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Genetic aspects of tropical calcific pancreatitis

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Abstract Tropical calcific pancreatitis (TCP) is a subtype of chronic pancreatitis which is unique to tropical regions. Patients present at young age with recurrent abdominal pain, nutritional deficiencies, and insulin-requiring diabetes. For a long time, the aetiology of this disorder was poorly understood. Several environmental factors, such as malnutrition or the consumption of toxic food components such as cyanogenic glycosides, were proposed as pathogenic factors. In the last decade, a major impact on the understanding of the aetiology of TCP has come from genetic studies on hereditary and idiopathic chronic pancreatitis. Genetic alterations in at least five genetic loci are clearly associated with chronic pancreatitis in the Western world. These include alterations in genes coding for trypsinogens, the most abundant digestive enzymes (*PRSS1* and *PRSS2*), the trypsin inhibitor (*SPINK1*) and the trypsin-degrading enzyme, chymotrypsinogen C (*CTRC*). In addition, alterations in the cystic fibrosis (*CFTR*) gene are associated with idiopathic pancreatitis. TCP clinically resembles non-alcoholic chronic pancreatitis of Western countries, suggesting that similar genetic defects might also be of importance in this disease entity. Indeed, alterations in at least two genes, *SPINK1* and *CTRC*, are strongly associated with TCP. The current review focuses on the recent developments in the understanding of the genetic basis of inherited pancreatitis, with special emphasis on TCP.

Keywords Tropical calcific pancreatitis · Chronic pancreatitis · Genetics · SPINK1 · CTRC

Abbreviations

FCPD	fibrocalculous pancreatic diabetes
TCP	tropical calcific pancreatitis
CP	chronic pancreatitis
ICP	idiopathic chronic pancreatitis
CF	cystic fibrosis
CTRC	chymotrypsinogen C
PRSS1	cationic trypsinogen
PRSS2	anionic trypsinogen
SPINK1	serine protease inhibitor, Kazal type 1

Tropical calcific pancreatitis (TCP) is a subtype of chronic pancreatitis (CP) of unknown aetiology, unique to third world countries located in the tropics [1–7]. Patients present at a young age with recurrent abdominal pain and nutritional deficiencies. The chronic pancreatitis leads to progressive beta-cell deficiency and patients often develop insulin-requiring diabetes (fibrocalculous pancreatic diabetes, FCPD) before the age of 30 years [1–4, 7].

Till recent years, the aetiology of TCP was obscure [1, 2]. Specific predisposing factors such as alcohol abuse, biliary tract stones or metabolic diseases are absent in these subjects. It has been proposed that environmental factors such as

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protein energy malnutrition [8] or the consumption of cassava (a source of cyanogenic glycosides) [1, 2, 9] may play a pathogenic role. However, TCP is frequently found in regions where cassava is not consumed [6, 7], and is also observed in patients of a higher economic background, in whom malnutrition is unlikely. The familial clustering of TCP suggests that genetic defects may predispose to the disease [10]. This observation indicates that environmental factors may be operative on the background of a genetic predisposition to pancreatitis.

During the last 12 years, several genetic defects have been discovered to be associated with hereditary as well as idiopathic chronic pancreatitis (ICP) in patients from Europe or the United States. These studies have changed our understanding of the pathogenesis of chronic pancreatitis. TCP resembles hereditary and idiopathic CP of Western countries in many clinical features. Thus, genetic defects similar to those found in ICP might also play a role in TCP. Recent investigations indicate that indeed ICP does share common genetic attributes with TCP, although some important differences have also been noted. However, it is worthwhile to underline that genetic studies on TCP were performed mostly on patients originating from India and Bangladesh. Genetic data on CP from other regions is mostly lacking.

In the current review, we will briefly summarize the information on clinical features of TCP, its epidemiology, and the role of environmental factors in its aetiology. The genetics of CP, with special emphasis on TCP, will be reviewed in detail.

1 Clinical features

Patients are diagnosed at an early age, usually between 15–30 years [1–7]. A history of alcohol intake is absent. The most common presenting feature is recurrent severe abdominal pain, which is present in 80–90% of patients [1–6]. Many patients have significant weight loss and vitamin deficiencies. Patients can also commonly present with, or develop on follow-up, insulin-requiring but ketosis-resistant diabetes, usually before the age of 40 years [2, 3, 7, 11, 12]. Despite different genetic and environmental influences, the clinical features of the disorder appear to be remarkably similar throughout the subcontinent. In contrast to earlier reports, where the disease affected mainly poorer segments of the population, more recent studies suggest that the disease occurs in all socio-economic groups [7, 12]. TCP has been previously reported to have a dismal prognosis, with a high mortality due to poor nutrition, infections and acute complications related to pancreatitis and diabetes [3, 5]. This was due in part to poor nutrition and lack of medical care available to these patients. In more recent reports, the life expectancy has improved and mortality is mostly related to chronic complications of diabetes and pancreatic malignancy [7, 13].

Patients have an atrophic pancreas, dilated pancreatic ducts and large intra-ductal stones visible on ultrasonography, even at the time of diagnosis [1–7, 11]. At presentation, almost all patients have markedly diminished exocrine function, though clinical steatorrhoea is uncommon [12, 14, 15]. Diabetes develops in >50% of patients with normal glucose tolerance over a 5-year period [16] and is associated with severe reductions in C-peptide [7, 11, 12, 14]. Thus, in contrast to early-onset ICP seen in the West [17], TCP is a more rapidly progressive disorder.

2 Epidemiology

TCP has been reported from many different third world countries [1, 2, 4]. While most studies are from the Indian sub-continent, it has also been reported from other countries in Asia (Thailand, Malaysia, China), Africa (Nigeria, Uganda, Kenya), Middle East and South America (Brazil) [1, 2, 4]. Almost all reports are from countries which are within the tropics. The disease is reported from all parts of the Indian subcontinent [1–7, 11], but the greatest number of cases have been reported from the small south Indian state of Kerala [2, 3, 5, 6].

