Pancreas

ORIGINAL ARTICLE

CFTR, SPINK1, CTRC and PRSS1 variants in chronic pancreatitis: is the role of mutated CFTR overestimated?

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ABSTRACT

Objective In chronic pancreatitis (CP), alterations in several genes have so far been described, but only small cohorts have been extensively investigated for all predisposing genes.

Design 660 patients with idiopathic or hereditary CP and up to 1758 controls were enrolled. PRSS1, SPINK1 and CTRC were analysed by DNA sequencing, and cystic fibrosis transmembrane conductance regulator (CFTR) by melting curve analysis.

Results Frequencies of CFTR variants p.R75Q, p.I148T, 5T-allele and p.E528E were comparable in patients and controls. We identified 103 CFTR variants, which represents a 2.7-fold risk increase (p<0.0001). Severe cystic fibrosis (CF)-causing variants increased the risk of developing CP 2.9-fold, and mild CF-causing variants 4.5-fold (p<0.0001 for both). Combined CF-causing variants increased CP risk 3.4-fold (p<0.0001), while non-CF-causing variants displayed a 1.5-fold over-representation in patients (p=0.14). CFTR compound heterozygous status with variant classes CF-causing severe and mild represented an OR of 16.1 (p<0.0001). Notably, only 9/660 (1.4%) patients were compound heterozygotes in this category. Trans-heterozygosity increased CP risk, with an OR of 38.7, with 43/660 (6.5%) patients and 3/1667 (0.2%) controls being trans-heterozygous (p<0.0001).

Conclusions Accumulation of CFTR variants in CP is less pronounced than reported previously, with ORs between 2.7 and 4.5. Only CF-causing variants reached statistical significance. Compound and trans-heterozygosity is an overt risk factor for the development of CP, but the number of CFTR compound heterozygotes in particular is rather low. In summary, the study demonstrates the complexity of genetic interactions in CP and a minor influence of CFTR alterations in CP development.

Significance of this study

What is already known on this subject?
▶ In addition to variants of PRSS1, SPINK1 and CTRC, cystic fibrosis transmembrane conductance regulator (CFTR) variants contribute to the pathogenesis of chronic pancreatitis (CP).
▶ Patient and control cohorts in studies that have screened for variants in all known genes were small, and CFTR variants in most cases have not been analysed comprehensively.
▶ Data obtained for cystic fibrosis (CF) indicate that predominantly mild CFTR variants with residual CFTR function can be expected in CP.

What are the new findings?
▶ CF-causing CFTR variants increase the risk of CP development (CF-causing severe OR 2.9; CF-causing mild OR 4.5), while non-CF-causing variants are more common in patients (1.5-fold), but this does not reach statistical significance; the overall risk contribution of CFTR variants to the pathogenesis of CP seems to be lower than previously reported (OR 2.7).
▶ All CFTR compound heterozygotes carry at least one mild CFTR allele, and compound heterozygotes with two CF-causing variants are rare (1.4%), which shows that atypical CF is present in a small minority of CP patients only.
▶ Complex genotypes such as homozygous, compound heterozygous and trans-heterozygous carriers are significantly more common in patients with ORs for distinct complex genotypes of 16.1—135.4, indicating that CP in many cases is a complex inherited disease.

How might it impact on clinical practice in the foreseeable future?
▶ In patients with early-onset CP and no identifiable aetiological factor, genetic testing for distinct variants (PRSS1, SPINK1, CTRC and CFTR) should be offered after comprehensive education.
▶ It seems that, for the pathogenesis of CP, the influence of CFTR variants has so far been overestimated, but a diagnosis of CF has to be ruled out, especially in patients with early-onset CP.

INTRODUCTION

Chronic pancreatitis (CP) is a relapsing or continuing inflammatory disease characterised by recurrent or persisting abdominal pain leading to permanent impairment of pancreatic function in some patients.[1 2] A genetic background for CP was first described in a pedigree with an obviously autosomal dominant inheritance pattern in 1952.[3] Depiction of the underlying defects in different genes established a model in which premature trypsin activation plays a central pathogenic role.

Genetic variations in cationic trypsinogen (PRSS1) (OMIM *276000) were predominantly found in families with a positive history of CP.[4] Functional analyses indicate that gain-of-function—that is, premature trypsinogen activation—might be the underlying mechanism of mutated PRSS1.[5] This concept of gain-of-function is further supported by a description of duplication and triplication of the trypsinogen locus in some CP families originating from France.[6]

In addition, alterations in the serine protease inhibitor, Kazal type 1 (SPINK1) (OMIM *167790), a potent trypsin inhibitor, have been described in patients with different forms of CP.[7–10] In contrast with PRSS1, SPINK1 variants seem to induce a loss of SPINK1 inhibitor function. Even though there is a strong genetic association of the most commonly found SPINK1 alteration, p.N34S, with CP, functional data revealed no reduced inhibitory capacity of this variant.[11] Following the pathophysiological concept, a loss-of-function variant (p.G191R) in the anionic trypsinogen (PRSS2) (OMIM *601564), the second major trypsin isoform was detected more commonly in controls.[12] Variations in chymotrypsinogen C (CTRC) (OMIM *601405), an enzyme that promotes degradation of trypsin, have been associated with idiopathic and alcoholic CP. On a functional level, CTRC mutations exhibited diminished secretion and/or activity.[13]

Cystic fibrosis (CF) (OMIM 219700) is an autosomal recessive inherited disorder characterised by chronic endobronchial infection, pancreatic insufficiency and male infertility. Approximately 1–2% of patients with CF have recurrent attacks of pancreatitis. Similarly to CF, some patients with CP show raised sweat chloride levels. Moreover, in both disorders, obstruction of the pancreatic duct due to dense secretion can be found, suggesting a possible link between these different disease entities.[14–18] Subsequently, variants of the cystic fibrosis transmembrane conductance regulator (CFTR) (OMIM *602421) were found to be associated with idiopathic and alcoholic CP.[19 20]

The complexity of the role of CFTR variants in CP and other diseases is disclosed by the large number of about 1900 variants that have so far been described (http://www.genet.sickkids.on.ca/cftr). Analysis of CFTR is further complicated by its size, comprising 27 exons. Moreover, the functional effects of CFTR variants are poorly understood in most cases.

Analysis of chloride channel properties has revealed abnormal sweat chloride levels and nasal potential differences in patients with CP compared with healthy controls when at least one CFTR variant was reported. The degree of impairment correlated with the number and type of CFTR variants detected, and was less pronounced than in pancreatic-sufficient or -insufficient CF patients.[21] In CF, genotype–phenotype correlation has been described for exocrine pancreatic function. Patients with exocrine insufficiency in most cases carry two 'severe' CFTR variants, while patients with sufficient exocrine function bear at least one 'mild' CFTR variant. Pancreatic insufficiency prevalence scores in CF patients with pancreatitis were most likely 'mild', in contrast with patients without pancreatitis who displayed 'moderate–severe' scores.[22] A possible conclusion is that, for the development of CP, residual CFTR function and pancreatic acinar cells are essential, which might be missing in CF patients with 'moderate–severe' pancreatic insufficiency prevalence scores and/or two 'severe' CFTR mutations.

These facts raise the question whether predominantly 'mild' CFTR variants can be found in CP and whether CFTR variants, like variants of other known pancreatitis-causing genes, are more common in different age groups. Owing to the limitations mentioned above, the number of patients and controls, if investigated at all, were rather small in previous studies, and a complete analysis of CFTR was performed in small cohorts only. To investigate the complex genetic basis of CP, we examined CFTR as well as PRSS1, SPINK1 and CTRC in a large cohort of patients with CP and in controls.

PATIENTS AND METHODS
Study population
The study protocol was approved by the medical ethics review committees of the University of Berlin (Charité) and the University of Leipzig. All participants gave informed consent. If patients were under the age of 18 years, parents gave informed consent.

