# Towards the pharmacogenomics of cystic fibrosis

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Cystic fibrosis (CF) is the most common lethal recessive genetic disease affecting children in Europe and the US. CF is a multiorgan disease and may present a variety of clinical symptoms, like chronic obstructive lung disease, exocrine pancreatic insufficiency (PI) and elevated sweat chloride concentration. CF mutations have also been found in other related clinical diseases such as congenital bilateral absence of the vas deferens (CBAVD), disseminated bronchiectasis and chronic pancreatitis. These clinical overlaps pose etiopathogenetic, diagnostic and therapeutic questions. Despite stunning advances in genomic technologies and drug discovery, drug therapy often improves disease symptoms but does not cure the disease. One of the main causes of this failure in CF cure may be attributable to genetic variability and to the scarce knowledge of CF biochemistry. Therefore, knowing the genotype of a patient might help improve drug efficacy, reduce toxicity and suggests innovative genomic-based therapy approaches.

**Keywords:** cystic fibrosis, cystic fibrosis transmembrane conductance regulator, CFTR mutations, gene therapy, genotype-phenotype correlation, ion channel therapy, pharmacological treatment, protein-assist therapy

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## Introduction

CF is a multi-system disorder characterized by defective electrolyte transport in epithelial cells and abnormally viscous mucus secretions from glands and mucus epithelia (OMIM #219700). Symptoms are pancreatic insufficiency associated with neonatal meconium ileus (~10% of patients) and chronic obstructive lung disease superimposed with recurrent opportunistic infections that progressively destroy lung tissue. The inflammatory response is a primary cause of irreversible lung damage. Inflammation is present in CF before the appearance of bacterial infections, as demonstrated by increased levels of neutrophils and IL-8 [1]. Other complications include liver disease, chronic sinusitis, infertility in male patients and elevated sweat concentrations. While the pulmonary aspect of CF is serious enough to lead to an average life expectancy of about 30 years, there can be a remarkable variability in the presence or severity of each clinical manifestation [2].

CF is an autosomal recessive disease caused by nearly 1000 different mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, located on 7q35. [4]. These mutations can be grouped into different classes based on their known or predicted molecular mechanisms of dysfunction and functional consequences for the CFTR protein [5]. A genotype-phenotype correlation has been reviewed classifying all CFTR variants and their potential pathogenetic mechanisms. However, since the disease is characterized by a complex and multi-organ involvement, these studies are conducted with respect to specific clinical components of the CF phenotype. The precise knowledge of the functional consequences of CFTR mutations breaks the ice in developing focused therapeutic approaches, even if there are multiple targets to hit in this disease, from the regulation of gene expression to the protein itself.

The purpose of this review is to provide an insight into the molecular mechanisms underlying CF and summarize the literature with respect to current clinical, molecular and pharmacological treatment leading to prevent CF complications.

## CFTR gene and protein function

The CF gene was identified on the long arm of chromosome 7 (7q31.2) and isolated by positional cloning in 1989. It consists of 27 exons spanning ~230 kb of genomic sequence and transcribing 6100 bases mRNA [4-7] (**Figure 1A**). CFTR is the protein product, a 1480 amino acid integral membrane protein of the ATP-binding cassette family [4]. It is composed of two repeated motifs, each with a transmembrane domain (TMD) and a cytoplasmic nucleotide-binding fold (NBF) separated by a hydrophilic regulatory domain (R) (**Figure 1B**). CFTR acts as a chloride channel activated by a cAMP-mediated PKA phosphorylation of the R domain and ATP binding/hydrolysis in the NBFs [8]. Chloride permeation through this pore is a diffusive process that is driven by the electrochemical gradient for this anion. ATP hydrolysis by NBDs probably controls channel gating by providing an

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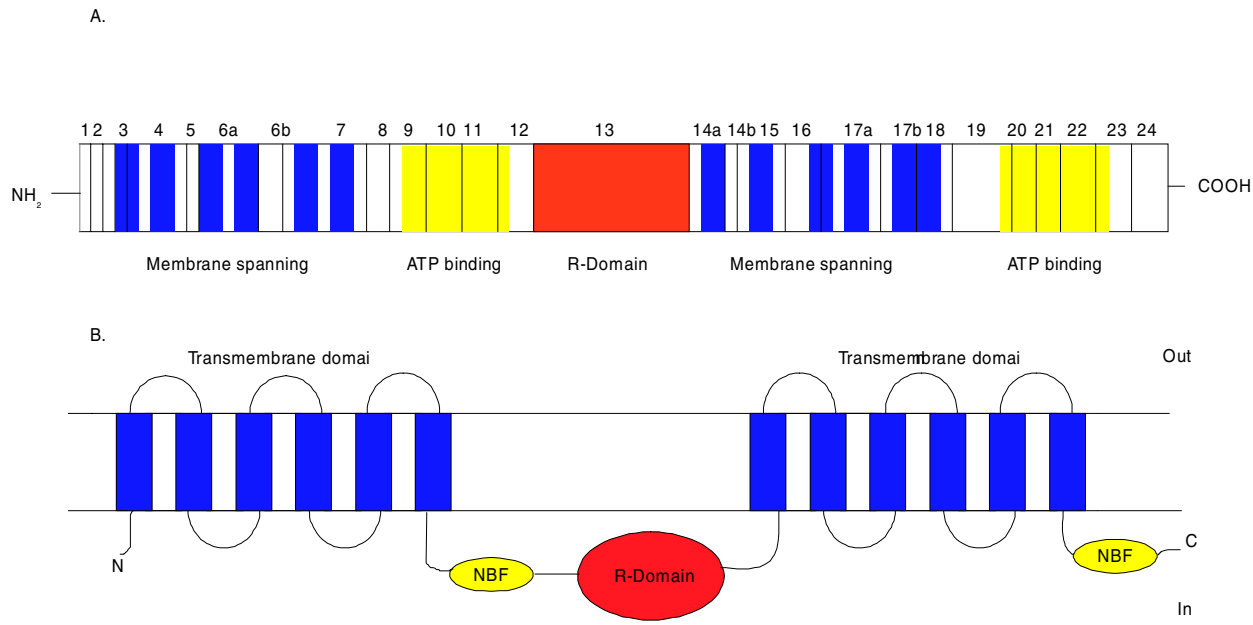
**Figure 1. CFTR gene structure and protein model.**




