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New pharmacological approaches for cystic fibrosis: Promises, progress, pitfalls

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ABSTRACT

With the discovery of the *CFTR* gene in 1989, the search for therapies to improve the basic defects of cystic fibrosis (CF) commenced. Pharmacological manipulation provides the opportunity to enhance CF transmembrane conductance regulator (CFTR) protein synthesis and/or function. CFTR modulators include potentiators to improve channel gating (class III mutations), correctors to improve abnormal CFTR protein folding and trafficking (class II mutations) and stop codon mutation read-through drugs relevant for patients with premature stop codons (most class I mutations). After several successful clinical trials the potentiator, ivacaftor, is now licenced for use in adults and children (>six years), with CF bearing the class III G551D mutation and FDA licence was recently expanded to include 8 additional class III mutations. Alternative approaches for class I and class II mutations are currently being studied. Combination drug treatment with correctors and potentiators appears to be required to restore CFTR function of F508del, the most common *CFTR* mutation. Alternative therapies such as gene therapy and pharmacological modulation of other ion channels may be advantageous because they are mutation-class independent, however progress is less well advanced. Clinical trials for CFTR modulators have been enthusiastically embraced by patients with CF and health care providers. Whilst novel trial end-points are being evaluated allowing CFTR modulators to be efficiently tested, many challenges related to the complexity of CFTR and the biology of the epithelium still need to be overcome.

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Abbreviations: Amil, Amiloride; AMP, Accelerating Medicine Partnership; ANO, anoctamins; AONs, antisense oligonucleotides; ASL, airway surface liquid; ATP, adenosine triphosphate; BMD, Becker muscular dystrophy; CaCC, Ca^{2+}-activated Cl^- channel; Cas, CRISPR-associated systems; CBAVD, congenital bilateral absence of vas deferens; cDNA, complementary DNA; CF, cystic fibrosis; Cl^-, chloride; CFRD, CF-related diabetes; CFTR, cystic fibrosis transmembrane conductance regulator; CRISPR, clustered regularly interspaced short palindromic repeats; DAG, diacylglycerol; DGK, diacylglycerol kinase; DMD, Duchenne muscular dystrophy; ECFSPR, European Cystic Fibrosis Patient Registry; ENaC, epithelial sodium channel; EU, European Union; ER, endoplasmic reticulum; FRT, fisher rat thyroid; GWAS, genome-wide association studies; HBE, human bronchial epithelial; hESCs, human embryonic pluripotent stem cells; HGF, hepatocyte growth factor; HRQOL, health-related quality of life; iPS, induced pluripotent cells; IP_3, D-myo-inositol trisphosphate; IQR, interquartile range; KvLQT1, cAMP-regulated K^+ channels; LCI, lung clearance index; ORCC, outwardly rectifying Cl^- channel; mdx mice, X chromosome-linked muscular dystrophy mice; *P. aeruginosa*, *Pseudomonas aeruginosa*; PDE, phosphodiesterase; PS, pancreatic sufficiency; PIP_2, phosphatidylinositol bisphosphate; PKC, protein kinase C; $P2Y_2$, purinergic receptors; UTP, uridine triphosphate; siRNA, small interfering RNA; SFHR, small fragment homologous replacement; T, thymidine; TALEN, transcription activator-like effector nuclease; WES, whole exome sequencing; WT, wild type.

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1. Cystic fibrosis — the disease in 2014

Cystic fibrosis (CF) is the most common life shortening condition in Caucasians and affects approximately 70,000 people around the globe including ~30,000 in North America and more than 30,000 in Europe (Sosnay et al., 2013). CF is an autosomal recessive disease which is caused by a mutation in each of the 2 *CFTR* genes.

So far, almost 2000 different mutations have been reported to the original *CFTR* mutation repository (www.CFTR.2.org, 214). F508del is by far the most common mutation. It is found in about 70% of CF chromosomes worldwide and is present in ~85% of patients on at least one allele. Whilst prevalence is broadly similar in populations which had their origin from northern Europe, there are considerable variations through Europe from as high as 1 in 1400 live births in Ireland, 1 in 4200 in Italy and 1 in 25,000 in Finland (Anonymous, 2002; O'Sullivan & Freedman, 2009). Prevalence rates are much lower in non-Caucasian populations (e.g. 1 in 4000–10,000 in Latin Americans, 1 in 15,000–30,000 in Africans and ~1 in >100,000 in people of Asian origin) (Anonymous, 2002; O'Sullivan & Freedman, 2009).

Cystic fibrosis is a multisystem disease affecting organs and tissues where CFTR is expressed. The common clinical manifestations are related to impact of the defect gene on the airways (upper and lower respiratory tract), gastrointestinal tract including the biliary system and the reproductive tract (Table 1). About 85% of patients with CF have pancreatic insufficiency associated with nutrient malabsorption and often under-nutrition. Decreased reabsorption of chloride ions via the CFTR channel in the sweat duct can lead to salt loss syndromes. Increased concentration of chloride ions in sweat (>60 mmol/L) remains the best diagnostic test for CF. Therapies are complex and involve pancreatic enzyme supplementation, fat soluble vitamins, mucolytic (e.g. dornase-alpha) and hydrator therapies (e.g. hypertonic saline, mannitol), airway clearance, and frequent and often repeated courses of antibiotics. The vast majority of morbidity and mortality results from pulmonary disease associated with chronic bronchial infection and bronchiectasis. Lung disease remains a progressive condition and the burden of therapy is very significant for the patient, his family and the health care system.

When initially described in the 1930s, CF was universally fatal in infancy or early childhood. Until recently the majority of people with CF were children, though in the past decade in many parts of the world there are now more adults than children (Anonymous, 2012a, 2012b, 2013a, 2013b, 2013c, 2013d). In some countries, including Canada, Italy and Denmark numbers of adults approach or even exceed 60% of the total CF populations (Anonymous, 2012a, 2012b). The median survival from CF has dramatically increased. It approaches 40 years and is predicted to extend to 50 years for children born in the current era (Elborn et al., 1991; Dodge et al., 2007). Importantly, improvements in outcomes have not been universal. Between 2003 and 2007, in non-European Union (EU) countries, the median age of the CF population was 12.1 years (IQR 6.0 to 19.2 years) and only 28% was 18 or more years of age, which is significantly lower than for those living in EU-countries where the median age was 17.0 years (IQR 9.5 to 19.2 years) and 47% was 18 or more years of age (McCormick et al., 2010).

Improvement in survival is thought to be multifactorial and related to earlier diagnosis, better detection of milder disease phenotypes, improved nutritional status, more aggressive treatment of pulmonary infection, more effective approaches to airway clearance and the management of patients within CF specialist centres supported by multi-disciplinary health care professionals (Mahadeva et al., 1998; Tiddens, 2009; Colombo & Littlewood, 2011). Newborn screening programmes have been established for more than 30 years in some parts of the world, though in recent years have been adopted in many more health care settings (Dijk & Fitzgerald, 2012). Recognition of bronchial infection and its aggressive treatment with antibiotics may prevent and or delay the development of chronic infection particularly of the most common bacterial infection in CF, *Pseudomonas aeruginosa* (*P. aeruginosa*) (Ratjen et al., 2010; Taccetti et al., 2012; Mayer-Hamblett et al., 2013). The rates of chronic *P. aeruginosa* infection in children with CF have dramatically fallen over the past 20 years (Lee et al., 2004).

In parallel with the growth of adult numbers, the health of young adults has improved. This is supported by data from the US CF Patient Registry which demonstrates that the median FEV_1% predicted of 18 year olds was 84% predicted in 2012, which represented an increase by 20% over the last 20 years (Anonymous, 2013a). Similarly, nutritional status has increased in older patients. Improved health at transition to adult care, with more effective and broader-based therapies throughout adult life has led to a growing number of older adults (>40 years) (Hodson et al., 2008; Simmonds et al., 2009; Plant et al., 2013). Longer life span and quality of life for young adults with CF have resulted in greater prospects of employment or study, having long-term relationships and considering having their own children (Anonymous, 2012a, 2012b, 2013a, 2013b, 2013c). Furthermore, survival with advanced lung disease is much greater as evidenced at the Royal Brompton Adult CF Centre in London, where median survival in adults with FEV_1 < 30% predicted improved from 13 months in the early 1980s to 5.3 years 20 years later (George et al., 2010). However, the improved survival is at the cost of an ever increasing treatment burden for the patients.

