Classification of PRSS1 variants responsible for chronic pancreatitis: An expert perspective from the Franco-Chinese GREPAN Study Group

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Short title: Classification of *PRSS1* variants

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ABSTRACT

Background: PRSS1 was the first reported chronic pancreatitis (CP) gene. The existence of both gain-of-function (GoF) and gain-of-proteotoxicity (GoP) pathological *PRSS1* variants, together with the fact that *PRSS1* variants have been identified in CP subtypes spanning the range from monogenic to multifactorial, has made the classification of *PRSS1* variants very challenging.

Methods: All currently reported *PRSS1* variants (derived primarily from two databases) were manually reviewed with respect to their clinical genetics, functional analysis and population allele frequency. They were classified by variant type and pathological mechanism within the framework of our recently proposed ACMG/AMP guidelines-based seven-category system.

Results: The total number of distinct germline *PRSS1* variants included for analysis was 100, comprising 3 copy number variants (CNVs), 12 5' and 3' variants, 19 intronic variants, 5 nonsense variants, 1 frameshift deletion variant, 6 synonymous variants, 1 in-frame duplication, 3 gene conversions and 50 missense variants. Based upon a combination of clinical genetic and functional analysis, population data and *in silico* analysis, we classified 26 variants (all 3 CNVs, the in-frame duplication, all 3 gene conversions and 19 missense) as "pathogenic", 3 variants (missense) as "likely pathogenic", 5 variants (four missense and one promoter) as "predisposing", 13 variants (all missense) as "unknown significance", 2 variants (missense) as "likely benign", and all remaining 51 variants as "benign".

Conclusions: We describe an expert classification of the 100 *PRSS1* variants reported to date. The results have immediate implications for reclassifying many ClinVar-registered *PRSS1* variants as well as providing optimal guidelines/standards for reporting *PRSS1* variants.

Keywords: Chronic pancreatitis; Genetic predisposition to disease; *PRSS1* gene; Trypsinogen/trypsin; Variant classification

List of abbreviations

ACMG/AMP, the American College of Medical Genetics and Genomics/Association for Molecular Pathology

ACP, alcoholic chronic pancreatitis

AIP, autoimmune pancreatitis

AP, acute pancreatitis

CFTR, cystic fibrosis transmembrane conductance regulator

CI, confidence interval

CP, chronic pancreatitis

CNV, copy number variant

CTRC, chymotrypsin C

ER, endoplasmic reticulum

FCP, familial chronic pancreatitis

gnomAD, the Genome Aggregation Database

GoF, gain-of-function

GoP, gain-of-proteotoxicity

gpAF, global population allele frequency

GREPAN, Genetic REsearch on PANcreatitis

GWAS, genome-wide association study

HGMD, Human Gene Mutation Database

hspAF, highest subpopulation allele frequency

ICP, idiopathic chronic pancreatitis

8.3KJPN, 8.3 K Japanese population reference panel

KRGDB, Korean Reference Genome Database

LD, linkage disequilibrium

LoF, loss-of-function

NA, not available

OR, odds ratio

RAP, recurrent acute pancreatitis

1. Introduction

Chronic pancreatitis (CP) is a chronic inflammatory process leading to progressive morphological and functional changes of the pancreas [1, 2]. It has a prevalence of 36-125 per 100,000 individuals [3]. The process of CP is thought to be irreversible once initiated [4, 5] and there is currently no cure for the disease. Therefore, determining the genetic basis of CP holds out promise for developing new options in disease prevention and treatment. In 1996, a missense variant, p.R122H, in the *PRSS1* gene (encoding cationic trypsinogen) was identified as a cause of an inherited form of CP, namely autosomal dominant hereditary CP (HCP) [6]. This marked the beginning of a new era in CP research. To date, more than 10 CP-related gene loci have been reported (for references, see [7]). Moreover, studies of *PRSS1* variants led to the recognition of two distinct pathological pathways in the etiology of CP, namely the trypsin-dependent pathway [8] and the misfolding-dependent pathway [9]. *PRSS1* variants belonging to the former pathway include missense variants that increase trypsinogen (auto)activation and/or trypsin stability as well as copy number and regulatory variants that increase *PRSS1* dosage; these variants have been collectively termed gain-of-function (GoF) variants [10]. *PRSS1* variants belonging to the latter pathway include only missense variants that could induce the formation of misfolded proteins that would in turn elicit endoplasmic reticulum (ER) stress; these variants have been termed gain-of-proteotoxicity (GoP) variants [10]. The existence of both GoF and GoP pathologically relevant *PRSS1* variants, together with the fact that *PRSS1* variants have been identified in CP subtypes spanning monogenic to multifactorial, complicate the classification and interpretation of *PRSS1* variants [11].

The American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP)-recommended five-category scheme (i.e., “pathogenic”, “likely pathogenic”, “uncertain significance”, “likely benign” and “benign”) for classifying variants in Mendelian disease genes [12] has been widely used in the human genetics field. However, a serious drawback of this five-category scheme is that it cannot deal properly with variants that fall somewhere between “pathogenic” and “benign”. Employing CP as a disease model, and focusing on the four most studied CP-related genes (i.e., *PRSS1*, *CFTR* (encoding cystic fibrosis transmembrane conductance regulator) [13, 14], *SPINK1* (encoding pancreatic secretory trypsin inhibitor) [15] and *CTRC* (encoding chymotrypsin C [16, 17]), we have recently proposed a seven-category system (i.e., “pathogenic”, “likely pathogenic”, “predisposing”, “likely predisposing”, “unknown significance”, “likely benign” and “benign”) for classifying variants in any disease-causing gene [10]. In a preprint, we have provided evidence to support our contention that the newly added “*predisposing*” variant classificatory category

is an appropriate repository for the many intermediate variants that fall somewhere between "pathogenic" and "benign" [18].

As far as *PRSS1* is concerned, our previous study employed many of the experimentally demonstrated GoF and GoP variants to establish proof of concept for our seven-category variant classification system [10]. Prior to our own study, two papers were directly relevant to the classification of *PRSS1* variants. In 2014, Németh and Sahin-Tóth provided an early classification of the then published *PRSS1* variants [19]. Their classifications relied strongly upon functional analysis data. For example, all variants that had not been functionally analyzed (apart from nonsense and canonical GT-AG splice site variants) were classified as being of "unknown significance". Moreover, all missense variants that were experimentally shown to be compatible with a GoF or GoP mechanism were classified as "pathogenic". The second paper did not include 5' and 3' variants or intronic variants (except for those occurring within the GT-AG canonical splice sites) and designated specific terms for classifying pathologically relevant *PRSS1* variants (e.g., "pathogenic variants with established risk to be disease-causing") [11]. Herein, we describe a classification of all currently known *PRSS1* variants for CP within the framework of the ACMG/AMP guidelines-based seven-category variant classification system; an expert perspective from the Franco-Chinese GREPAN (Genetic REsearch on PANcreatitis) Study Group.

2. Methods

2.1. *PRSS1* variants

PRSS1 variants (and related references) were derived primarily from the Database of Genetic Risk Factors in Chronic Pancreatitis (will be described as the CP Database throughout the manuscript) [19, 20] and complemented by data from the Human Gene Mutation Database (HGMD) [21, 22]. Three variants were excluded from further consideration: one somatic variant (i.e., p.G191R identified in four tumors [23]; registered in the CP database) and two variants obtained via personal communications (registered in HGMD). Cross-reference examination and keyword search ("*PRSS1*" plus "variant" or "mutation") in PubMed identified 6 additional variants (Figure 1).