**A.** CFTR gene structure and its functional domains; **B.** Protein model localized within the epithelial membrane.
CTFR: Cystic fibrosis transmembrane conductance regulator; NBF: Nucleotide-binding fold; R-Domain: Regulatory domain.

energy source for channel transition between closed and open states. CFTR represents an important component in the co-ordination of electrolyte movement across membranes of epithelial cells. Defective electrolyte transport caused by a loss or dysfunction of cAMP-activated chloride channels and thus hyperactivity of sodium channels in epithelial cells are the underlying metabolic derangement in CF patients [9]. Abnormal electrolyte transport across epithelia leads to altered mucus viscosity and recurrent episodes of obstruction, inflammation and progressive destruction of affected organs. A regulatory relationship has been suggested between CFTR and epithelial $Na^+$ channels (ENaC) in human epithelial cells [10], that is activation of CFTR $Cl^-$ channel inhibits ENaC. This could influence the epithelial $Na^+$ conductance in CF airways as a secondary effect caused by the altered inhibition of ENaC due to a mutant CFTR [11].

Recently, Choi *et al.* [12] demonstrated that in addition to disrupting $Cl^-$ transport, CF-associated CFTR mutations also disrupt the transport of another biologically important ion, $HCO_3^-$. The authors provided evidence that CFTR is essential for more than just the transport of Cl- and that $HCO_3^-$ transport is independently affected by different CF mutations. Bicarbonate is the body's major buffer and contributes to physiological properties of great relevance to CF, such as mucous viscoelasticity and pancreatic enzyme activity. Two principal classes of proteins physically interact with the opposing tails of CFTR:

- syntaxin 1A
- proteins containing a PDZ-domain (an amino acid sequence that mediates protein-protein interaction through binding with specific peptides)

Syntaxin 1A is a component of the membrane traffic machinery that binds to the amino-terminal tail of the CFTR. Proteins containing a PDZ-domain bind to the carboxy-terminal tail of this ion channel. The negative modulation of CFTR currents by syntaxin 1A is mediated by a direct physical interaction between this membrane protein [13]. The PDZ-domain mediated interaction could link CFTR to a variety of proteins including signaling molecules, cytoskeletal elements and other transport proteins [14]. This interaction could facilitate cross-talk between the CFTR channels and parallel ion transport pathways. Protein-protein interactions may be considered as targets for pharmacological intervention. Peptides that block syntaxin 1A-CFTR interactions can increase the functional

**The phenotypic criteria for CF diagnosis.**

- Chronic sinopulmonary disease (chronic cough and sputum production)
- Persistent colonization or infection with *Pseudomonas aeruginosa* or *Staphylococcus aureus* bronchiectasis, nasal polyps
- Gastrointestinal and nutritional abnormalities (MI, PI, recurrent pancreatitis and chronic hepatic disease)
- Salt loss syndromes (acute salt depletion)
- Male urogenital abnormalities
- Elevated sweat chloride concentration (> 60 mM)
- Presence of CF-producing mutations in each CFTR gene
- Characteristic abnormalities on nasal PD measurement

activity of the most common CF-associated mutant (ΔF508) in a tissue culture model of epithelial cells [13]. Interaction blockers between the tail and the R domain might be expected to inhibit CFTR channel activity and could serve as novel compounds for treating secretory diarrheas. Compounds that stabilize this interdomain interaction might augment chloride channel activity in cases of CF caused by partial-loss-of-function mutations.

