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Deciphering the role of V200A and N291S mutations leading to LPL deficiency

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HIGHLIGHTS

- Two compound heterozygote patients for *LPL* mutations have a severe phenotype.
- Both patients have very low levels of circulating LPL and it is not active.
- LPL V200A, *in vitro*, is markedly less secreted and not enzymatically active.
- LPL N291S, *in vitro*, is secreted but not enzymatically active.
- V200A and N291S mutations do not affect LPL dimerization.

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ABSTRACT

Background and aims: Type I hyperlipoproteinemia is an autosomal recessive disorder of lipoprotein metabolism caused by mutations in the *LPL* gene, with an estimated prevalence in the general population of 1 in a million. In this work, we studied the molecular mechanism of two known mutations in the *LPL* gene in *ex vivo* and *in vitro* experiments and also the effect of two splice site mutations in *ex vivo* experiments.
Methods: Two patients with hypertriglyceridemia were selected from the Lipid Clinic in Vienna. The first patient was compound heterozygote for c.680T > C (exon 5; p.V200A) and c.1139 + 1G > A (intron 7 splice site). The second patient was compound heterozygote for c.953A > G (exon 6; p.N291S) and c.1019-3C > A (intron 6 splice site).

The *LPL* gene was sequenced and post-heparin plasma samples (*ex vivo*) were used to test LPL activity. *In vitro* experiments were performed in HEK 293T/17 cells transiently transfected with wild type or mutant *LPL* plasmids. Cell lysate and media were used to evaluate LPL production, secretion, activity and dimerization by Western blot analysis and LPL enzymatic assay, respectively.
Results: Our data show that in both patients, LPL activity is absent. V200A is a mutation that alters LPL secretion and activity whereas the N291S mutation affects LPL activity, but both mutations do not affect dimerization. The effect of these mutations in patients is more severe since they have splice site mutations on the other allele.
Conclusions: We characterized these *LPL* mutations at the molecular level showing that are pathogenic.

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1. Introduction

Lipoprotein lipase (LPL) is a key enzyme in triglycerides (TGs) catabolism, whose role is to catalyze the hydrolysis of TGs in TG-rich lipoproteins such as chylomicrons and VLDL [1]. LPL belongs to the mammalian lipase family that includes hepatic lipase, gastric lipase, pancreatic lipase and endothelial lipase [2]. Human *LPL* gene is located on chromosome 8p22, spans 30 kb and is divided into 10 exons [3] encoding for a secreted protein of 448 amino acids and a 27-amino-acid signal peptide. LPL is primarily synthesized in adipocytes, macrophages, muscle cells where the dimer formation is the key step for LPL activity. It is transported to the luminal surface of vascular endothelial cells [4]. Indeed, the enzymatically active dimers of LPL hydrolyze triglycerides of chylomicrons and VLDL, releasing free fatty acids [5]. Intravenous administration of heparin displaces LPL into circulation, allowing the study of genotype/phenotype relation by measurement of LPL activity and mass in post-heparin plasma [6]. More than 200 naturally occurring mutation in the *LPL* gene have been identified [7,8]. They are located in the promoter region, the coding exons, mainly exon 5 and 6 [9], or splice junction sites and affect LPL activity by interfering with secretion, heparin binding or enzymatic activity [10]. Among these, the loss of function mutations leads to the onset of type I hyperlipoproteinemia, also known as familial lipoprotein lipase deficiency or familial chylomicronemia syndrome (FCS), characterized by impaired TGs hydrolysis that causes accumulation of chylomicrons and extremely high levels of triglycerides in the plasma (880 mg/dL or 10 mmol/L) [11]. Main symptoms of type I hyperlipoproteinemia include severe abdominal pain such as repetitive colicky pain, hepatosplenomegaly, the hyperviscosity syndrome, eruptive xanthomas and lipaemia retinalis. However, the most debilitating symptom is the recurrence of severe potentially life-threatening acute pancreatitis attacks (PAs) occurring in 30% of patients [12].

Since TG-lowering drugs, such as niacin and fibrates, are not effective in patients with type I hyperlipoproteinemia, the main therapeutic approach consists of diet treatment to reduce TGs levels, which usually has poor compliance [13,14]. Acute plasmapheresis may help during an episode of acute pancreatitis, but there is a lack of proven evidence through randomized controlled trials [15–17]. Furthermore, for the treatment of adults with genetically confirmed type I hyperlipoproteinemia and with an LPL protein mass > 5% of normal, who suffer from severe/multiple pancreatitis attacks, the first gene therapy treatment has been approved by the European Medicines Agency [18]. Specifically, Alipogene tiparvovec (Glybera®) gene therapy is an adeno-associated virus (AAV) serotype 1 (AAV-1) based that results in sustained expression of the naturally occurring, gain-of-function S447X variant of the human *LPL* gene and subsequent synthesis and secretion of active LPL by the muscle cells [17,19]. Of note, the Glybera (alipogene tiparvovec) gene therapy, already approved in Europe, is being withdrawn not due to risk-benefit but relative to cost-effectiveness, *i.e.* an average of $1 million per treatment [20].

