GENETIC INVESTIGATIONS IN CHRONIC PANCREATITIS: COHORT STUDY AND META-ANALYSIS

PhD Thesis

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1. Introduction

1.1. Genetics of chronic pancreatitis

Chronic pancreatitis (CP) is an irreversible inflammatory disease of the pancreas, characterized by fibrosis and loss of exocrine and endocrine tissue [Kleeff et al. 2017]. Pain, maldigestion and weight loss are the leading symptoms of CP, which significantly worsen the quality of life and life expectancy. The disease affects around 50/100.000 individuals globally and is more common in men than in women. The pathomechanism leading to chronic pancreatitis is complex, environmental risk factors, such as alcohol abuse or smoking, and genetic risk factors contribute together to the development of the disease [Gupte et al. 2018]. Acute pancreatitis (AP), recurrent AP (RAP), and CP form a disease continuum [Yadav and Lowenfels, 2013]. The progression of AP to RAP and eventually to CP is often caused by chronic alcohol consumption or genetic risk factors. The genetic risk of RAP and CP overlap, while genetic testing for AP is difficult to interpret due to inadequate follow-up to exclude cases of RAP and CP [Mayerle et al. 2019].

Hereditary pancreatitis (HP) is a rare autosomal dominant genetic disorder of the pancreas, first reported in 1952 [Comfort and Steinberg 1952], however its causing gene mutation on the serine protease 1 (*PRSS1*) gene, that encodes human cationic trypsinogen, was detected 4 decades later [Whitcomb et al. 1996]. This discovery highlighted the importance of trypsin in the pathogenesis of CP. Over the past 20 years, several genetic association studies identified multiple different gene mutations contributing to the disease, the most important affected genes are serine protease inhibitor Kazal-type 1 (*SPINK1*), chymotrypsin C (*CTRC*), cystic fibrosis transmembrane conductance regulator (*CFTR*), carboxypeptidase A1 (*CPA1*) and carboxyl ester lipase (*CEL*) [Mayerle et al. 2019]. Based on functional studies, alterations of these protein coding genes have been classified into 3 different pathological pathways leading to the development and progression

of the disease [Hegyi and Sahin-Tóth 2018, Sahin-Tóth 2017, Mayerle et al. 2019]. In the trypsin-dependent pathway, early intrapancreatic trypsinogen activation to trypsin plays the key pathogenic role; in the misfolding-dependent pathway, misfolding of digestive enzymes leads to endoplasmic reticulum stress; in the ductal pathway, the participating CFTR, claudin-2 (CLDN2), transient receptor potential vanilloid subfamily member 6 (TRPV6) and probable calcium-sensing receptor (CASR) variants contribute to impaired ductal secretion [Hegyi and Sahin-Tóth 2018, Sahin-Tóth 2017, Mayerle et al. 2019]. Despite the significant progress in the identification of CP genetic risk factors, there are many cases, where the researchers did not find genetic variants in the known risk genes. Thus, it is inevitable to think, that other, previously unreported gene alterations may have an impact on the pathomechanism of CP.

1.2. The role of the calcium-sensing receptor (CASR) in chronic pancreatitis

The calcium-sensing receptor is a family C G protein-coupled receptor. It is highly expressed in the parathyroid glands and detects reductions in extracellular Ca^{2+} concentration, which leads to the release of parathyroid hormone (PTH). The CASR regulates bone and mineral metabolism, urinary Ca^{2+} excretion by influencing parathyroid hormone secretion. PTH also promotes the generation of the physiologically active form of vitamine D. *CASR* mutations cause hypercalcaemic disorders like familial hypocalciuric hypercalcaemia (FHH), neonatal severe hyperparathyroidism (NSHPT), primary hyperparathyroidism (PHPT), or can cause hypocalcaemic disorders such as autosomal dominant hypocalcaemia (ADH) [Hannan et al. 2018].

An increase in serum calcium levels activates the receptor, triggering intracellular signaling to inhibit parathyroid hormone (PTH) secretion and calcium resorption. A decrease in serum calcium releases these inhibitions resulting in higher PTH secretion and increased calcium resorption in the kidneys. Similar to PTH regulation in the parathyroid glands, the expression of CASR in the

mammary epithelia negatively regulates the secretion of PTH-related peptide, which can mobilize calcium from bones for milk production. Heterozygous inactivating mutations in *CASR* cause FHH, an autosomal dominant disorder characterized by elevated serum calcium and decreased urinary calcium excretion [Hannan et al. 2018, Lee and Shoback 2018]. *CASR* mutations may also cause neonatal severe hyperparathyroidism, typically as a recessive disorder. In contrast, *CASR* mutations are rarely observed in adult-onset hyperparathyroidism and reduced expression of CASR in parathyroid adenomas is the likely explanation for the increased PTH secretion and hypercalcemia in this disease. Finally, activating mutations in *CASR* are associated with autosomal dominant hypocalcemia [Hannan et al. 2018].

The CASR is also expressed in human pancreatic ductal cells, where it may monitor and regulate the Ca^{2+} concentration in pancreatic juice. In case of high Ca^{2+} concentration, CASR takes action by triggering ductal electrolyte and fluid secretion, thereby it may help to prevent stone formation in pancreatic ducts. Hence, mutations of *CASR* are possible risk factors of pancreatitis [Rácz et al. 2018].