Variant nomenclature employed here was in accordance with [24]; NM_002769.5 and GRCh37/hg19 chromosome 7 sequence were used as reference *PRSS1* mRNA and DNA sequences, respectively. Herein, we would like to emphasize two points. First, multiple variant nomenclature errors have been corrected by the CP Database [20]. Second, NG_001333.2 (*Homo sapiens* T cell receptor beta locus on chromosome 7) has been

used by many investigators and the CP Database as reference *PRSS1* DNA sequence, resulting in alternate names for five common 5' variants (see subsections 3.2.1 and 3.4).

2.2. Approaches to, and principles for, variant classification

Functionality, a prerequisite for pathological relevance, is closely bound up with the type of variant or the variant's location within the genomic sequence of the gene in question. This is of particular importance for classifying *PRSS1* variants because both GoF and GoP variants are of pathological relevance whereas loss-of-function (LoF) variants are benign in relation to CP [10]. We therefore classified them by variant type and pathological mechanism within the framework of our recently proposed seven-category framework (Figure 1). Here, it is worth emphasizing three points. First, we previously proposed an allele frequency threshold, 0.001 as an aid to distinguish *PRSS1* "pathogenic" variants from "predisposing" variants with respect to CP [10]. Thus, any pathologically relevant *PRSS1* variant having an allele frequency of ≥0.001 in the general population would be considered to be too common to cause CP; rather, it would be regarded as predisposing to CP. This proposition, which was based on the allele frequency cutoff recommended for filtering dominant Mendelian disease-causing variants [25], and validated against most of the experimentally confirmed *PRSS1* GoF and GoP variants [10], will be adopted in the current study. Second, we previously proposed to classify LoF *PRSS1* variants (e.g., nonsense and canonical GT-AG splice site variants) as "benign" whilst specifying their *protective* nature in parentheses after the primary variant classificatory category [10]. Herein, we will classify all predicted and experimentally demonstrated LoF variants as "benign" whilst using the complementary term *protective* only for those variants causing a complete or almost complete LoF of the affected allele. Third, *PRSS1* variants were not only reported in subjects with CP but also in subjects with other diseases (see subsection 2.3.1). Here we included all known germline *PRSS1* variants in our classification. However, for those variants that were reported only in the context of non-CP diseases, their classification was carried out solely in relation to their pathological relevance to CP rather than to the non-CP diseases.

2.3. Factors taken into consideration for variant classification

2.3.1. Clinical genetic data

Clinical genetic data refer to whether or not the variant in question has been found in subjects with CP or other diseases, or in controls. Data from some original reports were reinterpreted according to our working definitions of HCP, familial CP (FCP) and idiopathic CP (ICP), in line with our previous publications [17, 26].

For variants that were reported in ≤3 studies, all studies were cited. For variants that were reported in >3 studies, only the first three publications or the first publication plus two other selected publications were cited along with the CP Database [20] (N.B. the CP Database collated comprehensively the related original reports and extracted the numbers of CP carriers and non-CP carriers for each variant). For any variant, a publication that possibly overlapped with a prior one from the same group (as annotated by [20]) was not cited here. Definitions of a variant as common (allele frequency of ≥0.05), low frequency (≥0.005 to <0.05), rare (≥0.001 to <0.005) or very rare (<0.001) are in accordance with Manolio et al. [27].

PRSS1 variants have been confirmed to play a pathological role (either causative or predisposing) in not only HCP, FCP and ICP but also in alcoholic CP (ACP) (e.g., [28, 29]). *PRSS1* variants may also play a role in autoimmune pancreatitis (AIP) (e.g., [30]) or asparaginase-associated pancreatitis [31] but definitive conclusions cannot be drawn at this stage owing to the limited data available and/or lack of replication. Here, clinical genetic findings made in these two rather specific manifestations of pancreatitis will not be considered as being informative with regard to the pathological role of *PRSS1* variants in the etiology of CP.

Whilst GoF and GoP *PRSS1* variants predispose to CP, CP itself increases the risk of pancreatic cancer [32]. However, to date, a direct causal link between GoF and GoP *PRSS1* variants and an increased risk of pancreatic cancer has not been established. Thus, germline *PRSS1* variants found in pancreatic cancer will not be considered as equivalent to variants found in CP, let alone for those found in cancers affecting other organs/tissues.

PRSS1 variants have sometimes been identified in patients with acute pancreatitis (AP), particularly recurrent AP (RAP) without known etiological factors. Given that 10% of subjects with a first attack of AP and 36% of subjects with RAP would progress to CP [33], such findings will be considered here as clinical genetic evidence pertinent to the pathological role of *PRSS1* variants in the etiology of CP.

2.3.2. Functional analysis data

Functional analysis data (with respect to the functional effect of the variant in question) refer to laboratory findings obtained from either biochemical analysis, cell transfection experiments, transgenic mouse studies or analyses performed using patient-derived material.

2.3.3. Population allele frequency data

Global population allele frequency (gpAF) and highest subpopulation allele frequency (hspAF) of studied variants were obtained from the Genome Aggregation Database (gnomAD) [34, 35].

2.3.4. In silico analyses and evidence-based conjecture

The linkage disequilibrium (LD) between two variants was evaluated by means of the LDpair Tool available on the LD link website [36]. The regulatory potential of 5' and 3' variants was evaluated in terms of their RegulomeDB probability scores [37, 38], a model that integrated functional genomics features along with continuous values such as ChIP-seq signals, DNase-seq signals, information content change, and DeepSEA scores. The score ranges from 0 to 1. The higher the score, the more likely the variant is to be a functional regulatory variant [39]. SpliceAI [40, 41], the currently most accurate tool for predicting splicing variants (e.g., [42, 43]), was used to classify intronic, nonsense and synonymous variants in terms of their contribution to aberrant splicing. Evidence-based conjecture was employed for classifying variants without supporting functional data whenever appropriate.

3. Results

A total of 100 distinct germline *PRSS1* variants, all reported in peer-reviewed papers, were derived from a combination of data from the CP Database, HGMD and literature search (Figure 1). Mindful of the existence of two discrete pathological mechanisms underlying CP-related *PRSS1* variants, these were divided into five subcategories for classificatory purposes (Figure 1). Before going into the details, we reiterate that some *PRSS1* variants have so far only been reported in subjects with diseases other than CP; these variants were classified with respect to their relevance to CP rather than the non-CP diseases.

3.1. Classification of gain of trypsinogen CNVs

Consistent with previous publications [10, 19], all three gain of trypsinogen CNVs were classified as "pathogenic" because (i) they have been identified in multiple ICP patients and/or HCP or FCP families; (ii) they are absent or extremely rare in the general population; and (iii) their presumed dosage effect on the etiology of CP was confirmed by transgenic mouse studies (Table 1).

3.2. Classification of 5' and 3' variants

For ease of discussion, the 12 5' and 3' variants will be divided into common and uncommon variants.

