Published in final edited form as:

*Am J Gastroenterol*. 2019 June ; 114(6): 974–983. doi:10.14309/ajg.0000000000000051.

# Protease-sensitive pancreatic lipase (*PNLIP*) variants are associated with early onset chronic pancreatitis

**Denise Lasher**[1,16], **András Szabó**[2,3,16], **Atsushi Masamune**[4,16], **Jian-Min Chen**[5,16], **Xunjun Xiao**[6], **David C. Whitcomb**[7], **M. Michael Barmada**[8], **Maren Ewers**[1], **Claudia Ruffert**[9], **Sumit Paliwal**[10,11], **Prachand Issarapu**[10], **Seema Bhaskar**[10], **K Radha Mani**[10], **Giriraj R Chandak**[10], **Helmut Laumen**[1], **Emmanuelle Masson**[5], **Kiyoshi Kume**[4], **Shin Hamada**[4], **Eriko Nakano**[4], **Katharina Seltsam**[9], **Peter Bugert**[12], **Thomas Müller**[13], **David A. Groneberg**[14], **Tooru Shimosegawa**[4], **Jonas Rosendahl**[9,15], **Claude Férec**[5], **Mark E. Lowe**[6], **Heiko Witt**[1,17], and **Miklós Sahin-Tóth**[2,17]

[1]Else Kröner-Fresenius-Zentrum für Ernährungsmedizin (EKFZ), Paediatric Nutritional Medicine, Technische Universität München (TUM), Freising, Germany

[2]Center for Exocrine Disorders, Department of Molecular and Cell Biology, Boston University Henry M. Goldman School of Dental Medicine, Boston, MA 02118, USA

[3]Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

[4]Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan

[5]UMR1078 “Génétique, Génomique Fonctionnelle et Biotechnologies”, INSERM, EFS - Bretagne, Université de Brest, CHRU Brest, Brest, France

[6]Department of Pediatrics, Washington University School of Medicine, St. Louis, MO 63110, USA

[7]Departments of Medicine, Cell Biology & Molecular Physiology, and Human Genetics, University of Pittsburgh and UPMC, Pittsburgh, PA, USA

[8]Departments of Human Genetics, University of Pittsburgh, Pittsburgh, PA, USA (MMB deceased December 2, 2016)

**Corresponding authors:** Heiko Witt, M.D., Paediatric Nutritional Medicine, Else Kröner-Fresenius-Zentrum für Ernährungsmedizin (EKFZ), Technische Universität München (TUM), Gregor-Mendel-Str. 2 85354 Freising, Germany Tel.: +49 8161 71 2466 Fax: +49 8161 71 2465, heiko.witt@tum.de, Miklós Sahin-Tóth, M.D., Ph.D., Department of Molecular and Cell Biology, Boston University Henry M. Goldman School of Dental Medicine, Boston University Medical Campus, 72 East Concord Street, Evans-433, Boston, MA 02118, USA, Tel.: +1 617 358 3790, Fax: +1 617 414 1041, miklos@bu.edu.

AUTHOR CONTRIBUTIONS

HW and MST conceived, designed and directed the study. DL, AM, ME, CR, KK, SH and EN performed genotyping and interpreted genetic analyses. MST, AS and MEL designed and supervised functional analyses. AS and XX carried out functional analyses. All other co-authors recruited study subjects, collected clinical data and/or provided genomic DNA samples or genotype data. HW and MST drafted and revised the manuscript with substantial help from MEL. All authors approved the final manuscript and contributed critical revisions to its intellectual content.

ACCESSION CODES

The genomic sequence of pancreatic lipase (*PNLIP*) used was ENSG00000175535 (GRCh38:CM000672.2) (Ensemble).

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

[9]Department of Internal Medicine, Neurology and Dermatology, Division of Gastroenterology, University of Leipzig, Leipzig, Germany

[10]Genomic Research on Complex diseases (GRC Group), CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India

[11]Epithelial Carcinogenesis Group, Cancer Cell Biology Programme, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain

[12]Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, Heidelberg University, German Red Cross Blood Service of Baden-Württemberg, Mannheim, Germany

[13]Department of Pediatrics I, Medical University, Innsbruck, Austria

[14]Institute of Occupational Medicine, Social Medicine and Environmental Medicine, Goethe-University, Frankfurt, Germany

[15]Department of Internal Medicine I, Martin Luther University, Halle, Germany

[16]These authors contributed equally to this work

[17]These authors jointly supervised the work

## Abstract

**Objectives.—**Premature activation of the digestive protease trypsin within the pancreatic parenchyma is a critical factor in the pathogenesis of pancreatitis. Alterations in genes that affect intra-pancreatic trypsin activity are associated with chronic pancreatitis (CP). Recently, carboxyl ester lipase (CEL) emerged as a trypsin-independent risk gene. Here, we evaluated *PNLIP* encoding pancreatic lipase as a potential novel susceptibility gene for CP.

**Methods.—**We analyzed all 13 *PNLIP* exons in 429 German non-alcoholic CP patients and in 600 German control subjects, in 632 patients and 957 controls from France, and in 223 patients and 1070 controls from Japan by DNA sequencing. Additionally, we analyzed selected exons in further 545 CP patients and 1849 controls originating from Germany, USA and India. We assessed the cellular secretion, lipase activity and proteolytic stability of recombinant PNLIP variants.

**Results.—**In the German discovery cohort, 8/429 (1.9%) patients and 2/600 (0.3%) controls carried a *PNLIP* missense variant ($P$=0.02, OR=5.7, 95% CI=1.1–38.9). Variants detected in patients were prone to proteolytic degradation by trypsin and chymotrypsin. In the French replication cohort, protease-sensitive variants were also enriched in patients with early-onset CP (5/632 [0.8%]) versus controls (1/957 [0.1%]) ($P$=0.04, OR=7.6, 95% CI=0.9–172.9). In contrast, we detected no protease-sensitive variants in the non-European populations. In the combined European data, protease-sensitive variants were found in 13/1163 cases (1.1%) and in 3/3000 controls (0.1%) (OR=11.3, 95% CI=3.0–49.9, $P$<0.0001).

**Conclusions.—**Our data indicate that protease-sensitive PNLIP variants are novel genetic risk factors for the development of chronic pancreatitis.

### Keywords

chronic pancreatitis; pancreatic lipase; hereditary pancreatitis; digestive enzyme mutations

## INTRODUCTION

Chronic pancreatitis (CP) is a continuing or relapsing inflammatory disorder characterized by progressive destruction of the pancreatic parenchyma. As a consequence, in advanced stages of the disease, maldigestion and diabetes mellitus develop due to exocrine and endocrine insufficiency of the gland [1]. Development and progression of a single episode of acute pancreatitis to recurrent acute pancreatitis (RAP) and CP is frequently driven by alcohol abuse or genetic risk factors, especially in early-onset disease.

Studies on the genetic basis of non-alcoholic CP identified variants mostly in digestive proteases or their inhibitor. Some of these mutations were primarily detected in patients with a strong family history of pancreatitis (hereditary CP), whereas other variants were mainly found in patients without any prominent etiological factors including alcohol and heredity (idiopathic CP). So far, genetic alterations in cationic trypsinogen (serine protease 1, *PRSS1*), anionic trypsinogen (serine protease 2, *PRSS2*), serine protease inhibitor Kazal type 1 (*SPINK1*), chymotrypsinogen C (*CTRC*), carboxypeptidase A1 (*CPA1*), and chymotrypsinogen B1 and B2 (*CTRB1-CTRB2*) have been found to predispose to or protect from CP [2–7]. In addition to protease and protease-inhibitor genes, mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, in the claudin 2 (*CLDN2*) locus and a hybrid allele of carboxyl ester lipase (*CEL*) were found significantly enriched in patients with CP [8–11].

A substantial fraction of patients with non-alcoholic CP, even in the presence of a strong family history, do not carry mutations in the above-mentioned genes, indicating that defects in additional genes contribute to disease pathogenesis. In the present study, we investigated the role of pancreatic lipase (PNLIP) in the development of CP. The *PNLIP* gene is located on chromosome 10, contains 13 exons and spans approximately 22 kB [12]. We considered *PNLIP* a likely candidate for a pancreatitis risk gene because it is expressed solely and abundantly in the exocrine pancreas. Furthermore, the discovery that genetic alterations in *CEL* increase CP risk highlighted the potential role of pancreatic lipases in CP susceptibility [11, 13].

## METHODS

### Study population.

The medical ethical review committees of all participating study centers approved this study. All study subjects gave informed consent. The non-alcoholic CP study cohorts included patients with a history of RAP and/or pathological imaging findings consistent with CP. We enrolled 531 unrelated German individuals with non-alcoholic CP (429 recruited in Munich and 102 recruited in Halle; 289 females; mean age ± S.D. 13.0 ± 6.8 years; age range: 0–30 years). In the replication study, we investigated 632 unrelated non-alcoholic CP patients originating from France (294 females; mean age ± S.D. 13.7 ± 5.7 years; age range: 0–20 years). We also investigated unrelated subjects affected with non-alcoholic CP from the United States ($n$ = 143), India ($n$ = 300) and Japan ($n$ = 223). Control subjects were recruited from Germany ($n$ = 2043), France ($n$ = 957), the United States ($n$ = 168), India ($n$ = 238), and Japan ($n$ = 1070) [14]. In addition, we compared the genetic data with the Exome

Aggregation Consortium (ExAC, http://exac.broadinstitute.org) and the 1000 Genomes Project (http://www.internationalgenome.org).

### Mutation screening.

Oligonucleotide sequences, PCR and cycle sequencing conditions are described in the Supplementary material.

