

RESEARCH ARTICLE

Cell and Animal Models of Gastrointestinal Disease

Reevaluating the role of TRPV6 deficiency in human and mouse pancreatitis

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Abstract

Functionally defective genetic variants of the calcium channel transient receptor potential vanilloid type 6 (TRPV6) have been shown to markedly increase the risk for chronic pancreatitis in humans. Genetic inactivation of *Trpv6* in mice worsened the severity of pancreatitis induced by secretagogue hyperstimulation. The aims of the present study were to reexamine the role of loss-of-function *TRPV6* gene variants in an adult Hungarian cohort with nonalcoholic chronic pancreatitis and to characterize cerulein-induced pancreatitis in a novel *Trpv6*-deleted mouse strain. We found that 2.1% of chronic pancreatitis patients carried defective *TRPV6* variants, whereas no such variants were identified in control subjects. Homozygous deletion of *Trpv6* in C57BL/6N mice (*Trpv6*-KO) caused no spontaneous pancreatitis but slightly increased the severity of cerulein-induced acute pancreatitis and delayed recovery after an acute episode in a subset of mice. Cerulein-induced intrapancreatic trypsin and chymotrypsin activity was unchanged in *Trpv6*-KO mice relative to C57BL/6N controls. Crossing homozygous *Trpv6*-KO mice with heterozygous *Spink1*-KO mice did not result in spontaneous pancreatitis. Our results confirmed the association of defective *TRPV6* variants with human chronic pancreatitis in a nonalcoholic cohort. Furthermore, we found that *TRPV6* played a relatively minor role in cerulein-induced murine pancreatitis, highlighting the need for better animal models for studying human *TRPV6* variants.

NEW & NOTEWORTHY Prior studies indicated that defective variants of transient receptor potential vanilloid type 6 (*TRPV6*) encoding a constitutive calcium channel increased the risk of chronic pancreatitis in humans, and genetic inactivation of *Trpv6* aggravated the severity of experimental pancreatitis in mice. We reexamined these findings by conducting a genetic case-control study and characterizing novel *Trpv6*-deleted mice. We confirmed the association of defective *TRPV6* variants with human chronic pancreatitis but found that *Trpv6* deficiency had limited impact on secretagogue-induced pancreatitis in mice.

calcium channel; cerulein; chronic pancreatitis; mouse model; *SPINK1*

INTRODUCTION

Chronic pancreatitis (CP) often develops due to underlying genetic risk factors (1). Genetic variants that increase CP risk cause increased intrapancreatic trypsin activity (trypsin-dependent pathway), misfolding of digestive enzymes

(misfolding-dependent pathway), or affect ductal function (ductal pathway) (2–4). The trypsin-dependent pathway of genetic risk includes variants in the human cationic trypsinogen (*PRSS1*), the serine protease inhibitor Kazal type 1 (*SPINK1*), and chymotrypsin C (*CTRC*). The misfolding-dependent pathway includes variants in carboxypeptidase



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A1 (*CPA1*), *PRSSI*, and the carboxyl ester lipase (*CEL*). Altered ductal function associated with cystic fibrosis transmembrane conductance regulator (*CFTR*) variants increases disease risk (4). The ductal pathway may also include rare variants of the calcium-sensing receptor (*CASR*), common variants at the claudin 2 locus (*CLDN2*), and variants in *TRPV6*, which encodes the epithelial calcium channel named transient receptor potential vanilloid type 6 (5).

TRPV6 was recognized as a CP susceptibility gene in 2020, when Masamune et al. (6) reported that rare loss-of-function variants in *TRPV6* markedly increased risk for early-onset nonalcoholic CP in cohorts from Japan, Germany, and France. The frequency of these variants in cases and controls was around 2%–4% and 0%–0.1%, respectively, resulting in high odds ratios (ORs). The authors also observed that a few functional variants were associated with CP, with lower odds ratios, such as p.I223T and p.D324N in Japanese or p.A18S and p.L299Q in Europeans. The pathogenic *SPINK1* variant p.N34S was found in 20% of cases with defective *TRPV6* variants and in almost 30% of p.L299Q carriers, suggesting that *TRPV6* variants may be mechanistically linked to the trypsin-dependent pathway. In agreement with the proposed pathogenic role in humans, a mouse model carrying the homozygous p.D581A inactivating pore mutation in the mouse *Trpv6* (corresponding to p.D582A in human *TRPV6*) exhibited more severe pancreatitis in response to cerulein hyperstimulation. However, the number of experimental animals was small, and the mice were on a mixed genetic background, which may have confounded the results.

In a replication study, Zou et al. (7) analyzed CP cases and controls from China and confirmed the association of defective *TRPV6* variants with CP, although the frequency in cases was lower (1.35%) than previously reported. Subsequently, Oracz et al. (8) detected 3.9% defective *TRPV6* variants in a Polish pediatric CP cohort. The same study analyzed additional German cases and confirmed the previously observed 2% frequency of defective *TRPV6* variants. Enrichment of the functional variant p.L299Q in CP was also confirmed. A French replication study in 2022 investigated not only idiopathic but also familial and hereditary CP cases (9). Frequencies of defective *TRPV6* variants were higher in the familial and hereditary cohort (3.2%) versus the idiopathic cohort (1.5%). Finally, several case reports and cohort studies described defective *TRPV6* variants in CP without analyzing controls (10–15). To date, pancreatitis genetics studies have reported 133 *TRPV6* variants with 110 analyzed functionally, 37 of which had less than 25% activity, while 23 have not been tested yet.

More recently, an important study by Masamune et al. (16) compared the clinical course of *TRPV6*-associated CP with cases carrying *PRSSI* or *SPINK1* variants or no known pathogenic mutations. The authors found similar disease characteristics for *TRPV6*-, *PRSSI*-, and *SPINK1*-related CP, while mutation-negative cases showed a less severe course. The investigators also described a new mouse model with *Pdx1-Cre*-driven pancreas-specific deletion of *Trpv6*. They observed that *Trpv6* deletion resulted in increased severity of cerulein-induced acute and chronic pancreatitis, although the number of experimental mice was not reported, and a *Pdx1-Cre* control was not tested. Interestingly,

cerulein upregulated *Trpv6* mRNA expression, a curious phenomenon also observed by Shah et al. (10). Finally, organoid cultures derived from wild-type and *Trpv6*-deleted mice showed no functional differences, suggesting that *TRPV6* is important for acinar rather than ductal function in the pancreas.

The aims of the present replication study were to investigate the significance of defective *TRPV6* variants in an adult Hungarian cohort with nonalcoholic CP and to generate and characterize a novel *Trpv6*-deleted mouse strain.

MATERIALS AND METHODS

Accession Numbers and Nomenclature

Nucleotide numbering corresponds to the coding DNA sequence of *TRPV6*, with the first nucleotide of the unconventional ACG translation initiation codon denoted as +1. Amino-acid numbering starts with the initiator methionine of the primary translation product. The human *TRPV6* genomic reference sequence was NC_000007.14, the human *TRPV6* mRNA reference sequence was NM_018646.6, and the mouse *Trpv6* genomic reference sequence was NC_000072.7.

Subjects

The study was conducted in accordance with the principles and guidelines of the Declaration of Helsinki and approved by the Institutional Review Board and Ethics Committee of the National Center for Public Health and Pharmacy [Ethical Approval Nos. 22254-1/2012/EKU (391/PI/2012), TUKEB 36305-1/2016/EKU, and NNK 17787-8/2020/EÜIG; and Biobanking Approval No. IF702-19/2012]. Deidentified genomic DNA samples were obtained from the Hungarian National Pancreas Registry. Subjects were recruited from 11 Hungarian centers between 2012 and 2019, including 145 unrelated patients with nonalcoholic CP (age at recruitment: 59.8 ± 12 yr; means ± SD; range: 21–85 yr), and 296 control subjects (47.3 ± 10.1 yr; range: 20–66 yr) with no pancreatic disease. All participants provided informed consent. Diagnosis of CP was based on the history of recurrent acute pancreatitis or recurrent abdominal pain typical for CP with pathological imaging findings consistent with CP, such as pancreatic calcifications, duct dilatation, or irregularities, with or without exocrine pancreatic insufficiency or diabetes.

Genotyping

Exonic and adjacent intronic sequences of *TRPV6* were analyzed by Sanger sequencing after PCR amplification. The PCR mix consisted of 0.6 U HotStarTaq DNA Polymerase (catalog no. 203205, Qiagen, Germantown, MD), 0.2 mM dNTP, 0.5 μM forward and reverse primers, 10× PCR buffer, and 10–50 ng genomic DNA template, in a final volume of 20 μL. Primer sequences are described in Oracz et al. (8). PCR amplicons (5 μL) were treated with 1 μL FastAP thermosensitive alkaline phosphatase (catalog no. EF0651, Thermo Fisher Scientific) and 0.5 μL exonuclease I (catalog number EN0581, Thermo Fisher Scientific) for 15 min at 37°C, and the reaction was stopped by heating the samples to 85°C for 15 min. The PCR primers were used as sequencing primers.

Meta-Analysis

Formal meta-analysis of case-control studies investigating *TRPV6* variants in CP cases and healthy controls (6–9) was carried out using the Review Manager 5.4 software (<https://www.cochrane.org>). For each variant, residual *TRPV6* function was calculated from the published graphs after subtraction of the background and was expressed as a percentage of the wild-type activity. Pooled odds ratio (OR) and 95% confidence interval (CI) values were calculated with the random effects model (Mantel-Haenszel method). Heterogeneity between studies was judged by the I^2 and χ^2 tests.

