The Common Chymotrypsinogen C (*CTRC*) Variant G60G (C.180T) Increases Risk of Chronic Pancreatitis But Not Recurrent Acute Pancreatitis in a North American Population

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OBJECTIVES: Recurrent acute pancreatitis (RAP) is a complex inflammatory disorder that may progress to fibrosis and other irreversible features recognized as chronic pancreatitis (CP). Chymotrypsinogen C (CTRC) protects the pancreas by degrading prematurely activated trypsinogen. Rare mutations are associated with CP in Europe and Asia. We evaluated the occurrence of *CTRC* variants in subjects with RAP, CP, and controls from the North American Pancreatitis Study II cohort.

METHODS: CP ($n = 694$), RAP ($n = 448$), and controls ($n = 1017$) of European ancestry were evaluated. Subgroup analysis included *CFTR* and *SPINK1* variants, alcohol, and smoking.

RESULTS: We identified previously reported rare pathogenic *CTRC* A73T, R254W, and K247_R254del variants, intronic variants, and G60G (c.180 C > T; rs497078). Compared with controls (minor allele frequency (MAF) = 10.8%), c.180T was associated with CP (MAF = 16.8%, $P < 0.00001$) but not RAP (MAF = 11.9% $P = $ NS). Trend test indicated co-dominant risk for CP (CT odds ratio (OR) = 1.36, 95% confidence interval (CI) = 1.13–1.64, $P = 0.0014$; TT OR = 3.98, 95% CI = 2.10–7.56, $P < 0.0001$). The T allele was significantly more frequent with concurrent pathogenic *CFTR* variants and/or *SPINK1* N34S (combined 22.9% vs. 16.1%, OR 1.92, 95% C.I. 1.26–2.94, $P = 0.0023$) and with alcoholic vs. non-alcoholic CP etiologies (20.8% vs. 12.4%, OR = 1.9, 95% CI = 1.30–2.79, $P = 0.0009$). Alcohol and smoking generally occurred together, but the frequency of *CTRC* c.180 T in CP, but not RAP, was higher among never drinkers–ever smokers (22.2%) than ever drinker–never smokers (10.8%), suggesting that smoking rather than alcohol may be the driving factor in this association.

CONCLUSIONS: The common *CTRC* variant c.180T acts as disease modifier that promotes progression from RAP to CP, especially in patients with *CFTR* or *SPINK1* variants, alcohol, or smoking.

INTRODUCTION

The chronic pancreatitis (CP) syndrome (ICD9 577.1, ICD10 K86.x) encompasses inflammatory disorders of the pancreas characterized by recurrent acute pancreatitis (RAP) or chronic pancreatic inflammation with variable fibrosis, calcifications, morphologic changes, and progressive loss of functional parenchyma, leading to endocrine and exocrine insufficiency, pain and risk of pancreatic adenocarcinoma.[2] CP is responsible for some of the worst quality-of-life scores of any chronic disease.[3] Based on the imaging criteria, the prevalence of CP in the United States is 41 cases per 100,000 people,[4] and we estimate that the prevalence of cases with a history of RAP is

about three times higher.[4] Thus, the RAP and CP syndrome is not a rare disorder.

There are important distinctions between acute pancreatitis (AP), RAP, and CP. AP is a syndrome of sudden pancreatic injury followed by an acute inflammatory response. The diagnosis of AP is based on clinical criteria of pancreatic inflammation leading to typical pain, release of pancreatic digestive enzymes into the blood stream and/or detection of edema or other markers of inflammation of the pancreatic gland and surrounding tissue using abdominal imaging methods.[5–7] AP can be a serious medical problem, because the magnitude of the inflammatory response is typically many

times greater than is expected from similar amount of injury to other tissues because of the activation of trypsinogen to trypsin within the pancreas.[5,8]

Trypsin is a serine protease of broad specificity that is normally activated in the duodenum to serve as a nutrient protease and as the primary activator of other pancreatic digestive enzymes. Premature activation of trypsin in the pancreatic acinar cell or within the pancreatic duct can lead to direct cell injury, indirect tissue injury through the effect of activating other pancreatic digestive enzymes, and cross-activation of the immune system. In addition to the acute inflammatory response, AP events initiate the activation, proliferation, and survival of pancreatic stellate cells, which are normally involved in pancreatic tissue healing, and under pathological conditions, fibrosis.[9,10]

Patients with one episode of AP are susceptible to RAP. The risk of RAP can often be reduced if the proximal etiological factor is removed. Examples include removal of the gallbladder to reduce the risk of recurrent biliary AP[11,12] and stopping alcohol consumption to prevent recurrent alcoholic pancreatitis.[13,14] Effective treatments are not yet available for patients with genetic variants that increase susceptibility to AP such as cationic trypsinogen gene (PRSS1) mutations,[15,16] and therefore patients with genetic risk or idiopathic pancreatitis typically suffer from RAP. Patients with RAP from any etiology may, but will not always, develop CP. RAP typically precedes CP by 2–5 years, but the rate of progression is highly variable,[17–20] indicating both a sequenced process and the effect of modifying factors. About a quarter of patients with CP do not have clinically recognized AP prior to diagnosis (below), but the fact that the majority of CP patients do have such a history suggest that this is a major pathological pathway as envisioned in the Sentinel Acute Pancreatitis Event model.[21–23]