There is only one community-based epidemiological study on the prevalence of TCP, which was conducted in the state of Kerala [18]. The prevalence of tropical pancreatitis was 126/100,000 and of calcific pancreatitis 98/100,000. This prevalence is far higher than has been reported for CP (mainly alcoholic) in the West (10–15/100,000). However, since this region of Kerala is believed to be endemic for the disease, it is likely that the prevalence of TCP in other regions of the subcontinent is lower.

There are numerous hospital/clinic based reports of TCP as a common aetiology of early-onset diabetes. The proportion of FCPD in these studies varies from 10–30% [19, 20], and is an indicator of the high frequency of this disease in the subcontinent. In a survey of chronic pancreatitis in different Asian countries, TCP was estimated to constitute 30% of CP in Malaysia, 46% in China and 58–70% in India [21]. A recent study from Kerala reports that while TCP is most common form of chronic pancreatitis at younger ages, the proportion of alcoholic CP is now increasing [22].

3 Aetiology

The aetiology of TCP is still unclear. As is true for other complex diseases, there is evidence that both environmental

and genetic factors play an important role in its causation (Table 1).

3.1 Environmental factors

Evidence that environmental factors play a role in the aetiology of TCP includes the fact that the disease is reported from many different third world tropical regions, where genetic backgrounds are different, but similar environmental factors (such as poor nutrition, infections) are prevalent [1, 4]. Their role is also supported by the presence of TCP in an endemic form in certain districts in the state of Kerala, where both the intake of cassava as staple food and poor nutrition has coexisted in the past [2, 5–7, 9].

The two environmental agents which have been most often implicated in the aetiology of TCP are cassava intake and protein deficiency. Cassava roots are a carbohydrate-rich but protein-deficient food, some varieties of which contain large amounts of cyanogenic glycosides [23]. On ingestion, these may release hydrogen cyanide which is postulated to be toxic to the pancreas. It is in those regions of Kerala where cassava is the staple food that the highest prevalence of TCP has been noted [5, 9]. There is also experimental evidence that cyanide will lead to transient diabetes in rats [9]. However, TCP is also found in many regions of the world and Indian subcontinent where cassava is not consumed [1, 2, 7, 11]. In an epidemiological study from Africa, the prevalence of diabetes was not increased in regions where cassava intake was high [24]. Also, in rats fed cassava for up to a year, no changes of CP were observed [25].

Some earlier publications have suggested that severe protein deficiency can lead to CP [4, 8]. In these reports, TCP patients were invariably emaciated and came from a poor economic background [3–5]. However, TCP is uncommon in many regions of the world where malnutrition is rife [1–4], and furthermore, it is reported in all socio-economic strata [2, 12, 14]. In addition, in monkeys fed low protein diets changes of pancreatic atrophy, but no inflammation, was observed [26]. The role of other environmental agents including oxidative stress related to xenobiotics and micronutrient deficiency, infections and autoimmunity has been proposed. However, thus far there is no conclusive evidence for their role.

In summary, while it is unlikely that the above-mentioned factors directly lead to CP, they might increase the possibility of disease in individuals who have increased genetic susceptibility.

3.2 Genetic factors

A familial aggregation of the disease suggests a genetic aetiology. However, in contrast to hereditary pancreatitis, there is no definite mode of inheritance [10]. The early age at onset and similarities with ICP, which has been shown to have a genetic basis, also suggest that similar genetic loci may be involved in its aetiology. Much of the recent advances in our knowledge of TCP have come from studies on the genetic aetiology of the disease in the past decade (Table 1).

4 Genetic alterations in the trypsinogen pathway

4.1 Cationic trypsinogen (PRSS1)

In 1896, Hans Chiari postulated that pancreatitis results from pancreatic autodigestion [27]. An inappropriate conversion of pancreatic zymogens to active enzymes

Table 1 Aetiological risk factors for tropical calcific pancreatitis

Aetiological agent	Remarks
Environmental	
Cassava intake (cyanogenic glycosides)	Release HCN which may be toxic to pancreas; some epidemiological and experimental evidence
Protein malnutrition	Severe deficiency causes pancreatic atrophy; unlikely to lead to chronic pancreatitis
Genetic	
Serum protease inhibitor Kazal type 1 *(SPINK1)*	Specific pancreatic trypsin inhibitor, very strong association of p.N34S variant with TCP
Chymotrypsin C	Trypsin-degrading enzyme; association of different variants (p.A73T, p.I64LfsX69) with TCP
Anionic trypsinogen	Major trypsinogen, p.G191R confers protection to chronic pancreatitis
Cystic fibrosis transmembrane regulator (CFTR)	Chloride channel, homozygous or compound heterozygous mutations cause cystic fibrosis
Cationic trypsinogen	Major trypsinogen, association not found in patients with TCP
Cathepsin B	Lysosomal protease, association uncertain (only 1 report so far)
Calcium sensing receptor	Important in calcium homeostasis, association uncertain (only 1 report so far)

TCP Tropical calcific pancreatitis

within the pancreatic parenchyma was proposed to initiate the inflammatory process. A key role has been attributed to the activation of trypsinogen to trypsin, converting all proteolytic proenzymes to their active form. Three different trypsinogens have been described in human pancreatic juice and have been designated, according to their electrophoretic mobility, as cationic trypsinogen (PRSS1), anionic trypsinogen (PRSS2), and mesotrypsinogen (PRSS3). Compared to the anionic isoenzyme, the cationic trypsinogen autoactivates more easily and is more resistant to autolysis.

By linkage analysis, several groups located a gene for hereditary pancreatitis on the long arm of chromosome 7 (7q35). Subsequently, a mutation in the cationic trypsinogen gene, also referred to as serine protease 1 (*PRSS1*) (OMIM 276000), was identified as the underlying defect. In 5 families, a c.365G>A transition leading to a substitution of arginine by histidine at residue 122 (p.R122H) segregated with the disease [28]. R122H appears to be the most common *PRSS1* mutation observed world-wide.