1.2.1. *CASR* genetic association studies in chronic pancreatitis

In recent years, the role of *CASR* mutations in the development of CP has emerged and has been investigated by several research groups. *Muddana et al.* analyzed *CASR* mutations in the US population and found that the p.R990G polymorphism is associated with CP. Moreover, the risk of CP with *CASR* p.R990G was increased in subjects with moderate to heavy alcohol consumption [Muddana et al. 2008]. In contrast to the American study, in a French cohort, *Masson et al.* did not identify *CASR* p.R990G as a pancreatitis risk factor, but they found that another polymorphism, the p.A986S is associated with the disease, but only in homozygous state [Masson et al. 2015]. *Sofia et al*. examined an Italian population by Next Generation Sequencing and could only identify two rare variants [Sofia et al. 2016]. Taken together, only three case-control studies

investigated *CASR* mutations in CP, and their results are conflicting, thus the role of *CASR* variants in the pathogenesis of CP remained controversial.

1.3. The role of chymotrypsin C (*CTRC*) in chronic pancreatitis

CP often develops in the background of genetic predisposition [Kleeff et al. 2017]. A number of susceptibility genes have been identified and many of these have been shown to influence the activation of the digestive protease precursor trypsinogen to its active form trypsin [Hegyi and Sahin-Tóth 2017]. Pathological, intrapancreatic activation of trypsinogen can occur through autoactivation, a reaction in which trypsin activates trypsinogen. Defense mechanisms that protect the pancreas against trypsinogen autoactivation and trypsin activity include the serine protease inhibitor Kazal type 1 (SPINK1) and chymotrypsin C (CTRC), which can readily degrade trypsinogen and thereby suppress its activation [Hegyi and Sahin-Tóth 2017, Witt et al. 2000, Rosendahl et al. 2008]. Gain-of-function mutations in the serine protease 1 (*PRSS1*) gene encoding human cationic trypsinogen and loss-of-function mutations in the *SPINK1* and *CTRC* genes stimulate autoactivation of trypsinogen and increase the risk for CP [Hegyi and Sahin-Tóth 2017]. The most common *PRSS1* mutations exert their stimulatory effect on autoactivation by blocking CTRC-dependent trypsinogen degradation [Hegyi and Sahin-Tóth 2017, Szabó and Sahin-Tóth 2012]. Genetic changes can be also protective, as exemplified by the p.G191R variant in the *PRSS2* gene encoding human anionic trypsinogen [Witt et al. 2006]. This variant causes autodegradation of anionic trypsinogen and thereby decreases the risk for CP. Similarly, a common inversion at the chymotrypsin B1-B2 (*CTRB1-CTRB2*) locus increases the expression of CTRB2, which leads to more effective degradation of anionic trypsinogen and reduced CP risk [Rosendahl et al. 2018].

CTRC as a pancreatitis risk gene was identified in 2008 [Szabó and Sahin-Tóth 2012, Masson 2008] and studies to date have described a large number of missense mutations and a microdeletion found in CP cases [Derikx et al. 2009, Paliwal et al. 2012, Masamune et al. 2012, Schubert et al. 2014, LaRusch et al. 2015, Koziel et al. 2015, Sofia et al. 2016, da Costa et al. 2016, Grabarczyk et al. 2017, Phillips et al. 2018, Zou et al. 2018, Cichoż-Lach et al. 2019, see also www.pancreasgenetics.org]. The majority of these variants were detected in a few cases only and only four variants were found to associate with CP in a statistically significant manner: variants c.738_761del24 (p.K247_R254del) and c.760C>T (p.R254W) were found primarily in European cohorts with approximate carrier frequencies of 1-2%, while variants c.217G>A (p.A73T) and c.703G>A (p.V235I) were detected in Indian cohorts with carrier frequencies of 1-5%. As judged by the odds ratio (OR) values, the effect sizes reported for these *CTRC* variants were variable, mostly in the range of 3-10-fold, however, a lack of effect and an OR of 19 were also described. Functional studies demonstrated that these four variants caused loss of *CTRC* function by various mechanisms that included reduced secretion from cells, decreased catalytic activity and increased sensitivity to degradation by trypsin [Rosendahl et al. 2008, Beer et al. 2012, Szmola and Sahin-Tóth 2010]. Furthermore, variant p.A73T was shown to induce endoplasmic reticulum (ER) stress [Beer et al. 2012, Szmola and Sahin-Tóth 2009]. Similar functional defects were observed with many of the rare CTRC variants [Rosendahl et al. 2008, Beer et al. 2012, Szmola and Sahin-Tóth 2009, Szabó et al. 2015].

In addition to the low-frequency missense and microdeletion variants, a common haplotype (circa 30% carrier frequency in CP cases) consisting of the c.180C>T (p.G60=) variant in exon 3 and the c.493+52G>A variant in intron 5, was found reproducibly to increase CP risk by about 2-fold in the heterozygous state and perhaps as much as 10-fold in homozygous carriers [Rosendahl et al. 2018, Masson et al. 2008, Derikx et al. 2009, Paliwal et al. 2012, Masamune et al. 2012, LaRusch et al. 2015, Grabarczyk et al. 2017]. In our recent meta-analysis, loss-of-function

heterozygous missense *CTRC* variants increased CP risk around 5-fold, on average [Berke et al. 2023]. The functional impact and the disease-relevant variant within the haplotype remain unclear; however, reduced CTRC expression possibly due to altered pre-mRNA splicing was hypothesized [Hegyi and Sahin-Tóth 2017, Berke et al. 2023]. Unlike the low-frequency missense and microdeletion CTRC variants, the p.G60= haplotype variants do not alter the amino-acid sequence of CTRC and cannot cause enzyme activity changes by any of the mechanisms described for the other variants.

