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A simple method to measure CFTR-dependent iodide transport in peripheral blood leukocytes: mutant HS-YFP assay

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A simple method to measure CFTR-dependent iodide transport in peripheral blood leukocytes: mutant HS-YFP assay-Silvia Vercellone

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Abstract

Cystic Fibrosis is a multi organ hereditary disease caused by a mutation in the gene coding for Cystic Fibrosis Transmembrane conductance Regulator protein (CFTR).The different mutations of the CFTR gene were divided into 6 classes on the basis of molecular defects and the consequent functional alterations. Depending on the type of mutation, the protein may be more or less expressed and functioning. This involves varying degrees of disease severity and not only between different mutations but also between patients having the same mutation. Research carried out to date to try to cure the basic defect has led to good but not sufficient results. In order to test promising drugs able to rescue the expression and function of CFTR are necessary easy and fast methods and the use of primary cells from the individual patients. Leukocytes are recognized in the scientific literature as key component of the pathogenetic events associated to cystic fibrosis. Leukocytes, especially monocytes, represent an easily accessible source of primary cells that might be exploited to monitor CFTR expression and activity. Based on previous work I was involved in the development of a new assay to measure CFTR activity suitable to be used in primary cells. The readout of this method is based on residue quantity of iodide present in the supernatant, after proper stimulation, quantified by the quenching of the fluorescence of Halide Sensitivity Yellow Fluorescence Protein (GST-HS-YFP). The specificity of the assay for CFTR activity was tested using two different CFTR inhibitors, CFTR-172 and PPQ-102, in MM6 cells and after silencing of MM6 with CFTR siRNA. These data were confirmed also in primary Leukocytes, where we recorded a significant difference between WT and CF PBMCs and Monocytes ($p<0,005$). GST-HS-YFP assay can represent easy and fast methods to measure the CFTR function in Leukocytes from CF patients during clinical trials and another useful technique applicable in different cell types to perform new drugs screening.

Riassunto

La fibrosi cistica è una malattia ereditaria multiorgano causata da una mutazione nel gene codificante per la proteina di regolatore di conduttanza transmembrana della fibrosi cistica (CFTR). Le diverse mutazioni del gene CFTR sono state divise in 6 classi sulla base di difetti molecolari e delle conseguenti alterazioni funzionali. A seconda del tipo di mutazione, la proteina può essere più o meno espressa e funzionante. Questo comporta diversi gradi di gravità della malattia e non solo tra diverse mutazioni ma anche tra pazienti che hanno la stessa mutazione. La ricerca effettuata fino ad oggi per cercare di curare il difetto di base ha portato a risultati buoni ma non sufficienti. Al fine di testare farmaci promettenti in grado di aumentare l'espressione e la funzione di CFTR sono necessari metodi facili e veloci e l'uso di cellule primarie derivanti dai singoli pazienti. I leucociti sono riconosciuti nella letteratura scientifica come componente chiave degli eventi patogenetici associati alla fibrosi cistica. I leucociti, in particolare i monociti, rappresentano una fonte facilmente accessibile di cellule primarie che potrebbero essere sfruttate per monitorare l'espressione e l'attività di CFTR. Sulla base di lavori precedenti sono stata coinvolta nello sviluppo di un nuovo test per misurare l'attività di CFTR. Il risultato di questo metodo si basa sulla quantità residua di ioduro presente nel surnatante, dopo opportuna stimolazione, quantificata dalla estinzione della fluorescenza della proteina a fluorescenza gialla ad alta sensibilità (GST-HS-YFP). La specificità del dosaggio per l'attività CFTR è stata testata utilizzando due diversi inibitori CFTR, CFTR-172 e PPQ-102, in cellule MM6 e dopo il silenziamento di MM6 con siRNA CFTR. Questi dati sono stati confermati anche nei leucociti primari, dove è stata registrata una differenza significativa tra WT e CF PBMC e Monociti ($p < 0,005$). Il dosaggio GST-HS-YFP può rappresentare un metodo semplice e veloce per misurare la funzione CFTR nei leucociti da pazienti CF durante gli studi clinici e un'altra tecnica applicabile in diversi tipi di cellule per eseguire lo screening di nuovi farmaci.

Introduction

Cystic Fibrosis (CF) is one of the most common multi-organ hereditary diseases in the Caucasian population, with an incidence equal to one case per 2500-2700 births (Ratjen and Döring 2003). This pathology is caused by a mutation in the gene coding for Cystic Fibrosis Transmembrane Regulator protein (CFTR), a chloride channel that is expressed in different cell types. Cystic fibrosis leads to pathological changes in organs that express CFTR, including secretory cells, sinuses, lungs, pancreas, liver, and reproductive tract. The absence of functional CFTR in airway epithelia leads to abnormal airway surface liquid, which favors chronic infection with Pseudomonas aeruginosa (PA), Staphylococcus aureus, Burkholderia cepacia and other bacteria. This chronic infection in CF lungs is associated with an exaggerated inflammatory response leading to pulmonary disease; the first cause of death in CF. The pancreas is affected in 85% of cases with a stagnation of pancreatic juices in the ducts. The lack of pancreatic juices in the intestinal canal leads to a malabsorption of fats, and to the consequences of fat-soluble vitamins, proteins and, to a lesser extent, sugars. Over time, the pancreas, more and more affected, secretes in smaller quantities, is a form of diabetes usually insulin-dependent. The disease affects the reproductive tract:98% of male subjects are sterile for azoospermia (Dodge 1995); women can have children but fertility is still reduced (Kopito, Kosasky, and Shwachman 1973).

Structure and function of CFTR

Between the end of the '70s and the beginning of the' 80s numerous investigations were carried out to try and identify the gene responsible for CF (Mayo et al. 1980). The analysis of polymorphisms carried out using restriction enzymes (RFLP) has mapped the locus CF on chromosome 7q31 (Knowlton et al. 1985; Tsui, Buetow, and Buchwald 1986; Klinger et al. 1986) (NCBI reference sequence NC_000007.13 , accession number NM_000492.3). The gene is 188703 base pairs long (Figure 1) and encodes a 1480 amino acid protein (NP_000483.3) known as the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (Riordan et al. 1989). CFTR belongs to the MRP subfamily involved in drug resistance mechanisms and together with six other subfamilies (ABC1, MDR /

TAP, ALD, OABP, GCN20, White) constitutes the superfamily of genes that encode proteins commonly referred to as ATP-binding cassette (ABC) transporters. This superfamily includes proteins that are important from a clinical point of view, such as glycoprotein P (P-gp), the protein responsible for resistance to many drugs (MDR) and tapasin (TAP) transporters (Gros, Croop, and Housman 1986; Higgins et al. 1988).

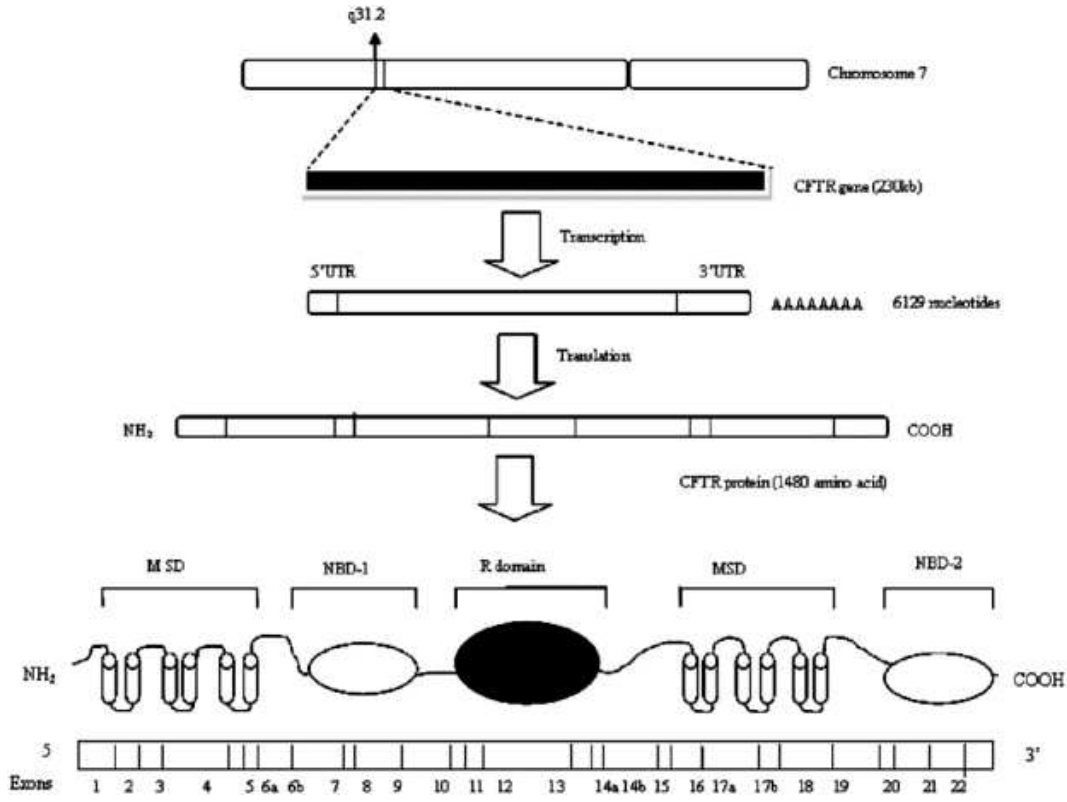


Figure 1. Diagram showing CFTR gene and resulting protein (CFTR, cystic fibrosis transmembrane conductance regulator; MSD, membrane spanning domain; NBD, nucleotide binding domain; R domain, regulatory domain)(Maurya, Awasthi, and Dixit 2012).

CFTR is a glycosylated protein with relative mass (Mr) of about 170 kDa in which several regions shown in Figure 2 have been identified. The protein is composed of two regions, MSD1 and MSD2, each of which includes 6 transmembrane domains (Figure 2). Each of the two MSD regions is connected to a nucleotide binding domain (NBD1 and NBD2) that binds ATP. The regulatory

domain (R-domain) is rich in polar amino acids and interposing between NBD1 and NBD2 connects the two MSD regions of CFTR. It is the presence of this R-domain that places the CFTR protein in the ABC transporters superfamily. In fact, the CFTR protein has channel functions that transports chloride ions through the plasma membrane, but can also transport other anions, for example iodide and bicarbonate.

