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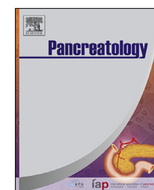

Risk of chronic pancreatitis in carriers of the c.180C>T (p.Gly60=) *CTRC* variant: case-control studies and meta-analysis

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ABSTRACT

Chymotrypsin C (CTRC) is a digestive serine protease produced by the pancreas that regulates intrapancreatic trypsin activity and provides a defensive mechanism against chronic pancreatitis (CP). CTRC exerts its protective effect by promoting degradation of trypsinogen, the precursor to trypsin. Loss-of-function missense and microdeletion variants of *CTRC* are found in around 4% of CP cases and increase disease risk by approximately 3-7-fold. In addition, a commonly occurring synonymous *CTRC* variant c.180C>T (p.Gly60=) was reported to increase CP risk in various cohorts but a global analysis of its impact has been lacking. Here, we analyzed the frequency and effect size of variant c.180C>T in Hungarian and pan-European cohorts, and performed meta-analysis of the new and published genetic association data. When allele frequency was considered, meta-analysis revealed an overall frequency of 14.2% in patients and 8.7% in controls (allelic odds ratio (OR) 2.18, 95% confidence interval (CI) 1.72–2.75). When genotypes were examined, c.180TT homozygosity was observed in 3.9% of CP patients and in 1.2% of controls, and c.180CT heterozygosity was present in 22.9% of CP patients and in 15.5% of controls. Relative to the c.180CC genotype, the genotypic OR values were 5.29 (95% CI 2.63–10.64), and 1.94 (95% CI 1.57–2.38), respectively, indicating stronger CP risk in homozygous carriers. Finally, we obtained preliminary evidence that the variant is associated with reduced *CTRC* mRNA levels in the pancreas. Taken together, the results indicate that *CTRC* variant c.180C>T is a clinically relevant risk factor, and should be considered when genetic etiology of CP is investigated.
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1. Introduction

Chymotrypsin C (CTRC) is a digestive serine protease, which is produced by the pancreas as an inactive precursor, chymotrypsinogen C [1]. In the duodenum, the CTRC proenzyme becomes activated by trypsin, and contributes to digestion by cleaving dietary proteins mainly after Leu, Phe, and Tyr amino-acids. The relatively strong Leu specificity is a unique feature of CTRC among other chymotrypsins such as CTRB1, CTRB2, and CTRL [2]. In addition to its digestive function, CTRC also regulates activation of other digestive proteases. Thus, CTRC is required for full activation of human pro-carboxypeptidases A1 and A2, which contain large activation peptides that are degraded during the activation process by the concerted action of trypsin and CTRC [3]. Furthermore, CTRC stimulates autoactivation of human cationic trypsinogen by processing the activation peptide to a shorter form [4,5]. CTRC-mediated cleavage of the activation peptide in human anionic

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trypsinogen slightly inhibits autoactivation [6]. Importantly, CTRC promotes degradation of trypsinogens, in a manner that is also dependent on autolytic cleavage by trypsin [5,7,8]. CTRC-dependent trypsinogen degradation serves as an essential protective mechanism in the pancreas against unwanted trypsin activity.

Development of intrapancreatic trypsin activity due to autoactivation of trypsinogens causes chronic pancreatitis (CP), the progressive, relapsing-recurring fibro-inflammatory disease of the pancreas. CTRC and the serine protease inhibitor Kazal type 1 (SPINK1) represent the anti-trypsin defenses of the pancreas that ward off unwanted trypsin activity and protect against pancreatitis [9]. In 2008, genetic variants in *CTRC* were identified in association with CP, including a large number of heterozygous missense mutations and an in-frame microdeletion [10,11]. Functional studies revealed that *CTRC* variants caused loss of function by multiple mechanisms that involved diminished cellular secretion, reduced catalytic activity, resistance to activation, and susceptibility to degradation by trypsin [10,12,13]. A subset of *CTRC* variants was also shown to induce endoplasmic reticulum stress in cell culture experiments [12,14]. To define the effect size of *CTRC* variants on CP risk, we recently performed a meta-analysis of all published case-control studies [15]. We examined 4 variants that were clinically relatively common (global carrier frequency in CP>1%), reproducibly showed association with CP, and their functional defect was verified experimentally. We found that heterozygous missense variants, p.A73T, p.V235I, and p.R245W, increased CP risk by 6.5, 4.5, and 2.6-fold, respectively, while the microdeletion p.K247_R254del had an effect size of 5.4, as judged by the calculated allelic odds ratios (OR). Global (average) carrier frequencies in CP for these 4 variants ranged from 1.0% to 2.4%, whereas the highest reported frequency in a single study ranged from 4.6% to 5.6%. Overall, contribution of *CTRC* missense variants and the microdeletion to the genetic etiology of CP can be observed in around 4% of cases.

The 2008 Nature Genetics article that described the discovery of *CTRC* as a CP risk gene focused on missense variants and did not report commonly occurring synonymous *CTRC* variants [10]. First, Masson et al. (2008) described c.180C>T (p.Gly60=, rs497078) in exon 3 and c.285C>T (p.Asp95=, rs41307798) in exon 4, with minor allele frequencies (MAF) of 11.9% and 4.3% in the French population, respectively [11]. Association of c.180C>T with familial CP (OR 2.46) but not with idiopathic or hereditary CP was also reported. Derikx et al. (2009), and later Paliwal et al. (2013) detected the same two variants in Indian cohorts, and confirmed association of c.180C>T with tropical CP [16,17]. Paliwal et al. (2013) also noted that CP risk was higher in homozygous (OR 9.89) versus heterozygous (OR 2.46) carriers. Masamune et al. (2013) and Zou et al. (2018) reported that the c.180C>T variant is rare in the Japanese and Chinese populations, respectively [18,19]. Association of the c.180C>T variant with CP was subsequently confirmed by Larusch et al. (2015) in US, Grabarczyk et al. (2017) in Polish, and by a GWAS study (2018) in pan-European cohorts [20–23]. The Polish study is notable because of the unique pediatric population it investigated and the large frequency (14.7%) of homozygous carriers in CP patients (OR 23). Both Grabarczyk et al. (2017), and Rosendahl et al. (2018) noted that variant c.180C>T was in linkage disequilibrium with variant c.493+52G>A (rs545634) in intron 5.

