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Variants in *CPA1* are strongly associated with early-onset chronic pancreatitis

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Abstract

Chronic pancreatitis is an inflammatory disorder of the pancreas. We analyzed *CPA1* encoding carboxypeptidase A1 in subjects with non-alcoholic chronic pancreatitis and controls in a German discovery cohort and three replication cohorts. Functionally impaired variants were present in 29/944 (3.1%) German patients and in 5/3,938 (0.1%) controls (odds ratio [OR] = 24.9; $P = 1.5 \times 10^{-16}$). The association was strongest in subjects aged 10 years (9.7%; OR = 84.0; $P = 4.1 \times 10^{-24}$). In the replication cohorts, defective *CPA1* variants were observed in 8/600 (1.3%) patients and in 9/2,432 (0.4%) controls from Europe ($P = 0.01$), in 5/230 (2.2%) patients and 0/264 controls from India ($P = 0.02$), and in 5/247 (2.0%) patients but 0/341 controls from Japan ($P = 0.013$). The mechanism of increased pancreatitis risk by CPA1 variants may involve misfolding-induced endoplasmic reticulum stress rather than elevated trypsin activity as seen with other genetic risk factors.

Chronic pancreatitis is an inflammatory condition characterized by abdominal pain and progressive damage to both exocrine and endocrine components of the pancreas resulting in insufficiency of the organ with maldigestion and diabetes. Although alcohol abuse has been long recognized as a major risk factor for chronic pancreatitis, genetic susceptibility has emerged during the last two decades as a strong determinant of disease risk, particularly in the pediatric population[1].

Genetic studies performed to date suggest that development of intra-pancreatic trypsin activity plays a central role in disease pathogenesis. Thus, gain-of-function mutations in cationic trypsinogen (*PRSS1*, OMIM 276000) as well as loss-of-function variants in the pancreatic trypsin inhibitor (*SPINK1*, OMIM 167790) and the trypsinogen-degrading enzyme chymotrypsin C (*CTRC*, OMIM 601405) increase the risk for chronic

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AUTHOR CONTRIBUTIONS

H.W. and M.S.-T. conceived, designed and directed the study. G.R.C., J.-M.C., J.R., A.M. and H.W. designed, performed and interpreted genetic analyses with significant contributions from D.B., F.B., M.B. (Frankfurt), S.B., C.D., D.L., E.M., S.P., S.S., A.S.-T, K.K., E.N., Y.K., T.S., J.T. and, A.Sc., A.Sz., S.B. (Boston), M.B. (Boston), R.S. and M.S.-T. carried out functional characterization of CPA1 variants. H.W., M.S.-T. and S.B. (Boston) wrote the manuscript with significant contributions from G.R.C., J.-M.C., J.R. and A.M. O.L. provided oligonucleotides. All other co-authors recruited study subjects, collected clinical data and provided genomic DNA samples. All authors approved the final manuscript and contributed critical revisions to its intellectual content.

Competing interests statement:
The authors declare that they have no competing financial interests.

Accession codes. Entrez nucleotide: carboxypeptidase A1 (*CPA1*): NT_007933.15 (Homo sapiens chromosome 7 genomic contig, GRCh37.p5); NM_001868.2 (human CPA1 mRNA sequence).

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pancreatitis[2-8]. Consistent with the proposed pathogenic role of trypsin, a rapidly auto-degrading variant of anionic trypsinogen (*PRSS2*, OMIM 601564) and a common *PRSS1* promoter variant protect against chronic pancreatitis[9,10].

Despite these recent advances, many patients do not carry mutations in any of the known susceptibility genes, suggesting the involvement of other yet unidentified genes. In the present study, we investigated the role of *CPA1* encoding carboxypeptidase A1 in chronic pancreatitis. Digestive carboxypeptidases are pancreatic metalloproteases, which hydrolyze C-terminal peptide bonds in dietary polypeptide chains[11]. Three different isoforms have been described in human pancreatic juice. A-type carboxypeptidases (CPA1 and CPA2) act on aromatic and aliphatic amino acid residues exposed by the action of chymotrypsins and elastases, whereas the B-type carboxypeptidase (CPB1) hydrolyzes C-terminal Lys and Arg residues generated by tryptic cleavages[11]. The gene encoding human *CPA1* (OMIM 114850) maps to 7q32.2, spans approximately 8 kb, and contains 10 exons. The inactive preproprotein comprises 419 amino acids, including a 16 amino-acid secretory signal peptide and a 94 amino-acid long propeptide. Activation of human proCPA1 to CPA1 is catalyzed by the sequential action of trypsin and CTRC, which cleave and degrade the propeptide[12]. After trypsinogens, proCPA1 is the second largest component of pancreatic juice, contributing more than 10% of the total protein[13].