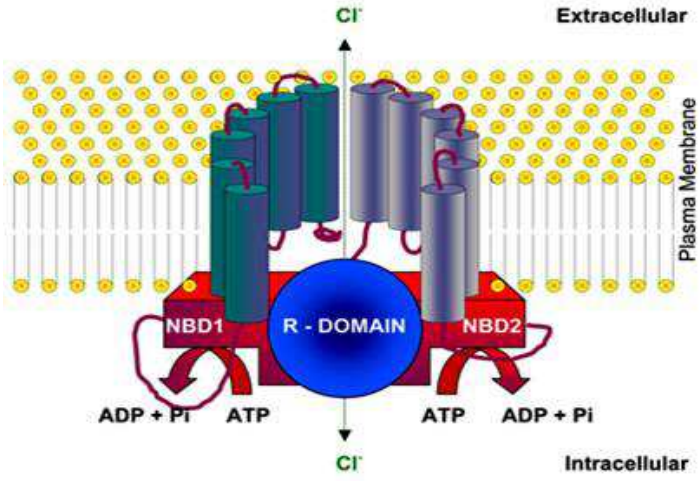


Figure 2.The CFTR channel. Two-dimensional representation of the CFTR protein (NP_000483.3) in the plasma membrane (courtesy of Oxford University Gene Medicine, UK).

Many of the mutations identified in CF patients are positioned in NBD1, while few have been identified in NBD2. This characteristic location of mutations is common in the ABC superfamily and indicates a separate role for the two binding domains. The most commonly found mutation is the deletion of 3 base pairs that code for the phenylalanine residue at position 508 of the amino acid sequence (F508del) in the exon that code for NBD1. The absence of this amino acid prevents the protein being formed from assuming a correct conformation ("folding"). The defective CFTR protein does not continue its maturation path from the endothelial reticulum to the Golgi where it is normally glycosylated, but is instead ubiquitinated and directed towards its degradation in the proteasome. Therefore most of the defective protein is degraded and only a small part is expressed on the plasma membrane where it exerts its channel function. Facilitating the folding of the nascent protein through the use of pharmacological

correctors, F508del / F508del CFTR is able to reach the plasma membrane and function as a chloride channel, albeit less efficiently than the non-mutated CFTR protein. The NBDs contain a series of highly conserved motifs able to bind and subsequently hydrolyze ATP. Site-specific mutagenesis studies in these regions have indicated that ATP must bind to both NBDs to cause channel opening (Jordan et al. 2008). Exon 13 encodes the R domain that is located between NBD1 and NBD2 (Figure 2). It contains several cAMP-dependent phosphorylation sites catalyzed by protein kinase A or C (PKA or PKC). The regulation of the activity of the CFTR ion channel in part depends on the degree of phosphorylation of the R domain and partly on the binding of ATP to the NDBs domains. The N-terminal portion of the R (RD1) domain is highly conserved among the species. About 20% of the CFTR protein is composed of transmembrane domains (M1 - M12). In these domains the typical secondary α-helix structure prevails. Many of the residues within these domains form the channel lining and play an important role in regulating pore function. Six of these positively charged residues are well conserved between species. Mutations in two of the six residues were found in CF patients, for example R334Q / W and R347C / H / L / P.

CFTR Mutation

So far, 1964 different mutations in the CFTR gene have been identified: 786 missense (40%), 311 frameshift (16%), 228 splicing (11%), 162 nonsense (8%), 39 in / del (2%) frames, 50 large in / del (3%), promoter (1%), 269 sequence variations (14%), 104 unknown (5%). A first attempt to group the mutations of the CFTR gene on the basis of the molecular defects and consequent functional alterations produced the 6 classes listed in Table I. CF patients with mutations included in one or more of these classes would be differently sensitive to treatment with chemical compounds able to correct the molecular defect. Class I include nonsense mutations with premature stop codons that lead a mRNA degradation and consequent no protein expression (Bobadilla et al. 2002). Class II included a most common mutation F508del that affect CFTR protein processing due to misfolding which is recognized by endoplasmatic reticulum (ER) quality control retention and which targets protein with abnormal conformation to

degradation (Amaral 2004). Class III mutations disrupt channel regulation through impaired gating (G551D) (Bell, De Boeck, and Amaral 2015). Class IV mutations decrease Cl^- ion conductance, Class V mutations reduce normal protein levels, often by affecting splicing and generating both aberrant and normal transcripts, whose levels vary amongst patients (Ramalho et al. 2002). Class VI mutations leads to decreased retention/anchoring at the cell surface, often associated with decreased protein stability at the plasma membrane or in a deletion mutant that takes out the CFTR protein initiation codon, so that the resultant protein lacks the N-tail required for cytoskeleton anchoring (Ramalho et al. 2009).

Tab I

Class	**Molecular defect**	**Conseguence**	**Exemple**
I	Premature termination stop codon (PTC).	Reduced or absent synthesis of CFTR	W1282X, R553X, G542X
II	Mysfolding protein	Abnormal CFTR conformation; reduced expression	F508del, N1303K
III	Imparied regulation of CFTR	Reduced or absent function	G551D, G551S, G1349D
IV	decreased retention/anchoring at the cell surface	Reduced channel conductance	R117H, R334W, R347P
V	Alternative spicing of mRNA	Reduced synthesis and expression of CFTR	2789+5G→A, A455E
VI	high turnover of CFTR on plasma membrane	Decreased protein stability	120del23, N287Y...

Screening and diagnosis of CF disease

Prenatal diagnosis. It is recommended for couples of healthy carriers who want a child, having a high risk of generating an affected individual (25% risk at each pregnancy). It is performed through the removal of corial villus (villocentesis) at the tenth week of pregnancy. The genetic test for cystic fibrosis is applied to the villus taken (analysis of the mutations of the CFTR gene carried by the parents).

Neonatal screening. It has been introduced in most Italian regions. A drop of blood is taken from all infants on whom the immunoreactivetrypsin test - IRT is performed. If IRT has a value beyond the reference threshold in the healthy population, the suspicion of disease arises and the genetic test is performed on the same drop of blood. This is diagnostic of CF if two mutations are identified in the CFTR gene, one on each allele. The definitive confirmation of the diagnosis is in any case entrusted to the sweat test, carried out already at 20-30 days of life in the newborn (Geokas et al. 1979; King et al. 1979; Crossley, Elliott, and Smith 1979; Simpson et al. 2005). Birth screening allows early diagnosis of CF disease and at the same time initiates the program of controls and therapies, which have significantly improved the quality of life in patients, as soon as possible.

Measurement of salt concentration, NaCl, in secretions of sweat glands. A concentration above the threshold equal to 60 mEq of Cl (-) per liter after 6 months and at 50 mEqCl (-) / L in the first 6 months of life strongly orientates towards the diagnosis of cystic fibrosis. Chloride values below 40 mEqCl (-) / L (30 in the first months of life) exclude the disease, albeit with some rare exceptions. Intermediate values (also called "borderline") are not conclusive, because they may be affected by interferences, for example pharmacological, both in healthy and sick subjects. In these individuals it is therefore necessary to carry out genetic analysis which, if performed correctly, turns out to be a diagnosis of CF disease. In the last few years a new sweat test, named “bubble test” (Wine et al. 2013), is under investigation that might provide some advantages with respect to the standard Gibson and Cook method (Bergamini et al. 2017).

Nasal potential difference (NPD).In addition to measuring the concentration of NaCl in the sweat, a second diagnostic test can be performed: the measurement of the potential difference in the cells of the nasal mucosa (NPD) that is particularly useful in cases where the sweat test has given uncertain results (Naehrlich et al. 2014). The mucosa of a CF patient's airway secretes less chloride and absorbs more sodium than normal; this imbalance involves measuring a more negative value of the tension through the nasal epithelium measured in the CF patient than that measured in a healthy individual. This test, certainly not very comfortable, also requires an important collaboration from the patient. Thus the patient's "compliance" can greatly influence the reproducibility of the results obtained with the measurement of NPD. Furthermore, its reference values have not yet been standardized globally and therefore each center has its own reference range of NPD values.

Genetic analysis. Methods to identify mutations in the CFTR gene are based on the sequencing of gene fragments, have high specificity, and a discrete sensitivity of 70-80% of cases. The analysis of the whole gene, instead, requires long time, has high costs, needs equipped laboratories and experienced personnel in the interpretation of the results and therefore it is carried out only in selected cases and in highly specialized centers.

Symptomatic therapy

The patient with CF is advised to follow a high-calorie, high-fat diet associated with pancreatic enzymes at every meal, replacing those that the pancreas does not produce, and supplemented with fat-soluble vitamins (vit A, D, E, K), mineral salts, especially in the young child, in hot weather and during sustained exercise. In addition, a strict therapeutic plan is applied which includes the following treatments:

Physiotherapy and respiratory rehabilitation to remove mucus from the respiratory system.

Education to a lifestyle with an aptitude for movement and also for sport: physical exercise promotes a harmonious development of the sick person, facilitates the removal of secretions and the effectiveness of respiratory function.

Aerosol therapy is applied to CF patients to thin the mucus, to dilate the bronchi or to administer antibiotics during acute and / or chronic lung infections. One of the drugs used for aerosol is human recombinant DNAs (Pulmozyme, Roche SpA). It acts by fragmenting the extracellular DNA resulting from the lysis of bacteria and granulocytes, decreasing the viscosity of the secretions and making antibiotic therapy more effective (Jones et al. 2005). This therapy is able to induce only a modest recovery of lung function by decreasing in some individuals the frequency of severe and recurrent acute respiratory attacks.

Antibiotic therapy. Administered orally or systemically, in cycles or for prolonged periods, serves to reduce the charge and to contain bacterial aggressiveness.