3.2.1. Common variants

All six common variants are in high LD, as indicated by the R^2 values in Table 2. The first reported variant, c.-408T>C (rs10273639; known as c.-408C>T in accordance with NG_001333.2), will henceforth be used as a tag for this common haplotype. c.-408T>C has a gpAF of 0.5189 in gnomAD (v2.1.1) [35]. A previous meta-analysis showed that c.-408T>C had a pooled odds ratio (OR) of 1.28 (95% confidence interval (CI) 1.17-1.40; p<0.00001) for ICP [44].

c.-204A>C (rs4726576; known as c.-204C>A in accordance with NG_001333.2) has been shown, both *in silico* and *in vitro*, to be a functional regulatory variant [45, 46]. It has previously been classified as "predisposing" in the context of CP [10]. Of the remaining five variants, only c.-408T>C was subjected to a promoter reporter gene assay, and was shown to have no effect on gene expression [46].

Herein, we assessed the regulatory potential of the six common variants in terms of the RegulomeDB probability score [37, 38]. As shown in Table 2, the experimentally demonstrated functional c.-204A>C variant has a RegulomeDB probability score of 0.75713 whereas the other five variants have scores of <0.14. We further correlated the six common variants with DNase I-accessible DNA regions in pancreatic tissue using data available from the NIH Roadmap Epigenomics Mapping Consortium website [47]. Only c.-204A>C was found to be located within a significant DNaseI-accessible DNA region within the *PRSS1* locus (Supplementary Figure 1). Additionally, c.-204A>C occurred within a MAF transcription factor binding site motif, according to RegulomDB [38] (Supplementary Figure 2a). Although MAF protein is highly expressed in the pancreas [48], to date, no studies have investigated its role in the exocrine pancreas. MAF proteins can act as transcriptional activators or repressors depending upon the target genes [49].

The above cross-variant comparisons have demonstrated a clear difference between c.-204A>C and the other five common variants in terms of their potential regulatory features. This has served to strengthen the previous classification of c.-204A>C as "predisposing" while suggesting that the other five variants could be classified as "benign". It should however be emphasized that c.-204A>C may not be the sole functional variant underlying the increased risk of the c.-408T>C-tagged haplotype. This risk haplotype has been recently shown to contain the *PRSS3P2* and *TRY7* pseudogenes whereas the alternative haplotype has lost the two pseudogenes [50, 51]; this alternative haplotype was reported to be associated with a protective effect against CP [52]. Interestingly, the risk haplotype appears to contain still functional *PRSS3P2/TRY7* pseudogene enhancers that serve to upregulate pancreatic *PRSS2* expression [53], which is consistent with the increasingly appreciated role of *PRSS2* in

pancreatitis (see Masson et al. [52] and references therein). It is plausible that the risk conferred by the c.-408T>C-tagged haplotype is attributable to two non-mutually exclusive mechanisms.

3.2.2. Uncommon 5' variants

Except for c.-338T>G, which was identified in 3 of 65 Chinese patients with pancreatic cancer but without pancreatitis [54], all six uncommon 5' variants have been reported only once, either in CP patients or controls (Table 3). Herein, it should be noted that whereas c.-338T>G is very rare in combined gnomAD populations, it occurs as a low frequency variant in the Japanese and Korean populations according to their respective population-specific databases (i.e., 0.01468 in accordance with the 8.3 K Japanese population reference panel (8.3KJPN) project and 0.0109 in accordance with the Korean Reference Genome Database (KRGDB); Table 3).

Four variants (c.-184G>A, c.-173C>T, c.-147C>T and c.-30_-28delTCC) were subjected to a promoter reporter gene assay but only c.-147C>T exhibited a significant (and negative) effect on gene expression [55]. Notably, c.-147C>T was identified as affecting a binding site for ATF4 [55]; ATF4 is highly expressed in the pancreas [48] and knockout of ATF4 has been reported to cause pancreatic deficiency in mice [56].

We further performed a cross-variant comparison in terms of regulatory potential. All six uncommon 5' variants are located within the significant DNase I-accessible DNA region that contains the common c.-204A>C variant (refer to Supplementary Figure 1a). Presumably, the high RegulomeDB probability score for the functional c.-147C>T variant, 0.98, would be largely attributable to its location within this significant DNase I-accessible DNA region and its disruption of the binding site for transcription factor ATF4 that has an experimentally confirmed role in the exocrine pancreas (Supplementary Figure 2b). By contrast, the identical and lower RegulomeDB probability score, 0.60906, for the other five variants would be accounted for by their location within the same significant DNase I-accessible DNA region even though they do not affect any known transcription factor binding sites (Table 3).

Taken together, all six uncommon 5' variants could be classified as "benign" (Table 3).

3.3. Classification of intronic variants

There existed no functional analytic data for all 19 *PRSS1* intronic variants with the exception of c.200+64_68delCCCAG.

3.3.1. Two canonical splice site variants

Neither of the two canonical splice site variants, c.40+1G>A and c.200+1G>A, was found in subjects with pancreatitis (Table 4). Both of them represent predicted LoF (pLoF) variants in accordance with [34]. Herein, we confirmed their LoF nature by means of SpliceAI [40, 41]. c.40+1G>A was predicted to disrupt the splice donor site of intron 1 whilst activating a nearby downstream cryptic splice donor site; the mutant transcript, which would be predicted to encode a product comprising only 19 amino acids (Supplementary Figure 3), is likely to be subject to significant degradation by nonsense-mediated mRNA decay [57]. c.200+1G>A was predicted to disrupt the splice donor site of intron 2 whilst activating an upstream cryptic splice donor site within exon 2; this would be predicted to lead to the loss of the terminal 33 bp of exon 2, which would in turn lead to the translation of a protein with an internal deletion of 11 amino acids; the mutant protein cannot be functional owing to the loss of histidine 63, one of trypsin's catalytic triad residues (Supplementary Figure 4).

Consistent with our previous publication [10], these two pLoF variants were classified as "benign (protective)".

3.3.2. A pentanucleotide deletion variant with accompanying *in vivo* functional data

c.200+64_68delCCCAG was detected in a Chinese AIP family but did not segregate with the disease [all three patients (one homozygote) and 3 of 7 genetically analyzed healthy members carried the variant]; it was also detected in a Chinese idiopathic AIP patient but was absent from 520 unrelated healthy controls [58]. RNA analysis using patient blood cells revealed a mutant transcript harboring an in-frame deletion of the first 141 nucleotides of exon 2 [58]. *In vitro* biochemical characterization demonstrated that the mutant protein had lower trypsin activity than the wild-type protein with or without enterokinase treatment [58]. However, as illustrated in Supplementary Figure 5, the mutant transcript would encode a protein lacking amino acids 14-60 in the context of pretrypsinogen and the first 37 amino acids in the context of trypsin. Such a significantly truncated protein at the N-terminal end is unlikely to have any trypsin activity. These, together with the fact that *PRSS1* has no confirmed role in AIP, would suggest that the detection of c.200+64_68delCCCAG in AIP was most probably a chance finding. Consequently, we think that it is reasonable to classify c.200+64_68delCCCAG as "benign" in the context of CP.

3.3.3. The remaining intronic variants

Two of the remaining 16 entries are worthy of mention. The first is c.41-49C>T, which was identified in 2 patients (father and son) of an FCP family. However, c.41-49C>T has a rather high allele frequency in the

general population (i.e., gpAF, 0.001122; hspAF, 0.005499 (Table 4)). Applying our previously proposed allele frequency threshold of 0.001 [10] suggests that it is unlikely to be a "pathogenic" variant. The second is c.454+172C>T, which was found in a HCP family. However, it was co-inherited with *PRSS1* p.N29I, the second most frequent *PRSS1* variant causing HCP.

