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Title: CLINICAL AND GENETIC FEATURES OF THREE PATIENTS WITH FAMILIAL CHYLOMICRONEMIA DUE TO MUTATIONS IN GPIHBP1 GENE

Article Type: Original Contribution

Keywords: Familial Chylomicronemia; Hypertriglyceridemia; Pancreatitis; Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1).

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Abstract: Background. Familial chylomicronemia is a recessive disorder that may be due to mutations in lipoprotein lipase (LPL) as well as in other proteins such as apolipoprotein C-II and apolipoprotein A-V (activators of LPL), GPIHBP1 (the molecular platform required for LPL activity on endothelial surface) and LMF1 (a factor required for intracellular formation of active LPL).
Methods. We sequenced the familial chylomicronemia candidate genes in two adult females presenting long standing hypertriglyceridemia and a history of acute pancreatitis.
Results Both probands had plasma TG >10 mmol/L but no mutations in the LPL gene. The sequence of the other candidate genes showed that one patient was homozygous for a novel missense mutation p.(Cys83Arg) and the other was homozygous for a previously reported nonsense mutation p.(Cys 89*), respectively, in GPIHBP1. Family screening showed that the hypertriglyceridemic brother of the p.(Cys83Arg) homozygote was also homozygous for this mutation. He had no history of pancreatitis. The p.(Cys83Arg) heterozygous carriers had normal triglyceride levels. The substitution of a cysteine residue in the Ly6 domain of GPIHBP1 is predicted to abolish one of the disulfide bridges required to maintain the structure of GPIHBP1. The p.(Cys89*) mutation results in a truncated protein devoid of function.
Conclusions. Both mutant GPIHBP1 proteins are expected to be incapable of transferring LPL from the sub-endothelial space to the endothelial surface.

Dear Editor

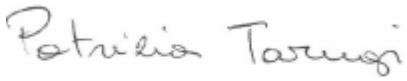
The manuscript entitled " Clinical and genetic features of three patients with familial chylomicronemia due to mutations in GPIHBP1 gene", is submitted for consideration for publication in Journal of Clinical Lipidology

The work described in the article has not been published previously in any form.

The article is not under consideration for publication elsewhere. Publication of the article is approved by all authors and tacitly by the responsible authorities where the work was carried out. If the article is accepted, it will not be published elsewhere by the authors, including electronically in the same form, in English or in any other language, without the written consent for the copyright-holder

Best regards,

Patrizia Tarugi



On behalf of all authors

REPLY TO REVIEWERS

All changes in the text are marked in red.

Reviewers' comments:

Reviewer #1: Summary

The authors report two homozygous GPIHBP1 mutations - the known p.C89X mutation and the novel p.C83R mutation - found in two patients with "familial chylomicronemia" and absence of LPL gene mutations. The brother of the p.C83R homozygote was also found to be a homozygote, and had severe hypertriglyceridemia of relatively late onset. The novel p.C83R is absent from public databases including 1000 genomes, ESP and ExAC. Both mutations were located in the Ly6 domain of GPIHBP1 gene which is important for binding and transporting LPL. The p.C83R mutation substitutes one of ten cysteines in the Ly6 domain, which predicted to disrupt disulfide bond and protein structure. Although no functional experiments were performed in this article, a previous in vitro study from another group (Beigneux, Young, 2011 JBC) used alanine scanning mutagenesis and showed the substitution of an alanine for cysteine at this position causes decrease in LPL binding ≥ 90%.

Comments

The new GPIHBP1 mutation provides a novel increment to the field, although the authors provide only indirect evidence of its dysfunction. It would have been better had the authors performed an LPL-GPIHBP1 binding assay for p.C83R to support their conclusion and increase the impact of this study.

We agree with reviewer. At present we do not have this assay running in our laboratory. However, we thought that the data available in literature (Beigneux et al. J Biol Chem 2011; 286:19735-43 and Beigneux et al. Circ Res. 2015; 116: 624-32) provided strong support to the pathogenicity of any substitution of Cys83.

The other striking finding is that the phenotype of homozygous GPIHBP1 deficiency is milder and of later onset than that of LPL deficiency, which often presents in childhood. It is striking that the homozygous brother of one proband was ascertained and characterized as part of this study rather than through presentation to the health care system, with features such as pancreatitis or even severe enough hypertriglyceridemia to have been concerning to his providers.

I think the authors need to make this point in the Abstract, Highlights and Discussion, i.e. severe HTG due to presumed GPIHBP1 deficiency is relatively less severe than the phenotype seen with more classical homozygous LPL deficiency.

Taking into account the reviewer's suggestion that the phenotype of homozygous GPIHBP1 deficiency might by milder than that observed in LPL deficiency, we reviewed all patients with homozygous/compound heterozygous GPIHBP1 deficiency (reported so far in literature) and compared their phenotype with what we observed in 35 patients with LPL deficiency (homozygote/compound heterozygotes) of our series (Rabacchi et al Atherosclerosis 2015; 241: 79-86). As shown in supplemental Table 3 this comparison failed to detect a difference between these two groups of patients. Therefore, at this stage, by looking at patients referred to Lipid Clinics in Hospital settings we cannot say that GPIHBP1 deficiency is milder than LPL deficiency. We mention this point in the discussion

Also, the authors' final sentence, namely that GPIHBP1 deficiency may be more common than assumed is not supported by their own data - it seems they had to search their clinic population pretty strenuously to find these cases.

We agree than our statement is not evidence based as we had not conducted a systematic analysis of GPIHBP1 gene in a large number of individuals with severe/moderate HTG. Our suggestion simply derives from the recent observation of five patients with GPIHBP1 deficiency among HTG patients referred to three different tertiary Lipid Clinics in University hospital settings (see supplemental references). On the other hand, as stated by the other reviewer of this manuscript, a large epidemiological study seems to indicate that GPIHBP1 deficiency is more common that LPL deficiency. To avoid possible misunderstanding we delete the final sentence.

Specific concerns

There are a number of concerns with grammar, spelling and typographical errors. Also, there are no page numbers - so I numbered the pages starting with 1 on the title page. I did not have time to document every single error, so the paper needs to be very carefully double-checked and proof read again.
page 2, line 2: "Three patients" - state whether related or unrelated - i.e. "Two siblings and a third patient"
page 2: perhaps in the "Highlights" you can mention whether heterozygotes have a phenotype
page 3 (Abstract), line 8: insert "the" before "LPL gene"
page 3, line 10: insert "a" between "for" and "previously"
page 3, line 12: replace "p.Cys83Arg carrier" with "p.Cys83Arg homozygote"
page 5, line 1: "two of whom are siblings"
page 6, line 1: delete period after "METHODS"
page 7, line 6: provide reference ranges for apolipoproteins
page 8, line 18: "the proband's father had type 2 diabetes ....atherosclerosis. The proband's parents ..."
page 8, line 8: spelling "gemfibrozil"
page 8, line 23: spelling "amylase"
page 9, last sentence: please add period
page 10, line 2: "Here we describe two ..."
page 10, line 5: "...revealed that the proband's brother also had severe..."
page 10, line 16: "Since LPL gene sequencing was negative...'
Please check format of references
Please check nomenclature format for naming mutations.