Numerous complications previously either unrecognized or rare resulting from CF and its treatment are now common in the CF adult clinic, adding significantly to the complexity of the care of the adult with CF. CF-related diabetes (CFRD), multi-resistant infections of the lung, metabolic bone disease leading to reduced bone density which may in part be contributed to by therapies and toxicity resulting from therapies such as aminoglycosides and drug allergies, gastrointestinal malignancy and the psychosocial consequences (such as depression and anxiety) are prevalent in adults with CF (Quon & Aitken, 2012; Plant et al., 2013; Bell & Reid, 2014). Lung transplantation is an option for those with progressive respiratory failure and adequate management of the above complications is increasingly important for patients who are likely to require assessment for transplantation in the future (Meachery et al., 2008; Lobo et al., 2013; Plant et al., 2013; Hollander et al., 2014). Many of these complications require additional therapy adding further to the treatment burden for the patient.

The past three decades have seen exciting developments in the outcomes for people with CF, though pre-mature death before 50 years remains the norm. Therefore, to see major additional improvements in survival and quality of life of the person with CF, it is vital that new therapies and particularly those which prevent early lung disease in children, those which alter the natural history of progressive airway disease of CF and those which change the basic defect in the epithelium of CF tissues are developed and trialled. The potential for CFTR specific therapies has been much discussed and studied by the CF scientific

Table 1
Clinical features of cystic fibrosis.

Sinopulmonary	Gastrointestinal/hepatobiliary	Reproductive and endocrine	Salt loss syndromes	Other
Chronic bronchial infection leading to bronchiectasis	Pancreatic exocrine insufficiency	Obstructive azoospermia in males	Acute dehydration due to heat prostration	Difficult vascular access
Chronic infection with multi-resistant pathogens	Recurrent acute pancreatitis in those with pancreatic sufficiency	Reduced fertility in women	Hyponatraemic, hypochloraemic metabolic alkalosis	Hypersensitivity reaction to antibiotics
Haemoptysis	Fat soluble vitamin deficiency	Delayed puberty	Pseudo-Bartter syndrome	CF arthropathy/hypertrophic pulmonary osteoarthropathy
Pneumothorax	Distal intestinal obstruction syndrome	Oligomenorrhea		Chronic kidney disease
Respiratory failure	Intussusception	Cystic fibrosis-related diabetes		Nephrolithiasis/oxalate nephropathy
Allergic bronchopulmonary aspergillosis	Appendiceal abscess	Metabolic bone disease (reduced bone mineral density)		Depression
Chronic rhinosinusitis and nasal polyposis	Cirrhosis with portal hypertension			Anxiety
	Gastroesophageal reflux			
	Constipation			
	Bacterial overgrowth including pseudomembranous colitis			

and medical community and much anticipated by the wider CF community since the discovery of the CF gene in 1989 (Riordan et al., 1989).

2. *CFTR* gene mutations and *CFTR* mutation classes

Generally, a higher frequency of the F508del mutation is observed in northern than southern European populations (Fig. 1A).

By comparison all other mutations are relatively rare. However, the relative frequency of specific *CFTR* mutations varies greatly between countries and even between regions within countries (Bobadilla et al., 2002), such is the case for G551D which also show heterogeneous geographic distribution (Fig. 1B).

In most countries, only 10 to 15 *CFTR* mutations occur at a frequency above 1%. Many *CFTR* mutations are very rare, only occurring in a few or even a single person. Many papers describe these country or region specific mutations that are sometimes 'nicknamed' after their origin (e.g. Dutch mutation, Mediterranean mutation, 'Slavic mutation' — see Table 2). The majority of information on the relative occurrence of specific mutations is comprehensively reviewed by Bobadilla et al. (2002).

According to the respective gene defect, the nearly 2000 *CFTR* gene alterations have the following distribution: missense (42%); frameshift (15%); splicing (13%); nonsense (10%); large (3%) and in-frame (2%) deletions/insertions; and promoter (0.5%); plus 15% of presumably non-pathological variants (www.CFTR.2.org, 214; Bobadilla et al., 2002). Ultimately, however, all CF disease-causing mutations result in defective cAMP-regulated Cl^- secretion by epithelial cells, but this is due to various reasons (Welsh & Smith, 1995). A major step forward was achieved by grouping *CFTR* mutations with a similar effect on CFTR protein synthesis or function in the same mutation class. Indeed, elucidation of the molecular and cellular effects of mutations is likely to be a rich source of information to predict disease severity and can also provide the scientific basis for development of targeted compounds for mutation-specific correction (Amaral & Kunzelmann, 2007; Amaral & Farinha, 2013). *CFTR* mutations have thus been classified according to their functional defect (Zielenski & Tsui, 1995; Amaral & Farinha, 2013), as follows (Table 3, Fig. 2):

Class I mutations impair protein production, and being often nonsense mutations (with premature stop codons) they lead to mRNA degradation by a process called nonsense-mediated decay. Common mutations in class I include G542X (common in Brittany and Southern France), R1162X (common in Austria and Northern Italy), or W1282X (reaching 48% amongst Ashkenazi Jews) (Bobadilla et al., 2002).

Class II mutations which besides F508del, include R560T (Roxo-Rosa et al., 2006), A561E (Mendes et al., 2003), R1066C (Seibert et al., 1996) and N1303K (Gregory et al., 1991) amongst others, affect CFTR protein processing due to misfolding which is recognized by endoplasmic reticulum (ER) quality control retention and which targets proteins with abnormal conformations to degradation (Amaral, 2004).

Class III mutations (e.g., G551D) disrupt channel regulation through impaired gating.

Class IV mutations (e.g., R334W) decrease Cl^- ion conductance (i.e. flow) through the Cl^- channel.

Class V mutations significantly reduce normal protein levels, often by affecting splicing and generating both aberrant and normal transcripts (e.g. 3272−26A>G), whose levels vary amongst patients (Ramalho et al., 2002) and in different organs of each patient.

Class VI mutations lead to decreased retention/anchoring at the cell surface, often associated with decreased protein stability at the plasma membrane, e.g. F508del-CFTR after rescuing to cell surface (Farinha et al., 2013; He et al., 2013) 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).

The major virtue of this classification lies in adapting strategies of drug development to the specific defects caused by groups of mutations. In view of drug development and drug distribution, it is therefore also useful to know the relative prevalence of these mutation classes. Therefore we include these data in Fig. 3 and Table 2 and refer to two papers describing the distribution of *CFTR* mutation classes across Europe, US and Australia (Boyle & De Boeck, 2013; de Boeck et al., 2014). Like most classifications, this CFTR mutation classification has limitations: these include a) at present for many mutations it is not yet known which mutation class they belong to and b) some mutations have characteristics of more than one mutation class (e.g., F508del is considered a class II mutation but especially rescued F508del CFTR also has characteristics of classes III and VI, drug).