In addition to these principal classes of interacting proteins, CFTR molecules are transiently associated with molecular chaperones (HSP70, calnexin, CHIP), which actively participate in CFTR biogenesis [15,16]. The effect of these interactions is crucial for the biosynthesis and degradation of the protein. Mutations in the cytoplasmic nucleotide binding domains, including the common allele ΔF508, decrease the following:

- efficiency of CFTR folding
- the probability of its dissociation from molecular chaperones
- maturation through the secretory pathway to the plasma membrane

### Clinical phenotype and diagnostic criteria

In addition to phenotypic criteria, a CF history in the family and/or a positive test for hypertrypsinogenemia in neonatal period, may be useful in delineating a CF diagnosis. The only manifestation of CF in the upper respiratory tract consists of a thickening of the mucosal lining of the sinuses (> 90%) and nasal polyps (10–30%), that can be removed surgically, even if their recurrence rate is high. Morbidity and mortality in CF is caused by recurrent and chronic infections of the lower respiratory tract. Different theories are being illustrated for explaining this link. One is that low fluid volume interferes with mucociliary clearance, or that increased salt concentrations in the periciliary fluid layer impair antimicrobial peptide function. Moreover CF shows a pronounced propensity for inhaled pathogens to colonize and subsequently infect the bronchi leading to recurrent and chronic bronchopulmonary infections. *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* [17] and *Streptococcus aureus* are the most recurrent pathogens.

Bacterial invasion stimulates a vigorous and excessive primarily neutrophil-driven inflammatory response throughout the lungs. Inflammation products not only damage incoming bacteria but also the host tissue itself. In fact inflammation precedes bacterial colonization. Almost all patients have chronic sinopulmonary disease and, in postpubertal men, obstructive azoospermia. Approximately 85–90% of all patients have exocrine PI. Early CF diagnosis is important to provide appropriate therapeutic interventions, prognostic and genetic counseling and to ensure access to specialized medical services. In the majority of cases the diagnosis of CF is confirmed by demonstrating an elevated (> 60 nmol/l) sweat chloride concentration. Around 10% of CF is diagnosed at birth because of MI but the majority is diagnosed in early infancy because of recurrent lower respiratory tract infections or malnutrition or both.

Molecular diagnosis is based on CFTR mutation screening that presents a rate of false-negative results dependent on geographic areas and relative frequency of mutation screened. In the absence of two clearly identifiable mutations a sweat test is mandatory (a chloride concentration of > 60mmol/l and a sodium concentration of > 70 mmol/l are found in ~98% of CF patients), even for an atypical CF phenotype (1–2% of patients) consisting of chronic sinopulmonary disease, pancreatic sufficiency and 'borderline' values of sweat chloride concentrations. This phenomenon could be partially explained by the so-called 'mild mutations' which, when present on one allele, may be associated with less increased or borderline values of sweat electrolytes. Early diagnosis is important since early treatment is associated with improved prognosis.

The most widely used test in neonatal screening is the detection of increased blood levels of immunoreactive trypsin (IRT) combined with mutational analysis. Abnormally high levels of IRT represent the basis for a molecular screening test, although an acceptable rate of repeat testing and false-positive and false-negative results. The vast majority of patients with CF have an abnormal pancreatic acinar and ductular function [18].

A number of direct and indirect tests are available to evaluate exocrine pancreatic function; the most widely used and informative is faecal fat analysis with minimum 72 h of pooled stool collection. Direct tests are highly specific and capable of evaluating the entire range of pancreatic function but their invasive nature precludes their use in routine clinical practice.

The presence of *P. aeruginosa,* if persistent, in the respiratory tract is highly suggestive of CF [19] and can be diagnostically helpful in the evaluation of patients with atypical features of CF. One of the most consistent features of the CF phenotype in postpubertal male subjects is obstructive azoospermia, a finding present in 98% of affected individuals [20]. In the majority of patients with CF, azoospermia occurs as a result of absent or rudimentary *vas deferentia*. Usually CBAVD patients have no evidence of respiratory tract or pancreatic abnormalities and may have normal or elevated sweat concentrations. The prognosis for these patients is excellent but it is recommended that they be closely monitored for the development of other CF-related complications [21].

Lung disease is the primary cause of death in CF but pulmonary manifestations show a high degree of intrafamiliar and intraphenotypic variability. Pancreatic disease ranges from a complete loss of exocrine and endocrine functions in some CF patients to a partial function in others and only to pancreatitis in others.

CF phenotype is highly variable among unrelated individuals and within families. The composition, frequency and type of CFTR mutations/variants parallel the spectrum of CFTR-associated phenotypes, from classic CF to mild monosymptomatic presentations, like idiopathic pancreatitis [22], chronic rhinosinusitis [23], nasal polyposis [24], asthma [25] or disseminated bronchiectasis and sarcoidosis [26]. In all these pathologies the presence of CF mutations among patients is statistically higher than in normal controls, indicating that CFTR variants may be associated with the disease development in the general population. Molecular analysis revealed the presence of two CF mutations:

- one that is observed in CF patients
- a second that is a variant not associated with CF

At the biochemical level the second mutation permits CFTR to function sufficiently to escape the CF phenotype but some epithelial tissue dysfunction does occur.

### CFTR mutation classification and genotype-phenotype correlation

Alterations in the CFTR gene designated as CF-causing mutations should fulfil at least one of the following criteria:

- The mutation has to cause a change in the amino acid sequence that severely affects CFTR synthesis and/or function.
- Introduce a premature termination signal (insertion, deletion, nonsense mutations).
- Alter the 'invariant' nucleotides of intron splice sites.
- Cause a novel amino acid sequence that does not occur in the normal CFTR gene from at least 100 carriers of CF mutations from the patient's ethnic group [27].

