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CFTR function is impaired in a subset of patients with pancreatitis carrying rare *CFTR* variants

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ABSTRACT

Background: Many affected by pancreatitis harbor rare variants of the cystic fibrosis (CF) gene, *CFTR*, which encodes an epithelial chloride/bicarbonate channel. We investigated CFTR function and the effect of CFTR modulator drugs in pancreatitis patients carrying *CFTR* variants.
Methods: Next-generation sequencing was performed to identify *CFTR* variants. Sweat tests and nasal potential difference (NPD) assays were performed to assess CFTR function *in vivo*. Intestinal current measurement (ICM) was performed on rectal biopsies. Patient-derived intestinal epithelial monolayers were used to evaluate chloride and bicarbonate transport and the effects of a CFTR modulator combination: elexacaftor, tezacaftor and ivacaftor (ETI).
Results: Of 32 pancreatitis patients carrying *CFTR* variants, three had CF-causing mutations on both alleles and yielded CF-typical sweat test, NPD and ICM results. Fourteen subjects showed a more modest elevation in sweat chloride levels, including three that were provisionally diagnosed with CF. ICM indicated impaired CFTR function in nine out of 17 non-CF subjects tested. This group of nine included five carrying a wild type *CFTR* allele. In epithelial monolayers, a reduction in CFTR-dependent chloride transport was found in six out of 14 subjects tested, whereas bicarbonate secretion was reduced in only one individual. In epithelial monolayers of four of these six subjects, ETI improved CFTR function.
Conclusions: CFTR function is impaired in a subset of pancreatitis patients carrying *CFTR* variants. Mutations outside the *CFTR* locus may contribute to the anion transport defect. Bioassays on patient-derived intestinal tissue and organoids can be used to detect such defects and to assess the effect of CFTR modulators.

1. Introduction

Pancreatitis is an inflammatory disorder of the acinar and ductal epithelia of the exocrine pancreas. It causes considerable discomfort and suffering and is among the most common gastrointestinal diagnoses associated with hospital admission [1]. Conventionally, inflammation is thought to be initiated by the premature

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Abbreviations

acute pancreatitis (AP)
bubble sweat test (BST)
CFTR bicarbonate conductance defect (CFTR-BD)
CFTR-related disorders (CFTR-RD)
chronic pancreatitis (CP)
cystic fibrosis (CF)
elexacaftor (ELX)
elexacaftor/tezacaftor/ivacaftor (ETI)
endoscopic retrograde cholangiopancreatography (ERCP)
European Cystic Fibrosis Society (ECFS)
exocrine pancreatic insufficient (PI)
exocrine pancreatic sufficient (PS)
forced expiratory volume in one second (FEV_1)
Gibson and Cooke sweat test (GCST)
idiopathic pancreatitis (IP)
intestinal current measurement (ICM)
intra-pancreatic activation of trypsin (IPAT)
3-isobutyl-1-methylxanthine (IBMX)
ivacaftor (IVA)
nasal potential difference (NPD)
next-generation sequencing (NGS)
recurrent acute pancreatitis (RAP)
short-circuit current (Isc)
tezacaftor (TEZ)

conversion of the zymogen trypsinogen, produced by acinar cells, into the active protease. This inappropriate activation of proteolysis within the pancreas itself leads to tissue injury, which, in turn, triggers immune cell infiltration. Inflammation may be contained to the pancreas, but in severe cases a systemic response may ensue that can culminate in multiple organ failure [2,3]. Acute pancreatitis may resolve spontaneously, but if the underlying causes driving tissue inflammation are not adequately addressed, episodes of acute pancreatitis can recur, or inflammation may become chronic [4–6].

Multiple genetic and environmental factors contribute to the etiology of pancreatitis [3,7–9]. In cases where no obvious environmental or metabolic trigger (e.g. alcohol abuse, hyperlipidemia, biliary disease, autoimmunity) is suspected, it is often challenging to identify the cause of acute (recurrent) or chronic pancreatitis [6]. However, mutation of one of several genes thought to be involved in the intra-pancreatic activation of trypsin (IPAT) has been shown to confer an increased risk of pancreatitis [7–9]. One such IPAT gene is *CFTR*, which encodes a phosphorylation-regulated anion channel that mediates chloride and bicarbonate transport across various epithelia [10].

Loss-of-function mutations in *CFTR* on both alleles cause cystic fibrosis (CF), a complex multisystem disorder characterized by loss of anion and fluid secretion across epithelia of the respiratory and gastrointestinal tract, including that of the intestine, and the biliary and pancreatic ductal tree. In pancreatic ducts, CFTR-dependent bicarbonate and osmotic fluid secretion drives the rapid passage (flushing) of digestive enzymes to the intestine [11,12]. In CF patients carrying minimal function mutations, obstruction of the pancreatic ducts with viscid mucus and protein aggregates can already develop *in utero*, leading to exocrine pancreatic insufficiency at an early stage of life [13]. Only those who carry *CFTR* mutations that permit residual anion channel function may preserve adequate exocrine pancreatic function. Still, these pancreatic sufficient CF subjects are more likely to develop pancreatitis [14]. *CFTR* variants leading to a more moderate impairment in CFTR function do not cause CF, but are associated with several CFTR-related disorders (CFTR-RD), often with a predominant presentation in a single organ. Typical manifestations of CFTR-RD are bronchiectasis, congenital bilateral absence of the vas deferens, and pancreatitis [15,16].

It has been estimated that up to 30% of those with idiopathic pancreatitis carry mutations in the *CFTR* gene [17,18]. These numbers suggest a critical role of CFTR-dependent ductal anion and fluid secretion in containing the deleterious effects of pathogenic stimuli. In fact, several typical stressors (e.g. bile acids, ethanol, trypsin) were shown to directly impact ductal CFTR function [19–21]. The consequent reduction in ductal bicarbonate and fluid secretion is thought to precipitate ductal cell injury and inflammation [22]. These findings not only imply that impairment of CFTR function is crucial in the development of pancreatitis, but also suggest that, for some forms, loss of ductal CFTR function initiates pancreatitis, and precedes acinar pathology [3,22,23].

