2.4 Nasal brushing and culture of epithelial nasal cells

Nasal epithelial cells were collected by nasal brushing. After nasal washings with physiological saline in order to remove mucus (two washings per day in the week before and one washing immediately before the sampling), the brushing was performed by a soft sterile interdental brush with 2.5 to 3 mm bristles (Paro-Isola) scraping along the middle portion of the inferior turbinate by gentle backward–forward and rotatory movements in each nostril, under direct visualization, using a headlamp without decongestant or local anesthesia.

The sample obtained from each nostril was immediately conserved in a 15 mL tube containing 2.5 mL of RPMI 1640 medium, complemented with 3% antibiotics and then cultured. The methods for culture are detailed as supplementary methods.

Cells were placed on Eppendorf Thermomixer, in agitation at 700 rpm for one hour to remove all cells from brushes. Then, cells were centrifuged at 2000 rpm for 20 minute, supernatants were discarded and cells were resuspended in serum-free bronchial epithelial cell growth medium BEGM (Clonetics). After centrifugation at 2000 rpm for 10 minutes, cells were plated in CELL T 25 flasks (Sarstedt Ltd). At confluence of 60%, cells were transferred in new flasks after count using Invitrogen Cell Countess.

2.5 Quantitative analysis of CFTR channel activity on epithelial nasal cells

To test the activity of the CFTR protein, we used the halide-sensitive fluorescent system. The iodide-sensitive fluorescent indicator, SPQ (Molecular Probes, Invitrogen, M440) was introduced into cells in a hypotonic solution of iodide buffer (in mM: 130NaI, 4 KNO_3, 1 $Ca(NO_3)_2$, 1

$Mg(NO_3)_2$, 10 glucose and 20 HEPES, pH 7.4) diluted 1:1 with water and containing a final concentration of 10 µm SPQ. Nasal cells were loaded for 15 min at 37°C in a humidified chamber with 5% CO2. The SPQ-loaded cells were plated in a *96-well plate and entered into the*EnSpire® Multimode Plate Reader, a reader of fluorescence intensity. Changes in CFTR-mediated SPQ fluorescence were monitored at the 445 nm wavelength in response to excitation at 345 nm. Fluorescent is constantly measured through the passage between different solutions containing halide anions. Cells were initially incubated with iodide buffer followed by incubation with nitrate buffer (NaI replaced with 130 mM $NaNO_3$) with the addition of specific activators of CFTR channel as forskolin (20 µM) (Sigma Aldrich) and genistein (50 µM) (Sigma Aldrich). The peak iodide efflux rate was calculated in accordance with the Stern-Volmer relationship as follows: $(F_o/F) - 1 = KC_Q$, where F is the observed fluorescence, F_o is the fluorescence in the absence of a quenching anion, C_Q is the concentration of the quenching anion, and K is the Stern-Volmer quench constant. The rates were calculated using SigmaPlot Version 7.1 for each mean fluorescence trace generated per well.

2.6 HEK 293 cell culture

Human Embryonic Kidney (HEK-293) cells were grown in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin (100 µU/mL – 100 µg/mL) and incubated in a humidified atmosphere of 5% CO2 at 37°C.

2.7 Plasmid Constructs and lentiviral vector production

The *wild-type* p.Asn1303Lys and p.Phe508del *CFTR* coding sequences were amplified from pTracer plasmid, gently provided by Prof. Galietta (Genoa, Italy). While, the genetic variation of interest either alone i.e., p.Asp1270Asn (D) or in different combinations, i.e.,

p.[Arg74Trp;Val201Met;Asp1270Asn] (DVR); p.[Arg74Trp;Asp1270Asn] (DV), were introduced into the *wild-typeCFTR* coding sequence using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) and the designed primers (available on request), in accordance with the manufacturer's protocol. All *CFTR* coding sequences, *wild-type* and all mutants, were cloned in the modified Lentiviral construct pMIRNA1 (SBI System Biosciences). This kind of plasmid is able to express the CFTR protein and the Yellow Fluorescent Protein (YFP) gently provided by Prof. Galietta) in an independent manner by the presence of a T2A sequence between YFP and *CFTR* coding sequence. Once checked by sequencing, the various *CFTR* constructs were packaged into VSV-G pseudotyped viral particles using the SBI pPACKH1 packaging plasmid mix. Both packaging and transduction of HEK293 cells were performed according to the manufacturer's instructions.

