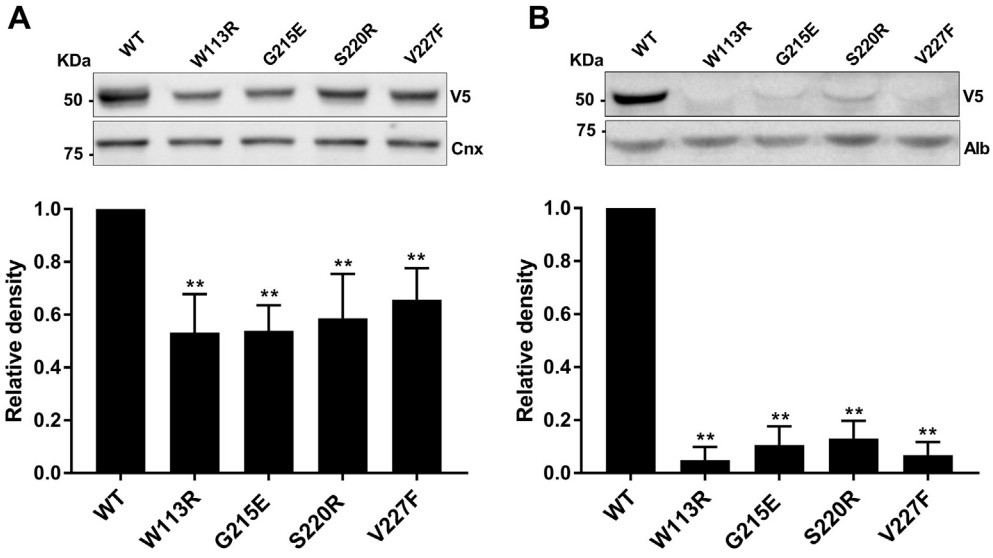

Figure 2 HEK 293T/17 cells transiently transfected show a reduction of the production and secretion of LPL variants. (A) Western blotting analysis of cell lysates (N = 6) shows a reduction in the production of LPL-V5 tagged protein of almost 50% in W113R and G215E LPL variants and of 35–40% in the S220R or V227F variants; calnexin was used as loading control. (B) Western blotting analysis of cell media (N = 5) shows a strong reduction in the secretion of LPL-V5 tagged protein of each *LPL* variant; albumin was used as loading control. WT: wild type; Cnx: calnexin; Alb: albumin. $^{**}P \leq 0.01$.

pcDNA3.1 expression vector and then all the mutants were obtained by mutagenesis in situ. A V5 tag at the C-terminus of each construct was added to ensure specificity. HEK 293T/17 cells were transiently transfected with the individual constructs and, after 48 h, cells were harvested and media were collected and concentrated 10 times.

HEK 293T/17 cells transfected with the mutant plasmids produced less LPL protein than cells transfected with the wild type *LPL* plasmid (Fig. 2A). Specifically, cells transfected with the W113R and G215E variants showed a reduction in protein synthesis of approximately 50%, whilst the protein synthesis was reduced by 35–40% in cells transfected with the S220R or V227F variant (Fig. 2A).

Furthermore, the amounts of secreted LPL in media of cells transfected with the mutant plasmids were 80% reduced compared to cells transfected with the wild type

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LPL plasmid (Fig. 2B). These data show that variants in the *LPL* gene reduce the production and secretion of LPL protein.

Next, HEK 293T/17 cells expressing the human wild type LPL or the other mutants were treated with cycloheximide to block protein synthesis (Fig. 3A). The degradation rate was not different between LPL wild type and the other mutants (Fig. 3B), suggesting that the variants do not affect protein degradation.

LPL enzymatic assay

We tested LPL activity in media of HEK 293T/17 transiently transfected with wild type *LPL* or the other four mutant plasmids. LPL activity was absent in media of cells overexpressing all the *LPL* variants while it was present in mouse post-heparin plasma and in the concentrated medium fraction of the cells overexpressing the wild type *LPL* (Fig. 4). All variants reduced the ability of LPL protein to hydrolyze triglycerides *in vivo* as shown by the presence of hypertriglyceridemia in the patients (Table 1), and with the markedly reduced activity against radiolabeled triglycerides in the LPL enzymatic assay.

