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TITLE

Nasal epithelial cells: an "ex vivo model" contributing to the diagnosis and evaluation of new drugs in Cystic Fibrosis

TUTOR
Prof. Giuseppe Castaldo

CANDIDATE
Antonella Miriam Di Lullo

COORDINATOR
Prof. Vittorio Enrico Avvedimento

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INDEX

NON STANDARD ABBREVIATIONS

ABC: ATP-binding cassette
ALI: air-liquid interface
AONs: antisense oligonucleotides
CF: Cystic Fibrosis
CBAVD: congenital bilateral absence of vas deferens
CFSPID: Cystic Fibrosis Screen Positive Inconclusive Diagnosis
CFTR: Cystic Fibrosis Transmembrane Conductance Regulator
CFTR-RD: CFTR-related disorders
CFRD: CF-related diabetes
Cl^- : chloride
DIOS: distal intestinal obstruction syndrome
DMSO: dimethyl sulfoxide
ENaC: epithelial sodium channel
ER: endoplasmic reticulum
FEV1: forced expiratory volume in 1 second
GAPDH: glyceraldehyde 3-phosphate dehydrogenase gene
HDACi: histone deacetylase inhibitor
HEK-293: Human Embryonic Kidney
HNECs: human nasal epithelial cells
IGT: impaired glucose tolerance
IBMX: 3-isobutyl-1-methylxanthine
MI: meconium ileus
NBDs: nucleotide-binding domains
NBS: newborn screening
NECs: nasal epithelial cells
PI: pancreatic insufficiency
PKA: protein Kinase A
PKC: protein Kinase C
PS: Pancreatic sufficiency
Real Time-PCR: real time polymerase chain reaction amplification
RT-PCR: reverse transcriptase polymerase chain reaction amplification
SCL: sweat chloride levels
SPQ, M-440: 6-methoxy-N-(3-sulfopropyl) quinolinium
STR: short tandem repeats
TMDs: transmembrane domains
3-bp: triple-base
Ub: ubiquitin
YFP: yellow fluorescent protein

ABSTRACT

Cystic Fibrosis (CF) is an autosomal recessive disease caused by mutations in the *cystic fibrosis transmembrane regulator* (*CFTR*) gene. About 2000 mutations have been described so far. We set up the *ex vivo* model of human nasal epithelial cells (HNECs) to test the effect of novel mutations and to evaluate the effect of molecular therapies in cells from patients with CF bearing specific genotypes. We improved the sampling (by brushing), culture and analysis of HNECs using several techniques to study the effect of *CFTR* mutations. We performed 223 brushings from patients with CF and controls. Using cultured cells we: i) demonstrated the widely heterogeneous expression of *CFTR* in patients and in controls; ii) defined the splicing effect of a *CFTR* mutation; iii) assessed the CFTR gating activity of HNECs from patients bearing different mutations; iv) demonstrated that butyrate significantly enhances *CFTR* expression; v) described the genotype-phenotype correlation and the results of either *in vitro* and *ex vivo* studies performed on HNECs in a large group of patients with CF carrying *CFTR* complex alleles.

According to our data we can conclude:

1) the HNEC brushing is performed without anesthesia and it is well tolerated by children and adults;

2) once sampled, HNECs may be stored up to 48 hours before culture. This allows multicenter studies;

3) the HNECs culture is a suitable model to study the molecular effect of novel *CFTR* mutations and/or mutations of uncertain significance;

4) the *ex-vivo* model of HNECs may be used to evaluate, before the use in humans, the effect of novel drugs on cells bearing specific *CFTR* mutations;

5) our procedure may be used for the quantitative measurement of the CFTR gating activity of the HNECs from patients with different genotypes. It may help to classify:

 i) CF patients bearing two severe mutations, with an activity <10%;

 ii) CF patients bearing at least a mild mutation, with an activity of 10-30%;

 iii) CF carriers (heterozygous subjects) with an activity between 40-70%.

1. BACKGROUND

1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive disease more frequent among Caucasians [1]. It depends on mutations in the *CFTR* gene that encodes the CF transmembrane conductance regulator (CFTR) membrane protein, a cAMP-activated chloride (Cl⁻) channel [1]. It is synthesized in the endoplasmic reticulum (ER) then is glycosylated at Golgi level and transported to the apical plasma membrane [1].

More than 2000 mutations have been reported so far in alleles from patients with CF and with milder CFTR-related disorders (CFTR-RD) [1,2]. However, only a few mutations have a clear molecular effect defined by functional studies (http://www.cftr2.org/index.php). Molecular analysis helps to confirm diagnosis, to identify carriers and to perform prenatal diagnosis in high-risk couples [3]. It is based on the analysis of a commercial panel of the most frequent mutations with a detection rate of about 80% [4]. The *CFTR* gene sequencing of the whole coding regions has a detection rate of about 95% [5,6]. However, such procedures frequently detect mutations without a clear functional effect [7-9] and novel mutations for which complex *in vitro* procedures would be necessary to define the pathogenicity [10]. In addition, complex alleles (i.e., more mutations on the same allele) further complicate the interpretation of molecular analysis [11,12].

CF is a systemic disease. Its hallmarks include elevated sweat chloride levels (SCL), chronic bacterial infections of lower airways and sinuses, pulmonary inflammation, bronchiectasis and male infertility caused by obstructive azoospermia. Although CF is a systemic disease, the main cause of mortality is lung disease with opportunistic bacterial colonizations and neutrophil-dominated chronic inflammation [1]. More than 80% of patients have pancreatic insufficiency (PI) requiring pancreatic enzyme supplementation while 10-15% have normal exocrine pancreatic function and show a milder disease. Typically, such latter patients have at least one *CFTR* mutation with a mild effect that is functionally dominant on the severe mutation [11,13]. Moreover, an increasing number of patients is diagnosed as CFTR-RD [14]. They show a later onset of symptoms often involving a single organ (i.e., pancreatitis, disseminated bronchiectasis, obstructive azoospermia secondary to congenital bilateral absence of vas deferens (CBAVD)) [15]. Patients with CFTR-RD usually show borderline SCL and mutations causing a variable degree of protein dysfunction, some of which in non-coding regions [2,15-17]. Furthermore, the spreading of newborn screening (NBS) reveals patients with discordance between immunoreactive trypsinogen, SCL, *CFTR* genotype and clinical phenotype. They are defined as Cystic Fibrosis Screen Positive Inconclusive Diagnosis (CFSPID) [18].

Only symptomatic therapies are available to treat patients with CF even if novel molecular drugs become available in the last years [19]. Such therapies, that may potentiate the activity or may correct

mislocalisation of the mutated protein, have an effect only in patients bearing specific mutations.

We set up the sampling, culture and analysis of human nasal epithelial cells (HNEC). This *ex vivo* model was applied to study the effect of novel mutations (among which *CFTR* complex alleles) and to assess the effect of novel molecular therapies on cells from patients bearing specific mutations.

1.2 CFTR structure and function

CFTR was revealed as disease-gene of CF in 1989. It encodes a membrane protein belonging to the ATP-binding cassette (ABC) transporter superfamily that acts as a chloride channel and participates in the transport of other ions like sodium and bicarbonate in epithelial cells [1,20]. The protein is expressed by the trachea, lung, pancreas and several tissues of the reproductive system [1,13,21]. The CFTR has the same domain structure as other ABC transporters including:

- two nucleotide-binding domains (NBD1 and NBD2), with ATP-hydrolytic activity, in tandem with
- two transmembrane domains (TMD1 and TMD2), each containing six membrane-spanning alpha helices and
- a 200 aminoacid regulatory domain or "R" region, largely unstructured. The R-region lies between the first TMD and the second NBD, within the cytoplasm [20,22]. It is phosphorylated by protein kinase A (PKA) and C (PKC) (Figure 1 - *from Meng et al. 2016*). The two halves of CFTR (each including a NBD and a TMD) homodimerize to form the transporter [1, 23]

The R-region contains several Protein Kinase A (PKA) phophorylation sites highly conserved in the phylogenesis [20,24]. PKA is a cAMP-activated kinase that phosphorylates CFTR and such process is triggered by cAMP increase [20]. The R-region blocks the NBDs from associating together keeping the channel in a closed conformation. Phosphorylation triggers a structural change that removes the R-region from its steric-interfering position and permits the dimerisation of NBD, causing a much larger conformational change [20]. Then, the binding of ATP promotes channel opening while ATP hydrolysis and release of ADP and inorganic phosphate trigger channel closure [25,26]. Recently, a more complex model involving the C-terminal region was proposed: the phosphorylation of the R-region causes a switch from NBDs interacting to C-terminal interacting region. So, the R-region acts as a global regulator of CFTR via its alternative interacting partners [27].
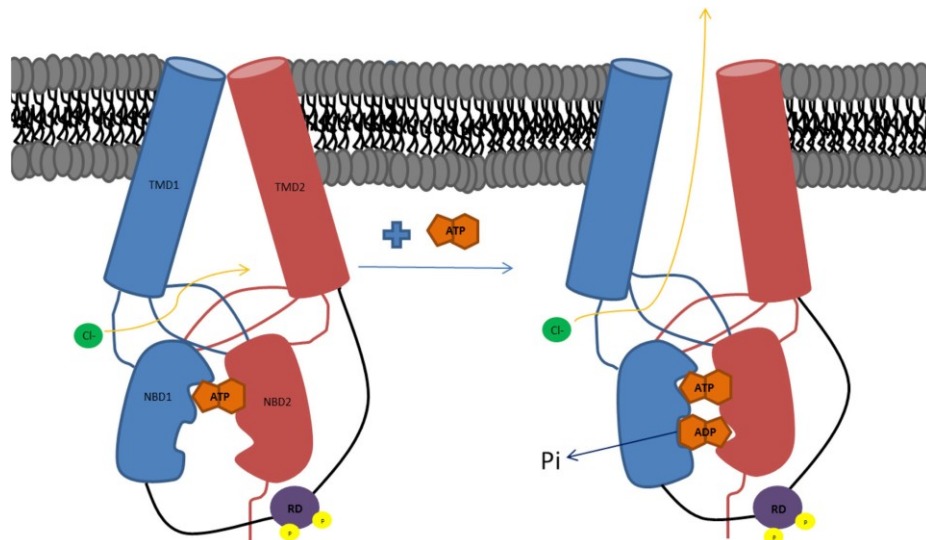
Figure 1: CFTR ATP binding and hydrolysis leads to channel opening triggering the flux of chloride ions (model proposed by Wang et al., from Meng et al. 2016).

In healthy subjects, a complex of various proteins that includes scaffold proteins (among which NHERF1) and signaling molecules (like cAMP and protein kinases) maintains CFTR in its correct position on the cell membrane and permits its activity [1]. In CF, the altered flux of ions and water into the lumen of bronchioles promotes bacterial infections and the influx of granulocytes into the airway lumen [1].

The actin cytoskeleton is essential to maintain the epithelium integrity and the activity of the CFTR protein: actin filaments control cell morphology, the adherence to extracellular matrix and prevent apoptosis [1]. The dysfunction of the CFTR protein causes the accumulation of sticky dehydrated mucus in various organs such as lungs, epididymis, biliary canalicula and pancreatic ducts. In the airways there is hyperabsorption of sodium and an abnormal ion and water flux consequent to decreased chloride secretion [28].

CFTR protein is also involved in the tonic negative regulation of the epithelial sodium channels (ENaC) activity [29]. The CFTR dysfunction triggers an increased ENaC activity.

Other pathological processes have been described in the airway of patients with CF, such as the reduced fluid secretion by airway submucosal glands and the altered secretion of mucous glycoproteins [30], reduced antimicrobial properties due to pH alterations or to altered ions concentration promoting bacterial colonization [31,32].

Chronic inflammation typically observed in patients with CF is characterized by high activity of cytokines (IL-1β, TNF-α, IL-8) in the bronchoalveolar lavage fluid and by a massive influx of granulocytes. This inflammatory response reduces the airway bacterial clearance [1]. In the intestine, meconium ileus at birth and distal intestinal obstructive syndrome in adults are due to dehydration of the mucus and may cause gut obstruction that, in most patients, requires surgery. All these alterations define the widely heterogeneous expression of the disease with progressive dysfunction of several organs [1].

1.3 CFTR gene mutations and classes

More than 2000 *CFTR* mutations have been described so far in patients with classic CF and CFTR-RD [1,2]. The most frequent mutation is p.Phe508del (F508del), found in about 66% of CF alleles worldwide. It is a class 2 mutation due to a triple-base (3-bp) deletion [1,33], causing the loss of the codon for phenylalanine at residue 508 of the protein sequence [34].