We thank the reviewer for the suggestions. We did our best to double- check the manuscript.

Reviewer #2:

1. Authors should discuss the recent work by Beigneux that shows that many mutations lead to dimers and multimers and that only monomers bind LPL. Your mutations will definitely elicit dimers and multimers.

We thank the reviewer for this suggestion. In general terms this study (Beigneux et al. Circ Res. 2015; 116: 624-32) shows that the substitution of some Cys residues of the Ly6 domain causes the generation of an unpaired thiol which may lead to intermolecular disulfide bonds formation and the production of dimers or multimers, with a concomitant reduction of the functional active monomeric form. With regard to the specific substitution of Cys83 (the residue converted to arginine in our patient) the study shows a decreased monomeric form of a GPIHBP1 mutant containing two substitutions Cys83Ala + Cys110Ala. Since the contribution of each mutation is not specified, it is difficult to say whether Cys83 substitution "per se" is sufficient to induce multimer formation - as it is clearly the case of the substitutions of other Cys residues (i.e. Cys65, Cys68 and Cys 89). We mention the possibility of dimer/multimer formation in the discussion

2. Do your mutations change restriction sites? If so, you should show this.

We made a computer search for changes in restriction sites induced by the two mutations. The c.247T>C substitution in exon 3 (Cys83Arg) introduces a restriction site for the enzyme BsrBI (BstD102I and AccBSI). Using our amplification conditions and primers, the digestion of the PCR amplified mutant exon 3 gives two fragments of 172 and 135 bp vs a single fragment of 307 bp in wild type exon 3. The c.267C>A in exon 3 (Cys89*) abolishes a restriction site for the enzyme BsgI. The digestion of amplified wild type exon 3 gives two fragments of 190 and 117 bp, whereas the digestion of wild type exon 3 results in a single fragment of 307 bp. Although we could have used this information for mutation screening, we preferred to use the direct Sanger sequencing of exon 3, which after all represents the gold standard for mutation detection. In addition the direct sequencing of exon 3 allowed the detection of both mutations found in our patients.

3. Giving heparin in the setting of GPIHBP1 deficiency works in mice, BUT THIS IS HIGHLY EXPERIMENTAL IN HUMANS AND IS PERHAPS NOT SUCH A GOOD IDEA. THIS FORM OF THERAPY WOULD BE EXPECTED TO LEAD TO STEATOSIS. This should be discussed.

In reviewing the medical records of proband #2 (homozygous for Cys89*), we discovered that the patient at the age of 44 (11 years ago) had been treated with intravenous injections of heparin presumably with the goal of releasing LPL from endothelial cells (on the reasonable assumption in pre-GPIHBP1 era that LPL might be entrapped within the cells). This treatment was discontinued after few months for the occurrence of endometrial bleeding. We have no record of the presence of hepatic steatosis in our patient after heparin treatment. One might expect this treatment to induce fatty liver as a consequence of massive increase of plasma free fatty acids induced by heparin-mediated release of LPL. High plasma level of fatty acid might cause overproduction of TG in the liver and possibly an insulin resistant state, conditions known to cause hepatic steatosis. We mention this point in the discussion.

4. The rationale for heparin therapy is NOT to release intracellular LPL; it is to release sub-endothelial LPL. Please discuss. Read the various Cell Metabolism papers.

In reporting heparin treatment of proband#2 we specified that the reasoning of the attending physicians (unaware of GPIHBP1 existence at that time) was based on the assumption that: i) the patient had a residual LPL activity; ii) heparin might facilitate not only the release of LPL from the endothelial surface but also from some other sites " within the endothelial cells". Today, in view of the documented role of GPIHBP1 in binding and trans-endothelial transport of LPL (Davis et al. Cell Metab 2010; 12: 42-52), our reasoning with regard to the effect of heparin treatment on LPL release is quite different.

5. The notion that giving CIII antisense inhibitors to chylomicronemia patients is a lousy idea, and this should be discussed. If you want to produce hepatic steatosis, this is the way to do it. This should be discussed.

The effects of short term treatment with apoC-III antisense oligo-nucleotide have been investigated in mice, non-human primates and humans. Although apoC-III ASO treatment tended to increase liver triglyceride levels in a mouse model, those changes did not achieve statistical significance. On the other hand apoCIII -/- mice fed a normal diet do not develop hepatic steatosis, which occurs if animals are fed a western type of diet.

In all preclinical rodent and nonhuman primate studies, there were no significant changes in plasma liver transaminases or other metabolic parameters when compared with controls. Experimental evidence indicates that this treatment does not reduce hepatic VLDL secretion in mice (Graham et al. Cir Res 2013; 112: 1479-90)

In the studies by Gaudet at al. in three patients with chylomicronemia due to LPL deficiency (N Engl J Med. 2014; 371: 2200-6) and 57 HTG patients (N Engl J Med. 2015; 373: 438-47) laboratory test results indicated that there was no significant effect of anti-apoCIII treatment on renal or hepatic function.

So these short term treatment studies do not raise safety concerns with regard to liver function. Whether long term treatment predisposes to hepatic steatosis (NAFLD or NASH) remains an open question. This point is raised in the discussion.

6. Discuss likelihood of nonsense mediated decay with your nonsense mutation, based on the "rules" for nonsense mediated decay.

We mention Nonsense Mediated mRNA Decay in the discussion. We agree with the reviewer that “some rules” have been proposed to predict nonsense-mediated mRNA decay (NMD) in mRNAs containing a premature termination codon (PTC). It is also true that there are several exceptions to these rules, depending on the gene and type of cells where a specific gene is expressed (Brogna S, Wen J. Nonsense-mediated mRNA decay (NMD) mechanisms. Nat Struct Mol Biol. 2009; 16: 107-13).

In general PTC in mammalian mRNA causes NMD when it is located upstream from an intron. Furthermore, for PTC to drive a strong NMD it should be located at least 50-55 bp upstream from the last exon/exon junction. In our case PCT position satisfies the first condition (upstream from an intron) but does not fully satisfy the second condition being located 28 bp from the last exon/exon junction. Therefore in our case we are not sure that NMD plays a relevant role.

7. Your review of the mutations is valuable. At this point, is there a shred of evidence that GPIHBP1 deficiency in humans is milder than LPL deficiency? Please discuss.

