# INVESTIGATION OF THE GENETIC BACKGROUND OF CHRONIC PANCREATITIS

**Doctoral (PhD) Thesis**

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Doctoral Council and Habilitation Committee of Medical and Pharmaceutical Sciences of the University of Pécs

2025

# I. INTRODUCTION

The inflammatory diseases of the pancreas represent a disease continuum – first appearing as an initial acute episode (acute pancreatitis; AP), followed by recurrent acute episodes (recurrent acute pancreatitis; RAP), and eventually progressing to chronic pancreatitis (CP) [1]. Chronic pancreatitis is characterized by longstanding morbidity due to progressive inflammation of the pancreas, resulting in irreversible morphological changes and functional deficit. The leading symptoms in majority of patients include abdominal pain, malabsorption, and ensuing weight loss, which significantly reduce quality of life.

CP is currently considered a multifactorial disorder, where the interplay of genetic alteration and environmental factors leads to the development of the disease. A highly significant etiological factor, identified as a culprit for more than 50% of CP cases, is chronic heavy alcohol consumption (alcoholic CP; ACP). About a third of CP cases are idiopathic, i.e. a specific etiological factor cannot be identified. When CP occurs in multiple generations within a family, it is referred to as hereditary CP. This form is associated with high-penetrance, disease-causing gene variants. However, it has become evident that genetic variations play a role not only in cases of hereditary CP but also in the development of sporadically occurring non-alcoholic or even alcoholic CP [2].

## I/1. Genetic risk factors in chronic pancreatitis

Hereditary CP was already recognized as an inflammatory disorder of the pancreas with autosomal dominant inheritance in the mid-20th century [3]. The first genetic variant, identified in 1996 by Whitcomb and colleagues, was located in the *PRSS1* gene, which encodes human cationic trypsinogen [4]. This discovery not only confirmed the central role of trypsinogen in the pathogenesis of CP but also initiated a series of studies aimed at mapping the genetic background of the disease, which continues to this day. Through candidate gene studies and genome-

wide association studies (GWAS), several susceptibility genes have been identified over the years [5].

Functional analysis of these variants outlined three possible pathways that either lead to the development or increase the risk of CP: i) the trypsin-dependent, ii) the misfolding-dependent, and iii) the ductal pathways [5]. The genes discovered so far have an undisputable role in disease development, however they are only identified in a fraction of idiopathic CP patients. Hence there is continuing emphasis on researching the genetic background and pathomechanism of CP, which is aimed at: i) identifying additional genetic risk factors; ii) determining the precise effects of genetic risk factors; and iii) describing the interaction between genetic and environmental risk factors.

## I/2. The trypsin-dependent pathway

The central mechanism in the so-called trypsin-dependent pathway of genetic risk of CP is premature intrapancreatic trypsinogen activation [6]. To prevent or eliminate unwanted intrapancreatic trypsin activity, the pancreas has two defense mechanisms. The first line of defense is a protease inhibitor termed serine protease inhibitor Kazal type 1 (SPINK1) [7], which is co-secreted from acinar cells with digestive enzymes. If the amount of active trypsin exceeds the amount of available SPINK1 protein, a second line of defense is triggered, starting with the trypsin-mediated activation of chymotrypsinogen C to active chymotrypsin C (CTRC) [8]. In the duodenum, CTRC acts primarily as a digestive enzyme. Inside the pancreas, however, the main function of CTRC lies in its ability to initiate the degradation of trypsinogen, thereby preventing further activation of trypsinogen molecules to trypsin. Mutations that reduce the secretion or function of defense mechanisms, namely SPINK1 or CTRC, predispose individuals to the development of CP.

### I/2.1. Chymotrypsin C (*CTRC*) in pancreatitis

The first line of genetic evidence for pathogenic *CTRC* variants was published in 2008 by Rosendahl et al., when a large-scale multicenter case-control study including more than 1300 patients and 2800 controls was conducted investigating

both hereditary, idiopathic, and alcoholic CP patients of German origin, and to a lesser extent tropical CP patients, which is a distinct form of the disease occurring predominantly in India [9]. Several missense variants which were overrepresented in CP patients compared to the general population were described, and the study underlined many important points about genetic variations of *CTRC*: i) the main pathologic effect of missense *CTRC* variants is either hindered secretion or reduced activity of the enzyme, decreasing the protective measures against trypsinogen autoactivation inside the pancreas; ii) heterozygous missense *CTRC* variants that occur relatively frequently among patients (global carrier frequency >1 %) increase the risk of developing the disease approximately 3-7-fold; iii) these variants seem to elevate the risk evenly in the alcoholic and idiopathic CP groups; iv) they are found at most in around 5% of patients.