A frequency gradient of the F508del mutation is observed from northern to southern European populations [19]. Similarly, the frequency of most *CFTR* mutations varies greatly between geographic areas such is the case for G551D [19] (Fig.2 A e B).
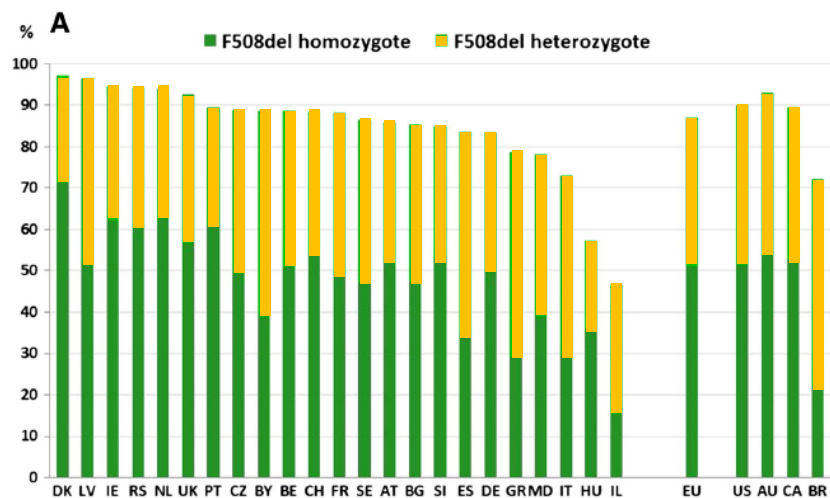
Figure 2A: Distribution of F508del *CFTR* mutation. Percent of patients homozygous (green) or heterozygous (yellow) for 508del mutation in different countries and regions. AT: Austria, BE: Belgium, BY: Republic of Belarus, BG: Bulgaria, CH: Switzerland, CZ: Czech Republic, DE: Germany, DK: Denmark, ES: Spain, FR: France, GR: Greece, HU: Hungary, IE: Ireland, IL: Israel, IT: Italy, LV: Latvia,MD: Republic of Moldova, NL:The Netherlands, PT: Portugal, RS: Serbia, SE: Sweden, SI: Slovenia, UK: United Kingdom. AU: Australia, EU: Europe, US: United States of America, BR: Brazil, CA: Canada. *(from Bell SC et al. 2015).*
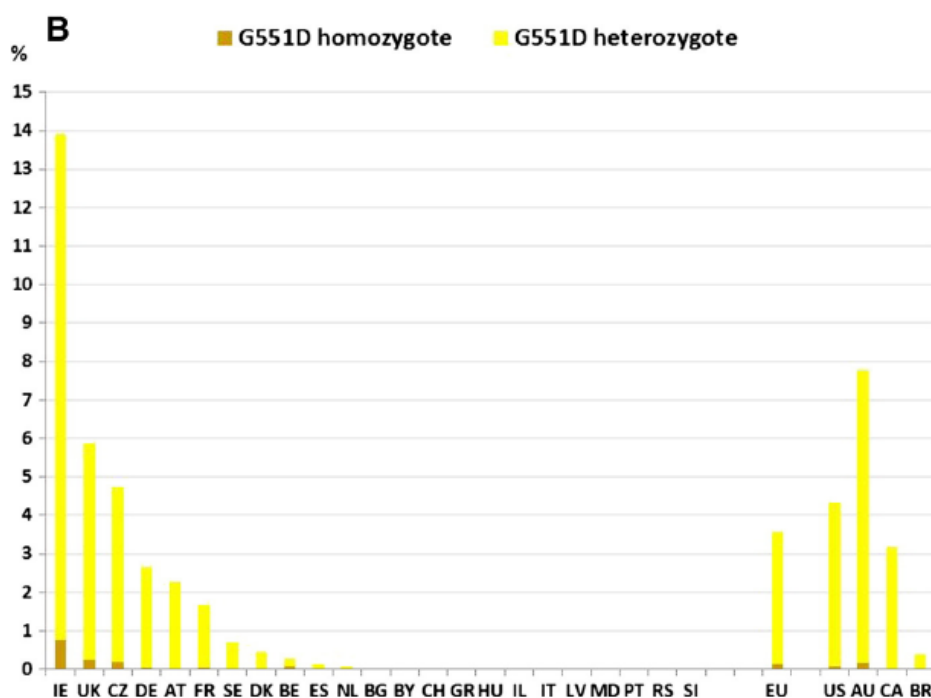
Figure 2B: Distribution of the G551D mutation. Percent of patients homozygous (dark) or heterozygous (light) for G551D mutation in different countries and regions. AT: Austria, BE: Belgium, BY: Republic of Belarus, BG: Bulgaria, CH: Switzerland, CZ: Czech Republic, DE: Germany, DK: Denmark, ES: Spain, FR: France, GR: Greece, HU: Hungary, IE: Ireland, IL: Israel, IT: Italy, LV: Latvia, MD: Republic of Moldova, NL:The Netherlands, PT: Portugal, RS: Serbia, SE: Sweden, SI: Slovenia, UK: United Kingdom. AU: Australia, EU: Europe, US: United States of America, BR: Brazil, CA: Canada. *(from Bell SC et al. 2015).*

In most ethnic groups only 10 to 15 *CFTR* mutations occur with a allelic frequency >1%. All the other mutations are very rare, mostly occurring in a few or a single patient. The 2000 *CFTR* mutations have the following distribution: missense (42%); frameshift (15%), splicing (13%), nonsense (10%), large (3%) and in-frame (2%) deletions/insertions, and promoter (0.5%); 15% are presumably non-pathological variants (www.CFTR.2.org) [19]. CFTR mutations are classified in six different classes according to the functional effect [19].

Class I mutations impair protein production. They include nonsense mutations (causing premature stop codons) that lead to mRNA degradation by nonsense-mediated decay. Examples of class I mutations are G542X (Britanny and Southern France), R1162X (Austria and Northern Italy), or W1282X (reaching a frequency of 48% among Ashkenazi Jews) [33].

Class II mutations affect the protein processing generating a misfolded protein which is recognized and degraded by endoplasmic reticulum (ER) quality control retention system [19].

Class III mutations impair the gating activity (e.g. G551D).

Class IV mutations decrease Cl⁻ ion conductance (e.g. R334W).

Class V mutations impair the splicing process generating variable percentages of aberrant and normal transcripts (e.g. 3272-26A>G). Their levels vary among patients and in different organs of each patient.

Class VI mutations decrease retention/anchoring to the cell membrane, often associated with decreased protein stability [19,35,36] (Figure 3).

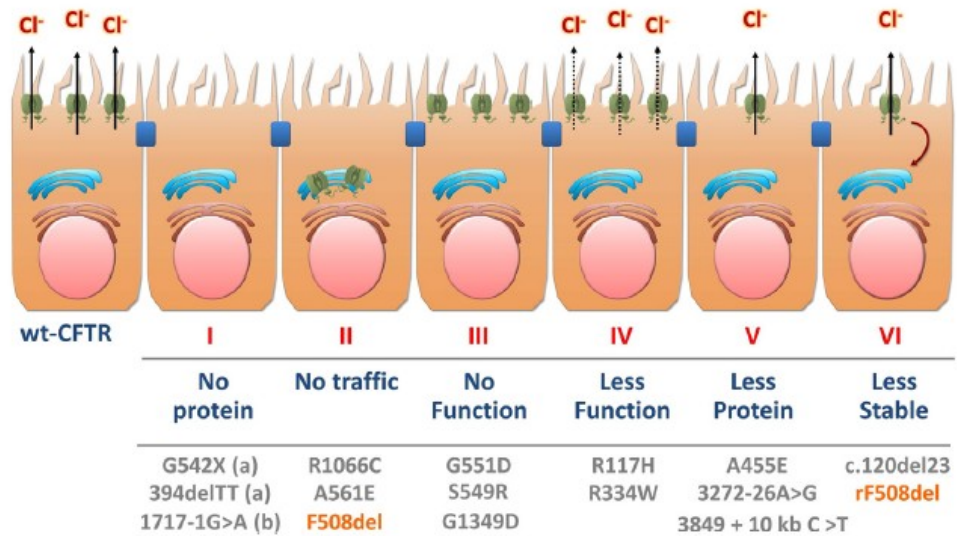| wt-CFTR	I	II	III	IV	V	VI
	No protein	No traffic	No Function	Less Function	Less Protein	Less Stable
	G542X (a)	R1066C	G551D	R117H	A455E	c.120del23
	394delTT (a)	A561E	S549R	R334W	3272-26A>G	rF508del
	1717-1G>A (b)	F508del	G1349D		3849 + 10 kb C >T	

Figure 3: Classes of CFTR mutations. Class I mutations, that completely abolish protein production, often include mutations that generate premature stop codons. Class II mutations (including the most prevalent F508del) cause retention of a misfolded protein in the ER, and subsequent degradation in the proteasome. Class III mutations affect channel regulation, impairing channel opening (e.g. G551D). Class IV mutations exhibit reduced conduction that causes decreased flow of ions (e.g. R334W). Class V mutations cause significant reduction in mRNA and/or protein levels – albeit with normal function – often impairing the splicing (e.g. 3272 − 26A>G). Class VI mutants cause significant plasma membrane instability and include F508del when rescued by most correctors (rF508del) *(from Amaral & Farinha, 2013)*.

This classification helps strategies of molecular drug development aimed to correct the effect of specific mutations, but has several limitations:
   a) for most mutations it is not yet defined the class;
   b) some mutations have characteristics of more than one class. An example is the F508del which, in addition to the trafficking defect (class II), displays also a gating (class III) and a cell surface stability defect (class VI). Another example is the R117H, classified as class IV due to a slight decrease in channel conductance but is not a CF-causing mutation alone. Indeed, it leads to CF when it is *in cis* with 5T which alone is a class V mutation, but not a mutation alone, again. So, the real CF causing mutation is the complex allele [R117H-5T] which can be considered as a class IV-V mutation.

In fact, the existence of complex alleles complicates the variability of the CF phenotype and the genetic counseling [11]. They are poorly defined for the lack of functional studies. Complex alleles result from the combination of two or more *CFTR* mutations *in cis* (i.e., on the same allele) that usually have a pathogenic effect while each single mutation has a milder or none effect [11]. So far, the following
the p.[Arg74Trp;Val201Met;Asp1270Asn],
the p.[Ile148Thr;Ile1023_Val1024del],

11

the p.[Arg117Leu;Leu997Phe] and
the c.[1210-34TG[12];1210-12T[5];2930C>T] [11]
complex alleles were described in patients with CF, but functional
studies on the effect of these mutations has been performed only in a few
cases [11].

1.4 Nasal epithelial cells

Airway epithelial cells play a relevant role in the first line of
antimicrobial defence: in fact, foreign particles and bacteria are trapped
in mucus and are removed by coordinated cilia beating and/or coughing
[36]. Human airways extend from the nose to trachea, bronchi,
bronchioles, and alveoli. Such epithelium includes a variety of cell types
with specialized functions and their relative distribution varies in the
different zones of the airways. The epithelium of the human airway is
columnar. It lays on a basal membrane and is lined by a mucus layer [37].
This epithelium is mainly constituted by ciliated cells. They are the main
cell type in nasal, tracheal and bronchial epithelium (85±2%, 81±2%,
and 83±2% respectively), whereas squamous cells dominated in
pharyngeal epithelium (87±3%) [34]. Other epithelial cells include basal,
goblet, serous and undifferentiated columnar cells; the relative
proportion of these cells is roughly similar among the various sites [34].
Interestingly, Trapnell et al, (1991) demontrated a striking discontinuity
in *CFTR* gene expression from nose to bronchus, with a sharp decrease
at the pharynx, and return to the levels of the nose in trachea and
bronchi. This pattern of gene expression is related to the number of
ciliated cells at each site, consistent with the concept that the *CFTR* gene
is highly expressed in ciliated cells [34]. The bronchial epithelia of
patients with CF is characterized by relatively less ciliated cells and a
greater basal undifferentiated and secretory cells [37]. While, no
differences in the cells distribution were found in the nasal epithelium of
patients with CF as compared to normal subjects.

Primary human epithelial cells can be obtained from nasal turbinates,
nasal polyps, trachea, bronchi or lung tissue specimens. However, such
tissues frequently contain yeast, fungi, or bacteria and media for cultures
should be supplemented with antibiotics for at least 3-5 days. Human
airway epithelial cell cultures are useful to study cell biology, disease,
and therapy related to respiratory tract diseases. Primary human airway
epithelial cells recapitulate the characteristic pseudostratified
mucociliary morphology and maintain most physiologic functions, thus,
represent an excellent model to study *in vivo* biology [38].

1.5 Epithelial cell culture

Different methods for primary human airway epithelial cell culture
were used so far like submerged, suspension, floating, and air-liquid
interface (ALI). These cultures reproduce the physiology of most *in vivo*
conditions [36]. Epithelial cells in culture show the characteristic
epithelial morphology, i.e., they appear isodiametric and compacted in
well-defined colonies with precise limits not spreading out of the
colonies. Cells of some CF airway epithelial lines are fibroblast-shaped,
aggregate in colonies with no precise limits and with cells spreading out

of the colonies. Epithelial cells express characteristic cytokeratins and form cell junctions typically observed in most epithelia *in vivo*. Monolayers formed by *in vitro* growth are real epithelial shetts that generate potential differences on both sides of the monolayer that can be easily measured. Primary cells are grown on collagen-coated dishes and usually on feeder layer. Most cells support only three to five passages [37].

1.6 CFTR mutation-specific therapies

The molecular effect of *CFTR* mutations is important for designing molecular treatments aimed to correct the defect (i.e. mutation-specific therapies) [19]. For istance, in class I, aminoglycoside antibiotics (e.g. gentamicin) and ataluren (PTC124) over-read the premature termination codons permitting the normal termination of the transcript ion. For Class II, chemical and molecular chaperones help to promote protein folding, allowing the mutant protein to avoid ER degradation and reach the cell membrane. These compounds have been called correctors (e.g. VX-809, VX-661). For class III mutations, CFTR channel activators defined potentiators, such as VX-770 (ivacaftor) had effect *in vitro* and in clinical trials in patients bearing at least one copy of the G551D mutation. For class IV mutations, the reduced conductance can be correct increasing the overall cell surface amount of the mutant protein with correctors, or enhancing the levels of channel activation (by potentiators). Class V mutations reduce normal protein levels often by affecting splicing and generating a variable percentage of aberrant and normal transcripts. Recent improvements in the use of antisense oligonucleotide (AONs) make this approach a very promising tool for the specific correction of splicing defects. Finally, compounds that enhance CFTR retention/anchoring at the cell surface partially correct the effect of class VI mutants. These include activators of Rac1 signaling which promote anchoring to actin cytoskeleton though NHERF1 [19].