In following studies, numerous other *PRSS1* alterations have been reported in families with suspected hereditary pancreatitis or in patients without a family history. With the exception of p.A16V, p.N29I, p.N29T, p.R116C, and p.R122C, however, these variations were found only in single patients or families, and a detailed clinical background was mostly not given. Thus, their pathogenic significance remains largely to be elucidated (for detailed information of the different variants see: www.uni-leipzig.de/pancreasmutation).

Although the precise disease mechanisms have not been unravelled, it is now a generally accepted model that an increased intrapancreatic trypsin activity results in pancreatitis (Fig. 1). Site-directed mutagenesis of recombinant human cationic trypsinogen revealed that while most mutations studied (e.g. N29I and R122H) significantly enhance autoactivation *in vitro*, some mutations such as R122H additionally inhibit autolysis of the active enzyme ([29], for review see also: [30]). Thus, gain-of-function mutations leading to enhanced intrapancreatic trypsinogen activation may be the common initiating step of pancreatitis caused by *PRSS1* mutations, whereas stabilization of trypsin may be an accessory mechanism.

So far, only two *PRSS1* variants, p.A16V and p.E79K, display unique features: Recombinant E79K trypsinogen showed *in vitro* unaltered catalytic activity, autolysis, and trypsin inhibitor inhibition. Instead, E79K activated anionic trypsinogen, PRSS2, at least twofold better than wild-type cationic trypsin. Thus, E79K leads to increased trypsinogen activation by trans-activation of PRSS2 instead of autoactivation [31]. However, since E79K was found in a similar frequency in control subjects, its pathogenic relevance remains to be elucidated.

Hereditary pancreatitis was initially defined as an autosomal dominant disease with a penetrance of 70% to

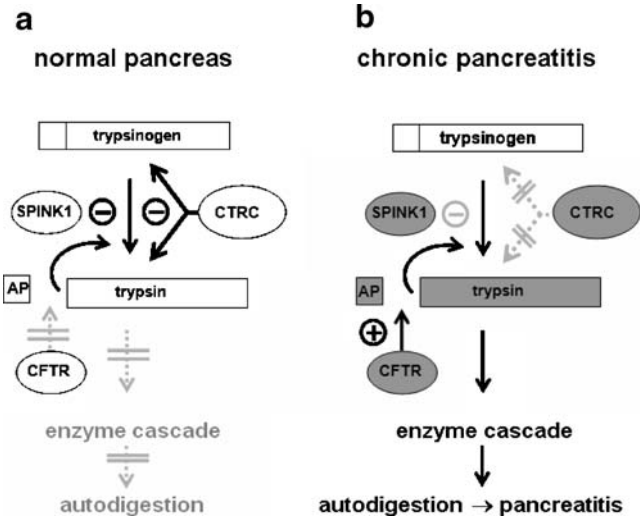

Fig. 1 Model of chronic pancreatitis. **a** Condition in the normal pancreas: trypsin resulting from autoactivation of trypsinogen within the pancreatic parenchyma is inhibited by SPINK1 and degraded by chymotrypsin C (CTRC). This defence mechanism prevents the pancreas from activation of the pancreatic enzyme cascade and autodigestion. **b** Condition in chronic pancreatitis: mutations in *PRSS1*, *SPINK1*, or *CTRC* lead to an imbalance of proteases and their inhibitors within the pancreatic parenchyma resulting in an inappropriate conversion of pancreatic zymogens to active enzymes with autodigestion and inflammation. Mutations in *CFTR* may disturb this delicate balance by intrapancreatic acidification or by a defective apical trafficking of zymogen granules and thus facilitate the intrapancreatic activation of digestive enzymes. *Dark boxes* represent products of mutated genes. (AP, activation peptide)

80% [32]. The clinical characteristics of most families with R122H or N29I are in line with this concept. In contrast, the third most common *PRSS1* variant, A16V, is almost exclusively found in patients without a family history of pancreatitis, indicating that *PRSS1* mutations do not exclusively follow a dominant inheritance pattern [33]. Thus, trypsinogen mutations display a considerable variability of penetrance. Initially, enhanced autoactivation was proposed as pathogenic mechanism. However, studies on recombinant A16V failed to demonstrate this effect. Instead, A16V *PRSS1* showed a fourfold increased rate of activation peptide processing mediated by chymotrypsin C (CTRC), resulting in accelerated trypsinogen activation *in vitro* [34].

Recently, a triplication of an approximately 605-kb segment containing *PRSS1* and *PRSS2* was reported in five families with hereditary pancreatitis. Thus, besides point mutations, a gain of trypsin through a gene dosage effect might also contribute to the disease pathogenesis [35].

The importance of *PRSS1* mutations as pathogenic mediators in hereditary pancreatitis is also underscored by a transgenic mouse model expressing mutant R122H mouse trypsinogen. Pancreata of the transgenic mice displayed early-onset acinar injury, inflammatory cell infiltration, and

enhanced response to caerulein-induced pancreatitis. With progressing age, pancreatic fibrosis and acinar cell dedifferentiation developed [36].

In marked contrast to chronic pancreatitis in Western countries is the complete absence of cationic trypsinogen (*PRSS1*) alterations in TCP. None of 283 Indian patients, including 43 subjects with a family history of the disease, who were investigated by two unrelated research groups showed a pathogenic *PRSS1* variant [37, 38]. Moreover, a recent study failed to detect any trypsinogen copy number variation in 268 Indian TCP patients [39].

4.2 Anionic trypsinogen (PRSS2)

Since increased proteolytic activity due to mutated *PRSS1* enhances the risk for CP, mutations in the isoenzyme, anionic trypsinogen, *PRSS2* (OMIM 601564), may also lead to the disease. Analyzing *PRSS2* in European CP patients and controls, however, revealed a c.571G>A transition resulting in a glycine by arginine exchange at codon 191 (p.G191R), which was over-represented in control subjects. G191R was found in 220/6459 (3.4%) controls but only in 32/2466 (1.3%) patients (odds ratio 0.37; $P=1.1\ 10^{-8}$) [40].

