

SHORT REPORT

Establishing the role of *PLVAP* in protein-losing enteropathy: a homozygous missense variant leads to an attenuated phenotype

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ABSTRACT

Background Intestinal integrity is essential for proper nutrient absorption and tissue homeostasis, with damage leading to enteric protein loss, that is, protein-losing enteropathy (PLE). Recently, homozygous nonsense variants in the plasmalemma vesicle-associated protein gene (*PLVAP*) were reported in two patients with severe congenital PLE. *PLVAP* is the building block of endothelial cell (EC) fenestral diaphragms; its importance in barrier function is supported by mouse models of *Plvap* deficiency.

Objective To genetically diagnose two first-degree cousins once removed, who presented with PLE at ages 22 and 2.5 years.

Methods Family-based whole exome sequencing was performed based on an autosomal recessive inheritance model. *In silico* analyses were used to predict variant impact on protein structure and function.

Results We identified a rare homozygous variant (NM_031310.2:c.101T>C;p.Leu34Pro) in *PLVAP*, which co-segregated with the disease. Leu34 is predicted to be located in a highly conserved, hydrophobic, α -helical region within the protein's transmembrane domain, suggesting Leu34Pro is likely to disrupt protein function and/or structure. Electron microscopy and *PLVAP* immunohistochemistry demonstrated apparently normal diaphragm morphology, predicted to be functionally affected.

Conclusions Biallelic missense variants in *PLVAP* can cause an attenuated form of the PLE and hypertriglyceridaemia syndrome. Our findings support the role of *PLVAP* in the pathophysiology of PLE, expand the phenotypic and mutation spectrums and underscore *PLVAP*'s importance in EC barrier function in the gut.

INTRODUCTION

Protein-losing enteropathy (PLE) is caused by damage to the intestinal mucosa or endothelial cells (ECs), leading to increased permeability and consequent protein loss.¹ Patients with PLE usually present with hypoalbuminaemia and peripheral or generalised oedema, with or without diarrhoea. Hypogammaglobinaemia and other deficiencies may also be observed, depending on severity and mechanism of the underlying pathology.¹

Most PLE cases are secondary to common diseases, whether of intestinal origin (eg, inflammatory bowel

disease, coeliac, intestinal infection) or extraintestinal (eg, systemic lupus erythematosus, congestive heart failure, hepatic disease); rare disorders, such as primary intestinal lymphangiectasia, should also be considered in the differential diagnosis.¹ Identification of the PLE aetiology enables treatment of the underlying cause, such as diuretics in congestive heart failure or immunosuppressants in inflammatory bowel disease.¹

In recent years, several rare genetic disorders that include familial PLE have been described. Compiling evidence from independent families supports the causality of newly identified genes and enables refinement of the phenotypic spectrum, as observed in other PLE-related genes, for example, *CCBE1*, *FAT4* and *ADAMTS3* in Hennekam lymphangiectasia-lymphoedema syndrome (MIM #235510), *CD55* in complement hyperactivation, angio-pathic thrombosis, and protein-losing enteropathy (CHAPLE) syndrome (MIM #226300) and *DGAT1* in congenital diarrhoea with PLE (MIM #615863).^{2–6}

Recently, two patients have been described with severe congenital PLE attributed to homozygous nonsense variants in the plasmalemma vesicle-associated protein gene (*PLVAP*).^{7,8} Here, we provide a third independent report linking a *PLVAP* variant with PLE, demonstrating that rare missense pathogenic variants can also cause PLE, although with a milder phenotype compared with the null allele.

MATERIALS AND METHODS

Study participants

Patients were evaluated by a multidisciplinary team at Rambam Health Care Campus, including a paediatric gastroenterologist, internal medicine experts and medical geneticists. Following study approval by the Institutional Helsinki committee, written informed consent was obtained for all participants and blood samples were obtained for DNA extraction and molecular analyses.