In the present study, we sought to perform a systematic, global analysis on the effect size of variant c.180C>T in CP with the goal of better quantifying CP risk in heterozygous and homozygous carriers. We also obtained preliminary data indicating the c.180C>T variant is associated with lower *CTRC* mRNA levels in the pancreas, potentially explaining the mechanism of increased disease risk.

2. Methods

Nomenclature. Nucleotide numbering reflects coding DNA numbering with the first nucleotide of the ATG translation initiation codon designated as +1 in the *CTRC* reference sequence (genomic reference: NC_000001.11, *Homo sapiens* chromosome 1, GRCh38. p7 primary assembly). Amino acids are numbered starting with the initiator methionine of the primary translation product.

Study subjects and genotyping. De-identified genomic DNA samples were obtained from the Hungarian National Pancreas Registry (ethical approval: TUKEB 36305–1/2016/EKU, biobanking approval: IF702-19/2012). All participants gave informed consent according to the ethical guidelines of the Declaration of Helsinki. The Hungarian cohort analyzed by direct DNA sequencing consisted of 291 CP patients (mean age at recruitment 55.7±11.7 years), including 124 with nonalcoholic CP (NACP) and 167 with alcoholic CP (ACP), and 349 control subjects (mean age at recruitment 49.1±12.1 years) with no pancreatic disease. Diagnosis of CP was based on the history of recurrent acute pancreatitis or recurrent abdominal pain typical for CP and/or pathological imaging findings consistent with CP, such as pancreatic calcifications, duct dilatation or irregularities, with or without exocrine pancreatic insufficiency or diabetes. Alcoholic CP was diagnosed when the patient's history included alcohol consumption of more than 80 g/day (men) or 60 g/day (women) for at least two years. Genotyping data for the c.180C>T variant were also extracted from the GWAS dataset generated by Rosendahl et al. (2018) [22].

DNA sequencing. The c.180C>T variant located in exon 3 of *CTRC* was detected using the following primer pair; CTRC_e23_V2 S 5′-GTG ACA CAG TAA AAT ATC AAC CC-3′ and CTRC_e23_V2 AS 5′-GAG TAG ATT ATG TAG CCC AGT G-3'. For the amplification of exon 5 with its flanking intronic regions where variant c.493+52G>A is located the following primer pair was used; CTRC e5 S2 5′-GAT GAC ATG TGA GAA GAA GCT ATG-3′ and CTRC e5 AS2 5′-TGG GAC CTA TTT GGG CAT AC-3'. Polymerase chain reaction (PCR) was performed using 1.0 U HotStarTaq DNA Polymerase (Qiagen), 0.2 mM dNTP, 0.5 μM primers, 10x PCR buffer (Qiagen) and 10–50 ng of genomic DNA template in a volume of 20 μL. The reaction was started with a 15 min initial heat activation at 95 °C followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 57 °C or 60 °C, respectively and 60 s extension at 72 °C; and was completed by a final extension step for 5 min at 72 °C. PCR products were verified by 1.5% agarose gel electrophoresis. The PCR amplicons (5 μL) were treated with 1 μL FastAP Thermosensitive Alkaline Phosphatase and 0.5 μL Exonuclease I (Thermo Fisher Scientific) for 15 min at 37 °C, and the reaction was stopped by heating the samples to 85 °C for 15 min. Sanger sequencing was performed using the forward and/or reverse PCR primers as sequencing primers.

Meta-analysis search strategy. Two authors independently performed a systematic search on August 2, 2022, in four databases (MEDLINE via Pubmed, Embase, Scopus, and CENTRAL via Cochrane Library) using the following search key: (CTRC OR "chymotrypsin C" OR "chymotrypsinogen C") AND pancreatitis AND ("gene" OR genetic* OR polymorphism* OR variant* OR mutation* OR G60G OR p. G60G OR G60= OR p. G60= OR c.180* OR 180T* OR 180C*). Filters or limits such as date or language were not applied. Citing (using MEDLINE via Pubmed and Google Scholar) and cited reference searches were performed on August 8, 2022.

Protocol registration. The present work is reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement (S1 Table) [24]. The protocol of the meta-analysis was registered in advance in the PROSPERO database under the registration number CRD42019139280.

Study selection and data extraction. The multi-step study selection process was completed by two authors using a reference

management program (Endnote X7.5; Clarivate Analytics, Philadelphia, PA). Genetic association case-control studies with adequately defined CP patients and controls investigating the c.180C>T (p.Gly60=) *CTRC* variant were included. Studies (i) analyzing autoimmune, hereditary, or familial chronic pancreatitis; (ii) with overlapping cohorts; and (iii) without proper allele frequency or genotype distribution data were excluded. Eligible original studies were subjected to data collection onto a predefined Excel sheet by two authors independently. The following data were extracted: first author, publication year, cohort ethnicity, range and mean age of participants, etiology of CP, number of cases and controls, genotyping method, and c.180C>T mutational status. In some cases, allele frequencies were calculated from the reported genotype distribution.

Quality assessment. Two authors independently appraised the quality of the included studies using a modified version of the Newcastle-Ottawa Scale (NOS) and by calculating the Hardy-Weinberg Equilibrium with the $\chi2$ test (Table 1). Discrepancies during search, selection, data extraction, and quality evaluation between authors were resolved by the corresponding author or by mutual agreement.