2. Aims

Aim 1. *Investigation of the role of CASR polymorphisms in chronic pancreatitis.*

As the role of *CASR* polymorphisms in the development of chronic pancreatitis was controversial, we set out to clarify the association between common *CASR* variants and CP by analyzing an ethnically homogenous Hungarian CP cohort using direct sequencing and TaqMan genotyping assays.

Aim 2. *Investigation of the role of missense CTRC variants in chronic pancreatitis.*

To evaluate the risk effect of CTRC variants that (i) alter the amino-acid sequence of CTRC, (ii) are relatively frequent (>1% carrier frequency in CP cases), (iii) showed reproducible association with CP at least in 2 independent cohorts, and (iv) were demonstrated to cause a loss of CTRC function experimentally, we aimed to perform a systematic, global meta-analysis.

3. Materials and methods

3.1. Investigation of the role of *CASR* polymorphisms in chronic pancreatitis

3.1.1. Nomenclature

Nucleotide numbering reflects coding DNA numbering with the first nucleotide of the ATG translation initiation codon designated as +1 in the *CASR* reference sequence (genomic reference: NC_000003.12, Homo sapiens chromosome 3, GRCh38.p13 primary assembly; mRNA reference: NM_000388.4). Amino acids are numbered starting with the initiator methionine of the primary translation product of *CASR*.

3.1.2. Study subjects

De-identified genomic DNA samples were obtained from the Hungarian National Pancreas Registry (ethical approval: TUKEB 22254-1/2012/EKU, biobanking approval: IF702-19/2012). Subjects were recruited from 11 Hungarian centers between 2012 and 2018, and all gave informed consent according to the ethical guidelines of the Declaration of Helsinki. The discovery cohort analyzed by direct DNA sequencing consisted of 261 patients with CP (106 nonalcoholic and 155 alcoholic cases) and 224 controls. The expanded study cohort analyzed by TaqMan SNP genotyping contained additional 76 CP patients (36 nonalcoholic and 40 alcoholic cases) and 616 controls. In total, 337 unrelated patients with CP (mean age at recruitment 56.4±12 years), including 142 with nonalcoholic CP and 195 with alcoholic CP, and 840 control subjects (mean age at recruitment 39.3±14.6 years) with no pancreatic disease were enrolled. 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.

3.1.3. DNA sequencing

Exon 7 with the flanking intron 6 and 3' UTR regions was amplified using 3 primer pairs. Polymerase chain reaction (PCR) was performed using 0.5 U HotStarTaq DNA Polymerase (Qiagen), 0.2 mM dNTP, 0.5 μM primers, 10x PCR buffer (Qiagen) and 10 to 50 ng of genomic DNA template in a volume of 25 μL. The reaction started with a 15 min initial heat activation at 95°C followed by 35 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 61.1°C (amplicon 1 and 2) or 53.2 °C (amplicon 3), and 40 sec (amplicon 1 and 2) or 50 sec (amplicon 3) extension at 72 °C; and finished by a final extension for 5 min at 72 °C. PCR products were verified by 2% 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 (amplicon 1 and 3) and reverse (amplicon 2) PCR primers as sequencing primers.

3.1.4. TaqMan SNP genotyping

TaqMan SNP genotyping assays were used to investigate the p.A986S and p.R990G CASR variants in the expanded study cohort (Assay ID: CASR rs1801725_7504853_20 and CASR rs1042636_7504854_20) using a StepOne Real-Time PCR system (Applied Biosystems by Life Technologies). The 20 μL reaction consisted of TaqPath ProAmp Master Mix (2x), TaqMan SNP genotyping assay (20x) and 10-20 ng genomic DNA template. The cycling conditions were as follows: 30 sec holding stage at 60 °C followed by a 10 min holding stage at 95 °C; 50 cycles of 15 sec denaturation at 92 °C and 1 min annealing at 60 °C; and a final 30 sec holding stage at 60 °C. Allelic discrimination plots were evaluated using the StepOne software. To confirm the results, all homozygous samples and 4-6 heterozygous and wild-type samples from each plate were sequenced.

3.1.5. Statistical analysis

The significance of the differences in allele frequencies and genotype distribution between

cases and controls was assessed by Fisher's exact test using the GraphPad Prism9 software. $P < 0.05$ was considered statistically significant.

3.2. Investigation of the role of missense *CTRC* variants in chronic pancreatitis

3.2.1. Protocol registration

The present work is reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement [Moher et al. 2009]. The protocol of the meta-analysis was registered in advance in the PROSPERO database under the registration number CRD42018111537.

3.2.2. Search strategy

We performed a systematic search on April 5, 2022, in three databases (MEDLINE via PubMed Central, Embase, and Cochrane Central Register of Controlled Trials) using the following search keys: pancreatitis AND (CTRC OR "Chymotrypsin C" OR Caldecrin OR "Elastase 4" OR ELA4 OR CLCR) AND (polymorphism OR polymorphisms OR variant OR variants OR mutation OR mutations) OR "p.A73T" OR "p.V235I" OR "p.K247_R254del" OR "p.R254W". No filters were applied. As an additional data source, we used the www.pancreasgenetics.org database of genetic risk factors.

3.2.3. Study selection and data extraction

Genetic association case-control studies investigating the following low-frequency CTRC variants were included: p.A73T, p.V235I, p.K247_254del, and p.R254W. Articles identified by the initial search were imported into a reference management program (EndNoteX7.4; Clarivate Analytics, Philadelphia, PA). After removing overlaps between databases and duplicate references, the remaining records were screened by title, abstract and full text by two authors independently. Disagreements were resolved by the corresponding author. Eligible original studies were subjected to data collection onto a pre-defined Excel sheet. The following

data were extracted: first author, publication year, basic demographics (ethnicity, age), type of pancreatitis, number of cases and controls, carrier frequencies of the *CTRC* variants analyzed in this study, and type of the genetic screening method. In cases of suspected overlap between study populations, studies with the highest number of participants were included in the final analysis. Zero event studies were excluded from the statistical analysis.