Anti-inflammatory therapy. It begins early in the course of the disease, before extensive irreversible lung damage occurs. Anti-inflammatory drugs that appear to slow the progression of the disease include oral corticosteroids, ibuprofen, and azithromycin, which, in addition to its antimicrobial effects, also possess anti-inflammatory properties (Pressler 2011).

In adolescents and adults, pancreas involvement can also cause diabetes, which requires daily insulin injections. The various complications require specific treatments: fluidization of intestinal contents in obstructive syndromes, fluidization of bile in hepato pathology, treatments against osteoporosis.

In the case of very advanced lung disease with irreversible respiratory failure, lung transplantation is the last chance to lengthen life expectancy.

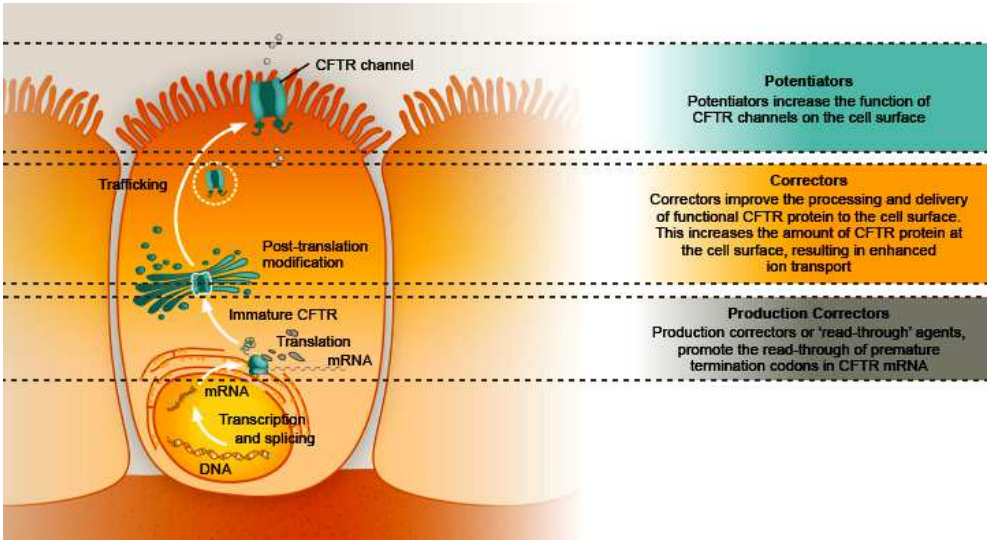
Therapies of the basic defect

Gene therapy. It involves inserting the CFTR gene without CF (wild type) mutations into cells of a CF patient who have a defective CFTR gene. In theory this result can be obtained through two methods. In the former, recombinant viral vectors, for example adenovirus, are used, which by penetrating into the host cell introduce the coding sequence of the wild type CFTR; in the second, the insertion of the wild type CFTR gene occurs through the use of a cationic lipid, such as GL67A, which carries DNA nanoparticles (DNA complexed with peptides) and self-assembling complexes of polyethylenimine (Derichs 2013; Rogan, Stoltz, and Hornick 2011; Davies and Alton 2010; Rosenecker, Huth, and Rudolph 2006). So far no satisfactory results have been obtained with these two approaches, the overall duration of expression of the correct gene is relatively short and numerous side effects have been observed both using the viral vector and adopting strategies not based on adenoviruses (Griesenbach, Pytel, and Alton 2015)..Since the in-depth study of the synthesis mechanisms of the CFTR protein and its chloride channel function, it was possible to identify new pharmacological targets useful to restore the correct synthesis and / or function of the mutated protein.

Through high-throughput screening assays, a technique to rapidly identify active compounds, antibodies, or genes involved in a particular biomolecular process, and other "drug discovery" techniques, small molecules have been identified which, by acting on the basic defect, are potentially able to re-establish CFTR expression on the plasma membrane and/or its channel activity. These substances have been divided into two categories: agents that target the genetic mutation and compounds that act on the consequences induced by specific mutations in the CFTR protein (Sloane and Rowe 2010).

In the last decade the researchers' attention has focused on the study of molecules called CFTR modulators. Starting from the study of the alteration mechanisms caused by the presence of particular classes of mutation of the CFTR gene, it was possible to identify active molecules on particular defects. It was observed that CF

individuals with particular mutations could benefit from treatment with molecules able to interfere with specific mechanisms of alteration of CFTR (Pettit 2012). Compounds capable of modulating the expression of CFTR and / or the function of the CFTR channel are classified into three main groups (Figure 3): enhancers, correctors and suppressors of early arrest codons (PTC readthrough) (Derichs 2013) .



PTC suppressors. The Class I mutations or nonsense, are present in about 5-10% of the CF population, although the incidence is about 60% of the Israeli population (Michael Wilschanski and Kerem 2011; M. Wilschanski et al. 2011). As described above, mutations of class I are associated with the presence of a premature stop codon (PTC), which terminates the translation of the CFTR protein early. The truncated protein is malfunctioned and does not reach the plasma membrane of the cell. In the past it was observed that gentamicin possessed the ability to reverse the effect of the presence of PTC by inserting a random amino acid that allow the ribosome to continue to read the mRNA and synthesize the entire protein. In vitro studies subsequently showed that the expression of CFTR on the cell membrane, when exposed to gentamicin, increased up to 35% (Howard, Frizzell, and Bedwell 1996; Bedwell et al. 1997). Furthermore, gentamicin was able to induce the expression of the CFTR protein in a homozygous mouse model for the G542X mutation (M. Wilschanski et al.

2011). Subsequent studies have confirmed these preliminary results, but the main problem turned out to be the high toxicity profile resulting from the prolonged exposure to aminoglycosides, so alternative molecules with less toxicity were sought. Ataluren (PTC-124, PTC Therapeutics, South Plainfield, NJ, USA) is one of these molecules currently under evaluation with a multicenter phase III study (ClinicalTrials.gov Identifier: NCT02139306, started June 2014) PTC-124 uses an action mechanism originally described for aminoglycosides allowing the ribosome to read premature stop codons without interfering with the true stop signal on the mRNA. Studies on CF mice have demonstrated the efficacy of this agent as well as the results of some phase II clinical trials (Konstan et al. 2011). PTC-124 not only increases the expression and activity of CFTR and improves clinical parameters, such as lung function and nasal potential in CF patients, but has also been shown to have good tolerability and reduced side effects in prolonged treatment of patients (M. Wilschanski et al. 2011). Cystic Fibrosis is not the only disease caused by the abnormal presence of PTC induced by the genetic defect. Hereditary diseases such as sickle cell anemia and Duchenne muscular dystrophy (DMD) share this pathogenetic mechanism. Therefore drugs that are able to circumvent the presence of PTC could be useful in the treatment of diseases other than CF. So far, clinical tests to assess the effectiveness of Ataluren in DMD patients have been rather disappointing (Finkel 2010). However, diseases with genetic mutations that induce the presence of PTC could benefit from pharmacological research on this class of molecules.

Correctors. Small molecules are defined CFTR correctors when can increase the amount of protein working on the surface of cells with CFTR class II mutations such as F508del (Verkman and Galietta 2009; Amaral 2011). To date, hundreds of molecular compounds have been identified with corrective characteristics (Pedemonte et al. 2005; Van Goor et al. 2006; Carlile et al. 2007; Kalid et al. 2010; Lin et al. 2010; Van Goor et al. 2011). Based on the mechanism of action, these correctors can be distinguished in pharmacological chaperones and in protein degradation regulators (proteostasis) (Hanrahan, Sampson, and Thomas 2013). Until now only a corrector Lumacaftor (VX 809) in association with Ivacaftor (VX 770), Orkambi, has been approved for patients aged 12 years and

above who have a the F508del mutation. However Orkambi in more than 1 in 10 of patient provokes a severe side effect that leads the suspension of administration (http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/003954/human_med_001935.jsp&mid=WC0b01ac058001d124). As such several trials are undergoing with the new corrector VX 661 or other alone or in combination with Ivacaftor for patients homozygous for F508del, but seems to be necessary the combination of multiple molecules to correct this type of mutations and many other compounds are in the pipelines of many biotech companies (Mijnders, Kleizen, and Braakman 2017)(Okiyoneda et al. 2013).

Potentiators. CFTR enhancers increase the likelihood of channel opening and are effective in correcting the effects of the presence of Class III (gating) or Class IV (conductance) mutations (Van Goor et al. 2009). As the cAMP-PKA signaling pathway constitutes the physiologically key step for CFTR activation, the first compound tested was IBMX (3-isobutyl-1-methylxan-thine). Drumm (Drumm et al. 1991) proposed that IBMX, by inhibiting phosphodiesterase, increases intracellular cAMP and consequently PKA activity so that DF508-CFTR can be activated to a higher level. Then was demonstrated that genistein, a tyrosine kinase inhibitor found in many foods, can activate CFTR in intact cells through a mechanism indistinguishable from that of forskolin, an adenylyl cyclase activator (B. Illek et al. 1995). Some later studies demonstrated that the CFTR protein itself is the target for genistein, establishing for the first time that CFTR itself can serve as a target for small-molecule CFTR potentiators (French et al. 1997). Besides genistein, other flavonoid molecules have also been shown to enhance CFTR function both in vitro and in vivo (Beate Illek and Fischer 1998).

In 2009, by using high throughput drug screening, Vertex Pharmaceuticals identified a compound, VX-770, that potently increases the activity of F508del CFTR and G551D-CFTR. The VX-770, or Ivacaftor, is a potentiator that effectively corrects the defect related to the class III G551D mutation. In vitro studies have shown that VX-770 has as its main target CFTR phosphorylated by PKA. It also seems to act through a dual mechanism: the first stabilizes the

channel in the open form and the second, unconventional, requires neither binding of the ATP on NBDs nor its hydrolysis to prolong the opening of the canal (Figure 4). This would explain why VX-770 is effective in correcting the CFTR defect caused by the G551D mutation that causes a decreased affinity of the ATP-binding site. Probably VX-770 binds to an allosteric site near the two NBDs, inducing a conformational change similar to the dimerization of NDB in the presence of ATP. Phosphorylation of CFTR would facilitate binding access (Eckford et al. 2012).