2.8 Western Blot analysis.

The HEK-293 cells stably expressing *wild-type* and mutated CFTR proteins were lysed in Triton lysis buffer (TLB: 1% Triton, 25 mmol/L Tris pH 7.4, 150 mmol/L NaCl) and protease inhibitors 2mg/ml, (Complete EDTA-free Protease Inhibitor Cocktail (Roche) for 1 hour at 4°C. The protein concentration was quantified by the Bradford assay (Biorad). All protein extracts were heated at 37°C for 20 minutes in SDS-PAGE solubilising buffer (57.85 mmol/L TrisHCl, 10% Glycerol, 2% SDS, 0.004% Bromophenol blue, pH 6.8) containing 125 mmol/L Dithiothreitol. 25 μg of total proteins were loaded in each lane and separated by SDS-PAGE-electrophoresis on a gradient polyacrylamide gel at 100 V for 2 hour. Following electrophoresis, proteins were transferred overnight onto immuno-Blot PVDF (Polyvinylidene Fluoride) (BIORAD) membrane.

The policlonal anti-CFTR antibody (Cell Signaling Tecnologies #2269) (diluted 1:1000) was used for CFTR protein detection and the anti-Tubulin TU-02 (Tubulin sc-8035, Santa Cruz Biotechnology) (diluted 1:4000) and the anti-GFP (GFP sc-81045, Santa Cruz) (diluted 1:2000) for western normalization and infection efficiency. Western blot quantification was performed using both ImageJ and Scion Image software.

2.9 CFTR activity assay on HEK293 cells.

The HEK-293 cells stably expressing the *wild-type* or mutant CFTR proteins were seeded in 96-well black microplates with a clear flat bottom (Corning Costar) until they reached the maximum confluence. After two washes with PBS, the 96-microplate with iodide-loading buffer (containing in mmol/L: 130 NaI, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 20 HEPES, pH 7.4) was loaded in the Enspire TM 2300 microplate reader for 20 minutes at 37 C° for fluorescence quenching. After substitution of the iodide-loading buffer with iodide-free buffer (same as the iodide loading buffer except NaCl replaced NaI) with 20 μM forskolin (Sigma) and IBMX 100 mM (Sigma) or DMSO as control, the 96-microplate was loaded in the Espire TM 2300 microplate reader for iodide efflux analysis. The rate of iodide efflux was calculated considering the maximal slope of the best fitting curve (fluorescence versus time).

Table 4: Demographic and clinical data of subjects bearing the [p.Arg117Leu;p.Leu997Phe] complex alleles or the p.Leu997Phe mutation

Gender	Current age (years)	Diagnosis	Age at diagnosis / enrolment (years/months)	Cause of diagnosis / enrolment	Allele 1	Allele 2	SCL* (mmol/L)	Current FEV_1 (%)	Pancreatic status	CFTR gating	Other
F	48	CF (?)	48y	Respiratory	p.[Arg117Leu;Leu997Phe]	p.[Arg117Leu;Leu997Phe]	90	113	PS	39.0	Pa col.; recurren pneumonit bronchiecta

M	58	CFTR-RD	58y	CBAVD	p.[Arg117Leu;Leu997Phe]	p.[Arg117Leu;Leu997Phe]	<u>88</u>	n.a.	PS	n.a.	
F	40	CF	33y	Respiratory	p.[Arg117Leu;Leu997Phe]	p.Arg334Trp	71	91	PS	19.5	Pa col.; na polyposi
M	35	CF	28y	Familiarity	p.[Arg117Leu;Leu997Phe]	p.Arg334Trp	75	120	PS	n.a.	Nasal polyposi
M	33	CF	1m	NBS	p.[Arg117Leu;Leu997Phe]	p.Gly85Glu	107	96	PS	n.a.	Nasal polyposi
M	24	CF	2y	Familiarity	p.[Arg117Leu;Leu997Phe]	p.Gly85Glu	80	90	PS	n.a.	Nasal polyposi
M*	40	CFTR-RD	40y	CBAV	p.Leu997Phe	p.Leu997Phe	50	95	PS	n.a.	

				D							
M*	21	Healthy	8y	Nasal polyposis	p.Leu997Phe	p.Leu997Phe	21	70	PS	28.9	Nasal polyposi
F	46	CFTR-RD	43y	Bronchiectasis	p.Leu997Phe	p.Asn1303Lys	55	85	PS	n.a.	
F*	22	CFTR-RD	11y	Recurrent pa	p.Leu997Phe	p.Gly542*	31	103	PS	24.8	Nasal polyposi

				ncreatitis							
M	38	CFTR-RD	32y	CBAVD	p.Leu997Phe	p.Phe508del	31	n.a.	PS	n.a.	
M	41	CFTR-RD	35y	CBAVD	p.Leu997Phe	p.Phe508del	31	n.a.	PS	n.a.	
M*	44	CFTR-RD	39y	CBAVD	p.Leu997Phe	p.Asn1303Lys	37	n.a.	PS	21.3	
M	36	CFTR-RD	30y	CBA	p.Leu997Phe	p.Asn1303Lys	50	n.a.	PS	n.a.	