### Plasmid construction and mutagenesis.

The expression plasmid pcDNA3.1(–) PNLIP was described earlier [15]. For this study, we engineered a 10-His tag to the C terminus of PNLIP. *PNLIP* mutations were generated by overlap extension PCR mutagenesis and cloned into the expression vector using EcoRI and HindIII restriction sites. The pTrapT7 PRSS1 plasmid carrying the coding DNA for human cationic trypsinogen, the pcDNA3.1(–) CTRB2 plasmid harboring the coding DNA for human chymotrypsinogen B2 with a C-terminal 10-His tag and the pcDNA3.1(–) CTRC plasmid encoding human chymotrypsinogen C with a C-terminal 10-His tag were described previously [16–18].

### Expression and purification of PNLIP.

Recombinant PNLIP was expressed in human embryonic kidney (HEK) 293T cells. Cells were grown in 75 $cm^2$ tissue culture flasks in 20 mL DMEM with 4.5 g/L glucose, 4 mM L-glutamine, 10% fetal bovine serum and 100 U/mL penicillin-streptomycin (final concentrations) in 5% $CO_2$ water jacketed incubator at 37 °C. At 70–90% confluence the cells were transfected with 30 μg plasmid DNA and 75 μL Lipofectamine 2000 in 20 mL DMEM medium with supplements. The cells were rinsed with 5 mL Opti-MEM after 16–20 h incubation, and covered with 20 mL Opti-MEM containing 100 U/mL penicillin-streptomycin. After 48 h incubation, the conditioned medium was harvested and the cells were covered with fresh Opti-MEM containing antibiotics and the medium was collected again after additional 48 h incubation. A typical protein expression experiment was performed with 5 tissue culture flasks. The harvested media were pooled and filtered with Steritop bottle top filters (Merck) to remove debris. PNLIP were purified from the conditioned media using a 5 mL Ni-NTA superflow affinity cartridge (Qiagen) attached to an Akta Purifier FPLC system. After sample loading, the cartridge was washed with 100 mL of 50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole (pH 8.0) to remove weakly binding proteins. PNLIP proteins were eluted with 50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole (pH 8.0) and 5 mL fractions were collected. Fractions containing pure PNLIP were pooled and dialyzed against 3 × 3 L of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl with a 10,000 Da molecular weight cutoff (MWCO) Spectra/Por Float-A-Lyzer Dialysis Device (Spectrum Labs). PNLIP samples were concentrated with a Vivaspin 2 Centrifugal Concentrator (MWCO 10,000) to a final volume of 1.5–2.0 mL. The concentration of PNLIP samples was determined from their ultraviolet absorbance at 280 nm using the molar extinction coefficient value 61,725 $M^{-1}$ $cm^{-1}$ with the exception of PNLIP mutants p.H42Y, p.D72Y and p.D264Y (63,215 $M^{-1}$ $cm^{-1}$), p.Y58H (60,235 $M^{-1}$ $cm^{-1}$), p.C198Y (63,090 $M^{-1}$ $cm^{-1}$) and p.C254R (61,600 $M^{-1}$ $cm^{-1}$). The concentration of PNLIP samples was typically 5.0–8.0 μM.

### Expression, purification and activation of human pancreatic proteases.

Human cationic trypsinogen was expressed in *E. coli* BL21(DE3) and purified with ecotin affinity chromatography [19]. Trypsinogen was activated with recombinant human enteropeptidase and the active trypsin concentration was determined by titration against ecotin.

CTRB2 and CTRC were expressed in HEK 293T cells, as described for PNLIP above. The His-tagged protease precursors were purified with a 5 mL Ni-NTA superflow cartridge on an Akta Purifier FPLC system. The protease precursors were eluted with 50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole (pH 8.0) and 5 mL fractions were collected. Fractions containing pure target proteins were pooled and dialyzed against 3 × 3 L of 100 mM Tris-HCl (pH 8.0), 150 mM NaCl; and concentrated with a Vivaspin 2 Centrifugal Concentrator (MWCO 10,000). Protease precursors were activated with trypsin beads, which were later removed by centrifugation. The concentration of active CTRB2 and CTRC was determined by titration against ecotin.

### Digestion of PNLIP with pancreatic proteases.

Purified wild-type and mutant PNLIP (2 μM) were incubated at 37 °C with 200 nM human PRSS1, CTRC or CTRB2 in 0.1 M Tris-HCl (pH 8.0) and 1 mM $CaCl_2$ (final concentrations). At the indicated times, aliquots (100 μL) were precipitated with trichloroacetic acid (10%) and analyzed by SDS-PAGE, Coomassie Blue staining and densitometry.

### SDS-PAGE and densitometry.

PNLIP samples were precipitated with 10% trichloroacetic acid (final concentration) and incubated for 5–10 min on ice. The PNLIP precipitate was recovered with centrifugation at 13,000 rpm for 10 min in a tabletop microcentrifuge and solubilized in 15 μL Laemmli sample buffer supplemented with 100 mM dithiotreitol. After heat denaturation at 95 °C for 5 min, proteins were electrophoresed on 15% polyacrylamide minigels followed by Coomassie Blue R250 staining. After destaining with 30% methanol and 10% acetic acid solution, the gels were dried between two layers of cellophane and scanned. Quantitation of bands was performed with the Quantity One 4.6.9 (Bio-Rad) software. Rectangles were drawn around the bands and isometric rectangles placed within the same lanes were used for background subtraction.

### Enzyme activity measurement of PNLIP.

Lipase activities of wild-type PNLIP and variant proteins were assayed at room temperature with the standard 5-min pH-stat method as previously described [20, 21]. The assays included an emulsion of tributyrin (114 mM), trioctanoin (27 mM), or triolein (6.9 mM) with 4 mM sodium taurodeoxycholate, in a total volume of 15 mL. Two μg of lipase was added (2.6 nM final concentration) with or without a 5-fold molar excess (2 μg, 13 nM) of purified recombinant human colipase [20]. The lipolytic activities are expressed in international lipase units per mg of enzyme. One unit corresponds to one μmol of fatty acids released per min.

### Statistics.

The significance of the differences between mutation frequencies in affected individuals and controls was tested by two-tailed Fisher's Exact Test. Additional odds ratios (OR) were calculated using SAS/STAT software (v 9.1) and GraphPad Prism (v 4.03).

## RESULTS

### European discovery and replication cohorts.

In the German discovery cohort, we sequenced all coding exons and the flanking intronic regions in 429 cases with non-alcoholic CP and in 600 control subjects. We found heterozygous missense variants in 8/429 (1.9%) cases and in 2/600 (0.3%) controls (Table 1). All cases with missense variants had early-onset disease (mean age 12.3 years). The most frequently found variant was p.F300L (*rs890551695*), which was present in four cases but not in controls. To replicate the findings in an independent European cohort, we sequenced *PNLIP* in 632 early-onset (≤20 years) non-alcoholic CP cases and 957 controls of French origin (Table 2). Heterozygous missense variants were present in 10/632 (1.6%) cases and in 8/957 (0.8%) controls. The only variant that was detected in both cases and controls was p.I265R (*rs377358755*). Other variants were detected only once with the exception of p.F300L, which was present in three cases but not in controls.

When the two European cohorts were combined, missense variants were found in 18/1061 cases (1.7%) versus in 10/1557 controls (0.6%), indicating a slight enrichment in the patient population ($P$=0.01, OR=2.7, 95% CI=1.2–6.2). Remarkably, however, most variants were unique to either the patient or the control cohorts, suggesting that variants associated with pancreatitis might be functionally damaging whereas variants found in controls may be functionally innocuous. The exclusive occurrence of the p.F300L variant among early-onset cases (7/1061, 0.7%, $P$=0.002) was particularly striking in this regard.

### Non-European cohorts.

We also sequenced *PNLIP* in 223 subjects with non-alcoholic CP from Japan (Supplementary Table 2). As controls, we used data from the Tohoku Medical Megabank Organization (ToMMo) database, which contains exome sequencing results for 1070 healthy Japanese individuals. In contrast to the European cohorts, all missense variants found in the Japanese cases were also detected among controls without a significant enrichment in either cohort. If we excluded the relatively common p.I186T variant (*rs78536862*), missense variants were detected in 4/223 (1.8%) cases and in 35/1070 (3.3%) controls ($P$=0.29). Only one missense variant, p.V307I (*rs773774916*), was present in both the Japanese and European cohorts. Notably, the p.F300L variant was absent in the Japanese subjects analyzed. To investigate the frequency of the p.F300L variant in additional cohorts, we sequenced exons 7, 8, 9 and the flanking intronic regions in *PNLIP* in 300 cases and 238 controls of Indian origin and 143 cases and 168 controls in a cohort from the United States. No missense variants were found in these two cohorts.

### Functional effects of PNLIP variants.

To characterize the functional properties of the PNLIP variants identified in the European cohorts, we first determined their secretion from transiently transfected HEK 293T cells. The amount of secreted lipase in the conditioned medium was measured by SDS-PAGE and Coomassie Blue staining followed by densitometry of the lipase bands. As shown in Figure 1, the large majority (19 of 23) variants were secreted normally (>75% of wild-type levels) and only four variants exhibited markedly reduced secretion (<20% of wild-type). Secretion-defective variants were equally present in cases and controls. Thus, variants p.G233E (no rs#) and p.C254R (*rs750709623*) were each found once in French cases, whereas variants p.A174P (no rs#) and p.V454F (*rs148560679*) were found in a French and a German control, respectively (Tables 1, 2).

To test the effect of *PNLIP* variants on lipase activity, 20 of the 23 variants were purified from the conditioned medium of transfected HEK 293T cells and their activity was tested on three different triglyceride substrates with increasing chain length: tributyrin, trioctanoin, and triolein (Figure 2). Only variant p.H92N (*rs368162708*) exhibited impaired catalytic activity on all substrates. Variants p.C198Y (no rs#) and p.D264Y (*rs755039876*) readily hydrolyzed tributyrin and trioctanoin but showed diminished activity against triolein. All other variants exhibited high activity (≥50% of wild-type) on all three substrates. None showed significantly increased activity relative to wild-type PNLIP. Considering the three variants with low activity, all three were found once in French controls, indicating that loss of PNLIP activity does not alter pancreatitis risk. Since the variants are heterozygous, clinical lipase deficiency should not occur in these carriers.