Statistical analysis. The effect of the minor T allele, the TT (TT vs. CC) and the CT (CT vs. CC) genotypes of the c.180C>T *CTRC* variant was assessed separately by calculating pooled odds ratios (OR) with 95% confidence intervals (CI) using the random-effects model with Der-Simonian Laird estimation. Results were displayed on forest plots. Heterogeneity between studies was investigated with the I^2 ($p \geq 0.1$) and χ^2 tests, interpretation of results was based on the Cochrane Handbook for Systematic Reviews of Interventions version 6.3 [25]. Sensitivity analysis was carried out by repeating the quantitative synthesis while leaving out one study at a time (leave-one-out method). Statistical analyses were performed with the Stata 15 (Stata Corp) program. LD statistics was performed using the CubeX software [26].

Allele-specific expression studies. De-identified human pancreatic cDNA samples from heterozygous carriers (n=10) were used to study the impact of the c.180C>T variant on *CTRC* expression. A Restriction Fragment Length Polymorphism (RFLP) assay was designed to measure the allele specific expression of the c.180C>T *CTRC* variant relative to the wild-type allele. A portion of the *CTRC* coding region was PCR-amplified using the following primer pair: forward 5′-CCC TGG CAG ATC TCC CTC C-3’; reverse 5′-CCA CCT GGA TGG TGT CAC TC-3’. The 295 bp amplicon was digested with FauI restriction enzyme for 1 h at 55 °C. FauI cleaves the c.180C wild-type allele to 244 and 51 bp products while the c.180T variant allele is not digested. A capillary electrophoresis system (Fragment Analyzer, Advanced Analytical Technologies) was used to analyze the undigested and FauI-digested PCR products. To calibrate the RFLP assay, we prepared cDNA from human embryonic kidney (HEK) 293 cells transiently transfected with wild-type or variant c.180C>T *CTRC* constructs. After RNA isolation and reverse transcription, the cDNA preparations were mixed in different proportions, resulting in mixtures containing 0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5, and 100% of the c.180T allele. The calibrator samples were PCR amplified, digested with FauI, and the amount of FauI-resistant PCR amplicons (corresponding to the c.180T allele) was calculated as percent of the total undigested *CTRC* amplicon, and plotted as a function of the nominal c.180T allele content. This calibration curve was then used to calculate the actual c.180T allele content of the pancreatic cDNA samples.

3. Results

Case-control studies. First, we investigated the c.180C>T *CTRC* variant status in 291 CP patients (124 NACP and 167 ACP) and 349 control subjects from Hungary. We observed a significant enrichment of the c.180T allele in CP patients (18.7%) in comparison to controls (10.2%), yielding an OR of 2.04 (95% CI 1.47–2.81). Genotype distribution analysis revealed that the homozygous c.180TT genotype was present in 3.8% of CP patients and in 0.3% of controls, while the heterozygous c.180CT genotype was found in 29.9% of CP patients and in 19.8% of controls (Table 2). Relative to the c.180CC genotype, the genotypic OR values for c.180TT and c.180CT were 15.9 (95% CI 2.04–124.18) and 1.82 (95% CI 1.26–2.63), respectively, indicating a stronger effect size of c.180TT homozygosity. We performed subgroup analysis of NACP and ACP patients and found similar minor allele frequencies in both cohorts (19.4% and 18.3%, respectively). However, the homozygous c.180TT genotype was more prevalent in NACP patients (5.7%) than in the ACP group (2.4%).

It has been previously reported that the c.180C>T and the intronic c.493+52G>A variants are in linkage disequilibrium [21–23,27]. We performed LD statistics by assessing the haplotype frequencies for both variants in the Hungarian cohort. We found that these variants are in strong linkage (D’ 0.98, r^2 0.94). From 11 patients carrying the homozygous c.180TT genotype 10 were homozygous for the c.493+52G>A variant, whilst all homozygous c.493+52G>A carriers were also homozygous for the c.180C>T variant.

Next, to extend our analysis, we extracted the c.180C>T genotype data from the pan-European GWAS dataset of 2336 CP patients (544 NACP and 1792 ACP) and 5768 controls reported by Rosendahl et al. (2018) [22]. Considering allele frequency, the c.180C>T variant was present in 16.6% of CP patients and in 9.7% of controls (OR 1.85, 95% CI 1.68–2.05). When genotype distribution was assessed, the homozygous c.180TT genotype was detected in 2.9% of CP patients and in 1.2% of controls, whereas the heterozygous c.180CT genotype was observed in 27.4% of patients and in 17.1% of controls (Table 2). Using the c.180CC genotype as reference, the genotypic OR values for c.180TT and c.180CT were 2.9 (95% CI 2.06–4.07), and 1.89 (95% CI 1.68–2.11), respectively, confirming the higher risk associated with c.180TT homozygosity. Subgroup analysis revealed no major differences regarding the minor allele frequency in the NACP and ACP groups (18.5% and 16.1%, respectively). However, similarly to the Hungarian cohort, the homozygous c.180TT genotype occurred more frequently in the NACP cohort (4.6%) than in the ACP cohort (2.4%).

Meta-analysis. The new Hungarian cohort data, the GWAS-derived pan-European analysis [22], and five other published case-control studies were used for quantitative synthesis (Fig. 1, Table 3) [16,17,19–21]. Altogether, 5379 patients and 9675 controls were analyzed to determine the effect of the minor c.180T allele, the c.180TT (TT vs. CC) and the c.180CT (CT vs. CC) genotypes of the

Table 1
Quality evaluation of studies included in meta-analysis using the Newcastle-Ottawa Scale.

Study	Selection				Comparability	Exposure		HWE
	CD	REC	SC	DC	CCC	AE	SMA	
Masson et al., 2008	+	+	+	+	+	+	−	−
Paliwal et al., 2013	+	+	+	+	+	+	+	+
Larusch et al., 2015	+	+	+	+	+	+	−	+
Rosendahl et al., 2018	+	+	+	+	+	+	+	−
Grabarczyk et al., 2017	+	+	+	+	+	+	−	+
Zou et al., 2018	+	+	+	+	+	+	+	+
Hungarian CP cohort	+	+	+	+	+	+	+	+

CD, cases defined adequately; REC, representativeness of the cases; SC, selection of controls; DC, definition of controls; CCC, comparability of cases and controls; AE, ascertainment of exposure; SMA, same method of ascertainment for cases and controls; HWE, Hardy-Weinberg Equilibrium in controls.