All types of mutations in CFTR are represented (missense (50%), frameshift (22%), nonsense, splice, small and large in-frame deletions or insertions) and distributed throughout the entire gene. These mutations affect CFTR through a variety of molecular mechanisms, that produce little or no functional CFTR. Genotypic variation provides a rationale for phenotypic effects of specific mutations.

Various mutations can be grouped into six different classes based on their known or predicted molecular mechanisms of dysfunction and functional consequences for the CFTR protein [28] (**Table 1**).

With regards to pancreatic phenotype, two categories of mutant alleles can be described: severe and mild [29]. There is a relationship between specific CFTR alleles and exocrine pancreatic function. A severe allele confers pancreatic insufficiency only if paired with another severe allele, whereas a mild allele sustains pancreatic function in a dominant fashion, even if the second mutation is severe. All mild mutations are associated with residual chloride channel activity at the epithelial apical membranes to compensate for a lack of an active CFTR corresponding to a severe allele. This is not true for the respiratory system where genotype-phenotype correlations are poor with a few exceptions. Severity cannot be predicted on the basis of genotype, even if the progression of pulmonary disease is less severe in patients with mild CFTR mutations in comparison with patients carrying severe genotypes. It is anticipated that new treatments will become available within the next few years, which will give maximal benefit to young infants if instituted before lung damage is evident. In this context, presymptomatic diagnosis

**Table 1. Protein-assisted and chloride channel therapies in CF.**

| Type | Genotype | Phenotype | Defect | Drugs that may improve phenotype |
|---|---|---|---|---|
| ***Class I*** Nonsense, frameshift, splice, large in-frame deletion or insertion mutations. | G542X<br>621 + 1 G→T<br>3905insT<br>W1282X<br>R553X<br>1717 - 1 G→A | PI | Lack of CFTR biosynthesis or defective biosynthesis producing abnormal protein variants.<br>No functional CFTR is present at the apical membrane of epithelial cells.<br>No cell surface chloride transport.<br>Phenotypic consequences tend to be severe. | Gentamicin<br>Neomicin (G418) |
| ***Class II*** Mutations that fail to be properly processed to a mature glycosylated form and transported to the apical membrane | ΔF508<br>N1303K<br>P574H<br>A455E | PI | Defective CFTR processing and trafficking. No cell surface chloride transport | Chemical chaperones<br>CPX<br>Phenylbutyrate<br>Deoxyspergualin |
| ***Class III*** Mutations preventing mechanisms required for the channel activation (ATP binding and hydrolysis at the nucleotide binding domains). | G551D<br>G551S | PI | Defective chloride channel Regulation<br>Reduced or absent cell surface chloride transport | Genistein<br>Pyrophosphate<br>UTP<br>INS36217<br>Moli1901 |
| ***Class IV*** Mutations located within membrane spanning domain, implicated in forming the pore of the channel. | R117H<br>R334W<br>G314E<br>R347P<br>ΔF508<br>P574H | PS | Reduced chloride conductance<br>Reduced levels of cell surface chloride transport | Genistein<br>Milrinone<br>Phenylbutyrate<br>UTP<br>INS36217<br>Moli1901 |
| ***Class V*** Mutations causing defects in CFTR channel expression levels. | 3849 + 10 kb C→T<br>2789 + 5 G→A<br>3272-26 A→G<br>A455E<br>3120 +1 G→A<br>1811 + 1.6 kb A→G<br>5T | PS | Normal CFTR channnels<br>Reduced numbers of normal CFTR<br>Reduced cell surface chloride transport | Genistein<br>Milrinone<br>Phenylbutyrate |
| ***Class VI*** Nonsense or frameshift mutations causing a 70–100 bp truncation of the C-terminus of the CFTR mutations that impair regulation of other types of ion channels. | Q1412X, 4326delTC, 4279insA | Severe | Functional but unstable CFTR at the apical membrane | See text |

*Modified from [56,102]*

*CFTR: Cystic fibrosis transmembrane conductance regulator; UTP: Uridine triphosphate.*