3.2.4. Quality assessment

A modified version of the Newcastle-Ottawa Scale (NOS) was used for the quality assessment of the included case-control studies [Lo et al, 2014].

3.2.5. Statistical analysis

The effect of the different *CTRC* variants was assessed by calculating pooled odds ratios (OR) with 95% confidence intervals (CI). Random-effects model with Der-Simonian Laird estimation was applied. Heterogeneity was examined with the I^2-test ($p \geq 0.1$). Sensitivity analyses (leave-one-out method) were also performed. Publication bias was ruled out by visual inspection of funnel plots and by Egger's test. Statistical analyses were performed with Stata 15 (Stata Corp).

4. Results

4.1. Investigation of the role of *CASR* polymorphisms in chronic pancreatitis

4.1.1. DNA sequence analysis of exon 7 of human *CASR* in a discovery cohort

To investigate whether common *CASR* variants alter risk for CP, we initially sequenced exon 7 and flanking intron 6 and 3' UTR regions of *CASR* in 261 patients with CP (106 nonalcoholic CP and 155 alcoholic CP) and 224 controls from the Hungarian National Pancreas Registry. We identified 2 common missense variants (allele frequency >5%), c.2956G>T (p.A986S) and c.2968A>G (p.R990G) and 3 low-frequency variants (allele frequency 1-5%), which included

a missense variant c.3031C>G (p.Q1011E), a synonymous variant c.2610G>A (p.E870=) and a 3’ UTR variant c.*60T>A, which was in linkage disequilibrium with p.Q1011E. In addition, we found 8 rare variants (allele frequency <1%), 7 of which were detected in one subject each. The rare variants included 3 missense variants; the c.1895G>A (p.G632D) variant was detected in a CP patient, while two previously reported missense variants, c.1775A>G (p.N592S) and c.2405A>G (p.N802S) were present in controls. The novel p.G632D variant was found in a male, nonalcoholic patient who developed CP at the age of 37. He had no history of smoking, and carried no pathogenic SPINK1 variants. His total serum calcium level was in the normal range (2.39 mmol/L). The serum calcium levels were not available for the carriers of the p.N592S and p.N802S variants.

In silico analysis using the “PredictSNP Consensus classifier for prediction of disease-related mutations” tool classified the p.G632D and p.N802S variants as potentially disease causing and the p.N592S variant as benign.

When allele frequency was considered, distribution of the variants between CP patients and controls showed no significant differences. Genotype distribution of common missense variants p.A986S and p.R990G and the low-frequency variant p.Q1011E was also assessed using dominant and recessive models of inheritance, but no significant differences between CP patients and controls were found (Exon 7 / c.2956G>T / GG: OR = -, 95% CI = -, p-value = -; Exon 7 / c.2956G>T / GT: OR = 1.03, 95% CI = 0.79-1.34, p-value = 0.89; Exon 7 / c.2956G>T / TT: OR = 1.19, 95% CI = 0.52-1.92, p-value = 0.68; Exon 7 / c.2956G>T / T: OR = 1.03, 95% CI = 0.82-1.30, p-value = 0.81; Exon 7 / c.2968A>G / AA: OR = -, 95% CI = -, p-value = -; Exon 7 / c.2968A>G / AG: OR = 1.13, 95% CI = 0.78-1.62, p-value = 0.57; Exon 7 / c.2968A>G / GG: OR = -, 95% CI = -, p-value = -; Exon 7 / c.2968A>G / G: OR = 1.05, 95% CI = 0.75-1.49, p-value = 0.79). In this analysis, a non-significant enrichment of the homozygous p.A986S variant was observed in patients (3.4%) versus controls (0.9%).

However, we noticed a deviation from Hardy-Weinberg equilibrium (HWE) in the control population, probably due to the limited sample size.

4.1.2. TaqMan SNP genotyping for the p.A986S and p.R990G variants in an expanded cohort

Since the homozygous p.A986S variant and the p.R990G variant were previously reported to associate with CP [Muddana et al. 2008, Masson et al. 2015], we expanded our study and investigated these two variants using TaqMan SNP genotyping assays in additional 76 CP patients (36 nonalcoholic and 40 alcoholic cases) and 616 controls. Taken the direct sequencing and genotyping results together, allele and genotype frequency of the common p.A986S and p.R990G variants were determined in 337 CP patients (142 nonalcoholic CP and 195 alcoholic CP) and 840 controls. Neither of these variants associated with CP (Exon 7 / c.2956G>T / GG: OR = -, 95% CI = -, p-value = -; Exon 7 / c.2956G>T / GT: OR = 1.11, 95% CI = 0.76-1.6, p-value = 0.63; Exon 7 / c.2956G>T / TT: OR = 1.58, 95% CI = 0.63-4.06, p-value = 0.37; Exon 7 / c.2956G>T / T: OR = 1.13, 95% CI = 0.82-1.55, p-value = 0.46; Exon 7 / c.2968A>G / AA: OR = -, 95% CI = -, p-value = -; Exon 7 / c.2968A>G / AG: OR = 0.92, 95% CI = 0.53-1.58, p-value = 0.89; Exon 7 / c.2968A>G / GG: OR = -, 95% CI = -, p-value = -; Exon 7 / c.2968A>G / G: OR = 0.88, 95% CI = 0.52-1.47, p-value = 0.70). Notably, in the combined results, there was no appreciable enrichment of the homozygous p.A986S variant in patients versus controls (2.7% versus 2.3%, respectively; OR=1.19, 95% CI 0.52-1.92, p=0.68). Subgroup analysis of nonalcoholic CP and alcoholic CP revealed no disease association either (Exon 7 / c.2956G>T / GG: OR = -, 95% CI = -, p-value = -; Exon 7 / c.2956G>T / GT: OR = 0.97, 95% CI = 0.70-1.35, p-value = 0.87; Exon 7 / c.2956G>T / TT: OR = 0.91, 95% CI = 0.33-2.57, p-value > 0.99; Exon 7 / c.2956G>T / T: OR = 0.97, 95% CI = 0.72-1.29, p-value = 0.88; Exon 7 / c.2968A>G / AA: OR = -, 95% CI = -, p-value = -; Exon 7 / c.2968A>G / AG: OR = 1.28, 95% CI = 0.83-1.97, p-value = 0.29; Exon 7 / c.2968A>G / GG: OR = -, 95% CI = -, p-value = -;