Table 2
Genotype distribution of the c.180C>T (p.Gly60=) *CTRC* variant in the Hungarian and pan-European CP cohorts. Genotypic OR values were calculated for the c.180TT and c.180CT (shown in italics) genotypes. OR, odds ratio, CI, confidence interval.

Hungarian CP cohort	c.180C>T genotype			OR	95% CI	*p*-value
	CC	CT	TT			
ACP (n=167)	110 (65.9%)	53 (31.7%)	4 (2.4%)	10.15 *1.95*	1.12–91.79 *1.28–2.97*	0.039 *0.002*
NACP (n=124)	83 (66.9%)	34 (27.4%)	7 (5.7%)	23.53 *1.66*	2.85–194.01 *1.03–2.67*	0.003 *0.039*
Total CP (n=291)	193 (66.3%)	87 (29.9%)	11 (3.8%)	15.90 *1.82*	2.04–124.18 *1.26–2.63*	0.008 *0.001*
Controls (n=349)	279 (79.9%)	69 (19.8%)	1 (0.3%)			
Pan-European CP cohort (Rosendahl et al., 2018)						
ACP (n=1792)	1259 (70.3%)	490 (27.3%)	43 (2.4%)	2.37 *1.86*	1.61–3.49 *1.64–2.11*	<0.0001 *< 0.0001*
NACP (n=544)	368 (67.6%)	151 (27.8%)	25 (4.6%)	4.71 *1.96*	2.94–7.54 *1.61–2.40*	<0.0001 *< 0.0001*
Total CP (n=2336)	1627 (69.7%)	641 (27.4%)	68 (2.9%)	2.90 *1.89*	2.06–4.07 *1.68–2.11*	<0.0001 *< 0.0001*
Controls (n=5768)	4715 (81.2%)	985 (17.1%)	68 (1.2%)			

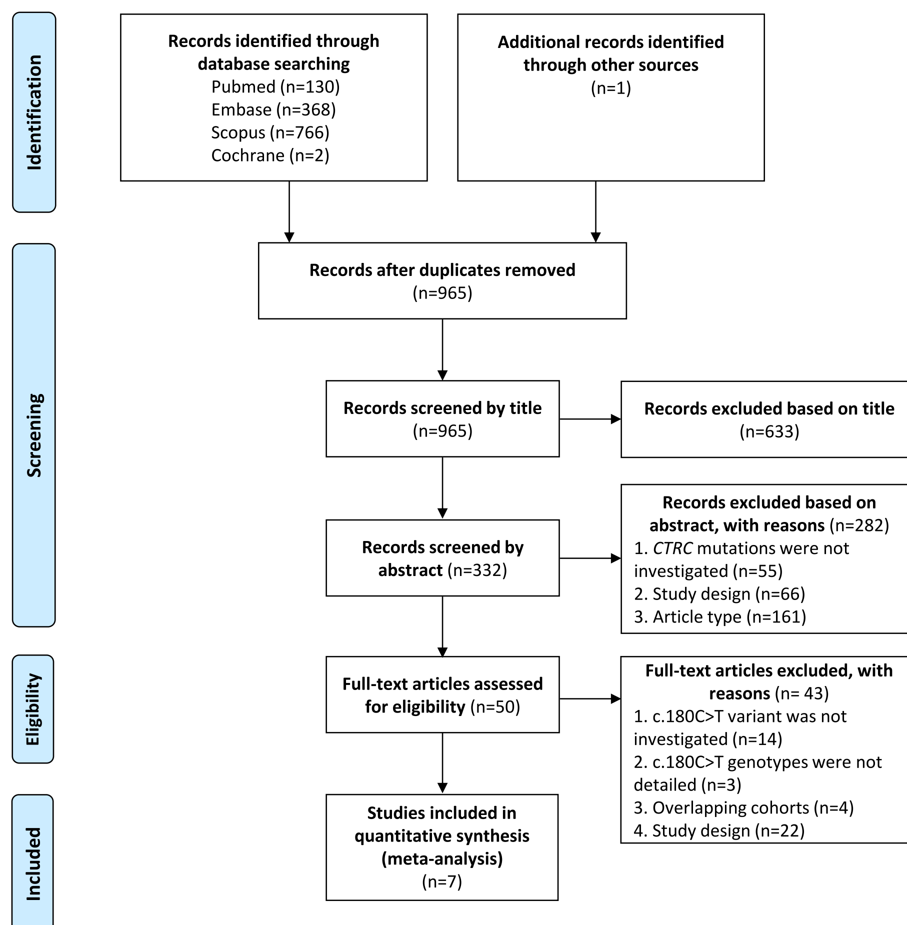

Fig. 1. Flow chart of the study selection process.

c.180C>T *CTRC* variant. In the global cohort, the minor c.180T allele was found significantly more frequently in CP patients (14.2%) than in controls (8.7%), yielding an allelic OR of 2.18 (95% CI 1.72–2.75) (Fig. 2A). Genotype analysis revealed that the global homozygous c.180TT and heterozygous c.180CT carrier frequencies were 3.9% and 22.9% in CP patients and 1.2% and 15.5% in controls, respectively. Thus, the effect size, as judged by the genotypic OR values calculated relative to the c.180CC genotype, is considerably higher in the homozygous state (OR 5.29, 95% CI 2.63–10.64) than in the heterozygous state (OR 1.94, 95% CI 1.57–2.38) (Fig. 2B and 2C).

Table 3
Characteristics of studies included in meta-analysis.