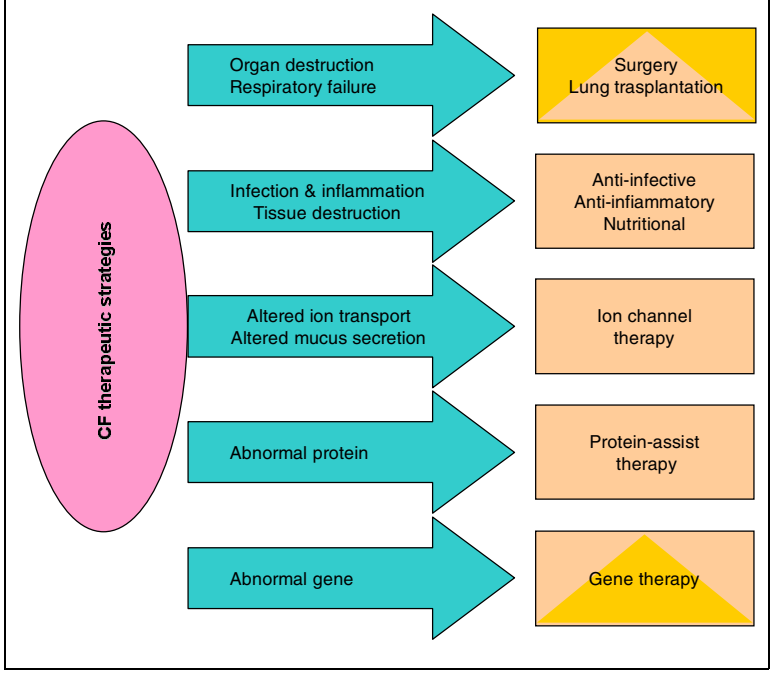
based on DNA analysis greatly improves the doctor-parent relationship. It is not clear how CFTR alleles contribute to pulmonary disease, or whether the carrier status acts as a predisposing factor in conjunction with other genetic and environmental factors, which determine the clinical outcome of CF. The poor correlation between CFTR genotype and the severity of

lung disease strongly suggests an influence of environmental and secondary genetic factors (CF modifiers) [30].

### CF pharmacological treatment

In the past, CF was an exclusively paediatric disease since only a few patients survived childhood. At present, a growing number of patients reach adulthood, representing around 50% of all CF patients up to now. This is due to a correct diagnosis of a mild CF form and an effective therapeutic approach directed towards disease complications, like chronic bronchopulmonary infections and malnutrition. The complexity of the disease requires extensive knowledge and experience in a wide range of medical issues (**Figure 2**). Nearly all CF patients need pancreatic enzyme substitution with meals, using microsphere preparations with acid-resistant outer surfaces that release enzymes in the duodenum. Steatorrhoea could be controlled by an adjuvant therapy with cimetidine, ranitidine or omeprazole. There is a universal agreement that a high-fat, high-calorie food intake is essential in patients with CF. A calorie intake of 150% of the normal recommendations or even higher may be necessary to secure normal growth and nutrition. Aggressive antimicrobial treatment leads to near normal growth and nutrition.

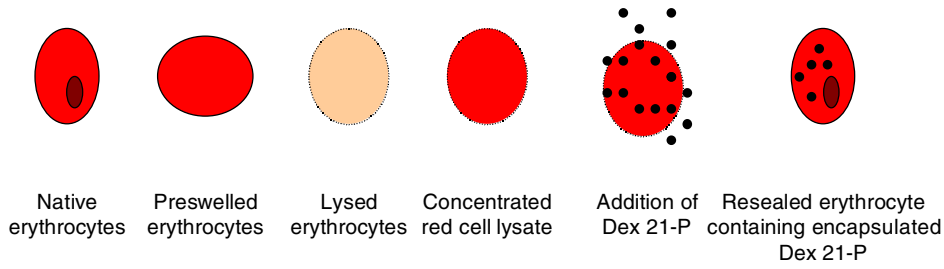
**Figure 2. Diagram of current CF therapeutic strategies.**




Insulin-dependent diabetes becomes prevalent with increasing age and beyond the age of 15 years, preceded by several years of a gradual decline in weight and lung function. Liver enzymes are elevated in 10–20% of patients but symptomatic liver disease is uncommon. The underlying pathology that leads to liver and bile duct complications is thought to be a reduced flow of altered bile fluid, which reflects that a defective CFTR function in the epithelium of the bile duct system interferes with development of the sperm duct *in utero*. Oral intake of supplemental ursodeoxycholic acid can lead to biochemical improvement.

The development of new treatments, including pharmacological agents which are expected to have a major impact on pulmonary hyperactivity, may be important for the life expectancy of patients and alleviate the problems of gene therapy. A new procedure has been to developed to deliver corticosteroids to interfere with some pathogenetic steps involved in pulmonary inflammation and alleviate the evolution of lung injury. This treatment is based on encapsulation of dexamethasone 21-phosphate (Dex 21-P) in autologous erythrocytes that, once removed from circulation, selectively deliver the encapsulated drug to macrophage cells (**Figure 3**) [31,32]. The major advance in the use of erythrocytes as drug-delivery systems is that they deliver drugs only to target cells [31]. Macrophages, play a central role in the inflammatory process, since once activated (i.e., by infectious agents) they produce inflammatory cytokines, IL-1, IL-6 and TNF-α (also responsible for the systemic and local syndrome following administration of adeno-CFTR during gene therapy) which in turn induce cyclo-oxygenase-2 (COX-2) associated with the production and release of prostaglandines from macrophages. Nine CF patients were treated by this approach without *P. aeruginosa* and *Aspergillus fumigatus*, by performing three infusions of erythrocytes loaded with Dex 21-P (mean 5.77 mg to each patient every 3 weeks). Preliminary data showed constant blood levels of Dex 21-P up to 10 days without toxic side effects [32]. Long-term administration of steroids to all patients to modulate or prevent airway inflammation remains controversial [33,34]. Non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen have also been used in CF because of their ability to inhibit neutrophil migration. Widespread use of ibuprofen for CF has been limited by a lack of long-term safety data [35].