The paradigm of the RAP-to-CP sequence seen in genetic disorders such as hereditary pancreatitis has been replicated in population-based epidemiological studies of non-genetic etiologies, such as alcoholic AP and idiopathic AP. After an incident of AP, the risk of developing CP over the next 10 years is five times higher in patients with RAP then those without RAP,[19] indicating that RAP is a major risk factor for the later development of CP.[13,18,19,24]

Although there is no consensus definition of CP, recent practice guidelines[25–28] have adopted a pragmatic definition of CP based on signs of pancreatic fibrosis and gland distortion following recommendations proposed by the Pancreatic Society of Great Britain and Ireland, known as the Cambridge Classification.[29] The Cambridge definition of CP is based on abdominal imaging criteria with identification of duct system distortion associated with fibrosis, supported by features of calcifications or atrophy, but not other complications of chronic injury and inflammation, such as gland dysfunction or pain. Fibrosis represents an abnormal immune response to injury mediated through macrophages and stellate cells, which are scarce in the native pancreas prior to an initial episode of AP.[9,10] Thus, in the majority of cases, clinical evidence of fibrosis follows two distinct steps, each with different risk factors. The first is recurrent injury, such as RAP, which is linked to trypsin activation in the pancreatic acinar and duct cells. The second step is an extreme or abnormal immune response to pancreatic injury[30,31] mediated through activated pancreatic stellate cells.

Based on a review of clinical studies, about a third of patients with AP will develop RAP and about a third of RAP patients will develop CP.[4,19,32] The risk of progression is the highest in patients with hereditary pancreatitis and those with exposure to environmental factors, such as alcohol and tobacco. Thus RAP is a risk factor of future development of CP, but RAP alone is not sufficient to cause CP.

Within established academic clinical pancreas centers, two subgroups of patients can be identified, those with well-established RAP and no significant evidence of fibrosis on abdominal imaging studies such as CT scan (defined as RAP patients), and those with evidence of pancreatic fibrosis on abdominal imaging (defined as CP patients).[1] Based on the known relationship between RAP and CP, the factors associated with susceptibility to RAP should also be associated with CP, but not all factors associated with CP are associated with RAP. Factors only associated with CP are linked to the second step, which involves the immune response and generation of fibrosis and presumed exposure to immune-modifying factors, whether environmental, genetic, or both. Although this is a dominant theme, there is clearly heterogeneity, which we believe is a function of both the severity and/or repetition of the injury and the individual's immune response to (recurrent) pancreatic injury. Thus, in the North American Pancreatitis Study II (NAPS2) study, we test for pancreatitis susceptibility using controls vs. RAP+CP and for susceptibility to fibrosis by comparing RAP and CP. If a potential risk factor is identified, then the nature of it being a protective factor or a CP risk factor can be explored by comparing RAP with controls (differences indicate protection from progression) and CP with controls (differences indicate risk of progression).

Alcohol and smoking are risk factors for CP, but other risk factors are also required as most people who smoke and drink do not develop CP. Most genetic factors associated with RAP/CP are also linked to mechanisms that normally protect the pancreas from intra-pancreatic trypsin activation. These include the cationic trypsinogen gene (PRSS1),[15,16] the cystic fibrosis transmembrane conductance regulator gene (CFTR),[33,34] and the serine protease inhibitor Kasal type 1 (SPINK1).[35,36] Other trypsin-regulating genes appear to be disease modifiers with a lower independent risk such as the calcium-sensing receptor gene (CaSR),[37,38] the gamma-glutamyltransferase 1 gene (GGT1),[39] and the chymotrypsinogen C gene (CTRC)[40,41] as recently reviewed.[23,42–44] Exceptions to the trypsin activation pathway include the CLDN2 locus, which is a risk factor for progression from RAP to CP, especially in men with heavy alcohol use[45] and rare carboxypeptidase A (CPA) variants, which appear to drive fibrosis through the unfolded protein response.[46]

Possible associations between CTRC variants and RAP/CP have not been reported in North American Populations. Genetic association studies from Europe and Asia have identified rare CTRC variants that are associated with CP, but they differ between populations, and associations with specific rare variants have been difficult to replicate.[40,41,47,48] The most commonly reported rare variants (minor allele frequency <5%) across all these studies have been R254W and

K246_R254del, although multiple novel mutations have been identified.[40,41,47,48]

The strongest evidence for a role of CTRC in pancreatitis is from functional studies. CTRC is a Ca^{2+}-dependent serine protease that can rapidly degrade all three human trypsinogen and is likely the protective agent identified by Rinderknecht as enzyme Y.[49] Functional studies on the CTRC protein by the Sahin-Tóth laboratory[40,50,51] have shown convincingly that CTRC has a key role in a major mechanism of trypsin auto-digestion and that loss-of-function mutations in *CTRC* disrupt this mechanism. Thus the strong biological plausibility of a protective role for CTRC against trypsin-dependent pancreatic injury and pancreatitis is supported by both population genetic and functional studies.