Exon 7 / c.2968A>G / G: OR = 1.19, 95% CI = 0.77-1.78, p-value = 0.44).

4.2. Investigation of the role of missense *CTRC* variants in chronic pancreatitis

4.2.1. Study design

We found four variants that met our inclusion criteria, three missense mutations and a microdeletion: p.A73T, p.V235I, p.R254W, and p.K247_R254del. We performed a comprehensive database search and retrieved all published case-control studies that included any of these variants. The protocol employed for study selection resulted in the identification of 14 articles [Rosendahl et al. 2008, Masson et al. 2008, Derikx et al. 2009, Paliwal et al. 2012, Masamune et al. 2012, Schubert et al. 2014, LaRusch et al. 2015, Koziel et al. 2015, Sofia et al. 2016, da Costa et al. 2016] was suitable for inclusion in this meta-analysis.

All four *CTRC* variants were detected worldwide, however, with uneven geographic/ethnic distribution. Thus, variants p.A73T and p.V235I were most frequently reported in South-Asian cohorts whereas variants p.K247_R254del and p.R254W were typically found in subjects of European origin. Considering the relatively small number of studies available, we chose to perform a global meta-analysis of the selected *CTRC* variants without regional or ethnic subgrouping.

In our analysis, we included both alcoholic CP and non-alcoholic CP cohorts, such as idiopathic, hereditary, familial and tropical CP. If reported separately, RAP cases were pooled with CP cases, while acute pancreatitis cases were excluded. Due to the somewhat inconsistent classification and/or reporting, subgroup analysis of the various CP cohorts was feasible only for alcoholic CP. Different CP cohorts (e.g. alcoholic and non-alcoholic) from a given publication were combined if these were compared with the same control group. Alcoholic and tropical CP cohorts from certain studies were treated separately if distinct control groups were used for comparison.

4.2.2. Association analysis

All 4 *CTRC* variants analyzed were found significantly more frequently in patients with CP than in controls. The effect sizes, as judged by the OR values, were similar for the 4 variants and ranged from 2.6 to 6.5. Comparable OR values were calculated for variants p.V235I (OR 4.5, 95% CI 2.2-9.1), and p.K247_R254del (OR 5.4, 95% CI 2.6-11.0) while variant p.A73T (OR 6.5, 95% CI 2.4-17.8) was associated with slightly higher and variant p.R254W (OR 2.6, 95% CI 1.6-4.2) with slightly lower risk. Global carrier frequencies for variants p.A73T, p.V235I, p.K247_R254del, and p.R254W in CP were 2.4%, 1.2%, 1.1% and 1.0%, whereas the highest reported carrier frequencies in CP were 5.6%, 4.9%, 5.3% and 4.6%, respectively.

The vast majority of carriers had heterozygous *CTRC* variants, whereas homozygous and compound heterozygous cases were rare. When we considered all homozygous and compound heterozygous *CTRC* variant carriers together (n=13) versus controls, the meta-analysis yielded an OR value of 10.8 (95% CI 2.4-49.6), indicating a higher risk effect than seen with any of the heterozygous *CTRC* variants. However, the small event number and the arbitrary pooling of variants limits the usefulness of this estimate.

To address the role of *CTRC* variants in alcoholic CP, we performed a meta-analysis for variants p.K247_R254del and p.R254W, which were detected in association with alcoholic CP in a handful of studies. No data were available for variants p.A73T and p.V235I. For this calculation, we compared alcoholic CP cases versus healthy controls. We confirmed disease association of variants p.K247_R254del (OR 5.4, 95% CI 1.4-21.5) and p.R254W (OR 2.4, 95% CI 1.2-4.6) with effect sizes that were similar to those seen in the overall CP group.

4.2.3. Linkage disequilibrium between variant p.K247_R254del and the common c.180C>T (p.G60=) haplotype

During our review of unpublished *CTRC* sequencing data from the Hungarian Pancreatic Study Group, we noted that three patients carrying a heterozygous microdeletion variant were also

positive for the common p.G60= variant; including 2 homozygous cases. To explore whether these two variants are in linkage disequilibrium, we re-analyzed genetic data of 12 subjects of German origin from Rosendahl, et al. (2008) and 11 individuals of Polish origin from Grabarczyk, et al. (2017). Two unpublished cases of Turkish and Serbian origin sequenced in Germany were also included. Taken together, from the 28 subjects evaluated, 27 carried both variants, and in 9 cases the p.G60= variant was homozygous.