Further analyses showed that patients with G191R were older than those without the protective variant. In the idiopathic/hereditary pancreatitis group, G191R was found in 24/1256 (1.9%) patients older than 20 years compared to 3/601 (0.5%) patients of younger age ($P=0.021$). A similar but statistically insignificant tendency was observed in the alcoholic chronic pancreatitis group: none of the 162 patients with an age of 40 years or younger showed G191R compared to 5/447 (1.1%) of patients older than 40 years [40].

Upon activation by enteropeptidase or trypsin, purified recombinant G191R protein showed a complete loss of trypsin activity due to the introduction of a novel tryptic cleavage site that renders the enzyme hypersensitive to autocatalytic proteolysis [40]. Taken together, the G191R PRSS2 variant mitigates intrapancreatic trypsin activity and thereby plays a protective role against chronic pancreatitis. Albeit the overall contribution of G191R to disease pathogenesis is low, the functional characterization of G191R provided the first example in pancreatitis of a disease-protective genetic variant. This data were confirmed recently by another study investigating Hungarian patients with CP and controls [41]. In the pancreatitis group, 1/140 (0.7%) was heterozygous for G191R, while 19/350 (5.4%) heterozygous carriers were found among the control population ($P=0.0096$, OR 0.13 (95% 0.017–0.945).

Similar to patients in Western countries, the p.G191R variant might also confer a protective effect in TCP. In a recent study, Sundaresan and co-workers investigated 174 patients and 794 control subjects originating from two different Indian centres, Lucknow and Vellore [42]. Only one patient (0.6%) was heterozygous for G191R. Thirteen (1.6%) controls possessed this allele, one of whom was homozygous. Although G191R was 3 times more common in controls than in patients, the difference was statistically insignificant (OR 0.35, 95% CI 0.04–2.7; $P=0.48$). Another pilot study from India, evaluated 68 patients with TCP and 50 controls for *PRSS2* variants [43]. This study failed to detect G191R in their sample, but found one high frequency variant (p.A90A; c.270G>A), which was equally present in patients and controls (allele frequency 0.58 vs. 0.61).

The lack of a significant association does not necessarily exclude a protective role of *PRSS2* G191R in TCP. In contrast to the previously reported population frequency of 3.4% in Europeans, the G191R frequency in Indians (1.6%) is significantly lower (220/6,459 vs. 13/794; OR 0.47; 95% CI 0.26–0.83; $P=0.01$) [42]. Calculating the power for a one-sided significance level of 0.05, this study only reaches a power of 27%. Thus, despite analyzing almost 1,000 subjects, the present study appears still to be underpowered, in most part due to the low frequency of the G191R allele in the background population. The prevalence of G191R differed significantly in control subjects from different parts of the country (east, north and south), with the highest prevalence in north India (5/84, 5.9%) compared to 8/710, (1.1%) in east and south India ($P=0.0071$) [42]. This suggests that there is some heterogeneity in the predisposition to TCP in different parts of India. In summary, the G191R *PRSS2* variant is less frequent in some regions of India as compared to Europe. The variant may play a protective role in the genetics of TCP, though larger studies are necessary to delineate this further.

4.3 Serine protease inhibitor, Kazal type 1 (SPINK1)

The serine protease inhibitor, Kazal type 1 (SPINK1) (OMIM 167790), also known as pancreatic secretory trypsin inhibitor (PSTI), is a potent anti-protease that is thought to be a major inactivation factor of intrapancreatic trypsin activity. SPINK1 was first isolated in the bovine pancreas by Kazal and co-workers in 1948. SPINK1 possesses a reactive site that serves as a specific target substrate for trypsin. However, trypsin inhibition by SPINK1 is only temporary, because the trypsin-SPINK1-complex itself represents as substrate for trypsin, resulting in a subsequent degradation of the inhibitor molecule and in restoration of the original trypsin activity [44].

Since gain-of-function alterations in *PRSS1* leading to a "super-trypsin" cause pancreatitis, it was hypothesized that pancreatitis may also raised by loss-of-function variants in pancreatic trypsin inhibitors. By a candidate gene approach,

SPINK1 was identified as another pancreatitis gene. In 18 out of 96 unrelated paediatric patients, a c.101A>G transition leading to substitution of asparagine by serine at codon 34 (p.N34S) was found. This variant was present in only 1 out of 279 control subjects (OR 62.2, $P<1\ 10^{-10}$). Six patients were homozygous for this mutation [45]. No phenotypic differences between heterozygous and homozygous N34S patients were detected. The association between N34S and CP has been confirmed by numerous others (for review see: [46]). N34S is mostly found in patients without a family history of CP: 15–40% of patients with so-called idiopathic CP carry N34S on one allele or on both alleles. Data taken from 8 larger studies in Europe and US showed that 12.6% of patients with chronic pancreatitis were heterozygous for N34S, and 3.6% were homozygous [46]. In contrast, homozygous individuals were never identified among these controls, whereas heterozygosity was detected at 1.9%. In summary, currently *SPINK1* mutations represent the strongest genetic risk factor in so-called idiopathic chronic pancreatitis in the Western world.

The pathogenic mode of action of N34S, however, remains elusive. Recombinant N34S mutated human SPINK1 did not show any altered trypsin inhibitor capacity or secretion [47–49]. It is worthwhile to note that N34S is in complete linkage disequilibrium (LD) with four other intronic sequence variants: c.56–37T>C, c.87+268A>G, c.195–604G>A, and c.195–66_-65insTTTT [45]. One may speculate that one of these intronic alterations, and not N34S itself, is the pathogenic relevant mutation.