In an attempt to answer this question we reviewed all patients with homozygous/compound heterozygous GPIHBP1 deficiency (reported in literature so far) and compared their phenotype with that of 35 patients of our series with homozygous/compound heterozygous LPL deficiency (Rabacchi et al Atherosclerosis 2015; 241: 79-86). As shown in supplemental Table 3, we could not detect any substantial difference between these two groups of patients. Therefore, by looking at

patients referred to Lipid Clinics in hospital settings, at this stage, we cannot state that GPIHBP1 deficiency is milder than LPL deficiency. We make this point in the discussion.

8. The idea that 95% is caused by LPL deficiency: how strong is that data? Do we really know that? For all populations??? Please discuss. This reviewer ran into an epidemiologist from a huge consortium, who told me that in their hands GPIHBP1 deficiency was more common than LPL deficiency.

The idea that 95% of familial chylomicronemia is due to LPL deficiency has been proposed in a recent review (Brahm A, Hegele RA. Hypertriglyceridemia. Nutrients 2013; 5: 981-1001). I assume that this figure refers to patients with familial chylomicronemia seen in tertiary lipid clinics (selection bias) and does not derive from population studies. We mention this point in the discussion. The new data from a huge consortium indicating that GPIHBP1 deficiency is more common than LPL deficiency is very interesting. We look forward to seeing the results of this survey.

CLINICAL AND GENETIC FEATURES OF THREE PATIENTS WITH FAMILIAL CHYLOMICRONEMIA DUE TO MUTATIONS IN *GPIHBP1* GENE

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Key words

Familial Chylomicronemia; Hypertriglyceridemia; Pancreatitis; Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1).

HIGHLIGHTS

* Two siblings and a third patient with severe hypertriglyceridemia were investigated

* They were found to be homozygous for two mutations in *GPIHBP1* gene

* The mutations involved two cysteine residues of the Ly6 domain of GPHHBP1

* The p.(Cys83Arg) substitution is a novel mutation predicted to disrupt GPIHBP1 structure

* The previously reported p.(Cys89*) mutation results in a truncated protein devoid of function

* The p.(Cys83Arg) heterozygous carriers had normal plasma triglyceride level.

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ABSTRACT

Background. Familial chylomicronemia is a recessive disorder that may be due to mutations in lipoprotein lipase (LPL) as well as in other proteins such as apolipoprotein C-II and apolipoprotein A-V (activators of LPL), GPIHBP1 (the molecular platform required for LPL activity on endothelial surface) and LMF1 (a factor required for intracellular formation of active LPL).

Methods. We sequenced the familial chylomicronemia candidate genes in two adult females presenting long standing hypertriglyceridemia and a history of acute pancreatitis.

Results Both probands had plasma TG >10 mmol/L but no mutations in the *LPL* gene. The sequence of the other candidate genes showed that one patient was homozygous for a novel missense mutation p.(Cys83Arg) and the other was homozygous for a previously reported nonsense mutation p.(Cys 89*), respectively, in GPIHBP1. Family screening showed that the hypertriglyceridemic brother of the p.(Cys83Arg) homozygote was also homozygous for this mutation. He had no history of pancreatitis. The p.(Cys83Arg) heterozygous carriers had normal triglyceride levels. The substitution of a cysteine residue in the Ly6 domain of GPIHBP1 is predicted to abolish one of the disulfide bridges required to maintain the structure of GPIHBP1. The p.(Cys89*) mutation results in a truncated protein devoid of function.

Conclusions. Both mutant GPIHBP1 proteins are expected to be incapable of transferring LPL from the sub-endothelial space to the endothelial surface.

INTRODUCTION

Familial chylomicronemia (Type I hyperlipidemia) is due to a defect in the lipolytic cascade of TG-rich lipoproteins (chylomicrons and VLDL) which may result from mutations in at least five different genes: *LPL* (encoding the enzyme lipoprotein lipase, LPL; OMIM #238600), *APOC2* (encoding the apolipoprotein C-II, the activator of LPL; OMIM #207750), *APOA5* (encoding apolipoprotein A-V, also an activator of LPL; OMIM #144650), *GPIHBP1* (encoding glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1, the molecular platform which, on the endothelial surface of capillaries, allows the interactions of LPL with TG-rich lipoproteins, apolipoprotein C-II, and apolipoprotein A-V; OMIM #612757), and *LMF1* (encoding the Lipase Maturation Factor 1, a tissue factor which allows the secretion of functional LPL and Hepatic Lipase, HL; OMIM #611761) [1, 2]. Inactivating mutations of LPL are the most common cause of familial chylomicronemia (95 % of cases) investigated in hospital settings [1]. The clinical features of the disorder, often diagnosed in infancy, include failure to thrive, eruptive xanthomas, lipemia retinalis, hepatosplenomegaly, recurrent abdominal pain and episodes of acute pancreatitis [1, 3].

GPIHBP1 is a 184 amino acids endothelial cell protein which acts as a transporter for LPL across the endothelial cells to the capillary lumen and appears to be the main binding site for LPL on the endothelial surface [4]. GPIHBP1 belongs to the Ly6 protein family, so called because of a lymphocyte antigen 6 domain that contains 10 cysteine residues, forming disulfide bonds and creating a characteristic three-finger structural motif [5,6]. The Ly6 domain is crucial because it is involved in the binding to LPL and allows the interactions of LPL with apoC-II, apoA-V and TG-rich lipoproteins on the endothelial surface [5]. Recently several patients with familial chylomicronemia were reported to carry mutations in *GPIHBP1* gene (reviewed in Supplemental material).

Here we describe three patients (two of whom are siblings) manifesting severe hypertriglyceridemia incidentally discovered in adolescence, who were found to be homozygous for *GPIHBP1* mutations affecting two cysteine residues of the Ly6 domain.

METHODS

We investigated two adult patients with severe hypertriglyceridemia and suspected to have familial chylomicronemia (clinical features are given below).

Laboratory investigations

Plasma lipids and apolipoproteins were determined as previously reported [7]. The candidate genes for familial chylomicronemia (*LPL*, *APOC2*, *APOA5*, *GPIHBP1* and *LMF1*) were resequenced sequentially as previously reported [7-9]. The mutation was designated according to the Human Genome Variation Society, 2015 version (http://www.hgvs.org/mutnomen/recs-DNA.html). GPIHBP1 protein sequence variants were designated according to http://www.hgvs.org/mutnomen/recs-prot.html. The in silico prediction of the effect of the missense mutation of GPIHBP1 protein was performed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT Human Protein and SIFT BLink (http://sift.jcvi.org/), SNPs3D (http://snps3d.org), PMut (http://mmb2.pcb.ub.es/pmut) and SNAP (https://www.rostlab.org/services/snap/) [10].

All subjects investigated gave their written consent to take part in the study. The study was conducted according to the Helsinki Declaration (2013) and approved by the Institutional Etic Committees.