PNLIP undergoes proteolytic degradation and loss of activity during intestinal transit [22, 23]. To test whether the mutations alter the proteolytic stability of PNLIP, we first incubated wild-type PNLIP and 19 variants with human cationic trypsin and followed their degradation by SDS-PAGE and Coomassie Blue staining (Figure 3). For these experiments, we used lower protease-to-lipase concentration ratios than those present in the intestinal tract. Surprisingly, wild-type PNLIP was unaffected by trypsin while variants p.P245A (no rs#), p.I265R, p.F300L, p.S304F (no rs#), and p.F314L (no rs#) suffered proteolytic cleavage. The 14 other variants tested were as resistant to trypsin as wild-type PNLIP (Supplementary Figure 1). Strikingly, the same pattern emerged when PNLIP variants were digested with human chymotrypsin C (Figure 4) or human chymotrypsin B2 (Figure 5). The five trypsin-sensitive variants were readily cleaved by the human chymotrypsins whereas wild-type PNLIP and the other 14 variants were resistant or showed minimal digestion (Supplementary Figures 2 and 3).

To identify the proteolytic sites involved, the cleavage products of variants p.P245A and p.F300L were subjected to N-terminal sequencing by Edman degradation. Figure 6 demonstrates the cleavage sites deduced from the N-terminal sequencing data, which were identical in the two variants analyzed. Trypsin and chymotrypsin cleave PNLIP variants within the peptide segment composed of amino-acids 341–355, which is located in the N-terminal domain near the junction with the C-terminal domain of PNLIP (Figure 6). The five PNLIP variants seem to destabilize this region and render PNLIP sensitive to proteolytic

attack. Cleavage generates a stable C-terminal fragment whereas the N-terminal fragment suffers further degradation by chymotrypsin (Figures 3, 4, 5).

### Protease-sensitive PNLIP variants are associated with early onset chronic pancreatitis.

Variants p.P245A, p.I265R, p.F300L, p.S304F, and p.F314L that were prone to degradation by trypsin and chymotrypsin were all identified in the European cohorts (Tables 1 and 2). We extended the genetic analysis of exons 8 to 10 by sequencing additional 102 patients and 1443 control subjects originating from Germany. We found no new variants in patients but detected the p.I265R variant in two more controls. Taken together, Table 3 demonstrates that protease-sensitive variants were found in 13/1163 cases (1.1%) and in 3/3000 controls (0.1%). Enrichment of these variants among cases was more than 10-fold (OR 11.3, 95% CI 3.0 – 49.9, $P<0.0001$), indicating that protease-sensitive PNLIP variants are strong risk factors for CP.

Characteristics of the eight German and five French patients with protease-sensitive PNLIP variants are listed in Table 4. Remarkably, all carriers were children or young adults and all presented with RAP. None were diagnosed with CP yet and in three cases the referring physician indicated “mild disease” in their charts. With respect to family history of pancreatitis, one patient had a sister who carried a *PNLIP* variant and exhibited a similarly mild disease course. However, in the other 12 patients no family history was noted, indicating that *PNLIP* variants typically do not cause hereditary pancreatitis. Two patients carried mutations in other risk genes (*CFTR* and *SPINK1*) as well. As expected with pediatric patients, no history of smoking or alcohol abuse was documented.

## DISCUSSION

The pancreas synthesizes and secretes several lipases including phospholipase A2, carboxyl ester lipase (CEL), PNLIP and PNLIP related protein 2 (PNLIPRP2) [24]. PNLIP is critical for efficient digestion of dietary triglycerides. Because common constituents of the duodenum such as bile salts inhibit PNLIP, it requires another pancreatic protein, colipase, for activity [25]. The role of CEL and PNLIPRP2 in dietary fat digestion in humans is less clear. Animal data demonstrate that both contribute to dietary fat digestion in suckling newborns, but similar data in humans is scarce [26, 27]. Similarly, convincing data describing the function of CEL and PNLIPRP2 in adult animals or humans is minimal. CEL likely contributes to the digestion of cholesterol esters and fat-soluble vitamin esters [28]. Both may contribute to digestion of galactolipids based on *in vitro* activity [29].

To date, among the pancreatic lipases, only genetic variants in *CEL* have been demonstrated to alter risk for CP. Human CEL contains a domain with a variable number of tandem repeats at the carboxyl terminus. While the number of repeats does not influence risk for pancreatic disease, single base pair deletions in the repeat region cause CP with exocrine and endocrine insufficiency [13, 30]. Additionally, a recombined allele of *CEL* and its pseudogene *CELP* was found to confer risk to CP in Europeans while a different hybrid allele in Asians showed no association with CP [11, 31]. Although the role of *PNLIP* in CP has not been analyzed prior to this study, a homozygous missense variant was reported in two brothers with steatorrhea caused by PNLIP deficiency [32]. In cell culture experiments,

the variant caused PNLIP misfolding, loss of secretion and endoplasmic reticulum (ER) stress [15]. Interestingly, abnormal pancreatic function tests raised the possibility that the brothers may have had CP, suggesting a pathogenic role for PNLIP misfolding in CP.

In the present study, we extended our investigations into the potential role of lipases in CP and analyzed *PNLIP* by targeted DNA sequencing in European, Japanese, Indian and US cohorts. In the German discovery cohort, *PNLIP* missense variants, the p.F300L variant in particular, were significantly overrepresented in cases, however, a similar enrichment of missense variants and p.F300L did not reach significance in the French replication cohort. Missense *PNLIP* variants were found with higher frequency in controls versus patients in the Japanese replication cohort, but the difference was not significant. No missense variants were found in the Indian and US cohorts. All missense variants detected were subsequently subjected to functional analysis to identify alterations in folding, secretion, enzymatic activity and proteolytic degradation. Because *PNLIP* variants were rare and genetic data alone has been suggestive but inconclusive, we filtered the variants using various functional criteria, which might explain pathogenicity.

Mutations in digestive enzymes can alter their folding and result in reduced secretion with intracellular retention, degradation and resultant endoplasmic reticulum (ER) stress. This mechanism has been observed with a subset of PRSS1 variants and in pathogenic CPA1 variants [33]. Only four of 23 PNLIP variants showed reduced secretion consistent with the possibility of protein misfolding. Modeling these missense mutations in the structure of PNLIP suggests they significantly alter the structure. The Cys254 residue forms a disulfide bond with Cys278 at the base of the lid domain and loss of that bond would disrupt the structure of that domain [34]. Both p.G233E and p.A174P are near the active site and create steric hindrance affecting Asp193 and Ser169, respectively. These residues contribute to the catalytic triad. Finally, p.V454F could potentially alter the structure of the C-terminal domain including the β5’- loop, which contributes to substrate binding [35]. The distribution of these four variants was similar in patients (two variants) and controls (two variants) suggesting that the impact of PNLIP misfolding caused by heterozygous variants may not be sufficient to cause disease. This is likely due to the lower expression levels of PNLIP relative to CPA1 or PRSS1 in the pancreas [36].

We also speculated that increased lipase activity might contribute to CP onset. This does not seem to be the case, as only three PNLIP variants showed impaired catalytic activity and these were all found in control subjects. Two of these, p.C198Y and p.D264Y likely alter the structure of predicted acyl chain binding sites, which can explain why activity against triolein, a long-chain triglyceride was most affected. Mutation p.H92N, which showed impaired activity against all substrates, is predicted to alter the structure of the β5’-loop. The result confirms the importance of this region in the function of PNLIP. In a recent study of pancreatic cancer, the p.H92N variant was found in two affected subjects (0.1%) but not in healthy controls [37]. This represents a 17-fold enrichment over the ExAc database allele frequency. It was not reported if these two subjects had CP, a known risk factor for pancreatic cancer. Still, the findings suggest that variant with p.H92N may increase risk for pancreatic disease.

Finally, we considered the possibility that increased resistance to proteolytic degradation might lead to increased lipase activity. Such mechanism was described for the clinically most frequent PRSS1 variants such as p.R122H [17]. In contrast to our expectations, we found that five variants exhibited accelerated degradation by trypsin and chymotrypsin and these proteolytically unstable variants were almost exclusively detected in young European cases, yielding a respectable OR of about 11. Thus, protease-sensitive PNLIP variants are strong susceptibility factors for early-onset CP. These variants were absent in the Japanese, Indian and US cohorts. This resembles the distribution of the pathogenic *CEL* hybrid allele, which is frequent in Europeans but absent in Asian populations [11, 31].

The mechanism by which protease-sensitive PNLIP variants increase risk for CP is not readily apparent. One possibility is that increased proteolytic sensitivity is a marker for some other functional defect, e.g. altered protein dynamics resulting in a tendency to aggregate or form a complex with some other protein, perhaps other digestive enzymes. This, in turn, may result in trypsin activation or ER stress, depending on which pathway is activated. Another intriguing scenario is that premature degradation of lipases might trigger regulatory mechanisms resulting in unwanted pancreatic stimulation. Finally, we note that the C-terminal colipase-binding domain of PNLIP remains intact after degradation with trypsin and chymotrypsin. This fragment can bind colipase and inhibit intestinal lipolysis in animals, but this function does not provide an apparent explanation why protease-sensitive PNLIP variants increase risk for CP [38, 39]. We found no evidence that the C-terminal domain can cause acinar cell injury in preliminary cell culture experiments.

Patients with protease-sensitive PNLIP variants were mostly children or young adults with RAP, which is the typical clinical presentation in this age group. In several cases, the referring physician described the disease course as mild. Based on these observations and the well- documented role of lipase activity in disease severity [40, 41], it is intriguing to speculate that even though protease-sensitive PNLIP variants increase risk for pancreatitis onset, they also limit disease severity due to lipase degradation.