Several other *SPINK1* alterations have been described during the recent years, mainly in single patients or families only (for detailed information of the different variants see: www.uni-leipzig.de/pancreasmutation). With the exception of a few mutations which strongly suggest a loss of function by destruction of the ATG initiation codon (p.M1?), or by shift of reading frame with premature termination (c.27delC, c.98dupA), the functional consequences of most variants are unknown. Recently, expression studies of two dominantly inherited mutations affecting the signal peptide, c.41T>C (p.L14P) and c.41T>G (p.L14R), reported a rapid intracellular degradation of the mutant inhibitor molecules leading to abolished SPINK1 secretion [50]. Even more pronounced than in *PRSS1*, *SPINK1* mutations display a marked variability of penetrance and inheritance pattern. Some variants such as p.M1?, c.27delC, or the codon 14 mutations, that most probably lead to complete functional loss of the mutated allele, appear to follow a dominant trait. Other alterations such as N34S decrease the SPINK1 capacity to a lesser extent and are inherited as recessive or complex traits.

So far, two genetically engineered animal models have evaluated the role of SPINK1 in pancreatitis. Transgenic expression of rat *Spink1* in mice, which leads to an increased endogenous trypsin inhibitor capacity by 190%, significantly reduced the severity of caerulein-induced pancreatitis [51]. Targeted disruption of *Spink3*, the murine orthologue of human *SPINK1*, resulted in autophagic degeneration of acinar cells, impaired regeneration, and death within 2 weeks after birth [52]. In both models, significant inflammatory cell infiltration or enhanced acinar trypsin activity was not observed. By using a more sensitive assay, however, enhanced tryptic activity in pancreatic acini of *Spink3* −/− mice, prepared 1 day after birth, was detected [53].

Patients with TCP display, in contrast to trypsinogen alterations, a very high incidence of *SPINK1* variants, which is markedly more pronounced than that reported from Western countries. In 2002, four different research groups investigated Indian and Bangladeshi patients suffering from TCP, and all reported a strong association with the *SPINK1* p.N34S alteration [37, 54–56]. Altogether, 351 patients and 973 control subjects were investigated in these studies. Approximately 9% of patients were homozygous for N34S and 29% heterozygous, compared to 0.2% and 4.3% in control individuals (Table 2).

Interestingly, N34S is also very common in Indian subjects suffering from alcohol-related chronic pancreatitis. Ten out of 41 (24.4%) affected individuals carried at least one N34S mutated allele [38]. Together with reports from Western countries, this data underlines the flowing transitions between the different subtypes of chronic pancreatitis [57, 58].

The frequent c.163C>T exon 3 variant, which leads to a proline by serine exchange at codon 55 (p.P55S), was not associated with TCP, indicating that this alteration represents an innocuous variation [37, 54, 56]. This data is in line with the results from multiple genetic association studies obtained from white Caucasians as well as with the results from functional studies, which showed no effect of P55S on inhibitor activity or protein secretion [48, 49].

Beside a single TCP patient with c.160T>C transition, leading to a tyrosine by histidine exchange at codon 54, p.Y54H, no other exonic sequence variant was detected in the above-mentioned studies [37, 38, 54, 56]. Y54H occurs in a strictly conserved residue. Transfection experiments with HEK and CHO cells displayed a markedly diminished secretion of mutated H54 SPINK1, suggesting that this variant is a true pathogenic alteration [48, 49].

Chandak et al. observed in 3 out of 68 (4.4%), but in none of 100 control subjects, a G>T transversion in the *SPINK1* promotor region at position −215 relative to the ATG initiation codon (c.1–215G>T) [54]. So far, this variant has never been detected in any chronic pancreatitis patient from other countries. Interestingly, at the same nucleotide a G>A transition (c.1–215G>A) was described in numerous patients from Japan and Western countries, and is unambiguously associated with chronic pancreatitis

Table 2 Frequencies of the *SPINK1* p.N34S variant in TCP

Study	Patients				Controls			
	Number	Homo-zygous (%)	Hetero-zygous (%)	Wild-type (%)	Number	Homo-zygous (%)	Hetero-zygous (%)	Wild-type (%)
Chandak et al. [54]	68	11.8	33.8	54.4	100	0	3.0	97.0
Hassan et al. [55]	180	8.3	25.0	66.7	705	0.3	4.4	95.3
Bhatia et al. [37]	66	13.6	30.3	56.1	92	0	2.2	97.8
Schneider et al. [56]	37	2.7	37.8	59.5	76	0	1.3	98.7
Total	351	9.4	29.1	61.5	973	0.2	4.3	95.5

Figures in square brackets refer to the references for the study

in non-Indian populations. Chandak and colleagues concluded that the c1.-215G>T and c.1–215G>A variants may lie in an unknown *cis*-acting element and thereby affect transcription of mutated SPINK1 with an already compromised functional capacity. However, the c.1–215G>A alteration is, in contrast to c.1–215G>T, in complete linkage disequilibrium with another *SPINK1* variant, c.194+2T>C [59, 60 and own unpublished data]. This transition affects the splice donor site at position 2 of intron 3, which is highly conserved in eukaryotes [45]. Analysis of the mutated mRNA showed a truncated *SPINK1* due to skipping of exon 3 [61]. Thus, the pathogenic significance of the c.1–215 variants remains uncertain.

A sole report of mutated *SPINK1* in a TCP patient outside the Indian subcontinent exists [62]. Recently, a Thai group published a case report of a patient with fibrocalculous pancreatic diabetes (FCPD) and a heterozygous *SPINK1* variant, c.194+2T>C (IVS3+2T>C), which represents the second most common pathogenic *SPINK1* variant in white Caucasians.

In families studied, TCP was associated with different patterns of N34S inheritance [37]. In one family, two affected children were homozygous for N34S, whereas the unaffected parents were heterozygous. This finding could indicate a recessive inheritance pattern. However, in another family, the 3 affected members were heterozygous for N34S. In another family, only the index patient was N34S heterozygous, whereas 3 other affected family members carried a wild-type allele. Lastly, in a simplex family, the affected subject was heterozygous for N34S; his N34S homozygous offspring was found to have no evidence of TCP. This data suggests that despite the striking association between TCP and N34S, this genetic alteration acts in a manner which is presently unclear [37].