RESULTS

Clinical Features of the patients

Proband #1 was a 42 year-old female found to have severe hypertriglyceridemia at 18 years of age during a routine laboratory test. At that time her plasma lipid profile was the following: total cholesterol (TC) 5.0 mmol/L; triglycerides (TG) 13.3 mmol/L; HDL cholesterol (HDL-C) 0.67 mmol/L; ApoA-I 94 mg/dL; ApoB 100 mg/dL. She was asymptomatic and her clinical examination was negative. A low fat diet was strongly recommended and treatment with fenofibrate (140 mg/day) was prescribed. However, the patient was lost at follow up until the age of 40 when she was admitted to hospital for acute pancreatitis, which was attributed to the presence of gallstones. After cholecystectomy, in view of the persistently elevated plasma TG level, she was referred to the lipid clinic with the diagnosis of possible familial chylomicronemia. The 35 year-old proband's brother, apparently healthy, had been discovered to have hypertriglyceridemia at the age of 11 when his plasma lipid profile was the following: TC 8.1 mmol/L; TG 23.7 mmol/L; HDL-C 0.60 mmol/L; ApoA-I 111 mg/dL. At the age of 20 he started treatment with fenofibrate (280 mg/day) and a low fat diet supplemented with ω-3 fatty acids (6 g/day). However, he also was lost at follow up and was referred to the lipid clinic only recently. Both siblings admitted poor compliance with the prescribed diet and drug treatment. The sister and the mother of the proband were healthy; the proband's father had a type 2 diabetes and carotid atherosclerosis. The proband's parents, apparently unrelated, came from the same restricted geographical district in Southern Italy.

Proband #2 was a 55 year-old female, who at the age of 12 was found to have elevated plasma TG during a routine laboratory test. At the age of 17, during estrogen-progestin treatment for dysmenorrhea, she developed acute pancreatitis associated with a striking rise in plasma TG (~83 mmol/L). Subsequently, she had a partial pancreatectomy for the presence of a pseudocyst. At the age of 20 she had cholecystectomy for gallstones. Despite treatment (hypolipidemic diet

supplemented with MCT-oil 20-30 mg/day, ω-3 fatty acids 2-4 g/day, gemfibrozil 900-1200 mg/day or fenofibrate 145 mg/day), her plasma TG level remained persistently elevated (>6 mmol/L) with occasional increases above 20 mmol/L, often associated with recurrent episodes of abdominal pain. At the age of 36 she was operated on for bilateral ovarian cysts. At the age of 44, in view of the high TG levels, she was treated for several months with heparin on the assumption that heparin might release active LPL from endothelial surface and putative intracellular pools making it readily available for intravascular TG hydrolysis. The patient was treated with a subcutaneous injection of 5.000 IU of heparin every third day and gemfibrozil 600 mg/twice a day. During this treatment plasma TG level decreased to ~3.5 mmol/L, TC to ~2.75 mmol/L, while HDL-C level did not change (~0.35 mmol/L). No alterations of liver transaminases were documented during this treatment, which might suggest liver lipid accumulation induced by free fatty acid release in plasma. This treatment was interrupted after a few months due to the occurrence of metrorrhagia. More recently the patient was found to have arterial hypertension, chronic gastritis and a mild elevation of fasting blood glucose (110 mg/dL). Abdominal ultrasound examination revealed the presence of hepatic steatosis. The proband's family history was negative for the presence of hypertriglyceridemia. No family member was available for study. The proband's parents were apparently unrelated but came from a small village in a mountain district and shared the same surname.

Figure 1 shows the pedigree of proband #1 and the plasma lipid profile of her family members at the time of molecular investigations. The proband and her brother had severe hypertriglyceridemia, associated with a marked reduction of plasma HDL-C. Proband's brother also had high TC. The proband's parents had a normal lipid profile; the proband's sister had a normal TG level but a moderate elevation of TC.

At the time of genetic investigation proband #2 showed the following lipid profile: TC 6.2 mmol/L; TG 31.5 mmol/L; HDL-C 0.51 mmol/L. Other laboratory tests were within the normal range:

HbA1c 5.6%, AST 23 IU/L, ALT 42 IU/L, amylase 65 IU/L (n.v. <100), lipase 259 U/L (n.v. 73-393).

Molecular findings

In both probands the sequence of *LPL*, *APOC2*, *APOA5* and *LMF1* genes was either negative or showed only common variants with no pathogenic effect. The sequence of *GPIHBP1* gene revealed that proband #1 was homozygous for a single nucleotide substitution (c.247T>C) in exon 3, resulting in arginine for cysteine conversion at position 83 - p.(Cys83Arg) - of GPIHBP1 protein (**Figure 2**). Computer search indicated that the mutation introduces a restriction site for the enzyme BsrBI (BstD102I and AccBSI). Using our amplification conditions and primers (supplemental material), the digestion of the PCR amplified mutant exon 3 is predicted to generate two fragments of 172 and 135 bp vs a single fragment of 307 bp in wild type exon 3.

The mutation screening of family members (performed by direct sequencing of exon 3) revealed that proband's brother was homozygous for the same mutation, whereas the sister and the parents were heterozygotes (**Figure 1**). All algorithms used for in silico analysis indicated that this mutation was deleterious.

Proband #2 was found to be homozygous for a single nucleotide substitution (c.267C>A) in exon 3, resulting in the conversion of a cysteine codon into a termination codon p.(Cys89*) (**Figure 2**). This mutation abolishes a restriction site for the enzyme BsgI. The digestion of amplified wild type exon 3 is predicted to generate two fragments of 190 and 117 bp, whereas the digestion of mutant exon 3 results in a single fragment of 307 bp.

These GPIHBP1 mutations were not found in a groups of 150 subjects with primary hypertriglyceridemia and 300 normolipidemic subjects of the Italian population.

DISCUSSION

Here we describe two probands in whom severe hypertriglyceridemia was incidentally discovered during adolescence. Proband #1, lost at follow up for many years, was referred to the Lipid Clinic with the diagnosis of possible familial chylomicronemia at the age of 40 after an episode of acute pancreatitis. Family history revealed that the proband's brother also had severe hypertriglyceridemia discovered in childhood.

Proband #2 suffered from acute pancreatitis, associated with a striking elevation of TG during estrogen-progestin treatment for dysmenorrhea when she was 17. It is most likely that in this case estrogen treatment had induced a marked elevation of plasma TG (>80 mmol/L) which in turn might have been the primary cause of acute pancreatitis. This hypothesis is supported by the observation that estrogen treatment may induce a severe hypertriglyceridemia in otherwise normo-triglyceridemic women with a pre-existing genetic abnormality of plasma TG [11]. On the other hand, carriers of *LPL*, *APOA5* or *GPIHBP1* mutations may develop severe hypertriglyceridemia and pancreatitis during pregnancy, as the result of the elevated plasma estrogen levels [12-14].