**Figure 3. Procedure used for the encapsulation of Dex 21-P in human erythrocytes.**




Dex 21-P: Dexamethasone 21-phosphate.

### CF pharmacogenomics-based treatment

Searching for active compounds to restore transport function in CF is a priority for creating drugs for CF patients. New pharmacological treatments towards a specific class of mutations is being developed (**Table 1**) [36]. In general three sources of molecules are currently tested:

- synthesized compounds
- chemical libraries
- natural products

First generation compounds were addressed to repair stop codon mutations (class I). This suppression activity is mediated by certain aminoglycosides [37] that bind to a specific site in ribosomal RNA and disturb codon-anticodon recognition at the aminoacyl-tRNA acceptor site. In cultured cells treated with aminoglycosides (neomicin and gentamicin) full-length CFTR synthesis is restored to up to 10–20% of normal levels [38,39]. This treatment also causes extensive misreading of the RNA code, which enables the insertion of alternative amino acids at the site of the mutated codon. Phase I *in vivo* trials of iv. and inhaled gentamicin in CF patients with one or two class I stop mutations have been completed for determining tolerability and safety of this procedure [40-42]. Using immunocytochemical and functional [6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ)-based] techniques, *ex vivo* exposure of airway cells from stop mutation CF patients led to the identification of surface localized CFTR in a dose-dependent fashion. In a following study, five patients with CF with stop mutations and five CF control subjects were treated with parenteral gentamicin for 1 week and underwent repeated *in vivo* measures of CFTR function (nasal potential difference (PD) measurements and sweat chloride $Cl^-$ testing). Thus gentamicin treatment can suppress premature stop mutations in airway cells from patients with CF and produce small increases in CFTR $Cl^-$ conductance (measured by the nasal PD) *in vivo* [42]. Restoration of CFTR function is determined by measuring potential difference in nasal epithelia. G542X, 621+1 G→T, W1282X and R553X belong to the class I group of mutations. Respiratory epithelia regulate an active ion transport (sodium and chloride) that generates a transepithelial electrical PD. Nasal PD can be measured *in vivo* [43], in patients as young as a few hours or older children [43,44]. Three features distinguish CF patients from healthy people in this analysis:

- Higher basal PD (which reflects enhanced $Na^+$ transport across a relatively $Cl^-$ impermeable barrier).
- Greater inhibition of PD after nasal perfusion with the $Na^+$ channel inhibitor (amiloride).
- Little or no change in PD in response to perfusion of the nasal epithelial surface with a Cl-free solution (which reflects an absence of CFTR-mediated $Cl^-$ secretion) [45].

The most intense effort for CF is focused on class II mutations, which involve protein trafficking and folding, and ΔF508 is contained in this group. Overexpression of ΔF508 cDNA in mammalian cells leads to the appearance of functional CFTR $Cl^-$ channels in the plasma membrane, suggesting that the ΔF508 mutation is 'leaky' [46]. ΔF508 creates a misfolded protein, which is not properly glycosylated but allows chloride ions to permeate the channel [47,48]. Many CF patients have at least one copy of ΔF508, the observation on residual function in ΔF508 CFTR raises the possibility of developing a therapeutic strategy. The wild type protein proceeds from the endoplasmic reticulum (ER) through the Golgi apparatus where it acquires

terminal glycosylation and from the Golgi to the cell surface by a mechanism regulated by interactions of cellular proteins with the C terminus [24]. ΔF508 CFTR cannot be folded properly in the ER, is subsequentley destroyed and hence does not reach the apical membrane.

The ΔF508 maturation can be increased by treating cells with 'chemical chaperones' like glycerol, although its clinical utility is limited by the high concentration required to produce clinical benefit [15]. The possibility that small and cell-permeant chemical chaperones stabilize unstable CFTR folding intermediates is worthy of consideration towards innovative pharmacological treatment of the disease. A class of butyric acid-derived compounds has shown promise for the ΔF508 mutation. *In vitro* investigations have demonstrated an increased production of mature CFTR and chloride transport at the cell surface for both wild type, ΔF508 and G551D, by a mechanism that involves upregulation at the transcriptional level and modulation of protein folding steps [47]. One pilot clinical trial in CF using phenylbutirate over a 1 week period showed a partial restoration of chloride transport in nasal epithelia [50,51]. Flavonoid compounds like genistein activate ΔF508 CFTR chloride channel and stimulate the wild type CFTR channel through direct binding to CFTR, causing an increase in the channel open time for chloride ions. These compounds are nevertheless ineffective at trafficking the ΔF508 protein to the cell surface. Therefore, nasal PD assays on ΔF508 homozygous patients are not considered an adequate functional test. Another class of compounds that is under scrutiny are represented by α1-adenosine receptor antagonists (8-cyclopentyl-1,2-dipropylxanthine) [52] that specifically bind to regions structurally resembling α-adenosine receptors [53]. This interaction stimulates ΔF508 CFTR movement to the cell surface and induces cAMP-mediated chloride currents.