The primary aim of this study was to replicate the association between pathogenic CTRC variants and pancrea-titis in a North American population. An additional aim was to determine whether there were interactions between CTRC variants and other risk factors for RAP or CP, such as alcohol use or smoking.

METHODS

Study population. NAPS2 and NAPS2 validation (NAPS2-CV) cohorts have been described in detail.[1,45,52] All NAPS2 subjects were recruited and consented by expert physicians using IRB (Institutional Review Board)-approved protocols. Prospectively collected demographic and phenotypic infor-mation included race, sex, family history, exposure to environmental risk factors (including alcohol and smoking history[52]), natural history of the disease (chronology of change in symptoms and status), and physician assessment of etiology. RAP was defined by more than one episode of documented AP (typical abdominal pain with elevation of pancreatic enzyme $>3\times$ normal or imaging evidence of AP), and CP was defined using imaging criteria or histology.[1] Alcohol and smoking histories were taken in detail and were categorized by both usage (non-user, ex-user, or current) and average threshold (smoking pack years 0, 1–12, 12–35, ≥ 35; average weekly alcohol intake (quantity–frequency criteria, i.e., average amount consumed on a drinking day and the number of days per month that this amount was consumed): abstainers: no alcohol use or <20 drinks in lifetime; light drinkers: ≤ 3 drinks/week; moderate drinkers: 4–7 drinks/week for females; 4–14 drinks/week for males; heavy drinkers: 8–34 drinks/week for females; 15–34 drinks/week for males; and very heavy drinkers: ≥ 35 drinks/week.) An additional 296 de-identified healthy control DNA samples were provided by SomaLogic, Inc. (Boulder, CO) and included demographic information (sex, age, ethnicity), drinking status, smoking status, and personal medical history (described in Whitcomb *et al.*[45]). Additional healthy controls were also obtained from an inflammatory bowel disease (IBD) case–control study (Dr Richard Duerr, University of Pittsburgh) ($n = 309$ healthy non-IBD controls), variables included age, sex, race, and smoking data.

DNA preparation and mutations analysis. Peripheral blood leukocyte DNA was purified as described.[1] PCR primers were designed for CTRC gene exons 2, 3, and 7 and the corresponding intronic regions that contain most of the commonly reported mutations reported in Germany, India, France, and Taiwan.[40,41,47,48,53]

PCR was performed in a total volume of 25 µl; 200 nmol of forward and reverse primers, 200 µmol of dNTP and $1\times$ PCR buffer II (ABI, Foster City, CA) with 20 ng of DNA. Amplification settings were 95 °C for 12 min $\times$ 1 cycle, 95 °C for 30 s, annealing temperature $\times$ 30 s and 72 °C $\times$ 20 s for 35 cycles, and 72 °C for 2 min $\times$ 1 cycle. Annealing temperatures and magnesium concentrations for different primers are available on request. PCR amplification products were treated with exonuclease I (NEB, Beverley, MA) and shrimp alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) and then purified by ethanol EDTA precipitation, according to the manufacturer's recommendations. For restriction fragment length polymorphisms (RFLP) analysis, the 463-bp amplifica-tion product of exon 7 sequencing as described above was digested with SmaI (New England BioLabs, Frankfurt, Germany) at 37 °C for 2 h. The digestion products were analyzed by electrophoresis in 5% polyacrylamide gel (Bio-Rad, Hercules, CA) and visualized with ethidium bromide staining. For direct sequencing, the PCR products were run on an ABI Prism 3730 Genetic Analyzer, and the sequence was analyzed using Sequencher 4.7 (Gene Codes, Ann Arbor, MI; Cycle sequencing kit v3.1 diluted 1:8 (ABI) using the appropriate PCR primers).

Genotyping of CTRC was done using a multi-step process. An initial group of CP and unrelated controls enrolled sequentially in the NAPS2 cohort were selected for DNA sequencing of exons 2–3 and 7 of CTRC, with final genotypes obtained for 247 Caucasian samples (CP = 148, controls = 99). Based on the *CTRC* variants identified in this initial direct sequencing, we tested the remainder of the cohort for the most frequent variants, R254W and G60G. We genotyped the NAPS2 cohort and SomaLogic controls for p.G60G (rs497078) using Taqman single-nucleotide polymorphism genotyping assay (Life Technologies, Carlsbad CA; Assay ID C__2393026) and obtained final genotype data for 755 CP, 429 RAP, 1024 unrelated controls, and 183 family controls. We also extended the genotyping of R254W to all NAPS2 samples using RFLP, a technique that captured both the R254W and K247_R254del mutations, and confirmed all positive and random negative results by sequencing. In total, we collected final genotypes for the exon 7 region in a total of 1512 samples: 128 by sequencing alone, 961 by RFLP, and 423 by a combination of the two.