				VD							
M*	39	CFTR-RD	25y	CBAVD	p.Leu997Phe	p.Arg553*	31	n.a.	PS	n.a.	
M	28	CFTR-RD	25y	Recurrent pancreatitis	p.Leu997Phe	p.Phe316LeufsX12	31	n.a.	PS	n.a.	
M	44	CFTR-RD	27y	CBAVD	p.Leu997Phe	p.Arg334Trp	42	n.a.	PS	n.a.	

M	40	CFTR-RD	39y	CBAVD	p.Leu997Phe	p.Arg334Trp	46	n.a.	PS	n.a.	
M*	39	CFTR-RD	38y	CBAVD	p.Leu997Phe	p.Asp1152His	44	n.a.	PS	n.a.	
F	41	CFTR-RD	36y	Recurrent pancreatitis	p.Leu997Phe	p.Asp1152His	41	n.a.	PS	n.a.	
M	40	CFTR-RD	35y	CB	p.Leu997Phe	c.[1210-34TG[12];1210-12T[5]]	39	n.a.	PS	n.a.	

				AVD							
M	39	CFTR-RD	36y	Bronchiectasis	p.Leu997Phe	c.[1210-34TG[12];1210-12T[5]]	31	n.a.	PS	n.a.	
M	3	Healthy	1m	NBS	p.Leu997Phe	p.Phe508del	31	n.a.	PS	n.a.	
F	2	Healthy	1m	NBS	p.Leu997Phe	p.Lys684SerfsX38	37	n.a.	PS	n.a.	
M*	5	Healthy	1m	NBS	p.Leu997Phe	p.Phe508del	15	n.a.	PS	n.a.	

M	2	Healthy	1m	NBS	p.Leu997Phe	p.Gly542*	16	n.a.	PS	n.a.	
M	5	Healthy	1m	NBS	p.Leu997Phe	p.Arg117His	35	n.a.	PS	n.a.	
M	3	Healthy	1m	NBS	p.Leu997Phe	p.Asp1152His	26	n.a.	PS	n.a.	
M	5	Healthy	1m	NBS	p.Leu997Phe	p.Asp1152His	27	n.a.	PS	n.a.	
F	5	Healthy	1m	NBS	p.Leu997Phe	c.[1210-34TG[12];1210-12T[5]]	31	n.a.	PS	n.a.	
F	5	Healthy	1m	NBS	p.Leu997Phe	c.[1210-34TG[12];1210-12T[5]]	32	n.a.	PS	n.a.	
M	47	Healthy	44y	Familiarity	p.Leu997Phe	p.Phe508del	60	105	PS	n.a.	
M*	31	Healthy	23y	Familiar	p.Leu997Phe	p.Phe508del	29	n.a.	PS	36.9	

				ity							
M	32	Healthy	32y	Familiarity	p.Leu997Phe	p.Asn1303Lys	24	n.a.	PS	n.a.	
M	44	Healthy	40y	Familiari	p.Leu997Phe	c.489+1G>T	15	n.a.	PS	n.a.	

				ty							
M	41	Healthy	40y	Partner of CF carrier	p.Leu997Phe	c.[1210-34TG[12];1210-12T[5]]	60	100	PS	n.a.	
F*	32	Healthy	26y	Familiarit	p.Leu997Phe	p.Glu279Asp	20	n.a.	PS	n.a.	

				y							
F	38	Healthy	38y	Family	p.Leu997Phe	p.Val938GlyfsX37	23	n.a.	PS	n.a.	
M	46	Healthy	38y	Partner of CF carrier	p.Leu997Phe	p.Arg117His	20	n.a.	PS	n.a.	

F	43	Healthy	43y	Partner of CF carrier	p.Leu997Phe	p.Arg117His	11	n.a.	PS	n.a.	
F*	32	Healthy	30y	Familiarity	p.Leu997Phe	N	n.a.	n.a.	PS	86.4	
M	41	Healthy	37y	F	p.Leu997Phe	N	n.a.	n.a.	PS	78.9	

				a m i l i a r i t y							

*In the subjects marker by the asterisk we performed the analysis of CFTR STR (see text); SCL: sweat chloride level; we underlined SCL discordant with diagnosis; FEV_1: forced expiratory volume in the 1st second; PS: pancreatic sufficiency; N: wild type allele; n.a.: not assessed