Inhibition of phosphodiesterase could increase CFTR-mediated chloride transport in wild type cells. In fact, compounds such as milrinone can restore ΔF508-mediated chloride channel but are not able to impair cellular trafficking in the nasal mucous. For this purpose they are useful for those mutations that already reach the cell surface. Genistein has also been shown to restore G551D function (class III mutation) [54]. In this case, mutated CFTR normally trafficks to the cell membrane but results in lower levels at the cell surface and with a reduced chloride conductance compared to the normal protein. ATP binding and phosphorylation to G551D are severely affected but can be overcome by interaction with flavonoids. The use of genistein in combination with cAMP-regulating molecules such as forskolin or PKA can restore the channel open time. Class IV and V mutations represent defects in CFTR channel conduction and in CFTR expression levels, respectively. Augmentation of CFTR function may restore a sufficient level to ameliorate the disease. The somministration of adenosine and its nucleotides can activate wild type and R117H forms of CFTR in cell cultures binding to the A2B receptor, present in human bronchial epithelium [55]. Genistein can overcome this block in regulation. Mutations that partially reduce chloride conductance through CFTR (class IV) can be stimulated with milrinone, which is a phosphodiesterase inhibitor.

Defective ion transport in CF airways results in the impaired clearance of bacteria and inhaled particles in mucus lining the respiratory tract. In the airway the common cellular chemical uridine triphosphate (UTP) acts briefly on a protein receptor on the surface of respiratory tract cells to stimulate chloride and water transport and improve mucociliary clearance, which could provide significant benefits to individuals with CF.

Clinical trials are underway using purinergic compounds such as the P2Y2 receptor agonist INS365 (a UTP analog). Activation of P2Y2 receptors has been found to both activate $Cl^-$ secretion and inhibit $Na^+$ absorption. The ultimate goal is to recover $Cl^-$ channel activity of mutant CFTR by enhancing either synthesis and expression of the protein or by activating silent CFTR $Cl^-$ channels. Strategies combining these drugs with compounds facilitating $Cl^-$ secretion and inhibiting $Na^+$ absorption *in vivo* may have the best chance to counteract the ion transport defect in CF [56]. INS365 may offer chemical stability advantages over UTP, in fact aerosolized INS365 is safe when delivered at single doses of up to 40 mg in adults and children with CF but higher doses are unlikely to be tolerated [57]. Finally, mutations that lead to a severe reduction in the normal CFTR protein (class V) could be 'corrected' by phenylbutyrates and/or supplemented with gene therapy.

Class VI is a new category that includes mutations that impair regulation of other types of ion channels [57] or mutations that cause the C-terminal truncations leading to its accelerated degradation.

## Gene therapy

The discovery of the CFTR gene has enabled correction of the CF defect in cultured cells [59-63]. Different vectors (viral or not) are utilised for gene therapy protocols [64]. Adenovirus (AdV) was the first vector studied in the context of lung gene therapy: this method was very effective at producing high titers of CFTR, also if AdV usually lacks its integration into the host chromosome. Adeno-associated virus (AAV) vectors can integrate themselves randomly or remain episomal. AAV does not produce a significant cellular inflammatory response but presents difficulties in insertion of genes as big as CFTR.

Clinical trials performed throughout the 1990s provided evidence that delivery of DNA by either viral or non-viral means was safe, though not clinically efficacious [65] using many techniques and different vectors, like retrovirus, adenovirus and cationic liposome [59,65]. Despite these problems, > 20 trials using AdV, AAV and cationic liposomes have been completed [61,62]. These studies essentially have all been Phase I (for evaluating safety and dosing). Researchers have delivered both viral and non-viral vectors topically to the nose and/or lower airways *via* direct liquid instillation or *via* aerosol. To summarize the results: AdV has dose-specific and vector-specific toxicity in the form of lung inflammation.

At high vector concentrations some individuals have demonstrated transient pulmonary infiltrates. Most subjects also exhibited increased titers of neutralizing antibodies. They elicit an immune response limiting their repeated application. Trials with more highly engineered AdV vectors have not been reported. Recombinant AdVs have the advantage of high transduction efficiency and are potentially excellent vectors. On the other hand, aerosol delivery of lipid-DNA complex was studied in several trials: there appears to be less toxicity, thus allowing repeated administration and delivery of large quantities of DNA.

A cytokine-mediated response has been reported (E Alton, personal communication). Despite the promise of preclinical studies that established the optimal lipid/plasmid formulation in cell culture they have confirmed the function of a recombinant human CFTR protein after transfection and assessed the safety and efficacy of single dosing to lungs of rhesus monkeys. The investigator was unable to detect recombinant CFTR mRNA in nasal epithelial biopsies or to detect CFTR-mediated chloride transport by nasal PD measurements. Molecular results are encouraging but electrophysiological demonstration of the correction of the functional defect has generically been absent or very modest.