Diagnosis of CP was based on two or more of the following findings as published previously[13]: briefly, a history of recurrent pancreatitis or recurrent abdominal pain typical of CP, pancreatic calcifications and/or pancreatic ductal irregularities revealed by endoscopic retrograde pancreaticography or by MRI of the pancreas, and/or pathological sonographic findings. Hereditary CP (HP) was diagnosed when one first-degree relative or two or more second-degree relatives had recurrent acute or chronic pancreatitis without any apparent precipitating factor. Idiopathic CP (ICP) was diagnosed in the absence of a positive family history or possible precipitating factors, such as alcohol abuse, trauma, medication, infection and metabolic disorders.

In total, 660 unrelated patients with HP or ICP from Berlin and Leipzig were enrolled (347 female; 107 HP, 553 ICP; median age 16 years, mean age 20.1 years, age range 1–81 years). The study population from Berlin comprised 439 patients (244 female; 101 HP, 338 ICP; median age 13 years, mean age 17.1 years, age range 1–70 years). A total of 221 German patients were recruited from Leipzig (118 female; six HP, 215 ICP; median age 23 years, mean age 26 years, age range 5–81 years).

Healthy unrelated German controls recruited for genetic association studies (medical students/staff, healthy retirees and healthy blood donors; n=1758) originated from Berlin and Leipzig (961 female, 104 of unknown gender; median age 32 years, mean age 38.7 years, age range 17–101 years). The control group from Berlin included 1644 individuals (927 female, 52 of unknown gender; median age 31 years, mean age 38.5 years, age range 17–101 years), whereas the Leipzig controls consisted of 114 individuals (34 female, 52 of unknown gender; median age 44.5 years, mean age 43.6 years, age range 20–64 years).

METHODS
PCR
Genomic DNA was extracted from peripheral blood leucocytes. PCR was performed using 0.75 U AmpliTaq Gold polymerase (Applied Biosystems, Darmstadt), 400 μM dNTPs, 1.5 mM MgCl$_2$ and 0.1 μM each primer in a total volume of 25 μl. Cycle conditions were an initial denaturation for 12 min at 95°C followed by 48 cycles of 20 s denaturation at 95°C, 40 s annealing at specific temperatures, 90 s primer extension at 72°C, and a final extension for 2 min at 72°C in an automated thermal cycler.

Primers were synthesised according to the published nucleotide sequences (PRSS1: GenBank: NM_002769.3; SPINK1: GenBank: NM_003122.3; CTRC: GenBank: NM_007272.2; CFTR: GenBank: NM_000492.3). Primers for the analysis of PRSS1, SPINK1 and CTRC were used as published previously.[7 13 23] Oligonucleotide sequences and annealing temperatures are available upon request.

DNA sequencing of *SPINK1*, *CTRC* and *PRSS1*

PCR products were digested with shrimp alkaline phosphatase (USB) and exonuclease I (GE Healthcare, Munich). Cycle sequencing was performed using BigDye terminator mix (Applied Biosystems). Reaction products were purified by ethanol precipitation or on a Sephadex G-50 column (GE Healthcare, Munich) and loaded on to an ABI 3100-Avant or an ABI 3730 fluorescence sequencer (Applied Biosystems). DNA mutation numbering is based on cDNA sequences that use the A of the ATG start codon as nucleotide +1. Mutations are described according to the nomenclature recommended by the Human Genome Variation Society (http://www.hgvs.org/mutnomen).

Melting curve analysis of *CFTR*

Melting curve analysis for 37 *CFTR* variants was performed in the LightCycler instrument (Roche Diagnostics, Mannheim) using a pair of fluorescent resonance energy transfer (FRET) probes. The following *CFTR* variants were analysed with specific FRET probes: p.E60X, p.R75Q, p.G85E, p.R117H, p.I148T, c.621 +1G>T (IVS4+1G>T), c.711+1G>T (IVS5+1G>T), c.1078delT, p.R334W, p.R347P, *9-13TG*, *5-9T*, p.A455E, p.M470V, p.F508del, c.1716G>A (p.E528E), c.1717-1G>A (IVS10-1G>A), p.G542X, p.S549N, p.R553X, p.R560T, c.1898+1G>A (IVS12 +1G>A), c.2143delT, c.2183AA>G, c.2562T>G, c.2657+5G>A (IVS14B+5G>A), p.L997F, p.I1005R, p.Y1092X, p.D1152H, p.R1162X, c.3659delC, p.S1235R, p.S1251N, p.W1282X, p.N1303K, and c.4389G>A. Since FRET probes were designed in nearly all cases complementary to the mutant, the probes were able to detect more than 100 additional variants located adjacent to the specific target nucleotide (unpublished data). All probes were devised and synthesised by TIB Molbiol (Berlin, Germany). In complex target regions, locked nucleic acids were integrated for better discrimination. Sequences of the probes are available upon request. If melting curves differed from the expected curves for the wild-type or the specific variant, DNA sequencing was performed to identify the underlying alteration.

Statistical analysis

The significance of the differences between variant frequencies in affected individuals and controls was tested by two-tailed Fisher's exact test and was calculated using GraphPad Prism (v4.03). $p < 0.05$ was considered to be significant. We excluded *CFTR* variants p.R75Q, p.I148T, the poly-T tract and p.E528E, *PRSS1* variant p.S124F, *SPINK1* variants c.1-52G>T and p.P55S, and *CTRC* variants p.R37Q, p.K151N and p.K172E from all calculations, because of missing functional data or a missing genetic association.

RESULTS
Genetic analysis of *SPINK1*, *CTRC* and *PRSS1*

We found *PRSS1* variants in 8.3% of the patients (55/660), but in none of 1758 controls ($p < 0.0001$) (table 1). The following single variants were significantly more common in patients: p.A16V, p.N29I, p.R122C and p.R122H with ORs ranging from 29.5 to 141.1 ($p = 0.002$ for p.R122C; for all other variants $p < 0.0001$).

In *SPINK1*, 12 different variants were detected. In the patient group, c.(1-215G>A;194+2T>C) ($p < 0.0001$, OR 38.1) and p.N34S (homozygous/heterozygous: $p < 0.0001$, OR 95.6 and 10.5) were significantly accumulated. In summary, after exclusion of c.1-52G>T and p.P55S, 19.6% of the patients (129/660) carried a *SPINK1* variant compared with 1.5% of the controls (27/1758) ($p < 0.0001$) accounting for an OR of 15.6 (95% CI 10.2 to 23.9).

A *CTRC* variant was determined in 4% of patients (22/546) and in 0.8% of controls (13/1667). This represents a significant accumulation in patients ($p < 0.0001$; OR 5.3, 95% CI 2.7 to 10.7). Two single variants, p.R254W and p.K247_R254del, were more common in patients ($p = 0.0005$, OR 4; $p = 0.003$, OR 28.2, respectively). To obtain stringent results, calculations were based on the smallest number of controls that were analysed for *CTRC* exons 2, 3 and 7 (table 1).

Genetic analysis of *CFTR*

Melting curve analysis detected 103 variants and characterised frequencies of five common alterations (c.1210-34TG(9_13), c.1210-12T(5_9), p.M470V, c.2562T>G and c.4389G>A). In total, *CFTR* variants were found in 103/660 (15.6%) patients compared with 112/1758 (6.4%) controls (OR 2.7, 95% CI 2 to 3.6). In subgroups, OR was 2.9 (95% CI 2 to 4.3) for CF-causing severe variants, 4.5 (95% CI 2.3 to 8.8) for CF-causing mild variants, and 3.4 (95% CI 2.4 to 4.8) for all CF-causing variants, whereas, for all non-CF-causing variants, there was a 1.5-fold over-representation in patients only ($p = 0.14$) (table 2). Notably, p.I148T was never found in *cis* with c.3199del6, and p.S1235R was always associated with p.G628 (wild-type).