VX-770 increases the secretion of Cl^- in bronchial epithelial cells with the G551D mutation by 10 times, inducing about 50% of the total CFTR activity present in a healthy control (Van Goor et al. 2009).

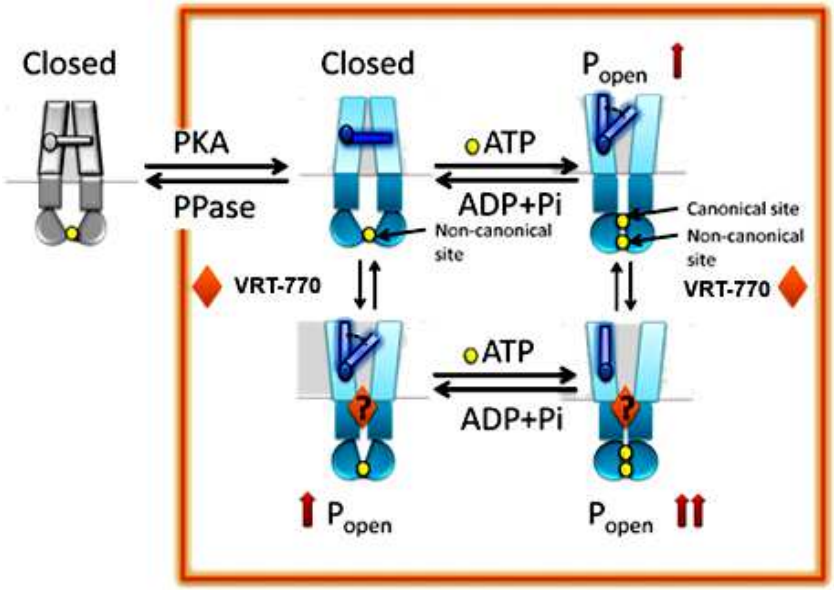


Figure 4. Mechanism of action of VX-770(Eckford et al. 2012)

Based on the results of pre-clinical studies, in 2012 it was approved by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) as an orphan drug for all patients with cystic fibrosis over 6 years of age with mutation G551D. It is marketed under the name Kalydeco (FDA Application No 203188). The drug is well tolerated, and at high concentrations (150 mg every 12h, oral administration), has proved effective in decreasing the concentration of Cl^- in sweat to a level close to normal (<60 mmol / L). It would also improve lung

function (mean increase of 9% FEV1) (Verkman and Galietta 2009). Ivacaftor restoring the balance of the flow of chloride through the canal would help to reduce the excessive activity of amiloride-sensitive channels and the absorption of water in epithelial cells with G551D mutation (Verkman and Galietta 2009). However, reagents other than VX-770 will be needed to rectify biochemical defects that are insensitive to VX-770 (Jih et al. 2017).

Models to study new chemical identity

Pre-clinical validation of novel compounds correcting CFTR in terms of their efficacy is required so that only the best candidates are trialled with patients. Unfortunately the impact of a defect in CFTR function differs among tissues and cell types, implying that no single CFTR-expressing cell model can accurately reproduce all aspects of CF. Moreover, although such tools may serve to facilitate personalized approaches to CF intervention, they are unable to report differences in corrector and potentiator response among individuals carrying the same CFTR mutation, such as caused by polymorphisms in the CFTR gene, CF modifier genes, of differences in the CFTR interactome (Ikpa, Bijvelds, and de Jonge 2014).

Animal models

A few years later after the discovery of *cftr* gene, a first mouse model for CF was created (Snouwaert et al. 1992) and start from this, the generation of CF animal models has greatly advanced our knowledge of the disease.

There are many advantages to the use of mice to study the pathology of CF disease, including the ease with which they can be genetically manipulated, short gestation times, and low cost of animal upkeep (Guilbault et al. 2007). However, there are also a number of limitations to their use, for example the mouse models fail to initiate spontaneous lung infection without pathogen challenge. Most models only display mild complications in the pancreas, liver, and vas deferens, which is in contrast to the severe abnormalities in human tissues (Lavelle et al.

2016). Although there is a 78% homology between the murine and human CFTR protein, a residual function of the channel seems to remain, and there is also another channel for chloride in epithelial tissue with a alternative signaling pathway that may compensate the lack of CFTR induced by different gene manipulations (Wilke et al. 2011). Pig seems to be a ideal models for study CF, because a lack of CFTR protein and function in CFTR−/−piglets impacts on the lungs, liver, pancreas, and GI tract, all organs which are similarly affected in humans with CF (Rogers et al. 2008; Ostedgaard et al. 2011). However the meconium ileus is present in 100% of CFTR−/− piglets (in contrast to its prevalence of 15% in infants with CF) and it is fatal without early surgical intervention (Rogers et al. 2008). The ferret model does demonstrate a large number of similarities to CF pathology in humans, particularly neonates. Ongoing steps to generate a CFTR−/− ferret that overcomes the GI pathology to enable animals to reach adolescence are needed to establish whether ferret lung pathology mirrors that of humans with CF. Nevertheless, it must be mentioned that the appearance of lung infections at both the start and end of the CFTR−/− ferret life span mirrors human disease, highlighting the potential benefits of this model. The development of F508del and G551D specific mutant is required to establish the ferret as an exemplary animal model for CF (Lavelle et al. 2016).The availability of mouse and other larger animal models expressing human CFTR provides the opportunity to unravel additional pathophysiological details and provides a robust platform to test novel therapeutics for many diseases, including cystic fibrosis (Rosen et al. 2017). However, it is necessary to consider the importance of finding alternative models to the animal model for ethical issues as well as to have models that are even closer to the patient's reality.

	Spontaneous lung infection	Pancreatic disease	Intestinal disease	Liver and gallbladder disease	Reproduction
Human	Yes	PI	MI	Biliary cirrhosis	Severe vas deferens defect
Mouse*	No	No	Intestinal obstruction, often fatal	No	Reduced fertility in females
Pig	Yes	PI	100% MI	Biliary cirrhosis	Severe vas deferens defect
Ferret	Yes	PI	75% MI	Liver disease	Severe vas deferens defect

*The phenotypic manifestations outlined above may differ between mouse models. PI: *pancreatic insufficiency*; MI: *meconium ileus*.

Tab III. Phenotypic manifestations of cystic fibrosis in humans and animal models(Lavelle et al. 2016)

In vitro models

There are different cell lines used in CF researcher, the most common are CFBE41o- and 16HBE14o-, bronchial epithelial cells generated by transformation of cystic fibrosis (CF) tracheo-bronchial cells with SV40 (Ehrhardt et al. 2006), other are cell lines derived from adenocarcinomas like Calu-3 (submucosal airway glands) and T84 (colon) cells are widely used because of their high level of CFTR expression, its apical localization when cultured on Transwell® filters, and their ability to develop a significant trans-epithelial resistance (Rosen et al. 2017). However cell lines are useful during the high throughput screening but they do not fully correspond to the reality of the patient. A major step forward was made by developing standard protocols for the extraction and maintenance in culture of primary Human Airway Epithelial Cells (HAEC) derived from pulmonary biopsies or nasal/bronchi brushes. When HAEC are seeded on Transwell® filters and exposed to an air-liquid interface for several weeks, progenitor cells differentiate into a pseudostratified epithelium that recapitulates the typical mucociliary structure of the airway epithelium (Randell et al. 2011).Among other in vitro-based assays to monitor CFTR function organoids have become a model of choice for CF research. Organoids are progenitor cells grown in a 3D-environment in the presence of conditioned medium or supporting cells that organize similarly to in vivo organs (with an internal lumen). Organoids representative of the intestinal and respiratory systems have been generated and these models represent robust systems for screening of drugs that may act alone or in combination to correct CFTR channels harboring different mutations (Dekkers et al. 2013; Tan et al. 2017). These models unfortunately are not applicable to monitoring the patient during a clinical trials or his life, because organoids, as HAEC, need several extraction process and some weeks of culture before it is possible to assay them. This is one of the reasons why in the last few years the interest about leukocytes and monocytes in CF is increased. Already back in 1991, (Yoshimura et al. 1991) mRNA encoding for CFTR was demonstrated in human leukocytes, but not until recently CFTR protein was detected in neutrophils, (Painter et al. 2006), lymphocytes (Shanshiashvili et al. 2012), and monocytes (Van de Weert–van Leeuwen et al. 2013; Johansson et al. 2014), in which it was

shown to be involved in membrane potential regulation (Sorio et al. 2011). More recently the relevance of a mutated CFTR in monocytes has been studied and it has been observed that the lack of the channel causes an exaggerated inflammatory response at the pulmonary level (Tarique et al. 2017), more in detail this phenomenon seems due to a critical regulatory role of CFTR in integrin activation by chemo-attractants in monocytes and identify CF as a new, cell type–selective leukocyte adhesion deficiency disease, providing new insights into CF pathogenesis (Sorio et al. 2016). So leukocytes and monocytes seems to be a good cellular model to investigate CFTR function in individual patients as these type of cells, are easy to collect with a good compliance for the donor. They can be readily isolated with minimal handling within a few hours, thus better mirroring the original conditions present in vivo. Other researchers start to consider leukocytes as a valuable cellular biomarker to be evaluated during the progress of a clinical trial (Guerra et al. 2017).

	Expression of CFTR	Easy to collect	Maintenances
Cell lines (CFBE41o-,Clau3,T84)	Yes	Yes (possible to buy)	several passages
HAEC	Yes	Yes (possible to buy)	few passages, expensive
Organoids	Yes	Long procedure	several passages, expensive
Leukocytes	Yes	Yes	max 24h

Tab.IV Summary of the various in vitro models discussed

Methods to measure CFTR activity in different models

Ussing Chamber.The Ussing chamber provides a physiological system to measure the transport of ions, nutrients, and drugs across various epithelial tissues (Clarke 2009). The method was developed in 1946 by the Danish biologist Hans H.