The present study was aimed to fully characterize two known missense mutations (p.V200A and p.N291S) in the *LPL* gene by *ex vivo* and *in vitro* experiments and tested the additional effect of two splice site mutations by *ex vivo* experiments. The splice site mutation c.1019-3C > A (intron 6) has been previously described [21], while the c.1139 + 1G > A (intron 7) has been described for the first time in this work. We found that the mutations are pathogenic.

2. Materials and methods

2.1. Subjects

Between April 2016 and April 2017, two patients from the Lipid Clinic in Vienna (Austria) with excessive primary hypertriglyceridemia with no other apparent cause were analyzed in more details during clinical workup since they were not carriers of well characterized *LPL* mutations. Routine clinical determination of serum lipid levels was performed by enzymatic methods (Roche reagents, run on a Cobas C111 analyzer) assessed as previously described [22]. Chylomicrons were analyzed by floating in microliter tubes for 10 min at 16.000 g (maximal) at 4°C prior to triglyceride determinations. Chylomicron triglyceride concentrations were determined by subtracting the cleared infranatant from original serum.

2.2. DNA sequencing

For massive-parallel sequencing, genes linked to hyperlipidemias were enriched from isolated genomic DNA using a customized gene panel (Nextera Rapid Capture Custom Enrichment; Illumina, San Diego, CA) and sequenced on a MiSeq sequencing platform (Illumina, USA) using 2 × 150-bp paired-end chemistry in accordance with manufacturer instructions. Sequenced reads were aligned to the GRCh37/hg19 human reference. Data analysis including copy number variation (CNV) analysis was performed with the SeqNext® software (JSI Medical Systems GmbH, Germany). The region of interest was the coding region ± 30 bp intronic flanking regions of the *LPL* gene (NM_000237.2). The classification of detected variants followed the consensus recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology [23]. In addition to *LPL*, also *APOC2*, *APOA5*, *LMF1* and *GPIHBP1* genes have been sequenced, without finding any other mutation.

2.3. Post-heparin plasma samples collection

Post-heparin plasma was obtained as previously described [14]. Briefly, pre-heparin blood sample was drawn at a fasting state followed by an intravenous bolus injection of heparin (60 IU/kg body weight) and collection of post-heparin blood 10 min later from the contralateral arm. Blood samples were centrifuged at 3000 rpm at 4 °C for 10 min to obtain plasma samples, and supernatants stored at −80 °C until analyzed.

2.4. Site-direct mutagenesis, cloning, and transfection

Wild type *LPL* cDNA was synthesized and cloned in pcDNA3.1 vector with a V5 epitope tag at the C-terminus by GeneArt Gene Synthesis (Thermo Fisher Scientific, Rockford, IL) as previously described [14].

To obtain the *LPL* V200A mutation, a single base-pair change from valine to alanine at nucleotide 200 was introduced using *in situ* mutagenesis technique (primer forward: CAG AAA CCA GCT GGG CAT GTT and primer reverse: AAC ATG CCC AGC TGG TTT CTG). To obtain the LPL N291S substitution (from asparagine to serine at nucleotide 291) the following primers were used: primer forward GCT ATG AGA TCA CTA AAG TCA GAG C, primer reverse GCT CTG ACT TTA CTG ATC TCA TAG G. A detailed protocol for mutagenesis is available on request. All the primers were purchased from Sigma Aldrich (St. Louis, MO) and the presence of the *LPL* mutation and fidelity of each construct were confirmed by DNA sequencing (Eurofins Genomic, Germany).

Human embryonic kidney 293T/17 (HEK 293T/17) cells from American Tissue Culture Collection (Manassas, VA) were cultured in high glucose Dulbecco's Modified Eagle's Medium containing 10% Fetal Bovine Serum (FBS), 5% penicillin-streptomycin and 2 mM L-glutamine and transfected with plasmids containing the human wild type *LPL* cDNA or carrying one of the other variants (3 mg/mL) as previously described [13].

After 48 h cells were lysed using mammalian protein extraction reagent (M-PER, ThermoFisher Scientific) containing complete protease inhibitor cocktail (Sigma Aldrich) and media were concentrated 10 times by centrifuging using VIVASPIN tubes (Sartorius Stedim Biotech, Göttingen, Germany). HEK 293T/17 lysates and media were used to analyze protein synthesis and secretion by Western blot; media

fractions were additionally used to measure LPL activity.