While the detection of *CFTR* variants is suggestive of CFTR-RD, in many of these cases, the functional and clinical consequences of these mutations are uncertain, and further diagnostic procedures to evaluate CFTR function are indicated. This has become even more pertinent now that CFTR modulators, i.e. small-molecule compounds that can restore the function of some (but not all) *CFTR* variants, have become clinically available [24]. CFTR modulators were shown to improve biomarkers of exocrine pancreas function in CF patients and animal models, and to ameliorate disease in an experimental model of autoimmune pancreatitis characterized by acquired CFTR dysfunction [23,25–27]. These data indicate that CFTR modulators may be applicable for treatment of those forms of pancreatitis in which CFTR dysfunction is suspected.

In this study we sought to assess CFTR function and the potential beneficial effects of CFTR modulator therapy in a group of subjects diagnosed with idiopathic pancreatitis, carrying mostly rare, poorly characterized *CFTR* variants. To evaluate CFTR function in this cohort, in addition to performing sweat tests and nasal potential difference (NPD) measurements, we used transepithelial current measurements to assess CFTR-mediated chloride and bicarbonate transport separately. Because pancreatic tissue cannot routinely be sampled from patients and no validated method is currently available that allows direct assessment of CFTR function in the pancreatic ductal epithelium, we used intestinal tissue and organoid-derived epithelial monolayers to evaluate the impact of mutations on CFTR-mediated anion transport. Intestinal organoids have been routinely used for assessing the response to modulators of *CFTR* variants in the context of CF research, and CFTR assays on intestinal organoids were shown to correlate with CFTR function in patient tissue [28,29]. Like the cells lining the pancreatic ductal tree, intestinal epithelial cells robustly express the WNK- and SPAK-type protein kinases, which, according to some studies, are required (and sufficient) for promoting CFTR-mediated bicarbonate secretion [30–32]. We considered CFTR-mediated bicarbonate transport of particular interest because mutations that were reported to interfere with the regulation of the bicarbonate permeability of CFTR by the WNK/SPAK pathway, were proposed to increase the risk of pancreatitis [31,33]. Furthermore, generation of organoid-derived intestinal epithelial monolayers allowed us to evaluate the effect of a CFTR modulator combination consisting of elexacaftor (ELX), tezacaftor (TEZ) and ivacaftor (IVA; when combined: ETI) on both CFTR-mediated bicarbonate and chloride transport.

2. Methods

2.1. Selection of patients

Patients were enrolled between July 2015 and June 2021 in two centers: the Cystic Fibrosis Unit of Bambino Gesù Children's Hospital, IRCCS and the Gastroenterology Unit, Department of Medicine of Borgo Roma Hospital, Verona. Inclusion criteria were: patient affected by acute, recurrent (≥2 episodes), or chronic pancreatitis, age ≥6 years [4,34]. Exclusion criteria were: pregnancy, solid organ or hematological transplantation, pancreatitis caused by autoimmune disease, gallstones, or hypertriglyceridemia, paraduodenal pancreatitis, consumption of >1 alcohol unit per day. Subjects were screened for *CFTR* mutations by targeted next-generation sequencing (see *Genetic Analysis* below), and included if at least one mutation was detected. Subjects were diagnosed with CFTR-RD, based on established diagnostic guidelines [35]. All participating subjects (or their legal representative) signed an informed consent document. The study was approved by the ethical committee of Bambino Gesù Children's Hospital, IRCCS (protocol #2183/2020), and the Borgo Roma Hospital, Verona (protocol #CFTR028).

2.2. Genetic analysis

Targeted Next-Generation Sequencing (NGS) was employed to sequence a panel of 9 IPAT (intra-pancreatic activation of trypsin) genes associated with pancreatitis: *CFTR* (NM_000492.3), *SPINK1* (NM_003122.3), *PRSS1* (NM_002769.4), *PRSS2* (NM_002770.2), *CTRC* (NM_007272.2), *CASR* (NM_001178065.1), *CTSB* (NM_147780.2), *CPA1* (NM_001868.2) and *KRT8* (NM_002273) [7,9]. Gene sequences were obtained through the Genome Browser online platform (https://genome.ucsc.edu/). Libraries were prepared using Twist Custom Panels (Twist Bioscience) and NGS was performed on a NovaSeq 6000 instrument (Illumina). Detected variants were identified using the Geneyx analysis platform (Geneyx) and were validated by Sanger sequencing. For all patients, the *CFTR* poly-T tract and TG tract were analyzed. Variants 7T and 9T in combination with any TG variant are reported as wild type (WT).

2.3. In vivo CFTR function measurements

Gibson and Cooke sweat test (GCST) was performed as routine procedure within the framework of regular patient care, according to established protocols [36]. Mean sweat chloride levels <30 mmol/L were considered within the normal range, whereas levels ≥60 mmol/L are considered pathognomonic for CF [37]. The bubble sweat test (BST) was performed according to a previously published protocol [38,39]. Briefly, the cholinergic agonist methacholine (M) and a cocktail (C) of β adrenergic agonists mixed with atropine were applied intradermally to stimulate CFTR-independent and -dependent sweating, respectively. C/M ratios ≥0.205 are considered to indicate normal CFTR function, while CF patients typically show ratios ≤0.0055. Nasal potential difference (NPD) measurement was performed according to the standard operating procedure of the European Cystic Fibrosis Society (ECFS) [40]. For discriminating CF patients, a locally validated Wilschanski Index cut-off value of 0.82 was used [41].

2.4. Intestinal organoid and epithelial monolayer culture

Organoids were generated from rectal forceps biopsies and maintained in medium containing the growth factors Wnt3a, Noggin, R-Spondin 1 and EGF in Matrigel matrix (Corning), according to established protocols [42]. For culture of epithelial monolayers, organoids were suspended in advanced DMEM (4 °C; Gibco) and washed by centrifugation (5 min, 400 g) to remove the extracellular matrix. Cells were dissociated by brief (45 s, 37 °C) incubation in trypsin solution (0.25%; Gibco), followed by repeated aspiration through a 200 μL pipette tip (Greiner). Trypsin activity was quenched by addition of fetal calf serum (10%) in advanced DMEM, and cells were washed in advanced DMEM and filtered through a cell strainer (70 μm; Falcon). Cells were collected by centrifugation and seeded (6.10^5 cells/cm^2) on permeable inserts (Transwell #3470; Corning) pretreated with diluted Matrigel (1:20 in phosphate buffered saline, 0.2 mL/cm^2, 2h, 37 °C). Culture medium was as for organoids, except that CHIR99021 (10 μmol/L; Sigma-Aldrich) and Y-27632 (10 μmol/L; R&D Systems) were added during the first 2 days after seeding. Cells were cultured until a confluent monolayer was obtained (10–14 days), which was monitored by measuring the transepithelial electrical resistance using an EVOM2 device (World Precision Instruments).