In summary, *SPINK1* alterations represent the most common and most important genetic risk factor for the development of TCP, at least in the Indian subcontinent. The frequency of the N34S mutation is higher in TCP patients and Indian controls, as compared to reports from the West. However, there is no specific pattern for inheritance of N34S in families and homozygotes do not reveal a greater severity in their clinical features, such as age of onset or presence of diabetes, when compared with heterozygotes. These data are analogous to that found in studies on ICP in Western countries.

4.4 Chymotrypsinogen C (CTRC)

Chymotrypsinogen C (CTRC) (OMIM 601405), also known as caldecrin, was first isolated from porcine pancreas in 1992 [63]. Recently, Szmola and Sahin-Tóth reported striking evidence that chymotrypsin C may be identical with the so-called enzyme Y, an obscure trypsinogen degrading activity in the human pancreatic juice [64]. Enzyme Y was described by Heinrich Rinderknecht in 1988 [65]. Szmola and Sahin-Tóth showed in their studies that CTRC is capable of degrading all human trypsin and trypsinogen isoforms with high specificity [64]. No other pancreatic protease tested (such as chymotrypsin B1 or B2 and elastase 2A, 3A or 3B) displayed trypsin or trypsinogen degrading activity. Although it is unlikely that CTRC mediated trypsinogen degradation possesses any physiological role in digestion, as high Ca^{2+} concentrations in the intestinal lumen would inhibit trypsinogen degradation and favour trypsinogen activation, Rinderknecht's hypothesis that enzyme Y protects the pancreas by degrading inappropriately activated trypsinogen might be valid. Thus, *CTRC* emerged as a strong novel candidate for a pancreatitis-associated gene.

Indeed, a recent study reinforces the concept that loss-of-function variations of trypsin inhibiting or degrading enzymes play a key role in the pathophysiology of CP [66]. Analysing German subjects with idiopathic or hereditary CP by direct DNA sequencing of all 8 exons of the 8.2 kb long *CTRC*, several *CTRC* variants were detected,

the large majority of which were in exons 2, 3, and 7. Altogether, 11 missense and 2 deletion variants in *CTRC* were identified. The two most frequent variants, c.760C>T (p.R254W) and c.738_761del24 (p.K247_R254del), both located in exon 7, were found in affected individuals with a frequency of 2.1% and 1.2%, respectively. Taken together, the two alterations were significantly over-represented in the pancreatitis group (30 out of 901; 3.3%) compared to controls (21 out of 2,804; 0.7%) (OR=4.6; CI=2.6–8.0; $P=1.3\times10^{-7}$) [66]. Variant c.738_761del24, which causes an in-frame deletion of 8 amino acids from Lys247 through Arg254 (p.K247_R254del), showed the strongest disease association (OR=11.5; CI=3.2–41.5; $P=0.00003$). Subgroup analysis of these two heterozygous variants revealed similar frequency in the hereditary 6/143 (4.2%; 6x p.R254W) and idiopathic pancreatitis (24/758 (3.2%; 13x p.R254W; 11x p.K247_R254del) groups. To confirm these findings in an independent cohort with another inflammatory pancreatic disease, a replication study on subjects with alcohol-related pancreatitis was performed. Again, the two CTRC variants, p.R254W and p.K247_R254del, were found in 10/348 (2.9%) individuals with CP, but only in 3/432 (0.7%) subjects with alcoholic liver disease without CP (OR=4.2; CI=1.2–15.5; $P=0.02$) [66].

To investigate the functional consequences of the *CTRC* missense alterations and the p.K247_R254del in-frame deletion, wild-type and mutant CTRC were expressed in human embryonic kidney (HEK) 293T cells and in the AR42J rat acinar cell line via transient transfection. The functional analyses of the CTRC variants revealed impaired activity and/or reduced secretion indicating that loss-of-function alterations in *CTRC* predispose to pancreatitis by diminishing its protective trypsin degrading activity.

In a cohort of 71 Indian TCP patients, 10 subjects carried 5 different *CTRC* alterations [66]. Interestingly, the frequency of *CTRC* alterations in subjects with pancreatitis was higher in Indians than in white Caucasians. Overall, 14.1% of Indian patients but only 1.2% (1/84) of controls carried a *CTRC* variant (OR=13.6; CI=1.7–109.2; $P=0.0028$). Two relatively frequent variants found in Indians were absent in Germans: the c.217G>A (p.A73T) missense alteration was found in 4 patients and the c.190_193delATTG (p.I64LfsX69) frame-shift deletion was found in 2 patients. Functional assays demonstrated that one of these mutants, p.A73T, led to abrogated CTRC secretion. c.190_193delATTG, leads to a shift of the reading frame with introduction of a premature stop codon and generation of a truncated protein (p.I64LfsX69). Alternatively, the mRNA of this variant may undergo nonsense-mediated decay resulting in no CTRC protein whatsoever. In either event, this frame-shift variant is expected to cause a complete loss of CTRC function.

On the other hand, the p.K247_R254del variant that was relatively frequent in affected individuals from Germany was not found in the Indian population and the enrichment of the p.R254W variant in subjects with tropical pancreatitis did not reach statistical significance. However, due to the significantly smaller size of the Indian cohort relative to the German cohort, caution is warranted in the interpretation of these differences. Nonetheless, the data clearly indicate that *CTRC* variants increase the risk for tropical pancreatitis as well [66].