TRPV6 Expression Plasmids

The wild-type *TRPV6* coding DNA was synthesized (GenScript, Piscataway, NJ) and cloned into the pcDNA3.1(-) vector using the *Xho*I and *Eco*RI restriction sites. In this construct, the native noncanonical ACG start codon was changed to ATG. The 5'-untranslated region (UTR) was shortened to seven nucleotides, and the native 3'-UTR was replaced with the 79-nucleotide-long 3'-UTR sequence from human *SPINK1*. The following five mutations were introduced (GenScript): c.428C>T (p.P143L), c.476C>T (p.T159I), c.580C>T (p.R194C), c.1346G>A (p.R449K), and c.1816G>A (p.A606T).

Western Blot

For immunological analysis by Western blot, HEK 293T cells (catalog no. Q401, GenHunter Corporation, Nashville, TN) were transfected with wild-type and mutant expression plasmids, as described previously (17). Cell lysates were prepared using RIPA Buffer (catalog no. R0278-50ML, MilliporeSigma) according to our published protocol (18). Aliquots containing 10 μ g total protein were resolved by 12% SDS-PAGE, transferred to an Immobilon-P membrane (catalog no. IPVH00010, MilliporeSigma), and probed with a rabbit polyclonal anti-TRPV6 antibody used at a 1:1,000 dilution (catalog no. ACC-036, Alomone Labs, Jerusalem, Israel). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (catalog no. HAF008, R&D Systems, Minneapolis, MN) used at a 1:10,000 dilution. Human α -tubulin was measured as a loading control using a mouse monoclonal antibody (DM1A) against α -tubulin (CP06, MilliporeSigma) at a 1:2,000 dilution, followed by HRP-conjugated goat anti-mouse IgG (HAF007, R&D Systems) at a 1:2,500 dilution. Incubations were carried out at 22°C. The primary antibodies were used overnight, while the secondary antibodies were applied for 1 h. Protein bands were detected using the SuperSignal West Pico PLUS Chemiluminescent Substrate (catalog no. 34580, Thermo Fisher Scientific). Densitometric evaluation of TRPV6 band intensities was performed with the Bio-Rad Image Lab 6.1 software.

Ca²⁺ Measurements

The functional activity of TRPV6 variants was assessed by measuring intracellular Ca²⁺ changes in HEK 293 cells as described previously (19). Briefly, cells (catalog no. CRL-1573, ATCC, Manassas, VA) were cultured in minimum essential medium (MEM; catalog no. 11095080, Thermo Fisher

Scientific) plus 10% FBS, and cotransfected with wild-type or mutant TRPV6 expression plasmids and the genetically encoded Ca²⁺ sensor GCaMP6f using the Effectene transfection reagent (catalog no. 301427, Qiagen). After 24 h, the transfected cells were plated in a poly-L-lysine-coated black-wall, clear-bottom, 96-well plate, and experiments were performed 48 h after plating. Thirty minutes before measurements, the serum-containing MEM media was replaced with a nominally divalent-free solution containing 5 mM KCl, 137 mM NaCl, 10 mM HEPES (pH 7.4), and 10 mM glucose, and the plate was kept at 37°C. Various concentrations of calcium (0.3, 1, 3, and 10 mM) were applied to initiate Ca²⁺ influx through TRPV6, and fluorescence changes were recorded at 37°C. At the end of the measurement, 5 μ M ionomycin was applied in the presence of 10 mM Ca²⁺ to determine the maximal achievable fluorescence. The GCaMP6f fluorescence was measured at an excitation wavelength of 485 nm, and emission was collected at 525 nm using the FlexStation 3 plate reader (Molecular Devices). For each TRPV6 construct, 4 separate transfections were performed, and measurements from 3 replicates per transfection were averaged and treated as a single data point for statistical analysis. To compare the intracellular calcium responses between wild-type and variant groups, area under the curve (AUC) calculations were carried out using Prism 10 (GraphPad Software, Boston, MA). The AUC between 0.3 mM and 10 mM Ca²⁺ was determined for each group from Fig. 1B. Mean values were compared between wild-type and variant groups using one-way ANOVA with Tukey's post hoc analysis.

Animal Studies Approval

Animal experiments were performed at the University of California, Los Angeles (UCLA) with the approval and oversight of the Animal Research Committee, including protocol review and postapproval monitoring. The animal care program is managed in full compliance with the US Animal Welfare Act, the US Department of Agriculture Animal Welfare Regulations, the US Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the National Research Council's *Guide for the Care and Use of Laboratory Animals*. UCLA has an approved Animal Welfare Assurance statement on file with the US Public Health Service, National Institutes of Health, Office of Laboratory Animal Welfare, and it is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Generation of the *Trpv6*-KO Mouse Strain

Mouse *Trpv6* is located in the vicinity of the trypsinogen locus on chromosome 6; it spans ~15.7 kb and comprises 15 exons. Deletion of 6079 nucleotides from c.249-1009 (intron 1) to c.1905 + 167 (intron 13) was accomplished by CRISPR/Cas9-mediated genome engineering on the C57BL/6N genetic background (Cyagen US Inc., Santa Clara, CA). In the resultant *Trpv6*-knockout mouse strain (*Trpv6*-KO), 12 of the 15 exons were excised, from exon 2 through exon 13. Because homozygous *Trpv6*-KO male mice were infertile, the *Trpv6*-KO strain was maintained by breeding heterozygous males with homozygous females, and the homozygous

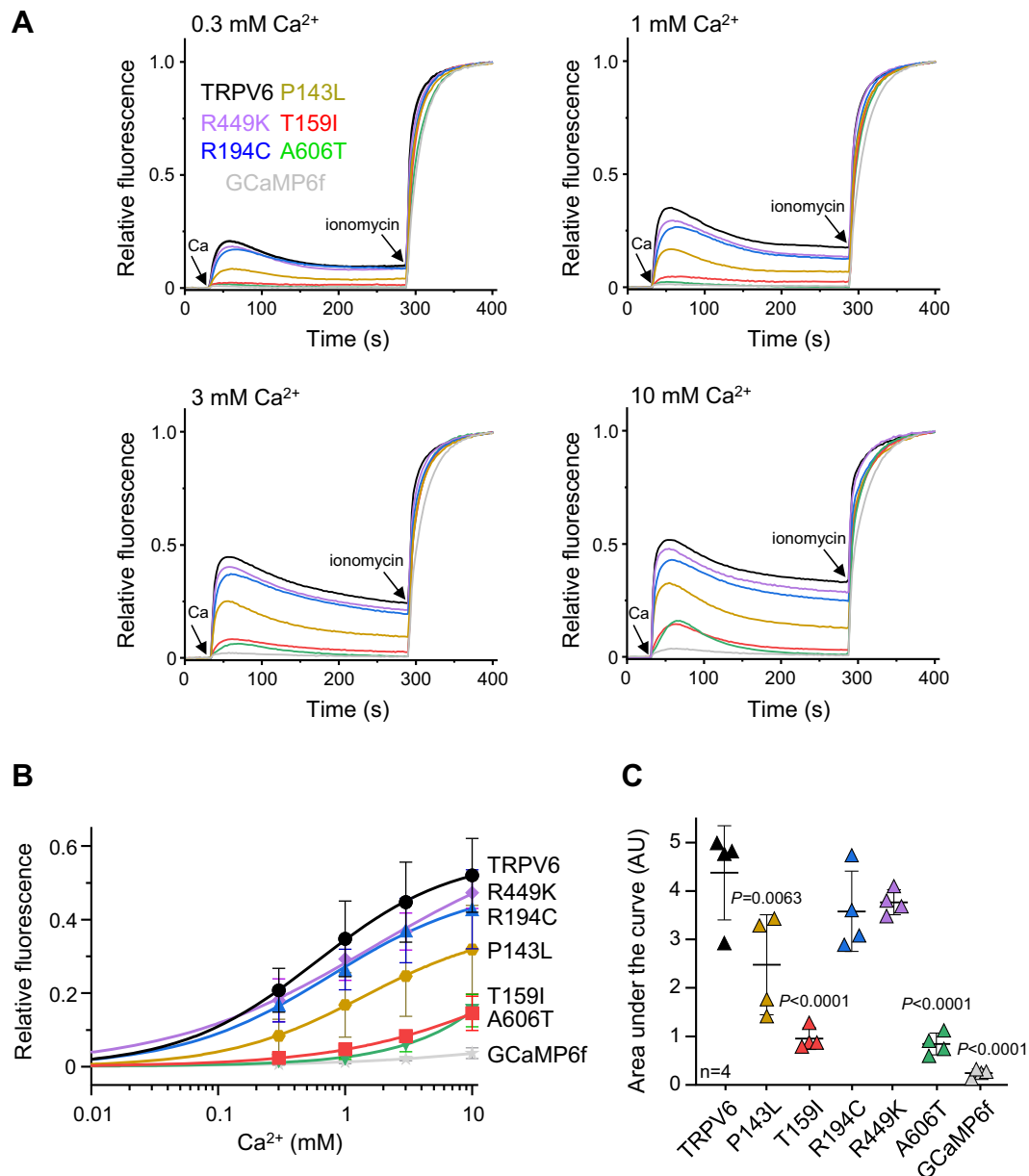


Figure 1. Effect of genetic variants on transient receptor potential vanilloid type 6 (TRPV6)-mediated Ca^{2+} influx in HEK 293 cells. Measurements were performed as described in MATERIALS AND METHODS. Cells were transiently transfected with the genetically encoded fluorescent Ca^{2+} indicator GCaMP6f, wild-type human TRPV6, or the indicated TRPV6 variants. **A:** representative measurements with 0.3 mM, 1 mM, 3 mM, and 10 mM Ca^{2+} . The arrows indicate when Ca^{2+} (Ca) and ionomycin were applied. The baseline fluorescence was subtracted, and traces were normalized to the maximum fluorescence measured after ionomycin addition. Cells transfected with GCaMP6f only were included as a negative control to measure endogenous basal Ca^{2+} influx in the absence of TRPV6. **B:** summary plot of Ca^{2+} influx as a function of the applied calcium concentration (means $\pm$ standard deviation; $n = 4$ transfections with 3 technical replicates each). **C:** statistical analysis of differences in Ca^{2+} influx mediated by wild-type TRPV6 and indicated variants. Individual data points for the area under the curve in arbitrary units (AU) from **B** were plotted for each construct with the mean and standard deviation indicated (see MATERIALS AND METHODS). The difference of means was analyzed by one-way ANOVA followed by Tukey's post hoc test.