2.5. Electrophysiological assessment of epithelial anion transport

Ion transport in rectal biopsies was assessed according to standard operating procedures for intestinal current measurement (ICM; SOP v.2.7; https://www.ecfs.eu/ctn/standardization-committees) [40,43]. After washing in ice-cold phosphate buffered saline, biopsies were mounted on P2407C type inserts (surface area: 0.011 cm^2; Physiologic Instruments), in low-volume chambers (P2250; Physiologic Instruments). Tissue was bathed in Meyler solution (mmol/L: 128 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4) supplemented with indomethacin (10 μmol/L), HEPES (10 mmol/L) and glucose (10 mmol/L), maintained at 37 °C, and gassed with 95% O_2, 5% CO_2. The transepithelial potential difference was clamped at 0 mV (EVC4000 module; World Precision Instruments), and the resulting short-circuit current (Isc) was recorded with a PowerLab 8/35 AD-converter and associated software (LabChart 8; AD Instruments). After a 10 min equilibration period, amiloride (100 μmol/L) was added to the luminal bathing medium to inhibit ENaC-mediated Na^+ currents. After a further 10 min period, the cAMP agonists forskolin (10 μmol/L) and 3-isobutyl-1-methylxanthine (IBMX; 100 μmol/L) were added to stimulate CFTR activity, followed by carbachol (100 μmol/L) and histamine (500 μmol/L). Cumulative Isc responses to forskolin/IBMX, carbachol and histamine were determined as described elsewhere [40,43,44]. Data shown represent the mean response of two biopsy specimens per subject.

Organoid-derived intestinal epithelial monolayers were incubated with ELX (VX-445, 3 μmol/L; MedChem), TEZ (VX-661, 3 μmol/L; SelleckChem) and IVA (VX-770; 0.3 μmol/L; SelleckChem), or vehicle (DMSO, 0.2%) for 20h. Subsequently, filters were mounted in P2302T/P2300-type Ussing chambers (surface area: 0.33 cm^2; Physiologic Instruments). Monolayers were initially bathed in Meyler solution supplemented with HEPES (10 mmol/L) and glucose (10 mmol/L). For assessing CFTR-dependent chloride transport, a similarly formulated solution was used, except that $NaHCO_3$ was replaced by Na-isethionate. The pH of this solution was set at 7.35 by NaOH titration. For assessing CFTR-dependent bicarbonate transport, NaCl and KCl were replaced by the isethionate salts, and $CaCl_2$ and $MgCl_2$ by acetic acid salts of these cations. The only CFTR-permeating anions in these solutions are chloride and bicarbonate, respectively. Where applicable, CFTR-modulator drugs were re-added. Solutions were maintained at 37 °C, gassed with 95% O_2, 5% CO_2 or, in case a bicarbonate-free solution was used, O_2. The transepithelial potential difference was clamped at 0 mV (VVC-MC8 module; Physiologic Instruments), and the Isc was recorded as for ICM. Forskolin (10 μmol/L) was used to stimulate CFTR activity. CFTR-dependence of the forskolin-dependent Isc

response was verified by addition of the CFTR inhibitor CFTRinh172 (20 μmol/L; Tocris Bioscience). Data shown represent the forskolin-dependent, CFTRinh172-sensitive change in Isc.

2.6. Statistical analysis

The statistical significance of mean differences in anion transport between epithelial monolayers and controls, and the effect of CFTR modulators on anion transport was evaluated by ANOVA, using Dunnett's test to correct for multiple comparisons (SPSS v.28; IBM).

3. Results

3.1. CFTR variants detected in pancreatitis patients

Thirty two subjects diagnosed with idiopathic pancreatitis were enrolled (Table 1). The age of subjects at the onset of pancreatitis ranged between 3 and 49 years. In 18 (56%) patients abdominal imaging revealed abnormalities: pancreatic duct dilatation (6, 19%) and pancreatic calcifications (4, 13%) were reported most frequently, and pancreas divisum was reported in one patient (S.32). Endoscopic retrograde cholangiopancreatography (ERCP) was performed on 22 (69%) patients: 16 (50%) underwent sphincterotomy and four (13%) recurrent stent placement. Two patients reported steatorrhea (S.14, S.18). These and three additional patients (S.01, S.03, S.11) had fecal elastase-1 concentrations below 100 μg/g indicating severe exocrine pancreas dysfunction, while two patients (S.10, S.25) had fecal elastase-1 concentrations between 100 and 200 μg/g indicating mild to moderate exocrine pancreas dysfunction. In one of these patients (S.11), the hemoglobin A1c (HbA1c) level was elevated, suggestive of endocrine pancreatic dysfunction. One patient, S.01, had a lowered forced expiratory volume in 1 s (FEV_1) of 48%, indicative of pulmonary disease.

Twelve (38%) patients carried CF-causing variants and 27 (84%) carried alleles with varying or unknown clinical consequences (Table 2) [45,46]. Among these, seven (22%) carried a *CFTR* variant that previously was classified as specifically defective in bicarbonate transport (CFTR-BD), namely R75Q (S.21), I148T (S.08) L997F (S.15, S.16, S.25), D1152H (S.05) and D1270N (S.24) [31,33]. Sixteen (50%) patients carried one *CFTR* allele on which no mutations were detected. To evaluate the complex genetic risk for pancreatitis, patients were tested for the presence of mutations in other genes involved in idiopathic pancreatitis (*SPINK1, PRSS1, PRSS2, CTRC, CASR, CTSB, CPA1* and *KRT8*) [7,9]. Eleven (34%) patients carried mutations in one of these genes, and one patient (S.14) carried mutations in both *CTRC* and *SPINK1* (Table 2). Three patients were eventually diagnosed with CF (S.01, S.02, S.05), and three additional patients, all displaying colonization of the respiratory tract by pathogenic bacteria, were provisionally diagnosed with CF (S.03, S.04, S.06; Table 2). Pending further CFTR functional assays to exclude CFTR-RD, three patients were provisionally diagnosed with chronic pancreatitis (S.13, S.14, S.16).