We performed direct DNA sequencing of all 10 *CPA1* exons in 944 individuals with non-alcoholic chronic pancreatitis and in 3,938 control subjects of German origin. Considering variants in the coding regions and flanking splice sites, we identified 31 missense variants, 1 nonsense variant, 1 frame-shift variant, and 1 splice-site variant; and found that 3 variants were significantly enriched in patients (Table 1). Functional analysis demonstrated that 17/34 (50%) variants resulted in a marked (>80%) loss of apparent CPA1 activity, a term we use to describe the combined effects of variants on secretion, proteolytic stability and catalytic competence (Table 1, Supplementary Figure 1, Methods). The vast majority of these variants were located in exons 7, 8, and 10. Remarkably, 14 out of 17 (82%) functionally impaired variants were found exclusively in patients, including the c.768C>G (p.Asn256Lys) variant, which was detected in 7 patients. Thus, *CPA1* variants with less than 20% apparent activity were significantly overrepresented in the chronic pancreatitis group (29/944; 3.1%) as compared to controls (5/3,938; 0.1%) (OR = 24.9; CI = 9.6-64.6; $P = 1.5 \times 10^{-16}$) (Table 1). No individual was compound heterozygous or homozygous for a defective *CPA1* variant. Variants found in non-coding regions and synonymous variants in coding regions are listed in Supplementary Table 1.

We observed that patients bearing a defective *CPA1* variant were younger than those without a *CPA1* alteration. In the German chronic pancreatitis group, the majority of *CPA1* variants with less than 20% apparent activity were observed in patients at or below 20 years of age (27/586 [4.6%]; OR = 38.0; CI = 14.6-99.1; $P = 6.8 \times 10^{-20}$]. This becomes even more significant in a subgroup of patients at or below 10 years of age. In this group, 22/228 (9.7%) carried an impaired *CPA1* variant (OR = 84.0; CI = 31.5-224.1; $P = 4.1 \times 10^{-24}$) (patients 10 yrs. vs. patients 20 years, $P = 0.007$; patients 10 yrs. vs. all patients, $P = 7.6 \times 10^{-5}$) (Table 2).

We also investigated all *CPA1* exons in 465 German patients with alcohol-related chronic pancreatitis. Only 2/465 (0.4%) patients were heterozygous for a defective *CPA1* variant: c.954_955delCA (p.Tyr318Ter) and c.811T>C (p.Cys271Arg), respectively. This indicates that loss-of-function *CPA1* alterations play a minor role in alcoholic pancreatitis.

To confirm the association of non-alcoholic chronic pancreatitis and *CPA1* in an independent European cohort, we sequenced all *CPA1* exons in 600 patients with non-

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alcoholic chronic pancreatitis and 2,432 control subjects originating from France, the Czech Republic and Poland. Again, variants with less than 20% apparent activity were significantly overrepresented in chronic pancreatitis patients (8/600; 1.3%) versus ethnically matched controls (9/2,432; 0.4%) (OR = 3.6; CI = 1.4-9.5; *P* = 0.01) (Table 3). One subject with chronic pancreatitis was homozygous for the c.1115G>A (p.Gly372Asp) variant.

In order to investigate the significance of *CPA1* variants in subjects of non-European descent, we sequenced all 10 exons in 230 individuals with non-alcoholic chronic pancreatitis and 264 controls of Indian origin and in 247 patients and 341 controls from Japan. Overall, 2.2% (5/230) of Indian patients but none of the controls carried a defective *CPA1* variant (*P* = 0.02) (Table 4). In the Japanese cohort, 2.0% (5/247) of patients but none of controls carried an impaired *CPA1* variant (*P* = 0.013) (Table 5). No individual from India or Japan was compound heterozygous or homozygous for a defective *CPA1* variant.

Chronic pancreatitis is a complex multi-genic disease and affected individuals often carry mutations in several disease-associated genes. To elucidate the relationship between *CPA1* alterations and *PRSS1, SPINK1, CTRC*, and *CFTR* variants, we investigated all German subjects with chronic pancreatitis for mutations in *PRSS1* (p.Ala16Val, p.Asn29Ile, and p.Arg122His), in *SPINK1* (p.Asn34Ser and c.194+2T>C), in *CTRC* (p.Arg254Trp and p.Lys247_Arg254del), and in *CFTR* (p.Phe508del). In total, 50/944 (5.3%) individuals carried a heterozygous *PRSS1* variant, 147/944 (15.6%) were positive for p.Asn34Ser (121 heterozygotes, 18 homozygotes) and c.194+2T>C (20 heterozygotes; 12/20 compound heterozygous with p.Asn34Ser), 28/944 (3.0%) were positive for a *CTRC* variant (21 instances of p.Arg254Trp and 7 occurrences of p.Lys247_Arg254del), and 42/944 (4.5%) were positive for *CFTR* p.Phe508del. Altogether, 273/944 (28.9%) of patients showed at least one of the above-mentioned genetic alterations and 24/944 (2.5%) were trans-heterozygous. However, only 1/29 (3.6%) patients with a defective *CPA1* variant was trans-heterozygous; this subject carried the *CPA1* c.1073-2A>G alteration (inherited from the mother) and the *SPINK1* p.Asn34Ser variant (inherited from the father). This suggests limited interaction of *CPA1* variants with variants in other susceptibility genes and stands in contrast with the high number of trans-heterozygotes for *SPINK1* and *CTRC* and/or *CFTR* variants, as described recently[14].