offspring were used for experiments. C57BL/6N mice obtained from Charles River Laboratories (Wilmington, MA) or produced in our breeding facility from the same stock were used as experimental controls. Both male and female animals (8–10 wk of age) were included in the studies. To evaluate older mice for spontaneous pancreatitis, a subset of homozygous *Trpv6*-KO animals was euthanized at 6 mo ($n = 12$) and 12 mo ($n = 7$) of age. Crossing homozygous *Trpv6*-KO mice with heterozygous *Spink1*-KO mice (20) was achieved by breeding double heterozygous males (*Trpv6*-

KO^{het} $\times$ *Spink1*-*KO*^{het}) with homozygous *Trpv6*-KO females. The *Trpv6*-*KO*^{hom} $\times$ *Spink1*-*KO*^{het} mice ($n = 11$) were euthanized at the age of 6 mo and evaluated for spontaneous pancreatitis only.

Genotyping Mice

The following primers were used to genotype mice for the *Trpv6*-KO allele. *Trpv6* forward primer: 5'-TTA AGG TGT GCT CTT TAG AGG CTG-3'; *Trpv6*-KO reverse primer: 5'-

CAC ATT CAG TTT TTC TGG GTC AAA-3'; and *Trpv6*-WT reverse primer: 5'-CAG AGA CTG GTG GGT TTT CTT CTT-3'. The PCR contained all 3 primers. The amplicon size from the *Trpv6*-KO allele was 361 bp, whereas the wild-type allele yielded a 474-bp product. Conditions for genotyping the *Spink1*-KO allele were reported previously (20).

Cerulein-Induced Pancreatitis

Cerulein (ammonium salt, catalog no. 4030451, Bachem Americas, Torrance, CA) was dissolved in sterile normal saline at a concentration of 10 µg/mL and filtered through a 0.2-µm syringe filter. To induce acute pancreatitis, mice were given 10 hourly intraperitoneal injections (10×) of saline or cerulein (50-µg/kg dose), or 8 injections on 2 consecutive days (2×8×), as indicated. Mice were euthanized 1 h after the last injection. To study recovery after the acute episode, mice were treated with the 2×8× injection protocol and were euthanized 3 days after the last injection.

Plasma Amylase Assay

Blood was obtained by cardiac puncture in heparinized syringes, and 1 µL of plasma was diluted to 10 µL with normal saline. Amylase activity was measured by adding 190 µL of the 2-chloro-*p*-nitrophenyl- α -D-maltotriose substrate (catalog no. A7564-60, Pointe Scientific, Canton, MI), as described previously (21). The rate of substrate cleavage was expressed in milli-optical density (mOD) per minute.

Histology

Pancreas tissue was fixed and stored in 10% neutral buffered formalin until processing. Paraffin blocks were sectioned and stained with hematoxylin-eosin at the Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center (Los Angeles, CA). Pancreas sections of cerulein-treated mice were scored by visual inspection for three parameters of acute pancreatitis (tissue edema, inflammatory cell infiltration, and acinar cell necrosis), as described recently (20). The degree of edema and inflammatory cell infiltration was expressed on an arbitrary scale of 0–3, while acinar cell necrosis was expressed as a percentage of the tissue area evaluated. Pancreas sections from saline-treated mice were not scored because they exhibited no histological changes. To quantitate recovery after an

acute pancreatitis episode, pancreas sections were scored for intact acinar cells, expressed as a percentage of the acinar tissue area examined.

Intrapancreatic Protease Activity

Mice were given a single injection of saline or cerulein (50-µg/kg dose) and euthanized 30 min later. Intrapancreatic trypsin and chymotrypsin activity was determined from freshly prepared pancreas homogenates using our published protocol (22). Activity was expressed in relative fluorescent units (RFU) per second, normalized to the total protein content in milligrams.

Statistics

Descriptive and inferential statistics were calculated using the Prism 10 software (GraphPad Software, Boston, MA). For the case-control study, OR and 95% CI values were determined by the Baptista-Pike method. The significance of differences in carrier frequencies between human cases and controls was assessed using Fisher's exact test. The difference in means between two experimental groups and within multiple groups was analyzed by two-tailed unpaired *t*-test and by one-way ANOVA with Tukey's post hoc analysis, respectively. *P* < 0.05 was considered statistically significant.

RESULTS

DNA Sequence Analysis of Human *TRPV6*

We used Sanger sequencing to analyze the 15 exons and flanking intronic sequences in 145 nonalcoholic CP cases and 296 controls of Hungarian origin. We found 12 nonsynonymous variants, including 4 novel missense variants: c.428C>T (p.P143L), c.476C>T (p.T159I), c.580C>T (p.R194C), and c.1346G>A (p.R449K) (Table 1). When considered individually, none of the variants was overrepresented to a statistically significant extent in cases or controls. The so-called ancestral *TRPV6* haplotype (p.C197R, p.M418V, p.M721T) (23) was found in 11.7% of cases and 14.5% of controls (*P* = 0.46). The p.A18S variant, which was enriched in European controls versus CP cases (6, 9), was present in 9% of Hungarian cases and in 6% of controls, including two homozygous subjects

Table 1. Nonsynonymous *TRPV6* variants in nonalcoholic chronic pancreatitis cases and healthy controls

Exon	Nucleotide Change	Amino Acid Change	CP (n = 145)	Controls (n = 296)	OR	95% CI	P
1	c.52G>T	p.A18S	13 (9%)	18 (6.1%)#	1.52	0.75–3.08	0.3213
3	c.428C>T	p.P143L	1 (0.7%)	0			
4	c.476C>T	p.T159I	1 (0.7%)	0			
4	c.580C>T	p.R194C	1 (0.7%)	0			
4	c.589T>C	p.C197R	17 (11.7%)	43 (14.5%)*	0.78	0.44–1.43	0.4626
5	c.658C>T	p.R220W	1 (0.7%)	0			
7	c.896T>A	p.L299Q	0	2 (0.7%)			
7	c.926C>T	p.T309M	1 (0.7%)	2 (0.7%)	1.02	0.07–8.84	>0.999
9	c.1252A>G	p.M418V	17 (11.7%)	42 (14.2%)*	0.8	0.45–1.48	0.5524
10	c.1346G>A	p.R449K	0	1 (0.3%)			
13	c.1816G>A	p.A606T	1 (0.7%)	0			
15	c.2162T>C	p.M721T	17 (11.7%)	43 (14.5%)*	0.78	0.44–1.43	0.4626
Functionally defective variants			3 (2.1%)	0			

Functionally defective variants are in boldface. CI, confidence interval; CP, chronic pancreatitis; OR, odds ratio; *TRPV6*, transient receptor potential vanilloid type 6. #Two controls were homozygous for the c.52G>T (p.A18S) variant. *Ancestral haplotype: 2 controls were homozygous.

Table 2. *Synonymous TRPV6 variants in nonalcoholic chronic pancreatitis cases and healthy controls*

Exon	Nucleotide Change	Amino Acid Change	CP (n = 145)	Controls (n = 296)	OR	95% CI	P
1	c.42G>A	p.G14=	0	1 (0.3%)			
1	c.78C>T	p.V26=	0	1 (0.3%)			
7	c.927G>A	p.T309=	1 (0.7%)	2 (0.7%)	1.02	0.07–8.84	>0.999
8	c.1056G>A	p.P352=	0	1 (0.3%)			
8	c.1200G>A	p.T400=	17 (11.7%)	42 (14.2%)*	0.84	0.45–1.48	0.5524
9	c.1263G>A	p.K421=	0	1 (0.3%)			
9	c.1323G>A	p.L441=	1 (0.7%)	0			
11	c.1512C>T	p.N504=	41 (28.3%)&	47 (15.9%)&	2.09	1.29–3.36	0.0034
13	c.1743C>T	p.I584=	0	1 (0.3%)			
14	c.1923G>A\$	p.T641=	3 (2.1%)	11 (3.7%)	0.55	0.16–1.8	0.5639
14	c.1998A>G	p.G666=	17 (11.7%)	43 (14.5%)*	0.78	0.44–1.43	0.4626
15	c.2202T>C	p.N734=	6 (4.1%)	16 (5.4%)	0.76	0.3–1.93	0.6479

CI, confidence interval; CP, chronic pancreatitis; OR, odds ratio; TRPV6, transient receptor potential vanilloid type 6. *Ancestral haplotype: 2 controls were homozygous. &One patient and 1 control were homozygous for c.1512C>T (p.N504=). \$National Center for Biotechnology Information (NCBI) reference sequence NC_000007.14 contains the minor allele A at this position.