All four subjects diagnosed with CFTR-RD were compound heterozygous, carrying one or two *CFTR* variants of varying clinical consequence (Table 2). These individuals had developed chronic pancreatitis, but showed no signs of CF-typical lung disease (e.g. lung colonization and/or lowered FEV_1). Of the eight other subjects (provisionally) diagnosed with chronic pancreatitis, seven carried a mutation in an IPAT gene, which, plausibly, had contributed to disease progression. Among these, S.18 carried a disease causing mutation in *PRSS1* (R122H), that is strongly associated with hereditary pancreatitis [47]. In all these subjects diagnosed with chronic pancreatitis, imaging revealed tissue lesions such as pancreatic duct dilation or calcification, but only two individuals in this group had thus far developed pancreatic insufficiency (S.14, S.18). Most (9/13) subjects diagnosed with recurrent acute pancreatitis carried a WT CFTR allele *in trans* of a variant of varying clinical consequence, or a variant known not to cause CF [45,46]. Mutation of an IPAT gene was detected in 3 out of 13 subjects diagnosed with recurrent acute pancreatitis.

3.2. Many subjects carrying CFTR variants present with intermediate sweat chloride levels

Whereas sweat chloride levels ≥60 mmol/L are considered pathognomonic for CF, intermediate values in the range of 30–59 mmol/L, while not fulfilling diagnostic criteria for CF, are nevertheless indicative of a reduced CFTR function [15]. Sweat chloride levels were obtained for all subjects included (Fig. 1). Three patients had sweat chloride concentrations typical of CF. Subject S.01 carried the common CF causing F508del mutation in combination with G85E, another CF-causing mutation [45,46]. S.02 combined the 5T; TG13 haplotype in *trans* of the F508del allele, which combination is also thought to be pathogenic and cause CF [48]. S.05 also carried a potentially pathogenic allele combination (L732X/D1152H), and presented with sweat chloride levels ranging

Table 1
Demographics and clinical characteristics of patients.

Demographic		**Assessed, n**
Females, n (%)	16 (50%)	32
Age at inclusion, mean (range)	26 (7–54)	32
Age at onset of pancreatitis, mean (range)	21 (3–49)	32
Positive family history, n (%)	5 (16%)	32
Clinical characteristics		
ERCP, n (%)	22 (68.8%)	32
Abdominal imaging abnormalities, n (%)	18 (56.3%)	32
Other GI symptoms present, n (%)	4 (12.5%)	32
Fecal elastase-1 <200 μg/g, n (%)	7 (25.0%)	28
HbA1c > 40 mmol/mol, n (%)	1 (3.8%)	26
FEV_1 <70%, n (%)	1 (6.7%)	15
Lung colonization, n (%)	9 (28.1%)	32
Diagnosis, n (%)	CF: 3 (9.4%) CFTR-RD: 4 (12.5%) CP: 5 (15.6%) RAP: 13 (40.6%) Provisional: 7 (21.9%)	32

A total of 32 subjects diagnosed with idiopathic pancreatitis was enrolled. CF: cystic fibrosis, CFTR-R: CFTR-related disease; CP: chronic pancreatitis, RAP: recurrent acute pancreatitis, Provisional: final diagnosis pending further assessment.

Table 2
Patient genotype, diagnosis and CFTR function measurements.

Subject	Diagnosis	CFTR genotype		IPAT genotype	GCST	BST	NPD	ICM	Monolayers		Pancreatitis
		Allele 1	Allele 2		[Cl^-] mmol/L	C/M ratio	Wilschanski Index	Isc $\mu A/cm^2$	Isc Cl^- (n) $\mu A/cm^2$	Isc HCO_3^- (n) $\mu A/cm^2$	Age at onset Years
S.01	CF	**F508del**	**G85E**	–	**125**	**0.0**	**32.1**	**4**			24
S.02	CF	**F508del**	5T;TG13	–	**84**	**0.0**	**1.77**	**9**			20
S.03	CF*	**3849+10kbC->T**	621+3A->G	CTRC: D190G	**35**	0.21	0.58	**63**			15
S.04	CF*	**3849+10kbC->T**	621+3A->G	CTRC: D190G	**32**	1.42	0.10	**69**			24
S.05	CF	**L732X**	D1152H	–	**59**				**27 ± 8** (4)	**4 ± 1** (3)	5
S.06	CF*	S158T	2752-15C->G	–	**43**	0.56	0.51	248			31
S.07	CFTR-RD	**F508del**	5T;TG12	–	**39**				**26 ± 1** (3)	14 ± 12 (3)	9
S.08	CFTR-RD	*I148T*	G1069R	–	**36**	0.31	0.38	298			23
S.09	CFTR-RD	*G576A*	R668C/I991M	–	27				113 ± 6 (3)	13 ± 4 (4)	4
S.10	CFTR-RD	G194V	*R1162L*	–	**52**				93 ± 12 (4)	16 ± 3 (4)	17
S.11	CP	**2183AA->G**	F1074L	PRSS1: E79K	**31**	**0.09**	0.24	**129**	65 ± 16 (3)	13 ± 1 (3)	18
S.12	CP	**2183AA->G**	WT	SPINK1: A56L	**30**	**0.07**	**34.3**	**41**	140 ± 8 (4)	16 ± 2 (4)	38
S.13	CP*	**R1162X**	WT	–	28						5
S.14	CP*	**G542X**	WT	CTRC: G217S; SPINK1: N34S	19						10
S.15	CP	*L997F*	WT	SPINK1: N34S	29			181	120 ± 42 (5)	19 ± 8 (4)	20
S.16	CP*	*L997F*	WT	CTRC: N78Q, FsX44	18						9
S.17	CP	5T;TG12	WT	CASR: V149I	**33**				119 ± 11 (4)	14 ± 1 (3)	7
S.18	CP	*R668C*	*G576A*	PRSS1: R122H	12						3
S.19	RAP*	**2183AA->G**	3500-57C->T	–	12		**1.72**	221			45
S.20	RAP	**2789+5G->A**	WT	–	**30**	0.21	0.10	178			8
S.21	RAP	*R75Q*	F1052V	–	**31**	0.32	0.32	**123**			30
S.22	RAP	V1379I	4095+63T->C	–	23	0.21	0.37	**116**	**57 ± 25** (5)	18 ± 6 (5)	36
S.23	RAP	R74W	V855M	–	**35**	1.04	0.21	**113**	103 ± 38 (4)	30 ± 13 (4)	41
S.24	RAP	D1270N	WT	SPINK1: N34S	24	0.99	0.33	**93**	179 ± 4 (2)	16 ± 8 (6)	13
S.25	RAP	*L997F*	WT	–	28	0.31	**3.10**	173			21
S.26	RAP	c.-812T->G	WT	–	**40**	0.40	**1.04**	257			9
S.27	RAP	S1426F	WT	SPINK1: N34S	11	**0.15**	0.01	**110**			27
S.28	RAP	4347+13A->G	WT	–	**37**	3.33	0.20	194	120 ± 5 (4)	16 ± 4 (4)	20
S.29	RAP	G1069R	WT	–	20	**0.09**	0.18		**29 ± 10** (7)	9 ± 2 (7)	21
S.30	RAP	G1069R	WT	–	18	0.82	0.08	**70**	**26 ± 10** (7)	11 ± 5 (7)	36
S.31	RAP	*R31C*	WT	CTRC: E225A	25	0.39	0.08	212			31
S.32	RAP	*5T;TG11*	WT	–	15	0.25	0.09	**93**			49