**I/2.2. The c.180C>T (p.Gly60=) variant of chymotrypsin C**

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 [9]. First, Masson et al. (2008) described the c.180C>T (p.Gly60=, rs497078) variant in exon 3 and the c.285C>T (p.Asp95=, rs41307798) variant in exon 4, with minor allele frequencies of 11.9% and 4.3% in the French population, respectively [10]. 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 [11, 12]. 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 both Japanese and Chinese populations [13, 14]. 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 [15-17]. 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). Interestingly, 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. Although the association of the p.Gly60= with CP is well established, the variant remains enigmatic for two reasons: first, it is a synonymous variant that does not alter the amino-acid sequence of the enzyme, therefore, its mechanism of action cannot be identified with biochemical and cell-biological approaches routinely used for missense *CTRC* variants. The second reason has to do with its frequency. Although the term is used less and less, not so long-ago p.Gly60= was called a polymorphism, because it is present to such a high extent in the general population. Usually, the frequency and the effect size of a mutation has an inverse correlation – the higher the frequency, the lesser the effect, otherwise it would be detrimental to the population. However, the problem with small effect sizes in genetics is that one needs a large enough cohort to determine the true effect, which is not always available in case of rare diseases. This is reflected in the results of previous studies investigating *CTRC* variants, where the calculated effect sizes for p.Gly60= varied greatly.

## I/3. The misfolding-dependent pathway

In 2009, a novel pathomechanism emerged as the functional analysis of some *PRSS1* mutations (p.R116C, p.C139S) yielded different outcomes compared to previously characterized pathogenic variants [18]. Neither of these variants exhibited the classic characteristics of *PRSS1* mutations in the trypsin-dependent pathway (increased autoactivation, decreased CTRC-dependent degradation). Instead, trypsinogen secretion from human embryonic kidney (HEK) 293T cells transiently transfected with these *PRSS1* variants was reduced by ~80%, the variants were retained intracellularly in an insoluble form, and the cells showed increased levels of immunoglobulin-binding protein (BiP, encoded by the gene heat shock protein family A (Hsp70) member 5; *HSPA5*) and spliced X-box binding protein-1 (XBP1s). These observations indicated misfolding of proteins

and their retention within the endoplasmic reticulum (ER), resulting in a state called ER stress, which is the presence of a significant amount of misfolded proteins in the ER. Prolonged and unresolved ER stress results in the overstimulation of the PERK (double-stranded RNA-activated protein kinase (PKR)–like ER kinase) brach of the unfolded protein response (UPR) ultimately leading the cell towards apoptosis [19].

Over the years a substantial amount of misfolding inducing nucleotide variants were found in different digestive enzymes that are overrepresented in chronic pancreatitis patients compared to the healthy general population [20]. These variants are less prevalent than those of the trypsin-dependent pathway, occurring in about 10% of genetically affected non-familial CP patients, but usually associate very strongly with the disease. Mutations exhibiting the 'misfolding phenotype' (where affected proteins are secreted poorly from cells yet they are detectable in cell lysates in an insoluble and protease-sensitive form) [20] are well characterized in abundantly expressed digestive enzymes, most notably in carboxypeptidase A1 (CPA1) [21] and carboxyl ester lipase (CEL) [22].

### I/3.1. Carboxyl ester lipase (*CEL*) in pancreatitis

CEL is a digestive enzyme which plays a role in the digestion and absorption of fats, cholesterol esters, and fat-soluble vitamins present in food [23]. It constitutes about 4% of the pancreas juice proteins. The *CEL* gene is made up of 11 exons, of which the last one contains a variable number of tandem repeats (VNTR) sequence [24]. Single base-pair deletions in the first 5 repeats cause maturity onset diabetes of the young type 8 (MODY8) [25], which contrary to its name, has been characterized as of late as a subtype of chronic pancreatitis. The deletion not only introduces and early STOP codon, but also repeating cysteine residues which make the enzyme prone to misfolding, causing intracellular retention and ER stress. Several other frameshift mutations have been described in the VNTR region of CEL, however the current belief is that they only lead to misfolding induced proteotoxicity when i) found in proximal VNTR segments, ii) and cause

early truncation and consequently a significant shortening of the C-terminal tail of CEL.

### I/3.2. Carboxyl ester lipase hybrid allele 1 (*CEL-HYB1*)

The *CEL* gene in the genome is found in tandem with its pseudogene (carboxyl ester lipase pseudogene, *CELP*) [26]. As the name implies, CELP does not encode a functional protein, as it contains only the first (1') and eighth through eleventh (8'-11') exons of *CEL*, and the mRNA is predicted to go through nonsense-mediated decay due to an early STOP codon found in the exon 8' of *CELP*. Since *CEL* and *CELP* are in proximity and highly similar in their sequences, the likelihood of genomic rearrangements at this locus is increased. In 2015, when a Norwegian study group examined the distal exons of *CEL* in healthy blood donors to search for such rearrangements, they identified a hybrid allele (later termed *CEL-HYB1*) which was generated by non-allelic homologous recombination between intron 10 and the adjacent exon boundaries of *CEL* and *CELP* [26]. The hybrid gene contained exons 1-10 of *CEL* and exon 11' of *CELP* and encoded a much shorter tail region than normal due to an early STOP codon in the third VNTR of the *CELP* gene. When expressed in transfected cells, the CEL-HYB1 protein exhibited characteristic signs of misfolding, such as reduced secretion, intracellular retention and aggregation, cellular ER stress [27]. Initial analysis of a small CP cohort with familial disease indicated significant enrichment of *CEL-HYB1* in CP cases (14%) relative to healthy controls (1%), suggesting that *CEL-HYB1* might be a strong risk factor for CP, as judged by the odds ratio (OR) of 15.5 [26]. Replication studies in 3 European cohorts from Germany and France demonstrated smaller but still impressive effect sizes with an average OR of 5.2 [26].