Three common variants, c.1210-34TG(9_13), p.M470V and c.4389G>A, were differently distributed in patients and controls (table 3). *TG10*-allele of c.1210-34TG(9_13) was found more commonly in patients (435/1320, 33%) than in controls (983/3516, 28%) ($p = 0.0008$, OR 1.3), whereas allele *TG11* was found more often in controls (2137/3516, 60.8%) than in patients (750/1320, 56.8%) ($p = 0.014$, OR 0.9). With regard to p.M470V, the *470M* allele accumulated in patients (607/1320, 46%) in comparison with controls (1480/3516, 42.1%) ($p = 0.02$, OR 1.2). Analysis of c.4389G>A displayed an over-representation of the *4389A* allele in patients (366/1320, 27.7%; controls: 544/2330, 23.3%; $p = 0.004$, OR 0.8). Genotype data of these variants confirmed results for *TG10/10*, p.470MM and c.4389AA ($p < 0.0001$, $p < 0.009$ and $p < 0.0005$, respectively).

To confirm that association of these variants is not due to co-segregation with p.F508del, which is found in 6.7% of patients and in 2.7% of control subjects, we calculated data for the variants after exclusion of p.F508del carriers in both patients and controls. Association of c.1210-34TG(9_13) (*TG10*-allele and *TG10/10* genotype) and c.4389G>A (*A*-allele and c.4389AA genotype) persisted after correction ($p = 0.001$, $p = 0.002$; $p = 0.0005$, $p = 0.0003$). In contrast, association of p.M470 did not withstand correction with regard to the genotype ($p = 0.1$), while the *470M* allele was still over-represented in patients ($p = 0.01$) (data not shown). The allele frequency for p.M470V did not withstand Bonferroni correction.

Homozygous and compound heterozygous carriers of *CFTR*, *SPINK1* and *CTRC* variants

The *SPINK1* combination p.N34S/p.N34S was found in 17/660 (2.6%) and p.N34S/c.(1-215G>A;194+2T>C) in 7/660 patients (1.1%) (total 24/660, 3.6%), but never in controls ($p < 0.0001$; OR 135.4, 95% CI 8.2 to 2231). One patient was compound heterozygous for *CTRC* p.V235I/p.R254W and one homozygous for p.R254W (1/546; 0.2% each). None of the 1700 controls was homozygous or compound heterozygous for *CTRC* ($p = 0.2$).

Compound heterozygous *CFTR* carriers with severe and mild CF-causing variants were found in 9/660 (1.4%) patients and in 1/1758 (0.06%) controls ($p = 0.002$; OR 16.1, 95% CI 1.9 to 134.2). Combinations of CF-causing severe or CF-causing mild or non-CF-causing with non-CF-causing variants were present in 17/660 (2.6%) patients and in 14/1758 (0.8%) controls ($p = 0.001$,

Table 1 Distribution of *SPINK1*, *CTRC* and *PRSS1* variants in patients and controls

Gene	Variant	Patients	Controls	p Value	OR (95% CI)
PRSS1	p.A16V	14/660 (2.1%)	0/1758	<0.0001	78.9 (4.7 to 1325)
	p.N29I	8/660 (1.2%)	0/1758	<0.0001	45.8 (2.6 to 795.4)
	p.N29T	1/660 (0.2%)	0/1758	NS	—
	p.R116C	2/660 (0.3%)	0/1758	NS	—
	p.R122C	5/660 (0.8%)	0/1758	0.002	29.5 (1.6 to 534.8)
	p.R122H	25/660 (3.8%)	0/1758	<0.0001	141.1 (8.6 to 2323)
	p.S124F*	1/660 (0.2%)	0/1758	NS	—
	Total	55/660 (8.3%)	0/1758	<0.0001	322.4 (19.8 to 5230)
SPINK1	c.(1-215G>A;194+2T>C)	14/660 (2.1%)	1/1758 (0.06%)	<0.0001	38.1 (5 to 290.3)
	c.1-53C>T	1/660 (0.2%)	0/1758	NS	—
	c.1-52G>T*	1/660 (0.2%)	0/1758	NS	—
	p.M1?	1/660 (0.2%)	0/1758	NS	—
	c.27delC	1/660 (0.2%)	0/1758	NS	—
	p.L14P	1/660 (0.2%)	0/1758	NS	—
	p.L14R	1/660 (0.2%)	0/1758	NS	—
	p.N34S (hom)	17/660 (2.6%)	0/1758	<0.0001	95.6 (5.8 to 1594)
	p.N34S (het)	90/660 (13.6%)	26/1758 (1.5%)	<0.0001	10.5 (6.7 to 16.4)
	p.P55S*	4/660 (0.6%)	16/1758 (0.9%)	NS	—
	p.R65Q	1/660 (0.2%)	0/1758	NS	—
	p.R67Q	1/660 (0.2%)	0/1758	NS	—
	p.V60YfsX35	1/660 (0.2%)	0/1758	NS	—
	Total	129/660 (19.6%)	27/1758 (1.5%)	<0.0001	15.6 (10.2 to 23.9)
CTRC	p.R37Q*	3/546 (0.6%)	7/1680 (0.4%)	NS	—
	p.Q44Q	1/546 (0.2%)	0/1680	NS	—
	p.K151N*	1/546 (0.2%)	0/1667	NS	—
	p.K172E*	0/546	1/1667 (0.06%)	NS	—
	p.G217S	1/546 (0.2%)	1/1700 (0.06%)	NS	—
	p.V235I	1/546 (0.2%)	1/1700 (0.06%)	NS	—
	p.R254W (het)	14/546 (2.6%)	11/1700 (0.7%)	0.0005	4 (1.8 to 9)
	p.R254W (hom)	1/546 (0.2%)	0/1700	NS	—
	p.K247_R254del	4/546 (0.7%)	0/1700	0.003	28.2 (1.5 to 525.2)
	Total	22/546 (4%)	13/1667 (0.8%)	<0.0001	5.3 (2.7 to 10.7)

In *CTRC*, not all patients and controls were analysed completely.
*Excluded from calculations because of missing or unknown significance: *PRSS1* p.S124F (no functional data); *SPINK1* c.1-52G>T (no functional data); *SPINK1* p.P55S (similar distribution in patients and controls; functional data showed no effect on inhibitor activity); *CTRC* p.R37Q, p.K151N and p.K172E (all: functional data indicate no significant alteration of activity or secretion; also for p.R37Q: similar distribution in patients and controls).
het, heterozygous; hom, homozygous.

OR 3.3, 95% CI 1.6 to 6.7). Except one (p.R117H (7T/7T)/p.S1235), these compound heterozygotes were excluded in the overall computations, because *CFTR* variant p.R75Q, p.I148T, 5T or p.E528E was present in at least one allele. In total, 10/660 (1.5%) patients and 1/1758 (0.06%) controls were compound heterozygous *CFTR* carriers (p<0.0001, OR 27, 95% CI 3.5 to 211.7). Individually, none of the combinations of two *CFTR* variants was significantly over-represented in patients or controls (table 4).

Trans-heterozygous carriers of *CFTR*, *SPINK1*, *CTRC* and *PRSS1* variants

Trans-heterozygotes were found in 43/660 (6.5%) patients and 3/1667 (0.2%) controls (p<0.0001, OR 38.7, 95% CI 12 to 125.1). After exclusion of carriers in which a non-CF-causing variant was essential for trans-heterozygous status, 33/660 (5%) patients and 2/1667 (0.1%) controls were trans-heterozygous (p<0.0001, OR 43.8, 95% CI 10.5 to 183.2). In addition, 24/660 (3.6%) patients and 2/1700 (0.1%) controls were trans-hetero-zygotes in that *CFTR* variants p.R75Q, p.I148T, 5T and p.E528E were essential for trans-heterozygous status (p<0.0001, OR 30.8, 95% CI 7.3 to 131). These samples were not integrated into our computations, because the over-representation of these combinations is explained by the accumulation of the concom-

itant variant (eg, p.N34S, see below) and the mentioned *CFTR* variants were found with similar frequencies in patients and controls. It is noteworthy that nine of the patients carried a severe *PRSS1* variant (p.N29I, p.R122C and p.R122H). As such in these cases the *CFTR* variants should be of minor or no importance for the development of CP. Calculations in patients were referred to the total number of 660 patients, although 114 patients were not analysed for *CTRC* variants, and in controls to 1667 individuals who were investigated completely for variants in all four genes to obtain more rigorous results (table 5).