2.5. Immunoblotting

Post-heparin plasma samples were diluted 1:50 in 0.5-M Tris-HCl (pH 7). The diluted plasma samples, cell lysates, and concentrated media samples were mixed with Laemmli buffer 5X (SDS 10%, Tris HCl 62.5 mM pH 6.8, glycerol 50%, bromophenol blue 0.01% and β-mercaptoethanol 25%) and boiled for 5 min at 95 °C. Proteins were size-separated by SDS-PAGE (10% acrylamide gel, SDS 0.1%, 100 V, 90 min, using running buffer containing 0.1% SDS) and transferred onto a nitrocellulose membrane (400 mA, 60 min). Membranes were incubated for 1 h with primary antibodies, washed 2 times for 10 min with 0.2% tris-buffered saline containing 0.2% tween (TBST), incubated 1 h with HRP-conjugated secondary antibodies, then washed 3 times for 10 min with 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-LPL (Sigma-Aldrich) (1:1000), mouse anti-APOA1 (AbD Serotec, Oxford, UK) (1:500), mouse anti-V5 (Invitrogen) (1:5000), rabbit anti-Calnexin (Sigma-Aldrich) (1:2000), mouse anti-Albumin (Sigma-Aldrich) (1:1000).

2.6. Lipoprotein lipase enzymatic activity

LPL enzymatic activity, in pre-heparin and post-heparin human plasma samples and in media fractions of HEK293T/17 cells transfected with *LPL* wild type plasmid and the two plasmids containing *LPL* mutants, was measured as previously described [13,14]. Briefly, 7.5 μL of each plasma sample was mixed with 42.5 μL of 0.2-M Tris HCl buffer (pH 8.0). The diluted plasma samples and 50 μL of each concentrated medium sample were incubated for 15 min at 37 °C with a reaction mix containing radiolabeled substrate [9,10-^{3}H(N)]-triolein (Perkin Elmer, Waltman, MA), cold triolein and phosphatidylcholine (Sigma-Aldrich, St Louis, MO), heat-inactivated FBS and bovine serum albumin (Sigma-Aldrich, St Louis, MO) in presence or absence of 1 M NaCl. The reaction was stopped and lipids were extracted using a solution of heptane/chloroform/methanol (1:1.25:1.41). Samples were then centrifuged at 3000 g for 15 min and the amount of [^{3}H]-oleic acid released was analyzed by scintillation counting in the upper phase. A mouse (C57BL/6) post-heparin plasma was used as a positive control, whereas 1 M NaCl was used for LPL specificity.

2.7. Analysis of LPL dimerization

Equal volumes of post-heparin plasma samples diluted 1:50 and conditioned media were mixed with non-denaturing loading buffer 5X (Tris HCl 62.5 mM pH 6.8, glycerol 50%, bromophenol blue 0.01%, β-mercaptoethanol 5% and Coomassie blue G250 0.05%). Samples were not boiled. Proteins were separated by non-denaturing PAGE (4–15% gradient acrylamide gel without SDS, 100 V, 90 min), and transferred onto a nitrocellulose membrane (400 mA, 60 min). Then, western blotting was performed as described into immunoblotting section.

2.8. Homology modeling of LPL

The homology model of human LPL was created with the protein fold recognition server Swiss-Model. The 3D structure of the human pancreatic lipase-related protein 1 was selected as template. Calculated models were visualized by Swiss PDB Viewer 4.1.0. LPL model was mutated on the computer at position 200 by substituting valine (V), a non-polar aliphatic residue, for alanine (A), another non-polar aliphatic residue. The same procedure was applied to N291S mutation, substituting asparagine (N), an uncharged polar residue, at position 291 for serine (S), another uncharged polar residue.