Ussing as a means to understand the phenomenon of active NaCl transport. The technique is used to measure the short-circuit current as an indicator of net ion transport taking place across an epithelium. Ussing chambers are used to measure ion transport in native tissue, such as gut mucosa (rectal biopsy), and in a monolayer of airway cells grown on permeable supports (HAEC). When the tissue is on the Ussing chamber each side, apical and basal are in contact with the same solution, so paracellular ion movements driven by the passive forces of transepithelial concentration and osmotic and hydrostatic gradients were eliminated. The passive transepithelial driving force created by the spontaneous electrical potential across the epithelium is eliminated by clamping the potential to zero with an external current passed across the epithelium. This current, known as the short-circuit current (Isc), is equivalent to the algebric sum of electrogenic ion movement by active transport (i.e., when using conversion with the Faraday constant). Thus, by eliminating transepithelial diffusion forces (osmotic and electrochemical gradients), the movement of ions as measured by isotopic tracers or the short circuit current (Isc) in the Ussing chamber resulted from active transport(Clarke 2009). This technique is exploited both to help the diagnosis of cystic fibrosis in specialized centers, through rectal biopsies, and for the study of new compounds using HAEC.

Forskolin Induced Swelling (FIS) assay. This recently developed technique is used to measure the CFTR activity in organoids derived from CF patients. Organoids derived from rectal biopsies after two o more weeks culture, are seeded in a proper support, multiwell or chamber slide, the day after are incubated with a fluorescent probe, calcein green, for 30 minutes and then analyzed by confocal microscopy. After the first acquisition a proper amount of Forskolin is added in all well and then a picture is taken after 10 minutes until 60-90 minutes. If the treatment is effective, the corrected organoids will show an increase of their volume, named swelling. FIS assay was originally developed and proposed by Dekkers and colleagues (Dekkers et al. 2013), and is now tested in few specialized centers for validation in a clinical context. This technique derives from the development of suitable protocols for intestinal stem cell culture and protocols

are still being perfected. In addition, qualified personnel and specialized infrastructures are required.

Patch clamp. This is an electrophysiology technique useful to measure ionic current in living cells, tissue section or patches of cell membrane. In cystic fibrosis it is a validated method to analyze the activity of the CFTR channel in different cell types, to deepen its physiological functioning and in the presence of different mutations (Linsdell 2016), to verify the response to certain drugs or to characterize CFTR in new models (Gonçalves et al. 2017; Salomon et al. 2016; Ettorre et al. 2014). It allows obtaining accurate results on the functionality of CFTR, however it is low throughput assay, requires high technical skill and profound knowledge of electrophysiology as well as a very precise experiment setup for each type of cell that is going to be measured.

Fluorescence probes. Numerous methods have been developed that exploit different fluorescent probes to measure the activity of ion channels, often they are techniques that require materials and instruments easily available in every laboratory and relatively easy to set up and interpret. They are not always able to provide with a definitive result, but are useful for the screening of new molecules. Among the most used are: MQAE is a 6-methoxyquinolinium derivative and a fluorescent indicator for intracellular Cl^-, This dye detects the ion via diffusion-limited collisional quenching (Gonçalves et al. 2017); the potential-sensitive probe bis- (1,3-diethylthiobarbituric acid) trimethineoxonol (DiSBAC2(3), Invitrogen, USA) can enter in depolarized cells where it binds to intracellular proteins or membrane and exhibits enhanced fluorescence and a red spectral shift. Increased depolarization results in additional influx of the anionic dye and an increase in fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence (Renier et al. 1995; Sorio et al. 2011). However, these techniques require an incubation time with the probe that goes in direct contact with the cells and therefore the interference that the probe with cellular homeostasis must be taken into consideration, besides they are methods best suited to be applied on adherent cells.

Aim of the thesis

A major challenge for rare diseases such as cystic fibrosis is finding a strategy to correct the basic defect and be able to personalize the treatment for each patient. Promising drugs able to rescue the expression and function of CFTR are now available and many are under advance state of development. These treatments are costly and sometimes give rise to serious side effects, setting the need for easy and fast methods to be used in primary cells from the individual patients that are suitable to predict and monitor their effectiveness as CFTR potentiator/correctors in a timely manner. Based on previous work I was involved in the development of a new assay to measure CFTR activity. The readout of this method is based on residue quantity of iodide present in the supernatant, after proper stimulation, quantified by the quenching of the fluorescence of Halide Sensitivity Yellow Fluorescence Protein (GST-HS-YFP). The specificity of the assay for CFTR activity was tested in CFTR expressing MM6, an acute human monocytic leukemia derived cell line, using two selective CFTR inhibitor,CFTR-172 and PPQ-102, cells and after silencing of MM6 with CFTR siRNA. I also tested primary leukocytes, where we recorded a significant difference between WT and CF PBMCs and Monocytes ($p<0,005$).

Materials and Methods

Reagents

CFTR activators :

Forskolin, a cell-permeable diterpenoid that possesses anti-hypertensive, positive ionotropic, and adenylyl cyclase activating properties. Many of its biological effects are due to its activation of adenylyl cyclase and the resulting increase in intracellular cAMP concentration

Genistein, inhibitor of tyrosine protein kinase; competitive inhibitor of ATP in other protein kinase reactions and a CFTR potentiator (French et al. 1997) and N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (cAMP). To verify the accuracy of the assay we treated the cells also whit two different CFTR inhibitors: 5-[(4-Carboxyphenyl)methylene]-2-thioxo-3-[(3-trifluoromethyl)phenyl-4-thiazolidinone (CFTR-inh 172), Ki = 300 nM, IC50=0,38μM. CFTR(inh)-172 leads to rapid, reversible and voltage-independent inhibition. The 6,7-Dihydro-7,9-dimethyl-6-(5-methyl-2-furanyl)-11-phenylpyrimido[4',5':3,4]pyrrolo[1,2-a]quinoxaline-8,10(5H,9)-dione (PPQ-102) a cell-permeable pyrimido-pyrrolo-quinoxalinedione (PPQ) compound that targets the intracellular nucleotide binding domain(s) of CFTR and inhibits CFTR-mediated chloride current in a reversible and voltage-independent manner with a IC50=90nM. For some experiment the corrector Lumacaftor (VX-809) and the potentiator Ivacaftor (VX-770) were used at the final concentration of 5μM.

Preparation of recombinant Glutathione S-transferase-Halides sensitive Yellow Fluorescent Protein (GST-HS-YFP)

The recombinant GST-HS-YFP was gently produced and provided by Dr. Monica Averna, Department of Experimental Medicine-Biochemistry Section, University of Genoa, Italy. The nucleotide sequence of the YFP was amplified by PCR from pEYFP-C1 plasmid (Clontech) with the following primers: Sn YFP-BamHI: 5’ AA-GGATCC-ATGGTGAGCAAGGGC and Asn YFP-EcoRI: 5’ A-GAATTC-

TTACTTGTACAGCTCGTCCATGC. Reaction was cycled at an initial denaturation temperature of 94°C for 1 min followed by 35 cycles at 94°C for 30 s, 58°C annealing temperature for 30 s and 68°C extension time for 1 min. A 5-min extension period at 68°C was incorporated after the last cycle of PCR. Then the amplicon has been ligated into pGex6P1 expression vector that contains GST (Glutathione S-transferase) as tag protein. To obtain YFP sensitive to halides both the mutation YFP-H148Q (Jayaraman et al. 2000) and I152L (Galietta, Haggie, and Verkman 2001) were introduced by mutagenesis. Mutagenesis has been performed using the polymerase chain reaction-based QuickChange site-directed mutagenesis kit (Stratagene) as indicated by the manufacturer. The identity of the mutated YFP has been confirmed by sequencing with CEQ 2000XL DNA analysis system. (Beckman Coulter). Mutated YFP has been produced in *E. Coli* DH5α as GST-HS-YFP fusion protein and purified to homogeneity following affinity chromatography with GSH-agarose. Briefly, transformed DH5α cells were grown in Super Broth medium (3.5% tryptone, 2.0% yeast extract, 0.5% NaCl, pH 7.0) containing 100 μg/mL ampicillin at 37 °C for 16 h. Cells were then ten-fold diluted and grown at 37 °C until optical density at 600 nm was ≥ 0.6. Recombinant protein expression was induced with 1.2 mM IPTG (Sigma-Aldrich) for 16 h at 25 °C. Cells were washed once with H_2O and lysed in the following lysis buffer: 0.1 M Tris/HCl pH 8.3, 0.15 M NaCl, 1% Triton-X100, 10 mM EDTA, 2 mg/mL lysozyme, 2 μg/mL aprotinin, 100 μg/mL leupeptin, and 2 mM AEBSF (2 mL lysis buffer/g). After 20 min at 0 °C, 10 mM $MgCl_2$ and 10 μg/mL DNase were added and the lysate was incubated for additional 20 min at 0 °C. The lysate was cleared by centrifugation (100,000 ×g for 20 min at 4 °C) and the resulting supernatant was loaded onto 2 mL GSH-agarose (Sigma-Aldrich) column, pre-equilibrated with 50 mM sodium borate pH 7.5, containing 0.15 M NaCl and 5 mM DTT (buffer A). The resin was washed with 20 column volumes of buffer A and recombinant GST-HS-YFP was eluted with 50 mM sodium borate pH 9.0, containing 0.15 M NaCl, 5 mM DTT, and 10 mM GSH. The eluate was submitted to desalting procedure using a PD10 column (GE Healthcare) pre-equilibrated with 50 mM sodium borate pH 8.0. Purity of GST-HS-YFP was evaluated by SDS-10% PAGE followed by blue Coomassie staining.

The fusion protein has a K_I^-= 3.1±0.6 mM (mean±SD; n=8) and a K_{Cl}^-= 138±26 (mean±SD; n=4).

Cell culture

The MM6 (acute human monocytic leukemia) cell line was cultured at 37 °C (5% CO2) with RPMI 1640 (Sigma–Aldrich) growth medium containing 10% fetal calf serum, 10 U/ml penicillin (Sigma–Aldrich), 100 μg/ml streptomycin (Sigma–Aldrich) and 4 mM l-glutamine. The MM6 cell line was provided by Prof. Claudia Cantoni, Department of Experimental Medicine, University of Genoa, Italy. The FRT (Fisher rat thyroid) cell line over-expressing human CFTR (WT FRT cells) was cultured at 37 °C (under a 5% CO2 atmosphere) in Coon's F-12 growth medium, containing 10% (v/v) FBS, 10 units/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine and 0.6 mg/ml zeocin. FRT cells not expressing human CFTR (FRT non-transfected cells) were cultured in the same medium in the absence of zeocin. FRT cell lines were provided by Dr L.J. Galietta, Molecular Genetics Laboratory, Giannina Gaslini Institute Genova, Italy.