3. The complex CF disease spectrum and the benefits of the CFTR2 project

Most of the nearly 2000 *CFTR* mutations described so far are likely pathogenic, since they are found in subjects with disease characteristics of CF. However, after the identification of the *CFTR* gene, an increasing number of *CFTR* mutations were described, also in subjects with milder disease characteristics such as isolated bronchiectasis and male infertility due to congenital bilateral absence of vas deferens (CBAVD). Data from CF newborn screening equally confirm the variability of the CF phenotype, as well as the importance of considering the possibility of "complex alleles" (i.e. alleles containing more than 1 *CFTR* mutation or polymorphism). In most CF newborn screening programmes a surplus of "patients" carrying the R117H mutation in *trans* with F508del were identified (Scotet et al., 2006; Thauvin-Robinet et al., 2009; Lilley et al., 2010) and many of these subjects did not develop phenotypic features of CF. Although R117H by itself somewhat reduces CFTR conductance and gating (Sheppard et al., 1993), it was found that the

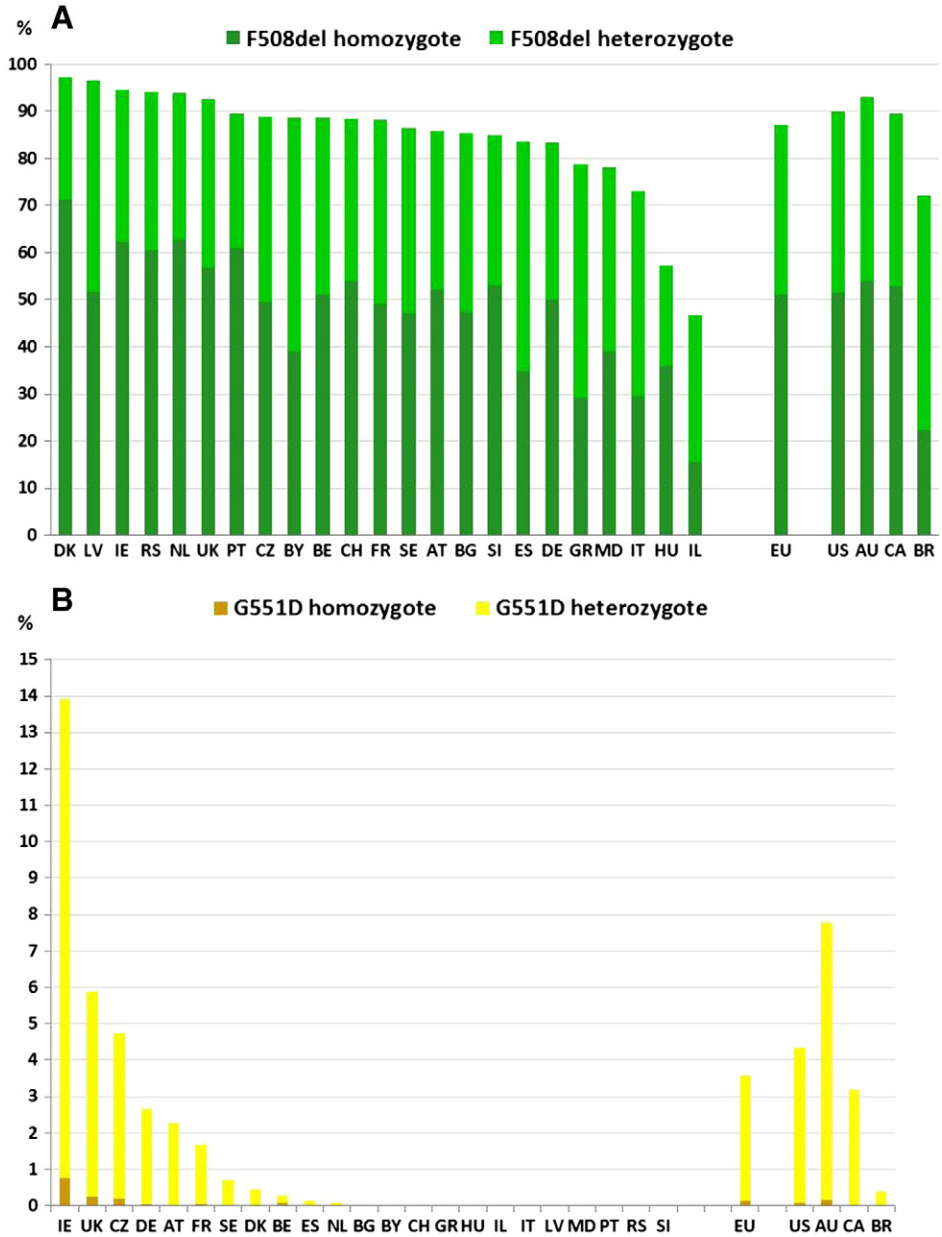


Fig. 1. Mutation distribution of F508del (**A**) and G551D (**B**) — (**A**) Percent of patients homozygous (dark) or heterozygous (light) for F508del mutation in different countries and regions. (**B**) Percent of patients homozygous (dark) or heterozygous (light) for G551D mutation in different countries and regions. AT: Austria, BE: Belgium, BY: Republic of Belarus, BG: Bulgaria, CH: Switzerland, CZ: Czech Republic, DE: Germany, DK: Denmark, ES: Spain, FR: France, GR: Greece, HU: Hungary, IE: Ireland, IL: Israel, IT: Italy, LV: Latvia, MD: Republic of Moldova, NL: The Netherlands, PT: Portugal, RS: Serbia, SE: Sweden, SI: Slovenia, UK: United Kingdom. AU: Australia, EU: Europe, US: United States of America, BR: Brazil, CA: Canada.

number of thymidine (T) repeats in intron 8 (IVS8) in *cis* with R117H explains the variability of the phenotype. Indeed, the number of T repeats determines the efficiency of correct splicing of exon 9: for instance 5 T (five thymidine repeats) leads to aberrant splicing so that the resulting CFTR protein lacks exon 9; whereas 7 T and 9 T lead to normal splicing and are only rarely associated with disease manifestations and these are invariably mild (Chu et al., 1991).

Hence improved knowledge of the *CFTR* gene led to new challenges: defining which *CFTR* mutations are truly "CF disease causing". The CFTR2 project was set up to solve this dilemma (www.CFTR.2.org, 2014). CFTR2 collected data from 57% of the estimated 70,000 worldwide individuals with CF using genotype and phenotype data from CF registries in Europe and North-America. CFTR2 then determined the 'disease liability' (i.e. the probability that a given mutation is CF-causing) of *CFTR* mutations with an allele frequency of ≥0.01% representing 96% of all CF alleles (Sosnay et al., 2013). Mutations were considered as disease causing if they fulfilled clinical criteria, functional criteria and a penetrance analysis (Sosnay et al., 2013). In this study, the phenotype of subjects homozygous or heterozygous for F508del and any of the 22 other *CFTR* mutations previously defined as CF causing by the American College of Medical Genetics (ACMG) was compared to the phenotype of subjects carrying other variants. A variant was deemed disease causing by clinical criteria if the mean sweat chloride (Cl^-) concentration derived from at least three individuals carrying the variant was ≥60 mM/L. In the functional analysis, processing of normal CFTR (so called "wild type", or WT) and variant was compared in different cell lines and Cl^- conductance of WT and variant was compared in Fisher rat thyroid (FRT) cells. For the penetrance analysis, *CFTR* variants occurring on the non-transmitted allele in at least 2 fertile fathers of CF children were considered as non-penetrant for CF and CBAVD. By July 2013, the ongoing CFTR2 programme had labelled 177 *CFTR* mutations as disease causing, 12 mutations were considered non-disease causing, 12 mutations were considered of varying clinical consequence and for 6 mutations the disease liability was still

Table 2
Common CFTR mutations and regional variation in Europe.

Mutation	Alternative name	Allele frequency (% of total known) in ECFSPR 2010	Allele frequency (% of total known mutations) in 2010 ECFSPR
F508del		64.5	Most frequent mutation worldwide Southeast to Northwest increasing prevalence in Europe IL 25.5 to DK 82.6
Mutations with an overall EU prevalence above 1%			
G542X	Mediterranean mutation	2.5	GR 6.7, ES 6.0
N1303K	Ancient Phoenician mutation	1.9	IT 4.2
W1282X	Jewish Ashkenazi mutation	1.2	IL 22.4
G551D	Celtic mutation	1.1	IE 7.3
1717−1G>A	Italian mutation	1.0	IT 3.7
Mutations with an overall EU prevalence below 0.5%			
G85E			PT 3.5
A455E	Dutch mutation		NL 3.5
CFTR dele 2,3	Slavic mutation		CZ 5.2, BY 6.7
394delTT	Nordic mutation		SE 7.9, DK 2.0
3905insT	Swiss mutation		CH 2.4
R1162X	Italian mutation		IT 7.8
A561E	Portuguese mutation		PT 3.2

Abbreviations
ECFSPR — European Cystic Fibrosis Society Patient Registry.
Country codes: BY Belarus, CH Switzerland, CZ Czech Republic, DK Denmark, ES Spain, GR Greece, IE Ireland, IL Israel, IT Italy, NL the Netherlands, PT Portugal, SE Sweden.

unknown. The full mutation list is available on the CFTR2 website (www.CFTR.2.org, 2014).