The mechanism by which loss-of-function *CPA1* variants predispose to chronic pancreatitis is not intuitively apparent. We found no detectable effect of CPA1 on trypsinogen activation, trypsin activity or trypsinogen degradation by CTRC (Supplementary Figure 2), indicating that *CPA1* mutations do not exert their effect via increasing intrapancreatic trypsin activity. On the other hand, the low apparent activity of most defective variants was due to markedly reduced secretion (Tables 1-5, Supplementary Figure 1 and 3), raising the possibility that CPA1 mutants misfold in the endoplasmic reticulum (ER) and cause ER stress, as demonstrated previously for some *PRSS1* and *CTRC* mutants[15,16]. Indeed, expression of the most frequently found p.Asn256Lys variant in AR42J rat acinar cells resulted in ER stress, as evidenced by increased splicing of XBP1 and elevated mRNA levels of the chaperones BiP and calreticulin (Figure 1). Considering that CPA1 is one of the most abundant proteins synthesized by the pancreas, misfolding induced ER stress seems a plausible mechanism to explain the clinical effect of heterozygous CPA1 variants.

In summary, loss-of-function *CPA1* variants are strongly associated with non-alcoholic chronic pancreatitis, especially with early-onset disease. Although there was evidence of mutational heterogeneity, identification of functionally impaired *CPA1* variants in both the European and non-European cohorts establishes its global role in the pathogenesis of chronic pancreatitis.

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ONLINE METHODS

Study population

The medical ethical review committees of all participating study centers approved this study. All study subjects gave informed consent. We enrolled 944 unrelated German individuals with the diagnosis of non-alcoholic chronic pancreatitis and 465 patients with alcohol-related chronic pancreatitis. In the replication study, we investigated 600 unrelated non-alcoholic chronic pancreatitis patients originating from France (n = 456), the Czech Republic (n = 21), and Poland (n = 123). In addition, we also investigated unrelated subjects affected with non-alcoholic chronic pancreatitis from India (n = 230) and Japan (n = 247). The diagnosis of chronic pancreatitis was based on two or more of the following findings: presence of a typical history of recurrent pancreatitis, pancreatic calcifications and/or pancreatic ductal irregularities revealed by endoscopic retrograde pancreaticography or by magnetic resonance imaging of the pancreas and/or pathological sonographic findings. Alcoholic chronic pancreatitis was diagnosed in patients who consumed more than 60 g (females) or 80 g (males) of ethanol per day for more than two years. Control subjects were recruited from Germany (n = 3,938), France (n = 2,000), the Czech Republic (n = 235), Poland (n = 197), India (n = 264), and Japan (n = 341).

Mutation screening

We designed primers complementary to intronic sequences flanking *CPA1* exons based on the published nucleotide sequence (GenBank # NT_007933.15) (Supplementary Table 2). After PCR amplification, the entire coding region and the exon-intron boundaries were sequenced. All mutations were confirmed with a second independent PCR reaction. In the German laboratories, we performed PCR using 0.75 U AmpliTaq Gold polymerase (Perkin Elmer, Rodgau, Germany), 400 μmol/L deoxynucleoside triphosphates and 0.1 μmol/L primers in a total volume of 25 μL. Cycle conditions were as follows: initial denaturation for 12 min at 95°C; 48 cycles of 20 s denaturation at 95°C, 40 s annealing at 64°C and 90 s primer extension at 72°C; and a final extension step for 2 min at 72°C. PCR products were digested with Antarctic phosphatase (New England Biolabs, Ipswich MA) or shrimp alkaline phosphatase (USB, Santa Clara, CA) and exonuclease I (New England Biolabs, Ipswich MA). Cycle sequencing was performed using BigDye terminator mix (Applied Biosystems, Darmstadt, Germany) with 56° annealing temperature. The reaction products were purified with ethanol precipitation and loaded onto an ABI 3730 or an ABI 3100-Avant fluorescence sequencer (Applied Biosystems).

Functional characterization of CPA1 variants

We investigated the functional consequences of *CPA1* alterations by transient transfection of HEK 293T cells (#Q401, GenHunter, Nashville, TN) with wild-type and mutant constructs and analyzing the conditioned medium for the amount of proCPA1 protein constitutively secreted using densitometry of stained gels and CPA1 activity after activation with trypsin and CTRC.

Expression plasmids, mutagenesis, adenovirus

Construction of the pcDNA3.1(-) human CPA1 expression plasmid has been reported previously[12]. The coding DNA in this plasmid was derived from IMAGE clone #3949850 (GenBank accession BC005279), which contains a c.827A>G (p. H276R) alteration. This mistake was corrected by back-mutating Arg276 to His. CPA1 mutants were created by PCR mutagenesis and ligated into the pcDNA3.1(-) expression plasmid. Recombinant adenovirus carrying wild-type *CPA1* or the p.N256K mutant was generated by Viraquest (North Liberty, Iowa).