Subjects were coded, based on (provisional) diagnosis and *CFTR* genotype. CF: cystic fibrosis; CFTR-RD: CFTR-related disease; CP: chronic pancreatitis; RAP: recurrent acute pancreatitis. *Provisional diagnosis pending further assessment. *CFTR* alleles shown in bold typeface are considered to cause CF when combined with another CF-causing allele [45,46]. WT: wild type allele. Alleles shown in italics do *not* cause CF. Other alleles have variable or unknown consequences; those underlined were previously categorized as CFTR-BD variants [31,33]. For GCST, median values are shown when multiple assays were performed. Anomalous, pathological test results are depicted in bold face. For GCST, BST and NPD, empirical threshold values were based on previously reported data (see *Methods*, section 2.3). For ICM, cut-off values were obtained from a cohort of 10 CFTR WT individuals (207 ± 68 $\mu A/cm^2$, CI: 159–255). Isc responses in monolayers are shown as mean ± SD.

between 31 (at age 4) and 79 mmol/L (at age 6). Based on genotype and clinical presentation, these subjects were retrospectively diagnosed with CF. In addition, three patients presenting with an intermediate GCST result and evidence of lung pathology where provisionally diagnosed with CF (S.03, S.04, S.06). Of the remaining 26 subjects, 11 had intermediate sweat chloride levels. Interestingly, whereas most of these were *CFTR* compound heterozygous, in five of these patients no second mutation was found (S.12, S.17, S.20, S.26, S.28). The remaining 11 subjects carrying WT alleles had normal sweat test results.

CFTR-dependent and -independent sweat secretion rates (BST) and NPD were examined in 21 and 22 subjects, respectively (Fig. 1B and C). Consistent with GCST, two patients diagnosed with CF (S.01, S.02), showed a strongly reduced level of CFTR-dependent sweat secretion (no BST was performed on the third CF patient, S.05). The majority of non-CF individuals in which BST was assessed, presented with normal secretion ratios (C/M ratio >0.205); only four additional patients (S.11, S.12, S.27, S.29) had sweat secretion ratios in the range typically encountered in heterozygotes carrying a minimal function allele (0.0055–0.205). NPD values were elevated in six patients (S.01, S.02, S.12, S.19, S.25, S.26), two of which were diagnosed with CF (S.01, S.02; no NPD was performed on patient S.05). Of the other four patients with a high NPD result, S.12 and S.26 yielded an intermediate GCST value, and S.12 also showed a lowered BST result.

3.3. Many subjects carrying CFTR variants show impaired CFTR-mediated intestinal ion transport

CFTR-mediated chloride and bicarbonate secretion was measured in rectal biopsies derived from 22 individuals in this cohort (Fig. 2). CFTR-dependent anion secretion was quantified as the cumulative, net Isc response elicited by forskolin, IBMX, carbachol and histamine. Established procedures for performing ICM were applied, as stipulated by the ECFS [40,43,44]. For comparison, we also performed ICM in a control cohort of 10 individuals that do not carry mutations in *CFTR*. ICM readily identified the anion secretory defects in S.01 and S.02, the two CF patients already singled out by sweat tests and NPD (no ICM data is available for the third CF patient identified, S.05). Of the remaining 20 subjects, 11 (55%) showed mildly impaired Isc responses, i.e. below the 95% confidence interval (CI) of the present control cohort, but higher than reported for CF subjects (Fig. 2) [43].

3.4. CFTR-mediated anion secretion across epithelial monolayers is impaired in a subset of patients

Intestinal monolayers were established from organoids cultured of biopsy specimens obtained from 14 subjects within this cohort. Previous studies have indicated that *CFTR* variants associated with pancreatitis may specifically impair CFTR-mediated bicarbonate

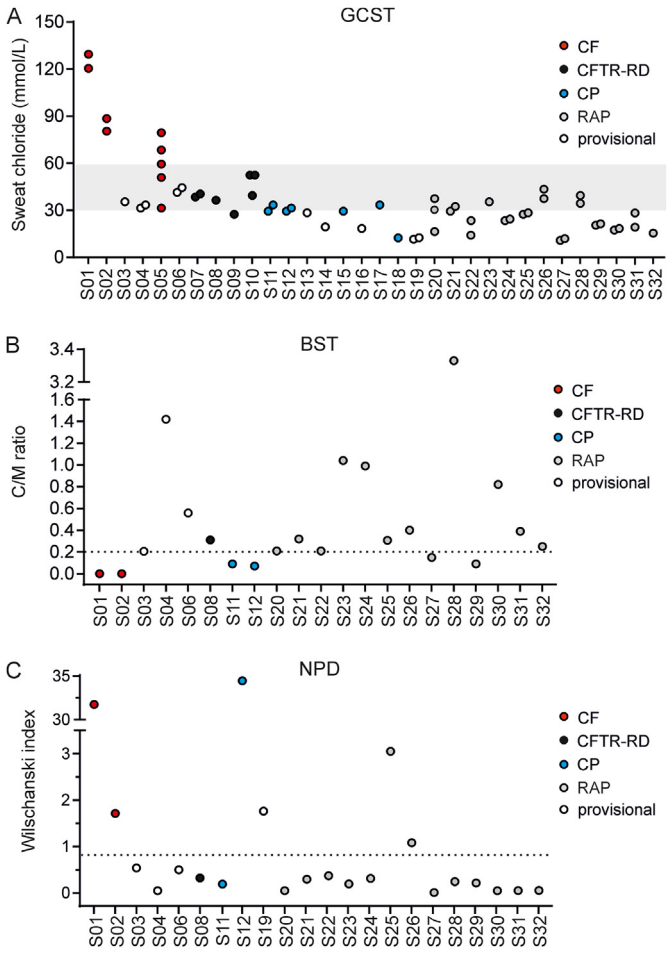

Fig. 1. In vivo CFTR function measurements. A. Gibson and Cooke sweat test (GCST). Data were collected of 32 subjects. Shaded area indicates range of intermediate sweat chloride levels (30–59 mmol/L). Each data point represents one technical replicate. **B.** Optical ratiometric sweat test (BST). The ratio of the CFTR-dependent (C) and CFTR-independent (M) rate of sweat secretion was determined in 21 subjects. Dashed line indicates the lower limit for normal values. **C.** Nasal potential difference (NPD) measurement. Data were collected of 22 subjects. Dashed line indicates the upper limit for normal values.