Even for the mutations classified as disease causing, a large variability in disease severity between patients is apparent. Following the discovery of the *CFTR* gene, it soon became obvious that specific *CFTR* mutations mainly determine the pancreatic phenotype (pancreatic sufficient or insufficient) but not the lung disease severity (Kerem et al., 1990b). Patients with 2 mutations of classes I to III are nearly always pancreatic insufficient, but having 1 milder mutation (usually of class IV or V) is sufficient to retain pancreatic sufficiency (PS). However, subjects carrying the same 'severe' genotype, e.g. F508del-homozygous, still have a large variation in the severity of lung disease (Kerem et al., 1990a). This pointed towards the strong influence of environmental factors and modifier genes (genes outside of the *CFTR* gene) on clinical outcomes (Collaco et al., 2010). Many environmental factors indeed have a proven effect on lung disease course: exposure to second-hand smoke (Collaco et al., 2008), and high environmental temperatures have a negative effect (Collaco et al., 2011). On the other hand, increased treatment intensity (Johnson et al., 2003), early referral to CF centre (Lebecque et al., 2009) and adherence to therapy (Com et al., 2014) have a positive effect on lung function.

Modifier genes are sought via a candidate gene approach or more recently via genome-wide association studies (GWAS) or whole exome sequencing (WES). Since CF lung disease is characterized by relentless cycles of lung infection and inflammation, the candidate gene approach focused on several genes involved in lung defence (e.g. mannose binding lectins (Yarden et al., 2004) and immune response (e.g. *TGF-beta*) (Drumm et al., 2005)). Using larger data sets and exploiting the model of extremes of phenotype, the genome wide association study identified new *loci* that modify lung disease severity (Wright et al., 2011). For more reading on modifier genes we refer to a review by Guillot et al. (2014). Importantly, the understanding how lung disease severity is influenced might lead to novel therapeutic strategies.

4. Mutation-specific therapies or CFTR repairing therapies

Examining the molecular and cellular basis of *CFTR* mutations has also become important for designing effective treatments correcting the basic molecular and cellular defects, i.e., mutation-specific therapies (Amaral & Kunzelmann, 2007). Examples include (see Fig. 4):

Class I: Aminoglycoside antibiotics (e.g. gentamicin), and ataluren (PTC124) to some degree 'over-read' the premature termination codons thereby permitting translation to continue to the normal termination of the transcript (Wilschanski et al., 2003; Kerem et al., 2008).

Class II: Chemical and molecular chaperones can potentially promote protein folding, allowing the mutant protein to escape ER degradation and reach the cell surface. These compounds have been termed correctors (Pedemonte et al., 2005). The corrector VX-809 which showed great success in vitro (Van Goor et al., 2011) is currently in clinical trial in combination with the potentiator ivacaftor (see below under "Clinical trials with CFTR modulators" section), as corrector monotherapy evidenced only modest results for F508del/F508del patients (Clancy et al., 2012). It is also envisaged that full correction of F508del-CFTR in CF patients will require double or even triple combination therapy [reviewed in: (Amaral & Farinha, 2013)]. Indeed, a recent report based on both experimental and protein modelling data proposes that the mechanism of action for VX-809 is compatible with putative binding of VX-809 to

Table 3
List of mutations by *CFTR* mutation class.

Class	Type of defect	List of mutations attributed to this class
Class I	Defective protein production	*Nonsense mutations*: G542X, R1162X, RW1282X *Deletions and insertions*: CFTRdele2,3; 1078delT; 1717−1G → A; 3659delC; 621+1G > T
Class II	Defective protein processing	G85E, F508del, I507del, R560T, A561E, R1066C, N1303K
Class III	Defective protein regulation (gating)	G178R, S549N, S549R, G551D, G551S, G970R, G1244E, S1251N, S1255P, G1349D
Class IV	Defective protein conductance	R334W, R347P, R117H
Class V	Reduced amount of functioning protein	2789+5G → A, 3272−26A>G, 3849+10KbC → T, A455E
Class VI	Reduced cell surface stability	Rescued F508del, c.120del23
Unclassified		All other mutations, including those unknown

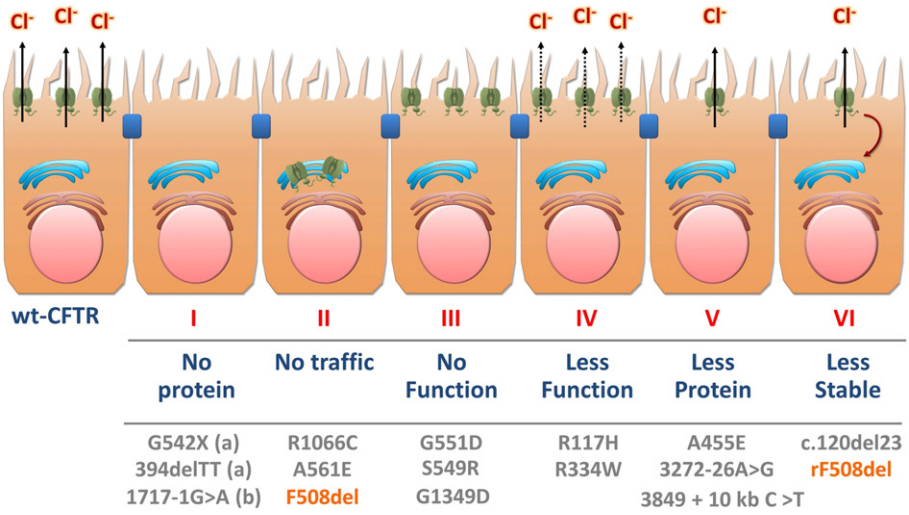


Fig. 2. Classes of *CFTR* mutations help to stratify correction of the basic defect by CFTR modulators through a "mutation class-specific" approach. Class I mutations, which abrogate protein production, often include mutations that generate premature stop codons, class Ia (e.g. G542X) that lead to mRNA degradation by nonsense-mediated decay or those affecting canonical splice sites class Ib (e.g. 1717 − 1G>A). Class II mutations (including the most prevalent, F508del) cause retention of a misfolded protein at the ER, and subsequent degradation in the proteasome. Class III mutants affect channel regulation, impairing channel opening (e.g. G551D). Class IV mutants exhibit reduced conduction: that is, decreased flow of ions (e.g. R334W). Class V mutants cause significant reduction in mRNA and/or protein levels – albeit with normal function – often through causing alternative splicing (e.g. 3272 − 26A>G). Class VI mutants cause significant plasma membrane instability and include F508del when rescued by most correctors (rF508del) (Amaral & Farinha, 2013).

a F508del-CFTR pocket (at NBD1:ICL4 interface) (Farinha et al., 2013). These models also indicate the existence of a second pocket in F508del-CFTR (between NBD1 and NBD2) to which chemically different compounds can still bind. These data, consistent with those from some (He et al., 2013) but not all studies (Ren et al., 2013), strongly support the hypothesis that there is potential for further synergistic F508del-CFTR correction by other compounds at distinct conformational sites. They also indicate requirement of combined therapies to fully rescue F508del-CFTR. Although based on specific conformational premises, a previous report (Okiyoneda et al., 2013) confirmed the requirement of two correctors to fully correct F508del-CFTR. Meanwhile another corrector out of came the pipeline and is under trial: compound VX-661 (sharing the molecular left-hand side structure with VX-809 but with a variant at the right-hand side) seems to have improved pharmacodynamic properties and less drug–drug interaction (Donaldson et al., 2013; Boyle et al., 2014) but probably shares the same mechanism of action.