Follow-up studies, however, painted a more complex picture. First, it became apparent that *CEL-HYB1* was not found in East-Asian populations where a different fusion, *CEL-HYB2* was prevalent [28]. The *CEL-HYB2* allele, in which exons 1-9 of *CEL* are fused with exons 10'-11' of *CELP,* showed no association

with CP, likely due to the degradation of its transcript via nonsense-mediated mRNA decay [28, 29]. Second, a replication study in a Polish pediatric cohort found no association of *CEL-HYB1* with CP, although a 2-fold enrichment in CP cases was observed with no statistical significance [30, 31]. Interestingly, this analysis also found a higher carrier frequency (2.4%) in the control population than the original report (range 0.7-1%). Third, a more recent study demonstrated that within the *CEL-HYB1* allele there are three different haplotypes defined by isoleucine (Ile) and threonine (Thr) amino-acids at the 488th and 548th position [27]. Remarkably, the Thr488-Thr548 haplotype was similarly found in *CEL-HYB1* positive CP patients (34/55) and controls (18/20), whereas the Thr488-Ile548 haplotype was found only in CP patients (20/55) and never in controls. Haplotype Ile488-Thr548 was rare, but present both in patients (1/55) and controls (2/20). Haplotypes Thr488-Thr548 and Thr488-Ile548 showed a similarly strong misfolding phenotype in cell culture experiments, while the Ile488-Thr548 haplotype had a minor effect, suggesting that Thr488 is the crucial determinant of misfolding. Introduction of Thr488 to full-length CEL also resulted in reduced secretion. The authors proposed that the Thr488-Ile548 haplotype of *CEL-HYB1* was pathogenic while the Thr488-Thr548 haplotype was benign or associated with much lower risk.

# II. AIMS

**Aim 1.** *Investigation of the c.180C>T (p.Gly60=) CTRC variant in chronic pancreatitis.*

Subaim 1: To determine the frequency and effect size of the p.Gly60= variant in Hungarian CP patients and healthy controls.

Subaim 2: To perform a systematic, global meta-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.

**Aim 2.** *Investigation of the carboxyl ester lipase hybrid allele 1 (CEL-HYB1) haplotypes in chronic pancreatitis.*

Subaim 1: To investigate the role of the *CEL-HYB1* allele in CP by performing a case-control study and haplotype analysis on a genetically well-characterized, ethnically homogenous Hungarian CP cohort.

Subaim 2: To ascertain the frequency and effect sizes of the two major disease-associated *CEL-HYB1* haplotypes by analyzing all available *CEL-HYB1* carriers from the published German, Polish, and French CP cohorts.

# III. METHODS

## III/1. Investigation of the c.180C>T (p.Gly60=) *CTRC* variant in chronic pancreatitis

### III/1.1. Study subjects and genotyping

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 (NACP) and 167 with alcoholic CP, and 349 control subjects (mean age at recruitment 49.1±12.1 years) with no pancreatic disease. Genotyping data for the c.180C>T variant was also extracted from the GWAS dataset generated by Rosendahl et al. (2018) [15].

### III/1.2. Meta-analysis process

Two authors independently performed a systematic search in four databases (MEDLINE via Pubmed, Embase, Scopus, and CENTRAL via Cochrane Library) using a search key based on our PICO formatted question. Citing (using MEDLINE via Pubmed and Google Scholar) and cited reference searches were also performed. The multi-step study selection process was done independently by two authors to identify genetic association case-control studies with adequately defined CP patients and controls investigating the c.180C>T (p.Gly60=) *CTRC* variant. 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 $\chi 2$ test. Discrepancies during search,

selection, data extraction, and quality evaluation between authors were resolved by the principal investigator or by mutual agreement.

### III/1.3. 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. Heterogeneity between studies was investigated with the $I^2$ ($p \geq 0.1$) and $\chi 2$ tests. Sensitivity analysis was carried out by repeating the quantitative synthesis while leaving out one study at a time (leave-one-out method).

### III/1.4. 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. Following PCR amplification and FauI enzyme digestion, a capillary electrophoresis system was used to analyze the amounts of undigested and digested PCR products.