($P = 0.32$). The functional variant p.L299Q, reported to increase CP risk in European cohorts (6, 8, 9), was found in two controls but not in any cases. Excluding the common ancestral haplotype and the p.A18S variant, the combined frequency of missense variants in cases and controls was 4.8% and 1.7%, respectively.

We also identified 15 synonymous variants (Table 2). Two variants (p.T400= and p.G666=) were in linkage disequilibrium with the ancestral haplotype. Variant c.1512C>T

(p.N504=) was enriched in CP cases (28.3%) versus controls (15.9%), yielding an OR of 2.1 (95% CI 1.29–3.36, $P = 0.0034$). A review of unpublished German data for this variant from the Masamune et al. (6) study revealed equal distribution between cases (82/395 = 20.8%) and controls (153/765 = 20%), suggesting that our association is spurious due to multiple testing. None of the other 14 variants was overrepresented in cases or controls to a statistically significant extent. No splice-site variants were detected. The 33 intronic variants found in the flanking

Table 3. *Intronic TRPV6 variants in nonalcoholic chronic pancreatitis cases and healthy controls*

Intron	Nucleotide Change	CP (n = 145)	Controls (n = 296)	OR	95% CI	P
1	c.249-243C>G\$	19 (13.1%)	55 (18.6%)*	0.66	0.38–1.17	0.1752
1	c.249-236T>G\$	19 (13.1%)	55 (18.6%)*	0.66	0.38–1.17	0.1752
1	c.249-224C>T	1 (0.7%)	1 (0.3%)	2.05	0.11–39.04	0.55
1	c.249-140C>T	0	1 (0.3%)			
2	c.346 + 45C>T	0	1 (0.3%)			
3	c.469 + 21G>A	9 (6.2%)	28 (9.5%)#	0.63	0.29–1.35	0.2777
3	c.469 + 35G>A	17 (11.7%)	43 (14.5%)*	0.78	0.44–1.43	0.4626
4	c.607 + 51G>A	1 (0.7%)	5 (1.7%)	0.4	0.03–2.96	0.6686
4	c.607 + 90C>T	1 (0.7%)	0			
4	c.608-78G>A	7 (4.8%)	16 (5.4%)	0.89	0.33–2.13	>0.999
4	c.608-14_11del	0	1 (0.3%)			
5	c.706 + 17G>A	1 (0.7%)	1 (0.3%)	2.05	0.11–39.04	0.55
5	c.707-42A>G	0	1 (0.3%)			
5	c.707-19T>C	0	1 (0.3%)			
6	c.882 + 95T>C	0	1 (0.3%)			
6	c.882 + 183A>G	0	1 (0.3%)			
6	c.882 + 201A>C	7 (4.8%)	14 (4.7%)	1.02	0.39–2.6	>0.999
6	c.882 + 236C>T	17 (11.7%)	43 (14.5%)*	0.78	0.44–1.43	0.4626
6	c.882 + 240C>T	0	3 (1%)			
6	c.883-84T>C\$	3 (2.1%)	12 (4.1%)	0.5	0.15–1.58	0.404
10	c.1406 + 8T>C	17 (11.7%)	42 (14.2%)*	0.8	0.45–1.48	0.5524
10	c.1407-108G>C	0	1 (0.3%)			
10	c.1407-69C>T	3 (2.1%)	0			
10	c.1407-54G>A	0	6 (2%)			
12	c.1639 + 47G>A	17 (11.7%)	44 (14.9%)*	0.76	0.43–1.39	0.463
12	c.1640-154C>G	17 (11.7%)	44 (14.9%)*	0.76	0.43–1.39	0.463
12	c.1640-140G>A	1 (0.7%)	1 (0.3%)	2.05	0.11–39.04	0.55
12	c.1640-22C>T	1 (0.7%)	6 (2%)	0.34	0.03–2.06	0.4344
13	c.1908 + 74A>G	17 (11.7%)	44 (14.9%)*	0.76	0.43–1.39	0.463
14	c.2015 + 153C>T	0	1 (0.3%)			
14	c.2015 + 166G>A	0	1 (0.3%)			
14	c.2016-94G>A	1 (0.7%)	0			
14	c.2016-18T>A	1 (0.7%)	0			

CI, confidence interval; OR, odds ratio; TRPV6, transient receptor potential vanilloid type 6. *Two controls were homozygous for these variants. #One control was homozygous for c.469 + 21G>A. \$National Center for Biotechnology Information (NCBI) reference sequence NC_000007.14 contains the minor allele nucleotide.

intronic regions are listed in Table 3. No intronic variant was enriched significantly in cases or controls.

Functional Effects of Missense *TRPV6* Variants

Of the 12 missense variants detected in this study, 8 were functionally characterized previously. Only variant p.A606T was reported as defective (6), while variants p.A18S, p.R220W, p.L299Q, p.T309M, and the three variants of the ancestral haplotype were functionally normal (6, 8, 9). To test the functional effects of the four newly identified missense variants, we measured calcium influx in HEK 293 cells transiently transfected with the genetically encoded Ca^{2+} sensor GCaMP6f and the *TRPV6* variants. As a positive control, we used wild-type *TRPV6*, and as a negative control, we used the previously characterized defective variant p.A606T. At the beginning of the experiments, the cells were kept in nominally Ca^{2+} free medium, and calcium influx was initiated by increasing the extracellular Ca^{2+} to 0.3, 1, 3, and 10 mM (Fig. 1A). Averaging data from 4 transfections indicated that variants p.P143L, p.T159I, and p.A606T exhibited lower activity than wild-type *TRPV6*, which was statistically significant (Fig. 1C). In contrast, variants p.R194C and p.R449K showed nearly normal activity (Fig. 1, B and C). All 3 functionally defective variants had some activity, as indicated by the larger Ca^{2+} influx compared to cells transfected with the GCaMP6f Ca^{2+} sensor only, especially at 10 mM Ca^{2+} (Fig. 1, A–C). The p.P143L variant showed more residual activity (54%) than p.T159I (17.2%) or p.A606T (14.5%).

Western blot analysis of total cell lysates revealed reduced expression for variants p.P143L, p.T159I, and p.A606T, and normal *TRPV6* levels for variants p.R194C and p.R449K (Fig. 2A). Densitometric evaluation of Western blots from six transfections indicated that the average loss of expression was statistically significant for all defective variants (Fig. 2B). The extent of the expression reduction in variants p.P143L and p.T159I seemed consistent with the degree of functional loss. In contrast, variant p.A606T showed higher expression levels than expected based on its activity, suggesting that some of its functional loss is related to a transport defect. As a caveat, we note that recent studies indicated that a large fraction of *TRPV6* is localized intracellularly, and total expression levels may not correlate well with the plasma membrane fraction responsible for calcium uptake (24). Overall, there were 3/145 (2.1%) functionally defective *TRPV6* variants in the Hungarian CP cohort and none in controls. These results are consistent with those described previously in other European cohorts. None of the 3 subjects with a defective *TRPV6* variant carried the *SPINK1* p.N34S pathogenic variant.

Meta-Analysis of Case-Control Studies

To place the three defective variants in the context of previously described loss-of-function *TRPV6* variants, we performed a meta-analysis of all available case-control studies (Fig. 3). We obtained residual *TRPV6* activity levels of the various variants from the published graphs and subtracted the background activity. We grouped the variants according to their activity: <20%, 20%–60%, and 60% to <80%. The analysis demonstrated that the odds ratio (OR) was proportional to the degree of functional loss. Thus, variants with <20% activity yielded an OR of 23.7 (95% CI: 7.93–70.9) while