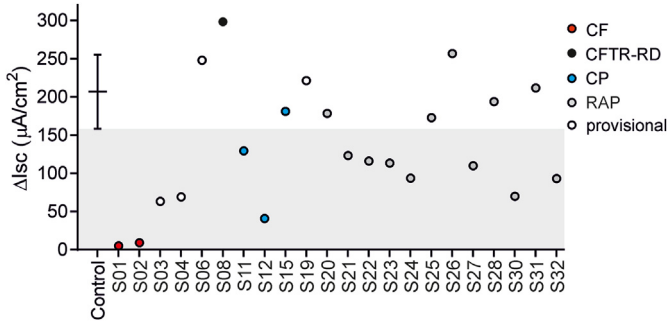

Fig. 2. CFTR function measurement in rectal biopsies by ICM. The cumulative Isc response upon forskolin, IBMX, carbachol and histamine administration was assessed in 22 subjects. Data points represent the mean of two technical replicates. For comparison, control values (*CFTR* wild type) are plotted as mean with 95% CI (n = 10). Shaded area denotes values < 95% CI of the control group.

transport [31]. To evaluate if any of the current subjects carry such variants, separate measurements of chloride and bicarbonate transport were performed. The forskolin-dependent, CFTRinh172-

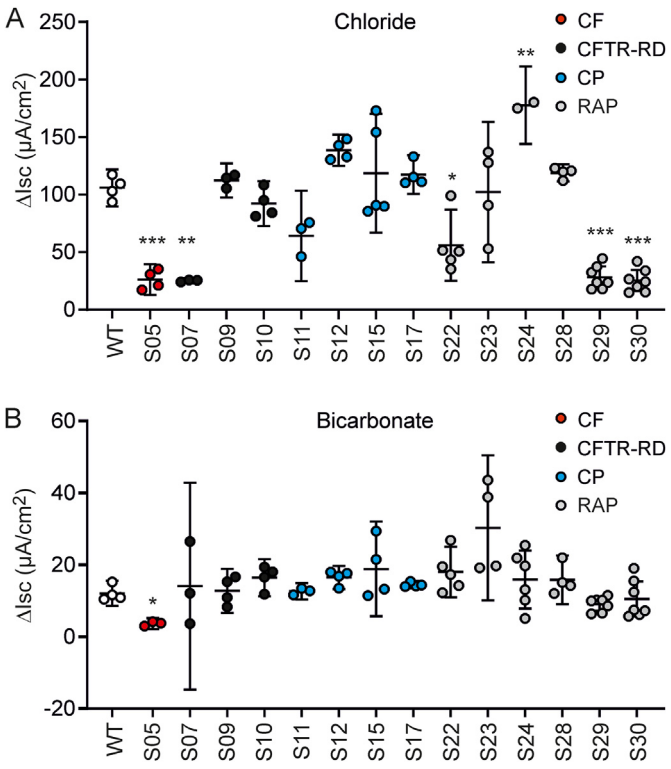

Fig. 3. CFTR-dependent chloride and bicarbonate transport in intestinal epithelial monolayers. A. Forskolin-dependent Isc responses in chloride solution. **B.** Forskolin-dependent Isc responses in bicarbonate solution. Data depict mean with 95% CI. Each data point represents one technical replicate. *P < 0.05, **P < 0.01, ***P < 0.001.

sensitive change in current reflects CFTR-mediated transepithelial chloride or bicarbonate secretion. Five patients (S.05, S.07, S.22, S.29, S.30) showed significantly impaired chloride secretion as compared to non-CF monolayers (Fig. 3A). In addition, monolayers of S.11 also tended to have low chloride secretory responses. In all three patients in this group for which data is available (S.11, S.22, S.30), ICM also indicated lowered anion secretory responses. Results of ICM and current measurements on monolayers corresponded for six out of a total of eight patients for which data of both assays was available. However, S.23 and S.24, while showing moderately lowered CFTR activity in biopsies, showed normal anion secretory responses *in vitro* in cultured cells. For patients S.05, S.07 and S.29 no ICM data was available. However, both S.05 and S.07, but not S.29, had produced intermediate or CF-like GCST results (Fig. 1, Table S1).

Of the epithelial monolayers investigated, three derived from patients carrying *CFTR* variants previously classified as defective in bicarbonate transport (S.05, S.15, S.24). Of these, only monolayers of S.05 displayed impaired bicarbonate secretion (as well as chloride secretion; Fig. 3B). In contrast, S.15 and S.24 showed normal anion secretory responses when assayed in either chloride- or bicarbonate-free solutions.

3.5. CFTR-modulators improve anion secretion in some pancreatitis patients

The intestinal organoid model allows evaluation of the effects of CFTR modulators on CFTR function. It is particularly suitable to test compounds that improve CFTR protein maturation and plasma membrane insertion (i.e. correctors), which may take several hours to take full effect. Because of the limited viability of native tissue

once removed from the body, such assays are impracticable in intestinal biopsies. The effect of IVA alone or ETI was evaluated in monolayers derived from six patients; those five which demonstrated lowered chloride transport in epithelial monolayers (S.05, S.07, S.22, S.29, S.30), and in patient S.11 who tended to a lowered response (see Fig. 3). IVA as well as ETI improved chloride transport in monolayers of S.05 (L732X/D1152H), but no significant improvement was observed in the other five patients (Fig. 4A). Both IVA or ETI treatment increased bicarbonate transport in patient S.05. ETI treatment enhanced bicarbonate transport in three additional patients (S.22, S.29, S.30). These results indicate that, in some patients, these modulators selectively improve CFTR-mediated epithelial bicarbonate secretion.