## III/2. Investigation of the carboxyl ester lipase hybrid allele 1 (CEL-HYB1) haplotypes in chronic pancreatitis

### III/2.1. Subjects

319 unrelated patients with CP, including 134 with nonalcoholic CP (age at recruitment 58.66 ± 13.6 years, mean ± SD, range 22-85) and 185 with alcoholic CP (55.52 ± 9.9 years, range 23-79 years), and 618 control subjects (40.98 ± 14.7 years, range 11-89 years) with no pancreatic disease were enrolled. For analyses of haplotype distribution, *CEL-HYB1* carriers from Germany (cases n=29,

controls n=13), France (cases n=17, controls n=9), and Poland (cases n=6, controls n=8) were used.

### III/2.2. Detection of the *CEL-HYB1* allele and haplotype indentification

Screening for the *CEL-HYB1* allele was performed using the LightCycler-based assay reported previously by Fjeld et al. (2015) [26], on a CFX96 Touch Real-Time PCR Detection System. Confirmation of *CEL-HYB1* positive samples was carried out by long-range, duplex PCR. To identify the haplotypes of positive subjects, Sanger sequencing was performed after selective PCR amplification of the hybrid allele.

### III/2.3. Genotyping the *CEL* c.1463T>C (p.Ile488Thr) variant

We used a restriction fragment length polymorphism (RFLP) assay to genotype the p.Ile488Thr variant in *CEL*. Briefly, exon 10 and flanking intronic sequences of the *CEL* gene were PCR amplified, the amplicon was digested with DpnII restriction enzyme (New England Biolabs), and the digestion products were visualized on a 2% agarose gel with ethidium bromide staining. p.Ile488Thr positive samples were also verified by Sanger sequencing.

### III/2.4. Functional studies

To study the effect of the identified haplotypes, HEK 293T cells were transiently transfected using previously published expression plasmids [27]. Total RNA was isolated from cells, reverse-transcribed, and *HSPA5* levels were measured by quantitative PCR, as reported previously (Sándor et al. 2022).

# IV. RESULTS

## IV/1. Investigation of the c.180C>T (p.Gly60=) *CTRC* variant in chronic pancreatitis

### IV/1.1. 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. 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 [15, 17, 32, 33]. 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) [15]. 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. 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%).

**IV/1.2. Meta-analysis**

The new Hungarian cohort data, the GWAS-derived pan-European analysis [15], and five other published case-control studies were used for quantitative synthesis [11, 12, 14, 16, 17]. 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 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). 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). Sensitivity analysis (leave-one-out method) supported the validity of the results.

Due to substantial between-study heterogeneity, we performed a subgroup analysis including only adult subjects of European origin. In this population, the minor c.180T allele was significantly over-represented in CP patients (16.8%) in comparison to controls (9.9%) yielding an OR of 1.77 (95% CI 1.59-1.98). 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. However,

moderate heterogeneity between the studies was noted for both genotypes. 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 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. We observed deviations from the Hardy-Weinberg equilibrium in two cohorts [10, 15]. In the pan-European GWAS by Rosendahl et al. (2018), this could be explained by the Wahlund effect [34] 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.

**IV/1.3. 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. 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, 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.

## IV/2. Investigation of the carboxyl ester lipase hybrid allele 1 (CEL-HYB1) haplotypes in chronic pancreatitis

### IV/2.1. Association of *CEL-HYB1* with chronic pancreatitis in Hungarians

We investigated the *CEL-HYB1* allele in 319 Hungarian CP patients (185 alcoholic, 134 non-alcoholic) and 618 ethnically matched controls without pancreatic disease. We found a significant overrepresentation of *CEL-HYB1* in patients *versus* controls (carrier frequency 9/319 vs. 5/618, OR=3.6, 95% CI 1.2-10.7, $P$=0.024). Subgroup analysis of alcoholic (6/185, 3.2%) and non-alcoholic (3/134, 2.2%) patients showed no substantial difference in *CEL-HYB1* carrier frequency. The lack of significance in case of NACP may be explained by the lower number of patients in this subgroup. All *CEL-HYB1* positive subjects were heterozygous. Sequencing exons 2 and 3 of *PRSS1*, exon 3 of *SPINK1*, exons 2, 3, and 7 of *CTRC,* exons 7, 8, and 10 of *CPA1*, and exons 4 and 11 of *CFTR* in the *CEL-HYB1* carriers revealed no CP risk variants, aside from the commonly occurring c.180C>T (p.Gly60=) synonymous *CTRC* variant, which was found in 2/9 patients (1 homozygous, 1 heterozygous) and 3/5 controls (heterozygous).