Importantly, none of these 16 variants were predicted by SpliceAI to affect splicing, supporting their "benign" nature from a mechanistic viewpoint.

3.4. Classification of nonsense, frameshift deletion and synonymous variants

In common with the two canonical splice site LoF variants (Table 4), none of the six pLoF variants including five nonsense (p.Y37*, p.Q56*, p.W57*, p.Q86*and p.C160*) and one frameshift deletion (p.P164Lfs*3), were found in patients with CP (Supplementary Table 1). They are unequivocal LoF variants and were thus classified as "benign (protective)".

The two common silent variants, c.486T>C (c.486C>T in accordance with NG_001333.2; p.D162=) and c.738T>C (c.738C>T in accordance with NG_001333.2; p.N246=), are in high LD with the previously discussed c.-408T>C variant. Of the four rare silent variants, only c.204C (p.R68=) was identified in a patient with idiopathic pancreatitis (Supplementary Table 1). All six silent variants could be reasonably classified as "benign" since none of them were predicted to affect splicing by SpliceAI.

3.5. Classification of missense, small in-frame duplication and gene conversion variants

3.5.1. Variants with supporting functional analysis

Thirty-six of the 54 missense, small in-frame duplication and gene conversion variants have been functionally analyzed, mainly by the Sahin-Tóth group. A functional test is generally regarded as the gold standard for classifying any variant. Therefore, it is relatively straightforward to classify these 36 variants on the basis of their supporting functional data.

All 17 experimentally demonstrated GoF variants had a hspAF of <0.001. Of these variants, 7 affected the activation peptide sequence (i.e., the first 7 variants in Table 5), 3 affected Asn29, 4 affected Arg122, p.V39A segregated perfectly with the disease in a large HCP family (i.e., the variant was identified in all seven genetically analyzed patients but not in any of the four healthy family members analyzed [59]) whilst p.E190K was identified in an 11-year-old girl with ICP. All these 16 variants were characterized by a GoF effect on PRSS1 itself. The remaining p.E79K variant, which was identified in a total of 17 patients with FCP, ICP or

ACP in multiple studies [20], may be regarded as an outlier with respect to the GoF mechanism. The p.E79K-cationic trypsin was shown to transactivate PRSS2 (encoding anionic trypsinogen, the second major trypsinogen isoform after PRSS1) more efficiently than the wild-type [60]; a finding compatible with the growing view that increased PRSS2 expression acts as an independent GoF mechanism underlying CP [52]. In short, the clinical genetic, functional and population data concurred, supporting a "pathogenic" classification for all 17 variants.

Of the 10 experimentally demonstrated GoP variants, 6 were characterized by a severe secretion effect and all these 6 variants had a hspAF of <0.001. Of the 6 variants associated with a severe secretion effect, 3 were further analyzed with respect to ER stress in transfected cells; all three were found to exhibit increased ER stress markers. Of these 3 variants, two had been identified in HCP families (Table 5). By contrast, none of the four variants characterized by a moderate secretion effect were found in HCP families. Most importantly, one of these variants, p.G208A, had an allele frequency of 0.009514 in East Asians; based upon data from a large Chinese cohort study [61], p.G208A had an OR of 4.72 for ICP (95% CI 2.88-7.72, p=9.2 × 10^{-11}) [62]. As a matter of fact, it was these two latter observations that prompted our proposal of the seven-category variant classification system [10]. Now that p.G208A is unequivocally categorized as a "predisposing" variant, it would appear reasonable to use "moderate secretion defect" and "severe secretion effect" as additional and functional criteria for classifying these GoF variants. Thus, the six variants with a severe secretion effect were classified as "pathogenic" whereas the four variants with a moderate secretion effect were classified as "predisposing" (Table 5).

All nine variants characterized by no effect or LoF were classified as "benign" (Table 5).

3.5.2. Variants without supporting functional data

The 18 missense variants lacking supporting functional data are summarized in Table 6. All were reported in single studies and were almost invariably found in single subjects. Moreover, eight of these variants were either found in controls or individuals with a disease that was not considered to be relevant to a pathological role for *PRSS1* variants in CP. Nonetheless, just as a rare benign variant may be found in a CP patient by chance (see Table 5), a rare pathological variant may also be found in a non-CP patient or control by chance for many reasons (e.g., asymptomatic disease, late disease onset, low penetrance, *de novo*). It should also be emphasized that *in silico* prediction tools tend to have relatively poor overall performance in relation to missense variants [63]. Their utility would be even more limited for the prediction of functional consequences of *PRSS1* variants owing to the existence of two possible pathological mechanisms. GoF variants are inherently refractory to

prediction [64] and we are not aware of any *in silico* tools that can distinguish GoP variants from LoF variants. Therefore, to be on the safe side, and following in the footsteps of Németh and Sahin-Tóth [19], all 18 missense variants could conservatively be classed as being of "unknown significance". We nevertheless attempted to improve upon the classification of five of these variants using evidence-based conjecture.

p.V39E was found in a patient with RAP and is absent from gnomAD (Table 6). p.V39A, which affected the same amino acid site as p.V39E, was a "pathogenic" GoF variant (see Table 5). p.V39A is thought to modify PRSS1 structure in such a way as to reduce trypsinogen cleavage at Arg122 by trypsin and at Leu81 by CTRC as well as reducing trypsin degradation by CTRC [65]. It is possible that p.V39E (as well as p.V39G that was identified serendipitously in a pediatric soft tissue sarcoma survivor; Table 6) has a similar effect to p.V39A. Taken together, we are minded to classify both p.V39E and p.V39G as "likely pathogenic".

p.Q98R was identified in a single patient with pancreatitis and has an extremely low allele frequency in gnomAD (Table 6). Interestingly, p.Q98K was a "benign" variant (see Table 5). Given that arginine and lysine have remarkably similar physicochemical properties (i.e., both are polar and positively charged) [66], we suggest classifying p.Q98R as "likely benign".

p.R122G was identified in a health control and has an extremely low allele frequency in gnomAD (Table 6). Given that both p.R122H and p.R122C are disease-causing, we would classify p.R122G as "likely pathogenic".

p.V123L was found in a subject without any overt pancreatic disease and is very rare in gnomAD (Table 6). p.V123M was a "benign" variant (see Table 5) and leucine is a conservative substitution for methionine [67], suggesting that p.V123L is a "likely benign" variant.