Class III: CFTR channel activators, which are termed potentiators (Moran & Zegarra-Moran, 2005), such as VX-770 (ivacaftor) which following demonstration of success in vitro (Van Goor et al., 2009) and in clinical trials with patients having at least one G551D mutation were already approved for the clinic by both FDA and EMA. More recently, the panel of mutations which can be rescued by ivacaftor in vitro has been expanded to include additional gating mutations (Yu et al., 2012). Following a successful clinical trial, FDA approval was extended to include 8 additional gating mutations (de Boeck et al., 2013b).

Class IV: Compensation for reduced conductance can be achieved by increasing the overall cell surface amount of these CFTR mutants with correctors. Alternatively, by increasing the levels of channel activation, at least a partial positive response to potentiators is also expected. However, both hypotheses require testing.

Class V: These mutations significantly reduce normal protein levels, often by affecting splicing and generating both aberrant and normal transcripts. Recent advances in the use of antisense oligonucleotides (AONs) make this promising approach look a very powerful tool for the specific correction of missplicing (Siva et al., 2014). Meanwhile however, it is anticipated that potentiators and correctors may also improve functional levels of CFTR for these mutants. Again, this hypothesis requires further testing.

Class VI: Compounds that enhance CFTR retention/anchoring at the cell surface will benefit these mutants. These may include activators of Rac1 signalling which promote anchoring to actin cytoskeleton via NHERF1, such as hepatocyte growth factor (HGF), already demonstrated to further enhance VX-809 recued F508del-CFTR (Moniz et al., 2013).

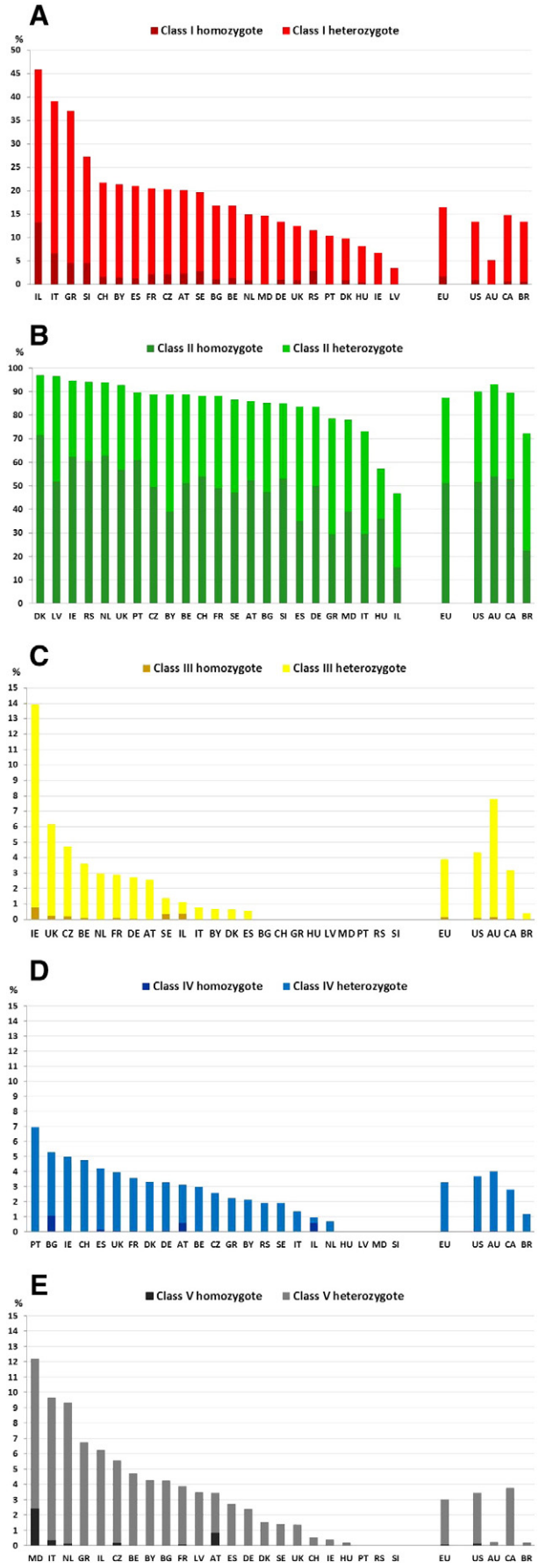
Despite the attractiveness of this *CFTR* mutation classification it should – previously alluded to – be emphasized that some mutations have more than one class defect. The most flagrant example is actually F508del, which besides the trafficking defect (Denning et al., 1992) (class II), also has a gating defect (Dalemans et al., 1991) (class III) and a cell surface stability defect (Sharma et al., 2004) (class VI). Another example is R117H which could be classified as class IV due to a slight decrease in channel conductance but is not a CF-causing mutation per se (Thauvin-Robinet et al., 2009; de Nooijer et al., 2011). Indeed, it only leads to CF when in *cis* with 5 T which alone is class V, but not a mutation per se either (Cuppens et al., 1994). So the "real CF-causing mutation" is the complex allele R117H-5 T which can be considered as a class IV/V mutation.

5. The complexity of assessment of efficacy of CFTR modulators in a wide range of *CFTR* mutations

5.1. Pre-clinical assessment of CFTR-repairing molecules

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. To this end investigational drugs should be tested ex vivo directly in native tissues from patients with CF or in cellular models with the rare mutations, towards a personalized-medicine approach.

Indeed, patients with CF begin to be in high demand for competing clinical trials. So far, efficacy testing on human bronchial epithelial (HBE) cells from patients with CF is considered to be the "gold standard"



for CFTR-repairing molecules going into clinical trial (Van Goor et al., 2009, 2011) and a good correlation has been found between data collected for VX-770 in HBEs and clinical trial outcomes (Ramsey et al., 2011). Despite this correlation demonstrated for this potentiator compound, it is probably insufficient to prove that primary HBEs are the gold standard for compound validation for all mutations. More data are required for additional compounds, especially for corrector candidates, in order to demonstrate that efficacy in primary HBE systems correlates well with clinical efficacy. As an example the bioavailability of small molecules, based exclusively on cell culture testing, may prevent good prediction of clinical efficacy. One example is given by the specific CFTR(inh)-172 (Ma et al., 2002) which is quite potent in inhibiting CFTR in primary HBE cultures (Van Goor et al., 2009; Fulcher et al., 2009) but has failed to demonstrate the same efficacy in human native sweat glands (Wang et al., 2004) or human intestinal tissue (MDA lab, unpublished observations).

Another example is the compound PTC-124. It was pre-validated for CF both in primary HBEs and mouse models (Du et al., 2008) and also showed initial promise for Duchenne/Becker muscular dystrophy (DMD/BMD) in both primary human muscle cells and in mdx mice expressing dystrophin nonsense alleles (Welch et al., 2007), yet PTC-124 has shown limited results in clinical trials for CF and DMD. For the treatment of nonsense mutation Duchenne muscular dystrophy (nmDMD) patients FDA has not approved of the drug, yet but the EU's Committee on Human Medicinal Products (CHMP) adopted a positive opinion for the conditional marketing authorization of ataluren in nmDMD patients age 5 years and older (www.actionduchenne.org, 2014; Kerem et al., 2014).

Accordingly, pre-clinical validation directly on native human tissues ex vivo, such as rectal biopsies already commonly used for the diagnosis of CF in several centres both in Europe and elsewhere (Hirtz et al., 2004; Derichs et al., 2010; Sousa et al., 2012; De Boeck et al., 2013a), or even samples of explanted CF airways, becomes alternative and attractive options to assess the efficacy of compounds. This approach may complement those on primary HBEs to achieve a better prediction of compound clinical value in human individuals.

Animal models have provided valuable insights into various aspects of CF and should also play an important role in pre-clinical validation of small molecules. However, since mouse models lacking functional CFTR do not develop the characteristic manifestations of human CF (Snouwaert et al., 1992; O'Neal et al., 1993; van Doorninck et al., 1995), the value of murine animals to predict the outcome of CFTR-repairing compounds may be somewhat limited. Moreover, given that human and murine CFTR exhibit different channel characteristics (Lansdell et al., 1998) and that different regulatory pathways operate in the murine and human tissues (Nadeau, 2001), it is predicted that efficacy of small molecules in mice may not translate into equivalent effectiveness in the human lung. Indeed, as above described for primary cultures, testing of the CFTR (inh)-172 compound in mice has also shown efficient inhibition (Ma et al., 2002) and yet the same effect is not observed in human tissues. This seems to suggest that prediction of clinical efficacy based on validation of CFTR-repairing compounds in mouse models may be of limited value.