### IV/2.2. *CEL-HYB1* haplotype analysis

To assess the haplotype distribution in the Hungarian cohort, we sequenced exons 10 and 11' of the *CEL-HYB1* allele in all carriers. Unexpectedly, we found that all cases and controls carried the Thr488-Thr548 haplotype of *CEL-HYB1*. No other *CEL-HYB1* haplotype was detected. To compare the *CEL-HYB1* haplotype distribution in Hungarians to other European populations, we sequenced the previously reported *CEL-HYB1* alleles from German, French, and Polish cohorts

[26, 30]. We found that approximately half of the CP patients from Germany (14/29) and one third of the Polish cases (2/6) carried the Thr488-Ile548 haplotype, while all French patients (17/17) carried the Thr488-Thr548 haplotype. All German (n=13), Polish (n=8), and French (n=9) controls carried the Thr488-Thr548 haplotype. Thus, as reported previously [27], the Thr488-Ile548 haplotype was never found in *CEL-HYB1* positive controls. Of the 29 German patients, family history was available for 21 subjects. At least one other family member was affected in 8 of these patients. Interestingly, we found the Thr488-Ile548 haplotype in 75% (6/8) of patients with a family history but only in 23% (3/13) without a family history. We did not identify the previously reported Ile488-Thr548 haplotype [27, 35] or the putative Ile488-Ile548 haplotype in any of the *CEL-HYB1* alleles sequenced.

**IV/2.3. *CEL* variant c.1463T>C (p.Ile488Thr) in the Hungarian population**

Since both pathogenic *CEL-HYB1* alleles contained Thr488, we tested whether this variation might increase CP risk when present in the full-length *CEL*, which typically contains Ile488. Using RFLP analysis, we investigated CP patients and controls that were negative for the *CEL-HYB1* allele and found the *CEL* variant p.Ile488Thr in 1/309 CP cases (0.3%) and in 2/611 controls (0.3%). Thus, the presence of Thr488 in *CEL* is rare and shows no association with CP (OR 1, 95% CI 0.1-11, $P$=0.993).

**IV/2.4. Functional analysis of *CEL-HYB1* haplotypes**

A previous study analyzed the functional effects of the Thr488-Ile548 and Thr488-Thr548 haplotypes in transfected cells and found largely comparable effects concerning defective secretion, intracellular aggregation, and induction of ER stress [27]. Using the same expression constructs, we re-analyzed the effects of the *CEL-HYB1* haplotypes on the expression of the ER master chaperone *HSPA5* (BiP) in transiently transfected HEK 293T cells. We used reverse-transcription quantitative PCR instead of the previously employed Western blotting because of its higher sensitivity to detect smaller differences. Relative to

cells transfected with empty vector, both *CEL-HYB1* haplotypes increased *HSPA5* expression significantly. Using either 1 µg or 4 ug expression plasmid for transfection, we consistently observed significantly higher *HSPA5* expression in cells transfected with the Thr488-Ile548 haplotype *versus* the Thr488-Thr548 haplotype, although the difference was small.

# V. DISCUSSION

Chronic pancreatitis is a recurring-relapsing complex inflammatory disorder, which besides the environmental risk factors has a wide array of genetic risk factors [5]. In this thesis we investigated two of these genetic changes, the commonly occurring CTRC c.180C>T (p.Gly60=) and the more infrequent carboxyl ester lipase hybrid allele 1 (CEL-HYB1).

## V/1. Investigation of the c.180C>T (p.Gly60=) *CTRC* variant in chronic pancreatitis

The aim of this study was to perform a systematic analysis on the global effect size of the *CTRC* variant c.180C>T in CP. In one of our previous studies we looked into the global prevalence and clinical impact of some relatively common (>1%) loss-of-function missense variants and a microdeletion [36]. We discovered that the global carrier rates for these individual variants ranged from 1.0% to 2.4%, with odds ratios varying between 2.6 and 6.5. Overall, it seems that around 4% of CP patients carry heterozygous missense *CTRC* variants, which indicates an about 5-fold increased risk towards CP, on average. In contrast to the missense *CTRC* variants, the CP-associated c.180C>T (p.Gly60=) *CTRC* variant investigated here is a commonly occurring synonymous mutation, meaning the amino-acid sequence of translated CTRC protein remains unchanged. To get a clearer picture of how the c.180C>T variant affects people worldwide, as the first step we conducted a new case-control study with individuals of Hungarian origin. We also gathered more genotyping data from the 2018 pan-European GWAS dataset [15]. 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).

However, upon completing the analysis of all the available data, we noticed a significant amount of between-study heterogeneity, which usually signals the presence of different subgroups in the data. As within genetic studies, different ethnicities usually carry different subsets of variations, 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. Even though this reduced the confidence interval, likely meaning that this effect size is closer to the actual risk, it has to be noted that heterogeneity between populations may have been caused by differences in the prevalence of other risk factors contributing to disease development, as the c.180C>T variant is not disease-causing itself.

Likewise, our previous analysis of *CTRC* missense variants where the increased risk was similar in NACP and ACP patients [36], the heterozygous c.180C>T variant was present with comparable allele frequencies in both cohorts. In what way variant c.180C>T increases CP risk has not been known. Here, by analyzing pancreatic cDNA from heterozygous carriers we offer preliminary evidence that expression of the minor c.180T allele is reduced relative to the more common c.180C allele. It has to be noted however, that the c.180C>T and the intron 5 variant c.493+52G>A are in linkage disequilibrium. Which of these variants is responsible for the observed reduction in mRNA expression remains to be determined.