Quality assessment and publication bias are shown in the full doctoral dissertation.

5. Discussion

5.1. Investigation of the role of *CASR* polymorphisms in chronic pancreatitis

The calcium-sensing receptor is a dimeric, G-protein coupled transmembrane receptor that is highly expressed in the parathyroid glands and the kidneys where it regulates systemic calcium homoeostasis [Hannan et al. 2018, Leach et al. 2020].

In addition to its systemic, “calcitropic” role, CASR is expressed in several tissues where it contributes to local regulation of various cellular processes. In the rat pancreas, CASR was found in acinar, ductal and islet cells, and activation of the receptor was shown to induce ductal bicarbonate secretion [Bruce et al. 1999]. CASR expression was also documented in all cell types of the human pancreas, including intrapancreatic nerves and blood vessels [Rácz et al. 2002]. Furthermore, the human pancreatic adenocarcinoma cell line Capan-1 was shown to express functional CASR [Rácz et al. 2002]. Based on these observations, it was suggested that CASR might regulate the calcium concentration of the pancreatic juice by increasing ductal fluid secretion, possibly through activating CFTR [Sahin-Tóth 2020]. We note, however, that evidence for the exact role(s) of CASR in the pancreas is limited and animal models with pancreas-specific *CASR* deletion or mutation have been lacking. The strongest indication that

CASR mutations may play a role in pancreatitis is the relatively frequent occurrence of pancreatitis in FHH [Pearce et al. 1996]. In a small number of FHH patients, trans-heterozygosity for *SPINK1* and *CASR* mutations was documented [Felderbauer et al. 2003, Felderbauer et al. 2006, Murugaian et al. 2008, Rajesh et al. 2009, Baudryet al. 2010]. However, it seems likely that FHH-associated pancreatitis is due to hypercalcemia rather than the local effects of inactivating CASR mutations in the pancreas.
Hypercalcemia is a well-known risk factor for pancreatitis. Hyperparathyroidism and malignancy-associated hypercalcemia are two commonly reported conditions in which pancreatitis frequently occurs in association with elevated serum calcium levels [Bai et al. 2012, Misgar et al. 2020, Imam et al. 2021]. Importantly, experimental studies in rats also demonstrated that hypercalcemia could induce pancreatitis [Frick et al. 1994, Mithöfer et al. 1995, Frick et al. 1995]. *SPINK1* variants represent an independent risk factor for CP, which often interact with other genetic and environmental risk factors to promote disease onset and progression. Thus, it is not surprising that some FHH patients with pancreatitis might carry *SPINK1* mutations as well.

Considering the role of commonly occurring *CASR* variants in CP risk, human genetic association studies yielded conflicting results. Therefore, in the present study, we examined the contribution of common exon 7 *CASR* variants to CP risk in a Hungarian cohort of nonalcoholic and

alcoholic CP cases.

A limitation of our analysis was the relatively small size of the patient cohorts. We identified 5 *CASR* variants with a population frequency above 1%, none of which showed an association with CP. No enrichment was observed when allelic or genotype distributions were considered or in a subgroup analysis of nonalcoholic and alcoholic patients. *SPINK1* mutation status was not analyzed, but we note that *SPINK1* variants p.N34S and c.194+2T>C are rare in this

Hungarian CP cohort [Hegyi et al. 2016].

We conclude that the previously reported associations between common *CASR* variants and CP were likely spurious due to chance and/or multiple testing. This conclusion is in agreement with the reported functional properties of these variants. Thus, in transfected HEK 293 cells variants p.A986S and p.Q1011E behaved exactly as wild-type CASR while variant p.R990G showed slightly enhanced function [Vezzoli et al. 2007]. The gain-of-function phenotype of variant p.R990G might explain its association with primary hypercalciuria [Vezzoli et al. 2007] but it seems difficult to reconcile with pancreatitis. Finally, we found a novel missense variant in a CP patient (p.G632D) and two previously reported rare missense variants in controls (p.N592S, p.N802S). Variants p.G632D and p.N802S were predicted to be functionally deleterious. Indeed, variant p.N802S was described as an inactivating mutation associated with FHH [Lia-Baldini et al. 2013]. No enrichment of rare missense variants was observed in the patient cohort. However, our analysis was limited to exon 7 and a direct comparison with the relevant results of the Férec group cannot be made [Masson et al. 2015].

At the same time we published our results, a German group presented their data on the three *CASR* single nucleotide polymorphisms (p.A986S, p.R990G and p.Q1011E) in German and French patients with CP [Ewers et al. 2021]. Similarly to our findings they did not reveal any significant association between the three common *CASR* polymorphisms and CP. Functional assays showed similar or slightly higher activity by the three variants p.A986S, p.R990G and p.Q1011E relative to WT which is consistent with the lack of correlation of these variants with pancreatitis.