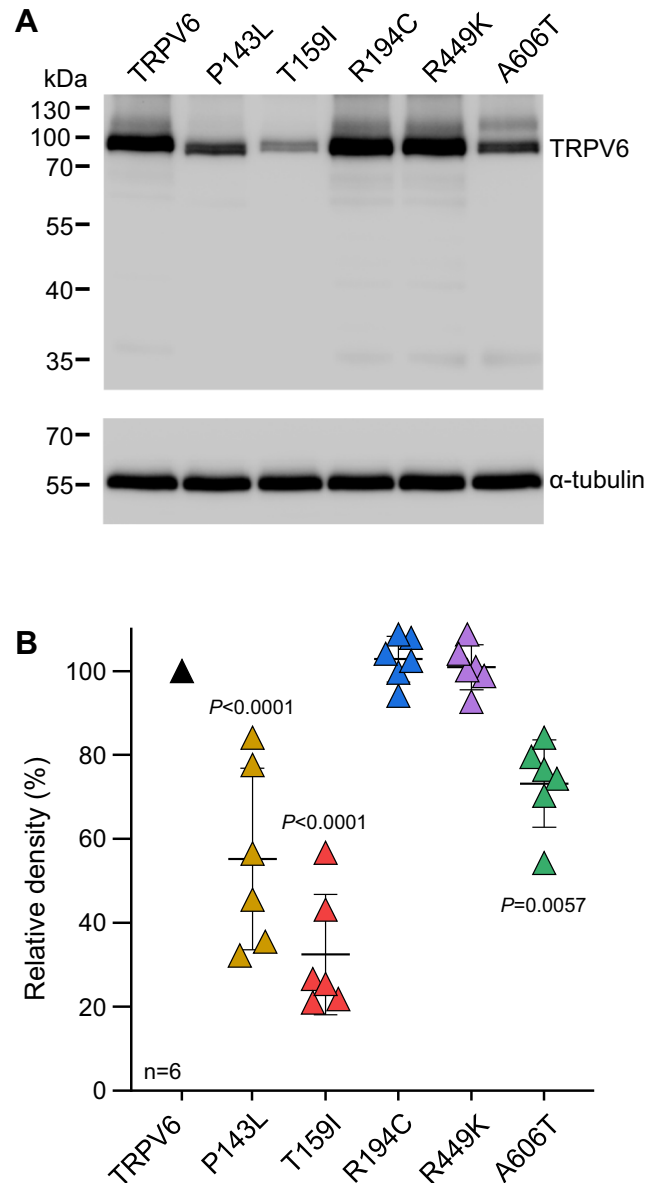


Figure 2. Effect of genetic variants on transient receptor potential vanilloid type 6 (*TRPV6*) expression in HEK 293 T cells. Lysates were prepared from cells transfected with wild-type *TRPV6* or the indicated variants and analyzed by Western blotting, as described in MATERIALS AND METHODS. A: representative Western blot. As a loading control, α -tubulin was measured. B: densitometric analysis of Western blots from multiple transfections ($n = 6$). Results were expressed as a percentage of the wild-type band intensity within each blot. Individual data points with the mean and standard deviation are shown. The difference of means was analyzed by one-way ANOVA followed by Tukey's post hoc test.

those between 20% and 60% activity produced an OR of 5.79 (95% CI: 1.64–20.37). Finally, variants with more than 60% residual function represented no significant risk (OR = 1.56; 95% CI: 0.35–6.96). We conclude that variants p.T159I and p.A606T increase CP risk strongly, whereas variant p.P143L has a less significant impact.

Generation of a New *Trpv6*-KO Mouse Model

We used CRISPR/Cas9 technology to delete exons 2–13 of the *Trpv6* gene in the C57BL/6N mouse genome. Except

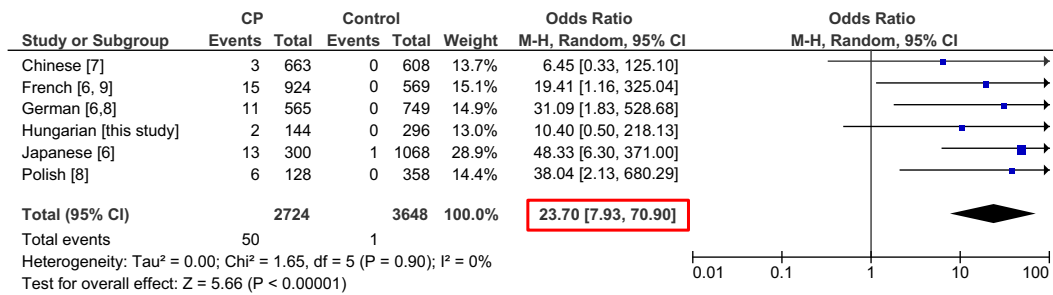
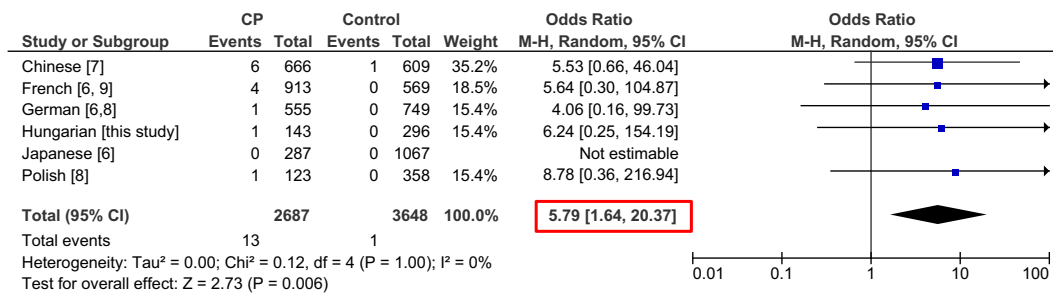
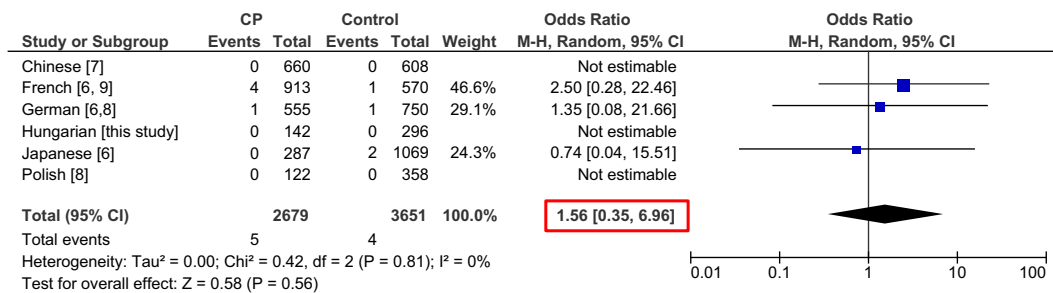
A Variants with <20% residual function**B** Variants with 20% - 60% residual function**C** Variants with 60% < - <80% residual function

Figure 3. Meta-analysis of the association between functionally defective transient receptor potential vanilloid type 6 (*TRPV6*) variants and chronic pancreatitis (CP) in global cohorts. Forest plots are shown. **A:** variants with <20% residual activity. **B:** variants with 20%–60% residual activity. **C:** variants with 60% to <80% residual activity. There was no heterogeneity between studies, as judged by the I^2 and χ^2 tests. Citations are indicated next to the cohorts. CI, confidence interval; M-H, Mantel-Haenszel.

for the previously reported male infertility (25, 26), homozygous *Trpv6*-KO mice showed no phenotypic or behavioral changes, and their pancreas histology at the age of 6 mo ($n = 12$) and 12 mo ($n = 7$) was normal (not shown).

Cerulein-Induced Acute Pancreatitis in *Trpv6*-KO Mice

To study the effect of *Trpv6* deletion on acute pancreatitis elicited by secretagogue hyperstimulation, we utilized two well-established protocols. First, we injected the mice 10 times hourly (10 $\times$) with cerulein and euthanized them 1 h after the last injection. Second, we used a protocol of 8 hourly injections on 2 consecutive days (2 $\times$ 8 $\times$) followed by euthanasia 1 h after the last injection on the second day. As expected, cerulein administration resulted in increased pancreas weight due to inflammatory edema (Fig. 4A) accompanied by markedly elevated plasma amylase activity (Fig. 4B), relative to control animals given saline injections. There was

no significant difference between wild-type C57BL/6N and *Trpv6*-KO mice. Histological examination of pancreas sections by hematoxylin-eosin staining revealed the known telltale signs of acute pancreatitis: widespread edema with inflammatory cell infiltration and scattered acinar cell necrosis (Fig. 5A). Histological scoring of multiple sections revealed a trend toward increased edema, higher number of inflammatory cells, and more necrosis in cerulein-treated *Trpv6*-KO mice relative to C57BL/6N animals (Fig. 5B). This trend was more pronounced in the experiments with the 2 $\times$ 8 $\times$ injection protocol, however, statistical significance was reached for necrosis only.

Cerulein-Induced Intrapaneatic Protease Activation in *Trpv6*-KO Mice

To evaluate the effect of *Trpv6* deletion on the secretagogue-induced activation of digestive proteases in the