Genotyping of PRSS1, SPINK1, and CFTR. The NAPS2 cohort was genotyped for known variants in related pancrea-titis risk genes *PRSS1*, *SPINK1*, and *CFTR* by a combination of direct sequencing and multiplex genotyping assays (Sequenom, San Diego CA) and individual taqman genotyp-ing assays (Life Technologies) as previously described.[54] *SPINK1* N34S taqman and *PRSS1* rs10273639 geno-typing was completed in the NAPS2-CV cohort. *PRSS1* rs10273639, *CFTR* and *SPINK1* N34S taqman genotyping was completed in the NAPS2, IBD controls, and SomaLogic control cohorts.

Statistical analysis. STATA SE12.0 (STATAcorp LP, College station, TX) was used to analyze and generate summary statistics of the data set. We compared variation frequency between cases and controls using either the Chi Square test (χ^2) or Fisher's exact test and used the Mann–Whitney test to compare age of onset. Odds ratios (ORs) and the corresponding 95% confidence intervals (95% CI) for CP risk were calculated using logistic regression. The Cochran–Armitage test for trend was used to investigate dose effects of c.180C>T. RAP and CP were modeled as having common susceptibilities, with CP occurring over time in the presence of additional disease-modifying factors to test whether associated risk were for pancreatic injury or fibrosis.[45,52] For all statistical comparisons, frequencies of CTRC variants in cases (CP and RAP) or CP or RAP were compared against unrelated controls only; family members genotyped were not included in this analysis. For all tests, $P < 0.05$ was considered significant.

RESULTS

Study population. We compared the demographics, alcohol and smoking classification, and reported etiology of pancreatitis for cases and controls (Table 1). The prevalence of very heavy alcohol use, ever smoking, and current smoking was significantly higher among CP cases than among either RAP cases or unrelated controls; a higher percentage of RAP cases never used alcohol or smoked compared with CP cases and controls. Alcohol usage was not available for the SomaLogic and IBD study healthy control samples.

Within the NAPS2-CV cohort ($n = 521$ CP cases) we found that a minority ($n = 134$; 25.7%) did not have a history of AP. We could not determine if there were subclinical episodes of AP, but a slight majority of these subjects with CP reported either no pancreatitis pain ($n = 46$; 34.3%) or only limited episodes of mild-to-moderate pain ($n = 25$; 18.7%) so that episodes of AP, typically recognized by pain, may not have been brought to medical attention.

Initial CTRC sequencing and RFLP genotyping of R254W. We identified rare exonic genetic variants in *CTRC* among the RAP and CP cases (Table 2); genotyping of exon 7 in the expanded cohort identified additional carriers of the rare variant R254W for a final count of 8/677 (1.2%) CP, 3/444 (0.7%) RAP, and 2/404 (0.5%) unrelated controls. The previously reported microdeletion in exon7, K247_R254del was identified in one RAP and one CP patient and no controls (0.1% CP vs. 0.2% RAP vs. 0.0% ctrl). All of the rare variations were found in the heterozygous state, and none were significantly associated with CP or RAP when compared with unrelated controls (Table 2). The mutation carriers had variable age of onset, gender, etiology, and alcohol/tobacco use (detailed in Table 3). Combined, disease-associated CTRC mutations (R254W, A73T, or K247_R254del) were identified in 1.33% of cases (1.62% CP) and 0.50% controls ($P = $ NS). Thus we were unable to confirm statistical association between these rare variants and RAP or CP in this cohort, either individually or in a group.

Direct sequencing of exon 3 in the initial cohort of 148 CP and 99 controls identified the common variant *CTRC* c.180 C>T/A (G60G) with non-significant overrepresentation of the minor T allele in CP, (CP T = 15.1%, A = 0.4%; unrelated controls T = 11.1%, A = 0%).

Analysis of *CTRC* c.180 C>T/A (G60G) among RAP and CP. In the expanded genotyping cohort, *CTRC* c.180T (G60G) was significantly overrepresented in CP (all etiologies) but not RAP patients as compared with unrelated controls (16.8% CP, 11.9% RAP, 10.8% controls) (Table 4). Comparing ORs indicated a dose effect of the minor allele with CT vs. CC OR 1.36, $P = 0.001$ and TT vs. CC OR 3.98, $P < 0.0001$.