Individually, we found a significant strengthening for the following combinations in patients: p.A16V(*PRSS1*)/p.N34S (*SPINK1*), p.R122H(*PRSS1*)/p.E528E(*CFTR*), p.N34S(*SPINK1*)/p.R75Q(*CFTR*), p.N34S(*SPINK1*)/5T(*CFTR*), p.N34S(*SPINK1*)/p.F508del(*CFTR*) and p.N34S(*SPINK1*)/p.S1235R(*CFTR*) (p=0.007, p=0.03, p=0.03, p=0.002, p<0.0001 and p=0.03; range of OR 10.7–45.8). In these cases, the over-representation is also most probably due to the strengthening of p.R122H (*PRSS1*) and p.N34S (*SPINK1*) as shown for *CFTR* p.R75Q (see below). One patient (p.N34S/p.R254W/p.R117H(7T/7T); 1/546, 0.2%) carried variants in three genes (p value 0.6).

SPINK1 variant p.N34S (heterozygous and homozygous) was found in combination with *CFTR* variants p.R75Q (heterozygous and homozygous) in 6/660 patients (0.9%), with 5T in

Table 2 *CFTR* variants detected by melting curve analysis

Gene	Variant	Patients	Controls	p Value	OR (95% CI)
CFTR (CF-causing, severe)	p.F508del	44/660 (6.7%)	48/1758 (2.7%)	<0.0001	2.5 (1.7 to 3.9)
	p.R117H (*5T/7T*)	2/660 (0.3%)	1/1758 (0.06%)	NS	—
	p.G542X	1/660 (0.2%)	1/1758 (0.06%)	NS	—
	c.1717-1G>A	3/660 (0.5%)	1/1758 (0.06%)	NS	—
	p.E585X	0/660	1/1758 (0.06%)	NS	—
	c.2183AA>G	0/660	1/1758 (0.06%)	NS	—
	p.R1158X	1/660 (0.2%)	0/1758	NS	—
	p.R1162X	1/660 (0.3%)	0/1758	NS	—
	p.N1303K	3/660 (0.5%)	0/1758	NS	—
	Total	55/660 (8.3%)	53/1758 (3%)	<0.0001	2.9 (2 to 4.3)
CFTR (CF-causing mild)	p.R117H (*7T/7T*)	13/660 (2%)	8/1758 (0.5%)	0.0009	4.4 (1.8 to 10.7)
	p.R117H (*7T/9T*)	3/660 (0.5%)	1/1758 (0.06%)	NS	—
	p.R347H	1/660 (0.2%)	0/1758	NS	—
	p.R347P	1/660 (0.2%)	0/1758	NS	—
	p.A455E	1/660 (0.2%)	0/1758	NS	—
	c.2657+5G>A	1/660 (0.2%)	0/1758	NS	—
	p.D1152H	3/660 (0.5%)	5/1758 (0.3%)	NS	—
	Total	23/660 (3.5%)	14/1758 (0.8%)	<0.0001	4.5 (2.3 to 8.8)
CFTR (non CF-causing)	p.R74Q	2/660 (0.3%)	0/1758	NS	—
	p.R75Q (het)*	29/660 (4.4%)	59/1758 (3.4%)	NS	—
	p.R75Q (hom)*	2/660 (0.3%)	1/1758 (0.06%)	NS	—
	p.Y84H	0/660	1/1758 (0.06%)	NS	—
	p.A120T	0/660	1/1758 (0.06%)	NS	—
	p.I148T*	4/660 (0.6%)	11/1758 (0.6%)	NS	—
	p.I507V	1/660 (0.2%)	2/1758 (0.1%)	NS	—
	p.F508C	1/660 (0.2%)	0/1758	NS	—
	c.1716+12T>C	0/660	1/1758 (0.06%)	NS	—
	p.E528E (het)*	36/660 (5.5%)	82/1758 (4.7%)	NS	—
	p.E528E (hom)*	0/660	2/1758 (0.1%)	NS	—
	c.1898+8C>G	0/660	1/1758 (0.06%)	NS	—
	p.H667Y	1/660 (0.2%)	0/1758	NS	—
	p.R668C	5/660 (0.8%)	3/1758 (0.2%)	NS	—
	p.G691R	0/660	1/1758 (0.06%)	NS	—
	p.L997F	5/660 (0.8%)	6/1758 (0.3%)	NS	—
	p.S1235R	10/660 (1.5%)	18/1758 (1.0%)	NS	—
	Total (excluded)*	25/660 (3.8%)	45/1758 (2.6%)	NS	—
CFTR (CF-causing)	Total (all)	78/660 (11.8%)	67/1758 (3.8%)	<0.0001	3.4 (2.4 to 4.8)
CFTR (all)	Total (excluded)*	103/660 (15.6%)	112/1758 (6.4%)	<0.0001	2.7 (2 to 3.6)

The table is divided into three parts. Upper section, CF-causing severe variants; middle section, CF-causing mild variants; bottom section, non-CF-causing variants according to a recent consensus statement.[24] Calculations to compare *CFTR* variant distribution in patients and controls were performed in the three different groups, for all CF-causing variants and for all *CFTR* variants in total.
*Variants p.R75Q, p.I148T and p.E528E were excluded from calculations because of their similar frequencies in patients and controls.
CF, cystic fibrosis; het, heterozygous; hom, homozygous.

8/660 patients (1.2%), and with p.E528E (heterozygous and homozygous) in 6/660 patients (0.9%) compared with 1/1758 (0.06%) controls for combination with p.R75Q and *5T*, and 2/1758 controls (0.1%) for combination with p.E528E (p.R75Q: p=0.002, OR 16.1, 95% CI 1.92 to 134; *5T*: p=0.0002, OR 21.6, 95% CI 2.7 to 172.8; p.E528E: p=0.008, OR 8, 95% CI 1.6 to 40). Since this might be explained by the over-representation of p.N34S in patients, we calculated frequencies of p.N34S carriers in patients (n=109) and controls (n=26). Thereafter, no significant association was obtained (p.R75Q: 6/109 patients, 5.5%, 1/26 controls, 3.9%, p=1.0; *5T*: 8/109 patients, 7.3%, 1/26 controls, 3.9%, p=1.0; p.E528E: 6/109 patients, 5.5%, 2/26 controls, 7.7%, p=0.7). In contrast, 6/31 (19.4%) patients with p.R75Q also carried p.N34S, whereas only 1/60 (1.7%) of controls with p.R75Q showed the p.N34S variant, representing the data expected in patients and controls. On analysis of about 200 parents for *CFTR*, p.R75Q was found to pass from one parent to the child in 6/13 cases (46%) only, which further underlines that this variant most likely does not contribute to disease pathogenesis.

Variant distribution in patients with HP and ICP

In the online supplementary tables 1–5, we have additionally divided the patient group into patients with HP and ICP and calculated p values and ORs for these groups. As expected, *PRSS1* variants were predominantly found in HP patients (30/107, 28%) compared with ICP patients (25/553, 4.5%). Frequencies of *SPINK1*, *CTRC* and *CFTR* variants did not differ significantly between HP and ICP. CF-causing severe variants were over-represented in HP patients (6/107, 5.6%), but this did not reach statistical significance in contrast with ICP patients (49/553, 8.9%). Compound heterozygous *CFTR* carriers were more common among HP (4/107, 3.7%) than ICP (5/553, 0.9%) patients.