Intracellular calcium signaling plays a critical role in pancreas physiology and aberrant calcium signaling is a hallmark of pancreatitis. Changes in extracellular calcium may have profound effects on calcium signaling and may directly promote activation of digestive proteases. Besides *CASR*, recent genetic studies focused on other calcium channels and receptors as well

[Kaune et al. 2020, Masamune et al. 2020, Zou et al. 2020, Burgos et al. 2021]. While the interesting preliminary findings with *GPRC6A* and *STIM1* await further replication [Kaune et al. 2020, Burgos et al. 2021], the *TRPV6* gene encoding a constitutive calcium channel was convincingly identified as a high-impact CP risk gene [Sahin-Tóth 2020, Masamune et al. 2020, Zou et al. 2020]. Loss-of-function mutations in *TRPV6* are strongly associated with CP with a large effect size. TRPV6 is expressed in both acinar and ductal cells and the disease-causing mechanism of *TRPV6* mutations has remained unclear so far. Because higher expression levels were reported in the ductal epithelium, the *TRPV6* gene was tentatively assigned to the ductal pathological pathway of CP risk [Sahin-Tóth 2020]. One can speculate that TRPV6 might regulate pancreatic juice calcium concentrations in concert with CASR. A recent example for functional interaction between these two molecules was described in the intestinal epithelium, where activation of CASR in the basolateral membrane attenuates TRPV6-dependent intestinal calcium absorption [Lee et al. 2019]. However, in light of our present data, TRPV6 more likely functions in a manner that is independent of CASR in the pancreas.

In summary, our results demonstrate that common *CASR* variants do not modify the risk for CP and should not be considered as genetic risk factors in the clinical setting. However, we cannot rule out that rare mutations that affect CASR function may contribute to the development of the disease.

5.2. Investigation of the role of missense *CTRC* variants in chronic pancreatitis

The aim of the present study was to obtain a formal estimate of the risk of CP in carriers of loss-of-function *CTRC* variants. To this end, we performed a meta-analysis of published studies that assessed the distribution of four low-frequency variants (>1%) between patients and controls. We chose these four variants, including three missense mutations and a microdeletion,

because their genetic association with CP has been reproducibly documented and experimental studies confirmed the variants caused loss of CTRC function. Thus, the effect size of these variants on CP risk should be generally applicable to other rare or private *CTRC* missense mutations as well. The commonly occurring *CTRC* risk variant c.180C>T (p.G60=) was excluded from this study, because it does not alter the CTRC amino-acid sequence, and its functional impact on CTRC has not been clarified yet.

Considering the relatively small number of publications suitable for meta-analysis, the lack of standardized cohort definitions with respect to etiology, and the variable reporting of homozygous and compound heterozygous carriers, we chose to perform a "global" analysis in which all CP cohorts were included and carrier frequencies (rather than alleles or genotypes) were considered. We note, however, that this global CP cohort mostly represents heterozygous *CTRC* variant carriers with non-alcoholic CP. Using this approach, the meta-analysis revealed that

CTRC variants increased CP risk by about 3-7 fold, as estimated by OR values. Homozygous or compound heterozygous carriers were rare but seemed to increase risk more significantly, by about 11-fold, which we consider a lower-end estimate. Subgroup analysis of alcoholic CP cases indicated a similar risk increase as seen in the global CP cohort. Recently, *Chen et al*. (2021) investigated gene-alcohol interactions in CP and reported higher risk in case of the c.760C>T (p.R254W) variant in alcoholic (OR=2.87) versus non-alcoholic CP (OR=1.98). We believe this may be a spurious finding due to the smaller case numbers analyzed than in our study. Importantly, in the same article, the cumulative analysis of rare pathogenic *CTRC* variants in exons 2,3 and 7 showed no difference between the alcoholic and non-alcoholic CP groups (OR=4.25 and 4.05, respectively), supporting the notion that *CTRC* variants contribute comparable risk effects to these two CP etiologies [Chen et al. 2021]. In contrast to *CTRC*, variants in *SPINK1* have a smaller impact in alcoholic than in non-alcoholic CP while gain-of-

function *PRSS1* variants are almost never found in alcoholic disease [Hegyi and Sahin-Tóth 2017, Chen et al. 2021].

The functional consequences of variants p.A73T, p.V235I, p.K247_R254del, and p.R245W were previously characterized in cell culture experiments with transfected HEK 293T cells and adenovirus-transduced AR42J cells [Rosendahl et al. 2008, Beer et al. 2012, Szabó et al. 2015]. In addition, enzymatic activity of CTRC variants was tested using purified CTRC protein. Based on these assays, variants p.A73T and p.K247_R254del were classified as high-risk variants and variants p.V235I and p.R245W as moderate-to-low risk variants [Beer et al. 2012]. Variant p.K247_R254del was found to exhibit a complete loss of function as it had no protease activity and trypsin rapidly degraded it. Similarly, nearly complete (80-90%) loss of function was observed with variant p.A73T, which was poorly secreted from cells. Furthermore, variant p.A73T induced endoplasmic reticulum (ER) stress indicating that mutation-induced misfolding and intracellular retention might explain its defective secretion. It remains unclear whether ER stress would contribute to CP risk in carriers of variant p.A73T. In contrast to variants p.A73T and p.K247_R254del, we found that the functional defect in variants p.V235I and p.R245W was less conspicuous. Variant p.V235I was secreted almost as well as wild-type CTRC from HEK 293T cells and the purified protein had circa 70% enzyme activity on a small peptide substrate. However, when this variant was tested in trypsinogen degradation experiments, it exhibited only about 50% activity relative to wild-type CTRC. Variant p.R254W was secreted to reduced levels (50% of wild type) from HEK 293T cells, while secretion from AR42J cells was almost normal (80%). The purified p.R254W protein was fully active on a small peptide substrate but its trypsinogen-degrading ability has not been tested so far. Variant p.R254W was also prone to degradation by high concentrations of trypsin. Taken together, available evidence indicates that variant p.V235I causes an about 50% loss of function, while the extent of the functional impairment in variant p.R254W remains hard to

define but appears relatively small.