Notwithstanding, better predictions and pre-clinical validation are expected from testing of small molecules in novel animal models for CF, e.g. the pig CF model, whose anatomy, biochemistry, physiology, size, lifespan and genetics are more similar to humans than mice (Rogers et al., 2008b). However, pre-clinical testing for correctors in

Fig. 3. Percent of patients having one (light) or two (dark) mutations belonging to mutation class I (panel A), II (panel B), III (panel C), IV (panel D) or V (panel E) in different countries and regions. AT: Austria, BE: Belgium, BY: Republic of Belarus, BG: Bulgaria, CH: Switzerland, CZ: Czech Republic, DE: Germany, DK: Denmark, ES: Spain, FR: France, GR: Greece, HU: Hungary, IE: Ireland, IL: Israel, IT: Italy, LV: Latvia, MD: Republic of Moldova, NL: The Netherlands, PT: Portugal, RS: Serbia, SE: Sweden, SI: Slovenia, UK: United Kingdom. AU: Australia, EU: Europe, US: United States of America, BR: Brazil, CA: Canada.

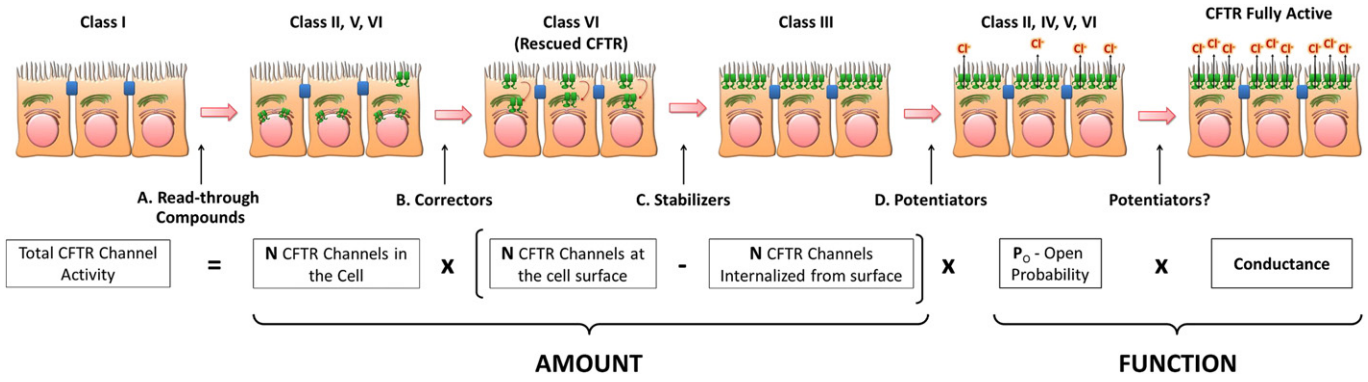


Fig. 4. CFTR pharmacological modulators have different modes of action. (**A**) Read-through compounds which include aminoglycoside antibiotics (e.g., gentamicin, tobramycin) act by suppressing premature termination codons (PTCs), thus permitting translation to continue to the normal termination of the transcript and thus increasing the total amount of complete CFTR being produced in the cell. (**B**) Correctors (e.g., VX-809 also known as lumacaftor; VX-661) potentially promote folding of mutant CFTR protein, allowing it to escape ER degradation and reach the cell surface, thus increasing the number of channels present at the plasma membrane. (**C**) Stabilizers include compounds (e.g., HGF) that enhance CFTR retention/anchoring at the cell surface thus also contributing to increase the number of channels present at the cell surface. (**D**) Potentiators (e.g., VX-770 also known as ivacaftor) activate CFTR, i.e., increase the open probability (P_o) of the channel by regulating its gating and possibly also the conductance.

the forthcoming F508del/F508del CF pig model (Rogers et al., 2008a) may also be hampered by the leaky processing of porcine F508del-CFTR, which is described to be even higher than murine F508del-CFTR (Ostedgaard et al., 2007). An ideal animal CF model to test for efficacy of correctors of human 508del-CFTR would thus be a *CFTR*−/− pig engineered to express two copies of the human F508del-*CFTR* gene (Klymiuk et al., 2010).

5.2. Endpoints for use in clinical trials

To support 'proof of concept' in phase 2 clinical trials with compounds aimed at improving CFTR function, it is logical to choose an outcome parameter that reflects CFTR function such as sweat chloride and nasal potential difference measurement (de Boeck et al., 2014). In phase 3 clinical trials the clinical benefit of a compound must be proven. Therefore, the advent of novel therapeutic options has been paralleled by an increase in interest for endpoints that can reliably measure treatment benefit (de Boeck et al., 2014). The centralization of CF care and the associated intensified treatment from diagnosis have led to a major improvement in lung disease course. Hence currently used clinical outcome parameters such as survival and FEV_1 are no longer appropriate to assess treatment benefit over the limited treatment period of a clinical trial, especially in subjects with normal baseline FEV_1. LCI, a measure of ventilation inhomogeneity derived from inert gas washout tests, is a promising new outcome measure (Kent et al., 2014) as it detects abnormalities in subjects with normal spirometry, is reproducible and responsive to changes in treatment. In addition, correlations of LCI with quality of life and longer-term outcomes such as time to pulmonary exacerbation are emerging (Vermeulen et al., 2014).

Sensitive imaging techniques have been developed that allow quantification of structural lung damage: chest CT scores for bronchiectasis correlate with LCI and quality of life measures (Tiddens et al., 2014). Chest CT scores for air trapping seem very useful in early disease, since they are the first abnormality detected (Mott et al., 2012). Magnetic resonance imaging is one of the other promising new outcome measures since it is not associated with radiation burden (Wielputz et al., 2014).

Many *CFTR* mutations are rare, some are even unique. Randomized controlled trials are appropriate for common mutations; cross-over designs may be more appropriate for less common mutations, but for rare mutations one really enters the area of personalized medicine and alternative trial designs are needed. In modified 'n-of-1' trials, the subject is exposed to treatment over variable blinded time slots, and outcome parameters are measured repeatedly (Duan et al., 2013). Treatment effect is then estimated by comparing outcome during repeated "on" versus "off" drug periods. For these patients with rare mutations, reliable ex vivo evaluation of treatment benefit from CFTR modulators directly on the patient tissues could complement or be an alternative for 'n-of-1' trials (Lillie et al., 2011).

Finally, as discussed in the pre-clinical assessment of compounds, airway epithelial cells derived from nasal brushings or organoids could be utilized (Dekkers et al., 2013), e.g. the mini intestinal organs grown from stem cells obtained by rectal mucosal sectional biopsies offer this possibility. Whether and how ex vivo treatment benefit translates into clinical efficacy may in the future and to some extent be extrapolated from results in patients with more common mutations.

5.3. Clinical trials with CFTR modulators

5.3.1. CFTR potentiators

Ivacaftor is the first drug on the market that is designed to improve the defective CFTR function. Ivacaftor is a CFTR potentiator that improves CFTR channel gating and is thus ideally positioned to treat patients with class III mutations (Yu et al., 2012). As described above, ivacaftor also improves gating and Cl^- current in cell lines with WT-CFTR and in cell lines with missense or splicing mutations associated with residual CFTR function (Van Goor et al., 2014). Ivacaftor might therefore also benefit subjects with these *CFTR* mutations.