Study	Demographic characteristics			p.G60= mutational status						
	Ethnicity	Age (mean ± SD/ range)	Etiology of CP	Cohort	Allele frequency		Genotype distribution			Genotyping method
					Major (C)	Minor (T)	Wild type (CC)	Heterozygous (CT)	Homozygous (TT)	
Masson et al., 2008	French	NA/≤20 (ICP); NA (FCP, HCP)	ICP FCP HCP	CP	483/574 (84.1%)	91/574 (15.9%)	209/287 (72.8%)	65/287 (22.7%)	13/287 (4.5%)	Direct sequencing/Sanger
				Controls	617/700 (88.1%)	83/700 (11.9%)	277/350 (79.1%)	63/350 (18%)	10/350 (2.9%)	Direct sequencing/Sanger or DHPLC
Paliwal et al., 2013	Indian	NA/NA	TCP ICP	CP	954/1168 (81.7%)	214/1168 (18.3%)	393/584 (67.3%)	168/584 (28.8%)	23/584 (3.9%)	Direct sequencing/Sanger
				Controls	1102/1196 (92.1%)	94/1196 (7.9%)	507/598 (84.8%)	88/598 (14.7%)	3/598 (0.5%)	Direct sequencing/Sanger
Larusch et al., 2015	North American (European ancestry)	50.5 ± 16.1/NA	ACP NACP	CP	1138/1368 (83.2%)	230/1368 (16.8%)	484/684 (70.8%)	170/684 (24.8%)	30/684 (4.4%)	Taqman SNP genotyping assay and/or direct sequencing/Sanger
				Controls	1807/2026 (89.2%)	219/2026 (10.8%)	804/1013 (79.4%)	199/1013 (19.6%)	10/1013 (1%)	Taqman SNP genotyping assay and/or direct sequencing/Sanger
Rosendahl et al., 2018	European	43 ± 17.1/1-98	ACP NACP	CP	3895/4672 (83.4%)	777/4672 (16.6%)	1627/2336 (69.7%)	641/2336 (27.4%)	68/2336 (2.9%)	Illumina BeadChip arrays
				Controls	10415/11536 (90.3%)	1121/11536 (9.7%)	4715/5768 (81.7%)	985/5768 (17.1%)	68/5768 (1.2%)	Illumina BeadChip arrays
Grabarczyk et al., 2017	Polish	NA/1.5−17.5	NA	CP	185/272 (68%)	87/272 (32%)	69/136 (50.7%)	47/136 (34.6%)	20/136 (14.7%)	Direct sequencing/Sanger
				Controls	718/802 (89.5%)	84/802 (10.5%)	321/401 (80%)	76/401 (19%)	4/401 (1%)	HRM-PCR
Zou et al., 2018	Chinese	40.7 ± 16/6-85	ICP ACP SCP	CP	2104/2122 (99.2%)	18/2122 (0.8%)	1043/1061 (98.3%)	18/1061 (1.7%)	0/1061 (0%)	Targeted next-generation sequencing
				Controls	2389/2392 (99.9%)	3/2392 (0.1%)	1193/1196 (99.7%)	3/1196 (0.3%)	0/1196 (0%)	Targeted next-generation sequencing
Hungarian CP cohort	Hungarian	55.7 ± 11.7/21-85	ACP NACP	CP	473/582 (81.3%)	109/582 (18.7%)	193/291 (66.3%)	87/291 (29.9%)	11/291 (3.8%)	Direct sequencing/Sanger
				Controls	627/698 (89.8%)	71/698 (10.2%)	279/349 (79.9%)	69/349 (19.8%)	1/349 (0.3%)	Direct sequencing/Sanger

CP, chronic pancreatitis; ACP, alcoholic chronic pancreatitis; NACP, non-alcoholic chronic pancreatitis; ICP, idiopathic chronic pancreatitis; FCP, familial chronic pancreatitis; HCP, hereditary chronic pancreatitis; TCP, tropical chronic pancreatitis; SCP, smoking-associated chronic pancreatitis; DHPLC, denaturing high performance liquid chromatography; HRM-PCR, high resolution melting polymerase chain reaction; NA, not available.