Genetic analysis

DNA samples of patients III-1 and IV-2 (figure 1A), healthy parents (II-1, III-5 and III-6) and a healthy sibling (III-2) were subjected to whole exome sequencing (WES) in collaboration with Regeneron Genetics Center (RGC). Sequencing was performed on the Illumina HiSeq2500 platform (Illumina, San



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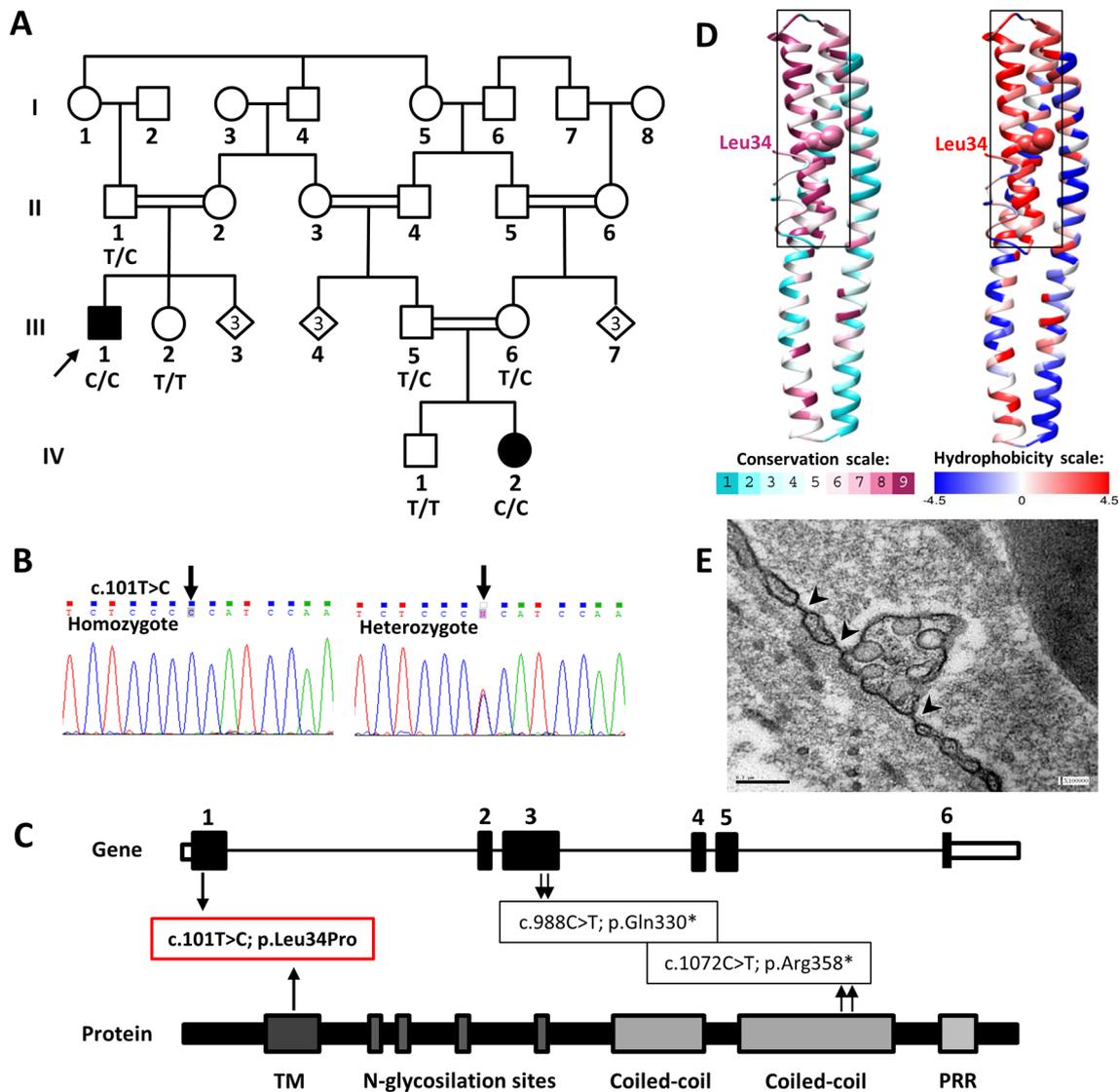