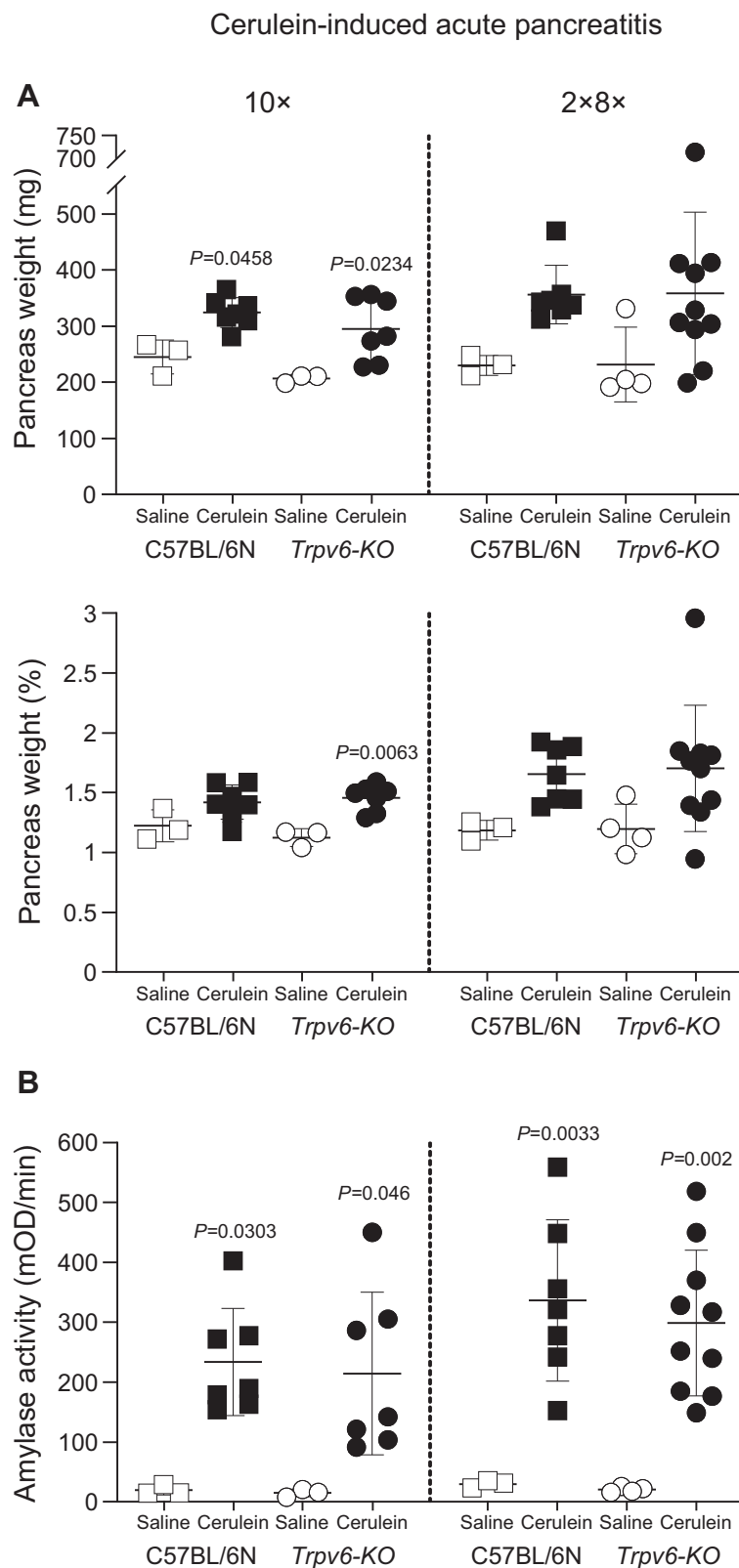


Figure 4. Cerulein-induced acute pancreatitis in transient receptor potential vanilloid type 6 knockout (*Trpv6-KO*) mice. C57BL/6N and *Trpv6-KO* mice were administered hourly injections of saline or cerulein using either a 10-injection protocol (10×; left) or an 8-injection protocol on 2 consecutive days (2×8×; right). Animals were euthanized 1 h after the last injection. **A:** pancreas weight in mg units and as percentage of body weight. **B:** plasma amylase activity. Individual data points with mean and standard deviation are shown. Saline, $n = 3-4$; cerulein, $n = 7-10$. The difference of means was analyzed by one-way ANOVA followed by Tukey's post hoc test. mOD, milli-optical density.

pancreas, we measured trypsin and chymotrypsin activity from pancreas homogenates. Mice were given a single cerulein injection, euthanized 30 min later, and the pancreas was freshly analyzed. As described previously,

cerulein induced significant elevations in the intra-pancreatic trypsin and chymotrypsin activity. However, there was no difference between C57BL/6N and *Trpv6-KO* mice (Fig. 6).

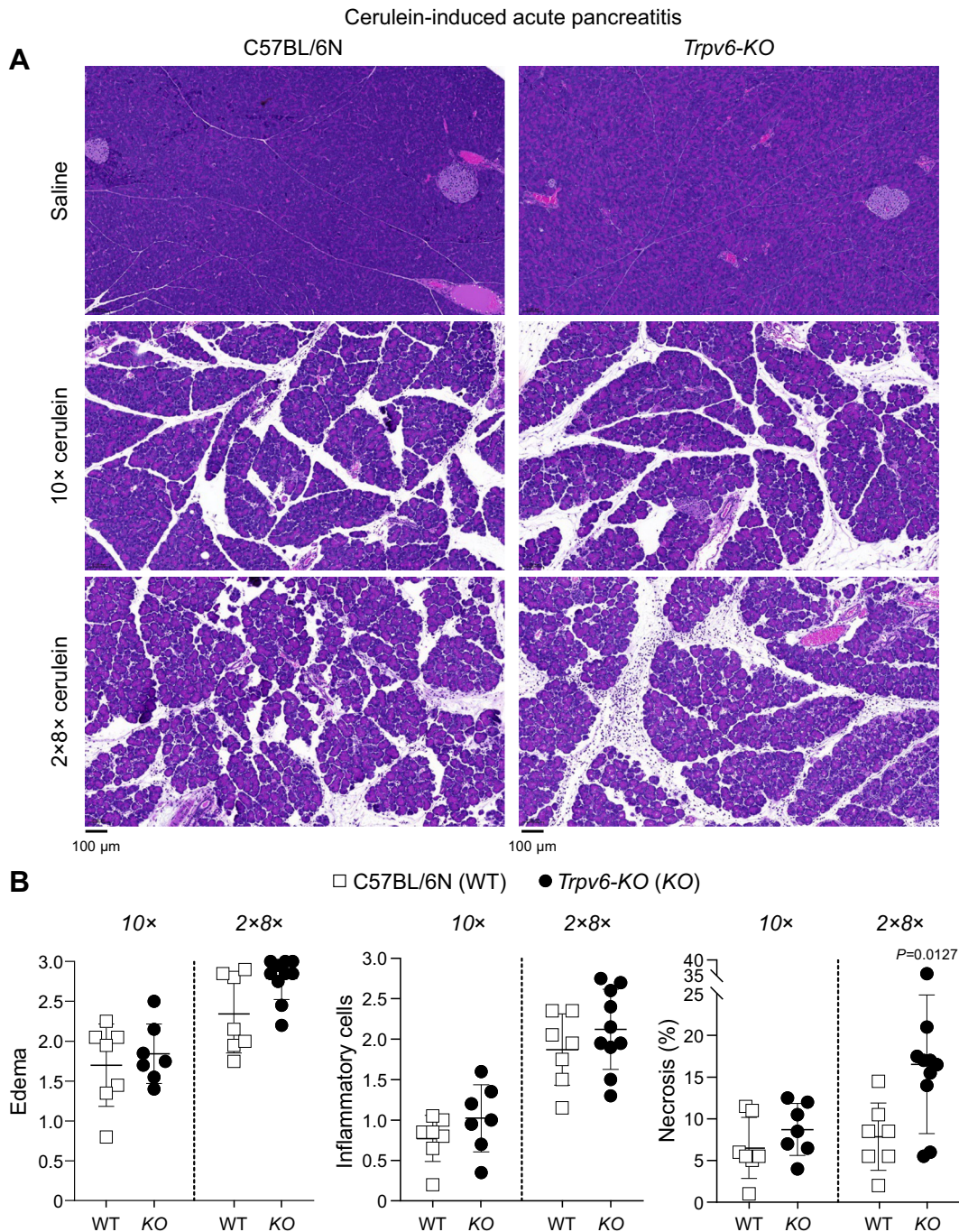


Figure 5. Histology of cerulein-induced acute pancreatitis in transient receptor potential vanilloid type 6 knockout (*Trpv6*-KO) mice. C57BL/6N and *Trpv6*-KO mice were administered hourly injections of saline or cerulein using either a 10-injection protocol (10×) or an 8-injection protocol on 2 consecutive days (2×8×). Animals were euthanized 1 h after the last injection. **A:** representative hematoxylin-eosin-stained pancreas sections. Scale bars are 100 μ m. **B:** histology scoring of pancreas sections ($n = 7-10$) from cerulein-treated mice for edema, inflammatory cell infiltration, and acinar cell necrosis. The pancreas of saline-treated mice showed no visible edema, inflammatory cells, or necrosis. Individual data points were graphed with the mean and standard deviation indicated. The difference of means was analyzed by a two-tailed unpaired *t* test. WT, wild type.

Recovery after Cerulein-Induced Acute Pancreatitis in *Trpv6*-KO Mice

Previous studies showed that genetically modified mice with trypsinogen mutations or chymotrypsin deletion rapidly progress to acinar atrophy and eventually to a CP-like state after an episode of acute pancreatitis, and recover

slowly and incompletely (21, 27, 28). To assess whether *Trpv6*-KO mice show a similar sensitivity, we treated mice with the 2×8× cerulein injection protocol and euthanized them 3 days after the last injection. Relative to saline controls, cerulein-treated mice had significantly reduced pancreas weight (Fig. 7A) and higher plasma amylase activity (Fig. 7B). A trend for lower pancreas weight (i.e., more