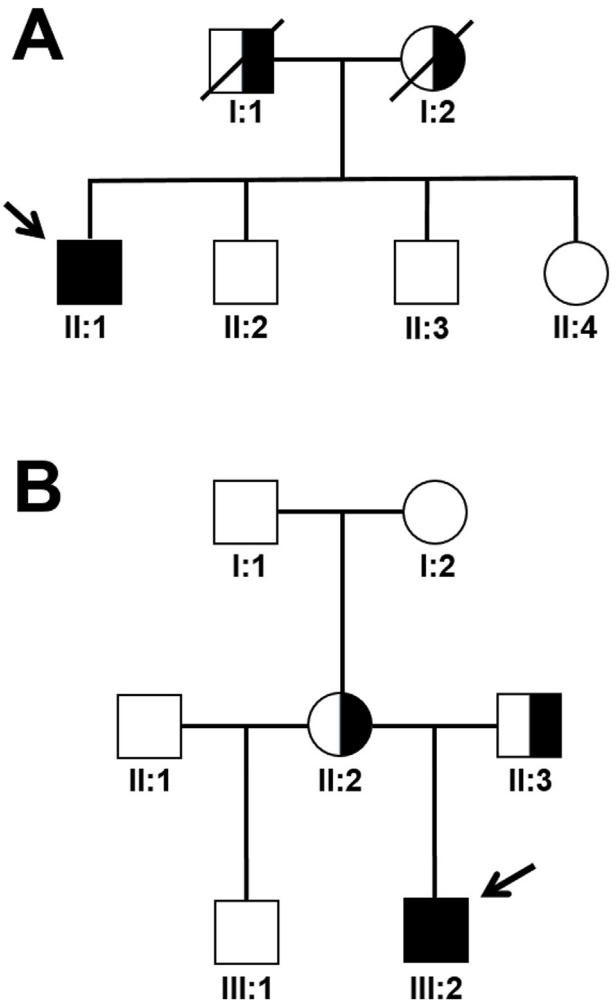

Fig. 1. Pedigree of families.
(A) Family tree of patient 1 (II:1) carrying two heterozygous mutations: c.680T > C (p.V200A) and c.1139 + 1G > A (intron 7 splice site). (B) Family tree of patient 2 (III:2) carrying two heterozygous mutations: c.953A > G (p.N291S) and c.1019-3C > A (intron 6 splice site). Only patient 1 and patient 2 have been genotyped in both families; open symbols indicate no known hyperlipidemia. The half-filled symbols are only by inference (but do not include the possibility of a *de novo* mutation). Squares and circles represent male and female family members, respectively, and filled symbols plus arrows denote affected individuals with LPL deficiency.

3. Results

3.1. Genetic screening

A 37-year-old normal-weight male patient (patient 1) of Indian origin and non-consanguineous parents, whose father died for myocardial infarction, was admitted to our metabolic clinic due to excessive hypertriglyceridemia (see family tree in Fig. 1A). The history revealed manifold bouts of pancreatitis and triglyceride levels > 2000 mg/dL, the last verified seven months ago. Alcohol consumption was denied, xanthomas were not present at the time of investigation. Serum triglyceride levels remained above 900 mg/dL under low-fat and carbohydrate-modified diet and the prescribed lipid lowering medication (atorvastatin 40 mg qd, fenofibrate 200 mg qd), but were reduced down to 250 mg/dL during pancreatitis attacks following prolonged fasting. Comedication was limited to calcium and vitamin D 1200 IU qd Lipoprotein ultracentrifugation revealed type I hyperlipoproteinemia with triglycerides of 1162 mg/dL, 635 mg/dL from which in chylomicrons, and cholesterol 223 mg/dL. Testing for *LPL* mutations revealed two

heterozygous mutations c.680T > C (p.V200A) and c.1139+1G > A (intron 7 splice site).

Patient 2 is a 40-year-old slightly overweight (BMI 27 kg/m^2) male of Caucasian origin and non-consanguineous parents (see family tree in Fig. 1B). He had TG-levels of approximately 3500 mg/dL, which were first discovered at 18 years of age and decreased to < 500 mg/dL under strict diet. He did not have a history of acute pancreatitis. His medication comprised bezafibrate 400 mg qd and vitamin D 2000 IU qd. Genetic analysis of the *LPL* gene revealed two following heterozygous mutations: c.953A > G (p.N291S) and c.1019-3C > A (intron 6 splice site). In addition to *LPL*, in both patients, *APOC2*, *APOA5*, *LMF1* and *GPIHBP1* have been sequenced without identifying pathogenic mutations.

3.2. Circulating LPL levels and LPL activity in post-heparin plasma

Post-heparin plasma samples were collected from two patients, both compound heterozygous for mutations in *LPL* gene, and used to analyze circulating LPL levels and to measure LPL activity. Circulating LPL levels were markedly low. In particular, in both post-heparin plasma samples the LPL protein was detected as a very faint band compared to the mouse post-heparin sample, which was used as positive control (Fig. 2A). Post-heparin plasma samples were used also to test the enzymatic activity of LPL. In both blood samples, no release of [^{3}H]-oleic acid from triolein was detected, indicating that there was no LPL activity (Fig. 2B).