Figure 1 A missense variant in plasmalemma vesicle-associated protein (PLVAP) segregates with protein-losing enteropathy (PLE) in a consanguineous family. (A) Pedigree of the extended family and genotypes of individuals tested for the c.101T>C variant. (B) Sequence chromatogram of the c.101T>C variant in a homozygous patient and heterozygous parent. (C) Structure of the *PLVAP* gene (top) and PLVAP (PV-1) protein (bottom), denoting the location of reported variants: the c.101T>C;p.Leu34Pro missense variant identified in this study, and the nonsense pathogenic variants described previously by Elkadri *et al*⁷ and Broekaert *et al*⁸ both occurring in the third exon and truncating the protein in the second coiled-coil domain. TM, transmembrane; PRR, proline-rich region. (D) PLVAP protein *ab initio* structure prediction and modelling by QUARK suggest that the Leu34 residue is located in a highly conserved and highly hydrophobic α -helical region. (E) Electron micrograph of duodenal biopsy from patient IV-2 shows preserved endothelial fenestral diaphragms (arrowheads) ($\times 100000$, scale bar 0.2 μ m).

Diego, California, USA) using the IDT xGen exome capture reagent (Integrated DNA Technologies, Coralville, Iowa, USA) and methods previously described.⁹ Parallel bioinformatics and analytical pipelines developed at the RGC and by Genoox data analysis platform (Tel Aviv, Israel) served for read mapping and alignment to the human reference genome, variant calling, annotation and data analysis.

We filtered WES data according to variant quality and frequency, eliminating variants with minor allele frequency >0.01 in public population databases (1000Genomes, dbSNP, ESP variant server, ExAc and gnomAD browsers and the Greater Middle-East Variome project),^{10–13} as well as in the internal Rambam Genetics Institute database, representing the Israeli population, and the RGC database. The remaining variants were filtered based on impact on the encoded protein (missense, nonsense, frameshift and splice site) and analysed under an autosomal

recessive disease model (homozygous or compound heterozygous in the patients, heterozygous in parents and either heterozygous or wild type in the healthy sister). Segregation analysis was performed for candidate variants by Sanger sequencing.

Computational analysis tools

The p.Leu34Pro variant was assessed with several sequenced-based pathogenicity predictors: PolyPhen2,¹⁴ SIFT,¹⁵ MutationTaster,¹⁶ Align-GVGD,¹⁷ Panther HMM,¹⁸ PredictSNP1,¹⁹ GenoCanyon²⁰ and fitCons.²¹ We also built a low-resolution three-dimensional (3D) structure model of PLVAP using QUARK, an *ab initio* 3D structure prediction method, which is employed for proteins that do not have homologous templates in the protein database (PDB) library, as is the case of PLVAP.²² Based on the best QUARK model, we assessed the impact of

p.Leu34Pro on protein stability, secondary structure, conservation and hydrophobicity with ConSurf,²³ I-Mutant3.0,²⁴ STRUM,²⁵ JPred,²⁶ Phyre2²⁷ and HOPE tools.²⁸

Electron microscopy (EM)

Duodenal biopsy from patient IV-2 was fixed in 3.5% glutaraldehyde in sodium cacodylate buffer, pH 7.2. Tissue was further processed in 2% OsO₄, followed by 4% uranyl acetate, dehydrated and embedded in Epon resin. Sections of 50 nm were obtained using Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). Sections were examined under JEOL JEM-1011 transmission electron microscope (JEOL, Peabody, Massachusetts, USA).

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded 4 µm sections of duodenal biopsies from both patients and two age-matched and sex-matched controls were immunostained with rabbit polyclonal anti-PLVAP antibody (NBP1-83911; Novus Biologicals, Littleton, Colorado, USA) and diluted 1:500, using BenchMark ULTRA autostainer (Ventana Medical Systems, Oro Valley, Arizona, USA).

RESULTS

Clinical report

Two patients (III-1 and IV-2, [figure 1A](#))—first cousins once removed from a Muslim Arab consanguineous kindred—presented with anasarca, severe hypoalbuminaemia (~0.5 g/dL) and hypogammaglobinaemia at ages 22 and 2.5 years, respectively; patient IV-2 also had highly elevated triglycerides (2800 mg/dL). There was no evidence of proteinuria and both patients were diagnosed with PLE. They are managed with a low-fat diet and middle-chain triglyceride-rich formula, with significant clinical and laboratory improvement, allowing them to resume normal lives. Exposure to high fat in their diet leads to rapid recurrence of disease symptoms. The full clinical characteristics are summarised in [table 1](#).