2. AIM

2.1 The model of HNECs obtained by nasal brushing

About 2000 mutations have been reported so far in the disease gene in patients with CF or CFTR-RD [2]. However, only for a few mutations the molecular effect has been defined with complex *in vitro* procedures [39]. We set up the sampling (by brushing), culture and analysis of HNEC using several techniques that help to test the effect of *CFTR* mutations. We used this *ex vivo* model either to study the effect of novel mutations and to assess the effect of butyrate on *CFTR* expression on nasal cells from patients bearing specific mutations.

2.2 The splicing effect of a *CFTR* mutation on HNECs by nasal brushing

We collected samples of HNECs from a CF patient heterozygous for the 711+1G>A mutation, that was predicted to have an effect on the splicing of exon 5. The analysis was performed by RT-PCR, using primers that included *CFTR* exon 4, 5 and 6. Electrophoretic analysis of the cDNA amplicon showed that the 711+1G>A mutation caused the retention of intron 5 due to altered splicing.

2.3 The CFTR gating activity in patients bearing different mutations

To test the activity of the CFTR protein, we used the halide-sensitive fluorescent system by the iodide-sensitive fluorescent indicator SPQ (Molecular Probes, Invitrogen, M440).

2.4 The effect of butyrate on CFTR expression

We treated cultured nasal epithelial cells from 5 controls and from 20 patients with sodium butyrate to assess the effect of the molecule on the levels of *CFTR* expression.

2.5 Genotype-phenoptype correlation and functional studies in CF patients bearing CFTR complex alleles

Complex alleles result from two or more *CFTR* mutations *in cis* (i.e., on the same allele) that usually have a pathogenic effect while each single mutation has only a minor or none effect. So far, few subjects bearing
the p.[Arg74Trp;Val201Met;Asp1270Asn],
the p.[Ile148Thr;Ile1023_Val1024del],
the p.[Arg117Leu;Leu997Phe] and
the c.[1210-34TG[12];1210-12T[5]; 2930C>T]
complex alleles were described [11] and a functional characterization of the effect of these mutations was performed in a limited number of cases. We studied a cohort of CF patients carrying *CFTR* complex alleles and described the genotype–phenotype correlation and the results of either *in vitro* and *ex vivo* studies performed on HNECs.

3. MATERIALS AND METHODS

3.1 Subject population

The study was approved by the Ethical Committee of the University of Naples Federico II. We performed nasal brushing to 123 CF patients with different genotypes and 100 healthy controls after a complete ear-nose-throat evaluation. Among these, we performed a retrospective analysis of all patients in follow-up at twelve Italian CF centres and included all subjects who were homozygous or compound heterozygous for the following complex alleles:

(1) p.[Arg74Trp;Val201Met;Asp1270Asn], n=8;
(2) p.[Arg74Trp;Asp1270Asn],n=2;
(3)p.[Ile148Thr;Ile1023_Val1024del],n=5;
(4) p.[Arg117Leu;Leu997Phe], n=6;
(5) c.[1210-34TG[12];1210-12T[5];2930C>T], n=3.

Furthermore, we studied subjects homozygous or compound heterozygous for the following mutations:

(1) (2) p.Asp1270Asn, n=2;
(3) p.Ile148Thr,n=4; and
(4) p.Leu997Phe, n=34.

Finally, we studied obligate carriers heterozygous for the p.Ile148Thr (n=2);
p.Leu997Phe (n=2) mutations and for the
p.[Arg74Trp;Asp1270Asn] complex allele (n=2).

We measured the CFTR gating activity on HNECs from 39 subjects and compared the data with those obtained from: (1) patients with CF with two class I–II mutations, n=8, and (2) carriers of class I–II mutations, n=4.

3.2 Clinical data

The diagnosis of CF, CFTR-RD and CFSPID was performed according to standard criteria [14,18,40]. From each subject, we collected clinical and genetic data at diagnosis and during the follow-up. SCL were analyzed using the Gibson and Cooke method [41,42,43,44]. SCL <40 mmol/L were considered normal, between 40 and 59 mmol/L were considered borderline and ≥60 mmol/L were considered pathological [42]. The last best forced expiratory volume in 1 second (FEV1) was recorded for patients > 6 years. It was expressed as percentage of predicted value for age, according to standardized reference equations for spirometry and was performed when patient was free from pulmonary exacerbations [45]. Given the interindividual variability of FEV1 and the evolution of lung damage with age, each patient was classified as severe or mild according to Schluchter et al. criteria that take into account both FEV1 value and age [46]. For patients who had died, we considered the last available value. *Pseudomonas aeruginosa* chronic infection was defined according to the modified Leeds criteria [47]. Pancreatic sufficiency (PS) was defined on the basis of at least two values of faecal

pancreatic elastase higher than 200 µg/g measured outside acute gastrointestinal diseases [48]. Faecal pancreatic elastase was evaluated annually in patients with PS and at least 3 months before enrolment. Pancreatitis was defined according to the report from the international study group of pediatric pancreatitis [49]. All patients performed annually a glucose tolerance test. CF-related diabetes (CFRD) was diagnosed according to the American Diabetes Association criteria [50]. CF-associated liver disease was defined by clinical laboratory and instrumental evaluations as previously described [51].

3.3 Molecular analysis of CFTR

We screened all patients using a commercial panel of mutations with a detection rate for CF alleles of about 80% [4]. Then, we tested for the most common rearrangements [6] and carried out gene sequencing (detection rate about 97% for classic CF) [52] in cases where one or both mutations resulted undetected after first-level analysis, according to European recommendations [53]. All laboratories involved in this study participate to the national project on standardization and quality assurance for molecular genetic testing [54]. For *CFTR* mutations, we used the nomenclature guidelines suggested by the Human Genome Variation Society.

3.4 Nasal brushing

Before sampling, the informed consent was required to all patients (legal guardian for minors), after a careful description of the aims of the study. All subjects underwent an ear-nose-throat evaluation. After nasal washings with saline in order to remove mucus (two washings per day in the week before and one washing immediately before the sampling), nasal brushing was performed by a soft sterile interdental brush with 2.5 to 3 mm bristles (Paro-Isola, Switzerland) scraping (Figure 4a) along the middle portion of the inferior turbinate by gentle backward–forward and rotatory movements (circular movement) in each nostril, under direct visualization, using a headlamp without decongestant or local anesthesia (Figure 4b). Patients were carefully monitored for vital and minor signs, comfort and pain. They were discharged on the same day.
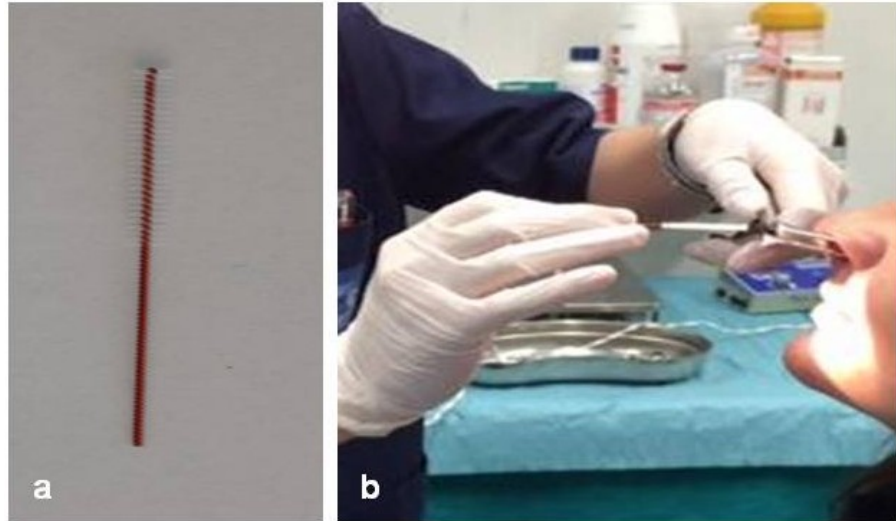
Figure 4: a) an example of soft sterile interdental brush with 2.5 to 3 mm bristles (Paro-Isola, Switzerland) scraping; b) ENT specialist operator during execution of nasal brushing in a patient.

3.5 Culture of nasal cells

The sample from each nostril was immediately stored in a 15 mL tube containing 2.5 mL of RPMI 1640 medium, complemented with 3% antibiotics. Cells were placed on Eppendorf Thermomixer, in agitation at 700 rpm for one hour to remove all cells from brushes. Cells were centrifuged at 2000 rpm for 20 minutes, supernatant were discarded and resuspended in serum-free bronchial epithelial cell growth medium BEGM (Clonetics, MD). Then, cells were placed in CELL T 25 flasks (Sarstedt Ltd, UK). At confluence of 60%, cells were passed in new flasks after count by Invitrogen (Italy) Cell Countess. Trypan blue exclusion test was used to establish total viable cell number and the percentage of viability. Nasal cells can be stored at 4°C up to 48 h before culturing (using the RPMI 1640 medium), and this permits to collect the samples from patients followed in other centres. At the confluence of >80%, cells were treated with 5 mM of sodium butyrate for 24 hours (Figure 5).

Figure 5: an example of HNECs placed in CELL T 25 flasks (Sarstedt Ltd, UK) at 5 days.

3.6 Nasal cytology

Epithelial cells include various types: ciliated, non-ciliated, striated and basal [55]. Ciliated cells (our target) represent more than 80% of cells obtained by nasal brushing; they have tall columnar shapes with distinct cilia. Non ciliated cel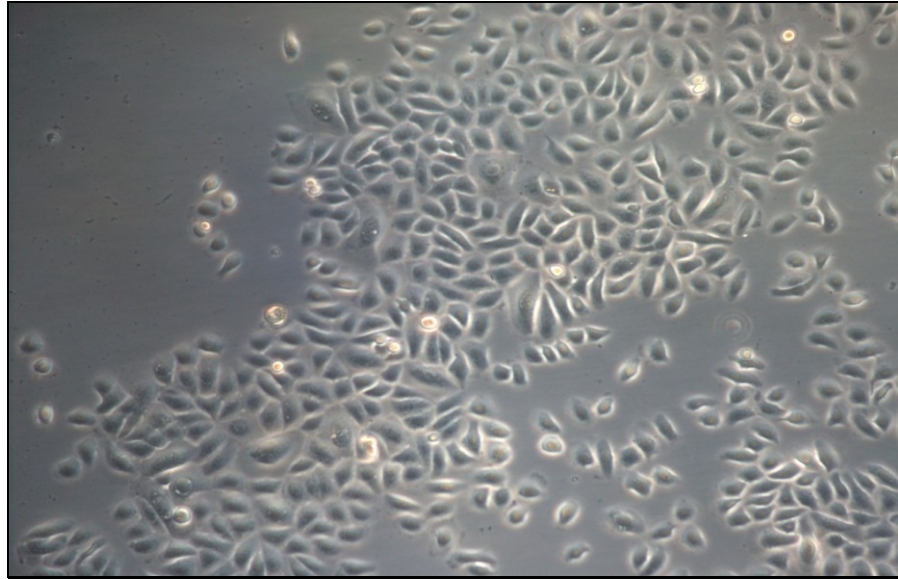ls, including secretory goblet cells, have similar shape but no cilia; striated and basal cells are smaller with dense, round nuclei, strongly stained cytoplasms, and a high nuclear-cytoplasm ratio. In addition, leukocytes or inflammatory cells may be found in the brushing sample if the patient would have inflammatory conditions. We used May-Grunwald-Giemsa stain of freshly obtained nasal cells to verify the presence of an adequate amount of ciliated cells and to exclude the presence of inflammatory cells. The freshly isolated human cells recovered from nasal brushings and spread on silane glass slides were stained by the May-Grunwald-Giemsa. After 5 minutes fixing in methanol, slides were immersed for 5 minutes in May-Grunwald's standard stain (Fluka Chemie, Switzerland), freshly diluted with an equal volume of phosphate buffer pH 6.8 and then, without washing, immersed for 10 to 15 minutes in Giemsa stain (Merck, Germany) diluted with nine volumes of phosphate buffer pH 6.8. After 3-4 rapid washes in phosphate buffer pH 6.8 and 2 to 5 minutes in water, slides were mounted with Entellan (Merck), covered with glass coverslips and dried for at least 1 hour before analysis. Samples on slides were evaluated for cell differential count and morphology using a conventional light microscope (Zeiss, Germany) (Figure 6).

The culture of nasal epithelial cells helps to selectively expand epithelial ciliate cells. To verify that such cells maintain their phenotype after prolonged culture (> 20 days) we used the cytokeratin staining. The staining with anti- KRT18/cytokeratin-18 (CK- 18; Abcam, Italy, ab52948) antibody was used to confirm epithelial cell purity, and that with anti-CD3+, (Abcam, ab5690),

CD4+ (Abcam, ab51312) or CD19+ (Abcam, ab25232) 1:500 antibodies was used to exclude the presence of lymphocytes or other inflammatory cells. Moreover, the cells were treated with anti-Pan-cytokeratin (C5992, Sigma Aldrich, Italy) 1:500 and MUC5AC (Abcam, ab3649) or MUC3B (Abcam, ab85006) 1:200 antibodies to exclude mucipar differentiation.
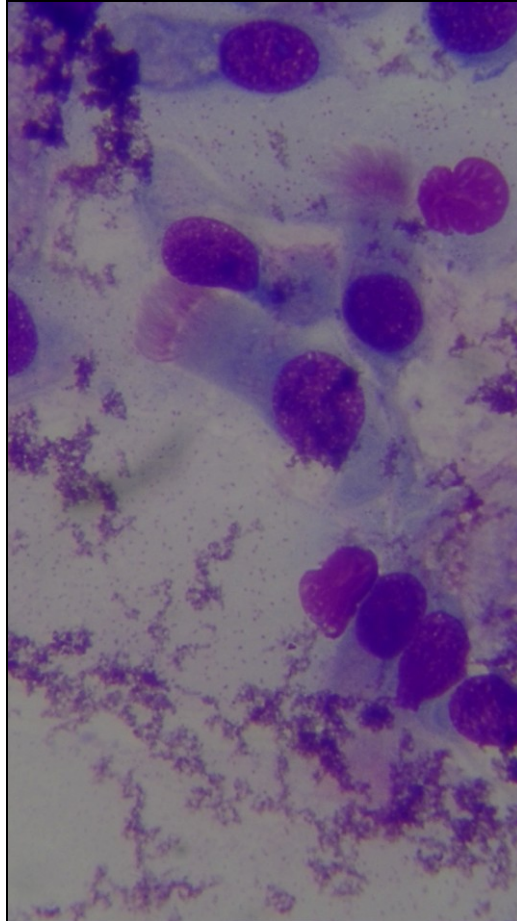
Figure 6: an example of ciliated cell's morphology stained by the May-Grunwald-Giemsa at a conventional light microscope - magnification 1000X (Zeiss, Germany).