Because of the original report by Masson et al.[41] linking c.180C>T with familial CP only, we analyzed the G60G *CTRC* variant specifically in the background of known genetic risk factors (disease-associated mutations in *SPINK1* (N34S), *CFTR*, *CTRC*, and *PRSS1*) and alcohol-associated etiologies.

Table 1 Demographics, self-reported alcohol and smoking, and physician-defined etiology in the study cohort

	Controls	CP	*P* value	RAP	*P* value	CP vs. RAP *P* value
Total number	1017	694		448		
Mean age—years (s.d.)	52.3 (15.8)	50.5 (16.1)	0.026	46.1 (15.5)	<0.0001	
Male—*n* (%)	440 (43.2)	364 (52.4)	0.0002	201 (44.8)	0.57	0.013
Drinking and smoking						
Ever drinker—*n/N* (%)[a]	552/700 (78.9)	544/683 (79.7)	0.76	332/442 (75.1)	0.14	0.078
Very heavy drinker—*n/N* (%)[b]	25/424 (5.9)	104/471 (22.1)	<0.0001	39/394 (9.9)	0.033	<0.0001
Ever smoker—*n/N* (%)	468/1009 (46.4)	491/684 (71.8)	<0.0001	246/441 (55.8)	0.0010	<0.0001
Current smoker—*n/N* (%)	149/1009 (14.8)	306/684 (44.7)	<0.0001	99/441 (22.5)	0.0003	<0.0001
Never smoke or drank—*n/N* (%)[a]	100/697 (14.3)	90/682 (13.2)	0.54	85/438 (19.4)	0.025	0.0068
Alcohol etiology—*n/N* (%)	—	284/694 (40.9)		109/448 (24.3)		<0.0001
Age of AP diagnosis, mean years (s.d.)	—	40.5 (17.9)		41.6 (16.2)		
Age of CP diagnosis, mean years (s.d.)	—	47.6 (16.5)		—		

AP, acute pancreatitis; CP, chronic pancreatitis; RAP, recurrent acute pancreatitis. Definitions: Ever drinker: consumption of ≥ 20 drinks during lifetime; very heavy drinker: ≥ 5 drinks/day during "the period of maximum drinking in life"; ever smoker: smoked ≥ 100 cigarettes during lifetime; current smoker: smoking at the time of study enrollment. Alcohol etiology for RAP and CP is based on the enrolling physician's assessment, with or without additional factors. Reported *P*-values are on demographics when compared with the control group.
[a]Alcohol status (current, ex, never) was not available for the Duerr controls.
[b]Alcohol usage (light, moderate, heavy, very heavy drinker) was not available for the inflammatory bowel disease study and SomaLogic controls.

Table 2 Rare genetic variations in chymotrypsinogen C (CTRC) gene in cases and controls

	DNA	Amino-acid change	Genotyping strategy	CP	RAP	Controls	*P* value[a]
Exon							
3	c. 217 G > A	p.A73T	Sequencing	1/148 (0.6%)		0/99 (0.0%)	NS
7	c.760 C > T	p.R254W	RFLP+Sequencing	8/677 (1.2%)	3/444 (0.7%)	2/404 (0.5%)	NS
7	c.738_761del24	p.K247_R254del	RFLP+Sequencing	1/677 (0.1%)	1/444 (0.2%)	0/404 (0.0%)	NS
Intron							
6	c.640-12G > A		Sequencing	1/148 (0.7%)		0/99 (0.0%)	NS
6	c.640-42G > C		Sequencing	2/148 (1.4%)		0/99 (0.0%)	NS

CP, chronic pancreatitis; NS, not significant; RAP, recurrent acute pancreatitis; RFLP, restriction fragment length polymorphisms. NS, *P* > 0.05. No homozygous or compound homozygous mutations above were identified.
[a]Fisher's test between CP vs. controls.

Table 3 Demographics, alcohol consumption, smoking, etiology, and status of other genetic mutations (*CFTR*, *SPINK1*) in carriers of CTRC mutations in NAPS2 cohort