4. Discussion

The Franco-Chinese GREPAN Study Group comprises clinicians, geneticists, bioinformaticists and basic researchers with diverse and complementary expertise in CP. In this study, the Group provides an expert classification of the currently reported 100 *PRSS1* variants with respect to their clinical relevance in relation to CP. Based upon the combined consideration of clinical genetic, functional analysis, population data and *in silico* analysis and evidence-based conjecture when appropriate, we classified 26 variants as "pathogenic", 3 variants as "likely pathogenic", 5 variants as "predisposing", 13 variants as of "unknown significance", 2 variants as "likely benign", and 51 variants as "benign". Apart from its completeness in terms of variants included, this study is characterized by two features. First, it employed *in silico* tools to aid the classification of 5' and 3', nonsense, silent and intronic variants but not missense variants. This decision was made on the basis of

balancing the biological plausibility of a given type of variant being pathologically relevant to CP or not with the reliability (and feasibility) of the *in silico* prediction tools on the other. For example, SpliceAI is a highly accurate tool for predicting the potential effects of single nucleotide substitutions or small indel variants on splicing. Therefore, a negative prediction for any silent or intronic *PRSS1* variant strongly supports a "benign" classification. Even in the case of a positive prediction (i.e., predicted to affect splicing), from a mechanistic viewpoint, the aberrantly spliced transcript would most likely lead to a LoF rather than a GoF or GoP. In this regard, it is worth mentioning that the vast majority of the silent and intronic *PRSS1* variants registered by ClinVar are classified as "uncertain significance", "conflicting interpretations of pathogenicity" or "likely benign" [68]. In line with our above reasoning, most, if not all, of these ClinVar-registered silent and intronic *PRSS1* variants should be reclassified as "benign" with respect to CP. By contrast, the existence of two types of *PRSS1* pathologically relevant missense variants, GoF and GoP, made *in silico* predictions undesirable because these predictions could be potentially misleading. Therefore, one may have to rely heavily on functional analysis to classify clinically relevant *PRSS1* missense variants. Second, having compared the GoP variants with respect to their clinical genetic, functional analysis and population data, we proposed to classify the variants with a "moderate secretion defect" as "predisposing" and the variants with a "severe secretion defect" as "pathogenic". This functional phenotype-based classification criterion, together with our previously proposed allele frequency threshold, promises to improve our understanding of the complexity underlying disease expression and the genotype-phenotype relationship in CP.

Variant classification is an important, complex and evolving issue in the field of human genetics, and this is reflected in the constantly refined variant classification standards/guidelines and the ever increasing number of reports of reclassified variants (for examples in both contexts, see [18]). In this vein, our proposed allele frequency threshold and functional phenotype-based classification criterion for distinguishing *PRSS1* "pathogenic" variants from "predisposing" variants (as well as our proposed classifications for some rare *PRSS1* missense variants) may need to be refined as more data become available. Indeed, separating "predisposing" variants from "pathogenic" variants is a difficult task, a process that would require us to make semi-arbitrary but nevertheless operational thresholds. As we noted previously, all such decisions "need to be made on a gene-by-gene basis and would require close collaboration between researchers and clinicians with specific expertise in the diseases/genes in question" [10]. Finally, it should be emphasized that some variants classified as "benign" may occur in *cis* with an as yet unidentified pathological variant.

In summary, this study provides a systematic classification of the so far reported 100 *PRSS1* variants for CP within the framework of the ACMG/AMP guidelines-based seven-category variant classification system. We believe that our study will have immediate implications for interpreting ClinVar-registered *PRSS1* variants and should serve to provide optimal guidelines/standards for reporting *PRSS1* variants in the future.

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Declaration of competing interest

The authors are unaware of any conflict of interest.

Appendix A. Supplementary data

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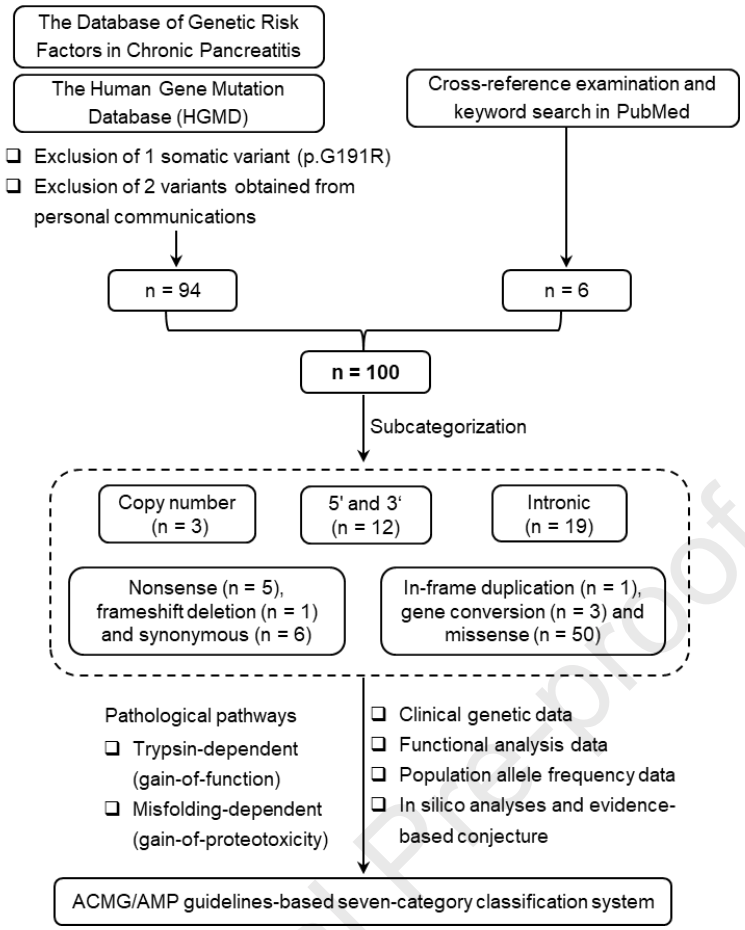

Figure 1. Variant collection and classification procedures.

Table 1. Classification of gain of trypsinogen CNVs

Variant	**Clinical genetic data**	**Functional analytic data**	**gpAF (hspAF)[a]**	**Classification (for CP)**
Triplication	Causes HCP in multiple families [69]; has also been identified in cases with FCP and ICP [45, 70]. Triplication CNVs identified in French patients [69, 70] were found to arise from a common founder chromosome [71])	GoF (three mouse transgenic studies [72-74] were retrospectively identified to be informative in relation to the pathogenic mechanism underlying the trypsinogen gene dosage effect in pancreatitis [45])	NA	Pathogenic
Duplication	Detected in multiple ICP patients and 2 FCP patients [20, 45, 70]; detected only in the youngest patient from an HCP family (the variant must have originated *de novo* since it was not detected in either parent) [75]. There existed at least five distinct duplication CNVs based upon breakpoint sequences characterized at the nucleotide sequence level [76]	Same as above	0.00004610 (0.0001049, African/African American)	Pathogenic
Double GoF hybrid variant	Identified in 1 HCP family with 6 patients across 3 generations [77]	GoF (gene dosage plus the effect of p.Asn29Ile) [77])	NA	Pathogenic

[a]In accordance with gnomAD SVs v2.1 [35]. NA, not available.