In summary, our study identified *PNLIP* as a novel pancreatitis-associated gene. Proteasesensitive PNLIP variants were significantly enriched in patients with non-alcoholic, early-onset CP. The mechanism by which these *PNLIP* variants drive the development of CP, however, remains enigmatic.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

The authors thank all study participants for providing clinical data and blood samples. This work was supported by the Else Kröner-Fresenius-Foundation (EKFS) (to HW), the Deutsche Forschungsgemeinschaft (DFG) grants Wi 2036/2–3 (to HW); National Institutes of Health grants R01DK058088 and R01DK095753 (to MST), R01DK808820 and R01DK097241 (to MEL), R01DK061451 (to DCW); the Hungarian Scientific Research Fund grant PD120960 (to AS); Grants-in-Aid for Scientific Research (KAKENHI) 16K15421 (to AM), 17K15916 (to EN), the Smoking Research Foundation (to AM); Conseil Régional de Bretagne, The Association des Pancréatites Chroniques Héréditaires, The Association de Transfusion Sanguine et de Biogénétique Gaetan Saleun, The Institut

National de la Santé et de la Recherche Médicale (INSERM), France (to CF); the Council of Scientific and Industrial Research, Govt of India, India (to GRC); and the OeNB Jubiläumsfonds 16678 (to TM).

## Abbreviations:

| | |
|---|---|
| **CP** | chronic pancreatitis |
| **OR** | odds ratio |
| **PNLIP** | pancreatic lipase |
| **RAP** | recurrent acute pancreatitis |

## REFERENCES

1. Witt H, Apte MV, Keim V, Wilson JS. Chronic pancreatitis: challenges and advances in pathogenesis, genetics, diagnosis, and therapy. Gastroenterology 2007; 132:1557–1573 [PubMed: 17466744]
2. Whitcomb DC, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, Martin SP, Gates LK Jr, Amann ST, Toskes PP, Liddle R, McGrath K, Uomo G, Post JC, Ehrlich GD. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. Nat Genet 1996;14:141–145 [PubMed: 8841182]
3. Witt H, Luck W, Hennies HC, Classen M, Kage A, Lass U, Landt O, Becker M. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. Nat Genet 2000; 25:213–216 [PubMed: 10835640]
4. Witt H, Sahin-Tóth M, Landt O, Chen JM, Kähne T, Drenth JP, Kukor Z, Szepessy E, Halangk W, Dahm S, Rohde K, Schulz HU, Le Maréchal C, Akar N, Ammann RW, Truninger K, Bargetzi M, Bhatia E, Castellani C, Cavestro GM, Cerny M, Destro-Bisol G, Spedini G, Eiberg H, Jansen JB, Koudova M, Rausova E, Macek M Jr, Malats N, Real FX, Menzel HJ, Moral P, Galavotti R, Pignatti PF, Rickards O, Spicak J, Zarnescu NO, Böck W, Gress TM, Friess H, Ockenga J, Schmidt H, Pfützer R, Löhr M, Simon P, Weiss FU, Lerch MM, Teich N, Keim V, Berg T, Wiedenmann B, Luck W, Groneberg DA, Becker M, Keil T, Kage A, Bernardova J, Braun M, Güldner C, Halangk J, Rosendahl J, Witt U, Treiber M, Nickel R, Férec C. A degradation-sensitive anionic trypsinogen (PRSS2) variant protects against chronic pancreatitis. Nat Genet 2006; 38:668–673 [PubMed: 16699518]
5. Rosendahl J, Witt H, Szmola R, Bhatia E, Ozsvári B, Landt O, Schulz HU, Gress TM, Pfützer R, Löhr M, Kovacs P, Blüher M, Stumvoll M, Choudhuri G, Hegyi P, te Morsche RH, Drenth JP, Truninger K, Macek M Jr, Puhl G, Witt U, Schmidt H, Büning C, Ockenga J, Kage A, Groneberg DA, Nickel R, Berg T, Wiedenmann B, Bödeker H, Keim V, Mössner J, Teich N, Sahin-Tóth M. Chymotrypsin C (CTRC) variants that diminish activity or secretion are associated with chronic pancreatitis. Nat Genet 2008; 40:78–82 [PubMed: 18059268]
6. Witt H, Beer S, Rosendahl J, Chen JM, Chandak GR, Masamune A, Bence M, Szmola R, Oracz G, Macek M Jr, Bhatia E, Steigenberger S, Lasher D, Bühler F, Delaporte C, Tebbing J, Ludwig M, Pilsak C, Saum K, Bugert P, Masson E, Paliwal S, Bhaskar S, Sobczynska- Tomaszewska A, Bak D, Balascak I, Choudhuri G, Nageshwar Reddy D, Rao GV, Thomas V, Kume K, Nakano E, Kakuta Y, Shimosegawa T, Durko L, Szabó A, Schnúr A, Hegyi P, Rakonczay Z Jr, Pfützer R, Schneider A, Groneberg DA, Braun M, Schmidt H, Witt U, Friess H, Algül H, Landt O, Schuelke M, Krüger R, Wiedenmann B, Schmidt F, Zimmer KP, Kovacs P, Stumvoll M, Blüher M, Müller T, Janecke A, Teich N, Grützmann R, Schulz HU, Mössner J, Keim V, Löhr M, Férec C, Sahin-Tóth M. Variants in CPA1 are strongly associated with early onset chronic pancreatitis. Nat Genet 2013; 45:1216–1220 [PubMed: 23955596]
7. Rosendahl J, Kirsten H, Hegyi E, Kovacs P, Weiss FU, Laumen H, Lichtner P, Ruffert C, Chen JM, Masson E, Beer S, Zimmer C, Seltsam K, Algül H, Bühler F, Bruno MJ, Bugert P, Burkhardt R, Cavestro GM, Cichoz-Lach H, Farré A, Frank J, Gambaro G, Gimpfl S, Grallert H, Griesmann H, Grützmann R, Hellerbrand C, Hegyi P, Hollenbach M, lordache S, Jurkowska G, Keim V, Kiefer F, Krug S, Landt O, Leo MD, Lerch MM, Lévy P, Löffler M, Löhr M, Ludwig M, Macek M, Malats

N, Malecka-Panas E, Malerba G, Mann K, Mayerle J, Mohr S, Te Morsche RHM, Motyka M, Mueller S, Müller T, Nöthen MM, Pedrazzoli S, Pereira SP, Peters A, Pfützer R, Real FX, Rebours V, Ridinger M, Rietschel M, Rösmann E, Saftoiu A, Schneider A, Schulz HU, Soranzo N, Soyka M, Simon P, Skipworth J, Stickel F, Strauch K, Stumvoll M, Testoni PA, Tönjes A, Werner L, Werner J, Wodarz N, Ziegler M, Masamune A, Mössner J, Férec C, Michl P, P H Drenth J, Witt H, Scholz M, Sahin-Toth M; all members of the PanEuropean Working group on ACP. Genome-wide association study identifies inversion in the CTRB1-CTRB2 locus to modify risk for alcoholic and non-alcoholic chronic pancreatitis. Gut 2018, 67:1855–1863 [PubMed: 28754779]

8. Sharer N, Schwarz M, Malone G, Howarth A, Painter J, Super M, Braganza J. Mutations of the cystic fibrosis gene in patients with chronic pancreatitis. N Engl J Med 1998;339:645–652 [PubMed: 9725921]
9. Cohn JA, Friedman KJ, Noone PG, Knowles MR, Silverman LM, Jowell PS. Relation between mutations of the cystic fibrosis gene and idiopathic pancreatitis. N Engl J Med 1998; 339:653–658 [PubMed: 9725922]
10. Whitcomb DC, LaRusch J, Krasinskas AM, Klei L, Smith JP, Brand RE, Neoptolemos JP, Lerch MM, Tector M, Sandhu BS, Guda NM,Orlichenko L; Alzheimer's Disease Genetics Consortium, Alkaade S, Amann ST, Anderson MA, Baillie J, Banks PA, Conwell D, Coté GA, Cotton PB, DiSario J, Farrer LA, Forsmark CE, Johnstone M, Gardner TB, Gelrud A, Greenhalf W, Haines JL, Hartman DJ, Hawes RA, Lawrence C, Lewis M, Mayerle J, Mayeux R, Melhem NM, Money ME, Muniraj T, Papachristou Gl, Pericak-Vance MA, Romagnuolo J, Schellenberg GD, Sherman S, Simon P, Singh VP, Slivka A, Stolz D, Sutton R, Weiss FU, Wilcox CM, Zarnescu NO, Wisniewski SR, O'Connell MR, Kienholz ML, Roeder K, Barmada MM, Yadav D, Devlin B. Common genetic variants in the CLDN2 and PRSS1-PRSS2 loci alter risk for alcohol- related and sporadic pancreatitis. Nat Genet 2012; 44:1349–1354 [PubMed: 23143602]
11. Fjeld K, Weiss FU, Lasher D, Rosendahl J, Chen JM, Johansson BB, Kirsten H, Ruffert C, Masson E, Steine SJ, Bugert P, Cnop M, Grützmann R, Mayerle J, Mössner J, Ringdal M, Schulz HU, Sendler M, Simon P, Sztromwasser P, Torsvik J, Scholz M, Tjora E, Ferec C, Witt H, Lerch MM, Njølstad PR, Johansson S, Molven A. A recombined allele of the lipase gene CEL and its pseudogene CELP confers susceptibility to chronic pancreatitis. Nat Genet 2015; 47:518–522 [PubMed: 25774637]
12. Sims HF, Jennens ML, Lowe ME. The human pancreatic lipase-encoding gene: structure and conservation of an Alu sequence in the lipase gene family. Gene 1993; 131:281–285 [PubMed: 8406023]
13. Raeder H, Johansson S, Holm PI, Haldorsen IS, Mas E, Sbarra V, Nermoen I, Eide SA, Grevle L, Bjørkhaug L, Sagen JV, Aksnes L, Søvik O, Lombardo D, Molven A, Njølstad PR. Mutations in the CEL VNTR cause a syndrome of diabetes and pancreatic exocrine dysfunction. Nat Genet 2006; 38:54–62 [PubMed: 16369531]
14. Nagasaki M, Yasuda J, Katsuoka F, Nariai N, Kojima K, Kawai Y, Yamaguchi-Kabata Y, Yokozawa J, Danjoh I, Saito S, Sato Y, Mimori T, Tsuda K, Saito R, Pan X, Nishikawa S, Ito S, Kuroki Y, Tanabe O, Fuse N, Kuriyama S, Kiyomoto H, Hozawa A, Minegishi N, Douglas Engel J, Kinoshita K, Kure S, Yaegashi N; ToMMo Japanese Reference Panel Project, Yamamoto M. Rare variant discovery by deep whole-genome sequencing of 1,070 Japanese individuals. Nat Commun 2015; 6:8018 [PubMed: 26292667]
15. Szabó A, Xiao X, Haughney M, Spector A, Sahin-Tóth M, Lowe ME. A novel mutation in PNLIP causes pancreatic triglyceride lipase deficiency through protein misfolding. Biochim Biophys Acta 2015;1852:1372–1379 [PubMed: 25862608]
16. Sahin-Tóth M Human cationic trypsinogen. Role of Asn-21 in zymogen activation and implications in hereditary pancreatitis. J Biol Chem 2000; 275:22750–22755 [PubMed: 10801865]
17. Szabó A, Sahin-Tóth M. Increased activation of hereditary pancreatitis-associated human cationic trypsinogen mutants in presence of chymotrypsin C. J Biol Chem 2012; 287:20701–20710 [PubMed: 22539344]
18. Szabó A, Sahin-Tóth M. Determinants of chymotrypsin C cleavage specificity in the calcium-binding loop of human cationic trypsinogen. FEBS J 2012; 279:4283–4292 [PubMed: 23035638]
19. Király O, Guan L, Sahin-Tóth M. Expression of recombinant proteins with uniform N-termini. Methods Mol Biol 2011; 705:175–194 [PubMed: 21125386]