Diagnosis	Enrollment age	Sex	CTRC	SPINK1	CFTR	Etiology	AP.dx age	CP.dx age	Smoker	Drinker	Drinker type
CP	53	F	K247_R254DEL	wt	wt	Alcohol	45	53	Current	Ex	Heavy
CP	61	M	R254W	wt	R75Q	Alcohol	—	51	Current	Current	Very heavy
RAP	42	F	R254W	wt	wt	Alcohol	39	—	Ex	Current	Heavy
CP	56	M	R254W	wt	wt	Alcohol	39	40	Ex	Ex	Very heavy
CP	31	M	R254W	N34S	F508del	Alcohol	—	20	Ex	Ex	Mod
RAP	32	F	K247_R254DEL	wt	wt	Gallstones	20	—	Current	Ex	Heavy
RAP	NA	M	R254W	wt	wt	NA	NA	—	NA	NA	NA
CP	46	M	R254W	wt	wt	Idiopathic	28	44	Current	Current	Light
CP	33	M	R254W	wt	wt	Idiopathic	27	28	Current	No	—
CP	66	M	R254W	wt	wt	Idiopathic	60	62	Ex	Ex	NA
CP	64	F	R254W	wt	wt	Idiopathic	—	61	Ex	No	—
RAP	43	F	R254W	wt	F508del	Idiopathic	42	—	No	Current	Light
CP	40	F	A73T	wt	wt	Idiopathic	—	40	No	Ex	Mod
CP	44	M	R254W	wt	wt	Genetic	42	44	No	No	—
CP	6	F	R254W	N34S/N34S	wt	Genetic	4	5	No	No	—
Family	37	F	R254W	N34S		—	—	—	Ex	Current	Heavy
Control	47	M	R254W	wt	wt	—	—	—	Ex	Ex	Very heavy
Control	29	F	R254W	wt	wt	—	—	—	No	Current	Light
CP	51	M	c.640-12G > A	wt	NA	Alcohol	49	49	Current	Current	Very heavy
CP	56	F	c.640-42G > C	wt	NA	Obstructive	56	56	Ex	Current	Light
CP	NA	M	c.640-42G > C	wt	R75Q	Alcohol	51	51	Current	Ex	Very heavy
Family	60	M	c.640-42G > C	wt	NA	—	—	—	Ex	Current	Light

AP, acute pancreatitis; CP, chronic pancreatitis; CTRC, chymotrypsinogen C; F, female; M, male; NA, data not available; RAP, recurrent acute pancreatitis; wt, genotyping revealed no mutation.

CTRC G60G was in epistasis with *CFTR* and *SPINK1* mutations as reflected by a higher frequency of the T allele among carriers (22.9%) but not with the limited number of *PRSS1* or *CTRC* mutations (10.2%).

We removed the samples with any known genetic risk factors and assessed the impact of c.180C > T within different etiologies. The c.180T allele was most frequent among alcohol-associated CP etiologies either when alcohol was reported as the only cause or with some additional etiological factor (20.8%). The T allele was less frequent among non-alcoholic etiologies (idiopathic, obstructive, autoimmune, etc.) (12.4%) and not significantly overrepresented in this group when compared with controls. The CP cases diagnosed by physicians as having alcohol-related pancreatitis were significantly more likely to have a high-risk G60G T allele than non-alcohol-related CP cases (Table 4, *P* = 0.0009).

To investigate whether alcohol and smoking modify the relationship between G60G and CP, we evaluated the risk of CP with and without alcohol and/or smoking by stratifying cases and controls with no mutations in *SPINK1*, *CFTR*, *CTRC*, and *PRSS1* based on self-reported drinking and smoking status (Table 5). Among patients with no known genetic mutations, the association of *CTRC* G60G with CP was influenced by drinking and smoking status, with the highest frequency seen in the small group of smokers who were never drinkers (22.2%). Although the T allele was significantly overrepresented among CP subjects who both smoke and drank at some point compared with healthy controls with the same history (OR 1.71, *P* = 0.0064), it was also significant among those with smoking only (OR 5.43, *P* = 0.0022) but not with those who drank alcohol but never smoked. The association between *CTRC* G60G and CP in subjects classified by physicians as having an alcohol-related etiology could be driven by smoking, which is tightly correlated with drinking,[24,52] making further analysis difficult due to sample size. Indeed, 91.8% of CP patients with alcoholic etiology in our cohort were ever smokers and 71.3% were

Table 4 CTRC G60G genotype frequencies in the study cohort stratification for physician-defined etiology and the presence of *PRSS1*, CTRC, *SPINK1*, and *CFTR* mutations

Analysis					TT vs. CC			CT vs. CC			TT/CT vs. CC		
G60G	TT	CT	CC	MAF%	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
Unrelated controls	10	199	804	10.8%									
Controls with any known mutation	0	12	56	8.8%									
All RAP	7	92	345	11.9%		NS			NS			NS	
All CP	30	170	484	16.8%	**3.98**	2.10–7.56	**<0.0001**	**1.36**	1.13–1.64	**0.0014**	**1.48**	1.23–1.77	**<0.0001**
Known risk mutation (CFTR/SPINK1)	4	42	63	22.9%	**4.32**	1.45–12.92	**0.0044**	**2.04**	1.39–3.00	**0.0002**	**2.15**	1.48–3.12	**<0.0001**
Known HP mutation (CTRC/PRSS1)	1	7	36	10.2%		NS			NS			NS	
No known mutations	25	123	390	16.1%	**5.15**	2.45–10.84	**<0.0001**		NS		**1.46**	1.15–1.86	**0.0022**
Alcoholic no mutation	16	66	154	20.8%	**8.35**	3.72–18.75	**<0.0001**	**1.73**	1.25–2.40	**0.0009**	**2.05**	1.51–2.78	**<0.0001**
Non-alcoholic no mutation	9	57	236	12.4%	**3.066**	1.23–7.63	**0.011**		NS			NS	
Alcoholic CP vs. non-alcoholic CP					**2.72**	1.17–6.32	**0.016**	**1.77**	1.18–2.67	**0.0056**	**1.90**	1.30–2.79	**0.0009**