In our patients *LPL* gene was sequenced first, on the assumption that most of familial chylomicronemia cases are due to inactivating mutations of LPL [1]. Since *LPL* gene sequencing was negative, we sequenced the other candidate genes and found that both probands were homozygous for mutations affecting two cysteine residues of Ly6 domain of GPIHBP1. Proband #1 was homozygous for a novel missense mutation p.(Cys83Arg), while proband #2 was homozygous for a nonsense mutation p.(Cys89*) previously reported in a compound heterozygote [15]. These findings extend the list of *GPIHBP1* mutations affecting the Cys residues of the Ly6 domain found in patients with severe hypertriglyceridemia **(Figure 3) (**Supplemental Tables S1 and S2 and supplemental references). The 10 cysteine residues of Ly6 domain (Cys65-Cys136) are disulfide bonded and disulfide bonds are crucial for forming a three-fingered structural motif. Interfering

with any of the disulfide bonds is predicted to induce gross alterations of the protein structure [4, 5]. A previous in vitro investigation showed that GPIHBP1 mutants in cysteine residues of Ly6 reached the surface of endothelial cells but had lost the ability to bind and transport LPL from the sub-endothelial space to the endo-luminal surface of the endothelial cells [16]. It is conceivable that GPIHBP1 p.(Cys83Arg) mutant does not bind LPL for three reasons: i) a previous in vitro mutagenesis study demonstrated that the substitution of cysteine 83 with alanine (a non-polar amino acid) reduced LPL binding by >90% [17], thus confirming the crucial role of this cysteine residue for GPIHBP1 function; ii) the substitution of Cys residues of the Ly6 domain causes the generation of an unpaired thiol which may lead to intermolecular disulfide bonds formation and the production of dimers or multimers (with a concomitant reduction of the functional active monomeric form) [18]; iii) the presence of an arginine residue at position 83 introduces a positive charge which might form salt bridges with the negative charged amino acids located in the Ly6 domain or in the acidic domain at the NH-2 terminal end of the GPIHBP1, thus disrupting the structure of the protein. In this context it is also possible that these conformational changes make the mutant GPIHBP1 more susceptible to intracellular degradation.

The p.(Cys89*) found in proband #2 is a nonsense mutation resulting in a truncated protein, expected to be devoid of function. It is also possible that the mRNA generated by the mutant allele, containing a premature termination codon (PTC), is subjected to a rapid degradation (nonsense mediated decay), even though the distance of the PTC from the last exon/exon junction is higher than that considered to be the gold standard for an efficient nonsense mediated decay [19].

A limitation of this study is that we did not measure LPL activity and mass in post-heparin plasma. However, a previous study documented a very low LPL activity in a familial chylomicronemia patient homozygous for a missense mutation p.(Cys65Tyr) in one of the cysteine residues of Ly6 domain [20].

From the clinical stand point our three patients raise two major questions concerning the phenotypic expression of the disorder and the most suitable therapeutic interventions, respectively. With regard to the first question: i) the late occurrence of a single episode of pancreatitis in proband #1 and the absence of this complication in her brother (despite a longstanding severe hypertriglyceridemia) and ii) the occurrence of single episode pancreatitis after estrogen treatment in proband #2, may suggest that GPIHBP1 deficiency has a milder phenotype with respect to that seen in LPL deficiency. To answer this question we compared the phenotype of patients with GPIHBP1 deficiency reported in literature with that of 35 patients with LPL deficiency of our cohort [10]. As shown in supplemental Table 3, we did not find a substantial difference between the two groups of patients. Thus, looking at patients referred to Lipid Clinics in a hospital setting it appears that the phenotype of GPIHBP1 is similar to that of LPL deficiency.

With regard to the second question, our patients being at high risk of pancreatitis, require therapeutic interventions and a strict follow up schedule. We have recommended a low fat diet, with restriction of saturated fat and carbohydrate intake (specifically simple sugars), supplemented or not with ω3-fatty acids (3-4 g/d). However, satisfactory TG levels may be difficult to achieve with diet alone. Drug therapy with fibrates or orlistat can be adopted but their use in chylomicronemia patients is controversial [1, 21-23]. If, despite this treatment, plasma TG level remains above 10 mmol/L, we might consider a new therapeutic intervention such as apoC-III-targeted antisense oligonucleotide [24-26]. Recently, three patients with the familial chylomicronemia syndrome due to an LPL mutation and TG levels ranging from 15.8 to 23.5 mmo/L were treated with this drug. After 13 weeks of study-drug administration plasma apoC-III levels were reduced from 71 to 90% and TG levels by from 56 to 86%. During the study, all patients on treatment had a TG level of less than 5.6 mmol/L [25]. It has been postulated that this treatment reduces the hepatic secretion of VLDL as well as increasing the hepatic removal of TG-rich lipoproteins through an LPL independent mechanism. Another recent study confirmed that a short term administration of anti

apoC-III antisense oligonucleotide efficiently reduced plasma TG in a group of 57 HTG patients without side effects, specifically with regard to liver and renal functions [26]. However, despite these encouraging results, it remains to be established whether a long-term treatment induces hepatic lipid accumulation as the result of the inhibition of the production of apoCIII, which is one of the major protein constituents of VLDL.

CONCLUSIONS

The present study, as well as other reports published over the last couple of years (see supplemental Tables S1 and S2), suggests that patients with the diagnosis of familial chylomicronemia may be homozygous or compound heterozygous for inactivating mutations of GPIHBP1.

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Conflicts of interest

The authors have no conflict of interest to disclose.

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C.R. and P.T. contributed to the design of the study and performed the analysis of candidate genes; S.D., S.P. and T.L were in charge of the patients management and clinical data collection; S.B. and S.C. coordinated the study and wrote the article. All authors read and approved the final version of the article.

LEGEND TO FIGURES

FIGURE 1.

Pedigree of proband #1. The proband is indicated by an arrow. Subjects homozygous for the GPIHBP1 mutation are indicated with full shaded symbols. Plasma lipids in mmol/L are shown below each subject.

FIGURE 2.

Partial nucleotide sequence of exon 3 in a control subject (upper panels), in proband #1 (A) and proband #2 (B).

FIGURE 3

Mutations in GPIHBP1 gene reported in patients with severe hypertriglyceridemia/ chylomicronemia.