4. Discussion

In this study, we investigated CFTR function in 32 individuals affected by recurrent acute, or chronic pancreatitis, carrying mutations in the *CFTR* gene that were mostly of unknown or variable clinical consequence. When such gene variants, suggestive of CF or CFTR-RD, are found, usually a sweat test is performed to evaluate CFTR function. However, mutations that cause only mild or moderate impairment of CFTR function frequently yield sweat test results that are inconclusive, and additional functional tests like NPD and/or ICM are used to further aid diagnosis [37,44]. Presently, we observed that GCST, BST, NPD and ICM yielded consistent data in cases where subjects carry minimal function mutations in *CFTR*, on both alleles. Accordingly, these individuals were retrospectively diagnosed with CF. In contrast, for the other *CFTR* genotypes in this cohort, the outcomes of these tests were often inconsistent. For instance, only three out of ten individuals with intermediate GCST results, presented with abnormal BST and/or NPD measurements. Similarly, in five patients with low responses in ICM, GCST results were in the normal range. These observations align with previous studies showing that in patients carrying alleles of variable clinical consequence, either in *trans* of a CF-causing or a WT allele, CFTR function, as assessed in established bioassays, may vary considerably at different points in time and between tissues [29,44]. Consequently, it is crucial that multiple diagnostic procedures are used to evaluate CFTR function in pancreatitis patients carrying *CFTR* variants. To further improve the utility of these diagnostic tools, a refinement of the criteria for CFTR dysfunction is warranted [15,37,40].

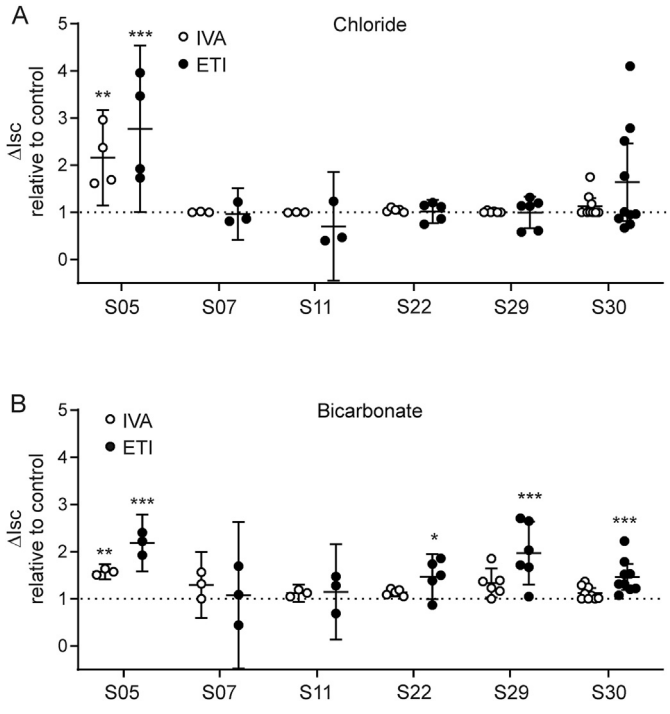

Fig. 4. Pharmacological correction of CFTR function assessed on intestinal monolayers. The effect of IVA or ETI treatment on forskolin-dependent, CFTR-mediated chloride (**A**) and bicarbonate (**B**) transport. Data depict Isc responses (mean with 95% CI) relative to a paired vehicle (DMSO)-treated control. Each data point represents one technical replicate. *P < 0.05, **P < 0.01, ***P < 0.001.

ICM indicated that CFTR-dependent anion transport was mildly impaired in 11 out of 22 individuals tested within this cohort, i.e. responses were below normal values, but not to the extent of CF patients carrying two minimal function alleles (e.g. S.01), who typically have negligible ICM responses [40,43]. In five of these subjects (S.12, S.24, S.27, S.30, S.32), next-generation sequencing, which covered not only exons but also the promoter and intronic regions of the *CFTR* gene, identified mutations on only a single allele. This is remarkable because obligate heterozygotes (CF carriers) typically display normal intestinal, CFTR-dependent anion secretion [43,49]. However, consistent with these results, it has been shown before that individuals carrying *CFTR* variants of varying clinical consequence can show considerable temporal and spatial variation in ICM responses, even when they also carry a WT allele. Not only did the ICM response of separate biopsies obtained from such subjects vary more than in wild types, but responses also tended to fluctuate between different points in time [44]. In addition, in some, CFTR function appeared to differ in intestinal *vs.* respiratory epithelia, i.e. they displayed CF-typical ICM responses, but NPD results in the normal range (or vice versa). This pattern was mirrored by the clinical manifestations in both organ systems, and low ICM responses were associated with gastrointestinal symptoms. Notably, it was found that all individuals in this particular cohort that were affected by pancreatitis, showed low CFTR activity in ICM [44]. It may be surmised that individuals in this category harbor mutations in separate genomic regions, not covered by the present sequencing protocol, that affect CFTR-dependent epithelial anion secretion in a tissue-specific manner. Conceivably, such “hidden” genetic modifiers affect the transcription of *CFTR* (variants), which is controlled by a combination of locally acting stimuli and transcription factors that often are particular for a given tissue (and cell type). At present, little is known about the transcriptional regulation of variant *CFTR* alleles in the pancreas, and its effects on ductal CFTR function are mostly uncharted. This implies that bioassays performed on other tissues (e.g. NPD, GCST, ICM) must be interpreted with some caution.

Among the currently available techniques ICM is one of a few capable of measuring CFTR function, *ex vivo*, in patient tissue. However, because only a small number of biopsies can be collected, and because ICM must be performed on freshly collected patient material, it may be challenging to perform sufficient replicate measurements to control for potential biological variation between tissue specimens [40,44]. To some extent, this limitation can be addressed by performing current measurements on intestinal organoids or organoid-derived epithelial monolayers. Other than ICM, the organoid model permits numerous measurements to be performed without time constraints, including evaluation of the effects of CFTR modulators on CFTR function at a personal level.

We succeeded in culturing intestinal organoid-derived monolayers from intestinal biopsies obtained from 14 individuals in this cohort. Measurement of CFTR-mediated anion secretion in these epithelial monolayers indicated that chloride transport was impaired in six patients out of these 14 tested. Remarkably, two subjects with low responses carried a *CFTR* WT allele (S.29, S.30), whereas another heterozygote (S.12) generated responses in the

normal range. This latter individual carried a minimal function (frameshift) mutation, demonstrating that one functional (WT) allele suffices for a normal anion secretory response. As already indicated by ICM, this suggests that heterozygous subjects with lowered anion secretory responses harbor mutations outside the genetic regions covered by our present sequencing protocol, that affect epithelial anion secretion.