Donor subject and sample collection/preparation

The blood sample from Healthy volunteer or CF patients was collected after informed consent by a specialized nurse. For every subject, CF or Healthy donor, a sample of 10 ml of blood was collect in two 5 ml BD Vacutainer® tubes contained 5mM EDTA. Then the sample was processed to extraction of PBMCs (peripheral blood mononuclear cells) or Monocytes. Briefly the whole blood was diluted in ratio 1:1 with this buffer: 0,14 M NaCl, 5mM KCl, 2g/L Glucose, 10mM HEPES in sterile water pH 7,4 and then loaded in a proper amount of FicollPaque™ and centrifuged. The PBMCs fraction was collected with a plastic pipette and washed two times with PBS buffer (Fig. 5). To purify the Monocytes we used the Monocytes isolation Kit II(MiltenyiBiotec). The magnetically labeled non-monocytes are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled monocytes pass through the column. After the purification, monocytes were plated in a 96 multi well plate

(costar 3596) without Fetal calf serum for 30 minutes and when the cells are adherent on plastic the complete medium was added. The day after Monocytes were washed twice with CFTR Buffer (0.25 M sucrose, 0,005 M Glucose, 20 mM sodium borate and 0.2 mM $CaCl_2$ in sterile water pH 7,5) and then the assay was performed.

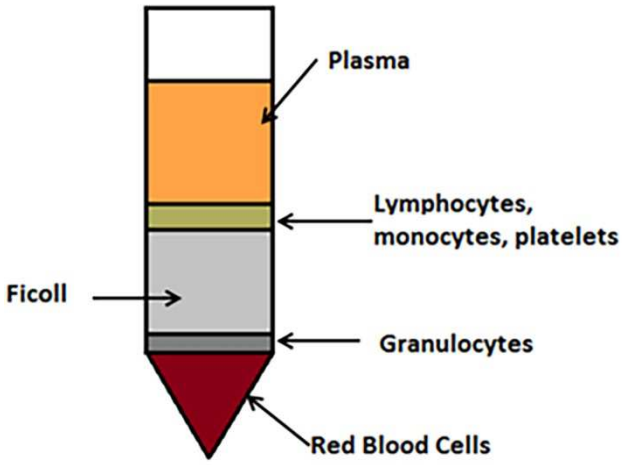


Figure 5. Representation of density gradient centrifugation with FicollPaque™

YFP Assay

The principle of this method to measure the CFTR activity in different cell type is based on the ability of GST-HS-YFP to change its fluorescence in relation to the concentration of iodide present in the medium. The method was originally proposed by (Averna et al. 2013) To perform this assay in non-adherent condition a proper amount of cells (tab.1) is resuspended in 200µL of CFTR buffer with or without stimulus, maintained at 37°C for 10-20 minutes and then exposed to 5mM NaI for 20 seconds. Cells are then removed by rapid centrifugation and the supernatant recovered. For adherent cells, seeded in 96 mw plate, the volume is 75µL. The supernatant is transferred in a black plate where 0,5µg of YFP is added. The plate is left for 10 minutes in slow agitation and then fluorescence is read at λ_{ex} = 485±15 nm and λ_{em}= 535±10 nm. The fluorescence recovery of GST-HS-YFP is directly related to the reduced levels of I^- due to channel activity. The read out of the assay is the difference of fluorescence between stimulus and

basal condition where, if CFTR channel is active, a positive difference (delta) is recorded; if CFTR channel is not active, no increase in fluorescence is detected.

Tab.II

	n° cell/cond
PBMC	$1,5*10^6$
MM6	200.000
Monocytes	200.000

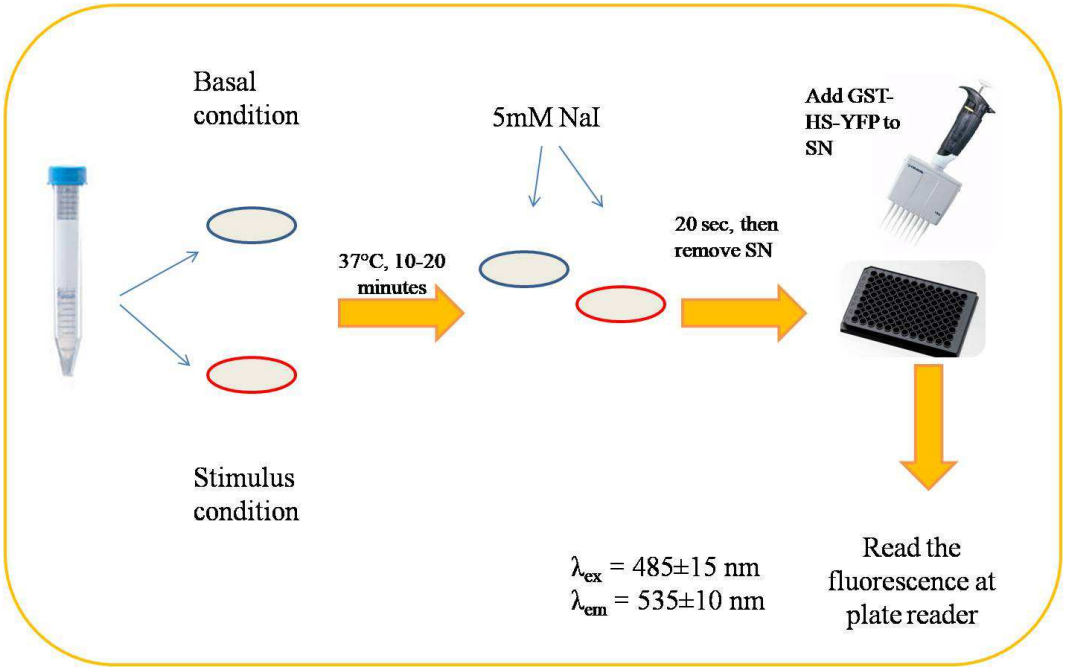


Figure 6. Schematic view of GST-HS-YFP assay

Western Blot

MM6 were lysed in lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100 pH 7.4) containing protease inhibitors (Roche,Inc.). 10µg of total protein were separated using 7,5 %(v/v) SDS-PAGE and transferred onto nitrocellulose membranes that were probed with monoclonal anti-CFTR antibody (Cell Signaling 2269) at a 1:500 dilution, overnight at 4°C. Membranes were re probed with monoclonal anti-actin (Sigma-Aldrich) to normalize for protein loading. The relative levels of CFTR were estimated by densitometry using the ImageJ program (http://rsb.info.nih.gov/ij/). The amount of band C is

calculated as a fraction of actin for the respective lane and reported as a fraction of the total (band C/actin). The values reported are expressed as means +/-SD (n=3). Data sets were compared by a t-test using GraphPad Prism.

Silencing

Gene silencing is mean an interruption or suppression of the expression of a gene at transcriptional or translational levels. This method generally is able to reduce the expression of a gene by at least 70% but do not completely eliminate it. RNA interference (RNAi) is a conserved biological response to double-stranded RNA that mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids, and regulates the expression of protein-coding genes. A simplified model for the RNAi pathway is based on two steps, each involving ribonuclease enzyme. In the first step, the trigger RNA (either dsRNA or miRNA primary transcript) is processed into a short, interfering RNA (siRNA) by the RNase II enzymes Dicer and Drosha. In the second step, siRNAs are loaded into the effector complex RNA-induced silencing complex (RISC). The siRNA is unwound during RISC assembly and the single-stranded RNA hybridizes with mRNA target. Gene silencing is a result of nucleolytic degradation of the targeted mRNA by the RNase H enzyme Argonaute (Slicer). If the siRNA/mRNA duplex contains mismatches the mRNA is not cleaved. Rather, gene silencing is a result of translational inhibition. (https://www.ncbi.nlm.nih.gov/probe/docs/techrnai/)

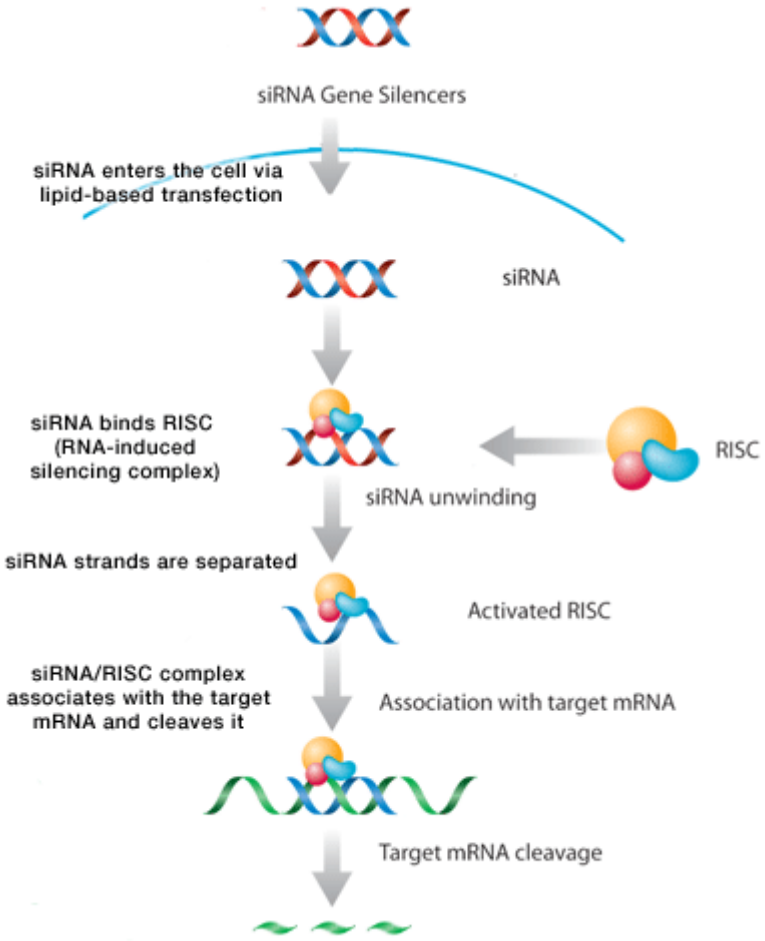


Figure 7. The image summarizes the process by which exogenous siRNA leads to silencing a desired mRNA. (https://www.scbt.com/scbt/whats-new/gene-silencers)

The MM6 cells line were transfected with a specific siRNA for CFTR protein (s2945, ThermoFisher scientific), or a Silencer® Negative Control (AM4611, ThermoFisher scientific) following manual instruction of siPORT™ NeoFX™ Transfection Agent (AM4510, ThermoFisher scientific). Briefly, the cells were suspended in a concentration of 90.000 cell/ml in a medium with 0,5 % FCS; the siPORT™ NeoFX™ Transfection Agent was diluted in RPMI without serum and incubated for 10 minutes at RT; then the RNA was diluted in RPMI serum free, the siPORT and RNA were mixed together and incubated for other 10 minutes. Finally the RNA/siPORT™ NeoFX™ Transfection Agent transfection complexes

were dispensed into 12 multi wells of a clean culture plate and the cells were added. After 72 hours the functional test YFP assay and Western blot analysis were performed.