Table 2. Classification of common *PRSS1* 5' and 3' variants

Location	Variant[a]			R^2 (with respect to rs10273639)[b]	Clinical genetic data	Functional data	RegulomeDB score[c]	Classification (for CP)
	c.nomenclature (NM_002769.5)	g.nomenclature (chr7, hg19)	rs number					
5'	c.-408T>C (c.-408C>T)[d]	g.142456928T>C	rs10273639		Association with CP discovered by means of GWAS [28]; replicated in multiple subsequent studies [20, 78, 79]; pooled odds ratio of the risk allele for ICP was 1.28 [44]	Not functional by means of a promoter reporter gene assay [46]; the risk haplotype is associated with slightly increased *PRSS1* and *PRSS2* mRNA expression in pancreatic tissue in a dosage-dependent manner [28, 44]; the risk haplotype contains still functional *PRSS3P2/TRY7* pseudogene enhancers that upregulate pancreatic *PRSS2* expression [52, 53]	0.13454	Benign
5'	c.-204A>C (c.-204C>A)[d]	g.142457132A>C	rs4726576	0.9365	Found to be in complete LD with c.-408T>C by resequencing the promoter region of *PRSS1* in French Caucasian individuals [46]; associated with CP in an Indian study [80] and a Chinese study [81]	GoF (increased gene expression by means of a promoter reporter gene assay [46])	0.75713	Predisposing
5'	c.-1809G>A[e]	g.142455527G>A	rs3757378	0.7111	Found by direct sequencing of 2.1 kb *PRSS1* 5' region; linked with c.-408T>C and c.-204A>C but not associated with tropical calcific pancreatitis [80]	No data available	0.00347	Benign
5'	c.-1798C>T	g.142455538C>T	rs3757377	0.7158	Same as c.-1809A>G	No data available	0.13454	Benign

5'	c.-1383A>G (c.-1383G>A)[d]	g.142455953A>G	rs9969188	0.9568	Same as c.-1809A>G	No data available	0.0	Benign
3'	c.*12,596G>A	g.142473466G>A	rs13228878	0.6593	Identified by GWAS; linked with c.-408T>C and associated with asparaginase-associated pancreatitis [31]	No data available	0.09659	Benign

[a]Variants are listed in the order of publication year.
[b]Data were obtained using the LDproxy Tool available on the LD link website [36] in the context of all populations.
[c]Data were obtained from the RegulomeDB website [38].
[d]Alternate name in accordance with NG_001333.2 (Homo sapiens T cell receptor beta locus on chromosome 7).
[e]This variant was incorrectly listed as c.-1,808G>A in the original report [80].

Table 3. Classification of uncommon *PRSS1* 5' variants

Variant			Clinical genetic data	Functional data	gpAF (hspAF)[a]	RegulomeDB[b]		Classification (for CP)
c.nomenclature (NM_002769.5)	g.nomenclature (chr7, hg19)	rs number				Score	Motif	
c.-338T>G	g.142456998T>G	rs184553357	Identified in 3 of 65 Chinese patients with pancreatic cancer (but without pancreatitis) [54]	No data available	0.00006389 (0.001284, East Asian) [N.B. allele frequency in the Japanese population, 0.01468 (8.3KJPN project) [82]; allele frequency in the Korean population, 0.0109 (KRGDB project) [83]]	0.60906	No	Benign
c.-184G>A	g.142457152G>A	rs139432246	Found in 1 of 242 French ICP patients (not in 384 controls) by resequencing [55]	No effect on reporter gene expression [55]	0.005121 (0.01849, African/African American)	0.60906	No	Benign
c.-173C>T	g.142457163C>T	rs572772014	Found in 1 of 384 French controls (not in 242 ICP patients) by resequencing [55]	No effect on reporter gene expression [55]	0.00003225 (0.00006529, non-Finnish European)	0.60906	No	Benign
c.-147C>T	g.142457189C>T	rs754367025	Found in 1 of 384 French controls (not in 242 ICP patients) by resequencing [55]	Significantly reduced reporter gene expression (a reduction of 86% of normal) by reducing the affinity of an ATF4 transcription factor binding site [55]	Absent	0.98[c]	ATF4[c]	Benign
c.-36G>A	g.142457300G>A	rs377134514	Found in 1 of 236 healthy Greek subjects [84]	No data available	0.00002475 (0.00005014, East Asian)	0.60906	No	Benign
c.-30_-28delTCC	g.142457306_142457308del	rs1287463139	Found in a single CP patient [85]	Mild effect on reporter gene expression (a	0.0004914 (0.004245, African/African American)	0.60906[d]	No[d]	Benign

				reduction of 24% of normal) [55]				

[a]In accordance with gnomAD v2.1.1 [35].
[b]Data were obtained from the RegulomeDB website [38].
[c]Used chr7(hg19):142457189-142457190 for querying RegulomeDB [38].
[d]Used chr7(hg19):142457306-142457307 for querying RegulomeDB [38].

Table 4. Classification of *PRSS1* intronic variants

Intron	Variant			Clinical genetic data	gpAF (hspAF)[a]	Classification (for CP)
	c.nomenclature (NM_002769.5)	g.nomenclature (chr7, hg19)	rs number			
1	c.40+1G>A	g.142457376G>A	rs149125789	Identified in a patient with benign pancreatic hyperenzymemia but without pancreatitis [86]	0.00006010 (0.0003204, African/African American)	Benign (protective)
1	c.40+40delC	g.142457415del	rs779579792	Identified in 1 of 381 patients with pancreatitis; no controls were analyzed [87]	NA	Benign
1	c.40+299G>A	g.142457674G>A	rs2011216	In LD with c.-408T>C [28]	0.3759 (0.6515, East Asian)	Benign
1	c.41-49C>T	g.142458357C>T	rs190942214	Detected in 2 patients (father and son) from a FCP family; no controls were analyzed [88]	0.001122 (0.005499, Ashkenazi Jewish)	Benign
2	c.200+1G>A	g.142458566G>A	rs143909348	Identified in 1 of 55 alcoholics without pancreatitis [89]	0.004579 (0.02871 African/African American)	Benign (protective)
2	c.200+64_68delCCCAG[b]	g.142458629_142458633del	rs377590054	Detected in all three patients (one homozygote) and in 3 of 7 healthy members of a Chinese AIP family; detected also in a Chinese idiopathic AIP patient [58]	0.00001422 (0.0002009, East Asian)	Benign
2	c.201-99G>C	g.142459526G>C	rs530207004	Identified in 1 of 50 patients with familial intestinal gastric cancer and 1 of 107 subjects from the normal Italian Tuscany population [90]	0.0007646 (0.001297, European (non-Finnish))	Benign
3	c.454+10A>C[c]	g.142459888A>C	Not available	Identified in 5 of 253 Han Chinese patients with pancreatitis (comprising 22 with alcohol-related diseases, 16 with idiopathic disease, 30 cases with hyperlipidemia, 35 with the hereditary form from six families, and 150 with gallbladder disease) [91]	NA	Benign
3	c.454+36T>C[d]	g.142459914T>C	rs761438114	Identified in 1 of 65 Chinese patients with pancreatic cancer [92]	0.0001275 (0.001655, East Asian)	Benign
3	c.454+75A>G	g.142459953A>G	rs1376416883	Identified in 27 of 253 Chinese patients with pancreatitis (comprising 22 with alcohol-related diseases, 16 with idiopathic disease, 30 cases with hyperlipidemia, 35 with the hereditary form from six families, and 150 with gallbladder disease) and in 4 of 320 controls [91]	NA	Benign
3	c.454+127A>T[e]	g.142460005A>T	rs376116875	Detected in 1 of 29 Chinese patients with pancreatitis; this patient also carried	NA	Benign

				c.454+157C>G (incorrectly named c.454+157G>C) [92]. Whether the two variants were in *cis* or in *trans* is unknown		
3	c.454+157C>G[f]	g.142460035C>G	rs371236770	Detected in 2 of 156 Chinese patients with pancreatic cancer [93] and 1 of 29 Chinese patients with pancreatitis [92]	NA	Benign
3	c.454+157C>A[g]	g.142460035C>A	Not available	Detected in 1 Chinese patient with CP [94]	NA	Benign
3	c.454+172C>T	g.142460050C>T	rs878977606	Co-inherited with *PRSS1* p.Asn29Ile in four patients from a HCP family [95]	NA	Benign
3	c.455-192T>A[g]	g.142460090T>A	Not available	Detected in 1 Chinese patient with CP [94]	NA	Benign
4	c.592-79G>A[h]	g.142460640G>A	rs531271210	Detected in 2 (one Italian male and one Turkish female) of 109 patients with ICP [96]	NA	Benign
4	c.592-78G>A[h]	g.142460641G>A	rs1337286040	Detected in 2 (one Italian male and one Turkish female) of 109 patients with ICP [96]	NA	Benign
4	c.592-24C>T	g.142460695C>T	rs192452846	Detected in 4 patient with pancreatitis [20, 87, 97, 98]	0.003316 (0.005373, non-Finnish European)	Benign
4	c.592[-11C>T;-8C>T][i]	g.[142460708C>T;142460711C>T]	rs183791770; rs200381474	Detected in 6 subjects with pancreatitis [20, 87, 98, 99]	0.003305 (0.01476, South Asian)	Benign