20. Cordle RA, Lowe ME. The hydrophobic surface of colipase influences lipase activity at an oil-water interface. J Lipid Res 1998;39:1759–1767 [PubMed: 9741688]
21. Yang Y, Lowe ME. The open lid mediates pancreatic lipase function. J Lipid Res 2000; 41:48–57 [PubMed: 10627501]
22. Layer P, Go VL, DiMagno EP. Fate of pancreatic enzymes during small intestinal aboral transit in humans. Am J Physiol 1986; 251:G475–480 [PubMed: 2429560]
23. Thiruvengadam R, DiMagno EP. (1988) Inactivation of human lipase by proteases. Am J Physiol 255, G476–481 [PubMed: 2459971]
24. Whitcomb DC, Lowe ME. Human pancreatic digestive enzymes. Dig Dis Sci 2007; 52:1–17 [PubMed: 17205399]
25. Lowe ME. The triglyceride lipases of the pancreas. J Lipid Res 2002; 43(12):2007–2016 [PubMed: 12454260]
26. Lowe ME, Kaplan MH, Jackson-Grusby L, D'Agostino D, Grusby MJ. Decreased neonatal dietary fat absorption and T cell cytotoxicity in pancreatic lipase-related protein 2-deficient mice. J Biol Chem 1998; 273:31215–31221 [PubMed: 9813028]
27. Miller R, Lowe ME. Carboxyl ester lipase from either mother's milk or the pancreas is required for efficient dietary triglyceride digestion in suckling mice. J Nutr 2008; 138:927–930 [PubMed: 18424603]
28. Hui DY, Howles PN. Carboxyl ester lipase: structure-function relationship and physiological role in lipoprotein metabolism and atherosclerosis. J Lipid Res 2002; 43:2017–2030 [PubMed: 12454261]
29. Andersson L, Carriere F, Lowe ME, Nilsson A, Verger R. Pancreatic lipase-related protein 2 but not classical pancreatic lipase hydrolyzes galactolipids. Biochim Biophys Acta 1996; 1302:236–240 [PubMed: 8765145]
30. Johansson BB, Fjeld K, El Jellas K, Gravdal A, Dalva M, Tjora E, Raeder H, Kulkarni RN, Johansson S, Njolstad PR, Molven A. The role of the carboxyl ester lipase (CEL) gene in pancreatic disease. Pancreatology 2018; 18:12–19 [PubMed: 29233499]
31. Zou WB, Boulling A, Masamune A, Issarapu P, Masson E, Wu H, Sun XT, Hu LH, Zhou DZ, He L, Fichou Y, Nakano E, Hamada S, Kakuta Y, Kume K, Isayama H, Paliwal S, Mani KR, Bhaskar S, Cooper DN, Férec C, Shimosegawa T, Chandak GR, Chen JM, Li ZS, Liao Z. No association between CEL-HYB hybrid allele and chronic pancreatitis in Asian populations. Gastroenterology 2016; 150:1558–1560 [PubMed: 26946345]
32. Behar DM, Basel-Vanagaite L, Glaser F, Kaplan M, Tzur S, Magal N, Eidlitz-Markus T, Haimi-Cohen Y, Sarig G, Bormans C, Shohat M, Zeharia A. Identification of a novel mutation in the PNLIP gene in two brothers with congenital pancreatic lipase deficiency. J Lipid Res 2014; 55:307–312 [PubMed: 24262094]
33. Sahin-Tóth M Genetic risk in chronic pancreatitis: the misfolding-dependent pathway. Curr Opin Gastroenterol 2017; 33:390–395 [PubMed: 28650851]
34. Winkler FK, D'Arcy A, Hunziker W. Structure of human pancreatic lipase. Nature 1990; 343:771–774 [PubMed: 2106079]
35. Freie AB, Ferrato F, Carriere F, Lowe ME. Val-407 and Ile-408 in the beta5'-loop of pancreatic lipase mediate lipase-colipase interactions in the presence of bile salt micelles. J Biol Chem 2006; 281:7793–7800 [PubMed: 16431912]
36. Scheele G, Bartelt D, Bieger W. Characterization of human exocrine pancreatic proteins by two-dimensional isoelectric focusing/sodium dodecyl sulfate gel electrophoresis. Gastroenterology 1981; 80:461–473 [PubMed: 6969677]
37. Tamura K, Yu J, Hata T, Suenaga M, Shindo K, Abe T, MacGregor-Das A, Borges M, Wolfgang CL, Weiss MJ, He J, Canto MI, Petersen GM, Gallinger S, Syngal S, Brand RE, Rustgi A, Olson SH, Stoffel E, Cote ML, Zogopoulos G, Potash JB, Goes FS, McCombie RW, Zandi PP, Pirooznia M, Kramer M, Parla J, Eshleman JR, Roberts NJ, Hruban RH, Klein AP, Goggins M. Mutations in the pancreatic secretory enzymes CPA1 and CPB1 are associated with pancreatic cancer. Proc Natl Acad Sci USA 2018 115:4767–4772 [PubMed: 29669919]

38. Ayvazian L, Kerfelec B, Granon S, Foglizzo E, Crenon I, Dubois C, Chapus C. The lipase C-terminal domain. A novel unusual inhibitor of pancreatic lipase activity. J Biol Chem 2001; 276:14014–14018 [PubMed: 11154696]

39. Sebban-Kreuzer C, Ayvazian L, Juhel C, Salles JP, Chapus C, Kerfelec B. Inhibitory effect of the pancreatic lipase C-terminal domain on intestinal lipolysis in rats fed a high-fat diet: chronic study. Int J Obes Relat Metab Disord 2003; 27:319–325 [PubMed: 12629558]

40. Navina S, Acharya C, DeLany JP, Orlichenko LS, Baty CJ, Shiva SS, Durgampudi C, Karlsson JM, Lee K, Bae KT, Furlan A, Behari J, Liu S, McHale T, Nichols L, Papachristou GI, Yadav D, Singh VP. Lipotoxicity causes multisystem organ failure and exacerbates acute pancreatitis in obesity. Sci Transl Med 2011; 3:107ra110

41. Patel K, Trivedi RN, Durgampudi C, Noel P, Cline RA, DeLany JP, Navina S, Singh VP. Lipolysis of visceral adipocyte triglyceride by pancreatic lipases converts mild acute pancreatitis to severe pancreatitis independent of necrosis and inflammation. Am J Pathol 2015; 185:808–819 [PubMed: 25579844]

## STUDY HIGHLIGHTS

**WHAT IS CURRENT KNOWLEDGE:**

Genetic risk for chronic pancreatitis is mainly associated with genes that encode pancreatic digestive proteases.

Studies to date highlighted the central role of trypsin in disease onset and progression.

**WHAT IS NEW HERE:**

Variants in pancreatic lipase that render the enzyme protease sensitive are highly enriched in chronic pancreatitis.

The finding indicates that mechanisms unrelated to trypsin activity can underlie pancreatitis pathogenesis.