Ivacaftor has proven efficacy in children over six year of age and adults (Accurso et al., 2010; Ramsey et al., 2011; J.C. Davies et al., 2013) with CF and at least one G551D mutation. The phase 3 studies demonstrated an average of 10% predicted improvement in FEV_1, a decrease in pulmonary exacerbations and an improvement in weight and health-related quality of life (HRQOL). Ivacaftor treatment improved lung clearance index (LCI, a measure of ventilation homogeneity) and further improved FEV_1 in patients with G551D who have preserved lung function (J. Davies et al., 2013). In "Named Patient Programs" ivacaftor also improved lung function in many of the subjects with G551D mutation who had advanced lung disease and very low FEV_1 (<40%) who had been previously excluded from participation in the randomized clinical trials (Hebestreit et al., 2013; Barry et al., 2014). In a cross-over study the efficacy of ivacaftor has also been proven in subjects who carry gating mutations other than G551D where the increase in FEV_1% predicted was in line with the G551D trials (de Boeck et al., 2013b). Furthermore, in patients with G551D, who have so far the longest ivacaftor treatment exposure, the long term benefit can be explored: treatment benefit seems to be sustained and the impact of treatment on acquisition of *P. aeruginosa* on the rate of decline of FEV_1 and on the occurrence of CF complications is being explored during continuous prospective open label follow up (McKone et al., 2013; Rowe et al., 2013).

Unfortunately, patients having at least one gating mutation are relatively rare: overall only 4% of patients but with marked differences between countries from less than 1% in Denmark, Italy and Portugal to 14% in Ireland (De Boeck et al., 2014) (Fig. 3).

In a randomized controlled trial including 69 children and adults with the R117H mutation (a class IV *CFTR* mutation), which is the most frequent mutation with residual function, the primary endpoint of improvement in FEV_1 was not met (www.ccf.org, 2014). In a pre-specified sub-analysis of subjects older than 18 years, a statistically significant improvement in FEV_1 of 5% predicted was demonstrated, as was an improvement in HRQOL score.

The efficacy and safety of ivacaftor are also currently being evaluated in children with G551D aged 2 to 5 years and in subjects with splicing and missense mutations (NCT01685801) associated with residual function.

5.3.2. Premature termination codon 'read-through' therapies

CFTR modulators also include treatments directed towards premature termination codons (class I mutations). Aminoglycoside antibiotics can induce read-through of premature termination codons, resulting in a full-length functional protein (Barton-Davis et al., 1999). This led to several studies administering topical aminoglycosides; and whilst conflicting results were reported, the work provided early experience evaluating modulator drugs (Wilschanski et al., 2000; Clancy et al., 2001; Wilschanski et al., 2003). Ataluren (PTC124 from PTC Therapeutics) was studied in two open label phase 2 trials with modest results in terms of efficacy (Kerem et al., 2008; Sermet-Gaudelus et al., 2010; Wilschanski et al., 2011). A long-term placebo controlled phase 3 study showed no improvement in the primary endpoint, FEV_1% predicted, but interestingly did demonstrate less drop in lung function compared to placebo in a predefined subset of subjects who were not treated with inhaled antibiotics, which are known to influence the efficacy of ataluren (Kerem et al., 2014). Given the in vitro impact on CFTR function (Takatori et al., 2007) and the potential to over-read premature termination codons in other genetic disease conditions which have (e.g. Duchene's muscular dystrophy) (Finkel et al., 2013) further refining and development of small molecules enhancing read-through of class I (stop) mutations is anticipated.

5.3.3. CFTR correctors

Class II mutations are the most common *CFTR* mutations globally. In an early CFTR modulator phase 2 study in patients homozygous for F508del, ivacaftor did not alter the primary endpoint and biomarker, sweat chloride, nor did it lead to improvements in secondary endpoints including FEV_1% predicted, HRQOL or weight when administered for 16 weeks (Flume et al., 2012). This was not an unexpected result given the mechanisms which are faulty in class II mutations. In a further phase 2 trial, corrector lumacaftor (VX-809) significantly lowered sweat chloride in a dose dependent fashion, but did not improve FEV_1% predicted in a 28 day study (Clancy et al., 2012). A phase 2 combination randomized control trial including lumacaftor (Vx809) and ivacaftor commenced in 2012 and the fourth cohort of this complex study evaluating higher drug doses in patients heterozygous for F508del is underway. The results of the first three cohorts have recently been published (Boyle et al., 2014). In cohort 1 (patients homozygous for F508del mutation), the co-administration of both drugs significantly improved CFTR function (measured by reduced sweat chloride) over levels with prior lumacaftor monotherapy. In cohort 2, patients homozygous for F508del-CFTR were randomized to receive placebo for 56 days or lumacaftor (200, 400 or 600 mg daily dose) for 28 days followed by lumacaftor in combination with ivacaftor 250 mg q12h for 28 days. In addition, a group of patients heterozygous for F508del-CFTR was randomized to receive placebo for 56 days or lumacaftor 600 mg daily for 28 days followed by lumacaftor with ivacaftor 250 mg q12h for 28 days. Treatment with lumacaftor alone reduced sweat chloride by day 28, but there was no significant further change during combination therapy. FEV_1 was unchanged during lumacaftor monotherapy. The short-term co-administration of lumacaftor with ivacaftor produced clinically meaningful improvements in lung function, both in absolute terms and relative to placebo. Treatment effects in heterozygous patients were less pronounced than were those in homozygous patients. Adverse events were comparable during combination therapy and placebo periods, however during lumacaftor monotherapy 12 of 97 participants experienced chest tightness or dyspnoea.

Cohort 3 evaluated 11 patients to assess the safety and pharmacokinetics of the 400 mg q12h dose of VX-809 for 28 days followed by VX-809 (400 mg q12h) in combination with ivacaftor (250 mg q12h) for 28 days. This cohort was designed to support inclusion of this dose in the phase 3 studies and showed a higher total exposure compared to 600 mg once daily dosing (details below). Safety results were similar to that of cohort 2. The most common adverse events in both groups were respiratory in nature (including one patient in the treatment group discontinuing treatment).

The pattern of lung function response observed in cohort 3 was similar to cohort 2, with a decline in FEV_1 during the VX-809 monotherapy dosing period followed by a statistically significant increase in FEV_1 during the VX-809 and ivacaftor combination dosing period. The within-group mean absolute improvement in FEV_1 observed during the combination-dosing period in cohort 3 was 6.6 percentage points.

The results of this phase 2 study provided the impetus to commence two-parallel international multi-centre phase 3 trials comparing lumacaftor (400 mg q12h or 600 mg once a day) in combination with ivacaftor (250 mg q12h) in people with CF aged 12 years and older homozygous for F508del-CFTR mutation. Recruitment of >1000 patients across the two trials was completed within 6 months and the first results are anticipated in mid-2014 (investors.vrtx.com/releasedetail.cfm?Releaseid=827435; investors.vrtx.com/releasesArchive.cfm?Year=&ReleasesType=&PageNum=2).

Another corrector compound developed by Vertex (VX-661) has shown promise in in vitro studies and a phase 2–28 day study of this compound in combination with ivacaftor has been completed. Preliminary analysis has been published in abstract form (Donaldson et al., 2013). The phase 2 randomized, double-blind, placebo-controlled study included 128 people with CF ages 18 and older with two copies of the F508del mutation (investors.vrtx.com/releasesArchive.cfm?Year=&ReleasesType=&PageNum=2). In this study with a complex dosing regimen patients were randomized to VX-661 (doses ranging from 10 to 150 mg once daily), or placebo, for 28 days. A further group of patients was randomized to receive VX-661 (doses ranging from 10 to 150 mg once daily) and ivacaftor (150 mg twice daily), or placebo, for 28 days. The primary endpoints of the study were safety, tolerability and change in sweat chloride. There were significant decreases in sweat Cl^-, both within-group and versus placebo. VX-661 was generally well-tolerated when given alone and in combination with ivacaftor. Increased FEV_1% predicted was seen for combination of VX-661 and ivacaftor for 28 days (two highest dose groups) with mean relative increases of 9.0% ($p = 0.01$) and 7.5% ($p = 0.02$) versus placebo.

Two corrector drugs have now been tested in phase 2 trials with ivacaftor although, ongoing and future trials will be required to determine if one combination provides better clinical efficacy.

6. Innovative non-CFTR based therapeutic approaches tackling the basic defect

6.1. CFTR by-pass therapies (ENaC, anoctamins)

The major virtue of these therapies is that they apply equally to all patients with CF. Whilst efforts proceed to identify novel correctors and to improve efficacy of correctors to rescue the most common defect F508del-CFTR, it is important to bear in mind that at least ~15% of all CF

patients will not benefit from F508del-CFTR corrector therapy, as they lack F508del in both alleles.