For eight subjects, both ICM and current measurements on monolayers were performed. For six of these, both assays yielded corresponding results. This is consistent with previous data showing that cAMP-dependent anion secretory responses in donor-matched rectal tissue and organoid-derived monolayers correlate, and that these responses are principally, if not fully, dependent on CFTR [40,50]. Notwithstanding, subjects S.23 and S.24 showed low responses in ICM but apparently normal responses *in vitro*, in monolayers. The cause of this disparity between measurements on rectal biopsies and epithelial monolayers made thereof is speculative. Conceivably, locally acting environmental cues modulate the anion secretory response in intestinal tissue of individuals carrying *CFTR* variants associated with CFTR-RD [44]. *In vitro*, in intestinal epithelial organoid cultures, such factors produced by other cell types are lost, which may explain why assays on epithelial monolayers do not necessarily mirror the results attained with ICM. This divergence emphasizes the relevance of preclinical CFTR assays in near native tissues (biopsies), to predict the impact of rare mutations on CFTR function in individuals affected by pancreatitis, CFTR-RD, or mild CF.

Only epithelial monolayers obtained from CF patient S.05 displayed impaired bicarbonate transport. This patient carried the CFTR-BD variant D1152H in *trans* of a minimal function allele (L732X). In contrast, other subjects carrying CFTR-BD variants (S.15, S.24) showed normal bicarbonate and chloride secretory responses, plausibly because they also carried a WT allele. Although categorized as a CFTR-BD variant, chloride secretion was also markedly reduced in S.05. Diagnosed with CF at the age of six based on a pathologic sweat test, this individual suffered two episodes of pancreatitis within 12 months. However, so far, no respiratory symptoms were observed. In line with our present results, it was previously shown that the D1152H mutation not only impairs bicarbonate conductance, but also leads to a substantial reduction (>40%) in CFTR-mediated chloride transport [51]. Previous studies have also shown that carriers of this allele can present with an intermediate GCST result and a BST result in the CF range, indicative of impaired chloride transport in the sweat glands [52]. In contrast, D1152H-CFTR demonstrated normal chloride transport function in human nasal epithelial cells and a heterologous expression system (HEK 293T cells), suggesting that a single *CFTR* variant can result in across-tissue, and perhaps across-subject, variation in residual CFTR activity [31,53].

Congruent with previous studies on the D1152H-CFTR variant, the intestinal epithelial monolayers derived from CF patient S.05 strongly responded to modulator treatment [51]. Either IVA or ETI treatment not only enhanced bicarbonate transport, but also significantly improved chloride secretion. Indeed, in some countries (including the U.S.), CF patients carrying this variant are eligible for modulator therapy. Importantly, in addition, three other individuals, two of which carried a WT allele, and all diagnosed with recurrent acute pancreatitis, showed a modest improvement in bicarbonate transport upon ETI treatment. Unexpectedly, although ETI enhanced bicarbonate secretion in this group of subjects, it did not improve chloride transport. This suggests that the rate of chloride secretion by intestinal epithelial cells in these individuals is limited by factors other than CFTR, such as the activity of the transporter(s) mediating cellular chloride uptake across the basolateral plasma membrane. Indeed, this is also indicated by the observation that S.30, even though carrying a WT allele, demonstrated low responses in ICM, unlike typical CF carriers [43,49].

An important limitation of this study is that we did not directly evaluate CFTR function in pancreatic tissue, and we cannot exclude that the effect of the presently investigated mutations on CFTR function differs between pancreatic ductal and intestinal cells, or that the response of the ductal cells to modulator treatment is different from intestinal cells. However, it has been shown that intestinal organoid-based assays correlate well with the efficacy of modulator therapy *in vivo*, and there is accruing evidence that modulator therapy improves pancreatic pathology in CF patients [26,27,54–57]. The increase in bicarbonate secretion elicited by ETI presently observed, therefore suggests that modulator therapy may improve ductal anion secretion in a select group of individuals affected by pancreatitis. A further limitation is that only a relatively small number of subjects with diverse genotypes was included in this study. The response to modulators, both *in vitro* and *in vivo*, may vary considerably between individuals with apparently identical *CFTR* genotypes [28,58]. Consequently, the effects of modulators observed in individual patients are not necessarily representative for a particular *CFTR* genotype, and drug responses may need to be evaluated on a personal basis, in patient-derived tissues or cells [15,44]. We surmise that, of the currently available techniques to assess CFTR function *in vivo* or in patient tissue, ICM is most suited to evaluate CFTR function in subjects affected by a variant of pancreatitis that is, potentially, associated with CFTR dysfunction. Subsequently, organoids generated from intestinal biopsies can be used to assess the response to CFTR modulators. Once cryopreserved, patient-derived organoids can henceforth be used to test novel drugs when they become available. Ultimately, clinical trials are warranted to establish which of these (compound) *CFTR* genotypes, responsive *in vitro*, truly benefit from modulator therapy.

5. Conclusion

In conclusion, our findings demonstrate that CFTR dysfunction in pancreatitis patients carrying *CFTR* variants is relatively prevalent, indicative of the critical role of CFTR dysfunction in the etiology of pancreatitis [18–23]. Impaired CFTR function was also observed in some carrying a WT *CFTR* coding sequence on one allele, suggesting that disease-causing mutations may also be located outside the *CFTR* locus. We show that patient-derived organoids may be used to assess the impact of *CFTR* genotype on epithelial bicarbonate and chloride transport, and the response to CFTR modulator therapy. Further, our data suggest that modulator therapy may be beneficial for carriers of *CFTR* variants that suffer from pancreatitis, but not CF. We propose that early detection of CFTR dysfunction, combined with personalized medicine approaches directed at restoring CFTR function, can potentially slow or even prevent disease progression in some individuals affected by pancreatitis.

Data sharing

Analytic methods, study materials and datasets will be made available to other researchers on request to the last author.

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Institutional Review Board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Bambino Gesù Children's Hospital, IRCCS (protocol number 2183/2020), and the Borgo Roma Hospital, Verona (number CFTR028).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2024.03.005.

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