## V/2. Investigation of the carboxyl ester lipase hybrid allele 1 (*CEL-HYB1*) haplotypes in chronic pancreatitis

In this study we sought to investigate the association of the *CEL-HYB1* allele with CP in the Hungarian population and characterize how different haplotypes might

be responsible for disease risk. Since earlier studies on *CEL-HYB1* did not look into the haplotype distribution, new replication studies and re-analysis of previously published data were warranted. In our case-control study, we observed a notable increase of *CEL-HYB1* in Hungarian CP cases with similar frequency in alcoholic and non-alcoholic CP. The effect size, as judged by the OR, was 3.6-fold, which is on-par with the impact of other important CP risk genes, such as heterozygous *CTRC* variants, or heterozygous severe *CFTR* variants such as p.Phe508del [5]. Our findings confirm that *CEL-HYB1* is an important risk factor and suggest that genetic testing of CP patients should include screening for this allele. Haplotype analysis found that only the Thr488-Thr548 haplotype was present in Hungarian *CEL-HYB1* carriers. This was surprising in light of a recent proposal that the Thr488-Thr548 haplotype was not associated with CP risk [27]. To clarify this inconsistency, we sequenced all available *CEL-HYB1* alleles from the published German, French, and Polish carriers [26, 30]. To our surprise, about half of the German and one third of the Polish subjects carried the Thr488-Ile548, while solely the Thr488-Thr548 haplotype was present in the French carriers. Similarly to what was reported previously, the Thr488-Ile548 haplotype was never found in *CEL-HYB1* positive controls, suggesting that this variant is a strong risk factor and may be considered disease-causing. As the Hungarian and French cohorts contained exclusively the Thr488-Thr548 haplotype and disease association of *CEL-HYB1* was clearly demonstrated in both cohorts, the observations confirm that the Thr488-Thr548 haplotype is pathogenic, though with a somewhat smaller effect size.

Several studies found family pedigrees where pancreatitis seemed to segregate with *CEL-HYB1* [26, 30, 37]. Fjeld et al. (2015) reported an OR value of 15 in their discovery cohort of familial CP cases [26]. Similarly, in the familial subgroup of Polish CP patients the *CEL-HYB1* allele frequency was relatively high (3/20) [30]. It is possible, even likely, that these families carried the Thr488-Ile458 haplotype, resulting in the relatively high penetrance observed.

We did not find the previously reported Ile488-Thr548 haplotype [27] in any of the *CEL-HYB1* positive samples analyzed in this study, which raises the possibility that this variant might not exist.

However, a recent US study on pancreatic cancer did report 12/1045 (1.1%) *CEL-HYB1* carriers with Ile488 in their control group, which likely corresponds to the Ile488-Thr548 haplotype [35]. The contradictory results reaffirm that genotyping *CEL-HYB1* variants might produce technical errors due to the complex and variable nature of the *CEL* locus. Finally, we note that although its functional properties have been reported, the putative Ile488-Ile548 *CEL-HYB1* haplotype has never been found [27].

The reason for the large difference in the clinical impact of the two *CEL-HYB1* haplotypes is not readily apparent. *CEL-HYB1* has been demonstrated to exhibit all characteristics of a misfolding risk variant, however, the difference between the effects of the Thr488-Thr548 and Thr488-Ile548 haplotypes is minimal [27]. We confirmed this notion, as both haplotypes caused marked ER stress in transfected cells with the effect of the Thr488-Ile548 haplotype being slightly higher. How such a small difference translates into a more severe clinical phenotype remains unsolved. Recent research into the pathogenic mechanisms of misfolding *CPA1* variants suggests that even minor functional differences between variants might result in dissimilar clinical outcomes. However, it is important to keep in mind that haplotypes may influence the mRNA expression of *CEL-HYB1* in the human pancreas, potentially affecting the levels of toxic protein products and associated ER stress [27]. Wild-type *CEL* contains Ile488, whereas both pathogenic *CEL-HYB1* haplotypes carry Thr488. To investigate whether Thr488 alone might render *CEL* pathogenic, we genotyped CP cases and controls for the p.Ile488Thr variant and found that the variant was rare (0.3%) and evenly distributed in the patient and control groups. The results indicate that Thr488 becomes pathogenic only in the context of *CEL-HYB1*. Kawamoto et al. (2022) reported the same variant in 5/1045 (0.5%) in a US population without pancreatic disease [35].