3.7 Real-time PCR for quantitative analysis of CFTR mRNA

Total RNA is isolated from HNECs using TRIzol (Invitrogen, Italy) as previously described [56]. RNA concentration and purity is evaluated using a NanoDrop ND-1000 spectrophotometer; reverse transcription is carried out on 1 μg of total RNA resuspended in DEPC-treated nano pure water using QuantiTect Rev Transcription Kit (Qiagen, CA) according to the protocol by the manufacturer. To check the levels of *CFTR* transcript in nasal epithelial cell (NEC), relative quantification by Real-time PCR is performed in duplicates using LightCycler 480 Probes Master containing *CFTR* primers (Roche Italy) and a TaqMan CFTR probe (ID. Assay 102716). Amplification is carried out with the LightCycler 480 Systems for Real-Time PCR (Roche) with a two-step PCR protocol

(preincubation of 10 min at +95° C followed by 45 cycles of amplification: 95°C for 10 sec, 60°C for 25s, 72°C for 1s). mRNA quantification results are normalized using *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene (Roche, ID. Assay 101128) as an endogenous control.

3.8 RT-PCR analysis to assess the effect of splicing mutations

Mutations within the exon-intron boundary were preliminarly analyzed by prediction softwares Alamut or NetGene2. Then, if the analysis predicted an alteration of the splicing pattern, we performed an electrophoretic analysis on cDNA obtained by RT-PCR from *CFTR* mRNA extracted from cultured nasal cells. We used different pairs of primers complementary to two (or more) subsequent exonic sequences. Using these primers, intronic DNA sequences retained in the mRNA due to the altered splicing (if present) were amplified, giving rise to one or more bands of greater size compared to the *wild type*.

3.9 Quantitative analysis of CFTR channel activity on HNEC

To test the activity of the CFTR protein, we used the halide-sensitive fluorescent system. The iodide-sensitive fluorescent indicator, 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ, M-440) (Molecular Probes, Invitrogen,) was introduced into cells in a hypotonic solution of iodide buffer (in mM:130 NaI, 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 20 min at 37°C in a humidified chamber with 5% CO_2. SPQ-loaded cells were then mounted on a LSM510 Meta confocal microscope with a 37°C heated stage and perfused with iodide buffer. Changes in CFTR-mediated SPQ fluorescence were monitored at the 445 nm in response to excitation at 340 nm. Fluorescent is constantly measured by the passage between different solutions contain halide anions. Cells were initially perfused with iodide buffer followed by perfusion 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 from the 50 cells examined per population per coverslip.

3.10 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.

3.11 Plasmid Constructs and lentiviral vector production

The *wild-type*, N1303K and F508del *CFTR* coding sequences were amplified from pTracer plasmid, gently provided by Prof. Galietta (Genua, Italy). While, the genetic variation of interest either alone i.e., D1270N (D) or in different combinations, i.e., [D1270N;V120M;R74W] (DVR); [D1270N;V120M] (DV), were introduced into the *wild-type CFTR* coding sequence using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) and the designed primers, 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) [57] 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.

3.12 Western Blot analysis

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 Tris HCl, 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 a immun-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.

3.13 CFTR activity assay on HEK-293 cells.

The HEK293 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, then 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 Espire 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 20μM forskolin (Sigma) and IBMX (100μM) (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).

3.14 Statistical analysis

For real-time PCR assay, the values of CFTR mRNA are reported as means +/- SD ratios to *GAPDH* housekeeping mRNA. Rate of chloride efflux measured in at least 50 cells for experiment. Mean ± SD of 3 experiments. Statistical significance was defined as p value of < 0.05 vs control subject.

4. RESULTS

4.1 Sampling and culture of HNECs

We performed nasal brushing to 100 healthy volunteers and 123 patients with CF (or carriers) with different *CFTR* genotypes. In all 223 cases HNECs were obtained successfully, with any complication nor discomfort for subjects. The May-Grunwald-Giemsa staining (performed on 30 samples) confirmed the presence of adequate amounts of ciliated epithelial cells. In all cases we cultured the cells and in 208/223 (93.2%) cases we obtained a positive culture. Figure 7 shows an example of the culture of HNECs at different days. In 16/223 (6.8%) cases the cells did not expand due to the strong contamination with mucus or with a high number of keratinocytes. To avoid contamination, we modified our original protocol, and now before the sampling we: i) carefully verify the absence of any clinical condition potentially associated with a higher mucus production; ii) perform washings with physiological solutions (see materials and methods).

To verify that the culture did not modify the phenotype of cells, we used a panel of anti-cytokeratin antibodies, specific for epithelial cells; we confirmed the same reactivity to antibodies of cultured cells at different days as compared to freshly sampled cells. Furthermore we assessed, by quantitative RT-PCR, the levels of *CFTR* transcript in cells before culture and at different days of culture until 20th day (in ten different experiments), and no significant changes were observed (data not shown). Finally, we assessed the effect of storage of cells in transport medium before culture, and in 10 different experiments we demonstrated that cells can be stored at least 48 hrs at 4°C before a positive culture.
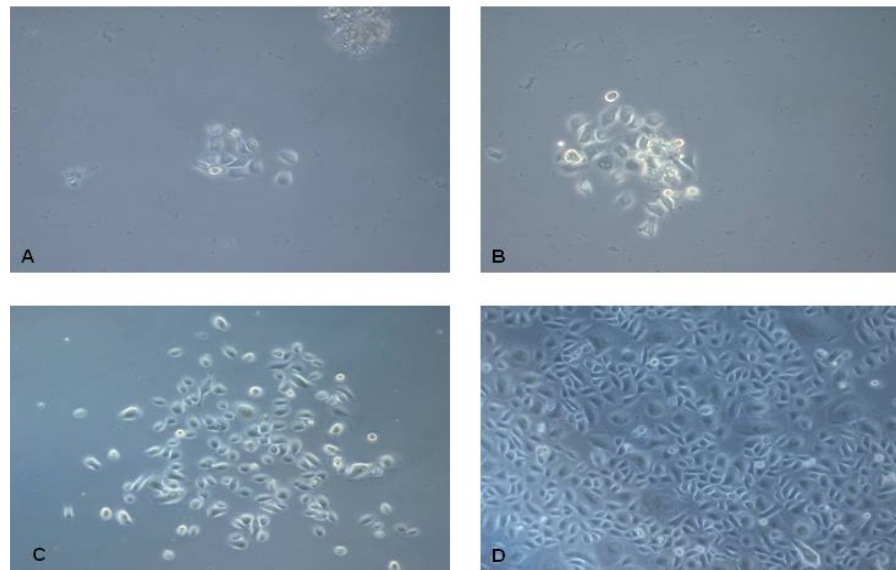
Figure 7: an example of HNECs expansion at different days of culture. **A**: 3 days; **B**: 4 days; **C**: 7 days; **D**: 10 days.

4.2 Analysis of CFTR mRNA levels

We analyzed, by quantitative RT-PCR, the levels of *CFTR* transcript as a ratio with the *GAPDH* housekeeping gene mRNA in samples from healthy subjects and from CF patients with different mutations (Figure 8); such analysis can be performed either on RNA from cultured cells and on RNA extracted from fresh sampled nasal cells entrapped in the brush before culture. The analysis showed a very heterogeneous basal expression of *CFTR* mRNA.
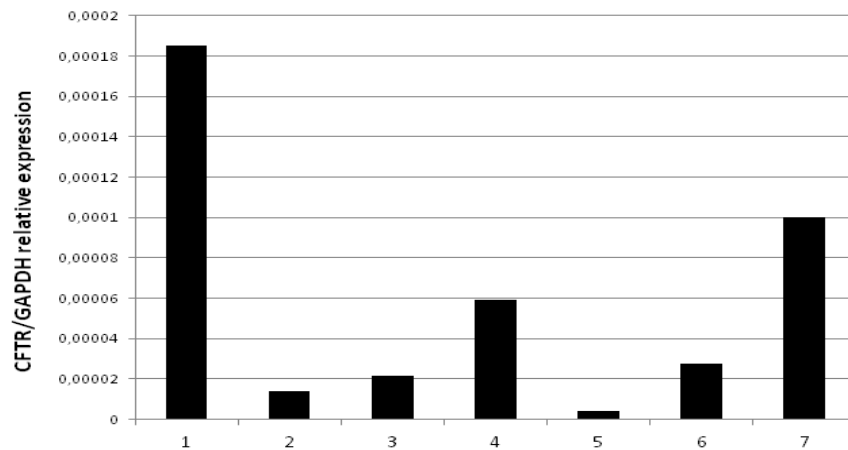
Figure 8: quantitative RT-PCR analysis of CFTR mRNA levels expressed as a ratio to the housekeeping GAPDH mRNA. 1: control sample from a healthy subject; 2 to 7: samples obtained from CF patients with different *CFTR* genotypes.

4.3 Treatment of HNECs with sodium butyrate

Then, we treated cultured nasal epithelial cells of 5 controls and of 20 patients with CF with sodium butyrate. The treatment enhanced *CFTR* mRNA levels in all cases (some examples are reported in Figure 9).
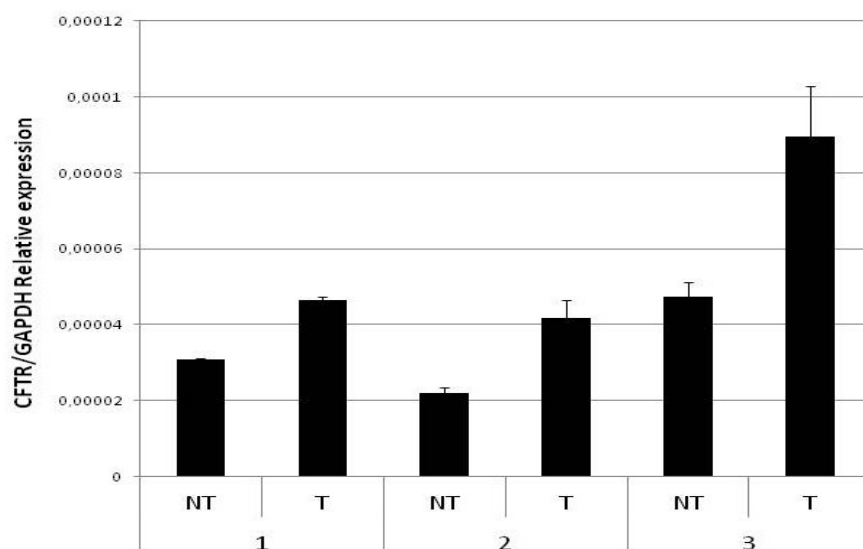
Figure 9: effect of butyrate on CFTR mRNA expression. The figure shows the quantitative RT-PCR analysis of CFTR mRNA levels expressed as a ratio to the

housekeeping GAPDH mRNA in three samples of nasal epithelial non-treated (NT) and butyrate-treated cells (T).

4.4 Analysis of the splicing effect of a *CFTR* mutation

We studied a patient with CF compound heterozygous for the 711+1G>A mutation, that was predicted by *in silico* analysis to cause the altered splicing of the exon 5. The analysis was performed by RT-PCR, using primers that included *CFTR* exons 4, 5 and 6. The electrophoretic analysis of the cDNA amplified product clearly showed that the 711+1G>A mutation impairs the splicing causing the retention of intron 5 (Figure 10).
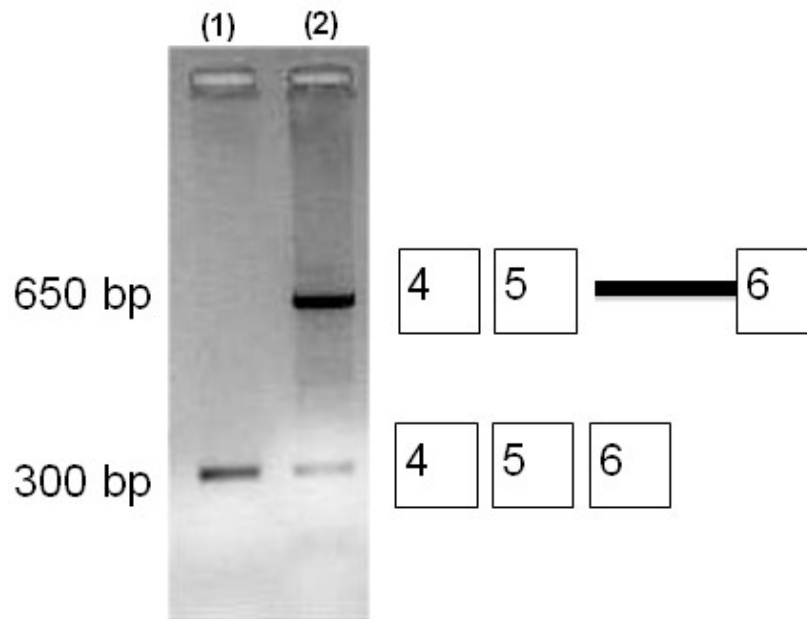
Figure 10: RT-PCR analysis of *CFTR* mRNA from a healthy control subject (1) and from a CF patient heterozygous for the 711+1G>A mutation (2). The mutation has a potential effect of altered splicing of the *CFTR* mRNA causing the retention of an intronic sequence that appears as an electrophoretic band with a higher molecular weight in addition to the normal band also present in the healthy subject.