3.3. LPL synthesis, secretion and activity in HEK293T/17 cells

To understand the molecular mechanism of these two mutations in the *LPL* gene, wild type *LPL* cDNA was cloned into pcDNA3.1 expression vector and the two mutants were obtained by *in situ* mutagenesis. To ensure the specificity for LPL, at the C-terminus of the protein a V5 tag was added. LPL V200A and N291S constructs were used to transiently transfect HEK293T/17 cells which were harvested after 48 h; media were collected and concentrated 10 times as previously described [14]. HEK293T/17 cells transfected with both mutant plasmids showed a very slight decrease in protein production (about 10%), compared to cells transfected with wild type *LPL* plasmid. However, no differences were found in protein synthesis (Fig. 3A).

As a further step, the secretion of LPL in the medium fractions was measured. In HEK293T/17 cells transfected with LPL V200A, LPL levels in the medium were significantly reduced by about 80% ($p=0.008$); conversely, cells transfected with LPL N291S did not show any fall in protein secretion (Fig. 3B). As a further step, LPL activity was evaluated in concentrated media of cells transfected with wild type or mutant plasmids were used. No LPL activity was detected in the media of cells transfected with LPL V200A, while a strong reduction was found with LPL N291S compared to LPL wild type (Fig. 4). Collectively, these data show that the V200A substitution does not affect protein synthesis but induces a strong reduction in protein secretion and enzymatic activity. On the other hand, N291S substitution strongly affects the proper functionality of LPL enzyme, despite it does not affect protein synthesis and secretion.

3.4. Analysis of LPL dimerization

To elucidate whether V200A and N291S mutations suppressed the dimerization of LPL, post-heparin plasma and conditioned media were analyzed by a non-denaturing gel Western blot. The results revealed that both mutations exerted no influence on the LPL dimerization (Fig. 5).

3.5. 3D structure of human LPL

The final model of the LPL molecule, in ribbon format, is illustrated in Fig. 6 by Swiss PDB Viewer. LPL is organized into two distinct structural domains the amino terminal (residues 1–312) and the carboxy terminal (residues 313–448). The lid structure (residues 216–239) covering the catalytic site is located in the N-domain. Since residue 200 and 291 are located in the coil region of loops, no changes were observed in the backbone of the molecules compared with the wild type LPL when the models were superimposed.

4. Discussion

Type I hyperlipoproteinemia is an autosomal recessive disorder of lipoprotein metabolism caused by mutations in the *LPL* gene with an estimated prevalence in general population of 1 in a million [24–26].

In this work, we studied the molecular mechanism of two known mutations in *LPL* gene by *ex vivo* and *in vitro* experiments and also the additional effect of two splice site mutations by *ex vivo* experiments. We described two patients carrying pathogenic mutations in the *LPL* gene: patient 1 compound heterozygote for c.680T > C (exon 5; p.V200A) and c.1139+1G > A (intron 7 splice site); patient 2 compound heterozygote for c.953A > G (exon 6; p.N291S) and c.1019-3C > A (intron 6 splice site). Furthermore, in both patients, no pathogenic mutations in other genes such as *APOC2*, *APOA5*, *LMF1* and *GPIHBP1* have been identified.

The V200A substitution has been previously identified in a cohort of Japanese patients with familial hyperlipidemia [27,28]. According to the recent pathogenic classification of *LPL* gene variants associated with LPL deficiency, the mutation c.680T > C (exon 5; p.V200A) has been classified as a variant of unconfirmed pathogenicity being this latter highly probable [1]. The frequency of the minor allele (C) in the overall population is 0.003% (gnomAD browser, Broad Institute). Conversely, concerning the intron 7 splice site mutation, no information regarding pathogenicity has been found. To our knowledge, this is the first time that this splice site mutation is described. Its frequency in the population is lower than 0.001% according to gnomAD browser of Broad