# VI. CONCLUSIONS

In the first part of this work we established that the *CTRC* variant c.180C>T is a risk factor for both ACP and NACP in Hungary using a case control study. After that we determined the global frequency and effect size of *CTRC* variant c.180C>T through meta-analysis. Relative to the c.180CC genotype, the homozygous c.180TT increases risk about five-fold (OR=5.29, 95% CI 2.63-10.64), and the heterozygous c.180CT about two-fold (OR=1.94, 95% CI 1.57-2.38). Restricting the analysis to people of European origin the analysis yielded a milder, but more accurate effect for both the c.180TT (OR=3.31, 95% CI 1.92-5.71) and the c.180CT (1.65, 95% CI 1.38-1.98) genotype. Finally we provided preliminary evidence that the expression of the minor c.180T allele is reduced compared to the more common c.180C allele.

In the second part of the thesis we determined that the Thr488-Thr548 haplotype of *CEL-HYB1* increases CP risk by 3.6-fold in Hungarians (OR=3.6, 95% CI 1.2-10.7), confirming that this haplotype is pathogenic. By assessing the haplotypes of the available European *CEL-HYB1* positive subjects we found that the Thr488-Thr548 haplotype is widespread in Europe, while the occurrence of the stronger, possibly causative Thr488-Ile548 haplotype, seems regionally restricted. Genotyping the Hungarian cohort for *CEL* p.Ile488Thr showed that this variant is rare and shows no association with CP (OR 1, 95% CI 0.1-11), and thus is likely not the culprit behind the pathogenicity of the *CEL-HYB1* allele.

We can conclude that both *CTRC* c.180C>T (p.Gly60=) and the *CEL-HYB1* hybrid allele are definite risk factors for developing chronic pancreatitis and should be included in the genetic screening of patients when searching for disease etiology.

# VII. REFERENCES


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# VIII. LIST OF PUBLICATIONS

## VIII/1. Publications related to the PhD thesis

**Berke, G.**, Beer, S., Gede, N., Takáts, A., Szentesi, A., Hegyi, P., Rosendahl, J., Sahin-Tóth, M., Németh, B. C., & Hegyi, E. (2023). Risk of chronic pancreatitis in carriers of the c.180C>T (p.Gly60=) CTRC variant: case-control studies and meta-analysis. *Pancreatology: official journal of the International Association of Pancreatology (IAP) ... [et al.],* 23(5), 481–490. https://doi.org/10.1016/j.pan.2023.05.013

**IF = 2.8 Q1**

**Berke, G.**, Sándor, M., Xiao, X. K., Lowe, M. E., Ewers, M., Erőss, B., Masson, E., Németh, B. C., Vincze, Á., Czakó, L., Rygiel, A. M., Rosendahl, J., Chen, J. M., Witt, H., Hegyi, P., Sahin-Tóth, M., & Hegyi, E. (2024). Carboxyl ester lipase hybrid 1 (CEL-HYB1) haplotypes confer varying risk for chronic pancreatitis. *Scientific reports*, 14(1), 30965. https://doi.org/10.1038/s41598-024-82077-4

**IF = 3.8 Q1**

## VIII/2. Publications related to the topic of the PhD thesis

**Berke, G.**, & Sahin-Tóth, M. (2024). Intron-mediated enhancement of SPINK1 expression for pancreatitis therapy. *Gut*, *74*(1), e9. https://doi.org/10.1136/gutjnl-2024-332818

**IF = 23.1 Q1**

Stefanovics, R., Sándor, M., Demcsák, A., **Berke, G.**, Németh, B. C., Zhang, W., Abu-El-Haija, M., & Sahin-Tóth, M. (2024). Novel chymotrypsin C (CTRC) variants from real-world genetic testing of pediatric chronic pancreatitis cases. *Pancreatology: official journal of the International Association of Pancreatology (IAP) ... [et al.]*, *24*(5), 690–697. https://doi.org/10.1016/j.pan.2024.06.003

**IF = 2.8 Q1**

**Berke, G.**, Gede, N., Szadai, L., Ocskay, K., Hegyi, P., Sahin-Tóth, M., & Hegyi, E. (2022). Bicarbonate defective CFTR variants increase risk for chronic pancreatitis: A meta-analysis. *PloS one*, *17*(10), e0276397. https://doi.org/10.1371/journal.pone.0276397

**IF = 3.8 Q1**

Takáts, A., **Berke, G.**, Gede, N., Németh, B. C., Witt, H., Głuszek, S., Rygiel, A. M., Hegyi, P., Sahin-Tóth, M., & Hegyi, E. (2022). Risk of chronic pancreatitis in carriers of loss-of-function CTRC variants: A meta-analysis. *PloS one*, *17*(5), e0268859. https://doi.org/10.1371/journal.pone.0268859

**IF = 3.8 Q1**

Takáts, A., **Berke, G.**, Szentesi, A., Farkas, G., Jr, Izbéki, F., Erőss, B., Czakó, L., Vincze, Á., Hegyi, P., Sahin-Tóth, M., & Hegyi, E. (2021). Common calcium-sensing receptor (CASR) gene variants do not modify risk for chronic pancreatitis in a Hungarian cohort. *Pancreatology : official journal of the International Association of Pancreatology (IAP) ... [et al.]*, *21*(7), 1305–1310. https://doi.org/10.1016/j.pan.2021.08.012