Results

Analysis of parameters that might affect the assay

The concentration of 5mM NaI is chosen based on a variation of GST-HS-YFP fluorescence according to Iodide concentration. Figure 8A shows a quenching curve and it is of note that from 5mM to 0 of NaI the slope of the curve is steepest, meaning that for small change in Iodide it is possible record a large variation in HS-YFP fluorescence. Furthermore there is no change in pH during the test (8B) and no change in fluorescence value in the presence of the stimuli without cells (8C).

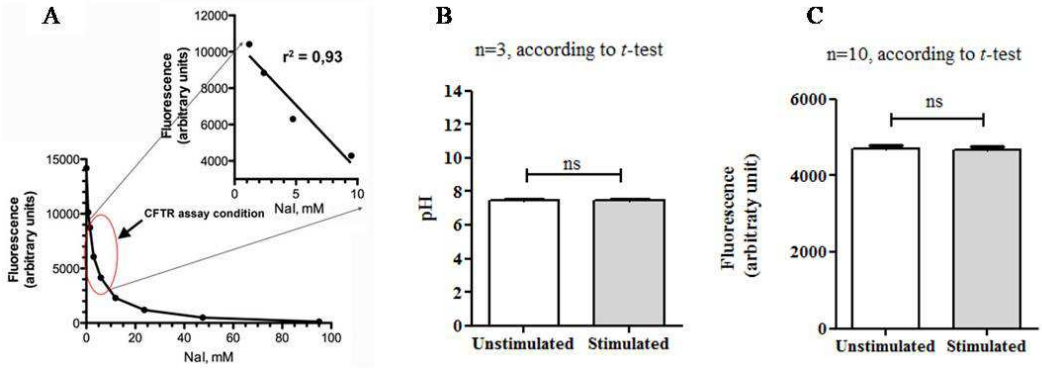


Figure 8.Panel A: quenching curve of YFP with different concentrations of NaI. Panel B: treatment does not alter pH during the test. Panel C: Additions of stimuli do not change the fluorescence value in absence of cells. The stimulus added to reproduce the experimental condition is 50µM Genistein and 100µM dibutirril-cAMP for 30 minutes.

The optimal time point for the iodide exchange was investigated in a time course experiment. MM6 cell line (fig 9A), PBMC and monocytes (fig 9B) from WT and CF individuals were exposed to stimuli from 5 to 20 minutes. Maximum signal was recorded at 10 minutes suggesting that after this time there is the maximum activation of the CFTR channel and therefore a greater uptake of iodide into the cell. So the 10 minutes time was chosen as duration of the stimulus as it provides a higher signal to noise ratio suitable to evaluate the increase of the channel

activity following different treatments.

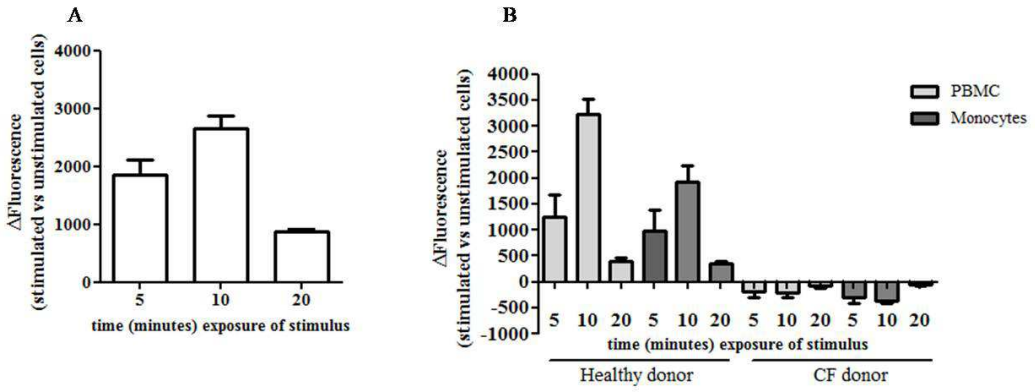


Figure 9.Panel A. Time course from 5 to 20 minutes of exposure to 10µM of Forskolin and 100µM of cAMP in MM6 cell line. Panel B shows the same condition applies of PBMC and Monocytes from healthy and CF donor.

HS-YFP assay is specific for CFTR activity

In order to test the specificity of the assay for CFTR activity, we tested different cell models and treatments: Fisher Rat Thyroid (FRT) cell line transfected with WT-CFTR, FRTnull clone and MM6 treated with specific siRNA or two different chemical inhibitors of CFTR activity. Figure 10, panel A shows the signals recorded in FRT cells, the average of three different experiment in FRT with wt CFTR is 500, in FRT null is below100, MM6 show a positive delta, while exhibit a negative value after siRNA treatment (n=4).Occurrence of siRNA-mediated silencing was confirmed by western blot analysis of whole cell lysate. Treatment of MM6 with two different CFTR inhibitors, CFTR-inh 172 or PPQ-102, both at 10µM, causes a significant decrease of the signal (panel C).Usually the accuracy of a test to discriminate diseased cases from normal cases is evaluated using Receiver Operating Characteristic (ROC) curve analysis (Zweig and Campbell 1993), in this case the analysis is applied to verify the robustness of the assay in discriminate the MM6 treated with specific inhibitors or vehicle(figure 10C). Accordingly, PPQ-102 inhibitor has a lower IC50 than CFTR-inh 172 ($IC50_{172}$=0,38µM, $IC50_{PPQ102}$=0,09µM).