Variant discovery

WES analysis yielded a single homozygous candidate variant: c.101T>C in the first exon of *PLVAP* (NM_031310.2) shared by both patients ([figure 1B](#)), which was absent from all public databases (minor allele frequency (MAF)=0.00) and appeared in our internal database in heterozygous state in one unrelated individual, also of Muslim Arab descent (MAF=0.001). This variant causes a substitution of a highly conserved leucine with proline at position 34 of the protein, which is located within the transmembrane domain ([figure 1C](#)). The variant was confirmed by Sanger sequencing and co-segregated with the disease in the family ([figure 1A](#)). No pathogenic variants in other PLE-related genes, including *CCBE1*, *FAT4*, *ADAMTS3*, *CD55* and *DGAT1*, were observed in WES of either patient. The rare homozygous variants for each patient are detailed in the online supplementary tables S1 and S2.

Computational analysis of p.Leu34Pro

The p.Leu34Pro variant was predicted deleterious by multiple pathogenicity prediction tools: PolyPhen2 – probably damaging (1.00), SIFT—deleterious (0.00), MutationTaster—disease causing (1.00), Align-GVGD—class C65 (most likely to interfere with function; GV: 0.00, GD: 97.78), Panther HMM—probably damaging (455), PredictSNP1—deleterious (87%), GenCanyon—deleterious (1.00) and fitCons—deleterious (0.55).

This consensus among eight different predictors supports an accurate prediction when compared with individual tools.¹⁹

Additional information about the structural impact of p.Leu34Pro variant was obtained by low-resolution 3D structure modelling of PLVAP using QUARK. The QUARK algorithm produced several models, which place Leu34 residue in a highly conserved and highly hydrophobic α -helical region ([figure 1D](#)). This information indicates that Leu34 is located in a functional region, that, given its high hydrophobicity and α -helical secondary structure, could be involved in protein–protein interactions, protein self-assembly or protein–membrane interaction. These physicochemical and 3D information suggest that Leu34 occupies a central location in the α -helix organisation, indicating that almost any mutation in this position will have functional and/or structural impact on the protein. More specifically, substitution of leucine by proline, a more polar and rigid residue, could compromise the α -helical packing, inducing structural and/or functional disturbances at the molecular level of the PLVAP protein.²⁹ Moreover, protein fold stability analysis of QUARK model with I-Mutant-3.0 and STRUM predicted a destabilising effect with $\Delta\Delta G$ of -1.27 and -2.09 kcal/mol, respectively. In summary, the extensive *in silico* analysis of p.Leu34Pro suggests that this variant will have a deleterious effect on protein folding and physicochemical characteristics, with probable disruption of protein function.

Fenestral diaphragms on duodenal biopsies

Electron micrograph of duodenal biopsy from patient IV-2 ([figure 1E](#)), as well as immunostaining of duodenal biopsies from both patients with anti-PLVAP antibody (online supplementary [figure S1](#)), revealed preserved endothelial fenestral diaphragms. No extracellular lipid droplets were detected in the biopsy of patient IV-2.

DISCUSSION

We describe a consanguineous family in which two patients with PLE are homozygous for a missense variant (c.101T>C; p.Leu34Pro) in *PLVAP*.