Cerulein-induced intrapancreatic protease activation

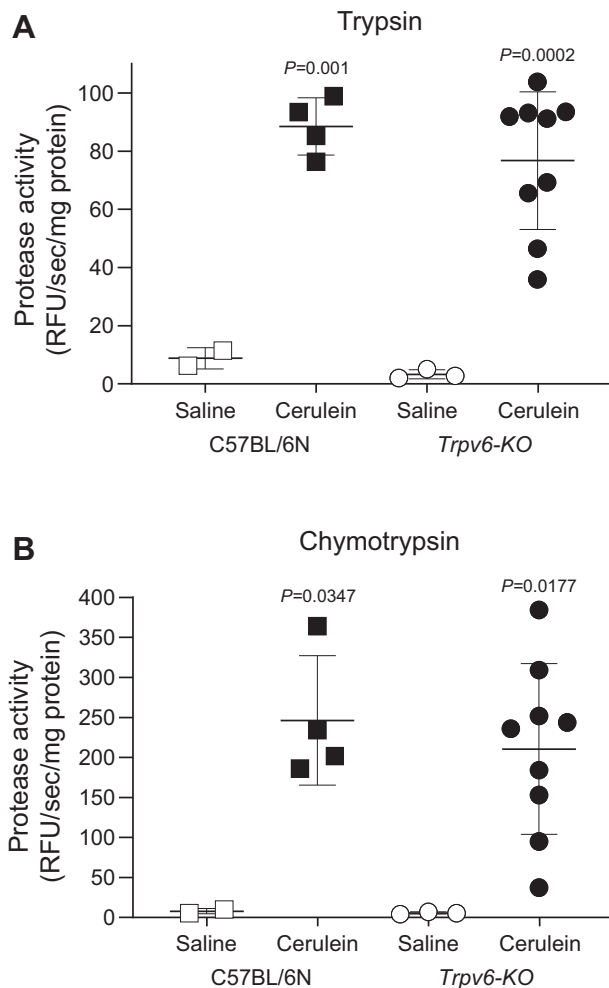


Figure 6. Cerulein-induced intrapancreatic protease activation in transient receptor potential vanilloid type 6 knockout (*Trpv6-KO*) mice. C57BL/6N and *Trpv6-KO* mice were given a single injection of saline or cerulein. Mice were euthanized 30 minutes after the injection, the pancreas was freshly homogenized, and protease activities were measured as described in MATERIALS AND METHODS. **A:** intrapancreatic trypsin activity. **B:** intrapancreatic chymotrypsin activity. Individual values with the mean and standard deviation were graphed. Saline, $n = 2-3$; cerulein, $n = 4-9$. The difference of means was analyzed by one-way ANOVA with Tukey's post hoc test. RFU, relative fluorescent units.

atrophy) and higher plasma amylase was observed in cerulein-treated *Trpv6-KO* versus C57BL/6N mice; however, the difference was statistically insignificant. Histological analysis of pancreas sections by hematoxylin-eosin staining showed a nearly normal pancreas with scattered small areas of residual scarring in C57BL/6N mice and slightly more in *Trpv6-KO* animals (Fig. 8A). Furthermore, focal acinar cell atrophy with inflammation (Fig. 8B) was apparent to a greater extent in *Trpv6-KO* mice than in C57BL/6N animals. Scoring of multiple sections ($n = 11$) for intact acini demonstrated that the average acinar cell damage was significantly higher in *Trpv6-KO* mice versus C57BL/6N controls (Fig. 8C).

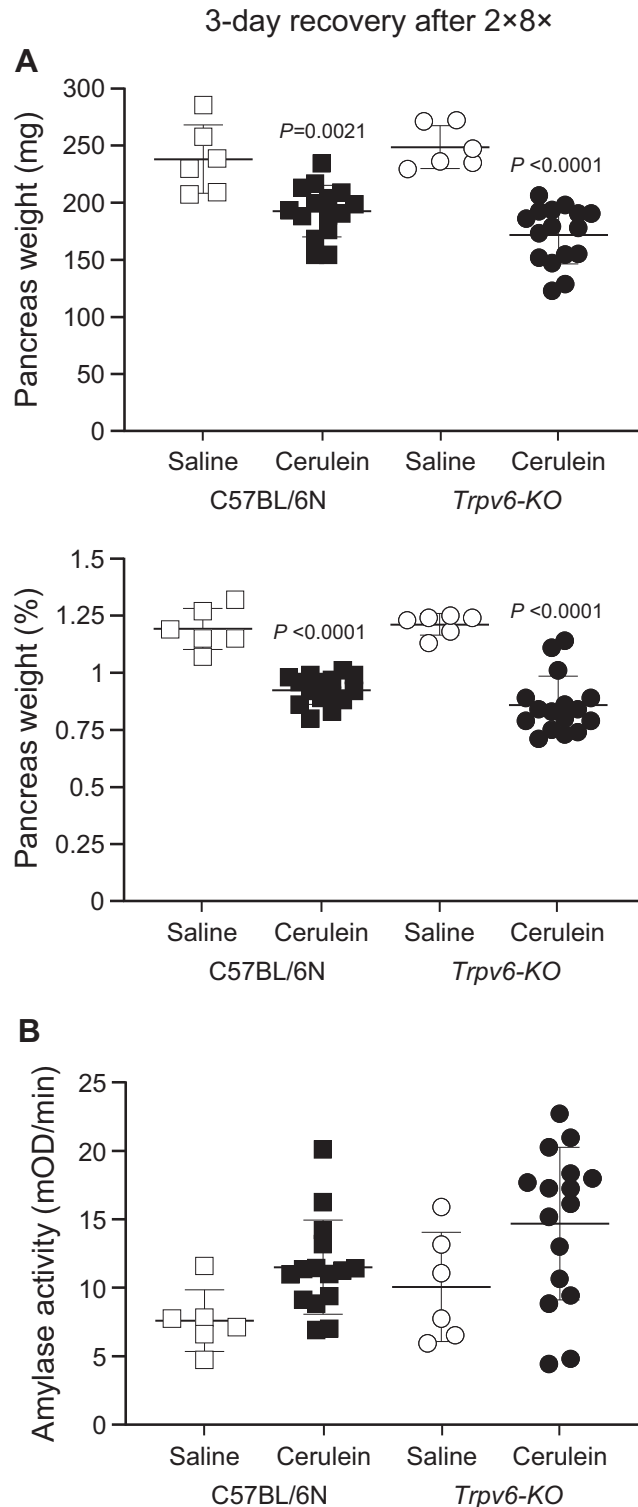
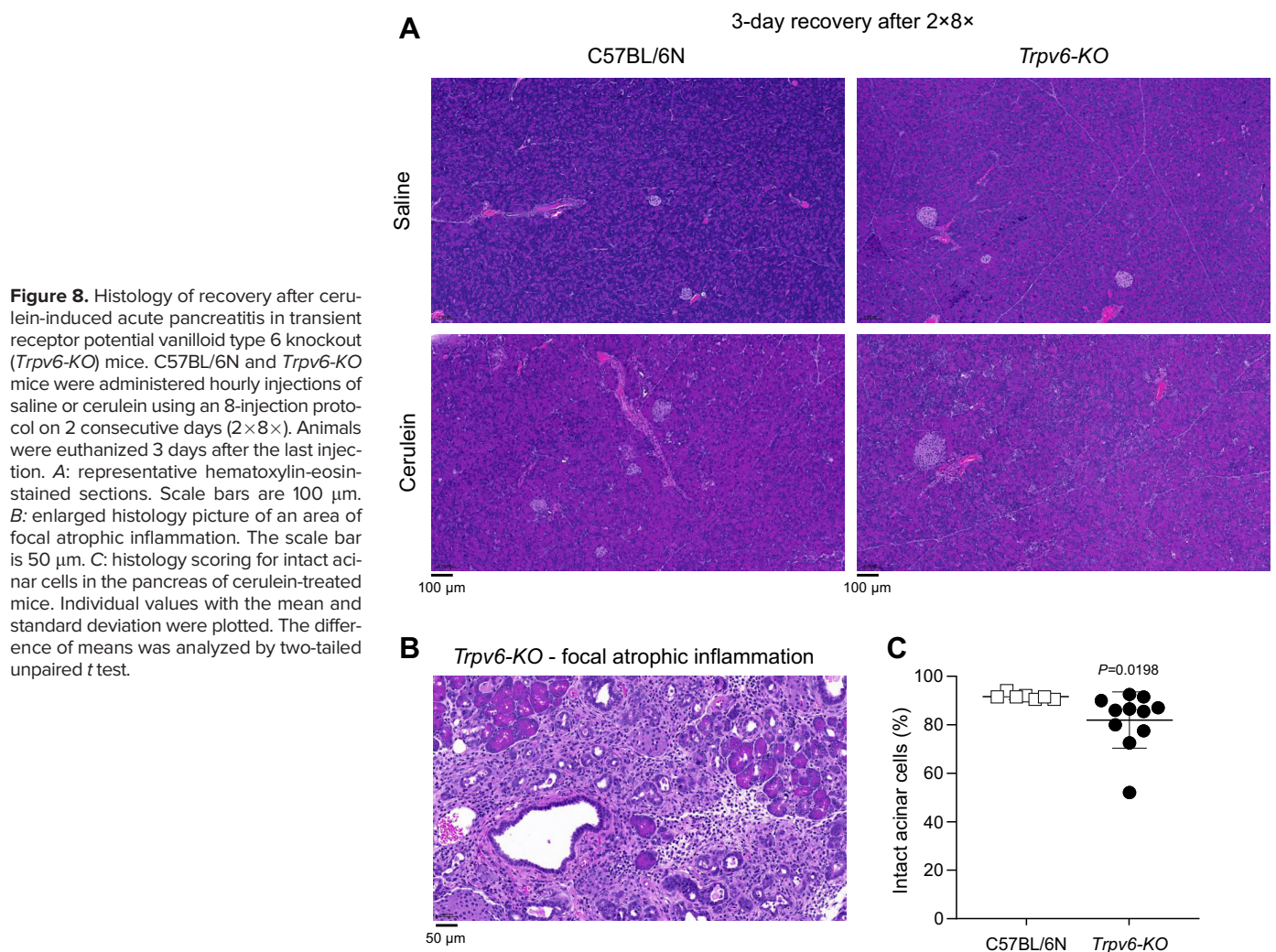


Figure 7. Recovery after cerulein-induced acute pancreatitis in transient receptor potential vanilloid type 6 knockout (*Trpv6-KO*) mice. C57BL/6N and *Trpv6-KO* mice were administered hourly injections of saline or cerulein using an 8-injection protocol on 2 consecutive days (2×8×). Animals were euthanized 3 days after the last injection. **A:** pancreas weight in mg units and as percentage of body weight. **B:** plasma amylase activity. Individual values with the mean and standard deviation were plotted. Saline, $n = 6$; cerulein, $n = 15-16$. The difference of means was analyzed by one-way ANOVA with Tukey's post hoc test. mOD, milli-optical density.