Figure 1

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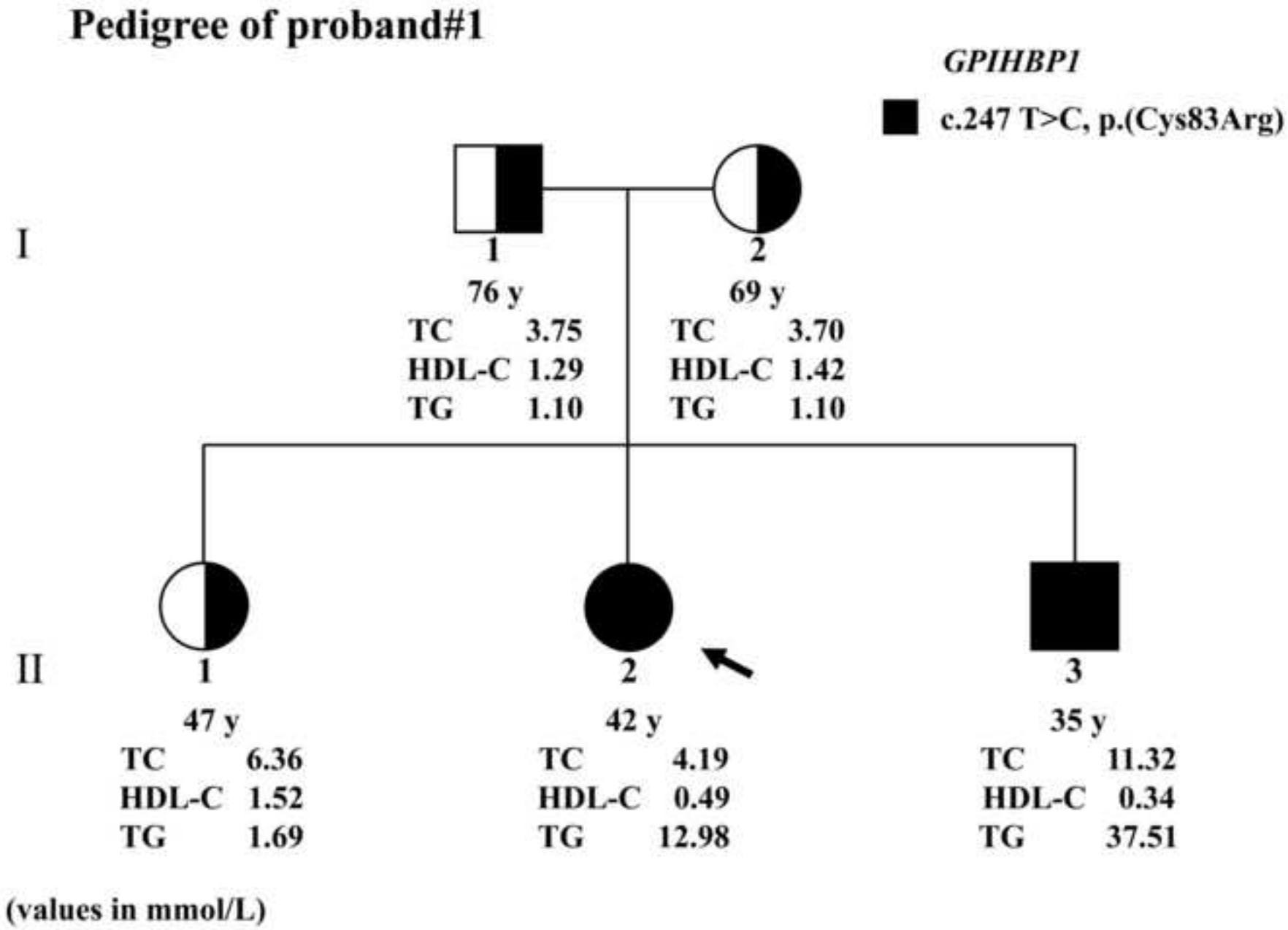

Figure 2

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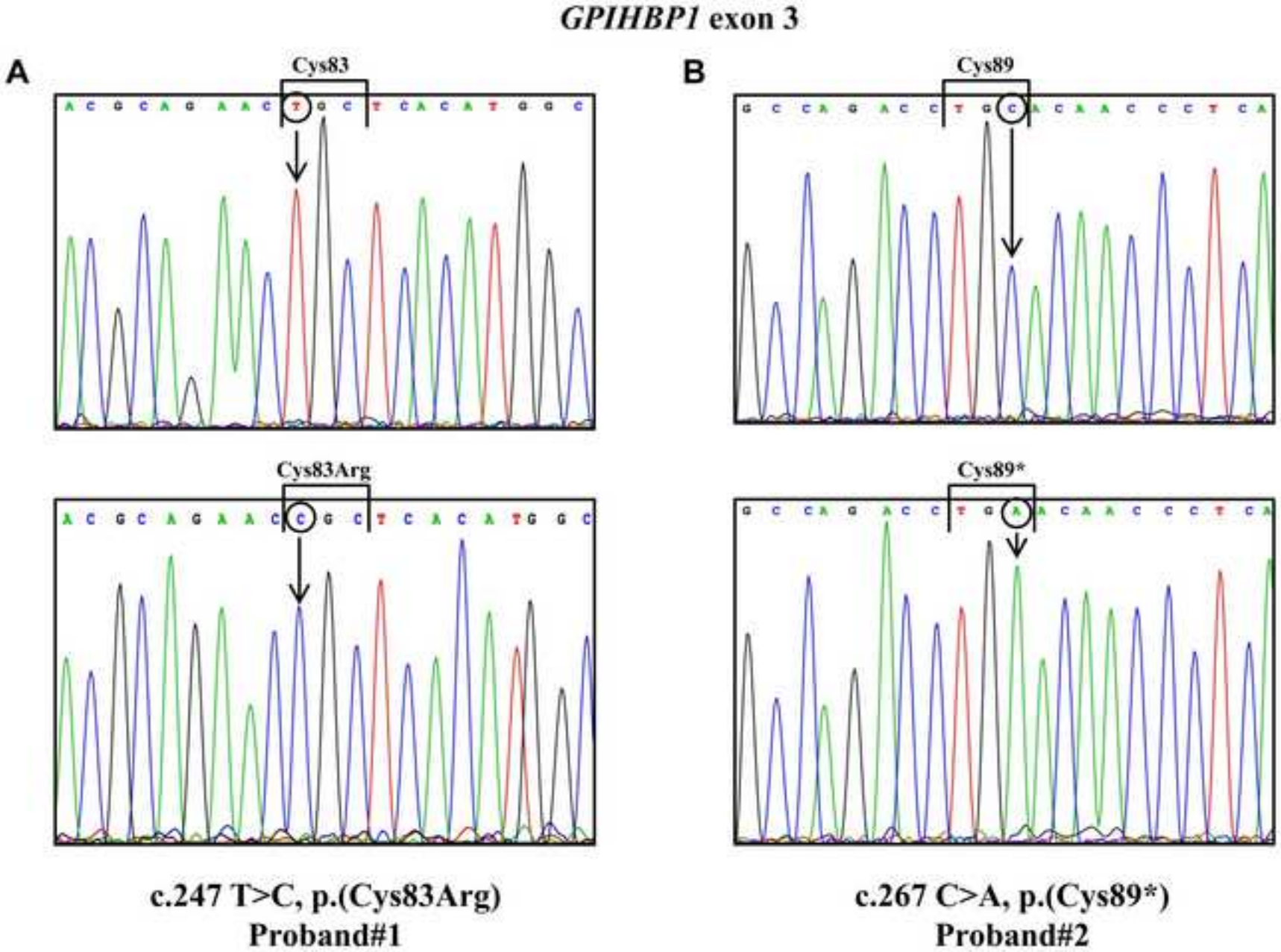