PLVAP (PV-1) is an endothelium-specific membrane-bound homodimeric glycoprotein. It is essential for the formation of fenestral diaphragms in blood vessels and lymphatic capillaries, in addition to other roles, such as leucocyte migration and angiogenesis. Therefore, PLVAP plays a central role in tissues, including the intestine, where absorption of interstitial molecules into the blood or lymphatics occurs.^{30–31} PLVAP diaphragms regulate EC permeability by providing a filtration system at the sinus-parenchyma barrier, which is highly dependent on molecule size, that is, only molecules smaller than ~70 kDa or ~30 nm can pass. Thus, PLVAP diaphragms maintain blood, lymph and tissue homeostasis, and block passage of macromolecules and pathogens.^{31–32} As expected, *Plvap*-deficient mice exhibit loss of fenestral diaphragms, causing increased EC permeability and leakage of protein-rich lymphatic fluid into the intestinal lumen and the peritoneum; the mice manifest with severe progressive PLE, generalised oedema and hypertriglyceridaemia, with decreased survival.^{31–33} The hypertriglyceridaemia in these mouse models was attributed to lipoprotein lipase depletion on ECs, hindering triglyceride hydrolysis and leading to increased plasma concentration of chylomicron remnants.^{7 31 34}

To date, two reports of patients with PLE with homozygous nonsense variants in *PLVAP* have been published; both patients presented in early infancy and their phenotype well-recapitulated the phenotype observed in knockout mouse models, including

Genotype-phenotype correlations

Table 1 Clinical characteristics of patients with *PLVAP* pathogenic variants

	Current study		Elkadri <i>et al</i> ⁷	Broekaert <i>et al</i> ⁸
	Patient 1 (III-1)	Patient 2 (IV-2)		
Gender	Male	Female	Male	Male
Age at onset	22 years	2.5 years	8 days	Prenatal, diarrhoea at 9 days
Consanguinity	Yes	Yes	Yes	Yes
Pathogenic variant	c.101T>C;p.Leu34Pro	c.101T>C;p.Leu34Pro	c.1072C>T;p.Arg358*	c.988C>T;p.Gln330*
Zygosity	Homozygous	Homozygous	Homozygous	Homozygous
Affected protein domain	Transmembrane domain	Transmembrane domain	Second coiled-coil domain	Second coiled-coil domain
Clinical characteristics				
Protein-losing enteropathy	Yes	Yes	Yes	Yes
Diarrhoea	Yes, resolved	No	Severe, haematochezia	Severe, haematochezia
Oedema/effusion	Anasarca, pleural and pericardial effusions	Anasarca	Anasarca, severe	Anasarca, moderate
Albumin (3.4–5 g/dL)	0.5 (L)	0.4 (L)	L	L
Total protein (6–8.2 g/dL)	3.1 (L)	3 (L)	L	NA
Triglycerides (30–170 mg/dL)	113 (N)	2800 (H)	H	NA
Total cholesterol (140–200 mg/dL)	305 (H)	383 (H)	N	NA
HDL cholesterol (35–80 mg/dL)	30 (N)	10 (L)	N	NA
Non-HDL cholesterol (100–170 mg/dL)	275 (H)	373 (H)	NA	NA
IgG (680–1560 mg/dL)	311 (L)	111 (L)	L	NA
IgA (70–350 mg/dL)	193 (N)	57.2 (L)	L	NA
IgM (40–280 mg/dL)	158 (N)	110 (N)	N	NA
Proteinuria	No	No	No	No
Abdominal imaging	Inflamed intestines with abdominal lymphadenopathy and mild ascites on CT	Moderate ascites without lymphadenopathy or enlarged intestines on US	NA	NA
Endoscopy	Duodenitis with duodenal ulcer, red and oedematous mucosa in proximal jejunum, nodular mucosa in terminal ileum, normal colon	Normal	Oedematous duodenum and stomach with normal colon	NA
Histopathology	Several focal lymphangiectasias in terminal ileum and ileo-cecal valve mucosa	Normal	Interstitial oedema	NA
Other	None	None	Dysmorphism, metabolic acidosis, lethargy, hyponatraemia, bilateral coloboma, dysplastic kidneys, hypothyroidism, venous thrombosis	Prenatal findings: cystic hygroma, nuchal and frontal oedema, polyhydramnios, cardiac white spot and postnatal findings: dysmorphism, metabolic acidosis, electrolyte deficiencies, elevated GGT, choroid plexus cysts, iris cysts, ASD, VSD, dilated megareuter with dilated renal pelvis, venous thrombosis
Management	Low-fat diet with MCT-rich supplementation (Nutren 2.0)	Low-fat diet with MCT-rich supplementation (Monogen)	NA	Total parenteral nutrition