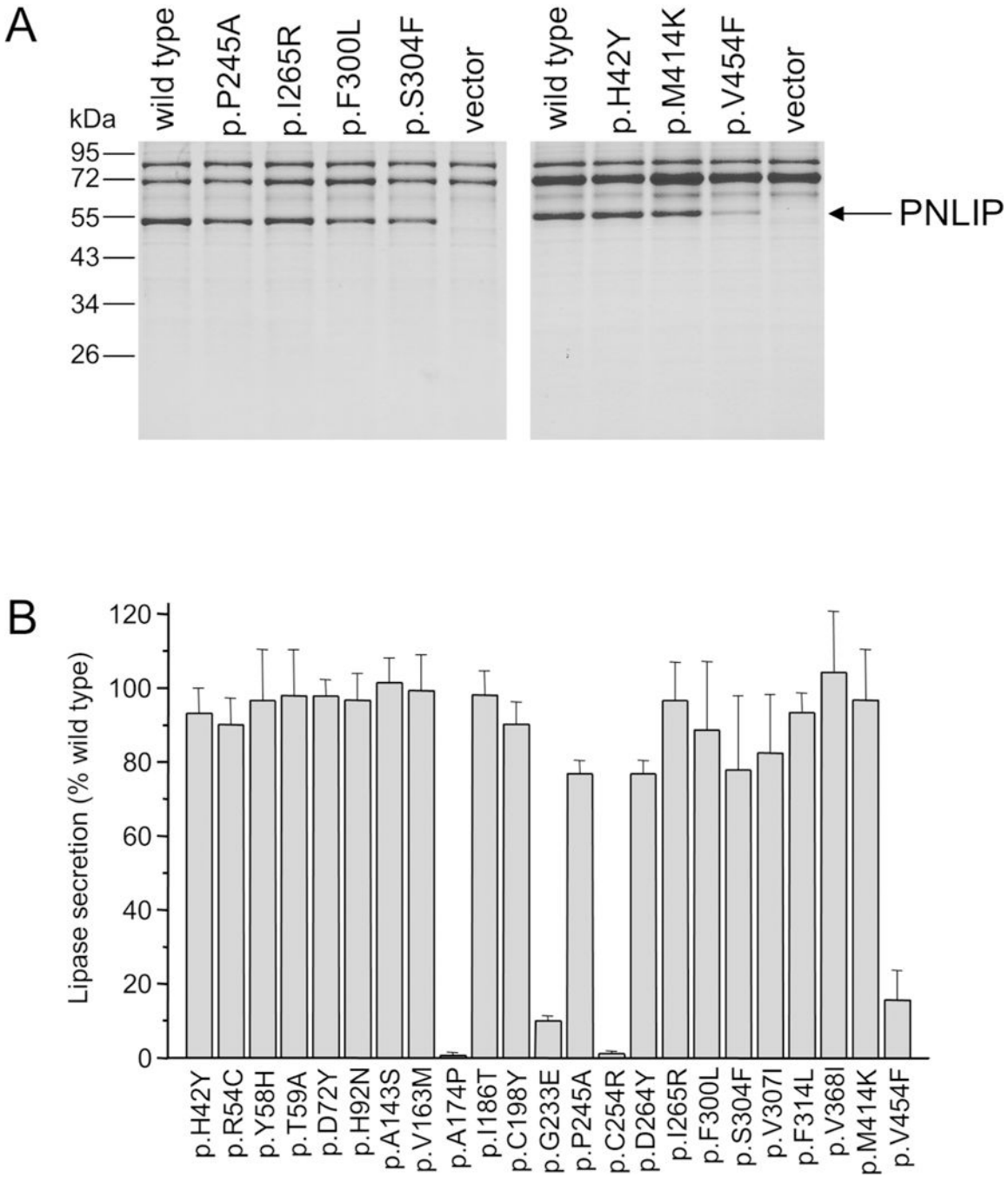


**Figure 1.**

Secretion of human pancreatic lipase (PNLIP) variants. HEK 293T cells were transiently transfected with the indicated constructs and conditioned media was collected after 48 h. Aliquots (200 μL) of conditioned media were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and Coomassie Blue staining. (**A**) Representative gels. (**B**) Densitometric evaluation of PNLIP band intensities. The mean and S.D. of three experiments are shown.

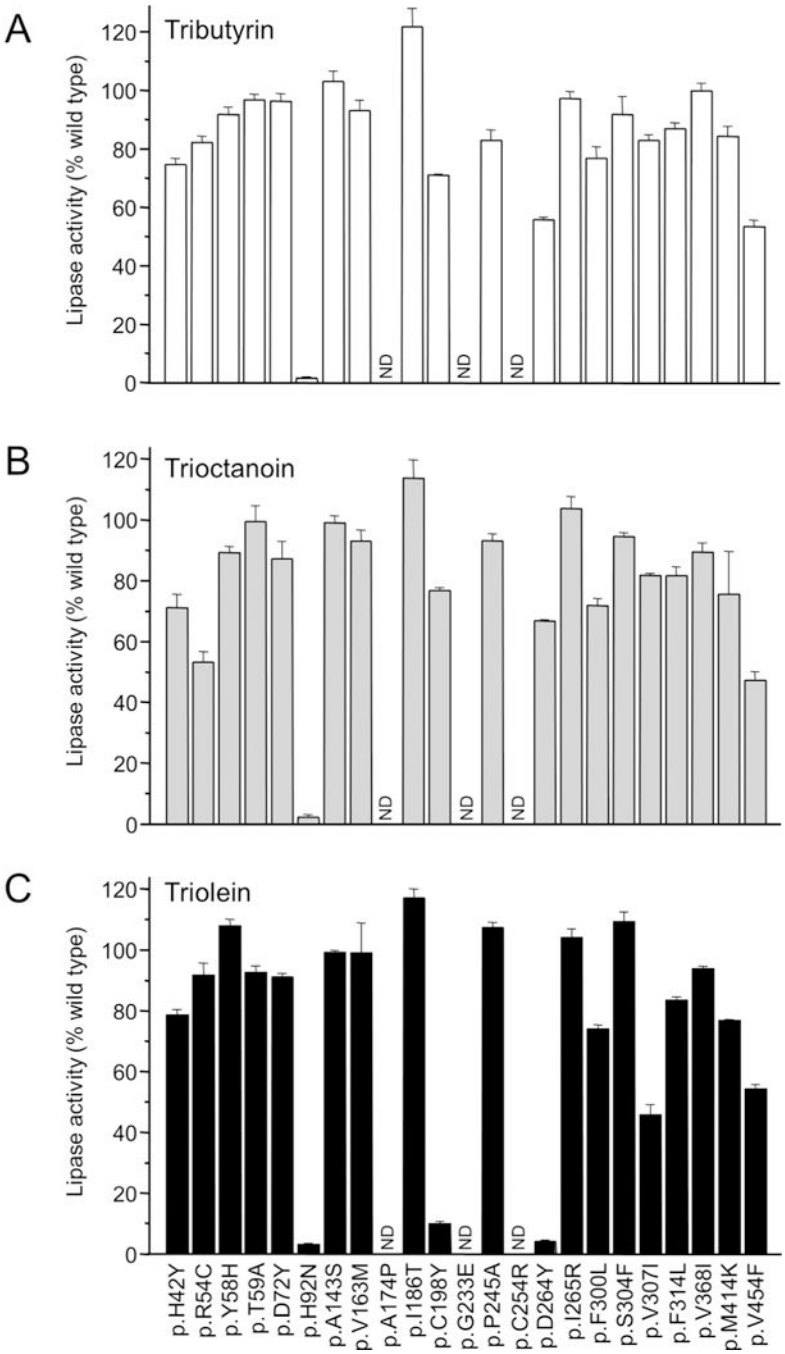


**Figure 2.**

Enzymatic activity of human pancreatic lipase (PNLIP) variants. The indicated lipase variants were purified and their activity was tested against three different triglyceride substrates; (**A**) tributyrin, (**B**) trioctanoin, (**C**) triolein. Mean activity values ± SD (n=3) were plotted. See Methods for experimental details. ND, not determined, because these mutants could not be purified due to their low expression levels.

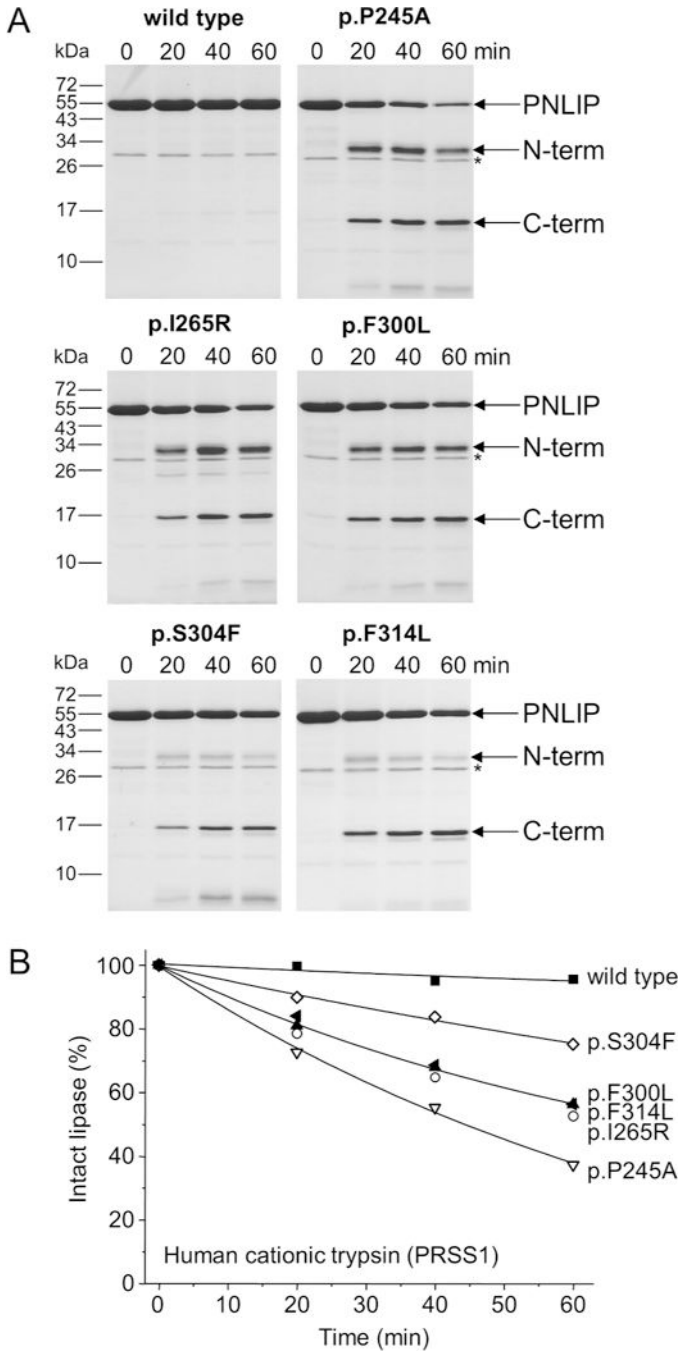


**Figure 3.**

Effect of human cationic trypsin (PRSS1) on human pancreatic lipase (PNLIP) variants. Digestion experiments were carried out as detailed in Methods. (**A**) Representative gels from two experiments. N-term, N-terminal PNLIP cleavage fragment. C-term, C-terminal PNLIP cleavage fragment. The asterisk indicates the PRSS1 band. (**B**) Densitometric evaluation of PNLIP band intensities. The average of two experiments is indicated. The figure shows the five PNLIP variants where significant degradation was observed. The effect of PRSS1 on all other PNLIP variants is shown in Supplementary Figure 1.