Figure 3
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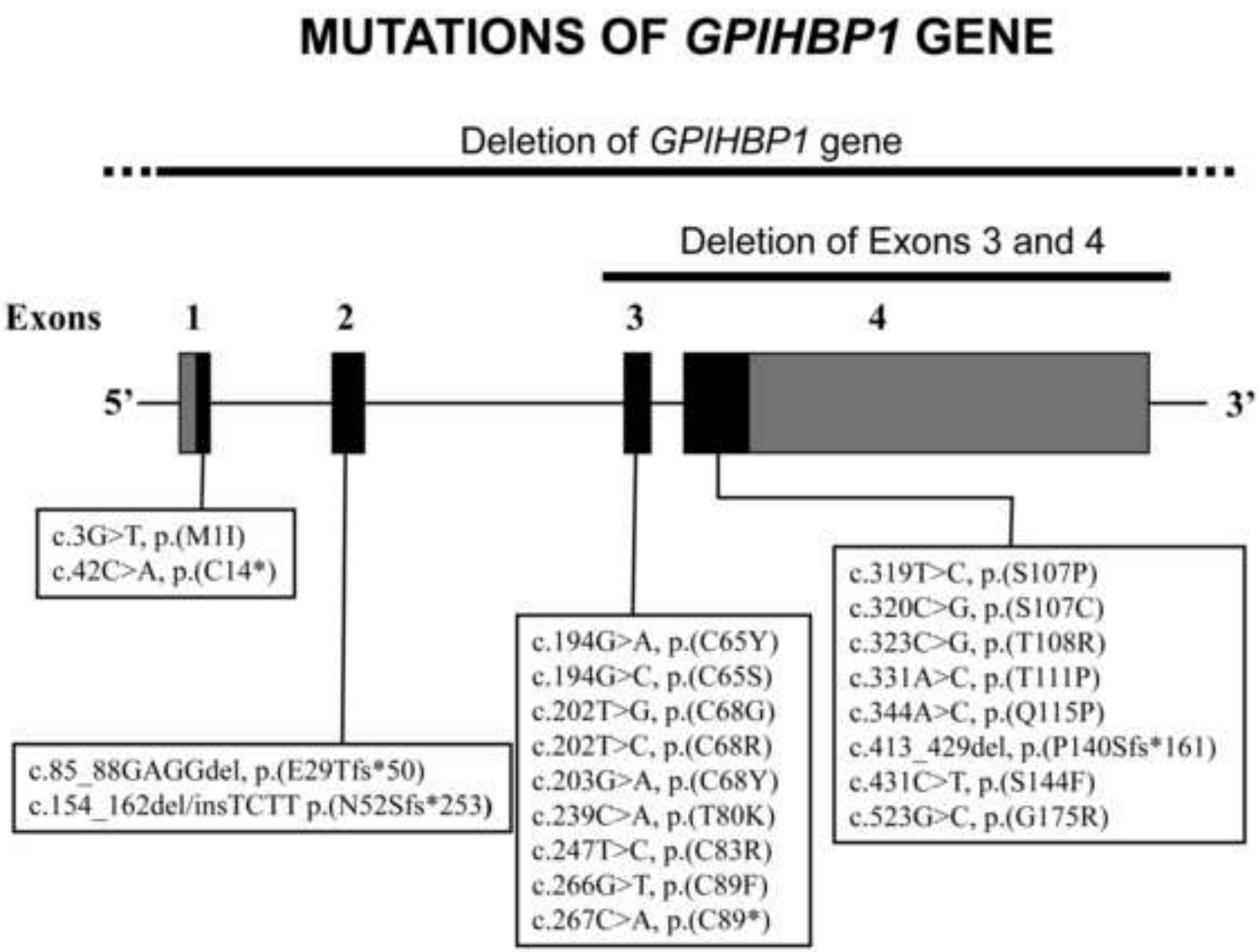

Supplementary Materials
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SUPPLEMENTAL MATERIAL

Primers used for amplification of *GPIHBP1* exon 3

3F 5'-AGGCTAGGCTTTGGGAGCACAG-3'

3R 5'-GTCTCTGAGGTGGCTCTGCAG-3'

Amplicon size=307 bp

Amplification conditions

Final concentration in a reaction volume of 50 µl :

Primer F/R [0.5 pmol/µl each], dNTP [0.2 mM each], $MgCl_2$ [2.5mM], DNA polymerase [2.4 U/µl], Buffer DNA polymerase 1X, DNA [2-4 ng/µl]

95°C x 3 min + 29 cycles (95°C x 30 sec – 63.6°C x 30 sec – 72°C x 30 sec) + 72°C x 5 min

SUPPLEMENTAL TABLES

GPIHBP1 (8q24.3) mutations

GPIHBP1 gene (GenBank-NCBI accession no.): ENSG00000182851

GPIHBP1 mRNA (GenBank-NCBI accession no.): NM_178172.5, GI 613410182, ENST00000330824

GPIHBP1 protein (GenBank-NCBI accession no.): NP_835466.2, GI 613410183, ENSP00000329266, UniPro Q8IV16

Table S1. Patients with severe hypertriglyceridemia/hyperchylomicronemia and mutations in GPIHBP1 gene

Ethnic origin (N. of families, N. of patients, genetic status and clinical findings)	Allele 1	Allele 2	See Suppl. References
Columbian (1 fam, 1HO: M 33y, HSM, LR, TG 38)	c.344A>C, p.(Q115P)	c.344A>C, p.(Q115P)	1
Arabian (1 fam, 1HO: M 3y: PC at 1y, LR, TG 45)	c.194G>A, p.(C65Y)	c.194G>A, p.(C65Y)	2
Swedish (1 fam, 3 CHE: F 9mo-27y, HSM, TG 22-57; M 10-13y, SM, TG 19.5; F 16mo-10y, RPC childhood, TG 20-48.5.	c.194G>C, p.(C65S)	c.202T>G, p.(C68G)	3
French (1 fam, 1 CHE: M 6mo, PC at 6mo, TG 19.6)	c.41G>T, p.(C14F) + c.266G>T, p.(C89F)	c.1-?_2282+?del (gene del), p.0	4
Algerian (1 fam, 1 HO: M 26y, RPC from 26y on, TG 26-65)	c.523G>C, p.(G175R)	c.523G>C, p.(G175R)	4
Spanish (1 fam, 1 HO: F 30y, PC at 6 and 22y, TG 15.8)	c.203G>A, p.(C68Y)	c.203G>A, p.(C68Y)	5