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Details regarding the construction of the CPA1 splice-site and duplication mutant expression plasmids are provided in the Supplementary Note.

Cell culture and transfection

HEK 293T cells were cultured in 6-well tissue culture plates (1.5×10^6 cells per well) in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad CA) supplemented with 10% fetal bovine serum, 4 mM glutamine and 1% penicillin/streptomycin at 37°C. Transfections were carried out at 90% confluence, using 10 μL Lipofectamine 2000 (Invitrogen) and 4 μg expression plasmid in 2 mL DMEM final volume. After overnight incubation, cells were washed and the transfection media was replaced with 2 mL OPTI-MEM I Reduced Serum Medium (Invitrogen). The conditioned OPTI-MEM media were harvested after 48 h incubation. AR42J rat pancreatic acinar cells (American Type Culture Collection #CRL-1492) were maintained in DMEM supplemented with 20% fetal bovine serum, 4 mM glutamine and 1% penicillin/streptomycin at 37°C. Prior to transfection, cells were plated in 6-well plates (10^6 cells per well) and were grown in the presence of 100 nM concentration of dexamethasone for 48 h to induce differentiation. Infections with adenovirus were performed using 4×10^7 plaque forming units (pfu) per mL final adenovirus concentrations in a total volume of 1 mL OPTI-MEM in the presence of dexamethasone (100 nM final concentration).

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CPA1 activity assay

Enzymatic activity of CPA1 was determined after activation with trypsin and chymotrypsin C (CTRC) using the N-[4-methoxyphenylazoformyl]-L-phenylalanine substrate[17], with minor modifications of our previously published conditions[12]. The CPA1 activity measured in the conditioned medium of transfected cells is referred to as "apparent activity" and reflects the combined effects of the variants on secreted proCPA1 levels, proteolytic degradation during activation and catalytic activity of the activated CPA1. To activate proCPA1, an aliquot (20 μL) of conditioned medium was supplemented with 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.05% Tween 20, 100 nM human cationic trypsin and 50 nM human CTRC (final concentrations in 40 μL final volume) and was incubated at 37 °C for 60 min. CPA1 activity was then measured by adding 50 μL assay buffer (0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.05% Tween 20) and 10 μL substrate (60 μM final concentration) to the activation mix. The decrease in absorbance was followed at 350 nm for 2 min. Rates of substrate cleavage were calculated from fits to the initial linear portion of the curves and were expressed as percent of the wild-type rate, which was set to 100%. The wild-type activity corresponded to 116 ± 34 mOD·min^{-1} (average ± S.D.), which equals to 262 ± 77 nM·s^{-1} substrate cleavage rate.

Measurement of proCPA1 secretion

Secreted proCPA1 protein levels in the conditioned medium were determined by SDS-PAGE and densitometry. An aliquot (200 μL) of the medium was precipitated with trichloroacetic acid (10% final concentration), the precipitate was recovered by centrifugation, dissolved in 20 μL Laemmli sample buffer containing 100 mM DTT (final concentration), and heat-denatured at 95 °C for 5 min. Electrophoretic separation was performed on 15% SDS-PAGE mini gels in standard Tris-glycine buffer and gels were stained with Brilliant Blue R-250. Quantitation of bands was carried out with the GelDocXR + gel documentation system and Image Lab 3.0 software (Bio-Rad, Hercules, CA).

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Measurement of ER stress

To study ER stress, we generated recombinant adenovirus carrying either wild-type proCPA1 or the p.Asn256Lys mutant, infected AR42J rat pancreatic acinar cells

(#CRL-1492, American Type Culture Collection [ATCC], Manassas, VA) and measured ER stress markers as described below.

Reverse transcriptase (RT)-PCR analysis and real-time PCR

Total RNA was extracted from AR42J cell lysates using RNeasy mini kit (Qiagen, Valencia, CA). RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). X-box binding protein 1 (XBP1) splicing was studied by PCR using a primer set that flanked the spliced region and amplified both spliced and unspliced forms (Supplementary Table 3). PCR was carried out using the *Taq* DNA Polymerase kit (Qiagen) with the following conditions: 10 min initial denaturation at 95°C followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 52°C, 30 sec extension at 72°C and a final extension at 72°C for 5 min. The PCR products were resolved on 2% agarose gels and stained with ethidium bromide. Quantification of mRNA expression was performed by real time PCR (7500 Real Time PCR System, Applied Biosystems). XBP1 expression was measured with SYBR Green (PCR Master Mix, Applied Biosystems) using different primer sets for the spliced, unspliced and total mRNA (Supplementary Table 3). Levels of immunoglobulin-binding protein (BiP) and calreticulin mRNA were determined using TaqMan primers (rat BiP, Rn00565250_m1; rat calreticulin, Rn00574451_m1) with TaqMan Universal PCR Mastermix (Applied Biosystems, Carslbad, CA). Real time PCR conditions were as follows: 2 min equilibration at 50°C, 10 min denaturation and enzyme activation at 95°C followed by 40 two-step cycles of 15 sec at 95°C and 60 sec at 60°C. Gene expression was quantitated using the comparative C_T method ($\Delta\Delta C_T$ method). Threshold cycle (C_T) values were determined using the 7500 System Sequence Detection Software 1.3. Expression levels of target genes were first normalized to the GAPDH internal control gene (ΔC_T) and then to expression levels measured in cells infected with empty adenovirus ($\Delta\Delta C_T$). Results were expressed as fold changes calculated with the formula $2^{-\Delta\Delta CT}$.