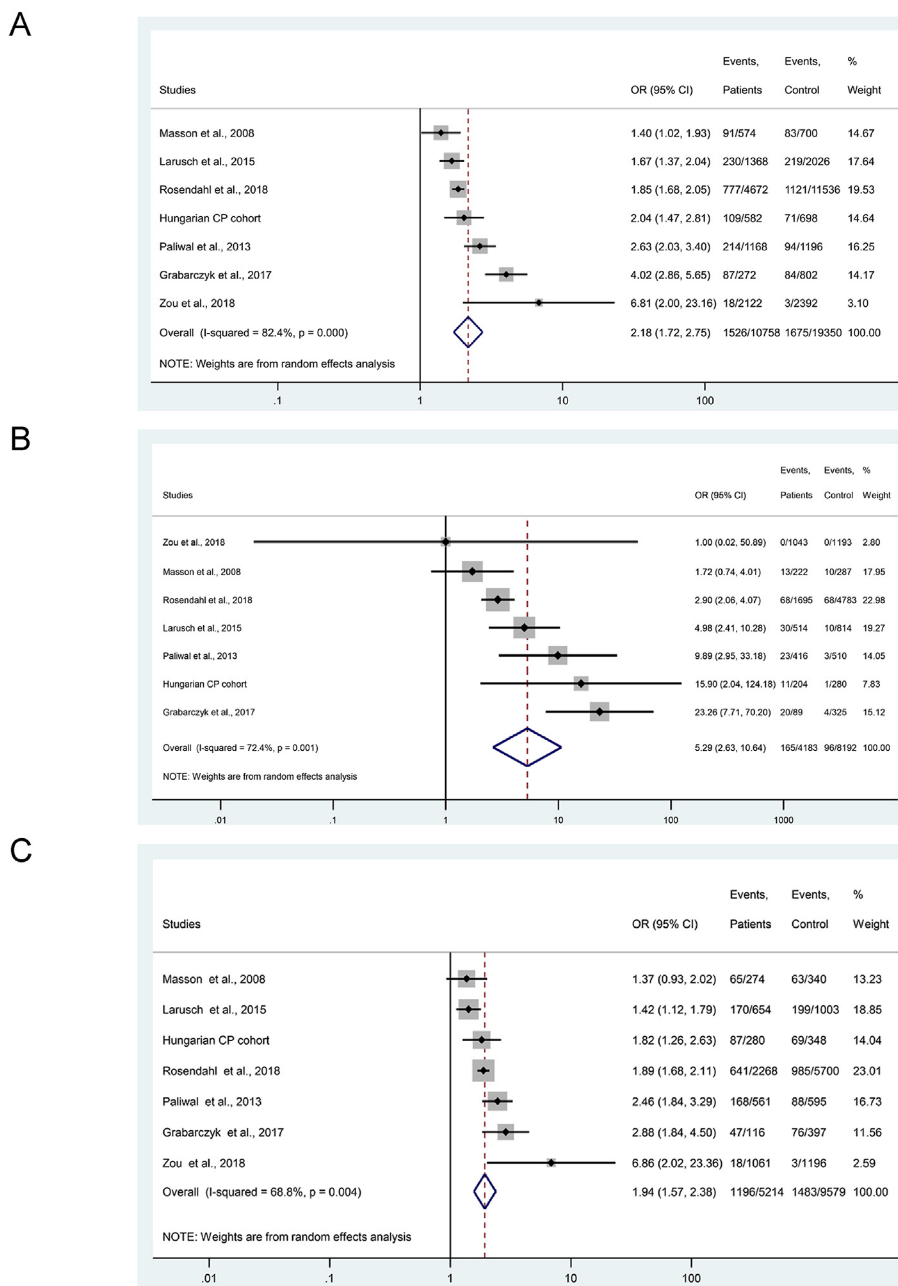

Fig. 2. Meta-analysis of the association between the c.180C>T *CTRC* variant and chronic pancreatitis (CP) in global cohorts. Forest plots are shown. **A,** Association between the c.180T minor *CTRC* allele and CP. **B**, Association of the c.180TT homozygous *CTRC* genotype with CP. **C**, Association of the c.180CT heterozygous *CTRC* genotype with CP.

Sensitivity analysis (leave-one-out method) supported the validity of the results. (S1 Fig). When assessing the between-study variation using I^2 statistic, we noticed substantial heterogeneity, probably due to ethnic and age differences between cohorts. The I^2 values obtained for the analyses shown in Fig. 2A, 2B, and 2C; were 82.4%, 72.4%, and 68.8%, respectively. Therefore, we performed a subgroup analysis including only adult subjects of European origin. In this population, the minor c.180T allele was significantly overrepresented in CP patients (16.8%) in comparison to controls (9.9%) yielding an OR of 1.77 (95% CI 1.59–1.98) (Fig. 3A). Heterogeneity between studies was low (I^2 21.4%). When assessing the impact of the c.180TT and c.180CT genotypes, both were significantly overrepresented in CP cases (4.6% and 27.7%) compared to controls (1.4% and 17.8%). Relative to the c.180CC genotype, the genotypic OR values for c.180TT and c.180CT were 3.31 (95% CI 1.92–5.71), and 1.65 (95% CI 1.38–1.98), respectively (Fig. 3B and 3C). However, moderate heterogeneity between the studies was noted for both genotypes (I^2 51.5% and 52.4%, respectively). When comparing the results of fixed-effect and random-effects estimates, no significant differences between the calculated odds ratios could be observed, suggesting the lack of small-study effect in our analyses.

To assess the role of the c.180C>T *CTRC* variant in alcoholic disease, we performed a combined analysis of the Hungarian and pan-European NACP and ACP cohorts. With respect to allele frequency, the minor c.180T allele was significantly overrepresented in both the NACP (OR 2.1, 95% CI 1.83–2.47) and the ACP (OR 1.8, 95% CI 1.62–2) cohorts. When genotype distribution was considered, the homozygous c.180TT genotype had a more pronounced impact