4.5 Gating activity of CFTR

We analyzed the quantitative gating activity of CFTR in all the 123 patients with CF or carriers. In all cases the analysis provided a clear result, and Figure 11 shows several examples: #1 is a healthy control subject (its activity is considered 100%); #2 and 3 are two CF patients with two severe mutations each (i.e., F508del/F508del for case #2 and G542X/4016insT for case #3): they show an activity of 9.9% and 10.4% as compared to the control, respectively; case #4 is a CF patient with a severe and a mild CF mutation (i.e., W1282X/D1152H): he shows an activity of about 20.3%. Finally, case #5 is a heterozygous carrier of the severe G542X mutation: he showed an activity of 76.8%. Finally, patients bearing two severe mutations (like the F508del, the G542X, the 4016insTc38) have an

activity ≤10%. Such patients usually show classic CF with PI and altered SCL. While, patients with CF bearing at least a mild mutation, such as D1152H show an activity between 10% and 30%. Such patients may have mild CF with PS or CFTR-RD with borderline or slightly enhanced SCL. Carrier subjects have a gating activity of 40 to 75%. Of course, the quantitative analysis of *CFTR* in nasal cells can also assess, in the *ex vivo* model from patients bearing specific mutations, the effect of potential drugs like potentiators and/or correctors [19] or molecular therapies before their use in humans [58, 59].
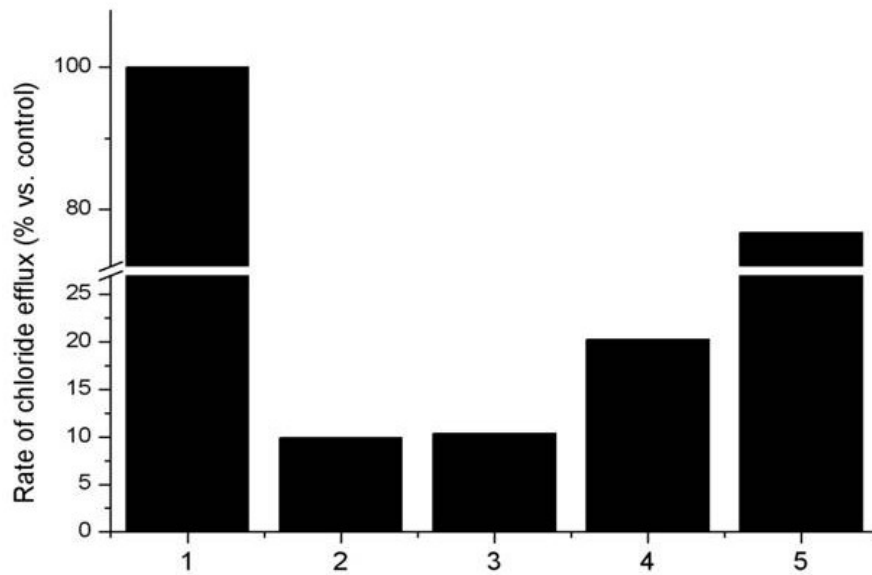
Figure 11: quantitative gating activity of *CFTR*. #1 is a healthy control subject (his activity is considered 100%); #2 and #3 are two CF patients compound heterozygous for two severe mutations each (i.e., F508del/F508del for case #2 and G542X/4016insT for case #3): they show an activity of 9.9% and 10.4% compared to the control, respectively; case #4 is a CF patient with a severe and a mild CF mutation (i.e., W1282X/D1152H) with 20.3% of activity. Finally, case #5 is a heterozygous carrier of the severe G542X mutation with an activity of 76.8%.

4.6 Genotype-phenotype correlation in CF patients bearing *CFTR* complex alleles:

p.[Arg74Trp;Val201Met;Asp1270Asn] and p.[Arg74Trp; Asp1270Asn] complex alleles and p.Asp1270Asn mutation

We studied eight subjects compound heterozygous for the p.[Arg74Trp;Val201Met;Asp1270Asn] complex allele triple mutant (table 1). Six had a class I–II (severe) mutation *in trans* (i.e., p.Phe508del: three cases; p.Asn1303Lys: two cases and p.Ser1206*: one case) and two had *in trans* a mild *CFTR* mutation with higher residual activity (i.e., p.Asp1152His and p.Asp579Gly). Among the six patients compound heterozygous with a severe mutation, four were diagnosed as CF. These patients had altered SCL, PS, mild lung disease and none was colonised by *P. aeruginosa*; two patients had impaired glucose tolerance (IGT). The mean CFTR gating activity

on HNEC (assessed in three cases) was 11.2% (range 9.8–12.0%), significantly higher (p<0.001) as compared with the mean activity of 6.9% found in eight patients with two class I–II mutations (figure 12 and table 2). The two other patients (one with the severe p.Phe508del mutation and the other with the severe p.Ser1206* mutation in *trans*), aged 12 and 13 years, were diagnosed as CFTR-RD. One had normal SCL (i.e., 37 mmol/L) and the other had borderline levels. The CFTR gating activity on HNEC, tested in one patient, was 15.0% (table1). The two remaining patients with the p.[Arg74Trp;Val201Met;Asp1270Asn] complex allele, carrying the p.Asp579Gly and the p.Asp1152His mutation *in trans*, were diagnosed as CBAVD at the age of 37 and 48 years, respectively. The patient carrying the p.Asp579Gly mutation had altered SCL (i.e.,118 mmol/L) despite the mild clinical course and a residual CFTR activity on HNEC of 19.1%; the other subject, carrying the p.Asp1152His mutation, had normal SCL and CFTR gating activity of 18.5% (table 1).

Of the two subjects with the p.[Arg74Trp;Asp1270Asn] complex allele (double mutant), one was classified as CFTR-RD and had the c.[1210-34TG;12 1210-12T[5]] complex allele (a mild mutation) *in trans*. He had CBAVD, normal SCL and a CFTR gating activity on HNEC of 18.9% (table 1). The other, revealed by NBS, was previously defined as CFSPID and now classified as healthy at the age of 5 years old. Finally, we analysed the residual CFTR gating activity on HNEC from two carriers of the p.[Arg74Trp;Asp1270Asn] complex allele. They had values of 92.6% and 94.0%, respectively.

In the two subjects with the p.Asp1270Asn single mutant (*in trans* with a class I–II mutation) both clinical data and SCL were normal. The CFTR gating activity on HNEC, available for one of them, was 44.0% (figure 12 and table 2).

Furthermore, we studied *in vitro* either the CFTR protein by western blot and the CFTR gating activity in HEK293 cells transfected with the different mutants of the p.[Arg74Trp;Val201Met;Asp1270Asn] complex allele. Western blot analysis revealed two bands (figure 13). The C band corresponds to the mature, fully glycosylated protein, while the B band is the core-glycosylated quite inactive protein. We calculated, for each mutant, the ratio between the C band and the total protein (band B+C). For the triple mutant, we obtained a ratio of 21%; the double mutant gave a ratio of 64% and, finally, the p.Asp1270Asn single mutant is associated with a ratio of 83% (figure 13). These data compare with a 58% and 40% ratio obtained for the severe p.Phe508del and p.Asn1303Lys mutants, respectively (figure 13). We then evaluated the CFTR activity, (figure 14) that was 38.6%, 42.8% and 45.4% of the wild type for the triple, the double and the single mutant, respectively. These data compare with the values of 2.9% and 0.2% obtained for the severe p.Phe508del and p.Asn1303Lys mutations, respectively.

Table 1: Demographic and clinical data of subjects bearing the [p.Arg74Trp;p.Val201Met;p.Asp1270Asn] or the [p.Arg74Trp;p.Asp1270Asn] complex alleles or the p.Asp1270Asn mutation

Gender	Current age (years)	Diagnosis	Age at diagnosis/ enrolment (years)	Cause of diagnosis/ enrolment	Allele 1	Allele 2	SCL* (mmol/L)	Current FEV_1 (%)	Pancreatic status	CFTR gating (%)	Other
M	36	CF	19	Hypochloremic alkalosis	p.[Arg74Trp;Val201Met;Asp1270Asn]	p.Phe508del	89	104	PS	n.a.	CBAVD
F	58	CF	53	Familiarity	p.[Arg74Trp;Val201Met;Asp1270Asn]	p.Phe508del	62	105	PS	12.0	
F	21	CF	9	Familiarity	p.[Arg74Trp;Val201Met;Asp1270Asn]	p.Asn1303Lys	109	119	PS	9.8	IGT
F	23	CF	10	Respiratory	p.[Arg74Trp;Val201Met;Asp1270Asn]	p.Asn1303Lys	65	95	PS	11.8	IGT; bronchiectasis
M	12	CFTR-RD	6	Respiratory	p.[Arg74Trp;Val201Met;Asp1270Asn]	p.Phe508del	*37*	87	PS	15.0	
M	13	CFTR-RD	1	Hypochloremic alkalosis	p.[Arg74Trp;Val201Met;Asp1270Asn]	p.Ser1206*	46	91	PS	n.a.	
M	37	CFTR-RD	32	CBAVD	p.[Arg74Trp;Val201Met;Asp1270Asn]	p.Asp579Gly	*118*	99	PS	19.1	
M	48	CFTR-RD	42	CBAVD	p.[Arg74Trp;Val201Met;Asp1270Asn]	p.Asp1152His	*31*	n.a.	PS	18.5	
M	40	CFTR-RD	34	CBAVD	p.[Arg74Trp;Asp1270Asn]	c.[1210-34TG[12];1210-12T[5]]	*32*	n.a.	PS	18.9	
F	5	Healthy	1	NBS	p.[Arg74Trp;Asp1270Asn]	p.Phe508del	11	n.a.	PS	n.a.	
M	40	Healthy	35	Familiarity	p.Asp1270Asn	p.Asn1303Lys	9	n.a.	PS	n.a.	
M	40	Healthy	35	Familiarity	p.Asp1270Asn	p.Phe508del	12	n.a.	PS	44.0	
M	50	Healthy	40	Familiarity	p.[Arg74Trp;Asp1270Asn]	N	n.a.	n.a.	n.a.	92.6	
F	47	Healthy	47	Familiarity	p.[Arg74Trp;Asp1270Asn]	N	n.a.	n.a.	n.a.	94.0	

*For patients with CF, we reported SCL at diagnosis while for CFTR-RD and for healthy subjects we reported current SCL; values in italics represent SCL discordant with diagnosis.

CBAVD, congenital bilateral absence of vas deferens; CF, cystic fibrosis; CFTR-RD, cystic fibrosis transmembrane conductance regulator -related disorders; FEV_1, forced expiratory volume in 1 second; IGT, impaired glucose tolerance; n.a., not assessed; N, wild-type allele; NBS, newborn screening; PS, pancreatic sufficiency; SCL, sweat chloride level.
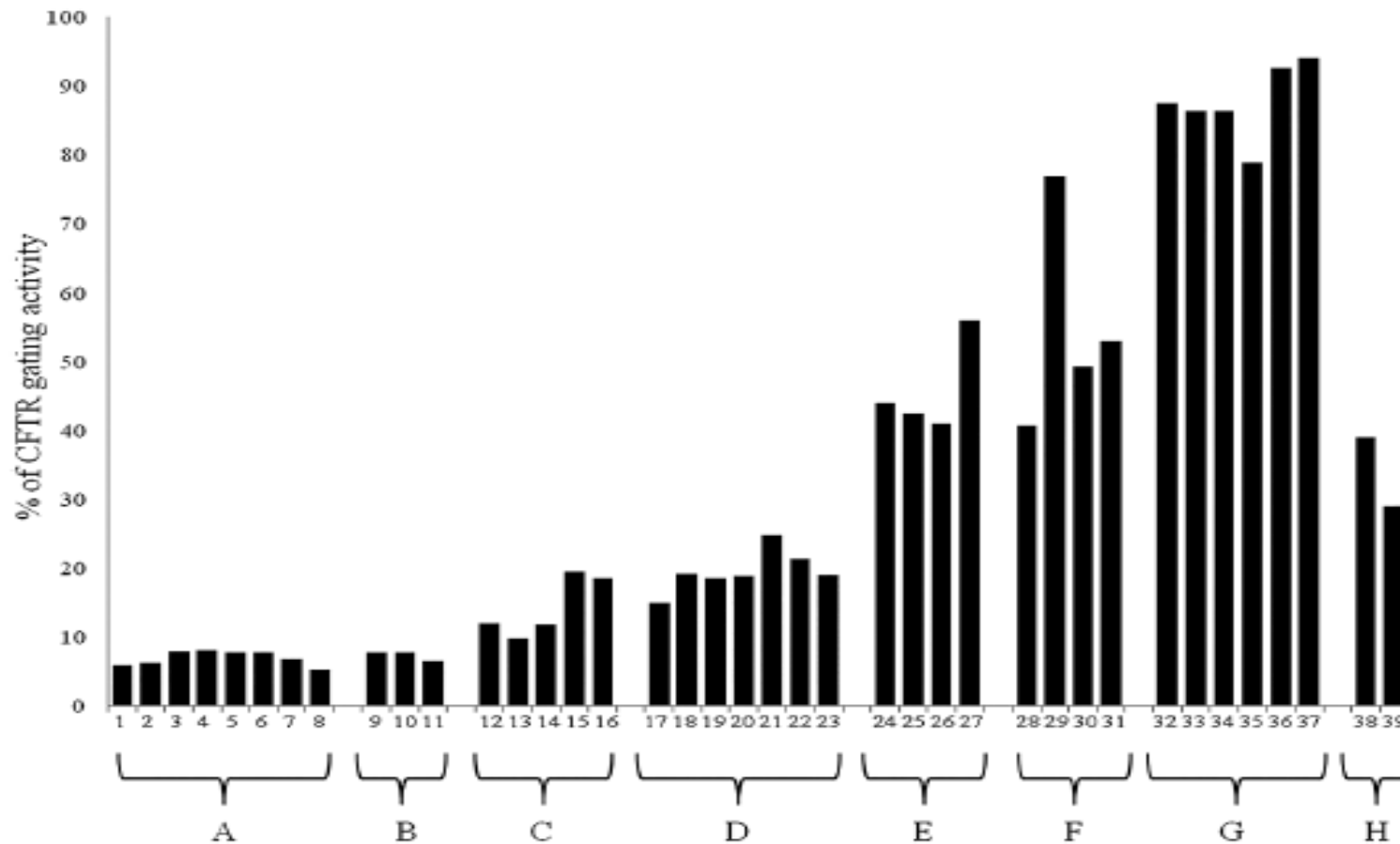
Figure 12: Cystic fibrosis transmembrane conductance regulator (CFTR) gating activity measured on human epithelial nasal cells in several groups of subjects. The values obtained for each sample and the groups are reported in table 2 *(from Terlizzi V. et al., 2016)*