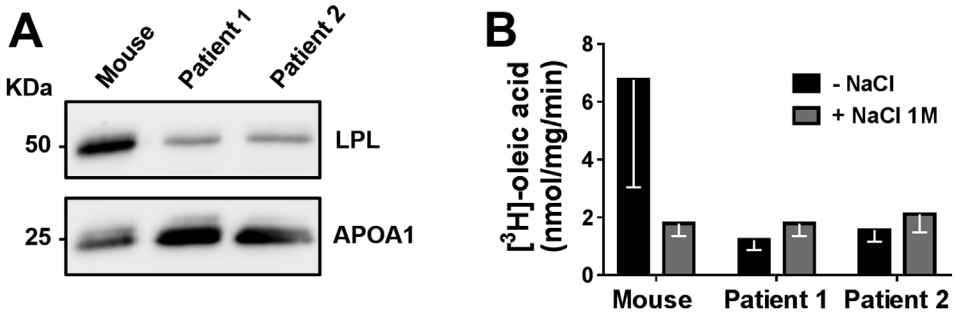

Fig. 2. Circulating LPL levels and LPL activity in post-heparin plasma from patient 1 and 2.
(A) Western blotting showing circulating levels of LPL in post-heparin plasma samples from patient 1 compound heterozygote for c.680T > C (p.V200A) and c.1139+1G > A (intron 7 splice site), patient 2 compound heterozygote for c.953A > G (p.N291S) and c.1019-3C > A (intron 6 splice site). Mouse post-heparin plasma was used as a positive control (mouse and human LPL amino acidic sequences show 90% homology). Anti-human APOA1 antibody was used as a loading control. Post-h, post-heparin; kDa, kilodalton; LPL, lipoprotein lipase; APOA1, apolipoprotein A1. (B) LPL induced release of radiolabeled oleic acid from triolein was measured in post-heparin plasma of patient 1 and 2. Mouse post-heparin plasma was used as a positive control. Sodium chloride 1 M (NaCl) was used to inhibit specifically LPL activity.

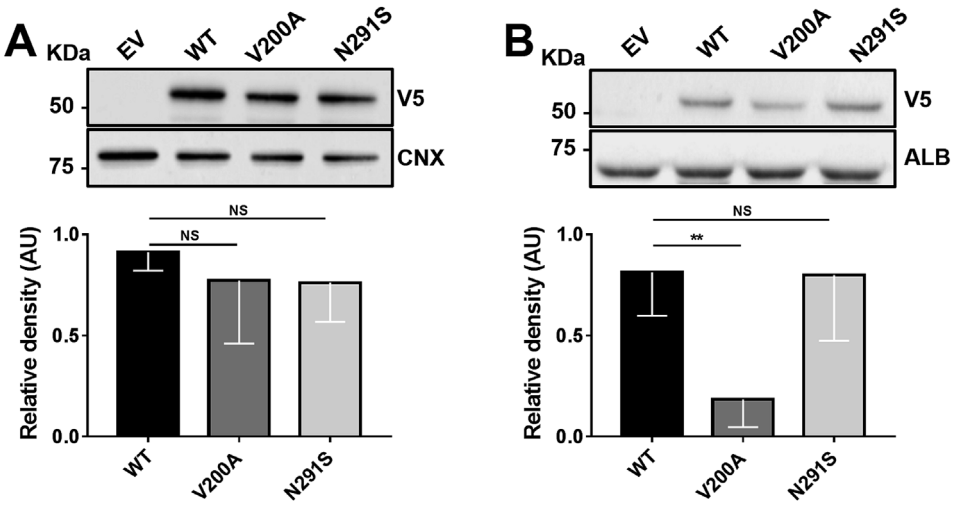

Fig. 3. HEK 293T/17 cells transiently transfected with wild type or mutants LPL (V200A and N291S).
(A) Western blotting analysis of cell lysates (N = 5) shows no reduction in the production of LPL-V5 tagged mutant forms compared to the wild type form (mean ± SD); calnexin was used as loading control. (B) Western blotting analysis of cell media (N = 8) shows a strong reduction in the secretion of LPL-V5 tagged V200A form, but not in the secretion of N291S LPL form (mean ± SD); albumin was used as loading control. EV: empty vector; WT: wild type; CNX: calnexin; ALB: albumin. *p* value was calculated by Mann Whitney test, ($p = 0.008$).

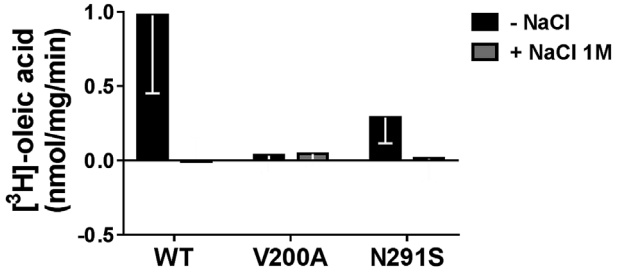

Fig. 4. LPL activity is absent in the media of HEK 293T/17 cells transfected with V200A and N291S LPL.
LPL induced release of radiolabeled oleic acid from triolein was measured in cell media of HEK 293T/17 cells transiently transfected with wild type, V200A or N291S LPL. Wild-type (WT) LPL medium was used as a positive control. Sodium chloride 1 M (NaCl) was used to inhibit specifically LPL activity.