Asian Indian (1 fam, 2 HO: M 2mo, LR, TG 421; F 44y, PC at 29y, TG 11)	17,499bp del including GPIHBP1 gene, p.0	17,499bp del including GPIHBP1 gene, p.0	6
Salvadoran (1 fam, 1 HO: F 36y, PC at 24y, LR, ERX, TG 73.2)	c.203G>A, p.(C68Y)	c.203G>A, p.(C68Y)	6
Caucasian (1 fam, 1 HO, TG >10)	c.194G>A, p.(C65Y)	c.194G>A, p.(C65Y)	7
Caucasian (1 fam, 1 HO: M 1y, PC at 1y, TG >10)	c.323C>G, p.(T108R)	c.323C>G, p.(T108R)	7
Caucasian (1 fam, 1 HO, TG >10)	c.344A>C, p.(Q115P)	c.344A>C, p.(Q115P)	7
Caucasian (1 fam, 1 HE: F 45y, PC, TG 35.8)	c.431C>T, p.(S144F)	-	7
Caucasian (1 fam, 1 HE: TG >3.37)	c.42C>A, p.(C14*)	-	8
Japanese (1 fam, 1 HO: F 54y, PC at 27y, CHD, TG 29.8)	c.41G>T, p.(C14F) + c.202T>C, p.(C68R)	c.41G>T, p.(C14F) + c.202T>C, p.(C68R)	9
Pakistani (1 fam, 4 HO: M 37y, RPC, TG 100; F 22y, RPC, TG 60; M 40y, TG >40; F 37y, RPC, TG 27)	c.182- ?_555+ ?del (Ex3_4del), p.0	c.182- ?_555+ ?del (Ex3_4del), p.0	10
Hispanic (1 fam, 1 CHE: F 5wks, TG 136, ERX, PC at 2y)	c.331A>C, p.(T111P)	c.413_429del, p.(P140Sfs*161)	11
Thai (1 fam, 3 HO: F 46y, TG 14.09-72.86; M 64y, TG 9.51; M 43y, TG 7.60)	c.320C>G, p.(S107C)	c.320C>G, p.(S107C)	12
Caucasian (1 fam, 1CHE: F 6mo, TG 30.09, PC at 6mo, ERX)	c.85_88GAGGdel, p.(E29Tfs*50)	c.267C>A, p.(C89*)	13
Italian (1 fam, 1 CHE: F 3days, TG 18.8)	c.154_162AACAGGCTCdelTCTTins, p.(N52Sfs*253)	c.319T>C, p.(S107P)	14
Pakistani (1 fam, 1 HO: M 39y, RPC from 23y on, TG 50.72)	c.239C>A, p.(T80K)	c.239C>A, p.(T80K)	15

Ecuadorian (1 fam, 1 HO: F 25y, RPC from 15y on, TG 43.16)	c.3G>T, p.(M1I)	c.3G>T, p.(M1I)	15
Italian (1 fam, 2 HO: F 18-42y, PC at 40y, TG 13.0; M 11-35y, TG 23.7-37.5)	c.247T>C, p.(C83R)	c.247T>C, p.(C83R)	PP
Italian (1 fam, 1 HO: F 12-55y, PC at 17, TG 31.5-83.0)	c.267C>A, p.(C89*)	c.267C>A, p.(C89*)	PP

HO = Homozygote; CHE = Compound heterozygote; HE = Heterozygote ; HSM = hepatosplenomegaly; SM = splenomegaly; LR = lipemia retinalis; ERX= eruptive xanthomas; PC = pancreatitis; RPC = recurrent pancreatitis; CHD = coronary heart disease; TG = mmol/L ; PP = present paper.

Table S2. GPIHBP1 pathogenic mutations found in patients with severe hypertriglyceridemia/hyperchylomicronemia

Exon	cDNA	Protein	Mutation location in GPIHBP1 domain	Suppl. References
Whole	Gene deletion	p.0	-	4, 6
1	c.3G>T	p.(M1I)	Signal peptide	15
1	c.42C>A	p.(C14*)	Signal peptide	8
2	c.85_88GAGGdel	p.(E29Tfs*50)	Acidic N-terminal	13
2	c.154_162AACAGGCTCdelTCTTins	p.(N52Sfs*253)	Acidic N-terminal	14
3_4	c.182- ?_555+ ?del (Ex3_4del)	p.0	-	10
3	c.194 G>A	p.(C65Y)	Ly6 domain	2, 7
3	c.194 G>C	p.(C65S)	Ly6 domain	3
3	c.202 T>G	p.(C68G)	Ly6 domain	3
3	c.202 T>C	p.(C68R)	Ly6 domain	9
3	c.203 G>A	p.(C68Y)	Ly6 domain	5, 6
3	c.239C>A	p.(T80K)	Ly6 domain	15
3	c.247T>C	p.(C83R)	Ly6 domain	NEW, PP
3	c.266 G>T	p.(C89F)	Ly6 domain	4
3	c.267 C>A	p.(C89*)	Ly6 domain	13, PP
4	c.319 T>C	p.(S107P)	Ly6 domain	14
4	c.320 C>G	p.(S107C)	Ly6 domain	12
4	c.323 C>G	p.(T108R)	Ly6 domain	7
4	c.331 A>C	p.(T111P)	Ly6 domain	11
4	c.344 A>C	p.(Q115P)	Ly6 domain	1, 7
4	c.413_429del,	p.(P140Sfs*161)	Linker and GPI-anchor deletion	11

4	c.431 C>T	p.(S144F)	Linker	7
4	c.523 G>C	p.(G175R)	GPI-anchor	4

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Table S3. Comparison between patients with LPL and GPIHBP1 deficiency

	LPL	GPIHBP1	P *
HO/CHE	26/9	21/7	NS
M/F	16/19	13/15	NS
Age (years) mean ± SD	22.8 ± 21.0	26.6 ± 19.7	NS
TG max (mmo/L) median (IQ range)	28.25 (21.11-56.72)	37.75 (19.52-50.16)	NS
TG max (Log) mean ± SD	3.48 ± 0.32	3.49 ± 0.36	
PC or RPC	19/35 = 54.3%	18/28 = 64.2%	NS
PC or RPC (M/F)	8/11	6/12	NS
PC or RPC (HO/CHE)	16/3	14/4	NS
Age (years) at PC or first episode of RPC	18.6 ± 15.9	15.6 ± 12.3	NS
TG max (mmo/L) at PC/RPC median (IQ range)	24.91 (18.42-38.42)	38.50 (24.4-63.3)	NS
TG max (Log) at PC/RPC mean ± SD	3.39 ± 0.25	3.52 ± 0.30	

* Statistical comparisons were performed by X^2 and Mann-Whitney tests; HO = Homozygotes, CHE = compound heterozygotes; TG = plasma triglyceride: PC = Pancreatitis: RPC = recurrent pancreatitis