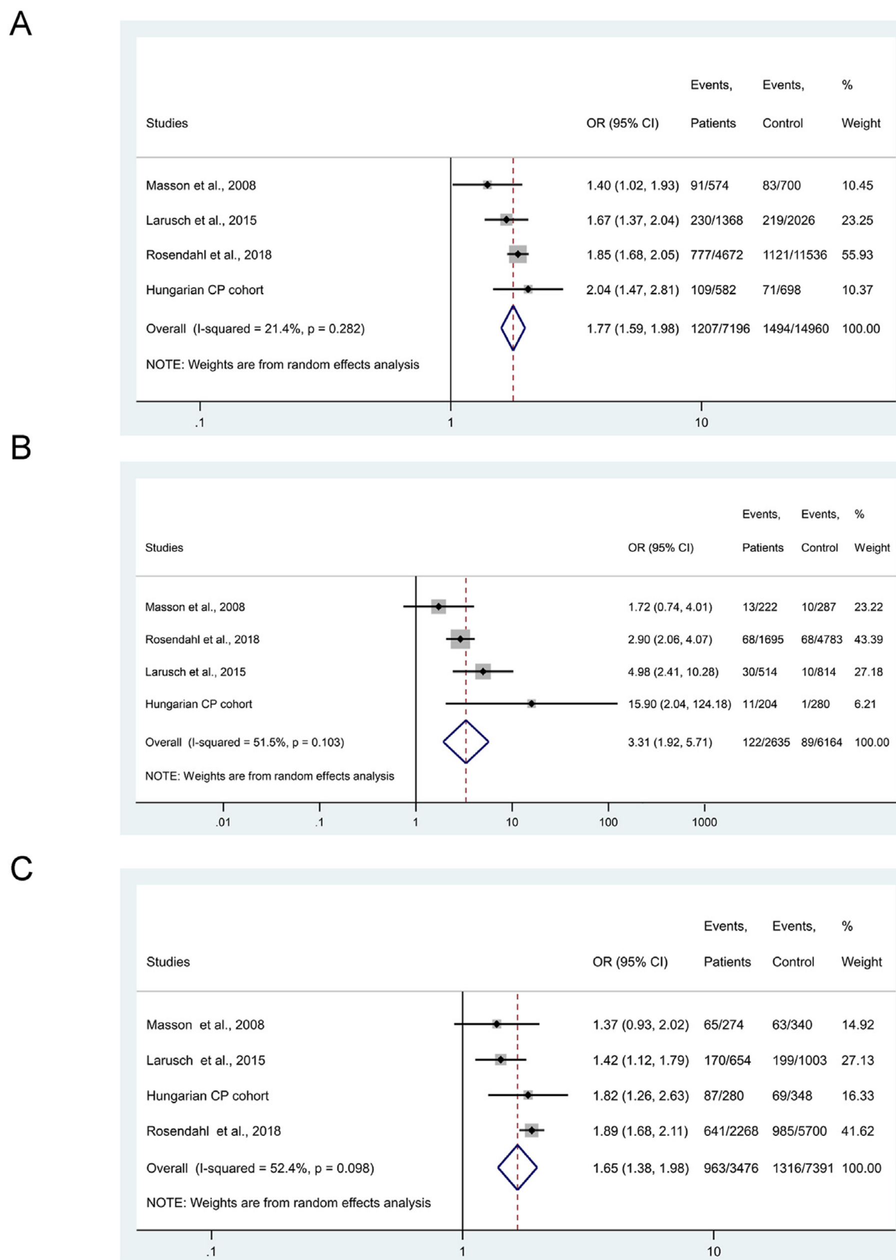

Fig. 3. Meta-analysis of the association between the c.180C>T *CTRC* variant and chronic pancreatitis (CP) in European adult cohorts. Forest plots are shown. **A**, Association between the c.180T minor *CTRC* allele and CP. **B**, Association of the c.180TT homozygous *CTRC* genotype with CP. **C**, Association of the c.180CT heterozygous *CTRC* genotype with CP.

on CP risk in the NACP cohort (OR 5.1, 95% CI 3.34–7.9) than in ACP patients (OR 2.5, 95% CI 1.71–3.62). However, the calculated effect sizes of the heterozygous c.180CT genotype were similar in both cohorts (NACP, OR 1.94, 95% CI 1.62–2.34; ACP, OR 1.88, 95% CI 1.67–2.12).

All the included studies had excellent quality based on the modified NOS scale (Table 1). We observed deviations from the Hardy-Weinberg equilibrium in two cohorts [11,22]. In the pan-European GWAS by Rosendahl et al. (2018), this could be explained by the Wahlund effect [28], since several cohorts were combined. In the French study by Masson et al. (2008), no explanation was apparent, however, in sensitivity analysis, after leaving this study out, no significant impact on the overall results was observed.

Effect of c.180C>T variant on *CTRC* mRNA expression. We performed mRNA expression analysis on cDNA samples obtained from surgically resected pancreas specimens from 10 patients heterozygous for the c.180C>T *CTRC* variant. First, we visually compared the signal heights at position c.180 on the Sanger sequencing electropherograms of pancreatic cDNA samples, and found that c.180T peaks were slightly smaller than the c.180C peaks (Fig. 4A). In contrast, the T and C signals were identical in size when genomic DNA was sequenced (not shown). Next, for quantitative analysis of allele specific expression, we used an RFLP assay and found that in heterozygous carriers, mRNA levels of the variant c.180T allele were 40.7±2% (mean±SD, n=10) of the total *CTRC* expression (Fig. 4B), instead of the expected 50% ($p<0.0001$). In heterozygous c.180CT and homozygous c.180TT carriers, this would translate to total *CTRC* mRNA expression levels of 84.3% and 68.6%, respectively, relative to the wild-type CC genotype.

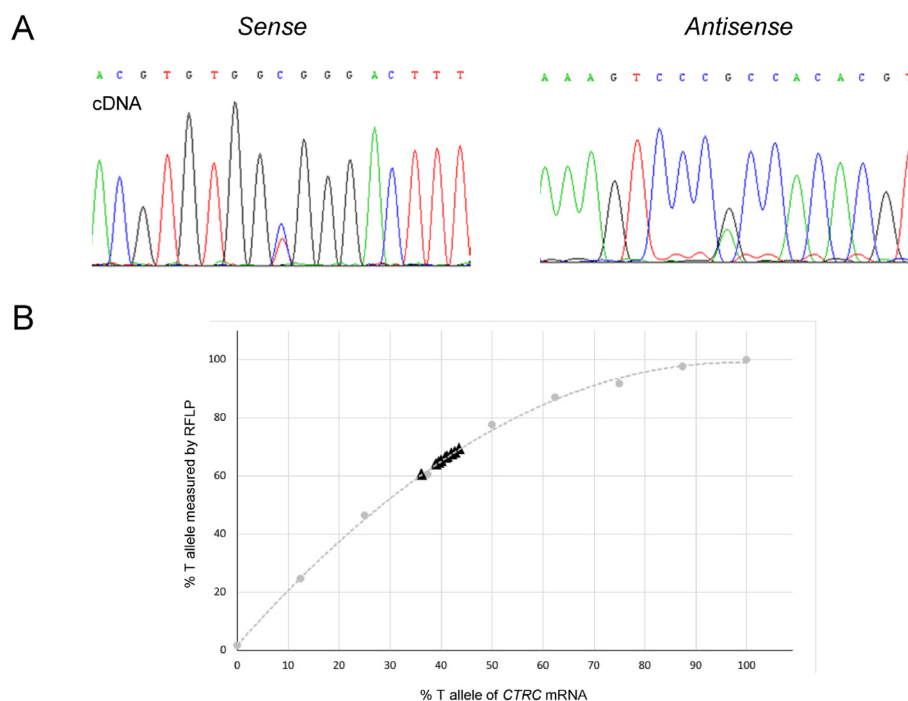

Fig. 4. Effect of c.180C>T variant on *CTRC* mRNA expression. **A**, Electropherograms showing Sanger sequencing of a cDNA sample from the pancreas of a heterozygous *CTRC* c.180C>T carrier. Note the difference in the peak heights at position c.180. **B**, Relative mRNA expression levels of the c.180T *CTRC* allele in the pancreas of heterozygous c.180C>T carriers. The gray line and symbols represent the calibration curve; the black triangles denote the pancreatic cDNA samples analyzed (n=10). See *Methods* for experimental details.