[a]In accordance with gnomAD v2.1.1 [35].
[b]Incorrectly named as c.200+56_60delCCCAG in the original report [58].
[c]Incorrectly named as c.454+10T>G in the original report [91].
[d]Incorrectly named as c.454+36A>G in the original report [92].
[e]Incorrectly named as c.454+127T>A in the original report [92].
[f]Incorrectly named as c.454+157G>C in the original reports [92, 93].
[g]These variants were incorrectly described as exon 3 variants in the original report and were identified in the same patient [94]. Whether the two variants were in *cis* or in *trans* is unknown.
[h]These two variants were detected together. They are most likely to have been generated simultaneously [100] given their occurrence at adjacent positions and their absence in gnomAD.
[i]The two variants were annotated here to be in *cis* since (i) they are in complete LD (R^2 = 1.0) using the LDpair Tool [36] in the context of all populations and (ii) they were detected together in all six carriers.

Table 5. Classification of functionally analyzed *PRSS1* missense variants, small in-frame duplication and gene conversion variants

Exon	Variant		Clinical genetic data[a]		Functional consequence	gpAF (hspAF)[d]	Classification (for CP)
	c.nomenclature (NM_002769.5)	Amino acid change	Number of HCP families (family description) reported[b]	Other[c]			
GoF							
2	c.47C>T	p.A16V	2 (4 patients across 2 generations; 3 patients across 2 generations) [101]	Frequently reported in patients with FCP or ICP [20, 102, 103]. It is the third most commonly detected rare *PRSS1* variant in CP [20]	Increased activation [65]	NA [104]	Pathogenic
2	c.49C>A	p.P17T		Arose *de novo* in a Hungarian CP patient [105]	Increased activation [105]	NA	Pathogenic
2	c.56A>C	p.D19A		Identified in a French ICP patient [106]	Increased autoactivation [106]	NA	Pathogenic
2	c.62A>C	p.D21A	1 (5 patients across 3 generations; all 3 tested patients carried the mutation) [107]	Also found in 1 patient with RAP [108] and 1 patient with early onset pancreatitis [98]	Increased activation [109]	NA	Pathogenic
2	c.65A>G	p.D22G		Identified in two patients of a FCP family [110]	Increased autoactivation [106]	NA	Pathogenic
2	c.68A>G	p.K23R		Reported in 12 CP patients with FCP, RAP or ICP [20, 85, 98, 108]	Increased autoactivation [106, 111]	NA	Pathogenic
2	c.63_71dup	p.K23_I24insIDK	1 (3 patients across 2 generations; all 3 patients carried the variant) [112]		Increased activation [112]	NA	Pathogenic
2	c.86A>T	p.N29I	The second most frequent variant causing HCP [20]; in the first report, one family had 19 patients across 7 generations [113]	Also frequently reported in patients with ICP [20, 61, 114]	Increased activation and stability [65]	NA	Pathogenic
2	c.[86A>T;161A>G] (gene conversion)	p.[N29I;N54S]		Identified in a patient with ICP [115]	Increased autoactivation (solely due to p.N29I) [115]	NA	Pathogenic

2	c.86A>C	p.N29T	1 (8 patients across 3 generations; all patients were mutation carriers) [116]	Also reported in patients with FCP, ICP or RAP [20, 117, 118]	Increased activation and stability [65]	NA	Pathogenic
2	c.116T>C	p.V39A	1 (9 patients across 3 generations; all 7 genetically analyzed patients carried the mutation whilst all 4 genetically analyzed unaffected members did not carry the mutation) [59]		Increased stability [65]	NA	Pathogenic
3	c.235G>A	p.E79K		Identified in a total of 17 patients with ICP, FCP or ACP [20], the first three reports being [60, 119, 120]	Increased transactivation of PRSS2 [60]; there is growing evidence that increased expression of PRSS2 is an independent mechanism underlying CP [52]	0.00003579 (0.0003076, African/African American)	Pathogenic
3	c.364C>T	p.R122C		Identified in cases with ICP or FCP worldwide [20, 121-123]	Increased autoactivation and stability [65, 122]	0.00001988 (0.00003517, European (non-Finnish))	Pathogenic
3	c.365G>A	p.R122H	The most frequent variant causing HCP [20]; in the discovery report, one family had 20 patients across 4 generations [6]	Also frequently reported in ICP patients [20, 114, 118]	Increased autoactivation and stability [65, 124, 125]	0.00001062 (0.00002327, European (non-Finnish))	Pathogenic
3	c.365_366GC>AT (gene conversion)	p.R122H	1 (4 patients across 4 generations; the two genetically analyzed patients carried the mutation) [126]	Identified in a Belgian patient with ICP [127]	Increased autoactivation and stability [65, 124, 125]	NA	Pathogenic
3	c.[343T>A;347G>C;365_366delinsAT] (gene conversion)	p.[S115T;R116P;R122H]		Identified in a 20-year-old German male with RAP; *de novo* occurrence in a 7-year-old Polish girl with CP [128]	A combination of increased trypsinogen activation (attributable to p.R122H) and secretion	NA	Pathogenic

					(attributable to p.R116P) [128]		
4	c.568G>A	p.E190K		Identified in an 11-year-old girl with ICP [129]	Increased autoactivation [129]	NA	Pathogenic
GoP							
3	c.276G>T	p.K92N		Identified in a Caucasian ICP patient [119] and a Chinese AIP patient [30]	Moderate secretion defect [130]	NA	Predisposing
3	c.298G>C	p.D100H		Identified in 1/109 [96] and 1/351 [131] patients with ICP; co-inherited with a *CTRC*-deleting complex rearrangement in a subject with diabetes and identified alone in two clinically unaffected family members [132]	Severe secretion defect [130]	NA	Pathogenic
3	c.311T>C	p.L104P	2 (both having 3 patients across 3 generations) [133, 134];	Identified in two ICP patients [20] and two FCP families [135]	Severe secretion defect and elevation of ER stress marker [136]	NA	Pathogenic
3	c.346C>T	p.R116C	2 (3 patients across 2 generations [137]; 3 patients across 3 generations [138])	Also reported in nearly 40 patients with FCP or ICP [20, 96]	Severe secretion defect and elevation of ER stress marker [138]	0. 00007072 (0.0007018, East Asian)	Pathogenic
3	c.371C>T	p.S124F		Identified in 1 of 660 German CP patients [118]	Moderate secretion effect [130]	0.000003977 (0.00005437, East Asian)	Predisposing
3	c.380C>G	p.S127C		Identified in an ICP patient and his unaffected mother [139]	Severe secretion defect and elevation of ER stress marker [139]	NA	Pathogenic
3	c.415T>A	p.C139S		Identified in 13 CP carriers [20], the first three reports being [87, 91, 140]	Severe secretion defect [138]	NA	Pathogenic
3	c.416G>T	p.C139F		Identified in 4 patients with ICP or RAP [99, 133, 141]	Severe secretion defect [130]	NA	Pathogenic
4	c.508A>G	p.K170E		Identified in 1 patient with CP [142] and 2 patients	Moderate secretion effect [130]	NA	Predisposing