Statistics

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

Supplementary Material

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Dedicated to Rudolf Ammann in commemoration of his 87th anniversary.

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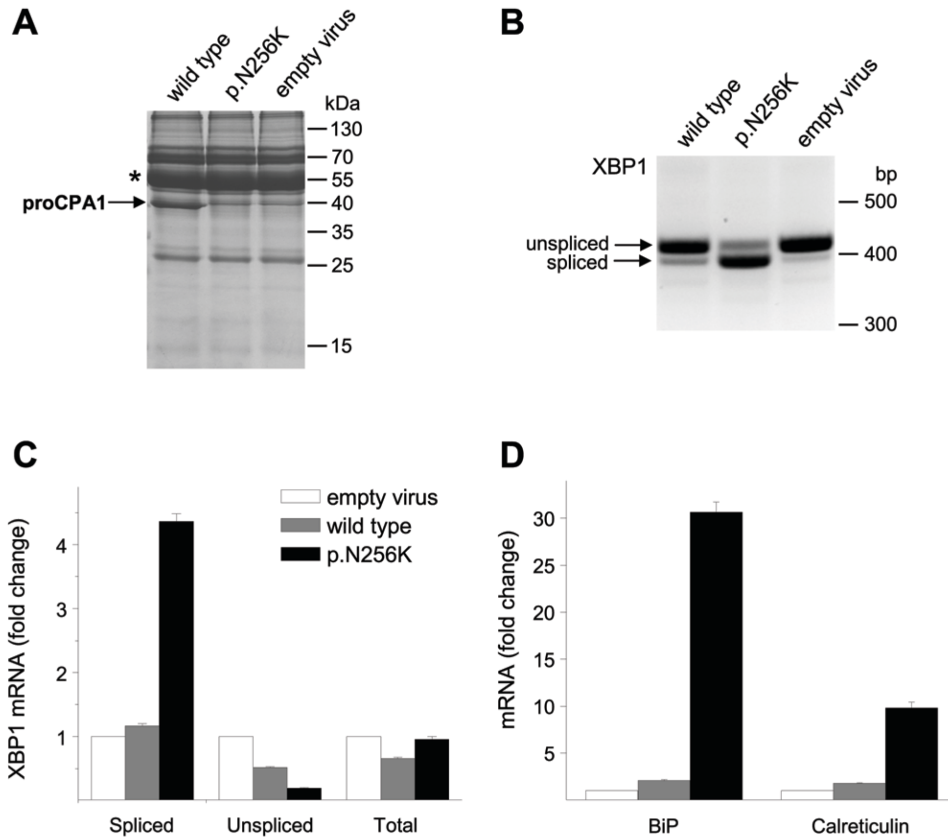

Figure 1.

Endoplasmic reticulum (ER) stress induced by the p.Asn256Lys CPA1 variant. **(A)** AR42J rat acinar cells were transfected with the indicated wild-type, mutant or empty adenovirus vectors for 24 h using 4×10^7 pfu per mL virus concentration. Conditioned media (200 μL) were precipitated with trichloroacetic acid (10% final concentration) and analyzed by SDS-PAGE and Coomassie blue staining. Note the complete lack of secretion of the p. Asn256Lys mutant. The faint band at 40 kDa represents an endogenous protein also found in the medium from cells infected with empty virus. The asterisk indicates the characteristically strong amylase band. See *Methods* for experimental details. A representative gel of three independent transfections is shown. **(B)** XBP1 splicing was assessed by RT-PCR and agarose gel electrophoresis with ethidium bromide staining. A representative gel of three independent experiments is shown. **(C)** Levels for spliced, unspliced and total XBP1 mRNA were measured by quantitative real-time PCR as described in *Methods* and expressed as fold changes relative to levels measured in cells transfected with empty adenovirus. **(D)** Quantitative real-time PCR measurement of BiP and calreticulin mRNA was performed as described in *Methods* and expressed as fold changes relative to levels measured in cells transfected with empty adenovirus. Error bars represent standard deviation ($n = 3$).