4. Discussion

The aim of the present study was to perform a systematic analysis on the global effect size of the *CTRC* variant c.180C>T in CP. Mechanistically, the *CTRC* gene and its variants belong to the so-called trypsin-dependent pathological pathway of CP risk, which describes the interactions of various genetic changes that control intrapancreatic trypsin activity [9]. Thus, gain-of-function mutations in the serine protease 1 (*PRSS1*) gene that increase autoactivation or block degradation of human cationic trypsinogen cause CP with high penetrance. Loss-of-function variants in the protective *SPINK1* and/or in *CTRC* genes increase CP risk significantly. Variant p.G191R in the serine protease 2 (*PRSS2*) gene sensitizes human anionic trypsinogen for autodegradation and thereby protects against CP [29]. Similarly, a frequent deletion polymorphism within the so-called *PRSS1-PRSS2* haplotype protects against CP by slightly reducing expression of *PRSS2* [30–32]. Finally, a common inversion allele at the *CTRB1-CTRB2* locus protects against CP by increasing expression of CTRB2, which results in more efficient degradation of anionic trypsinogen [22,33]. The frequencies and effect sizes of the various genetic alterations within the trypsin-dependent pathway vary considerably, which may be further compounded by variations due to ethnicity and CP etiology. To understand their clinical impact and disease relevance, each genetic variant must be precisely characterized with respect to allele frequency and genotype distribution (if applicable), which permits calculation of OR and 95% CI values. To obtain global estimates of effect sizes, studies can be pooled and synthesized by meta-analyses.

With respect to CTRC, we previously analyzed the global frequency and clinical effect of relatively frequent (>1%) loss-of-function missense variants and a microdeletion [15]. We found that global carrier frequencies of the individual variants were between 1.0% and 2.4%, and the OR values ranged from 2.6 to 6.5. Overall, it appeared that loss-of-function heterozygous missense *CTRC* variants are present in approximately 4% of CP patients and increase CP risk around 5-fold, on average. Although rare, homozygous and compound heterozygous missense *CTRC* variants were associated with higher risk.

In contrast to the missense *CTRC* variants, the CP-associated c.180C>T (p.Gly60=) *CTRC* variant studied here is a commonly occurring genetic change that does not alter the amino-acid sequence of CTRC. To better quantify the global impact of the c.180C>T variant, first we performed a new case-control study on subjects of Hungarian origin and extracted additional genotyping information from the 2018 pan-European GWAS dataset [22]. Next, we performed a meta-analysis that combined the new and the previously published data on global cohorts. Considering allele frequency, the c.180C>T variant was significantly overrepresented in CP patients (~14%) versus controls (~9%); and increased CP risk about 2-fold. Analysis of genotypes revealed that the homozygous c.180TT genotype increased CP risk more strongly (~5-fold) than the heterozygous c.180CT genotype (~2-fold). We observed considerable between-study variation when all available data were analyzed. Therefore, we confirmed the risk effect of the c.180C>T variant allele and genotypes in a subset of cohorts containing only adults of European origin. The heterogeneity between populations may be attributed to differences in the prevalence of other risk factors contributing to disease development, as the c.180C>T variant is not disease-causing itself.

Previously, we found that *CTRC* missense variants increased CP risk similarly in NACP and ACP patients [15]. Likewise, the c.180C>T variant was present with comparable allele frequencies in the NACP and ACP cohorts. Interestingly, however, the homozygous c.180TT genotype seemed to have a larger effect in NACP (~5-fold) than in ACP (2.5-fold), for reasons that are not readily apparent. The c.101A>G (p.N34S) *SPINK1* variant was also shown to impart larger risk in NACP than in ACP [34,35]. In contrast, the *PRSS1-PRSS2* haplotype was reported to increase ACP risk primarily with little, if any, effect in NACP [36–38]. Some of these differential effects may be due to changes in digestive enzyme expression induced by

chronic alcohol abuse; e.g. an increase in secreted anionic trypsinogen levels was documented in alcoholics [39].

The mechanism of action by which variant c.180C>T increases CP risk has been unknown. Here, we analyzed pancreatic cDNA from heterozygous carriers and offer preliminary evidence that the expression of the c.180T allele is reduced relative to the c.180C allele. We estimated the decreased mRNA levels to be around 68.6% in homozygous c.180TT and 84.3% in heterozygous c.180CT carriers relative to those of wild-type (c.180CC) *CTRC*. Similar reduction of *CTRC* mRNA transcripts with the c.180T allele is also reported by the Genotype-Tissue Expression (GTEx) project (https://gtexportal.org). Schmidt et al. (2022) using Bayesian colocalization analysis of genome-wide significant risk loci from the previously published GWAS study (2018) with pancreas expression quantitative trait loci data from the GTEx portal identified two shared causal variants, the c.180C>T and the intron 5 variant c.493+52G>A [23]. Both variants are in linkage disequilibrium with other variants flanking the *CTRC* gene (https://ldlink.nci.nih.gov). Which of these variants is responsible for the observed reduction in mRNA expression remains to be determined.

In summary, we determined the global frequency and effect size of *CTRC* variant c.180C>T (p.Gly60=) using case-control studies and meta-analysis. The results confirm that *CTRC* variant c.180C>T is a clinically relevant risk factor for both NACP and ACP, and should be included in genetic screening tests when CP etiology is investigated. However, asymptomatic populations should not be routinely screened for this variant since its positive predictive value is very low.

Author contributions

EH, MST, GB, PH, and SB conceived the study. EH, BCN, and MST directed the study. GB and AT performed the database search, selection and data extraction. NG and GB performed the statistical analysis. JR, BCN and ASZ provided genotyping data. SB performed the functional experiments. MST and EH wrote the manuscript. GB prepared the tables and figures. All authors approved the final manuscript.

Declaration of competing interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2023.05.013.

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