Table 2: CFTR gating activity measured on epithelial nasal cells in the following groups of patients: (A) CF with PI and two class I–II *CFTR* mutations; (B) CF with PI compound heterozygous for the [p.Ile148Thr;p.Ile1023_Val1024del] complex allele and a class I–II *CFTR* mutation; (C) CF with PS and compound heterozygous for a complex allele and a class I–II *CFTR* mutation; (D) CFTR-related disorders; (E) healthy subjects compound heterozygous for a class I–II mutation and a sequence variation with no functional effect; (F) healthy subjects heterozygous for a class I–II mutation; (G) healthy subjects heterozygous for a sequence variation with no functional effect; and (H) subjects with a undefined diagnosis *(from Terlizzi V, et al. J Med Genet 2016)*.

Group	n	CFTR genotype	Gating activity (%) Individual	Mean (SD)
A	1	p.[Phe508del];[Phe508del]	5.9	6.9 (1.1)
	2	p.[Phe508del];[Phe508del]	6.2	
	3	p.[Phe508del];[Phe508del]	7.9	
	4	p.[Phe508del];[Phe508del]	8.1	
	5	p.[Phe508del];[Phe508del]	7.7	
	6	p.[Gly542*];[Ser1297PhefsX5]	7.7	
	7	p.[Gly542*];[Ser1297PhefsX5]	6.8	
	8	p.[Asn1303Lys];c.[579+1G>T]	5.2	
B	9	p.[Ile148Thr;Ile1023_Val1024del];[Phe508del]	7.7	7.3 (0.7)
	10	p.[Ile148Thr;Ile1023_Val1024del];[Phe508del]	7.8	
	11	p.[Ile148Thr;Ile1023_Val1024del];[Asn1303Lys]	6.5	
C	12	p.[Arg74Trp;Val201Met;Asp1270Asn];[Phe508del]	12.0	14.3 (4.4)
	13	p.[Arg74Trp;Val201Met;Asp1270Asn];[Asn1303Lys]	9.8	
	14	p.[Arg74Trp;Val201Met;Asp1270Asn];[Asn1303Lys]	11.8	
	15	c.[1210-34TG[12];1210-12T[5];2930C>T];[1000C>T]	19.5	
	16	c.[1210-34TG[12];1210-12T[5];2930C>T];[579+1G>T]	18.5	
D	17	p.[Arg74Trp;Val201Met;Asp1270Asn];[Phe508del]	15.0	19.5 (3.0)
	18	p.[Arg74Trp;Val201Met;Asp1270Asn];[Asp579Gly]	19.1	
	19	p.[Arg74Trp;Val201Met;Asp1270Asn];[Asp1152His]	18.5	
	20	c.[220C>T;3808G>A];[1210-34TG[12];1210-12[5]]	18.9	
	21	p.[Leu997Phe];[Gly542*]	24.8	
	22	p.[Leu997Phe];[Asn1303Lys]	21.3	
	23	c.[1210-34TG[12];1210-12T[5];2930C>T];[Asn1303Lys]	19.0	
E	24	p.[Asp1270Asn];[Asn1303Lys]	44.0	45.8 (6.9)
	25	p.[Ile148Thr];[Phe508del]	42.3	
	26	p.[Ile148Thr];[Phe508del]	41.0	
	27	c.[443T>C];[579+1G>T]	56.0	
F	28	p.[Phe508del];[=]	40.7	54.9 (15.4)
	29	p.[Gly542*];[=]	76.8	
	30	p.[Asn1303Lys];[=]	49.3	
	31	p.[Gly542*];[=]	53.0	
G	32	p.[Ile148Thr];[=]	87.4	87.6 (5.4)
	33	p.[Ile148Thr];[=]	86.3	
	34	p.[Leu997Phe];[Asn1303Lys]	86.4	
	35	p.[Leu997Phe];[Asn1303Lys]	78.9	
	36	p.[Arg74Trp;Asp1270Asn];[=]	92.6	
	37	p.[Arg74Trp;Asp1270Asn];[=]	94.0	
H	38	p.[Arg117Leu;Leu997Phe];[Arg117Leu;Leu997Phe]	39.0	
	39	p.[Leu997Phe];[Leu997Phe]	28.9	

CF, cystic fibrosis; PI, pancreatic insufficiency; PS, pancreatic sufficiency.
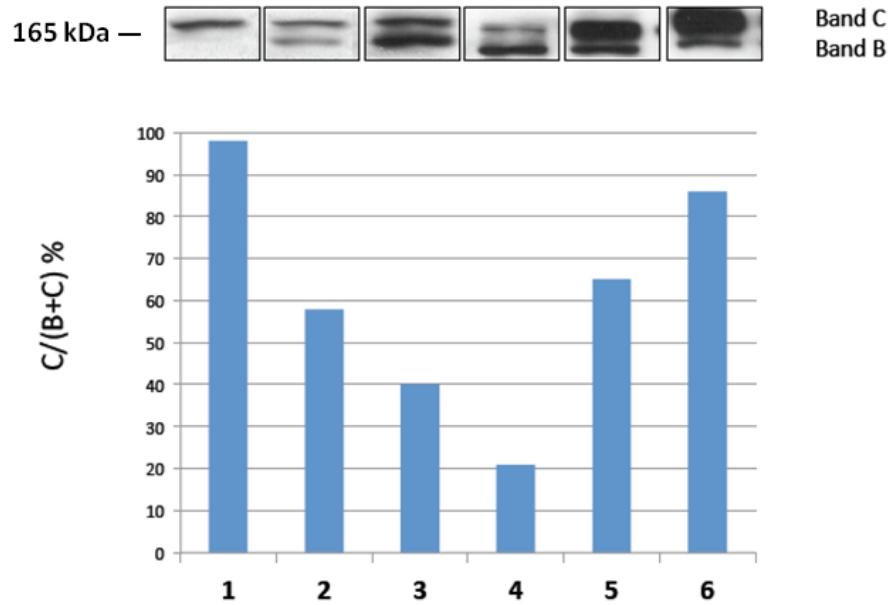
Figure 13: Western blot analysis of the cystic fibrosis transmembrane conductance regulator (CFTR) protein glycosylation in HEK293 cells stably expressing the wild type (1) or the mutant p.Phe508del (2), p.Asn1303Lys (3), p.[Arg74Trp; Val201Met;Asp1270Asn] (4), p.[Arg74Trp;Asp1270Asn] (5) and p.Asp1270Asn (6) proteins. Band C represents the mature, fully glycosylated protein, whereas band B represents the unglycosylated protein. The histogram shows the C/B+C ratio. The values are 1:98%, 2:58%, 3:4%, 4:21%, 5:65% and 6:86%.
Molecular Weight of CFTR:165kDa.
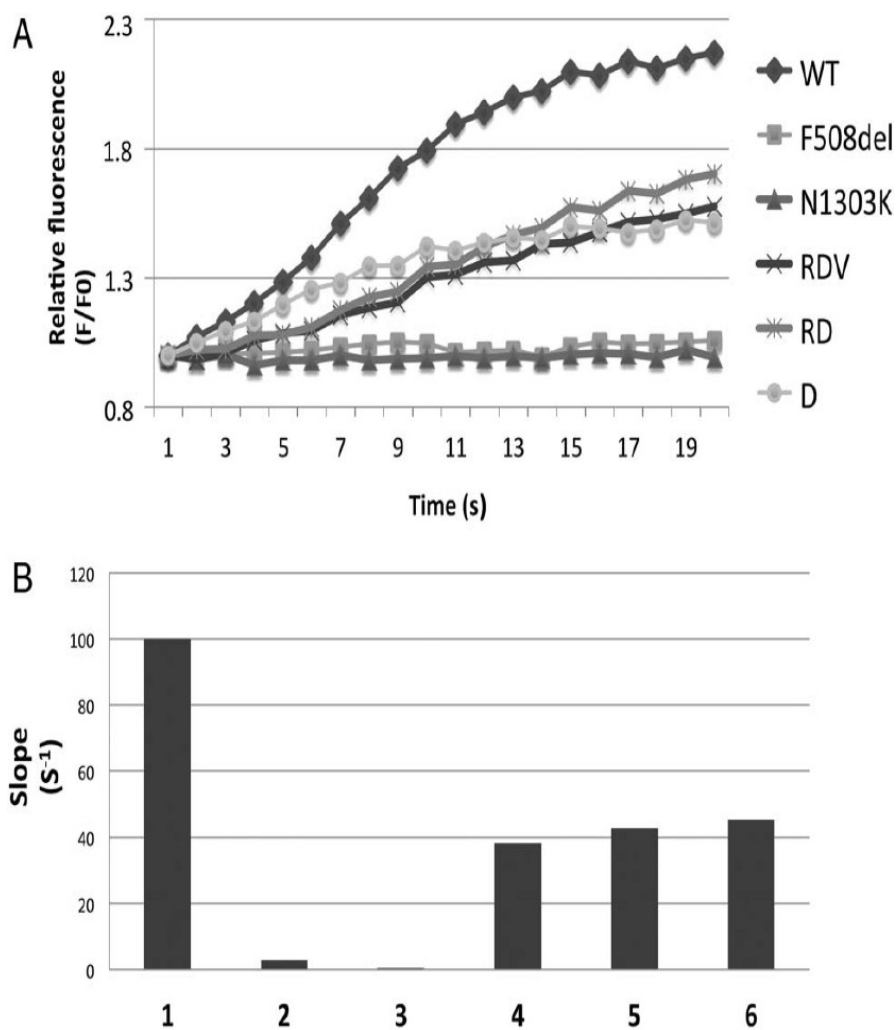
Figure 14: (A) Changes of fluorescence of stimulated HEK293 cells stably expressing the wild type (wt) or the mutants p.Phe508del, p.Asn1303Lys, p.[Arg74Trp;Val201Met;Asp1270Asn](RDV), p.[Arg74Trp;Asp1270Asn] (RD) and p.Asp1270Asn (D) CFTR protein (mixture of 20 mM forskolin and 100 mM IBMX).The values were expressed as relative fluorescence F/F0, where F is the change in fluorescence with time and F0 is the minimum fluorescence. (B) The rate of fluorescence change was quantified from the maximal slope using the best fitting of the fluorescence change and was (1) wt: 100%, (2) p.Phe508del: 2.9%, (3) p.Asn1303Lys: 0.2%, (4) RDV: 38.6%, (5) DV: 42.8% and (6) D:45.4%.

p.[Ile148Thr;Ile1023_Val1024del] complex allele and p.Ile148Thr mutation

We found five patients with the p.[Ile148Thr;Ile1023_Val1024del] complex allele (table 3). They had a class I–II severe mutation *in trans* (i.e., p. Phe508del: two subjects; p.Lys684SerfsX38: one subject; p. Asn1303Lys: one subject and p.Gly85Glu: one subject). All the five patients had classic CF with pathological SCL and PI; lung disease, assessed by FEV1% related to the age was mild (three cases) or severe (two cases). Three patients had CFRD and two had severe liver disease that was the cause of death (table 3). The mean CFTR gating activity on HNEC (available only for three patients) was 7.3% (range 6.5–7.8%; figure 12 and table 2), not significantly different as compared with the mean value of 6.9% found in patients with CF with two class I–II (severe) mutations.

All four subjects with the p.Ile148Thr mutation were compound heterozygotes with a class I–II mutation (severe) on the other allele. They were adults, asymptomatic and had normal SCL (table 3) and were identified by molecular analysis, being consanguineous of patients with CF. The CFTR gating activity on HNEC ranged from 41.0% to 56.0% (figure 12 and table 2) i.e., comparable to that found in carrier subjects. Finally, two healthy subjects were revealed as heterozygous for the p.Ile148Thr mutation being partner of CF carriers. They had a CFTR activity on HNEC of 87.4% and 86.3%, respectively.