To elucidate the relationship between *CTRC* alterations and *PRSS1* and *SPINK1* variants, affected subjects were investigated for p.N34S variation of *SPINK1*. Among the patients with tropical pancreatitis, 29/71 (40.9%) were positive for p.N34S. Interestingly, none of the 7 homozygotes, but 6/22 (27.3%) of the heterozygotes carried a *CTRC* variant (1 individual was compound heterozygous for p.A73T and p.D260N). In contrast, only 3/42 (7.1%) individuals with wild-type *SPINK1* were heterozygous for a *CTRC* variant (p.N34S heterozygous vs. p.N34S wild-type, $P=0.051$) [66]. This suggests a possible interaction between pathogenic variations in these 2 loci.

5 Genetic alterations in other genes

Besides genetic alterations in genes encoding enzymes or enzyme inhibitors of the trypsinogen pathway, several other genes have been investigated in TCP. The data obtained from these studies, however, were not investigated by a second independent research group or gave conflicting or negative results.

5.1 Cystic fibrosis transmembrane conductance regulator (CFTR)

Cystic fibrosis (CF) is an autosomal recessive inherited disorder characterised by chronic obstructive pulmonary disease with proximal bronchiectasis often resulting in lung failure, exocrine pancreatic insufficiency with maldigestion, and an elevated sweat chloride concentration. Other clinical features include meconium ileus, liver fibrosis, and male infertility due to obstructive azoospermia. The incidence of cystic fibrosis in Caucasians is approximately 1:2,500. In 1989, *CFTR* (OMIM 602421) was identified as the cystic fibrosis gene by linkage analysis and chromosome walking (positional cloning) [67, 68]. *CFTR* belongs to the ATP-binding cassette (ABC) super-family and encodes a transmembrane protein present at the surface of most epithelial cells, functioning as a cyclic adenosine monophosphate (cAMP)-responsive chloride channel [69]. CFTR has also been implicated in several other processes such as regulation of other ion channels and membrane trafficking.

In 1998, two studies described an association between CP and *CFTR* variants [70, 71]. The link between cystic

fibrosis and CP is supported by the findings that both conditions may show abnormal sweat chloride contents as well as pancreatic ductal obstruction due to inspissated secretions. Moreover, some CF patients suffer from recurrent attacks of pancreatitis.

One study tested 134 patients with CP, including 60 cases with idiopathic and 71 cases with alcohol-induced disease, for 22 *CFTR* mutations [70]. Eighteen patients (13.4%), including 12 with ICP (20%), were heterozygous for a *CFTR* mutation. The frequency of *CFTR* mutations in alcohol-related CP was twice, and in ICP four times higher, than expected. In the other study, 17 *CFTR* mutations in 27 patients with idiopathic CP were investigated [71]. Seven patients (25.9%) had at least one *CFTR* mutation and 1 patient was compound heterozygous. The frequency of *CFTR* mutations in idiopathic CP was six times higher than expected. Both studies, however, investigated the most common *CFTR* mutations only. Following studies showed that, in many instances, mild i.e. not classic CF-causing variants are associated with CP.

While the association between *CFTR* and ICP is now well established, the pathogenic mechanisms are poorly understood. One may speculate that idiopathic CP represents nothing else than "atypical" cystic fibrosis caused by the combination of two mild or of one mild and a severe *CFTR* mutation. Subsequent studies analyzing the complete *CFTR* coding sequence as well as *PRSS1* and *SPINK1* found that in Western countries 25% to 30% carried at least one *CFTR* mutation, but only few patients were compound-heterozygous [72, 73]. Several CP patients, however, were trans-heterozygous for a *CFTR* alteration and a *SPINK1* or *PRSS1* variant, respectively, illuminating the significance of the combination of mutations in different genes in the disease pathogenesis [72, 73].

Data on *CFTR* variants in TCP are very limited and so far only a single study exists. Although all 27 *CFTR* coding exons, including the *5T* allele, were analysed, the study population consisted of only 18 Indian TCP patients [74]. Two patients (11%) showed a *CFTR* variant: one subject was homozygous for *5T* and the other heterozygous for p.R1070Q, which is presumed to be a mild missense variant. Neither patient gave a history of chronic sinopulmonary disease, and chest x-rays and pulmonary function tests were normal. The overall frequency of *CFTR* alterations was 0.083 (3/36), which was far lower than that observed in white Caucasian subjects with CP (0.20–0.24). In summary, further genetic studies are necessary to elucidate the significance of *CFTR* variants in TCP.

5.2 Cathepsin B (CTSB)

The lysosomal cysteine protease cathepsin B (CTSB) (OMIM 116810) is presumed to perform an essential role in intracellular activation of trypsinogen. However, the mostly circumstantial evidence is based on its *in vitro* capability to activate trypsinogen and its co-localisation in secretory granules containing digestive enzymes [75, 76]. Moreover, after induction of experimental caerulein-induced pancreatitis, *Ctsb* knock-out mice displayed a lowered trypsin activity within the gland and a diminished acinar necrosis compared to wild-type animals [77]. However, inhibition of rat cathepsin B with synthetic inhibitors gave conflicting results, either increasing or decreasing zymogen activation, or failing to ameliorate the course of experimental pancreatitis [78, 79].

Recently, Mahurkar and co-workers performed a mutational analysis of *CTSB* and found a association between TCP and a c.76C>G transversion in exon 3, leading to leucine by valine exchange at codon 26 (p.L26V) [80]. Investigating 306 Indian patients with TCP and 330 controls, the *26V* allele was significantly overrepresented in patients (minor allele frequency 0.46) compared to control subjects (minor allele frequency 0.29) (P=0.013). Unfortunately, no data on the genotype distributions was given in this study. So far, functional studies on biochemical consequences of the L26V variant are lacking. According to own preliminary results, we were unable to replicate the findings of Mahurkar and colleagues. In a pilot study, we investigated 69 patients with TCP and 88 control subjects originating from North India for L26V and found similar allele frequencies in both groups (minor allele frequency 0.32 in TCP vs. 0.31 in controls) (unpublished data).