**IF = 3.8 Q1**

## VIII/3. Publications unrelated to the topic of the PhD thesis

Sindler, D. L., Mátrai, P., Szakó, L., Berki, D., **Berke, G.**, Csontos, A., Papp, C., Hegyi, P., & Papp, A. (2023). Faster recovery and bowel movement after early oral feeding compared to late oral feeding after upper GI tumor resections: a meta-

analysis. *Frontiers in surgery*, *10*, 1092303. https://doi.org/10.3389/fsurg.2023.1092303

**IF = 1.6 Q2**

Csontos, A., Németh, D., Szakó, L., **Berke, G.**, Sindler, D. L., Berki, D., Papp, C., Hegyi, P., Vereczkei, A., & Papp, A. (2024). Intraoperative pyloric drainage is unnecessary during esophagectomies: a meta-analysis and systematic review of randomized controlled trials. *Pathology oncology research : POR*, *30*, 1611823. https://doi.org/10.3389/pore.2024.1611823

**IF = 2.2 Q2**

**Sum of impact factors from publications related to the topic of PhD thesis: 6.6**

**Sum of all impact factors: 47.7**

# IX. LIST OF CONGRESS PRESENTATIONS AND POSTERS

**APA/JPS/CAP/IAP 2024 Meeting**, 2024, Lahaina, HI, US

Berke G, Sahin-Tóth M: Intron-mediated enhancement of SPINK1 expression for pancreatitis therapy – poster presentation

**55th Meeting of the European Pancreatic Club**, 2023, Alpbach, Austria

Berke G, Hegyi E, et al.: CEL-HYB1 haplotypes confer varying risk for chronic pancreatitis – poster presentation

**Third Scandinavian Baltic Pancreas Symposium**, 2023, Stockholm, Sweden

Berke G, Hegyi E, et al.: CEL-HYB1 haplotypes confer varying risk for chronic pancreatitis – poster presentation

**United European Gastroenterology Week 2022**, Vienna, Austria

Berke G, Hegyi E, et al.: The hybrid allele 1 of carboxyl-ester lipase (CEL-HYB1) elevates the risk of chronic pancreatitis in Hungary: a case-control study - oral presentation

**54th Meeting of the European Pancreatic Club**, 2022, Kyiv, Ukraine

Berke G, Hegyi E, et al.: The hybrid allele 1 of carboxyl-ester lipase (CEL-HYB1)

elevates the risk of chronic pancreatitis in Hungary: a case-control study - poster presentation

**64th Annual Meeting of the Hungarian Society of Gastroenterology**, 2022, Siófok, Hungary
Berke G, Hegyi E, et al.: The hybrid allele 1 of carboxyl-ester lipase (CEL-HYB1) elevates the risk of chronic pancreatitis in Hungary: a case-control study - oral presentation

**10th Conference of the Hungarian Pancreatic Study Group**, 2022, Vecsés, Hungary
Berke G, Hegyi E, et al.: Risk of chronic pancreatitis in carriers of loss-of-function CTRC variants: A meta-analysis – oral presentation

**United European Gastroenterology Virtual Week 2021**
Berke G, Hegyi E, et al.: Bicarbonate defective CFTR variants in chronic pancreatitis: A meta analysis - poster presentation

**53rd Meeting of the European Pancreatic Club**, 2021, Verona, Italy
Berke G, Hegyi E, et al.: Bicarbonate defective CFTR variants in chronic pancreatitis: A meta analysis - oral and poster presentation

**63rd Annual Meeting of the Hungarian Society of Gastroenterology**, 2021, Siófok, Hungary
Berke G, Hegyi E, et al.: Bicarbonate defective CFTR variants in chronic pancreatitis: A meta analysis - poster presentation

**9th Virtual Conference of the Hungarian Pancreatic Study Group**, 2020, Hungary
Berke G, Hegyi E, et al.: Clinical significance of bicarbonate defective CFTR variants in pancreatitis – oral presentation

# X. ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my mentor, **Eszter Hegyi**, whose invaluable guidance, unwavering support, and insightful advice have been instrumental throughout my Student Researcher and PhD journey. Her encouragement, expertise and French coffee press have shaped both my research and my academic growth, and for that, I am truly thankful.

I am also profoundly grateful to **Miklós Sahin-Tóth** for his guidance and thoughtful input, which have greatly enriched my work. His knowledge and mentorship have been a source of inspiration and have significantly contributed to the development of this thesis.

Additionally, I would like to extend my appreciation to **everyone who has collaborated with me on the various projects throughout my PhD**. Their dedication and contributions have played a crucial role in advancing this research.

Finally, I am thankful to **the Institute for Translational Medicine** for welcoming me and providing the resources and support necessary for my PhD journey. Working in such a stimulating and collaborative environment has been a privilege, and I am grateful for the friendships and professional relationships that have been forged along the way.

This work would not have been possible without the collective efforts of all those mentioned above, and I sincerely thank each of you for your support.