CI, confidence interval; CP, chronic pancreatitis; CTRC, chymotrypsinogen C; HP, hereditary pancreatitis; MAF, minor allele frequency; NS, not significant; OR, odds ratio; RAP, recurrent acute pancreatitis. CP patients with any CFTR or SPINK1 mutation (known risk) were investigated separately from those with HP causing mutations (either PRSS1 or CTRC rare alleles) and those with no identified mutations (which included both alcoholic and non-alcoholic etiologies). Reported OR were made by comparing against all unrelated controls, except for the final. Bold values indicate statistically significant comparison (OR, *P*-value).

Table 5 CTRC G60G carrier frequencies in CP cases and controls (no genetic mutation) with and without smoking or drinking history

Drinking and smoking analysis G60G	TT	CT	CC	MAF%	OR TT/CC vs. CC	95% CI	P value
+Ever drinkers+ever smokers							
CP	17	87	257	16.76%	**1.67**	1.15–2.42	**0.0064**
Controls	3	53	231	10.28%			
+Ever drinkers – never smokers							
CP	2	12	60	10.81%		NS	
Controls	4	37	178	10.27%			
– Never drinkers+ever smokers							
CP	1	14	21	22.22%	**5.43**	1.73–17.04	**0.0022**
Controls	1	4	38	6.98%			
– Never drinkers – never smokers							
CP	5	7	44	15.18%		NS	
Controls	0	24	63	13.79%			
Current smokers							
CP	16	68	163	20.24%	**2.08**	1.27–3.40	**0.0031**
Controls	1	27	113	10.28%			
Current drinkers							
CP	6	43	131	15.28%	**1.61**	1.06–2.42	**0.024**
Controls	4	71	322	9.95%			

CI, confidence interval; CP, chronic pancreatitis; CTRC, chymotrypsinogen C; MAF, minor allele frequency; NS, not significant; OR, odds ratio. Bold values indicate statistically significant comparison (RO, *P*-value).

current smokers, in contrast to non-alcoholic CP composing of 57.8% ever smokers and 28.2% current smokers.

DISCUSSION

In the current study, we demonstrate that the *CTRC* G60G (c.180C>T) variant significantly increases risk of alcohol/smoking or CFTR/SPINK1-associated CP, but not RAP. In addition, we found that the *CTRC* c.180C>T allele, in a dose-dependent way, significantly increased the risk of CP, but not RAP. These findings may be especially important to the field of

medicine, because the high-risk *CTRC* G60G T allele is common, with a frequency of approximately 10% among Caucasians, and the effect is strong and associated with frequent risk factors such as smoking, alcohol, and SPINK1 or CFTR mutations. Following the Sentinel Acute Pancreatitis Event model,[55] the pathogenic *CTRC* variants become important after an initial attack of AP, providing a window of opportunity for CP-preventing interventions.

The present study is the first from North America and provides several important observations. Our first aim was to replicate the European and South Asian findings of a statistical

association between rare variants (A73T, R254W, and K247_R254del) and CP. Although there was a trend, the frequency of these variants in the NAPS2 cohort was too low to confirm statistical association with CP. Our second aim was to evaluate more complex genetic interactions. We attempted to replicate the findings of Masson *et al.*[41] in reporting that *CTRC* G60G T allele is a risk factor for CP. An important new discovery was that the *CTRC* c.180C > T allele was not associated with RAP, suggesting that loss of CTRC function accelerates progression from RAP to CP. A second important discovery was demonstration of a strong association between *CTRC* c.180C > T and CP in subjects with trypsin-dysregulating genetic risks (*SPINK1* and *CFTR* mutations). Finally, we demonstrated that CTRC c.180T allele increases risk of CP in patients with a history of alcohol use and smoking, with the primary effect potentially linked to smoking.

Several important differences were noted between the results of our North American cohort and previous reports. The primary reason appears to be differences in the population demographics and patient characteristics. Masson *et al.*[41] first reported that the common polymorphism c.180 C > T CT genotype to be significantly overrepresented in a small European cohort of CP patients with a positive family history short of hereditary pancreatitis classification ($n = 42$); however, there was no significant association found in idiopathic CP ($n = 216$) or hereditary pancreatitis ($n = 29$) patients in the same study or with the collective cohort or the TT genotype. Follow-up studies in tropical CP[48,56] confirmed the G60G impact in a larger Indian cohort, while a Japanese study[57] reported no significance but very few carriers of the T allele (2/506 CP, 0/274 controls). Functionally deleterious CTRC mutations (i.e., R254W, K247_R254del and A73T) were collectively associated with CP in all previous studies, but we were unable to replicate this finding. Aside from the racial variability in c.180C > T frequency between Caucasian, South Asian, and Japanese populations, we note many other differences between the previous studies and ours.