Variant distribution in patients aged >20 and <20 years

In younger patients, overall *PRSS1* variants were 2.9-fold more common (>20 years: 9/239, 3.8%; <20 years: 46/421, 10.9%; p=0.001, OR 3.1, 95% CI 1.5 to 6.5), whereas overall *SPINK1* variants were similarly distributed (56/239, 23.4%; 73/421,

Table 3 Allele frequencies of *CFTR* variants c.1210-34TG(9_13), c.1210-12T(5_9), p.M470V, c.2562T>G and c.4389G>A

Variant	Allele	Patients	Controls	p Value	OR (95% CI)
c.1210-34GT(9_13)	*TG9*	1/1320 (0.08%)	4/3516 (0.1%)	NS	—
	TG10	435/1320 (33%)	983/3516 (28%)	0.0008	1.3 (1.1 to 1.5)
	TG11	750/1320 (56.8%)	2137/3516 (60.8%)	0.014*	1.2 (1 to 1.3)*
	TG12	130/1320 (9.8%)	385/3516 (10.9%)	NS	—
	TG13	4/1320 (0.3%)	7/3516 (0.2%)	NS	—
c.1210-12T(5_9)	*5T*	40/1320 (3%)	107/3516 (3%)	NS	—
	7T	1106/1320 (83.8%)	3003/3516 (85.4%)	NS	—
	9T	174/1320 (13.2%)	406/3516 (11.5%)	NS	—
c.1408A>G p.M470V	M	607/1320 (46%)	1480/3516 (42.1%)	0.02*	1.2 (1 to 1.3)*
	V	713/1320 (54%)	2036/3516 (57.9%)		
c.2562T>G	T	864/1320 (65.5%)	1530/2284 (67%)	NS	—
	G	456/1320 (34.5%)	754/2284 (33%)		
c.4389G>A	G	954/1320 (72.3%)	1786/2330 (76.7%)	0.004	1.3 (1.1 to 1.5)
	A	366/1320 (27.7%)	544/2330 (23.3%)		

c.2562T>G was investigated in 1142 controls and c.4389G>A in 1165 controls, respectively.
*No significant p value after Bonferroni correction.

17.3%; p=0.07). *CTRC* variants were also found in similar frequencies in both age groups (>20 years: 6/170, 3.5%; <20 years: 16/376, 4.3%; p=0.8). All *CFTR* variants combined were more common in younger patients (74/421, 17.6%) than in

older patients (29/239, 12.1%), but this difference was not significant (p=0.07).

Complex genotypes, which includes homozygous carriers of p.N34S and p.R254W and compound heterozygous and trans-

Table 4 Homozygous and compound heterozygous patients and controls with at least two *CFTR*, *SPINK1* or *CTRC* variants

Gene	Variant	Patients	Controls	p Value	OR (95% CI)
CFTR (CF-causing severe or CF-causing mild/CF-causing mild)	p.F508del/p.R117H (*7T/9T*)	2/660 (0.3%)	1/1758 (0.06%)	NS	—
	p.F508del/p.R347H	1/660 (0.2%)	0/1758	NS	—
	p.F508del/p.D1152H†	1/660 (0.2%)	0/1758	NS	—
	p.R117H (*7T/7T*)/c.2657+5G>A	1/660 (0.2%)	0/1758	NS	—
	p.R117H (*7T/7T*)/p.R1158X	1/660 (0.2%)	0/1758	NS	—
	p.R117H (*7T/7T*)/c.1717-1G>A	1/660 (0.2%)	0/1758	NS	—
	p.R117H (*7T/9T*)/p.N1303K	1/660 (0.2%)	0/1758	NS	—
	p.D1152H†/p.N1303K	1/660 (0.2%)	0/1758	NS	—
	Total	9/660 (1.4%)	1/1758 (0.06%)	0.002	16.1 (1.9 to 134.2)
CFTR (CF-causing severe or CF-causing mild or non-CF-causing/Non-CF-causing)	p.F508del/p.R75Q*	0/660	1/1758 (0.06%)	NS	—
	p.F508del/*5T**	2/660 (0.3%)	1/1758 (0.06%)	NS	—
	p.F508del/p.E528E*	2/660 (0.3%)	2/1758 (0.1%)	NS	—
	p.R75Q*/*5T**	1/660 (0.2%)	1/1758 (0.06%)	NS	—
	p.R75Q*/p.E528E*	2/660 (0.3%)	2/1758 (0.1%)	NS	—
	p.R117H (*7T/7T*)/p.R75Q*	1/660 (0.2%)	0/1758	NS	—
	p.R117H (*7T/7T*)/p.E528E*	1/660 (0.2%)	0/1758	NS	—
	p.R117H (*7T/7T*)/p.S1235R	1/660 (0.2%)	0/1758	NS	—
	p.I148T*/*5T**	0/660	1/1758 (0.06%)	NS	—
	p.R347P/p.E528E*	1/660 (0.2%)	0/1758	NS	—
	p.E528E*/*5T**	1/660 (0.2%)	4/1758 (0.23%)	NS	—
	p.H667Y/*5T**	1/660 (0.2%)	0/1758	NS	—
	p.L997F/*5T**	1/660 (0.2%)	0/1758	NS	—
	p.L997F/p.E528E*	0/660	1/1758 (0.06%)	NS	—
	p.D1152H†/*5T**	1/660 (0.2%)	0/1758	NS	—
	p.S1235R/*5T**	2/660 (0.3%)	1/1758 (0.06%)	NS	—
	Total	17/660 (2.6%)	14/1758 (0.8%)	0.001	3.3 (1.6 to 6.7)
CFTR	Total (all, excluded)*	10/660 (1.5%)	1/1758 (0.06%)	<0.0001	27 (3.5 to 211.7)
SPINK1	p.N34S (hom)	17/660 (2.6%)	0/1758	<0.0001	95.6 (5.7 to 1594)
	p.N34S (het)/c.(1-215G>A;194+2T>C)	7/660 (1.1%)	0/1758	<0.0001	40.4 (2.3 to 708.2)
	Total	24/660 (3.6%)	0/1758	<0.0001	135.4 (8.2 to 2231)
CTRC	p.R254W (hom)	1/546 (0.2%)	0/1700	NS	—
	p.R254W/p.V235I	1/546 (0.2%)	0/1700	NS	—
	Total	2/546 (0.4%)	0/1700	NS	—

For *CFTR* compound heterozygous carriers, calculations were performed for patients and controls carrying a combination of one CF-causing severe or a CF-causing mild in addition with one CF-causing mild variant (upper section).
In the lower section, the combinations CF-causing severe/non-CF-causing, CF-causing mild/non-CF-causing and non-CF-causing/non-CF-causing are presented.
All compound heterozygotes in this section except one (p.R117H (*7T/7T*)/p.S1235R) carry *CFTR* variants that were not over-represented in patients on at least one allele.
*Compound heterozygotes in that variants p.R75Q, p.I148T, *5T*-allele, and p.E528E were essential for compound heterozygous status were excluded from calculations.
†Variant p.D1152H can have a broad phenotype and might be classified as a CF-causing severe variant.
het, heterozygous; hom, homozygous.

Pancreas

heterozygous carriers, were found in similar distribution in younger (55/421, 13.1%) and older (24/239 (10%) patients (p=0.3), when summarised together (table 6).

DISCUSSION

This is the largest study to date that presents genetic data for *CFTR*, *SPINK1*, *CTRC* and *PRSS1* in patients with ICP and HP.