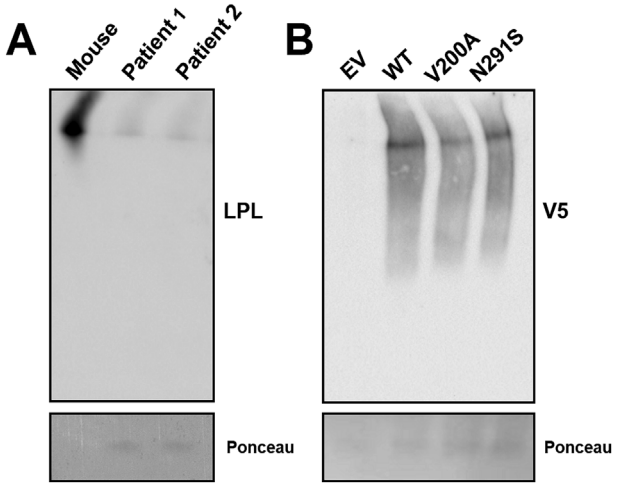

Fig. 5. A non-denaturing Western blot analysis of LPL dimerization in post-heparin plasma and HEK293 T/17 conditioned media.
(A) Non-denaturing Western blotting showing circulating levels of LPL in post-heparin plasma samples from patient 1 compound heterozygote for c.680T > C (p.V200A) and c.1139+1G > A (intron 7 splice site), patient 2 compound heterozygote for c.953A > G (p.N291S) and c.1019-3C > A (intron 6 splice site). Mouse post-heparin plasma was used as a positive control. (B) Non-denaturing Western blotting analysis of cell media shows no difference in dimerization of LPL-V5 tagged mutant forms compared to the wild type form. EV: empty vector; WT: wild type. Ponceau was used as loading control.

Institute. Hence, we describe a novel pathogenic *LPL* splice site mutation leading to virtual absence of LPL activity and protein.

Biochemical analysis of LPL activity was performed in post-heparin plasma (*ex vivo*) revealing absence of LPL activity in the sample. Immunoblot analysis showed only a faint LPL band despite normal presence of control loading protein (APOA1). Moreover, results in HEK293T/17 showed a marked reduction in LPL secretion although the dimerization analysis revealed that there was no difference compared to the wild-type.

The c.953A > G mutation (exon 6; p.N291S), found in patient 2 is considered as “pathogenicity is doubtful”, whereas the second mutation of the same patient c.1019-3C > A (intron 6 splice site) is considered as “pathogenic” [1]. The c.953A > G mutation (p.N291S) has been identified and described by several authors [29–33].

Reymer et al. found that the N291S mutation has a high frequency in a cohort of patients with coronary artery disease and it is associated with high levels of TGs and low levels of HDL cholesterol [29]. However, the frequency of the minor allele (G) in the overall population is 1.3%. In particular, Reymer and collaborators showed that, in approximately 1 in 20 males with proven atherosclerosis, the current variant is associated with significantly reduced HDL cholesterol levels ($p = 0.001$) and resulted in a significant decrease in LPL catalytic activity ($p < 0.0009$) [29]. Buscà et al. showed that both the wild-type and N291S human LPL expressed in COS-1 cells were secreted to the extracellular medium and presented similar intracellular distribution patterns consistently with our results. Furthermore, heparin-Sepharose affinity chromatography assays revealed normal heparin affinity of the N291S. In addition, both the mutant and the wild type proteins bound to the surface of human fibroblasts and of un-transfected COS-1 cells [31].

This mutation has also been described and associated with LPL deficiency by Mailly and colleagues, who investigated a patient with LPL deficiency with a history of acute pancreatitis [32]. *In vitro* mutagenesis and homozygous expression studies confirmed that the N291S substitution is associated with a significant reduction in LPL activity. This patient was also carrying a mutation of the *LPL* intron 6 splice site (frequency in the overall population 0.0016%). This mutation was identified for the first time by Hölzl and co-workers in three families living in the same geographic area, a secluded valley in Austria [21]. Interestingly, the splice site was found in homozygosity in some patients and heterozygosity in others. In particular, one patient was a compound heterozygote carrying the splice site mutation on one allele and a missense mutation (G188E) on the other allele. The blood sample of this patient did not show any LPL activity similarly to our compound heterozygote patient 2. Clearly, mutations of splice site of introns may have various consequences that cannot be predicted by the primary