Table 3: Demographic and clinical data of subjects bearing the [p.Ile148Thr;p.Ile1023_Val1024del] complex allele or the p.Ile148Thr mutation
(from Terlizzi V. et al. J Med Genet 2016)

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 complications
M	31	CF	8 years	Respiratory	p.[Ile148Thr; Ile1023_Val1024del]	p.Phe508del	66	91	PI	7.7	CFRD; Pa col.; nasal polyposis; bronchiectasis
F	Died (34)	CF	6 years	Respiratory	p.[Ile148Thr; Ile1023_Val1024del]	p.Phe508del	112	24	PI	7.8	CFRD; Pa col.
M	Died (14)	CF	2 months	NBS	p.[Ile148Thr; Ile1023_Val1024del]	p. Lys684SerfsX38	91	69	PI	n.a.	Cirrhosis; Pa col.
F	Died (13)	CF	2 months	NBS	p.[Ile148Thr; Ile1023_Val1024del]	p.Asn1303Lys	101	45	PI	6.5	Cirrhosis; CFRD; Pa col.
F	18	CF	6 months	Respiratory	p.[Ile148Thr; Ile1023_Val1024del]	p.Gly85Glu	80	67	PI	n.a.	Recurrent pancreatitis; IGT; Pa col.; DIOS
F	39	Healthy	37 years	Familiarity	p.Ile148Thr	P.Phe508del	12	n.a.	PS	n.a.	
M	46	Healthy	39 years	Familiarity	p.Ile148Thr	P.Phe508del	11	n.a.	PS	42.3	
F	41	Healthy	41 years	Familiarity	p.Ile148Thr	P.Phe508del	15	n.a.	PS	41.0	
M	50	Healthy	38 years	Familiarity	p.Ile148Thr	c.579+1G>T	15	n.a.	PS	56.0	
M	32	Healthy	32 years	Familiarity	p.Ile148Thr	N	n.a.	n.a.	n.a.	87.4	
M	28	Healthy	28 years	Familiarity	p.Ile148Thr	N	n.a.	n.a.	n.a.	86.3	

*For patients with CF, we reported SCL at diagnosis while for healthy subjects we reported current SCL.
†For patients who had died, we reported the last FEV$_1$ available.
CF, cystic fibrosis; CFRD, cystic fibrosis-related diabetes; DIOS, distal intestine obstruction syndrome; FEV$_1$, forced expiratory volume in 1 second; IGT, impaired glucose tolerance; n.a., not assessed; N, wild-type allele; NBS, newborn screening; Pa col., *Pseudomonas aeruginosa* chronic colonisation; PI, pancreatic insufficiency; PS, pancreatic sufficiency; SCL, sweat chloride level.

p.[Arg117Leu;Leu997Phe] complex allele and p.Leu997Phe mutation

We studied two siblings homozygous for the p.[Arg117Leu;Leu997Phe] complex allele (table 4). One is a female diagnosed as CF with PS (mild CF) at 48 years because of recurrent pneumonia and chronic colonization by *P. aeruginosa*. The CFTR gating activity on HNEC was 39.0% (figure 12 and table 2) thus in the range obtained in patients with mild CF or CFTR-RD. Her sibling is a 58-year-old male with CBAVD, and a SCL of 88 mmol/L.

Then, we studied two pairs of siblings compound heterozygous for the p.[Arg117Leu;Leu997Phe] complex allele and the pArg334Trp (1 sib-pair) or the p.Gly85Glu (the other sib-pair, table 2) mutations. All were affected by CF with PS (mild). The CFTR gating activity on HNEC, available only for one adult female (compound heterozygous for the p.Arg334Trp mutation), was 19.5% (figure 9 and table 2), again in the renge of patients with mild CF.

Moreover, we observed two subjects homozygous for the p.Leu997Phe mutation (table 4). The first has CBAVD with borderline SCL. The other, at the age of 21 years, has only chronic sinus disease with nasal polyposis (found at the age of 8 years old) and normal SCL. The CFTR gating activity on HNEC was 28.9% (range of mild CF).

Eight patients compound heterozygous for the p.Leu997Phe and a class I–II (severe) mutation and six patients compound heterozygous for the p.Leu997Phe and another (mild) mutation (table 4) had monosymptomatic CFTR-RD (CBAVD: nine cases; recurrent pancreatitis: three cases; isolated bronchiectasis: two). Six of them had borderline SCL and eight had normal SCL. In two patients from this group, both carrying a class I–II (severe) mutation on the other allele, the CFTR residual gating activity on HNEC was 24.8% and 21.3%, respectively (again, the range of mild CF).

Nine subjects (aged 2–5 years old) compound heterozygous for the p.Leu997Phe mutation and a class I–II (severe) mutation (four cases) or another (mild) mutation (five cases) had been classified as CFSPID. At present, all of them are asymptomatic (table 4) and have normal SCL.

Nine other subjects (aged 31–46 years old) compound heterozygous for the p.Leu997Phe and a class I–II (severe) mutation (four cases) or another (mild) mutation (five cases) were classified as healthy, being all asymptomatic. Seven of such nine subjects had normal SCL, in two the SCL were borderline. The nine subjects had been identified for familiarity with patients with CF (six cases) or being partner of CF carrier subjects (three cases). For one of them, the CFTR activity on HNEC was 36.9% (also in this case, the range of mild CF).

Finally, in two healthy subjects heterozygous for the p.Leu997Phe mutation the CFTR gating activity on HNEC was 86.4% and 78.9% (tables 2 and 4, and figure 12).

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 (?)	48 years	Respiratory	p.[Arg117Leu; Leu997Phe]	p.[Arg117Leu; Leu997Phe]	90	113	PS	39.0	Pa col.; recurrent pneumonitis; bronchiectasis
M	58	CFTR-RD	58 years	CBAVD	p.[Arg117Leu; Leu997Phe]	p.[Arg117Leu; Leu997Phe]	88	n.a.	PS	n.a.	
F	40	CF	33 years	Respiratory	p.[Arg117Leu; Leu997Phe]	p.Arg334Trp	71	91	PS	19.5	Pa col.; nasal polyposis
M	35	CF	28 years	Familiarity	p.[Arg117Leu; Leu997Phe]	p.Arg334Trp	75	120	PS	n.a.	Nasal polyposis
M	33	CF	1 month	NBS	p.[Arg117Leu; Leu997Phe]	p.Gly85Glu	107	96	PS	n.a.	Nasal polyposis
M	24	CF	2 years	Familiarity	p.[Arg117Leu; Leu997Phe]	p.Gly85Glu	80	90	PS	n.a.	Nasal polyposis
M*	40	CFTR-RD	40 years	CBAVD	p.Leu997Phe	p.Leu997Phe	50	95	PS	n.a.	
M*	21	Healthy	8 years	Nasal polyposis	p.Leu997Phe	p.Leu997Phe	21	70	PS	28.9	Nasal polyposis
F	46	CFTR-RD	43 years	Bronchiectasis	p.Leu997Phe	p.Asn1303Lys	55	85	PS	n.a.	
F*	22	CFTR-RD	11 years	Recurrent pancreatitis	p.Leu997Phe	p.Gly542*	31	103	PS	24.8	Nasal polyposis
M	38	CFTR-RD	32 years	CBAVD	p.Leu997Phe	p.Phe508del	31	n.a.	PS	n.a.	
M	41	CFTR-RD	35 years	CBAVD	p.Leu997Phe	p.Phe508del	31	n.a.	PS	n.a.	
M*	44	CFTR-RD	39 years	CBAVD	p.Leu997Phe	p.Asn1303Lys	37	n.a.	PS	21.3	
M	36	CFTR-RD	30 years	CBAVD	p.Leu997Phe	p.Asn1303Lys	50	n.a.	PS	n.a.	
M*	39	CFTR-RD	25 years	CBAVD	p.Leu997Phe	p.Arg553*	31	n.a.	PS	n.a.	
M	28	CFTR-RD	25 years	Recurrent pancreatitis	p.Leu997Phe	p.Phe316LeufsX12	31	n.a.	PS	n.a.	
M	44	CFTR-RD	27 years	CBAVD	p.Leu997Phe	p.Arg334Trp	42	n.a.	PS	n.a.	
M	40	CFTR-RD	39 years	CBAVD	p.Leu997Phe	p.Arg334Trp	46	n.a.	PS	n.a.	
M*	39	CFTR-RD	38 years	CBAVD	p.Leu997Phe	p.Asp1152His	44	n.a.	PS	n.a.	
F	41	CFTR-RD	36 years	Recurrent pancreatitis	p.Leu997Phe	p.Asp1152His	41	n.a.	PS	n.a.	
M	40	CFTR-RD	35 years	CBAVD	p.Leu997Phe	c.[1210-34TG[12];1210-12T[5]]	39	n.a.	PS	n.a.	
M	39	CFTR-RD	36 years	Bronchiectasis	p.Leu997Phe	c.[1210-34TG[12];1210-12T[5]]	31	n.a.	PS	n.a.	
M	3	Healthy	1 month	NBS	p.Leu997Phe	p.Phe508del	31	n.a.	PS	n.a.	
F	2	Healthy	1 month	NBS	p.Leu997Phe	p.Lys684SerfsX38	37	n.a.	PS	n.a.	
M*	5	Healthy	1 month	NBS	p.Leu997Phe	p.Phe508del	15	n.a.	PS	n.a.	
M	2	Healthy	1 month	NBS	p.Leu997Phe	p.Gly542*	16	n.a.	PS	n.a.	
M	5	Healthy	1 month	NBS	p.Leu997Phe	p.Arg117His	35	n.a.	PS	n.a.	
M	3	Healthy	1 month	NBS	p.Leu997Phe	p.Asp1152His	26	n.a.	PS	n.a.	
M	5	Healthy	1 month	NBS	p.Leu997Phe	p.Asp1152His	27	n.a.	PS	n.a.	
F	5	Healthy	1 month	NBS	p.Leu997Phe	c.[1210-34TG[12];1210-12T[5]]	31	n.a.	PS	n.a.	
F	5	Healthy	1 month	NBS	p.Leu997Phe	c.[1210-34TG[12];1210-12T[5]]	32	n.a.	PS	n.a.	
M	47	Healthy	44 years	Familiarity	p.Leu997Phe	p.Phe508del	60	105	PS	n.a.	

Continued

Table 4 *Continued*

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
M*	31	Healthy	23 years	Familiarity	p.Leu997Phe	p.Phe508del	29	n.a.	PS	36.9	
M	32	Healthy	32 years	Familiarity	p.Leu997Phe	p.Asn1303Lys	24	n.a.	PS	n.a.	
M	44	Healthy	40 years	Familiarity	p.Leu997Phe	c.489+1G>T	15	n.a.	PS	n.a.	
M	41	Healthy	40 years	Partner of CF carrier	p.Leu997Phe	c.[1210-34TG [12];1210-12T[5]]	*60*	100	PS	n.a.	
F*	32	Healthy	26 years	Familiarity	p.Leu997Phe	p.Glu279Asp	20	n.a.	PS	n.a.	
F	38	Healthy	38 years	Familiarity	p.Leu997Phe	p.Val938GlyfsX37	23	n.a.	PS	n.a.	
M	46	Healthy	38 years	Partner of CF carrier	p.Leu997Phe	p.Arg117His	20	n.a.	PS	n.a.	
F	43	Healthy	43 years	Partner of CF carrier	p.Leu997Phe	p.Arg117His	11	n.a.	PS	n.a.	
F*	32	Healthy	30 years	Familiarity	p.Leu997Phe	N	n.a.	n.a.	PS	86.4	
M	41	Healthy	37 years	Familiarity	p.Leu997Phe	N	n.a.	n.a.	PS	78.9	

*In the subjects marked with the asterisk, we performed the analysis of CFTR STR (see text).
CBAVD, congenital bilateral absence of vas deferens; CF, cystic fibrosis; CFTR-RD, cystic fibrosis transmembrane conductance regulator-related disorders; FEV$_1$, forced expiratory volume in 1 second; n.a., not assessed; N, wild-type allele; NBS, newborn screening; PS, pancreatic sufficiency; SCL, sweat chloride level; the values in italics represent SCL discordant with diagnosis; STR, short tandem repeats.

c.[1210-34TG;[12];1210-12T[5]2930C>T] complex allele

We studied three patients with the c.[1210-34TG[12];1210-12T [5];2930C>T] complex allele *in trans* with a class I–II (severe) mutation (table 5). One was diagnosed as CF with PS (mild CF), a mild pulmonary disease despite *P. aeruginosa* colonization and pathologic SCL. Two other patients had CFTR-RD. The CFTR gating activity measured on HNEC was 18.5% in the patient with CF and 19.0% in one of the two patients with CFTR-RD (figure 12 and table 2), i.e., in the range observed in patients with mild CF or with CFTR-RD.

Finally, a synopsis of CF clinical expression in patients with different *CFTR* complex alleles is reported in table 2.

Table 5: Demographic and clinical data of subjects bearing the c.[1210-34TG[12];1210-12T[5];2930C>T] complex allele.

Gender	Current age (years)	Diagnosis	Age at diagnosis (years)	Cause of diagnosis	Allele 1	Allele 2	SCL* (mmol/L)	Current FEV_1 (%)	Pancreatic status	CFTR gating	Other
M	22	CF	18	Respiratory symptoms	c.[1210-34TG[12];1210-12T[5];2930C>T]	c.579+1G>T	87	104	PS	18.5	Pa col.
M	43	CFTR-RD	25	CBAVD	c.[1210-34TG[12];1210-12T[5];2930C>T]	p.Asn1303Lys	50	110	PS	19.0	Recurrent pancreatitis
F	32	CFTR-RD	19	Recurrent pancreatitis	c.[1210-34TG[12];1210-12T[5];2930C>T]	p.Asn1303Lys	41	100	PS	n.a.	

*For the patient with CF, we reported SCL at diagnosis while for the patients with CFTR-RD we reported current SCL.
CBAVD, congenital bilateral absence of vas deferens; CF, cystic fibrosis; CFTR-RD, cystic fibrosis transmembrane conductance regulator-related disorders; SCL, sweat chloride level; FEV_1, forced expiratory volume in 1 second; PS, pancreatic sufficiency; Pa col, *Pseudomonas aeruginosa* chronic colonisation.