Although alcohol abuse is the predominant cause of CP, we decided to exclude patients with alcoholic CP because ICP/HP and alcoholic CP seem only share the same genetic risks to some extent.[9 13 25] Therefore, for alcoholic CP with expected modest genetic risk factors, a different approach such as genome-wide association studies is warranted. Extensive screening for all known CP predisposing genes allows us to display the complex

Table 5 Trans-heterozygous carriers of *CFTR*, *SPINK1*, *CTRC* and *PRSS1* variants

PRSS1	SPINK1	CTRC	CFTR	Patients	Controls	p Value	OR (95% CI)
p.A16V	p.N34S (het)			4/660 (0.6%)	0/1758	0.007	24.1 (1.3 to 448.7)
p.A16V		p.R254W		1/546 (0.2%)	0/1700	NS	—
p.A16V			p.F508C†	1/660 (0.2%)	0/1758	NS	—
p.A16V			p.L997F†	2/660 (0.3%)	0/1758	NS	—
p.R116C	p.N34S (het)		p.E528E*	1/660 (0.2%)	0/1758	NS	—
p.R122C		p.R254W		1/546 (0.2%)	0/1700	NS	—
p.R122C			p.F508del	2/660 (0.3%)	0/1758	NS	—
p.R122H		p.G217S	5T/7T*	1/546 (0.2%)	0/1700	NS	—
	p.N34S (het)/ c.(1-215G >A;194+2T>C)	p.R254W		2/546 (0.4%)	0/1700	NS	—
	p.M1?	p.R254W		1/546 (0.2%)	0/1700	NS	—
	p.L14P	p.R254W		1/546 (0.2%)	0/1700	NS	—
	p.N34S (het)	p.R254W		1/546 (0.2%)	0/1700	NS	—
	p.N34S (het)	p.R254W	p.R117H (7T/7T)/ p.R75Q	1/546 (0.2%)	0/1700	NS	—
	p.N34S (het)	p.K247_R254del	p.E528E*	1/546 (0.2%)	0/1700	NS	—
	p.N34S (het)	p.R254W	p.E528E*	1/546 (0.2%)	0/1700	NS	—
	c.(1-215G>A; 194+2T>C)		p.F508del	1/660 (0.2%)	0/1758	NS	—
	p.N34S (hom)		p.I507V†	1/660 (0.2%)	0/1758	NS	—
	p.N34S (hom)		p.S1235R†	1/660 (0.2%)	0/1758	NS	—
	p.N34S (het)		p.R117H (5T/7T)	1/660 (0.2%)	0/1758	NS	—
	p.N34S (het)		p.F508del/ p.R117H (7T/9T)	1/660 (0.2%)	0/1758	NS	—
	p.N34S (het)		p.F508del/p.E528E	1/660 (0.2%)	1/1758 (0.06%)	NS	—
	p.N34S (het)		p.F508del/ p.E528E/5T/7T	0/660	1/1758 (0.06%)	NS	—
	p.N34S (het)		p.R117H (7T/7T)	1/660 (0.2%)	0/1758	NS	—
	p.N34S (het)		p.F508del	8/660 (1.2%)	0/1758	<0.0001	45.8 (2.6 to 795.4)
	p.N34S (het)		p.R668C†	1/660 (0.2%)	0/1758	NS	—
	p.N34S (het)		p.S1235R†	3/660 (0.5%)	0/1758	0.03	18.7 (1 to 363.2)
	p.N34S (het)		p.N1303K	1/660 (0.2%)	0/1758	NS	—
		p.R254W	p.R117H (7T/7T)/ c.1717-1G>A	1/546 (0.2%)	0/1700	NS	—
		p.R254W	p.R668C†	0/546	1/1700 (0.06%)	NS	—
		p.R254W	p.L997F†	1/546 (0.2%)	0/1700	NS	—
Total (all)				43/660 (6.5%)	3/1667 (0.2%)	<0.0001	38.7 (12 to 125.1)
Total (CF-causing)				33/660 (5%)	2/1667 (0.1%)	<0.0001	43.8 (10.5 to 183.2)
p.N29I			p.R75Q	1/660 (0.2%)	0/1758	NS	—
p.R122C			5T/9T	1/660 (0.2%)	0/1758	NS	—
p.R122H			p.R75Q	2/660 (0.3%)	0/1758	NS	—
p.R122H			5T/7T	2/660 (0.3%)	0/1758	NS	—
p.R122H			p.E528E	3/660 (0.5%)	0/1758	0.03*	18.7 (1 to 363.2)
	p.N34S (het)/ c.(1-215G >A;194+2T>C)		5T/9T	1/660 (0.2%)	0/1758	NS	—
	c.(1-215G>A; 194+2T>C)		5T/9T	0/660	1/1758 (0.06%)	NS	—
	c.(1-215G>A; 194+2T>C)		p.E528E	1/660 (0.2%)	0/1758	NS	—
	c.1-53C>T		p.R75Q	1/660 (0.2%)	0/1758	NS	—
	p.N34S (hom)		p.R75Q (hom)	1/660 (0.2%)	0/1758	NS	—
	p.N34S (hom)		5T/7T	1/660 (0.2%)	0/1758	NS	—
	p.N34S (hom)		p.E528E	2/660 (0.3%)	0/1758	NS	—

Continued

Table 5 Continued

PRSS1	SPINK1	CTRC	CFTR	Patients	Controls	p Value	OR (95% CI)
	p.N34S (het)		p.R75Q	4/660 (0.6%)	1/1758 (0.06%)	0.03*	10.7 (1.2 to 96.1)
	p.N34S (het)		p.I148T	1/660 (0.2%)	0/1758	NS	—
	p.N34S (het)		5T/7T	5/660 (0.8%)	0/1758	0.002	29.5 (1.6 to 534.8)
	p.R65Q		p.R75Q	1/660 (0.2%)	0/1758	NS	—
	p.R67C		p.E528E	1/660 (0.2%)	0/1758	NS	—
		p.G217S	p.E528E	0/546	1/1700 (0.06%)	NS	—

To obtain stringent results, the total number of patients (n=660) was used for calculations of p values, although some of the patients were not completely analysed. Likewise, we used the number of controls that were analysed completely (n=1667). In the upper section, trans-heterozygotes who in parts carry a CF-causing severe, CF-causing mild and non-CF-causing CFTR variant are summarised.

*In these patients, the 5T allele and variant p.E528E are not essential for trans-heterozygous status. Computations were performed for all trans-heterozygous patients (Total all). In addition, a calculation was performed after exclusion of patients in whom non-CF-causing variants were essential for trans-heterozygous status.

†(Total CF-causing). In the lower section, trans-heterozygotes with non-CF-causing variants, p.R75Q, p.I148T, 5T-allele and p.E528E, are displayed, which were excluded from calculations because their over-representation is due to accumulation of the concomitant variant (eg, p.N34S) in patients (see Results section) or the CFTR variant displayed similar frequencies in patients and controls (p.R75Q, p.I148T, 5T-allel, and p.E528E).

het, heterozygous; hom, homozygous.