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Table 1

Non-synonymous *CPA1* variants in German subjects with non-alcoholic chronic pancreatitis and healthy controls

Exon	Nucleotide change	Amino acid change	Patients (n = 944)	Controls (n = 3938)	P value	OR	95% CI	Apparent activity	Secretion level
1	c.5G>A	p.Arg2Gln	0 (0%)	2 (0.05%)	1.0	-	-	103	92
2	**c.79C>T**	**p.Arg27Ter**	**0 (0%)**	**2 (0.05%)**	**1.0**	**-**	**-**	**0**	**0**
2	c.101C>T	p.Ala34Val	0 (0%)	1 (0.03%)	1.0	-	-	98	97
3	c.197G>A	p.Arg66Gln	0 (0%)	2 (0.05%)	1.0	-	-	60	55
3	c.281A>G	p.Gln94Arg	1 (0.1%)	13 (0.3%)	0.5	-	-	57	57
3	c.321C>G	p.Phe107Leu	0 (0%)	1 (0.03%)	1.0	-	-	112	100
3	c.371C>T	p.Thr124Ile	8 (0.9%)	45 (1.1%)	0.6	-	-	23	27
4	c.410C>G	p.Ala137Gly	1 (0.1%)	0 (0%)	0.2	-	-	52	56
5	c.497G>A	p.Gly166Asp	5 (0.5%)	20 (0.5%)	1.0	-	-	73	66
5	**c.542G>A**	**p.Arg181Gln**	**0 (0%)**	**1 (0.03%)**	**1.0**	**-**	**-**	**1**	**39**
6	c.622G>A	p.Ala208Thr (het)	71 (7.5%)	266 (6.8%)	0.4	-	-	81	73
6	c.622G>A	p.Ala208Thr (hm)	1 (0.1%)	1 (0.03%)	0.4	-	-	-	-
6	c.622G>T	p.Ala208Ser	0 (0%)	1 (0.03%)	1.0	-	-	91	83
6	**c.673G>A**	**p.Gly225Ser**	**1 (0.1%)**	**0 (0%)**	**0.2**	**-**	**-**	**4**	**12**
7	**c.710G>A**	**p.Arg237His**	**0 (0%)**	**2 (0.05%)**	**1.0**	**-**	**-**	**0**	**81**
7	**c.751G>A**	**p.Val251Met**	**2 (0.2%)**	**0 (0%)**	**0.1**	**-**	**-**	**0**	**0**
7	**c.758C>G**	**p.Pro253Arg**	**1 (0.1%)**	**0 (0%)**	**0.2**	**-**	**-**	**0**	**0**
7	**c.768C>G**	**p.Asn256Lys**	**7 (0.7%)**	**0 (0%)**	$\mathbf{9.9 \times 10^{-6}}$	**nc**	**nc**	**0**	**0**
7	c.775G>A	p.Ala259Thr	0 (0%)	1 (0.03%)	1.0	-	-	85	82
8	**c.811T>C**	**p.Cys271Arg**	**1 (0.1%)**	**0 (0%)**	**0.2**	**-**	**-**	**1**	**0**
8	**c.829G>A**	**p.Gly277Ser**	**1 (0.1%)**	**0 (0%)**	**0.2**	**-**	**-**	**0**	**0**
8	**c.839C>A**	**p.Ala280Asp**	**1 (0.1%)**	**0 (0%)**	**0.2**	**-**	**-**	**0**	**5**
8	**c.847G>A**	**p.Glu283Lys**	**2 (0.2%)**	**0 (0%)**	**0.1**	**-**	**-**	**0**	**0**
8	**c.982G>A**	**p.Glu328Lys**	**1 (0.1%)**	**0 (0%)**	**0.2**	**-**	**-**	**9**	**42**
9	c.1009G>C	p.Val337Leu	0 (0%)	1 (0.03%)	1.0	-	-	64	61
Intron 9	**c.1073-2A>G**	**p.Tyr358fs**[§]	**3 (0.3%)**	**0 (0%)**	**0.007**	**nc**	**nc**	**0**	**0**
10	**c.1085G>A**	**p.Gly362Glu**	**1 (0.1%)**	**0 (0%)**	**0.2**	**-**	**-**	**0**	**6**
10	**c.1126T>C**	**p.Ser376Pro**	**2 (0.2%)**	**0 (0%)**	**0.1**	**-**	**-**	**0**	**7**

Exon	Nucleotide change	Amino acid change	Patients (*n* = 944)	Controls (*n* = 3938)	*P* value	OR	95% CI	Apparent activity	Secretion level
10	**c.1144C>T**	**p.Arg382Trp**	**5 (0.5%)**	**0 (0%)**	**0.0003**	**nc**	**nc**	**0**	**31**
10	c.1157G>A	p.Arg386His	0 (0%)	1 (0.03%)	1.0	-	-	92	97
10	c.1193C>T	p.Pro398Leu	0 (0%)	1 (0.03%)	1.0	-	-	42	64
10	c.1217C>G	p.Ala406Gly	0 (0%)	1 (0.03%)	1.0	-	-	137	114
10	**c.1247delA**	**p.Asn416fs**	**1 (0.1%)**	**0 (0%)**	**0.2**	**-**	**-**	**11**	**15**
10	c.1251C>A	p.His417Gln	0 (0%)	1 (0.03%)	1.0	-	-	62	54
10	c.1253C>T	p.Pro418Leu	0 (0%)	1 (0.03%)	1.0	-	-	91	99
All variants with apparent activity <20%			**29 (3.1%)**	**5 (0.1%)**	**1.5×10^{-16}**	**24.9**	**9.6-64.6**	**-**	**-**