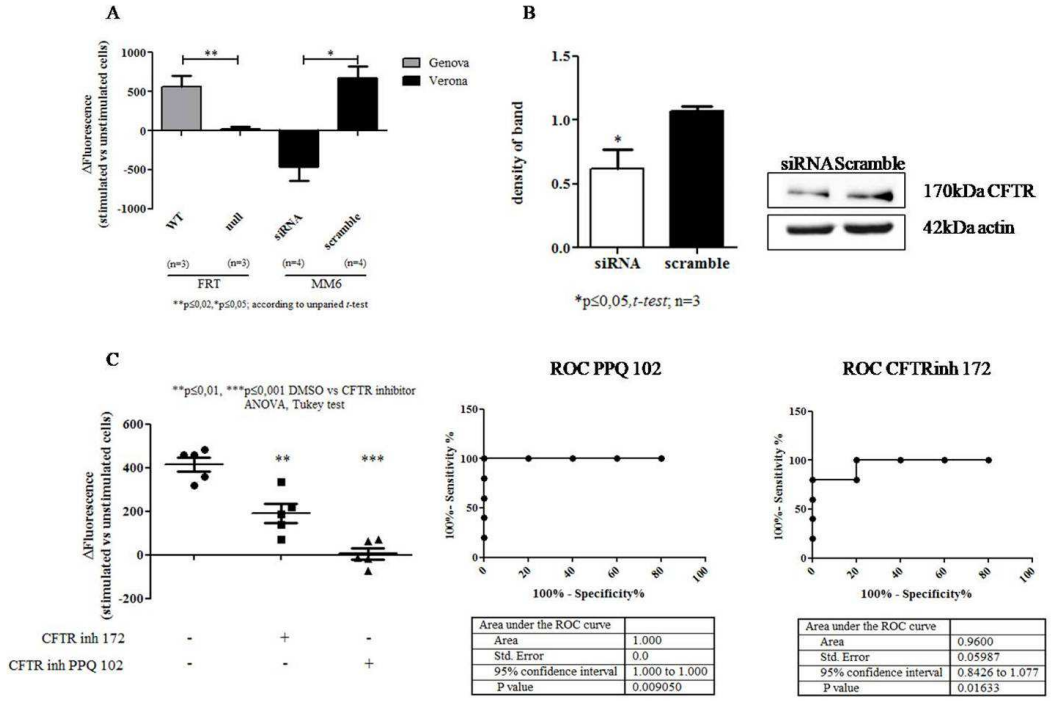


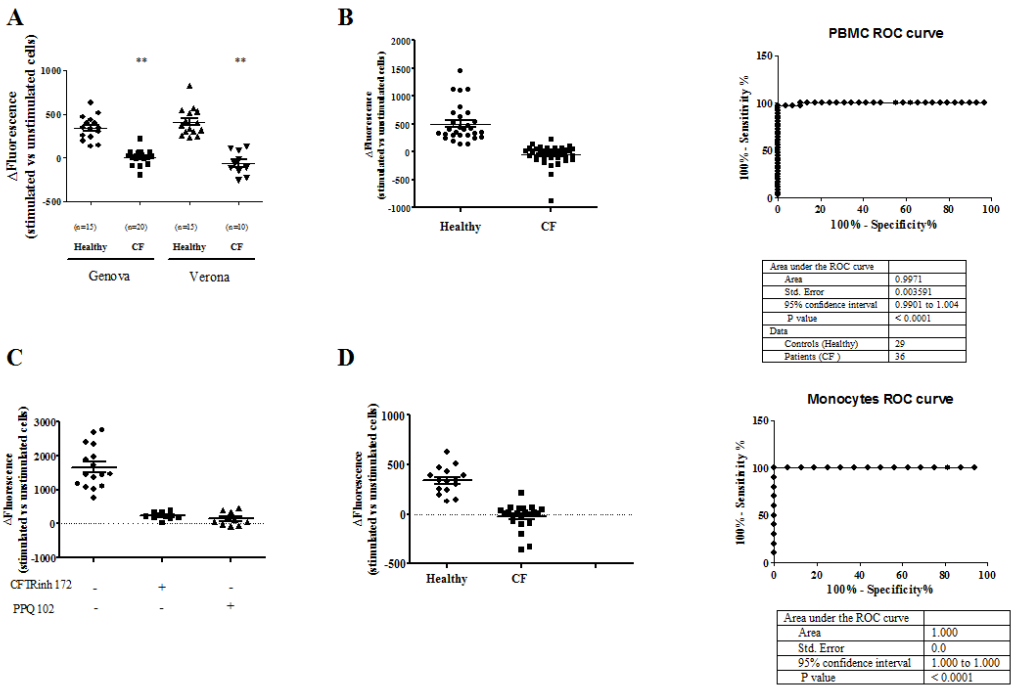
Figure 10. Analysis of assay specificity. Panel A: no positive fluorescence signal is detectable in FRT null (CFTR negative) or in MM6 treated with a siRNA for CFTR, at variance with FRT cell transfected with wt-CFTR (WT) or mock transfected MM6 (scramble) where a positive fluorescence value is detected. Stimulus: Forskolin 10µM +dibutyryl 100µM; time 10 minutes - siRNA s2945, final concentration 30nM, transfected with siPORTNeoFX transfection agent.

Panel B confirms the reduction of CFTR protein in MM6 following silencing with siRNA for CFTR (s2945). Control is represented by the same cells treated with a scramble sequence. CFTR is detected by the anti-CFTR antibody 2269(from Cell Signaling).

Panel C: Similar results were obtained following inhibition of CFTR function in MM6 by the chemical inhibitors CFTR-inh 172 or PPQ-102(10µM). ROC curves are reported.

From cell lines to patients

Having shown that the signal measured depends on CFTR expression or function we move on to further evaluate primary cells from control (Healthy) and CF patients. In two different laboratories, the values recorded for PBMCs from healthy donor is around 500 fluorescence unit, while PBMCs from CF patients present with null or negative values. The performance of a test is evaluated using Receiver Operating Characteristic (ROC) curve analysis (figure 11B). The assay displays high sensitivity and specificity being capable to discriminate PBMCs from CF and Healthy donors. Similar results are obtained using Monocytes (figure 11D): in this type of cells the value for Healthy control is around 1500 fluorescence units. If Monocytes from healthy donor are pre-treated with two different CFTR-inhibitors, the value is reduced from 1500 to 100 fluorescence units, similarly to MM6 cell lines (11C).



Area under the ROC curve	
Area	0.9971
Std. Error	0.003591
95% confidence interval	0.9901 to 1.004
P value	< 0.0001
Data	
Controls (Healthy)	29
Patients (CF)	36

Area under the ROC curve	
Area	1.000
Std. Error	0.0
95% confidence interval	1.000 to 1.000
P value	< 0.0001

Figure 11. Panel A**:** Results of YFP assay on PBMCs from healthy and CF donors, performed by two independent units (Genova and Verona). P<0,01 ANOVA, Tukey test. Panel B: all data are been grouped and ROC curve is shown.

Panel C: Results obtained in monocytes, treated with two different CFTR inhibitors

Panel D: Fluorescence values recorded from healthy and CF monocytes and the corresponding ROC curve.

We also evaluated the response of obligate heterozygous carrying one healthy and one mutated CFTR allele. While the test easily discriminate both healthy and obligate heterozygous patients, it is unable to identify a partial reduction of CFTR function, a condition typical of parents of affected individuals, healthy carriers of a mutation in one CFTR gene allele (Fig 12).

We are now developing a method of analysis that quantifies the exact amount of iodide exchanged through a reference curve that will be useful to normalize data among different conditions and operators and to reduce the consequences of batch-to batch variations of YFP preparations.

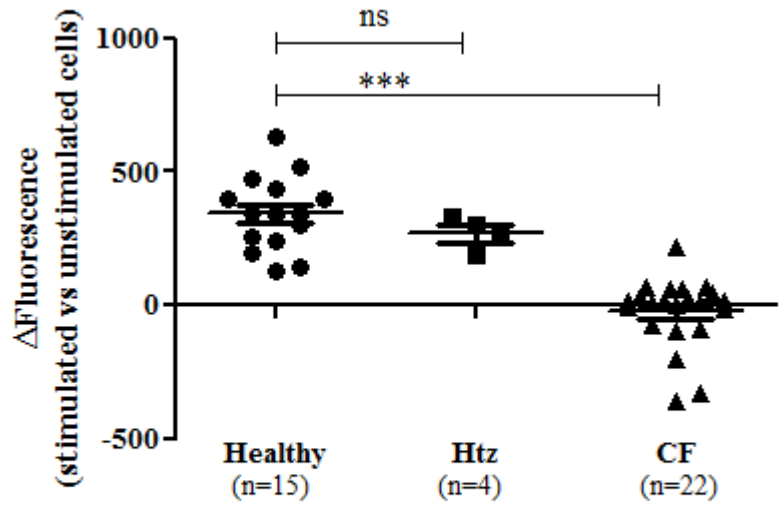


Figure 12. The graphic shows the differential fluorescence values recorded from PBMCs of non-CF, obligate heterozygotes (Htz) or CF individuals.

In support of the hypothesis that this test could be useful in evaluating the efficacy of a therapy, we collected samples of a homozygous F508del CF patient and evaluated the activity of the CFTR channel on PBMC before the start of Orkambi therapy, during and after the suspension. As shown in Figure 13, at time zero (no drugs taken) there was no increase of fluorescence, even if using different stimuli, after a week of treatment we recorded a positive delta and after one week from the suspension of the therapy the activity almost disappeared.

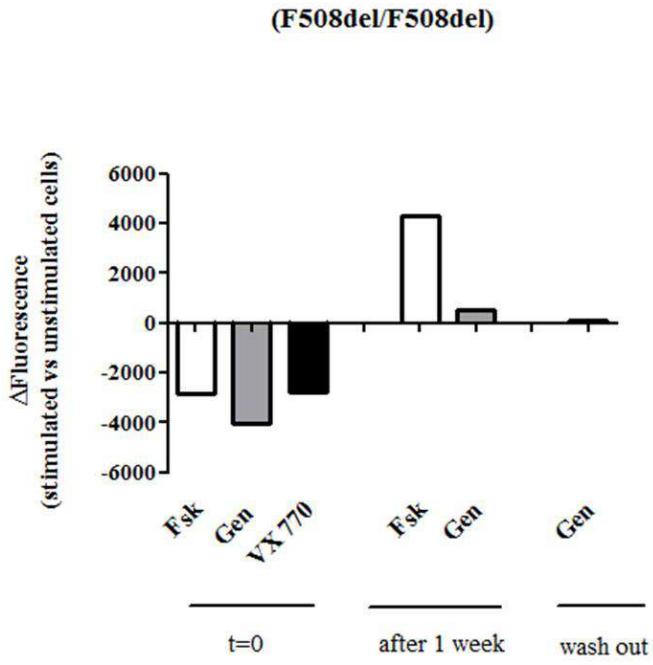


Figure 13. PBMC from CF patient are collected and YFP assay was performed before, during and after treatment in vivo with Orkambi therapy and following suspension of the therapy (wash out). Stimuli are dibutyryl 100µM; time 10 minutes for all the condition in combination with: Fsk: Forskolin10µM; Gen: Genistein (50µM), VX770 5µM as indicated.

Conclusion

The measurement of CFTR channel activity using this new assay appears specific for CFTR function, as demonstrated with the use of different disease models: FRT cells transfected with CFTR cDNA or the corresponding CFTR negative cells, MM6 cells whose CFTR gene expression is reduced by silencing technology and by the use of specific CFTR inhibitors. Finally the results were validated with the use of primary cells derived from controls and CF patients.

PBMCs represent convenient cells fast and easy to purify, and as such, appear suitable for monitoring patients during treatment with drugs acting as CFTR correctors or potentiators.

When purified monocytes are cultured these cells can be exposed to the compound of interest for a defined time (24-48 hours) and the evaluation of the response to CFTR agonist/s can indicate the potential capability of the treatment to correct CFTR function in the cells and, by inference, to the individual. Indeed CFTR activity in Monocytes was recently found to increase significantly at 3 and 6 months after Ivacaftor therapy and correlates with FEV1, FVC, BMI(Guerra et al. 2017). This suggests that myeloid cells might represent valuable predictive tool in CF patients.

Compared to other techniques already used to monitor the activity of the channel through fluorescent probes, such as DiSBAC2(3) or MQAE, this method does not require direct contact between the probe and the cells. This is an advantage as it is one less variable that can disrupt the system. In addition, the tools required for the analysis are easy to use and require short training, while for other techniques such as the patch clamp or Ussing chamber in-depth knowledge of electrophysiology and more sophisticated tools and expertise are required. GST-HS-YFP assay is a method that can find different applications and can be adapted to different cell types based on investigative needs, for example as screening tool performed on cell lines. Combined with other diagnostic tests already recognized by the scientific community, such as the sweat test or ICM, it could represent a valuable aid in the diagnosis of cystic fibrosis, particularly in monitoring the efficacy of

CFTR correcting therapies in individual patients. These results are still preliminary and precise cut off values need to be established along with reference curves and quantitative evaluation of iodide exchange.

The ultimate goal of this study is to develop a test that can accelerate the path toward the definition of targeted therapies for each CF patient in an era that witness an unprecedented development of combination treatments for this still incurable disease.

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