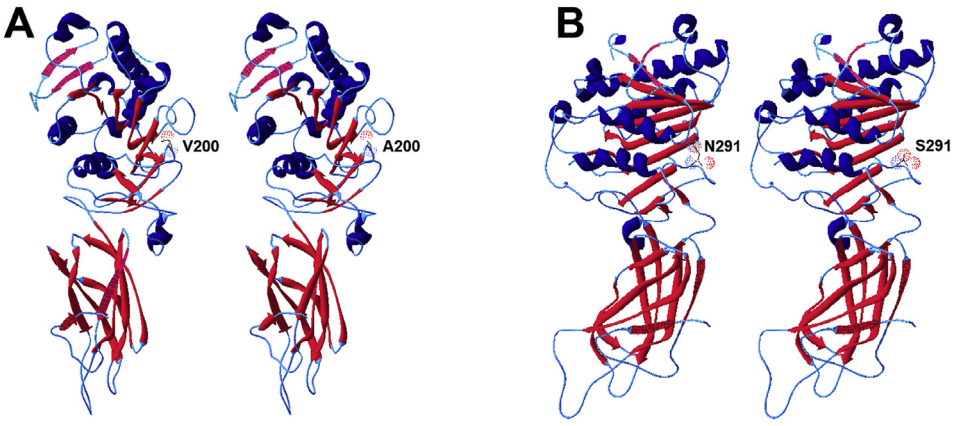

Fig. 6. Homology structural models of human wild-type LPL, V200A and N291S mutants. Ribbon diagram viewing the monomer of LPL organized into two distinct structural domains, amino terminal (residues 1–312) and carboxy terminal (residues 313–448). The model was obtained using the crystallographic structure of the human pancreatic lipase-related protein 1 as template. The calculated models were visualized by Swiss PDB Viewer 4.1.0. (A) The LPL model was mutated at position 200 by substituting valine (V), a non-polar aliphatic residue, for alanine (A), another non-polar aliphatic residue. (B) The same procedure was applied to the N291S mutation, substituting asparagine (N), an uncharged polar residue, at position 291 for serine (S), another uncharged polar residue.

sequence alone [21]. Functional analysis of LPL activity was performed on post-heparin plasma (*ex vivo*), revealing absence of LPL activity in the sample. Immunoblot analysis showed only a faint LPL band despite normal presence of control protein (APOA1). Moreover, *in vitro* experiments showed that the N291S, *per se*, does not affect protein synthesis and secretion although strongly affecting LPL enzymatic activity. Furthermore, compared to the wild-type protein, the dimer formation was not affected by the mutation.

Consistently with our findings, most of the deleterious missense mutations involve exons 5 and 6. Missense mutations in these exons have been suggested to cause LPL protein homo-dimer instability and, thus, LPL deficiency [34]. More specifically, LPL protein is organized into two distinct structural domains, N-terminal (residues 1–312) and C-terminal (residues 313–448) (Fig. 6). The lid structure (residues 216–239) that covers the catalytic site is located in the N-domain. Exons 1–4 and exons 5–6 make up the upper and lower parts of the N-domain, respectively, whereas exons 7–9 make up the C-domain. Razzaghi et al. found that more than 60% of the point mutations occurred in exons 5 and 6 contributing to the catalytic domain and the lid structure [35].

In general, lipoprotein lipase genes cluster in exons 5 and 6 leading to mutant proteins unable to achieve or maintain normal dimer conformation, thus, becoming inactive [7,34,36]. On the other hand, subjects with insertion/deletion, nonsense or splice site mutations usually are unable to produce the LPL protein and, thus, are deleterious mutations [1].

For fully enzymatic activity, LPL requires some important cofactors such as apolipoprotein C—II and A-V (APO-CII, APO-AV) [37,38], glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1) [25], lipase maturation factor 1 (LMF1) [39]. Although missense mutations may affect the interaction with these cofactors, in this work we did not test whether V200A or N291S mutations affected any interactions with LPL cofactors. This can be listed as limitation of our study.

In our patient, the heterozygous intron 6 splice site mutation c.1019-3C > A at least in conjunction with the N291S mutation clinically acts like a dominant negative variant.

Overall, this information clearly explains the severe phenotype in compound heterozygous patients due to the additional effect of both the mutations.

The identification of LPL mass in blood samples of patients affected by type I hypertriglyceridemia is a crucial information for the treatment of those patients. Treatment with alipogene tiparvovec in patients with no LPL mass may be not successful due to immune response to the injected functional LPL protein; however, Glybera (alipogene tiparvovec) is being withdrawn due to a cost-effectiveness analysis [20].

In conclusion, we have fully characterized two known missense mutations (p.V200A and p.N291S) by *ex vivo* and *in vitro* experiments and tested the additional effect of two splice site mutations by *ex vivo* experiments. The splice site mutation c.1019-3C > A (intron 6) has been already described while the c.1139+1G > A (intron 7) is identified and described in this work for the first time.

Conflict of interest

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

Authors contributions

ER performed genetic analysis. TMS performed clinical diagnosis. PP designed the study and performed the experiments. MB performed the experiments. All authors contributed to the manuscript preparation and interpretation of data.

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