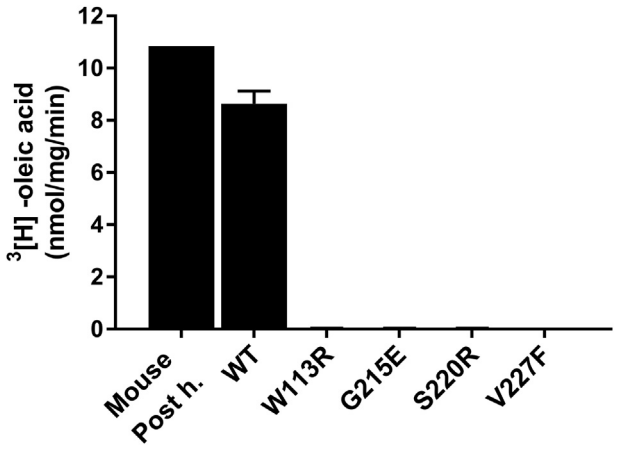

Figure 4 Lipoprotein lipase assay of cell media fractions. Lipoprotein lipase activity in cell medium of HEK 293T/17 transiently transfected with *LPL* wild type and variants. Mouse post-heparin plasma was used as positive control. Post-h: post-heparin; WT: wild type.

Discussion

In this study, we characterized at a molecular level three already known variants and identified and characterized one novel pathogenic variant in the *LPL* gene.

Type I hyperlipoproteinemia is a rare inborn error of metabolism caused most frequently by loss of function variants in the *LPL* gene [28]. Type I hyperlipoproteinemia prevalence is estimated to 1 in a million in the general population. Gothenburg lipid clinic provides tertiary care for the 1.6 million inhabitants of Västra Götaland [29]. We described four patients (including the brother of the third patient) in addition to the previously described three patients carrying pathogenic variants in the *LPL* gene, for a total of seven patients. Based on a frequency of 1 in a million we have a 4 fold enrichment of variants resulting in a prevalence of approximately 1 in 400,000. The enrichment of variants may be explained by the recent migratory flux of individuals from the Middle East. Indeed, 5 of our 7 patients were born or were descendant from this geographical area. Interestingly, all these 5 probands were from consanguineous families. Since the early 1970s, Sweden has had a large migratory flux from Middle East [30] and consanguineous marriages are relatively common in this geographical area [31], this could explain the excess of rare recessive disorders we observed.

Patient 1 was compound heterozygous for three pathogenic variants namely W113R, G215E and M404R. In particular, the W113R has been previously identified in a 1-year-old child [32] from England, in a 19-year-old female from Seattle [33] and in a 5-year-old male from Croatia [23]. The overexpression of this variant resulted in a total loss of LPL enzyme activity [32] *in vitro*. The G215E variant has been previously associated to lipoprotein lipase deficiency in several individuals of different ancestries [27]. The M404R variant, identified by our group [25], resulted