5.3 Regenerating islet-derived genes 1α (REG1A & REG1B)

Secretory pancreatic stone protein (PSPS) (OMIM 167770), also known as lithostathine or as REG1A (regenerating islet-derived 1-alpha), is the major component of the protein matrix of calculi in chronic pancreatitis [81]. The high abundance of PSPS in pancreatic juice (10–14% of total protein) suggests an important role in exocrine pancreatic secretion. Pancreatic juice is supersaturated with calcium carbonate, and *in vitro* experiments showed that PSPS inhibits crystal growth [82, 83]. Demonstration of decreased pancreatic mRNA levels and diminished PSPS in the pancreatic juice of patients with chronic calcific pancreatitis lead to the hypothesis that CP may be related to diminished crystal growth inhibition caused by defect PSPS [84]. However, the physiological relevance of PSPS in calcium carbonate inhibition has been questioned by others [85]. Thus, the precise function of *REG1A* remains obscure.

Several research groups have analysed *REG1A* and *REG1B* (partially) in patients affected by TCP [86–89]. None of these studies observed a significant association

between TCP and REG genes, rendering a pathogenic role of this gene family unlikely.

5.4 Angiotensin converting enzyme (ACE)

The systemic hormonal renin-angiotensin system (RAS) has important physiological functions in the regulation of blood pressure, fluid and electrolyte balance. They are mainly executed by angiotensin II, the product of hydrolysis of angiotensin I by angiotensin-converting enzyme (ACE). Apart from circulating RAS, many tissues and organs exhibit their own RAS products and activities. In the pancreas, major components of such a local pancreatic RAS have been implicated in the regulation of acinar digestive enzyme secretion, islet hormonal secretion, and ductal anion secretion [90]. Markedly up-regulated expression of RAS components has also been described in acute pancreatitis [91]. Several recent studies have demonstrated that ACE inhibitors and angiotensin II type 1 receptor blockers prevent pancreatic stellate cell activation and fibrosis [92, 93]. A frequent *ACE* insertion/deletion (I/D) alteration in intron 16 exists that accounts for nearly half of the variation of serum ACE levels [94].

A recent study investigated the *ACE* I/D variant in 171 Indian subjects with TCP and compared the genotype and allele frequencies with 99 ethnically matched healthy controls [95]. No significant differences with TCP *per se*, as well as with fibrosis or progression of the disease, were observed. The authors concluded that the *ACE* I/D variant does not play a role in the pathogenesis of TCP.

5.5 Calcium-sensing receptor (CASR)

The calcium-sensing receptor (CASR) (OMIM 601199) is a G protein-coupled plasma membrane receptor that is activated by high extracellular calcium levels. CASR adjusts the extracellular calcium set point, thus regulating PTH secretion and renal calcium excretion [96]. Mutations in *CASR* can result in gain or loss of receptor function. Gain-of-function alterations are associated with autosomal dominant hypocalcaemia and Bartter syndrome type V, while loss-of-function mutations are associated with familial hypocalciuric hypercalcaemia (FHH) and neonatal hyperparathyroidism.

Recently, Murugaian and co-workers screened the complete *CASR* coding region by direct DNA sequencing in 35 Indian TCP patients and in 35 controls [97]. This study was driven by two previous case reports of a patient with FHH and pancreatitis and of a patient with CP (without altered serum calcium levels), who were trans-heterozygous for a *CASR* and the *SPINK1* N34S variant [98, 99]. In the TCP study, the authors found 4 different heterozygous *CASR* variants in 5/35 patients (14.9%), but in none of control subjects. All alterations were hitherto undescribed [97]. It is worth to note that all *CASR* mutated TCP patients displayed calcium level within the normal range and that the functional consequences of these novel *CASR* variants remain to be determined.

5.6 Major histocompatibility complex (MHC) genes

A few studies have investigated the association of genes encoding the major histocompatibility complex (MHC), also designated as human leukocyte antigen (HLA), and TCP. One study reported an association with the DQ beta gene. DQ beta T2/T6 was present in 39% of patients compared to 19% in control subjects (Pc=0.04) [100]. Another study found a positive association with DQ9 (A*0201-B*0303) [101]. In a family-based study, using TDT analysis, an overall association between TCP and HLA-DQB1 (P=0.003) was reported, that was largely due to a positive association with HLA-DQB1*0302 and a negative association with HLA-DQB1*0202 [102]. However, these findings should be interpreted with caution. In the past, numerous different MHC antigens have been linked with different types of CP such as alcoholic or idiopathic disease. Most studies reported a positive association for one or more alleles, but these could not be confirmed by other groups Thus, the significance of these observed associations remains to be elucidated in further studies.

6 Conclusions

Recent genetics studies indicate that TCP and FCPD are diseases which share, at least in part, similar genetic susceptibility loci with ICP described in the West. Thus, it would be correct to view TCP and ICP as being related, rather than distinct entities. The higher prevalence of TCP in the Indian subcontinent, in comparison to ICP in the West, as well as areas of endemicity, still needs to be explained. Whether this is due to differences in genetic predisposition, specific environmental factors, or, as is most likely, a combination of both, still needs to be ascertained.

Genetic alterations in at least two genes encoding the serine protease inhibitor, Kazal type 1 (*SPINK1*) and chymotrypsinogen C (*CTRC*) are clearly associated with so-called idiopathic pancreatitis found in white Caucasians, as well as with so-called tropical calcific pancreatitis.

In contrast to Western countries, alterations in the cationic trypsinogen (*PRSS1*) are of minor or no importance in pathogenesis of so-called TCP. Variants in the genes coding for anionic trypsinogen (PRSS2), the cystic fibrosis transmembrane conductance regulator (*CFTR*), cathepsin B (*CTSB*), and the calcium-sensing receptor (*CASR*) might

play a role in TCP. However, further studies are required to clarify the significance of these genetic loci.

The majority of white patients with idiopathic/hereditary chronic pancreatitis, as well as the preponderance of patients suffering from TCP, do not harbour a mutation in one of the above-mentioned genes. Thus, it is likely that further genes will be involved in their pathogenesis.

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