CTRC is believed to provide protection from prematurely activated trypsin in the pancreas.[50] Previous studies and reviews documented a high concordance of CTRC variants with *CFTR* or *SPINK1* variants, suggesting that risk is conferred through complex genotypes.[56,58] In addition, alcohol and smoking are major risk factors for CP, but additional risk factors appear to be necessary to specifically target the pancreas.[22,45,52]

The mechanism underlying the pathology associated with *CTRC* G60G is unknown, but disruption of key regulator elements is likely. The *CTRC* G60G (c.180C > T, rs497078) variant disrupts a SP-1 nucleotide-binding site. In addition, this single-nucleotide polymorphism is in linkage disequilibrium ($R^2 \geq 0.8$) with other variants altering nucleotide-binding sites, including c.493+52G > A (rs545634) that disrupts an ATF3 site involved in the complex process of cellular stress response and (rs201599742) that disrupts a TATA box and other regulator motifs.[59] A disease mechanism linked to disruption of SP-1 and ATF3-binding sites is biologically plausible, as nucleotide-binding factors linked to this site are associated with pancreatic injury responses, including inflammation and fibrosis.[60]

CTRC clearly has an important role in protecting the pancreas from injury, and the present study clarifies the role. First, the lack of association between pathogenic *CTRC* variants and RAP indicates that *CTRC* is not an AP susceptibility factor such as *PRSS1* or *CFTR* but rather a disease modifier. Second, the pathogenic effects of *CTRC* variants are linked to pathogenic *CFTR* and *SPINK* variants resulting in multiple defects in regulating intra-pancreatic trypsin. Third, the higher prevalence of the *CTRC* G60G haplotype in smokers and alcoholics suggests that *CTRC* expression is positively regulated by stress. Finally, the high risk of CP in smokers with the *CTRC* G60G haplotype is provocative, because it suggests that chronic smoking increases stress and that smoking either causes injury through a trypsin-related process or that CTRC has broader functions than degrading trypsin in compartments with low calcium concentrations.

The study has some limitations. We have identified the well-known CTRC mutations R254W, K245_R254del and A73T in CP, but at a lower frequency than in other reported cohorts,[40,41,48,50,56,57] leading to a discrepancy in the impact of these mutations on North American CP as a whole. Our study was unable to demonstrate association of rare variants with disease despite being a larger cohort than other studies. In addition, we sequenced only three exonic regions in our initial screening study, thus a number of rare or common variants in the untested coding and regulatory regions would be missed. On the other hand, the detailed and mechanism-based phenotyping of the NAPS2 cohort allowed for simultaneous assessment of genetic and environmental factors in this cohort and provided new insights and hypotheses into the mechanism of a complex disorder. Future studies are needed to understand the dynamics of CTRC expression under stress, determining the specific single-nucleotide polymorphisms linked with altered expression, the mechanism of increased risk with alcohol and smoking, and modeling of genetic risk in the background of complex genomic data and environmental factors.

In conclusion, we found that the common synonymous variant *CTRC* c.180C > T (G60G) is associated with significant risk for CP, but not for RAP. We also demonstrated that the risk of CP in humans is associated with the presence of other genetic factors linked to disregulated intra-pancreatic trypsin activity and RAP and CP such as mutations in *CFTR* and *SPINK1*, alcohol, and smoking. As *CTRC* c.180C > T is in a genetic region that contains multiple nuclear-binding protein sites, we hypothesize that the risk of CP is enhanced by failed response to pancreatic stress, allowing trypsin-activating conditions to drive inflammation and rapid progression to CP.

CONFLICT OF INTEREST

Study Highlights

WHAT IS CURRENT KNOWLEDGE

✓ Chymotrypsinogen C (CTRC) is a proteolytic enzyme that is an important factor regulating intra-pancreatic trypsin activity by destroying activated trypsin in solutions with low calcium concentrations.

✓ Rare pathogenic variants of *CTRC* have been associated with chronic pancreatitis in Europe and Asia.

✓ The *CTRC* G60G (c.180T) variant has been associated with chronic pancreatitis in India and in familial pancreatitis in France.

WHAT IS NEW HERE

✓ Rare *CTRC* alleles are present in the North American population but less frequently than in populations from other continents.

✓ The common *CTRC* G60G (c.180T) variant is strongly associated with CP in North America, but is not associated with RAP, suggesting that CTRC modifies the risk of progressing from RAP to CP, but is not an acute pancreatitis susceptibility gene.

✓ The c.180C > T variant is associated with CP of two specific etiologies, either underlying genetic causes (CFTR and/or SPINK1 mutations) or alcoholic CP, which is also heavily associated with smoking.

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