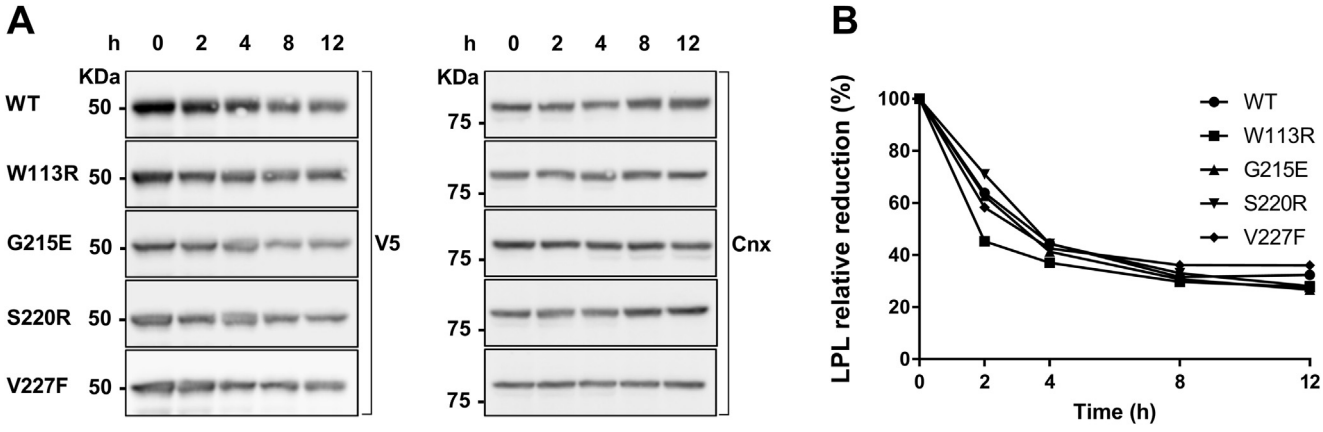

Figure 3 Degradation of LPL wild type and variant proteins after blocking protein synthesis with cycloheximide. (A) Western blotting analysis of cells transiently transfected with LPL wild type or mutant proteins and subsequently treated with cycloheximide (200 μg/mL) for the indicated time points. (B) Densitometry of western blotting bands (mean of three independent experiments) using Image Lab Software (Bio-Rad).

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in a marked reduction in protein secretion and a complete loss of function.

Patient 2 was heterozygous for the S220R variant. This variant has previously been reported in a Swedish patient [1]. No other variants in candidate genes (*APOC2*, *APOA5*, *GPIHBP1* and *LMF1*) were found. This patient was heterozygous for the *LPL* variant and he had a phenotype compatible with homozygosity. Indeed, his triglyceride levels were higher than 10 mmol/L on multiple occasions; he did not respond to fibrates and had several pancreatitis from age 18. However, because of the absence of a formal post-heparin plasma assay, a lipoprotein lipase deficiency diagnose cannot be made. The discrepancy between genotype and phenotype could be due to a single or combination of multiple factors: a) the variant interferes with LPL dimerization resulting in a dominant negative effect; b) the presence of diabetes mellitus and overweight; c) the presence of rare gain of function variants in genes involved in circulating triglycerides metabolism (i.e.: *APOC3*, *ANGPTL3*, *ANGPTL4*) [34]; d) the presence of several variants with a small effect size in increasing circulating triglycerides.

The prediction program SIFT predicts variant S220R to be probably damaging and not tolerated.

In patient 3 (and his brother) we identified a novel homozygous variant (V227F). *In silico* analysis, using the prediction programs SIFT predicts that the variant is pathogenic. Interestingly, another variant in the same codon (V227A) is a pathogenic variant of type I hyperlipoproteinemia [35].

To elucidate the molecular mechanism underlying the genetic results, we performed *in vitro* studies of the W113R, G215E, S220R and V227F variants. Cells overexpressing the W113R or G215E mutant proteins showed a reduction of almost 50%, whilst the cells transfected with the S220R or V227F variants showed a reduction in protein synthesis by 35–40%. Furthermore, the LPL protein secretion in medium was reduced by at least by 80% compared to cells transfected with wild type LPL. No differences were found in the intracellular degradation rate between wild type LPL and the mutant proteins suggesting that the variants affect protein synthesis. Consistently, no LPL activity was detected in the same medium fractions from the cells transfected respectively with all the tested variants. Taken together, these data demonstrate that these are *LPL* loss of function variants and therefore pathogenic.

In conclusion, we have characterized at a molecular level three known and one novel *LPL* variants causing lipoprotein lipase deficiency, showing that all these variants are pathogenic.

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Author contributions

All authors contributed to the manuscript preparation and interpretation of data. SR performed clinical diagnosis. SR and PP designed the study. AC and PP performed experiments. TPL performed genetic analysis.

Conflicts of interest

SR has been consulting for Chiesi Farmaceutici Group, Amgen, Sanofi, Novonordisk, Akcea therapeutics, Genzyme and AstraZeneca in the last 5 years.

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