5. DISCUSSION

5.1 Sampling and culture of HNECs

Culture of HNECs resulted a contributory model to study the molecular effect of *CFTR* mutations and novel drugs in cells from patients bearing specific *CFTR* genotypes. Of course, the model can be used for all genetic diseases in which the disease-gene is expressed by HNECs. In the present study, we improved and validated the procedures for sampling and culture on a wide number of cases and set up procedures for the analysis of the molecular effect of *CFTR* mutations.

The sampling was well tolerated by all 223 subjects studied, including about one third of pediatric patients; it does not need anesthesia nor hospitalization and (as we demonstrated during the study) it requires just several washings with saline in the days before the sampling. Of course, the sampling must be performed by a physician adequately trained in otolaryngology. Interestingly, we demonstrated that the transport medium permits to store the sampled cells before culture for up to 48 hours, allowing the analysis of cells sampled from other centres. In fact, in the study of CF patients bearing complex alleles [11], HNECs sampling was performed in patients followed in centres from 12 italian regions and then analyzed in our Lab.

The staining with May-Grunwald-Giemsa and with a panel of anti-cytokeratin antibodies [38] confirmed that we effectively obtained and cultured HNECs without contamination by inflammatory cells. HNECs can be cultured up to 15 days, confirming a previous study by our group in which we assessed the effect of mannose binding lectin on several types of cells (among which HNECs) during senescence [56]. Furthermore, in the present study we demonstrated that the culture does not modify the levels of *CFTR* expression.

Various studies used human *ex vivo* models of CF. Main limitations are the invasiveness and the risk of most techniques to collect human cells, the small number of cells collected and the limited number, poor quality, and non representative nature of samples resulting from surgery (like nasal polypectomies or lung transplants). Brushing of the respiratory tract allows easy sampling of numerous, representative, well-preserved and dissociated cells from the superficial mucosa. The group by Garratt et al., recently described the technique of bronchial brushing as a possible gold standard model of airway disease in CF. However, this sampling requires anaesthesia and less that 50% of samples were successfully cultured [55].

Other authors suggested the model of porcine nasal epithelial cells in culture to study the pathogenesis of sinusitis, but this model is limited by the possibility to study transgenic pigs with only a single or few *CFTR* genotypes [60]. Other Authors used cultured cells from nasal polyps for proteomic analysis[61] but the limit of such model is that only a few patients with CF (about 10-15%) undergo surgery for nasal polyps [62].

5.2 The study of *CFTR* mutations that impair the splicing process

The HNECs model permits to study cells from patients bearing specific mutations defining the molecular effects of mutations of

uncertain significance. For example, mutations within exon-intron boundaries may affect the splicing process (more than two dozens *CFTR* mutations are known to impair the splicing process), and more recently it was observed that also missense mutations that do not change the amino acid may impair the splicing process [63]. The study of the splicing effect of novel mutations would require a complex procedure to express the mutation *in vitro* followed by the mini-gene assay [63]. This is a rather complex and expensive procedure not available for routine use. Conversely, the availability of nasal cells directly from the patient with the mutation to be characterised permits assess the splicing effect with a simple RT-PCR reaction followed by electrophoresis. This analysis can be performed on HNECs without culture. Using this approach we demonstrated the splicing effect of the 711+1G>T mutation confirming its pathogenicity. Using the same procedure, in a previous study our group defined the pathogenic effect on the splicing of three mutations (i.e., the 504C>G, the 621+16G>T and the 1341+45T>G) [2] while another study defined the splicing effect of the 712-1G>T and of the 2789+5G>A mutations [64] demonstrating that the results obtained with this novel procedure fully match with those obtained with the classic minigene assay.

5.3 The study of *CFTR* gene expression

Quantitative RT-PCR analysis can be performed either on cultured HNECs and on freshly sampled cells and permits to reveal the level of *CFTR* expression in each subject. Such approach can be used to define the effect of mutations in the promoter region, that are described with a increasing frequency in CF patients [16]. Also in this case, a simple quantitative RT-PCR analysis of HNECs would avoid the complex and expensive procedure of *in vitro* expression and analysis of mutations in cell lines [16]. However, our study demonstrated that the *CFTR* gene expression levels are highly heterogeneous either in normal subjects and in patients with CF, thus it will be necessary to study a large number of healthy subjects to obtain reference values.

Quantitative RT-PCR analysis may be used also to assess the effect of potential drugs that may enhance gene expression, like butyrate. The mechanisms of butyrate action are multiple. It can modulate kinase and phosphatase proteins, stimulate microtubule and microfilament formation, and have regulatory effects on gene expression (including heat shock protein and alkaline phosphatase) [65, 66]. Butyrate is part of the well-known class of histone deacetylase inhibitors (HDACi) with epigenetic effects. In fact, histone tail acetylation is believed to enhance the accessibility of a gene to the transcription machinery, whereas deacetylated tails are highly charged and believed to be tightly associated with the DNA backbone, thus limiting the accessibility of genes to transcription factors. So, butyrate can enhance *CFTR* expression by inhibiting deacetylation. In our laboratory, we already studied the effect of butyrate on the expression of the *SLC26A3* gene in patients with congenital chloride diarrhoea, and to predict the patients that may benefit from the treatment [58]. Actually, we are evaluating the effect of butyrate on *CFTR* expression and the results obtained in the present

study are very encouraging since the treatment of HNECs from either 5 normal controls and from 20 patients with CF was invariably associated to a significant enhancement of *CFTR* expression. Once confirmed the effect of butyrate on a larger number of cases, it could be proposed as a therapeutic strategy, increasing *CFTR* expression in patients with CF that would be treated with potentiators and activators.

5.4 Quantitative analysis of CFTR gating

The quantitative analysis of CFTR gating activity may contribute either to confirm the diagnosis of CF and to predict the severity of the phenotype. Even if our data are preliminary and have been obtained only in patients with several genotypes, the gating activity of CFTR shows several ranges:

i) patients with two class I/II mutations (that are tipically classified as severe mutations) like the F508del, the N1303K, the G542X and several complex alleles (see below), display a gating activity <10%. Usually, such patients have classic CF with PI.

ii) patients with one or both mild mutations, like the D1152H, show a gating activity between 10 and 30%. Such patients usually appear with mild CF and PS or with CFTR-RD.

iii) carrier subjects display an activity between 40 and 70%.

Thus, the analysis of CFTR activity would be performed prospectically in patients bearing rare mutations for which the effect is not well-defined and may help to predict the clinical expression of the disease.

Of course, the analysis of CFTR gating activity on HNECs may be used in patients with CF that experience the treatment with novel drugs like activators or potentiators to assess the efficacy of the treatment [19]. And finally, the analysis permits to assess the effect of novel drugs on cells bearing specific mutations before their use in humans [58,59] as our group recently experienced in patients with the rare congenital chloride diarrhea [67].

Moreover, our was the first study on a large series of patients with CF bearing different *CFTR* complex alleles that were evaluated for the genotype-phenotype correlations and for the *CFTR* gating activity using the *ex vivo* model of HNECs. Among these mutations, the p.[Arg74Trp;Val201Met;Asp1270Asn] was found in eight patients. Six had a severe mutation *in trans*; four of them had mild CF and two had CFTR-RD. In two other patients, the mutations *in trans* were the p.Asp579Gly and p.Asp1152His that usually have a milder effect [68, 69]. Both the patients had CBAVD, the same clinical expression previously found in four patients with the same complex allele *in trans* with mild mutations [70]. Thus, the complex allele may be classified as a mild mutation and the clinical expression depends on the mutation *in trans*. This was confirmed by the mean CFTR gating activity on HNEC that resulted 11.2% in the patients with mild CF and 17.5% in those with CFTR-RD versus a mean of 6.2% (p<0.001) found in patients with CF and two class I–II severe mutations. A further confirmation to such data came from the *in vitro* expression study: the

p.[Arg74Trp;Val201Met;Asp1270Asn] construct causes a significant reduction in the processing of the mature protein (i.e., 21% of the normal), but it gives rise to a CFTR residual gating activity of 38%, while the severe mutations p.F508del and p.Asn1303Lys mutants had a residual activity of 2.9% and 0.2%, respectively.

The p.[Arg74Trp;Asp1270Asn] complex allele (i.e., the double mutant), was found in two patients: the first is a child diagnosed as CFSPID,[18] still asymptomatic at the age of 5 years old and the second is a patient with a normal SCL and CBAVD (with a residual CFTR activity on HNEC of 18.9%). Both the patients had a severe mutation in trans. These data indicate that the pathogenic effect of the double mutant is low (if any) in agreement with the report of two asymptomatic subjects with the same genotype [71]. Similarly, the p.Asp1270Asn (single mutant) was found *in trans* with the severe p.Phe508del and the p.Asn1303Lys, respectively in two adults both asymptomatic with normal SCL. Such mutation has never been described in subjects with CF or CFTR-RD so far, while it was found with a high frequency in the general population, suggesting that it could be a polymorphism with no pathogenic effect [72]. In agreement, either the [p.Arg74Trp;p.Asp1270Asn] complex allele and the p.Asp1270Asn mutation caused only a slight reduction in the synthesis of the mature protein (65% and 86%) *in vitro* and had a gating activity of 43% and 45%, respectively. All these data indicate that the double mutant and the p.Asp1270Asn alone have no pathogenic effect [73].

Our study revealed the p.[Ile148Thr;Ile1023_Val1024del] complex allele as a severe CFTR mutation. It was identified *in trans* with a class I–II severe mutation, in five patients with CF, pancreatic insufficiency and severe complications like diabetes and liver disease. The CFTR gating activity on HNEC was comparable with that observed in patients with CF with two class I–II mutations (<7.0%). While, the p.Ile148Thr mutation alone does not have a relevant pathogenic effect: in was found, *in trans* with a class I–II CFTR mutation, in four asymptomatic adults and, despite they had a severe mutation *in trans*, the gating activity of CFTR on HNEC ranged 41.0–56.0% (i.e., the same range of values observed in subjects heterozygous for a class I–II mutation). On the other hand, two healthy subjects heterozygous for the p.Ile148Thr (i.e., 87.4% and 86.3%) had a gating activity of 87.4% and 86.3%, further confirming that the I148T has a minimal functional effect, confirming previous *in vitro* studies [70] and in agreement with the high frequency of the I148T in healthy subjects [72]. Thus, we suggest that such mutation would not be tested in panels of mutations.

Furthermore, we studied two siblings homozygous for the p.[Arg117Leu;Leu997Phe] complex allele. The first had CF with a mild clinical expression at 35 years old, while the sibling had CBAVD. Both the patients had altered SCL. Similarly, four other patients compound heterozygous for the complex allele and another CFTR mutation have mild CF with PS. The residual CFTR gating activity on HNEC of 39.0% in one of the homozygous patients and that of 19.5% in one of the cases compound heterozygous for the complex allele and the p.Arg334Trp indicates that the p.[Arg117Leu;Leu997Phe] acts as a mild mutation.

These data are in agreement with those of four patients, compound heterozygous for the p.[Arg117Leu;Leu997Phe] and a severe mutation that had a mild CF with PS in two cases and a more severe form of the disease with or without PI in two others [74].

We found the single mutant p.Leu997Phe homozygous or *in trans* with a causing mutation either in patients with CFTR-RD (mainly CBAVD) and in healthy subjects, confirming previous reports [74, 75]. Furthermore, some subjects classified as CFSPID after the NBS, resulted free from symptoms during the follow-up in the successive years, again in agreement with previous studies [76]. The functional analysis of CFTR on HNEC was performed in two patients with CFTR-RD, both compound heterozygous for the p.Leu997Phe and a class I–II mutation: the activity was 21.3% and 24.8%, while the activity measured on an asymptomatic subject with the p.[Phe508del];[p.Leu997Phe] genotype was 36.9%. Thus, the p.Leu997Phe has a higher residual gating activity as compared with class I–II mutations, but with a wide variability. Other factors like environment and modifier genes contribute to modulate the disease's symptoms of each CF patient.

Finally, the c.[1210-34TG[12];1210-12T[5];2930C>T] complex allele was found in three patients. All of them had a severe mutation *in trans*. The first was diagnosed as CF since SCL were 87 mmol/L but the clinical expression of the disease was very mild. The two other patients were diagnosed as CFTR-RD. The CFTR gating activity of 18.5% and 19.0% found in the CF and in one of the CFTR-RD patients, confirm that the c.[1210-34TG[12];1210-12T[5];2930C>T] complex allele is a mild mutation. These results agree with those reported in a patient compound heterozygous for the c.[1210-34TG[12];1210-12T[5];2930C>T] complex allele and the p.Phe508del mutation that had a mild CF despite nasal potentials and monocyte functional assay were compatible with a CF phenotype [76].

6. CONCLUSIONS

The *ex vivo* model of cultured HNECs may help to study the pathogenetic mechanism of specific CF mutations directly on cells from the patient and permits to study the effect of novel therapies. Based on our results, we conclude that:

1) HNEC brushing can be performed without anaesthesia and is well tolerated by children and adults. It is slightly invasive, easily repeatable, and allows to sample a sufficient amount of representative, well-preserved HNECs, suitable to apply a wide range of techniques;

2) HNECs can be preserved for up to 48 hours before culture, thus allowing multicentre studies;

3) HNEC culture is a useful model to study the molecular effects of novel *CFTR* mutations including complex alleles;

4) the *ex-vivo* model of HNECs may be used to evaluate, before human use, the effect of new drugs on patients' cells bearing specific *CFTR* mutations; these drugs can modulate the effect of *CFTR* mutations opening new therapeutic frontiers;

5) our methodology is adequate for the quantitative measurement, by fluorescence, of the CFTR gating activity of patients HNECs with different genotypes helping to classify patients with severe or mild CF, CFTR-RD and carrier subjects.

Moreover, our procedure would allow monitoring patients during drug treatment, and evaluating the real effects of new molecular therapies.

7. ACKNOWLEDGMENTS

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