				with tropical calcific pancreatitis [80]			
5	c.623G>C	p.G208A		148 CP carriers reported [20], the first 3 reports being [87, 143, 144]. Based upon data from ref. [61], the allele-based OR of p.G208A for ICP was 4.72 (95% CI 2.88-7.72, $p = 9.2 \times 10^{-11}$) [62]	Moderate secretion defect [130]	0.0007141 (0.009873, East Asian)	Predisposing
No effect or loss-of-function							
2	c.107C>G	p.P36R		Identified in two patients with ICP [119, 130]	Loss-of-function (degradation by CTRC) [130]	0.00009908 (0.001353, East Asian)	Benign
3	c.241C>A	p.L81M		Not segregated with CP in a Chinese AIP family (i.e., the variant was identified in the index patient with CP (12 y, female), her grandmother with CP and her healthy aunt but not in her grandfather with CP) [145]	Biochemical analysis of recombined trypsinogen expressed in *E. coli* did not find differences in autoactivation or trypsin activity between the mutant and wild-type molecules [145]. Cleavage at Leu81 by CTRC is required for CTRC-dependent degradation of PRSS1 [65, 146]; methionine is one of the conservative substitutions for leucine [67] and is one of the preferential cleavage sites of CTRC [147]; and p.L81M mutant was cleaved by CTRC as efficiently as wild-type [148]	NA	Benign
3	c.248G>A	p.G83E		Identified in 1 patient with ICP [119]	Loss-of-function (degradation by CTRC) [130]	NA	Benign

3	c.263T>A	p.I88N		Identified in 1 patient with CP [87]	Loss-of-function (degradation by CTRC) [130]	0.000007953 (0.00004619, European (Finnish))	Benign
3	c.292C>A	p.Q98K		Identified in 1 patient with CP [142]	Functionally neutral [130]	0.0001494 (0.0005880, Ashkenazi Jewish)	Benign
3	c.361G>A	p.A121T		15 CP carriers and 6 non-CP carriers reported [20], the first 3 reports being [91, 149, 150]	Functionally neutral by biochemical analysis; and no secretion defect [151]	0.00001995 (0.00006172, African/African American)	Benign
3	c.367G>A	p.V123M		Identified in 1 patient with ICP [119] and five non-CP subjects (1 unaffected control and four cancer patients) [20, 152, 153]	Loss-of-function (degradation by CTRC) [130]	0. 00003183 (0.0001003, East Asian)	Benign
3	c.410C>T	p.T137M		Reported in a total of 12 CP carriers and 15 non-CP carriers [20], the first three reports being [87, 93, 140]	Functionally neutral [130]	0.0006792 (0.009425, East Asian)	Benign
4	c.541A>G	p.S181G		Identified in 6-year-old Italian boy with RAP; the patient also carried *CFTR* p.Phe508del; the two variants were present in the clinically normal mother [154]	Functionally neutral [130]	0.00006362 (0.0002613, South Asian)	Benign

[a]Data from some original reports were reinterpreted according to our working definitions of HCP, FCP and ICP [17, 26].
[b]Most of these data were taken from Masson et al. [10]. Segregation data were additionally provided for variants reported in single HCP families.
[c]For variants reported in >3 unrelated studies, only the first three publications or the first publication and two other selected publications were cited along with the CP Database [20].
[d]In accordance with gnomAD v2.1.1 [35].

Table 6. Classification of *PRSS1* missense variants without supporting functional data

Exon	Variant		Clinical genetic data	gpAF (hspAF)[a]	Classification
	c.nomenclature (NM_002769.5)	Amino acid change			
2	c.97A>G	p.N33D	Identified in 1 patient (African origin) with idiopathic pancreatitis[b] [11]	NA	Unknown significance
2	c.116T>A	p.V39E	Identified in 1 patient with RAP [155]	NA	Likely pathogenic
2	c.116T>G[c]	p.V39G	Identified in a pediatric soft tissue sarcoma survivor [156]	NA	Likely pathogenic
2	c.125A>G	p.N42S	Identified in 1 patient with RAP [155]	NA	Unknown significance
2	c.200C>T	p.S67F	Reported by a study of 10,389 cases from 33 cancer types [153]	0.00001593 (0.00002892, Latino/Admixed American)	Unknown significance
3	c.293A>G	p.Q98R	Identified in 1 patient with pancreatitis [98]	0.000003976 (0.000008790, European (non-Finnish))	Likely benign
3	c.296A>G	p.Y99C	Identified in a patient with pancreatic ductal adenocarcinoma [157]	0.000007953 (0.00001758, European (non-Finnish))	Unknown significance
3	c.303G>C	p.R101S	Identified in 1 patient (Caucasian origin) with idiopathic pancreatitis[b] [11]	NA	Unknown significance
3	c.310C>G	p.L104V	Identified in two patients and two healthy members of a family with solid pseudopapillary tumors [158]	NA	Unknown significance
3	c.364C>G	p.R122G	Identified in 1 health control [152]	0.00003193 (0.00006498, European (non-Finnish))	Likely pathogenic
3	c.367G>T	p.V123L	Found in 1/1000 German subjects without any pancreatic disease [130] and 1 patient with pancreatic cancer [152]	0.00003536 (0.00006970, European (non-Finnish))	Likely benign
3	c.403A>G	p.T135A	Found in a Chinese patient with pancreatic cancer who also carried c.410C>T (p.Thr137Met; see Table 5); whether the two variants are in *cis* or in *trans* remains unknown [93]	NA	Unknown significance
3	c.443C>T	p.A148V	Identified in a patient with benign pancreatic hyperenzymemia [86]	0.0003605 (0.002564, African/African American)	Unknown significance
4	c.487G>A	p.A163T	Identified in 1 ICP patient [99]	0.00004242 (0. 0002257, Latino/Admixed American)	Unknown significance
4	c.544A>T	p.N182Y	Identified in 1 of 1061 Chinese patients with ICP [61]	NA	Unknown significance
4	c.557T>C	p.V186A	Identified in 1 patient (Ashkenaze origin) with idiopathic pancreatitis[b] [11]	NA	Unknown significance

5	c.689C>T	p.T230I	Identified in a patient with idiopathic pancreatitis[b] [11]	NA	Unknown significance
5	c.721A>G	p.N241D	Identified in 1 patient (North Africa origin) with CP[b] [11]	0.000003981 (0.000008799, European (non-Finnish))	Unknown significance

[a]In accordance with gnomAD v2.1.1 [35].
[b]Thierry Bienvenu, personal communication.
[c]Converted from chr7(hg19):142458481T>G (refer to Supplementary Table S2 in the original report [156].