When the OR values are compared with the functional properties of the mutants, the difference between the high-risk *CTRC* variants p.A73T (OR 6.5) and p.K247_R254del (OR 5.4) versus the low-risk variant p.R254W (OR 2.6) is apparent and supports the validity of the results of our meta-analysis. Curiously, variant p.V235I (OR 4.5) is an outlier in this classification, as its risk effect would suggest a stronger functional defect than experimentally documented. We note, however, that the functional analysis published to date is limited in scope and variant p.V235I may cause loss of CTRC function by other mechanisms not tested yet, e.g. by affecting mRNA expression or splicing. Despite the imperfect correlation between the risk effects and the functional loss of *CTRC* variants, our current study establishes a solid baseline value for the effect size of heterozygous *CTRC* null variants, which is around 5-6-fold increased risk of CP. As noted above, homozygous and compound heterozygous *CTRC* variants are expected to impart substantially higher risk, however, available data are limited.

An unexpected "bonus" observation from this study is the linkage disequilibrium between the p.K247_R254del microdeletion variant and the common p.G60= haplotype. Even though several laboratories reported both variants in their cohorts; their association has never been described before. Genetic counselors of patients with *CTRC* variants need to take this new information into consideration when determining the overall risk of CP. Although the p.G60= haplotype increases CP risk about 2-fold, this effect will be negated by the presence of the microdeletion on the same allele and the overall risk will be solely determined by the heterozygous microdeletion variant. Furthermore, in case of a heterozygous microdeletion occurring together with p.G60= homozygosity, one should consider only one p.G60= allele when estimating compounded disease risk.

A limitation of the present study is the global nature of the analysis, which may mask higher or lower effect sizes associated with certain etiology, geography or demographics. In this

regard, it is noteworthy that variants p.A73T and p.V235I are prevalent in India, while variants p.K247_R254del and p.R254W were predominantly reported from Europe. Restricting the analysis to Indian versus European cohorts might yield higher OR values for given variants than the global approach. Similarly, the impact of *CTRC* variants may be higher in pediatric CP versus adult-onset CP, as suggested by a Polish study [Zou et al. 2018]. Despite these unresolved questions, our meta-analysis offers strong support for the notion that *CTRC* variants are relatively strong risk factors for CP and argues for routine genetic screening of patients in the clinical setting.

6. Conclusions

We aimed to explore the complex roles of the calcium-sensing receptor (*CASR*) and chymotrypsin C (*CTRC*) in pancreatitis, examining their genetic variations and potential links to chronic pancreatitis. Our findings offer valuable insights into the genetic landscape of CP, emphasizing the intricate relationship between genetic factors and pancreatic disease mechanisms.

The investigation into *CASR* variants, particularly those in exon 7, did not reveal any significant association with CP in our Hungarian cohort. This result challenges previous reports suggesting a link between common *CASR* variants and CP risk. Our analysis, supported by functional studies, indicates that common *CASR* variants are unlikely to be major contributors to CP susceptibility, emphasizing the importance of scrutinizing reported associations to avoid misleading conclusions.

Our meta-analysis on loss-of-function *CTRC* variants provides a comprehensive evaluation of the risk in patients carrying these variants. The observed 3-7-fold increase in CP risk for heterozygous carriers, and an even more pronounced 11-fold risk for homozygous or compound heterozygous carriers, establishes *CTRC* as a significant risk factor for CP. This

analysis refutes the notion that *CTRC* variants have a differential impact on alcoholic and non-alcoholic CP, suggesting a consistent risk associated with these variants across different etiologies.

Our study also highlights the functional consequences of specific CTRC variants, with variants p.A73T and p.K247_R254del classified as high-risk, while p.V235I and p.R245W are considered moderate-to-low risk. The correlation between the observed odds ratios and the functional characteristics of these variants supports the validity of our meta-analysis, providing clinicians with valuable insights into the potential severity of specific *CTRC* mutations.

Furthermore, the unexpected linkage disequilibrium between the p.K247_R254del microdeletion variant and the common p.G60= haplotype adds an important layer to the risk assessment process. Genetic counselors must consider these nuances to provide more accurate risk predictions for patients with *CTRC* variants.

In conclusion, our study contributes significantly to clarifying the role of *CASR* variants in CP and to risk assessment of *CTRC* variants in CP. While common *CASR* variants do not play a role in CP susceptibility, *CTRC* variants emerge as robust risk factors. This work underscores the importance of continued research into the genetic basis of pancreatic diseases, paving the way for more precise diagnostics and personalized interventions in the clinical setting.

7. Novel findings

1. The three *CASR* single nucleotide polymorphisms (p.A986S, p.R990G and p.Q1011E) are present in the Hungarian population.

2. The three *CASR* single nucleotide polymorphisms (p.A986S, p.R990G and p.Q1011E) are not associated with chronic pancreatitis in Hungary.

3. The loss-of-function *CTRC* variants (p.A73T, p.V235I, p.K247_R254del, and p.R245W) increase the risk of chronic pancreatitis by approximately 3-7-fold in heterozygous

carriers, while homozygous or compound heterozygous carriers have about an 11-fold increased risk. The risk elevation is similar in alcoholic and non-alcoholic chronic pancreatitis, and the degree of risk varies between variants depending on the extent of their functional impairment.

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10. Publications

Publications related to the PhD thesis

1. Takáts, A., Berke, G., Szentesi, A., Farkas Jr, G., Izbéki, F., Erőss, B., Czakó, L., Vincze Á., Hegyi, P., & Hegyi, E. (2021). Common calcium-sensing receptor (CASR) gene variants do not modify risk for chronic pancreatitis in a Hungarian cohort. Pancreatology, 21(7), 1305-1310.

2. 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.