P values were determined by Fisher's Exact Test. Apparent CPA1 activity and secretion level are expressed as percent of wild type. Apparent activity corresponds to the CPA1 activity measured in the conditioned medium of transfected cells after activation with trypsin and chymotrypsin C (CTRC) (See *Methods*). Thus, apparent activity reflects the combined effects of the variants on secretion, catalytic activity and degradation by trypsin and/or CTRC. Secretion level indicates the concentration of proCPA1 in the conditioned medium measured by SDS-PAGE and densitometry (See *Methods*). Alterations marked in bold indicate variants with less than 20% apparent activity.

§Splice site variant modeled functionally as intron retention as described in *Methods*.

OR, odds ratio, CI confidence interval, het, heterozygous; hm, homozygous; nc, not calculated as variant was not detected in controls rendering OR infinite.

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Table 2

Distribution of functionally impaired *CPA1* variants in different age groups of German subjects with non-alcoholic chronic pancreatitis

Age of Patients	Patients	Controls	*P* value	OR	95% CI
All	29/944 (3.1%)	5/3938 (0.1%)	1.5×10^{-16}	24.9	9.6-64.6
age > 20 years	2/358 (0.6%)	5/3938 (0.1%)	0.2	-	-
age 20 years	27/586 (4.6%)	5/3938 (0.1%)	6.8×10^{-20}	38.0	14.6-99.1
age 10 years	22/228 (9.7%)	5/3938 (0.1%)	4.1×10^{-24}	84.0	31.5-224.1

P values were determined by Fisher's Exact Test. Alterations with less than 20% apparent activity were included.

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Table 3

Non-synonymous *CPA1* variants in the European replication study

Exon	Nucleotide change	Amino acid change	Patients (n = 600)	Controls (n = 2432)	P value	OR	95% CI	Apparent activity	Secretion level
1	c.5G>A	p.Arg2Gln	0 (0%)	6 (0.2%)	0.6	-	-	103	92
2	**c.79C>T**	**p.Arg27Ter**	**1 (0.2%)**	**5 (0.2%)**	**0.7**	**-**	**-**	**0**	**0**
2	**c.80G>C**	**p.Arg27Pro**	**0 (0%)**	**1 (0.04%)**	**1.0**	**-**	**-**	**0**	**0**
Intron 2	**c.148-1G>A**	**p.Leu50_Glu127del**[§]	**0 (0%)**	**1 (0.04%)**	**1.0**	**-**	**-**	**0**	**0**
3	c.197G>A	p.Arg66Gln	0 (0%)	1 (0.04%)	1.0	-	-	60	55
3	c.241T>C	p.Ser81Pro	0 (0%)	1 (0.04%)	1.0	-	-	53	57
3	c.281A>G	p.Gln94Arg	0 (0%)	9 (0.4%)	1.0	-	-	57	57
3	c.313T>C	p.Phe105Leu	1 (0.2%)	0 (0%)	0.2	-	-	109	99
3	c.334C>T	p.Arg112Cys	1 (0.2%)	0 (0%)	0.2	-	-	69	78
3	c.371C>T	p.Thr124Ile	3 (0.5%)	14 (0.6%)	1.0	-	-	23	27
4	c.389A>C	p.Asp130Ala	0 (0%)	1 (0.04%)	1.0	-	-	77	68
5	c.497G>A	p.Gly166Asp	1 (0.2%)	11 (0.5%)	0.5	-	-	73	66
6	c.604C>A	p.Gln202Lys	1 (0.2%)	0 (0%)	0.2	-	-	114	104
6	c.622G>A	p.Ala208Thr	45 (7.5%)*	143 (5.9%)*	0.1	-	-	81	73
6	**c.686C>T**	**p.Thr229Met**	**0 (0%)**	**1 (0.04%)**	**1.0**	**-**	**-**	**0**	**0**
6	c.695C>T	p.Thr232Met	1 (0.2%)	0 (0%)	0.2	-	-	87	80
7	**c.751G>A**	**p.Val251Met**	**1 (0.2%)**	**0 (0%)**	**0.2**	**-**	**-**	**0**	**0**
8	**c.809C>G**	**p.Pro270Arg**	**1 (0.2%)**	**0 (0%)**	**0.2**	**-**	**-**	**9**	**14**
8	**c.941A>G**	**p.Tyr314Cys**	**1 (0.2%)**	**0 (0%)**	**0.2**	**-**	**-**	**0**	**23**
8	**c.954_955delCA**	**p.Tyr318Ter**	**2 (0.3%)**	**0 (0%)**	**0.04**	**nc**	**nc**	**0**	**0**
9	c.1010T>C	p.Val337Ala	0 (0%)	1 (0.04%)	1.0	-	-	63	90
Intron 9	**c.1072+1G>T**	**p.Asp330fs**[§]	**0 (0%)**	**1 (0.04%)**	**1.0**	**-**	**-**	**0**	**0**
Intron 9	**c.1073-2A>G**	**p.Tyr358fs**[§]	**1 (0.2%)**	**0 (0%)**	**0.2**	**-**	**-**	**0**	**0**
10	c.1115G>A	p.Gly372Asp	1[&] (0.2%)	0 (0%)	0.2	-	-	25	34
10	c.1203G>C	p.Lys401Asn	1 (0.2%)	0 (0%)	0.2	-	-	115	103
10	**c.1217C>T**	**p.Ala406Val**	**1 (0.2%)**	**0 (0%)**	**0.2**	**-**	**-**	**0**	**87**
All variants with apparent activity <20%			**8 (1.3%)**	**9 (0.4%)**	**0.01**	**3.6**	**1.4-9.5**	**-**	**-**