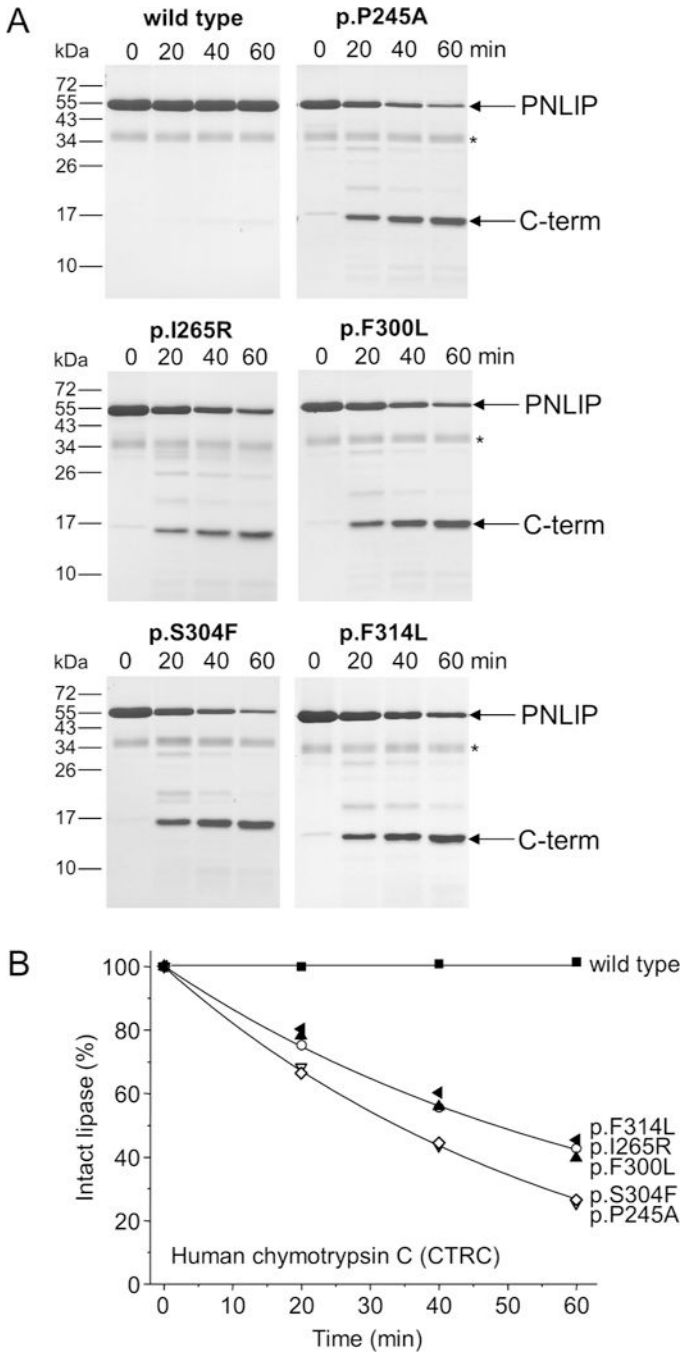


**Figure 4.**

The effect of human chymotrypsin C (CTRC) on human pancreatic lipase (PNLIP) variants. Digestion experiments were performed as described in Methods. (**A**) Representative gels from two experiments. C-term, C-terminal PNLIP cleavage fragment. The asterisk indicates the CTRC band. (**B**) Densitometric evaluation of PNLIP band intensities. The average of two experiments is indicated. The figure shows the five PNLIP variants where significant degradation was observed. The effect of CTRC on all other PNLIP variants is shown in Supplementary Figure 2.

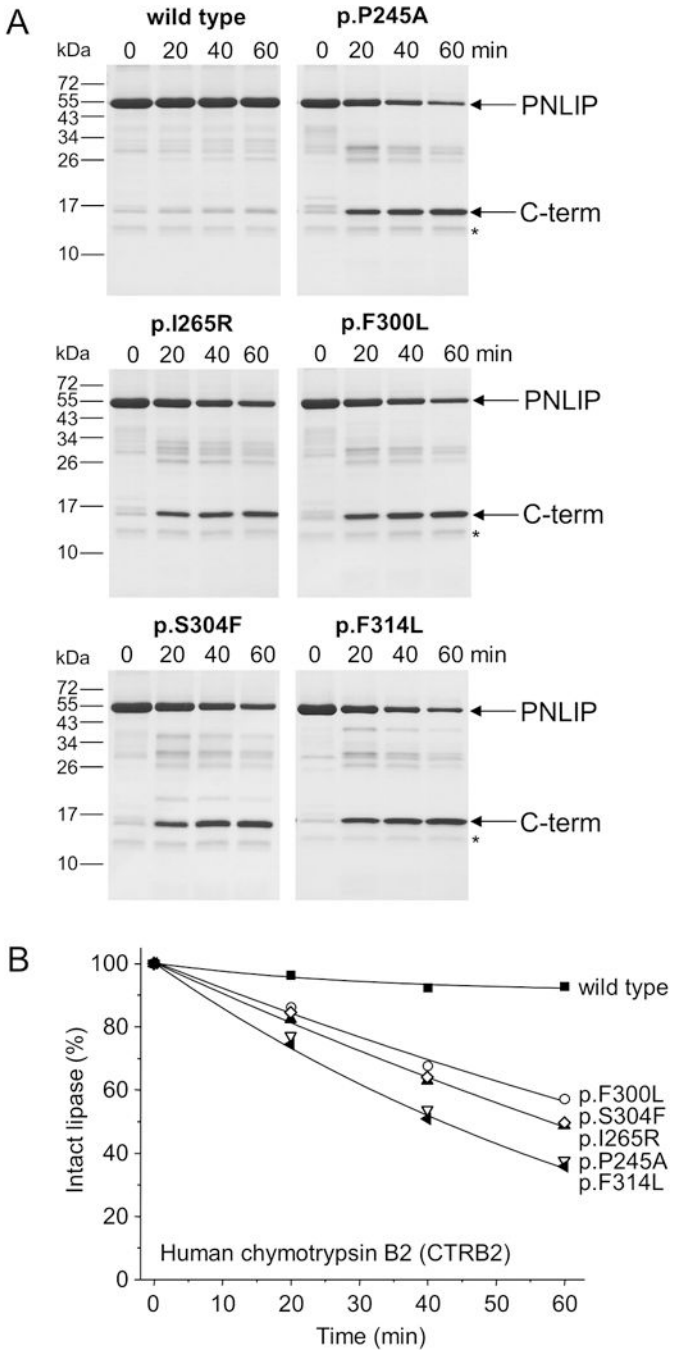


**Figure 5.**

The effect of human chymotrypsin B2 (CTRB2) on human pancreatic lipase (PNLIP) variants. Digestion experiments were carried out as detailed in Methods. (**A**) Representative gels from two experiments. C-term, C-terminal PNLIP cleavage fragment. Note that human CTRB2 migrates on reducing gels as two smaller bands due to autolytic cleavage of the Tyr164- Asn165 peptide bond. The N-terminal CTRB2 chain co-migrates with the PNLIP C-terminal fragment and the C-terminal CTRB2 chain is indicated by the asterisk. (**B**) Densitometric evaluation of PNLIP band intensities. The average of two experiments is

indicated. The figure shows the five PNLIP variants where significant degradation was observed. The effect of CTRB2 on all other PNLIP variants is shown in Supplementary Figure 3.