ASD, atrial septal defect; GGT, gamma-glutamyltransferase; H, high; HDL, high-density lipoprotein; L, low; MCT, middle-chain triglycerides; N, normal, NA, not available; PLVAP, plasmalemma vesicle-associated protein; US, ultrasound; VSD, ventricular septal defect.

loss of fenestral diaphragms as observed on EM of intestinal biopsies, and clinical findings of early-onset anasarca, hypoalbuminaemia and hypertriglyceridaemia.^{7,8} The patients we describe here presented with a similar, although milder and with later-onset age, phenotype of PLE and partial penetrance of hypertriglyceridaemia. The overlap in symptoms, the segregation of the c.101T>C;p.Leu34Pro variant within the family (as expected for an autosomal recessive disease) and it being the only rare variant shared between both patients, are strong evidence supporting pathogenicity. The later onset of symptoms in our patients could be attributed to the missense nature of the pathogenic variant we describe (p.Leu34Pro), compatible with residual PLVAP

function. Leucine and proline have several different biochemical and biophysical characteristics, for example, leucine is more hydrophobic and larger than proline. In addition, proline has several unique characteristics compared with other amino acids, such as its conformational rigidity, the tendency to disrupt or improve α -helical packing³⁵ and the ability to exist in both trans- and cis-configurations, all of which may affect protein folding, protein-membrane interaction and more. Therefore, the leucine-to-proline substitution is likely to affect the polarity, packing and organisation of the α -helix encompassing this highly conserved region (figure 1D). This is predicted to impact protein stability and protein structure and/or function within the EC membrane,

likely leading to a functional deleterious effect on the barrier of the fenestral diaphragms. The presence of apparently normal fenestral diaphragms on EM and IHC from a patients' duodenal biopsies (figure 1E, online supplementary figure S1) suggests that p.Leu34Pro is compatible with synthesis and localisation of mutant PLVAP in the fenestrae, likely allowing residual function that accounts for the milder phenotype in comparison to patients with null variants. Additional studies are required to assess the possible effect on protein stability and barrier functions of the fenestral diaphragms composed of mutant PLVAP.

Interestingly, intrafamilial disease heterogeneity is present. Disease onset in the proband (III-1) was in early adulthood, whereas patient IV-2 had childhood onset of PLE. Moreover, patient IV-2 had severe hypertriglyceridaemia, with plasma concentrations 16-fold higher than normal range, similar to the first reported PLVAP-null patient,⁷ while the proband had normal serum triglycerides; triglyceride levels were not reported in Broekaert *et al.*⁸ This phenotypic variability suggests the presence of other modifying factors. Review of the proband's WES results revealed that he carries a heterozygous nonsense variant in *CETP* (NM_000078.2:c.544C>T;p.Gln182*), which encodes the cholesteryl ester transfer protein, while patient IV-2 is wild type. CETP is involved in cholesteryl ester and triglyceride transfer among lipoproteins and promotes cholesterol uptake by the liver; heterozygous and homozygous variants in this gene have been associated with hyperalphalipoproteinaemia, characterised by elevated high-density lipoprotein concentration and particle size.³⁶ CETP loss-of-function in the proband may have affected the serum triglyceride and lipoprotein metabolism, which may have contributed to his normal triglyceride levels compared with other patients with PLVAP-PLE. Moreover, the reduced lipid load in the proband may have led to the reduction in the chylomicron burden on the intestines, allowing for the late disease onset. This theory is supported by the fact that a low-fat diet attenuates the symptoms in both patients, while high-fat diet exacerbates the PLE phenotype. Discovery of additional PLVAP-associated PLE patients will allow the assessment of genotype-phenotype correlations, as well as evaluation of modifying effects of other genes.

In conclusion, we provide evidence that missense variants in PLVAP in humans lead to an attenuated PLE syndrome characterised by later onset, accompanied by generalised oedema and high serum triglyceride concentration. This underscores the importance of PLVAP diaphragms in maintaining EC permeability and vascular and intestinal homeostasis.

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and HNB drafted the manuscript. All coauthors critically reviewed and approved the final version of the submitted manuscript.

Competing interests None declared.

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