Table 6 Distribution of PRSS1, SPINK1, CTRC and CFTR variants in different age groups (age >20 or <20 years)

Gene	Variant	Controls	Patients age >20	p Value	OR (95% CI)	Patients age <20	p Value	OR (95% CI)
PRSS1	p.A16V	0/1758	3/239 (1.3%)	0.002	52.1 (2.7 to 1012)	11/421 (2.6%)	<0.0001	98.5 (5.8 to 1677)
	p.N29I	0/1758	1/239 (0.4%)	NS	—	7/421 (1.7%)	<0.0001	63.6 (3.6 to 1117)
	p.N29T	0/1758	0/239	NS	—	1/421 (0.2%)	NS	—
	p.R116C	0/1758	0/239	NS	—	2/421 (0.5%)	0.05	21 (1 to 437.7)
	p.R122C	0/1758	0/239	NS	—	5/421 (1.2%)	<0.0001	46.4 (2.6 to 842.2)
	p.R122H	0/1758	5/239 (2.1%)	<0.0001	82.5 (4.5 to 1498)	20/421 (4.8%)	<0.0001	179.6 (10.8 to 2977)
	p.S124F*	0/1758	1/239 (0.4%)	0.1	—	0/421	NS	—
	Total	0/1758	9/239 (3.8%)	<0.0001	145 (8.4 to 2500)	46/421 (10.9%)	<0.0001	435.5 (26.8 to 7088)
SPINK1	c.(1-215G>A;194+2T>C)	1/1758 (0.06%)	9/239 (3.8%)	<0.0001	68.8 (8.7 to 545.4)	5/421 (1.2%)	0.0005	21.1 (2.5 to 181.3)
	c.27delC	0/1758	1/239 (0.4%)	NS	—	0/421	NS	—
	c.1-53C>T	0/1758	0/239	NS	—	1/421 (0.2%)	NS	—
	c.1-52G>T*	0/1758	0/239	NS	—	1/421 (0.2%)	NS	—
	p.M1?	0/1758	0/239	NS	—	1/421 (0.2%)	NS	—
	p.L14P	0/1758	0/239	NS	—	1/421 (0.2%)	NS	—
	p.L14R	0/1758	0/239	NS	—	1/421 (0.2%)	NS	—
	p.N34S (hom)	0/1758	4/239 (1.7%)	0.0002	67.2 (3.6 to 1253)	13/421 (3.1%)	<0.0001	56 (7.3 to 429.4)
	p.N34S (het)	26/1758 (1.5%)	40/239 (16.7%)	<0.0001	13.4 (8 to 22.4)	50/421 (11.9%)	<0.0001	9 (5.5 to 14.6)
	p.P55S*	16/1758 (0.9%)	0/239	NS	—	4/421 (1%)	NS	—
	p.V60YfsX35	0/1758	0/239	NS	—	1/421 (0.2%)	NS	—
	p.R65Q	0/1758	1/239 (0.4%)	NS	—	0/421	NS	—
	p.R67Q	0/1758	1/239 (0.4%)	NS	—	0/421	NS	—
	Total	27/1758 (1.5%)	56/239 (23.4%)	<0.0001	19.6 (12.1 to 31.8)	73/421 (17.3%)	<0.0001	13.5 (8.5 to 21.2)
CTRC	p.R37Q*	7/1680 (0.42%)	1/170 (0.6%)	NS	—	2/376 (0.5%)	NS	—
	p.Q44Q	0/1680	0/170	NS	—	1/376 (0.3%)	NS	—
	p.K151N*	0/1667	0/170	NS	—	1/376 (0.3%)	NS	—
	p.K172E*	1/1667 (0.06%)	0/170	NS	—	0/376	NS	—
	p.G217S	1/1700 (0.06%)	0/170	NS	—	1/376 (0.3%)	NS	—
	p.V235I	1/1700 (0.06%)	0/170	NS	—	1/376 (0.3%)	NS	—
	p.R254W (het)	11/1700 (0.7%)	5/170 (3%)	0.01	4.7 (1.6 to 13.6)	9/376 (2.4%)	0.004	3.8 (1.6 to 9.2)
	p.R254W (hom)	0/1700	0/170	NS	—	1/376 (0.3%)	NS	—
	p.K247_R254del	0/1700	1/170 (0.6%)	NS	—	3/376 (0.8%)	0.003	31.9 (1.6 to 618.8)
	Total*	13/1667 (0.8%)	6/170 (3.5%)	0.006	4.7 (1.8 to 12.4)	16/376 (4.3%)	<0.0001	5.7 (2.7 to 11.9)
CFTR	CF-causing severe	53/1758 (3%)	18/239 (7.5%)	0.001	2.6 (1.5 to 4.6)	37/421 (8.8%)	<0.0001	3.1 (2.1 to 4.8)
	CF-causing mild	14/1758 (0.8%)	5/239 (2.1%)	NS	—	18/421 (4.3%)	<0.0001	5.6 (2.7 to 11.3)
	Non CF-causing	45/1758 (2.6%)	6/239 (2.5%)	NS	—	19/421 (4.5%)	0.05	1.8 (1 to 3.1)
	Total	112/1758 (6.4%)	29/239 (12.1%)	0.003	2 (1.3 to 3.1)	74/421 (17.6%)	<0.0001	3.1 (2.3 to 4.3)
Complex genotypes	Homozygosity‡	0/1758	4/239 (1.7%)	0.0002	67.2 (3.6 to 1253)	14/421 (3.3%)	<0.0001	125.1 (7.5 to 2104)
	Compound heterozygous†	1/1758 (0.06%)	8/239 (3.3%)	<0.0001	60.9 (7.6 to 489)	10/421 (2.4%)	<0.0001	42.8 (5.5 to 335.1)
	Trans-heterozygous†	3/1667 (0.2%)	12/239 (5%)	<0.0001	29.3 (8.2 to 104.7)	31/421 (7.4%)	<0.0001	44 (13.4 to 145)
	Total	4/1667 (0.2%)	24/239 (10%)	<0.0001	46.4 (16 to 135.1)	55/421 (13.1%)	<0.0001	62.5 (22.5 to 173.5)

CFTR variants were grouped into three categories for clarity reasons as denoted in table 2: CF-causing severe, CF-causing mild, non-CF-causing. CFTR variants, p.R75Q, p.I148T and p.E528E, were excluded from computations as explained before. In the bottom section, complex genotypes—that is, homozygous (p.N34S and p.R254W), compound heterozygous (with inclusion of SPINK1 p.N34S/c.(1-215G>A;194+2T>C)) and trans-heterozygous—of all analysed genes were compared.

*Excluded from calculations because of missing or unknown significance: PRSS1 p.S124F (no functional data); SPINK1 c.1-52G>T (no functional data); SPINK1 p.P55S (similar distribution in patients and controls; functional data showed no effect on inhibitor activity); CTRC p.R37Q, p.K151N and p.K172E (all: functional data indicate no significant alteration of activity or secretion; also for p.R37Q: similar distribution in patients and controls).

†Compound and trans-heterozygous carriers with p.R75Q, p.I148T, 5T-allele and p.E528E were excluded as explained before.
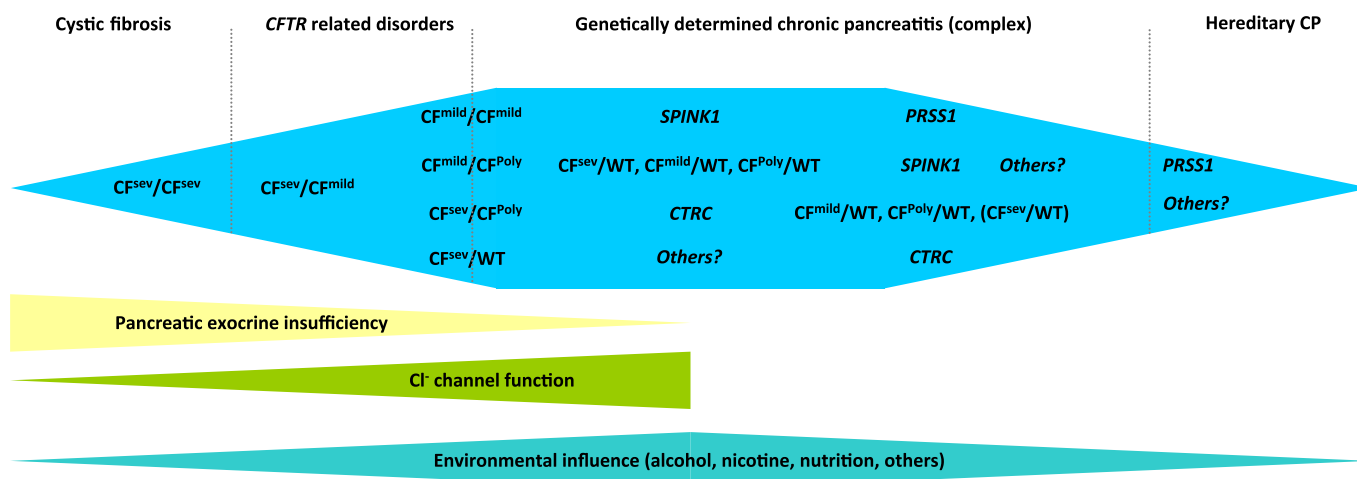
‡Homozygosity for p.N34S and p.R254W.