Pancreas Phenotype of *Trpv6-KO*^{hom} × *Spink1-KO*^{het} Mice

Because a significant fraction of patients with defective TRPV6 variants also carried a pathogenic *SPINK1* p.N34S variant (6), we crossed the homozygous *Trpv6-KO* strain with heterozygous *Spink1-KO* mice (20). We used heterozygous *Spink1-KO* mice because homozygous mice are not viable (20, 29, 30). We evaluated the *Trpv6-KO*^{hom} × *Spink1-KO*^{het} mice (*n* = 11) at the age of 6 mo for signs of spontaneous pancreatitis. Pancreas weight, plasma amylase, and pancreas histology were all normal, indistinguishable from those of C57BL/6N controls (not shown).

DISCUSSION

In the present study, we attempted to replicate previous findings showing that functionally defective TRPV6 variants increase the risk of CP in humans, and genetic inactivation of *Trpv6* in mice increases the severity of cerulein-induced pancreatitis (6–9, 16). We made the following observations: 1) in 2.1% of adult nonalcoholic CP patients of Hungarian origin, we found defective TRPV6 variants, whereas no such variants were identified in control subjects; 2) meta-analysis of case-control studies confirmed that TRPV6 variants with

<20% residual activity strongly increase CP risk; 3) we could not confirm enrichment of variant p.A18S in controls or variant p.L299Q in patients, as reported previously; 4) homozygous *Trpv6*-deleted mice did not develop spontaneous pancreatitis; 5) cerulein-induced acute pancreatitis was slightly more severe in *Trpv6-KO* versus C57BL/6N mice; however, the difference was not statistically significant for most parameters measured; 6) recovery after acute pancreatitis was focally delayed in a subset of *Trpv6-KO* mice but not in C57BL/6N mice; 7) cerulein-induced intrapancreatic protease activity was comparable in C57BL/6N and homozygous *Trpv6-KO* mice; and 8) crossing homozygous *Trpv6-KO* mice with heterozygous *Spink1-KO* mice did not result in spontaneous pancreatitis.

Our human genetic studies confirmed that defective TRPV6 variants occur with relatively low frequency in CP. The 2.1% incidence reported here is in line with prior European studies on German, French, and Polish cohorts that reported frequencies between 1.5% and 3.9% (6, 8, 9). Our results also suggest that TRPV6 variants may play a similar role in adult nonalcoholic CP as in pediatric cases. An intriguing and unresolved aspect of TRPV6 genetics is the apparent association of certain functional variants with CP. In Europeans, variant p.A18S was overrepresented in controls

(6, 9), whereas variant p.L299Q was enriched in patients (6, 8, 9). Here, we observed contradictory distributions; p.A18S was slightly enriched in cases, and variant p.L299Q was detected only in controls. Although these differences were not significant and our cohorts were smaller than those analyzed previously, the lack of association seems convincing and remains unexplained for the time being.

Our mouse model studies partly confirmed published claims that *Trpv6* deletion increases the severity of cerulein-induced pancreatitis (6, 16). The new *Trpv6*-KO strain did not develop spontaneous pancreatitis, not even when crossed with a heterozygous *Spink1*-KO strain. To elicit pancreatitis experimentally, we used various protocols of cerulein administration and observed similar outcomes in C57BL/6N and *Trpv6*-KO animals, with a nonsignificant trend toward increased severity. A statistically significant difference between the two strains was seen only with the 2×8× cerulein protocol for histological acinar cell necrosis (after 1 h) and acinar atrophy (after 3 days). Prior studies also used a 2×8× cerulein stimulation protocol and detected higher serum amylase/lipase in *Trpv6*-deficient animals, which we did not observe. Published histology findings, however, were similar to the focal atrophic inflammation we described here in a subset of cerulein-treated *Trpv6*-KO mice. We note again the low mouse numbers and the mixed genetic background used by the previous studies. It has been well documented that the genetic background of the mouse strain significantly impacts the severity of cerulein-induced experimental pancreatitis (Ref. 31 and references therein). We conclude that *Trpv6* function is dispensable for the physiology of the mouse pancreas and *Trpv6* deletion has a relatively small impact on experimental pancreatitis elicited with cerulein hyperstimulation. It is conceivable, however, that TRPV6 might play a more significant role in other experimental models of acute and chronic pancreatitis including those utilizing repeated cerulein hyperstimulation on a longer time scale (32). Furthermore, assuming TRPV6 functions primarily in the pancreatic ducts, experimental pancreatitis models that directly target ducts (duct ligation, retrograde infusion) may provide different results. Finally, one might argue that *Trpv6*-KO mice do not model loss-of-function TRPV6 missense variants adequately. Recently, we generated a mouse model carrying the loss-of-function p.R341Q variant, which corresponds to the human variant p.R342Q (6, 8). Preliminary data indicate that this strain exhibits a phenotype similar to that observed in the *Trpv6*-KO mice.

The mechanism by which defective TRPV6 variants increase pancreatitis risk remains enigmatic. There is no agreement on which cell type in the pancreas expresses pathologically relevant TRPV6. In a 2002 immunohistochemistry study, the authors detected strong labeling in the exocrine acinar cells of the mouse pancreas (33). No labeling was seen in the islets of Langerhans. Within the pancreatic acinar cells, the strong staining was restricted to the secretory pole, although much weaker basolateral staining was also observed. In the human exocrine pancreas, apical membrane and granular staining in acinar cells within the secretory pole were predominant, with very weak basolateral staining. A more recent 2021 study

used a murine *Trpv6*-GFP reporter strain to characterize TRPV6 expression in various mouse tissues (34). The presence of TRPV6 was then confirmed by immunoprecipitation and mass spectrometry analysis. In the adult (8–12 wk old) mouse pancreas, TRPV6 was exclusively found in the acinar cells. Interestingly, expression was reduced in the juvenile pancreas (postnatal day 5). Finally, a 2016 single-cell transcriptomic study reported higher *Trpv6* mRNA expression in ductal cells versus acinar cells (35). We emphasize, however, that the low levels of *Trpv6* mRNA and TRPV6 protein across tissues contribute to substantial variability among expression studies, as discussed in published reviews (36, 37).

TRPV6 expressed on duct cells was hypothesized to play a role in the removal of unwanted calcium that is released together with the digestive enzymes during secretion (5, 6). Defective calcium clearance due to TRPV6 mutations might stimulate trypsinogen activation in the ducts. A similar function was described for TRPV6 in the epididymis, where removal of luminal calcium is essential for normal sperm maturation and function (25, 26). Homozygous *Trpv6*-KO males have been reported infertile, a finding we also confirmed in the present study. TRPV6 expressed on acinar cells may play a role in calcium reuptake, which might affect the calcium signaling of the acinar cell indirectly. Defective calcium signaling due to TRPV6 mutations might interfere with secretion or stimulate intracellular trypsinogen activation. The possibility of such a scenario is supported by a recent study showing that in the mouse uterus TRPV6 modifies calcium signaling in endometrial cells (38). The situation is further complicated by new observations that the majority of TRPV6 protein is localized in intracellular vesicles (24), raising the possibility that TRPV6 may directly contribute to the robust calcium signaling pathways of the acinar cell.

The pancreas in humans seems to be uniquely sensitive to impairment of TRPV6 function. The only other disease associated with TRPV6 mutations in humans is transient neonatal hyperparathyroidism that often presents with skeletal deformities (39–42). This rare condition typically requires the presence of two defective TRPV6 alleles and develops due to the deficiency of placental calcium transport. Remarkably, once oral feeding begins after birth, the condition rapidly improves and skeletal changes often normalize, despite the continued presence of the pathogenic TRPV6 variants. No pancreatitis was observed in these patients or in their obligate heterozygous parents.

In summary, in the present study, we confirmed that defective human TRPV6 variants increase the risk for CP in an adult cohort and demonstrated that genetic deletion of mouse *Trpv6* does not result in spontaneous pancreatitis but slightly increases the severity of secretagogue-induced pancreatitis and delays recovery after the acute episode. Limitations of our study include the relatively small CP cohort studied in humans and the reliance on the cerulein-induced experimental pancreatitis model in mice.

DATA AVAILABILITY

Materials, experimental data, and protocols associated with the current study are available from the corresponding author upon request.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.H., T.R., E.H., and M.S.-T. conceived and designed research; G.B., M.S., S.R., N.C.M.G., Z.G.P., R.H., A.D., B.C.N., H.W., and A.S. performed experiments; G.B., M.S., S.R., N.C.M.G., Z.G.P., R.H., A.D., B.C.N., H.W., A.S., P.H., T.R., E.H., and M.S.-T. analyzed data; G.B., M.S., S.R., T.R., E.H., and M.S.-T. interpreted results of experiments; G.B., S.R., and T.R. prepared figures; M.S.-T. drafted manuscript; G.B., M.S., S.R., N.C.M.G., Z.G.P., R.H., A.D., B.C.N., H.W., A.S., P.H., T.R., E.H., and M.S.-T. edited and revised manuscript; G.B., M.S., S.R., N.C.M.G., Z.G.P., R.H., A.D., B.C.N., H.W., A.S., P.H., T.R., E.H., and M.S.-T. approved final version of manuscript.

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