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P values were determined by Fisher's Exact Test. Apparent CPA1 activity and secretion level were measured as described in Table 1 and expressed as percent of wild type. Alterations marked in bold indicate variants with less than 20% apparent activity.

[§] Functional effects of splice site variants c.148-1G>A, c.1072+1G>T, and c.1073-2A>G were modeled as skipping of exon 3, skipping of exon 9 and retention of intron 9, respectively, as described in *Methods*.

[*] One individual was homozygous for p.Ala208Thr.

[&] Individual was homozygous for this variant.

OR, odds ratio, CI confidence interval, nc, not calculated as variant was not detected in controls rendering OR infinite.

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Table 4

Non-synonymous *CPA1* variants in Indian subjects with non-alcoholic chronic pancreatitis and healthy controls

Exon	Nucleotide change	Amino acid change	Patients (n = 230)	Controls (n = 264)	P value	OR	95% CI	Apparent activity	Secretion level
2	c.94G>C	p.Asp32His	1 (0.4%)	0 (0%)	0.5	-	-	79	75
5	c.506G>A	p.Arg169His	4 (1.7%)	0 (0%)	0.046	nc	nc	24	23
6	c.622G>A	p.Ala208Thr	6 (2.6%)	7 (2.7%)	1.0	-	-	81	73
8	**c.922T>C**	**p.Tyr308His**	**5 (2.2%)**	**0 (0%)**	**0.02**	**nc**	**nc**	**3**	**17**
All variants with apparent activity <20%			**5 (2.2%)**	**0 (0%)**	**0.02**	**nc**	**nc**	**-**	**-**

P values were determined by Fisher's Exact Test. Apparent CPA1 activity and secretion level were measured as described in Table 1 and expressed as percent of wild type. Alterations marked in bold indicate variants with less than 20% apparent activity.

OR, odds ratio, CI confidence interval, nc, not calculated as variant was not detected in controls rendering OR infinite.

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Table 5

Non-synonymous *CPA1* variants in Japanese subjects with non-alcoholic chronic pancreatitis and healthy controls

Exon	Nucleotide change	Amino acid change	Patients (n = 247)	Controls (n = 341)	*P* value	OR	95% CI	Apparent activity	Secretion level
4	c.410C>G	p.Ala137Gly	1 (0.4%)	0 (0%)	0.42	-	-	52	56
7	**c.713A>T**	**p.Lys238Met**	**1 (0.4%)**	**0 (0%)**	**0.42**	**-**	**-**	**0**	**3**
7	**c.751G>A**	**p.Val251Met**	**2 (0.8%)**	**0 (0%)**	**0.18**	**-**	**-**	**0**	**0**
7	**c.764G>T**	**p.Arg255Met**	**1 (0.4%)**	**0 (0%)**	**0.42**	**-**	**-**	**0**	**86**
9	c.1021G>A	p.Ala341Thr	37 (15.0%)	53 (15.5%)	1.0	-	-	99	85
10	**c.1079-27_1111dup60**	**p.Thr368_Tyr369ins20**§	**1 (0.4%)**	**0 (0%)**	**0.42**	**-**	**-**	**0**	**49**
	All variants with apparent activity <20%		**5 (2.0%)**	**0 (0%)**	**0.013**	**nc**	**nc**	**-**	**-**

P values were determined by Fisher's Exact Test. Apparent CPA1 activity and secretion level were measured as described in Table 1 and expressed as percent of wild type. Alterations marked in bold indicate variants with less than 20% apparent activity.

§Functional effect of variant **c.1079-27_1111dup60** was modeled as insertion of 20 amino acids between Thr368 and Tyr369, as described in *Methods*.

OR, odds ratio, CI confidence interval, nc, not calculated as variant was not detected in controls rendering OR infinite.