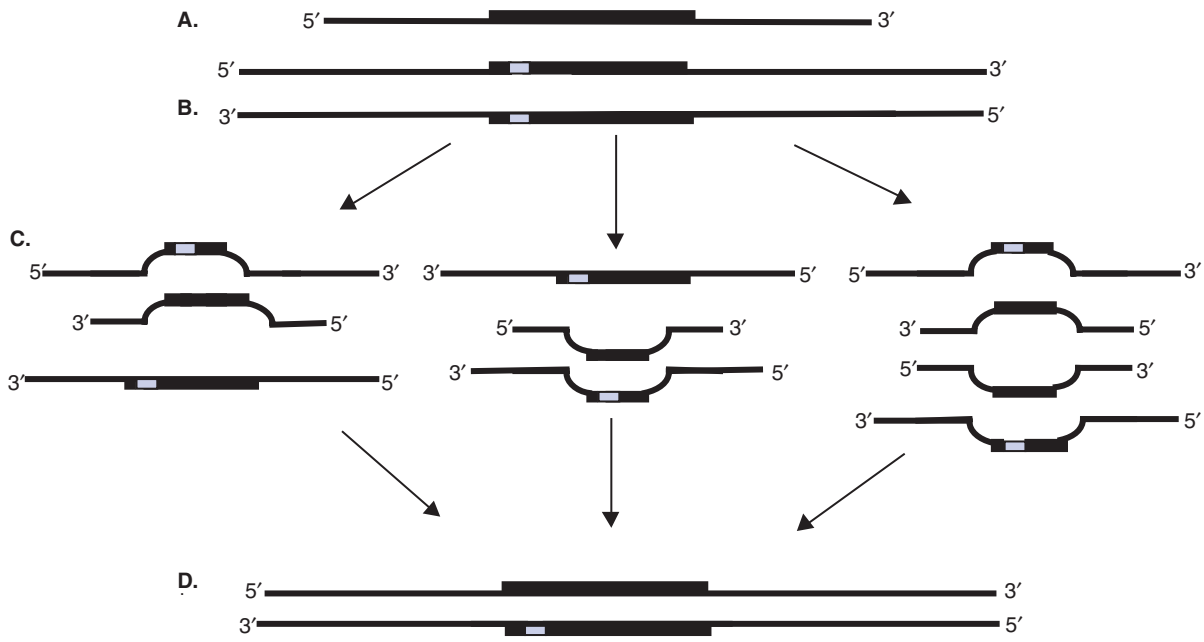
The limited success of the first-generation of applied gene therapy has stimulated researchers in developing alternative approaches for the treatment of this disease. The more promising techniques are based on gene-targeting [66]. This strategy is to insert a molecular mechanism into cells that target abnormal DNA sequences in the chromosome and correct the mistake. Different methodologies have been used to reach this objective: RNA-DNA chimeras [67] ribozymes [68] and small fragment homologous replacement (SFHR) [69-71].

The aim is to target the repair construct to the regions flanking errors in the native gene. RNA/DNA chimera then takes advantage of endogenous DNA repair enzymes to fix the resulting mismatch. Rybozymes effect similar repair to the aberrant DNA sequence. The SFHR approach uses small fragments of genomic DNA homologous to the wild type nucleotide sequence for *in vitro* transfecting of cells from mutated individuals (**Figure 4**) [69]. To date, SFHR has been used to correct the ΔF508 mutation *in vitro* in transformed human epithelial cells from CF patients [69,73] and recently to modify specific genomic sequences in exon 10 of the mouse CFTR *in vivo* [72]. The results of these studies showed that SFHR could be used as a gene therapy to introduce specific modifications into cells of clinically affected organs and cells expressing the new sequence. SFHR seems to be a promising technique towards a viable gene therapy for CF airways.

## Outlook

The clinical manifestations of CF include respiratory, reproductive and digestive system diseases. Although these clinical manifestations are determined by specific CFTR mutations, individual phenotypic variations exist between CF patients. This suggests that some phenotypic features are caused by the CFTR genotype (e.g., pancreatic status), others, such as pulmonary disease, are strongly influenced by both secondary genetic factors and the environment. Less common manifestations, such as MI, probably require synergistic involvement of distinct genes (e.g., CFTR and CFM1) for phenotypic expression (Lap Chee Tsui, personal communication). These considerations illustrate the genetic and clinical complexity of this disorder and suggest

**Figure 4. The SFHR technique.**




**A**. "Therapeutic" small DNA fragment. **B.** Genomic DNA containing the ΔF508 mutation in homozygotes. **C.** Configurations of homologous pairing during SFHR. **D**. Genomic DNA corrected by SFHR (heterozygous correction).
SFHR: Small fragment homologous replacement.

that a single pharmacological treatment will be unlikely to be used as a remedy for CF. There are multiple targets in CF from the regulation of gene expression to the protein itself. The rapid progress of the post-genomic project, will provide advances in high-throughput technologies such as combinatorial chemistry and high-density cDNA arrays will identify new targets and additional CF modifiers. We are confident that within the next 5 years many CF patients will benefit by a combination of pharmacogenomics and *gene pill* drugs.

## Highlights

- CF clinical heterogeneit poses etiopathogenetic, diagnostic and therapeutic questions.
- Early diagnosis of CF could greatly improve the life expectancy of CF patients.
- Genetic variability and the scarce knowledge of CF biochemistry up to now represent the main causes of failure in CF cure.
- Drug therapy has to be selected on the basis of different genotype, but genotype-phenotype correlation is not always easy to perform.
- Gene therapy combined with a pharmacogenomic approach is a promising approach for the cure of the disease.

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