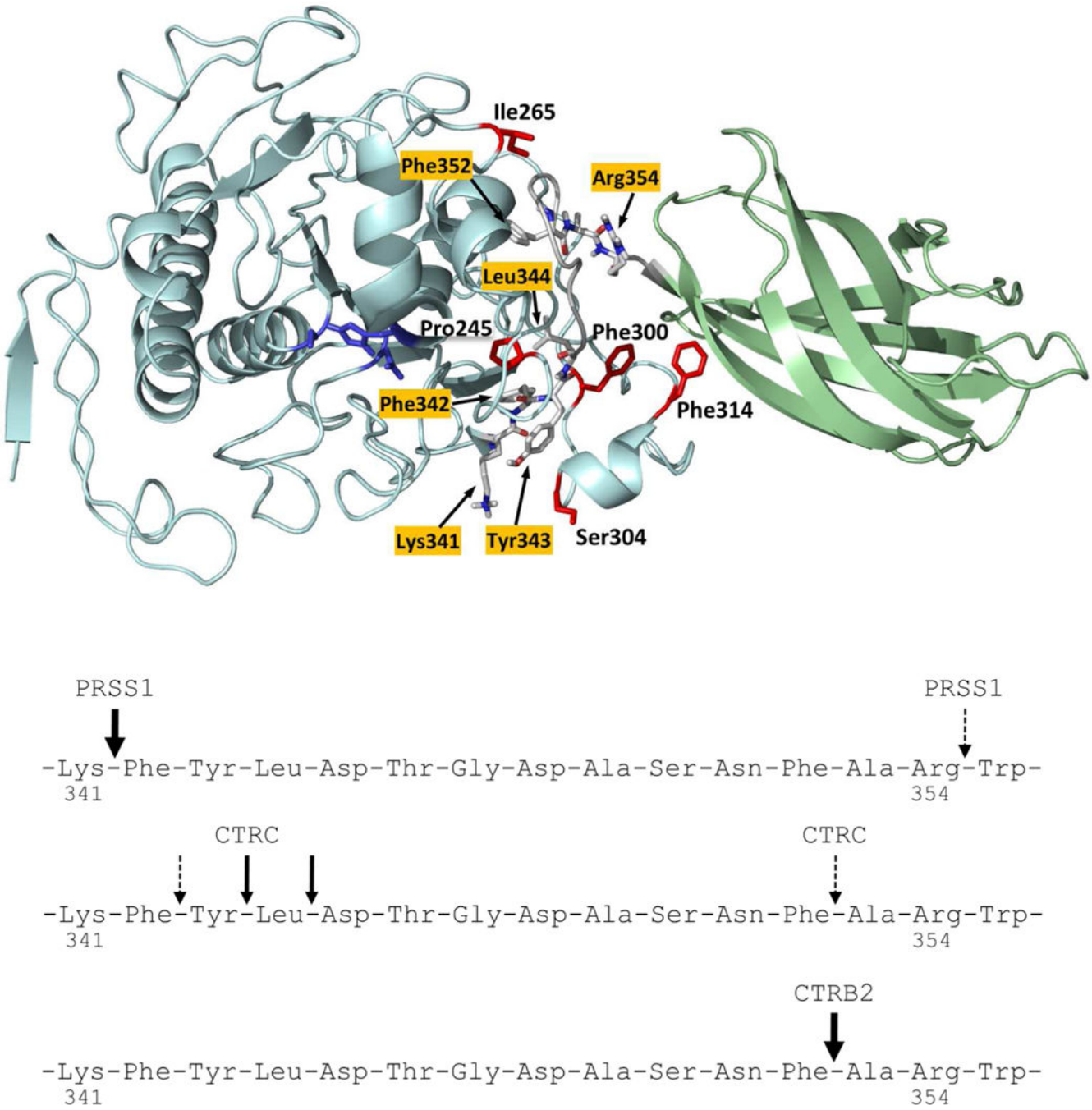


**Figure 6.**

Location of proteolytic cleavage sites in degradation-sensitive human pancreatic lipase (PNLIP) variants. Ribbon model of PNLIP (Protein Data Bank file 1LPB). Amino acids affected by mutations that promote PNLIP degradation are highlighted in red. Labels on orange background indicate the proteolytic cleavage sites identified in PNLIP variants p.F300L and p.P245A by N-terminal sequencing of cleavage fragments. The amino-acid sequence of PNLIP between positions 341 and 355 is also shown with human cationic trypsin (PRSS1), human chymotrypsin C (CTRC) and human chymotrypsin B2 (CTRB2)

cleavage sites indicated. Solid (versus dashed) arrows denote stronger cleavage. See text for further details.

**Table 1.**

Heterozygous *PNLIP* missense variants in German subjects with non-alcoholic chronic pancreatitis and healthy controls. Protease-sensitive variants are marked in bold. The enrichment of p.F300L (*P*=0.03), of all variants (*P*=0.02, OR=5.7, 95% CI=1. 1–38.9), and of all protease-sensitive variants (*P*=0.001) in cases is significant.

| Exon | Nucleotide change | Amino-acid change | Cases(%) | Controls (%) | *P* |
|---|---|---|---|---|---|
| 3 | c.124C>T | p.H42Y | 0/429 (0) | 1/600 (0.2) | |
| 8 | **c.733C>G** | **p.P245A** | **1/429 (0.2)** | **0/600 (0)** | |
| 8 | **c.794T>G** | **p.I265R** | **2/429 (0.5)** | **0/600 (0)** | |
| 9 | **c.900C>A** | **p.F300L** | **4/429 (0.9)** | **0/600 (0)** | 0.03§ |
| 9 | **c.911C>T** | **p.S304F** | **1/429 (0.2)** | **0/600 (0)** | |
| 13 | c.1360G>T | p.V454F | 0/429 (0) | 1/600 (0.2) | |
| **All variants** | | | **8/429 (1.9)** | **2/600 (0.3)** | **0.02$** |
| **All protease-sensitive variants** | | | **8/429 (1.9)** | **0/600 (0)** | **0.001&** |

§ *P*=0.03; OR= Inf. (95% CI = 0.9-Inf.)

$ *P*=0.02; OR= 5.7 (95% CI = 1.1–38.9)

& *P*=0.001; OR= Inf. (95% CI = 2.1-Inf.)

**Table 2.**

Heterozygous *PNLIP* missense variants in French subjects with non-alcoholic chronic pancreatitis and healthy controls. The enrichment of all protease-sensitive variants (p.I265R, p.F300L and p.F314L; marked in bold) in cases is significant (OR 7.6, 95% CI 0.9–172.9, *P*=0.04).

| Exon | Nucleotide change | Amino-acid change | Cases(%) | Controls (%) | *P* |
|---|---|---|---|---|---|
| 4 | c.214G>T | p.D72Y | 1/632 (0.2) | 0/957 (0) | |
| 4 | c.274C>A | p.H92N | 0/632 (0) | 1/957 (0.1) | |
| 5 | c.427G>T | p.A143S | 0/632 (0) | 1/957 (0.1) | |
| 6 | c.487G>A | p.V163M | 0/632 (0) | 1/957 (0.1) | |
| 6 | c.520G>C | p.A174P | 0/632 (0) | 1/957 (0.1) | |
| 7 | c.593G>A | p.C198Y | 0/632 (0) | 1/957 (0.1) | |
| 8 | c.698G>A | p.G233E | 1/632 (0.2) | 0/957 (0) | |
| 8 | c.760T>C | p.C254R | 1/632 (0.2) | 0/957 (0) | |
| 8 | c.790G>T | p.D264Y | 0/632 (0) | 1/957 (0.1) | |
| 8 | **c.794T>G** | **p.I265R** | **1/632 (0.2)** | **1/957** (0.1) | |
| 9 | **c.900C>A** | **p.F300L** | **3/632 (0.5)** | **0/957** (0) | 0.06 |
| 9 | c.919G>A | p.V307I | 0/632 (0) | 1/957 (0.1) | |
| 10 | **c.942C>G** | **p.F314L** | **1/632 (0.2)** | **0/957** (0) | |
| 11 | c.1102G>A | p.V368I | 1/632 (0.2) | 0/957 (0) | |
| 12 | c.1241T>A | p.M414K | 1/632 (0.2) | 0/957 (0) | |
| **All variants** | | | **10/632 (1.6)** | **8/957 (0.8)** | **0.17** |
| **All protease-sensitive variants** | | | **5/632 (0.8)** | **1/957 (0.1)** | **0.04**[&] |

[&] *P*=0.04; OR= 7.6 (95% CI = 0.9–172.9)

**Table 3.**

Protease-sensitive PNLIP variants in European subjects with non-alcoholic chronic pancreatitis and healthy controls. The enrichment of p.F300L (*P*=0.0001) and of all proteasesensitive variants in cases is significant (*P*<0.0001, OR=11.3, 95% CI=3.0–49.9).

| Exon | Nucleotide change | Amino-acid change | Cases(%) | Controls (%) | *P* |
|---|---|---|---|---|---|
| 8 | c.733C>G | p.P245A | 1/1163 (0.1) | 0/3000 (0) | 0.3 |
| 8 | c.794T>G | p.I265R | 3/1163 (0.3) | 3/3000 (0.1) | 0.4 |
| 9 | c.900C>A | p.F300L | 7/1163 (0.6) | 0/3000 (0) | 0.0001 |
| 9 | c.911C>T | p.S304F | 1/1163 (0.1) | 0/3000 (0) | 0.3 |
| 10 | c.942C>G | p.F314L | 1/1163 (0.1) | 0/3000 (0) | 0.3 |
| **All protease-sensitive variants** | | | **13/1163 (1.1)** | **3/3000 (0.1)** | **<0.0001** |

**Table 4.**

Characteristics of cases with protease-sensitive PNLIP variants identified in the German and French cohorts. All genetic variants indicated are heterozygous. The patient with the *SPINK1* variant also had autosomal dominant polycystic kidney disease. The patient with family history had a sister with the same *PNLIP* variant and similarly mild disease course. RAP, recurrent acute pancreatitis. Mild disease course denotes the overall clinical impression of the referring physician.

| *PNLIP* variant | Other variants | Age, years (study entry) | Age, years (diagnosis) | Gender | Family history | Smoking | Alcohol | Disease course |
|---|---|---|---|---|---|---|---|---|
| **German cohort** | | | | | | | | |
| p.P245A | *CFTR* p.F508del | 7 | 4.5 | male | no | no | no | RAP |
| p.I265R | - | 25 | unknown | female | no | unknown | unknown | RAP |
| p.I265R | *SPINK1* p.N34S | 16 | 8 | female | no | no | no | RAP, mild |
| p.F300L | - | 12 | 11 | female | no | no | no | RAP |
| p.F300L | - | 12 | 12 | female | sister | no | no | RAP, mild |
| p.F300L | - | 11 | 7 | male | no | no | no | RAP, mild |
| p.F300L | - | 8 | 7 | female | no | no | no | RAP |
| p.S304F | | 7 | 6 | female | no | no | no | RAP |
| **French cohort** | | | | | | | | |
| p.I265R | - | 15 | 15 | female | no | no | no | RAP |
| p.F300L | - | 17 | 16 | female | no | no | no | RAP |
| p.F300L | - | 19 | 19 | female | no | unknown | unknown | RAP |
| p.F300L | - | 23 | 16 | female | no | unknown | unknown | RAP |
| p.F314L | - | 4 | 3 | male | no | no | no | RAP |