Figure 1 Chronic pancreatitis (CP) is a complex genetic disorder that develops through the interaction of environmental and genetic factors. Cystic fibrosis and hereditary CP seem to require no, or little, environmental influence apart from their disease-causing genetic basis. Patients with CP compound-heterozygous for *CFTR* variants may in many cases be designated as *CFTR*-related disorders. *CFTR* function decreases, which is displayed in positive sweat chloride measurements, when *CFTR* variants accumulate in patients, whereas exocrine pancreatic insufficiency increases. CF, *CFTR* variant; *PRSS1*, cationic trypsinogen; *SPINK1*, serine protease inhibitor, Kazal type 1; *CTRC*, chymotrypsinogen C; CF^{sev}, CF-causing severe variant; CF^{mild}, CF-causing mild variant; CF^{Poly}, non-CF-causing variant.

genetic interactions in ICP and HP. Association of *CFTR* variants with CP is well established, but classification of some variants with regard to their influence on the development of CP and the aligned risk increase for *CFTR* carriers had not unambiguously been defined. In a recent consensus statement, the clinical consequences of some *CFTR* variants and their association with exocrine pancreatic function have been classified.[24] Accordingly, several common variants should be excluded from the computation, which will provide a more conservative and realistic calculation.

PRSS1, *SPINK1* and *CTRC* variants were over-represented in patients, as reported previously. *PRSS1* variants were predominantly found in younger patients (<20 years) (p=0.001), whereas *SPINK1* variants did not differ in the two age groups (p=0.07). In line with former studies, we detected the *SPINK1* p.P55S variant in similar frequencies in patients and controls.[7 8 26–28] Moreover, functional studies failed to show any effect of p.P55S on SPINK1 inhibitory capacity.[29] Consequently, we excluded p.P55S from all calculations.

In contrast with other authors, we excluded *CFTR* variants p. R75Q, p.I148T, *5T* and p.E528E from our calculations, because they were distributed similarly in patients and controls.[30 31] Thereby, overall, *CFTR* variants displayed a 2.7-fold risk increase for CP development. We found the strongest association with CF-causing mild *CFTR* variants (OR 4.5); however, these variants were found in only 3.5% of patients, while CF-causing severe variants were found in 8.3% of our patients and increased CP risk 2.9-fold. The importance of non-CF-causing variants is questionable. Although an association has been described in former studies, the overall effect of these variants is expected to be rather low, and, in our study, these variants did not reach significance (p=0.14). In total, *CFTR* variants displayed a 3.1-fold risk increase (p<0.0001) in young patients under 20 years of age, whereas in older patients the risk increase was only twofold (p=0.003) (comparison of both groups: p=0.07).

Some common variants such as the poly-T tract in intron 9 influence CFTR transcript levels.[32] In addition, common *CFTR* haplotypes seem to modulate susceptibility to CP.[33] A large number of our patients were part of a study that identified

a *CFTR* haplotype (*TG10-7T-M470*) that increases the risk of CP. Thus, it is not surprising that our analysis of these common variants is in line with the former finding. *TG10* and *c.4389A* accumulated in CP patients even after exclusion of p.F508del carriers, highlighting the potential importance of common variants in CP.

Compound heterozygotes for *CFTR* and *SPINK1* variants were found more commonly in patients than in controls. Overall, *CFTR* compound heterozygotes displayed an OR of 27 (95% CI 3.5 to 211.7), but this accounts for 10 patients (1.5%) only. In contrast, 24/660 (3.6%) patients were homozygous or compound heterozygous for a *SPINK1* variant (p<0.0001; OR 135.4, 95% CI 8.2 to 2231), but only 2/546 (0.4%) for a *CTRC* variant (p=0.2). In this regard, it is justifiable to compare ORs of *PRSS1* variant carriers (OR 322.4), homozygous and compound heterozygous *SPINK1* carriers (OR 135.4) and compound heterozygous *CFTR* carriers (OR 27), while overall carriers of *CTRC* variants (OR 5.3) can be compared with overall *SPINK1* (OR 15.7) and *CFTR* carriers (OR: CF-causing severe, 2.9; CF-causing mild, 4.5; all *CFTR* variants, 2.7) to classify their influence on the pathogenesis of CP. These data highlight the minor importance of *CFTR* variants in CP development.

Recent publications partly presented a stronger accumulation of *CFTR* variants and compound heterozygotes, but comprehensive screening of a circumscribed German group of patients and controls revealed comparable results.[34] Different results are attributable to smaller sample sizes analysed, a lack of screening of controls, and inclusion of common but innocuous variants, whereas we chose a very stringent and, in our eyes, reliable calculation by excluding these variants.

The complexity of CP pathogenesis is demonstrated when interactions of all so far known pancreatitis genes are investigated. Trans-heterozygotes accumulated in patients, and 6.5% of our patients carried variants in at least two genes (OR 38.7, 95% CI 12 to 125.1). We could not replicate the data of a former study that reported a 900-fold increase in risk for patients with two *CFTR* variants and *SPINK1* variant p.N34S, which is probably because we analysed control subjects, unlike the former

publication.[30] Nevertheless, trans-heterozygotes in particular demonstrate that carriage of different variants in different pancreatitis-associated genes increases the CP risk substantially and may in some individuals explain why the disease developed.

However, there is some dispute about how to interpret complex genetic results. In a recent study, trans-heterozygosity for *SPINK1* p.N34S with *CFTR* p.R75Q was reported to increase CP risk.[31] We also demonstrate a significant accumulation of p. N34S/p.R75Q trans-heterozygotes in patients (6/660, 0.9% vs controls 1/1758, 0.06%), and the data seem to portend an association for the combination of p.N34S with *CFTR* *5T*-allele and p.E528E also. Of note, the calculation of trans-heterozygotes in the overall cohorts, which are all patients in comparison with all controls, may be incorrect. From our point of view, the data have to be compared between p.N34S carriers in patients and p.N34S carriers in controls. When we performed the calculation in these analogous groups, the described association cannot be sustained. Frequencies of p.R75Q in patients and controls carrying p.N34S were 6/109 (5.5%) and 1/26 (3.9%), respectively (p=1.0). This is also true for the combinations of p. N34S with *5T* or p.E528E and supports the notion that the observed unequal distribution of trans-heterozygotes in the overall cohorts is due to the over-representation of p.N34S in the patient group.

In summary, we provide further evidence that CP is a complex genetic disorder characterised by different genetic alterations in different genes and a complex interplay of these alterations. *CFTR* variants found in CP patients are in most cases 'mild' variants with residual CFTR function, and in all *CFTR* compound heterozygotes at least one 'mild' *CFTR* allele was present. Frequencies of *CFTR* variants and *CFTR* compound heterozygotes in CP are lower than previously reported. With regard to the associated genes in CP, it seems conclusive to propose two terminal positions, which are CF on the one side and hereditary CP on the other (figure 1). Both forms will be characterised by 'severe' genetic changes sufficient for disease development in nearly all cases, while the influence of environmental factors may be minor. Pancreatitis will be rare in patients with two 'severe' CF-causing variants, and the numbers of patients with pancreatitis will increase when at least one 'mild' *CFTR* variant is present. In between, complex inherited CP including *CFTR*-related disorders and pancreatitis seems to be distributed, and there may be a floating transition to patients that carry variants in different pancreatitis-associated genes.

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CFTR, SPINK1, CTRC and *PRSS1* variants in chronic pancreatitis: is the role of mutated *CFTR* overestimated?

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