Moreover, only ~40–50% % of patients are F508del-homozygous and efficacy of correctors on patients with only one F508del is expected to be even lower than the already modest results on F508del-homozygous patients (Clancy et al., 2012). Thus, new therapies that correct the fluid and pH imbalance in CF by stimulation of non-CFTR Cl^- channels to compensate for the absence of functional CFTR are urgently needed (Fig. 5).

Current knowledge indicates that this can be achieved by normalizing ENaC, the sodium epithelial channel that is hyperactive in CF epithelia or through activation of alternative Cl^- channels, of which anoctamins, representing Ca^{2+}-activated Cl^- channels, are the most attractive candidates. Stimulation of a basolateral K^+ channel could also increase the driving force for Cl^- secretion and hence favour CFTR-mediated or CaCC-mediated Cl^- secretion. These approaches are reviewed here, as well as other approaches aimed at restoring ion homeostasis in CF.

The absence of CFTR from the apical membrane of epithelia leads to enhanced Na^+ conductance via ENaC in surface airway epithelial cells leading to excessive water absorption (Mall et al., 1998). So, along with the search for small molecules to restore CFTR activity, ENaC inhibitors have been sought for CF therapeutics to reduce ENaC-mediated Na^+ hyperabsorption and increase ASL hydration (Amaral & Kunzelmann, 2007). ENaC can be blocked by specific inhibitors such as amiloride, benzamil and phenamil and probably by activation of protein kinase C (PKC). Also, activation of purinergic receptors by ATP, UTP or denufosol inhibits ENaC, besides activating CaCC (Amaral & Kunzelmann, 2007). Despite the very promising phase II trial results (Deterding et al., 2007), this compound had variable results in two phase III trials (Accurso et al., 2011; Ratjen et al., 2012). Amiloride (Amil), used for the management of hypertension and congestive heart failure, was the first ENaC pharmacological inhibitor tested in CF, but studies showed no significant improvement potentially due to its short half-life in the lungs (Knowles et al., 1990). Longer-acting and more potent ENaC inhibitors (IC50 ≈ 10 nM) include Amil-derivatives

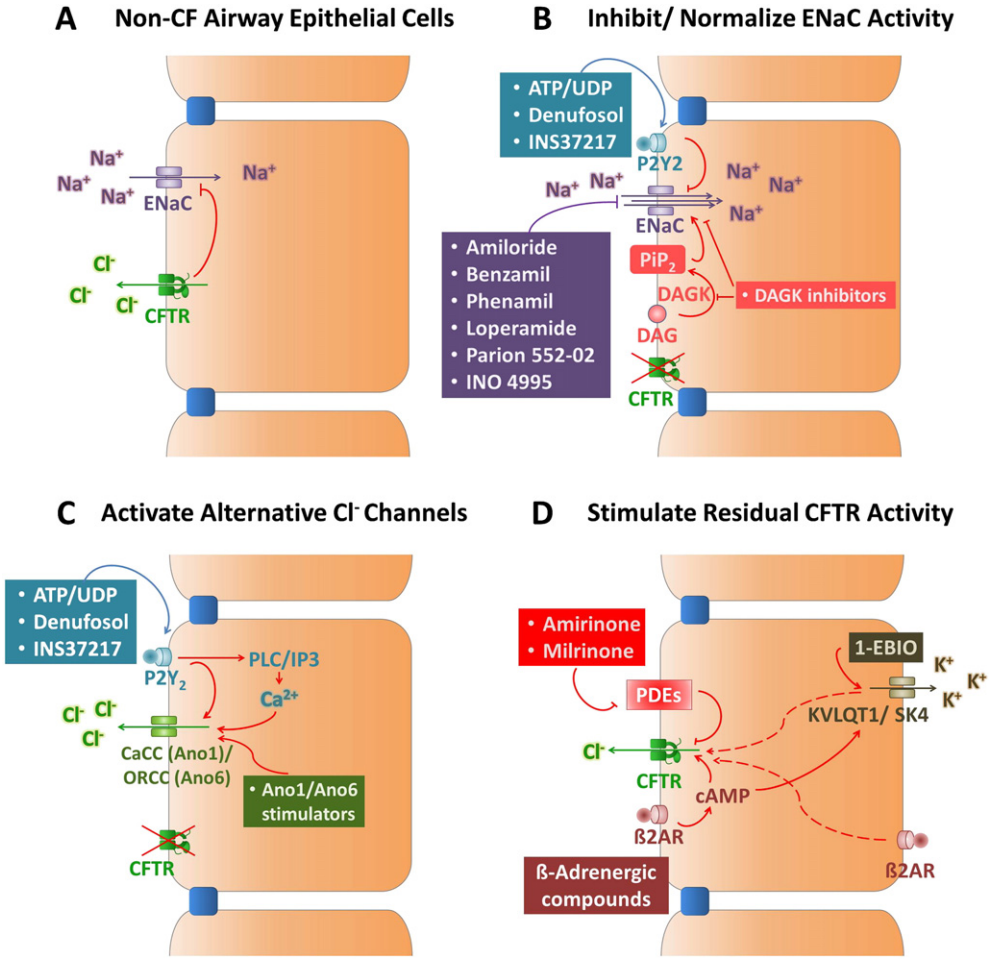


Fig. 5. Pharmacological compounds used in therapeutic strategies aimed at circumventing the CFTR ion channel defect in CF airways by influencing other ion channels, "by-pass therapies". (**A**) In A non-CF surface airway epithelial cells the CFTR-mediated Cl^- secretion keeps ENaC activity under normal level. (**B**) In CF, absence of CFTR-mediated Cl^- secretion leads to enhanced Na^+ conductance in surface airway epithelial cells through ENaC, which can be blocked by specific inhibitors such as amiloride, benzamil and phenamil, and probably by activation of protein kinase (PKC). The activation of purinergic receptors ($P2Y_2$) by ATP or UTP also inhibits ENaC. However, excessive blocking of ENaC may cause severe harm, via undesirable accumulation of fluid in the lungs, i.e., pulmonary edema. Instead, compounds normalizing airway surface liquid (ASL) homeostasis through physiological regulation of ENaC e.g., via compounds inhibiting diacylglycerol (DAG) kinase (DGK) which catalyses conversion of DAG to phosphatidylinositol (O'Sullivan & Freedman, 2009; Anonymous, 2012a)-bisphosphate (PIP_2) — an ENaC activator, thus restoring normal ENaC activity (Almaca et al., 2013). (**C**) Stimulation of alternative Cl^- channels such as the Ca^{2+}-activated Cl^- channels, like Anoctamin 1 (Ano1) can be achieved in CF airway epithelial cells by stimulation of luminal $P2Y_2$ purinergic receptors with ATP or UTP via a cascade of events that involves activation of phospholipase C (PLC) and breakdown of PIP_2 to D-myo-inositol (O'Sullivan & Freedman, 2009; Anonymous, 2012a; Sosnay et al., 2013)-trisphosphate (IP_3) or by stimulators of CaCCs like INO-4995 (Tian et al., 2013) or by yet unidentified agonists of outwardly rectifying Cl^- channels (ORCCs), recently identified as Anoctamin 6 (Ano6). (**D**) When there is residual CFTR-mediated Cl^- secretion (class IV–VI mutations), increasing the electrical driving force for luminal Cl^- secretion by stimulation of the basolateral Ca^{2+}-activated K^+ channel SK4 by the benzimidazol compound 1-EBIO, or activation of cAMP regulated K^+ channels (KvLQT1) by agonists of the cAMP pathway, such as ß-adrenoceptor compounds, may bring about benefit. Additionally, blockers of phosphodiesterases (PDE) such as amrinone and milrinone which prevent CFTR de-phosphorylation and hence its de-activation may also enhance the residual levels of CFTR activity. [Adapted from: (Amaral & Kunzelmann, 2007)].

such as benzamil and PS552 (Parion Sciences, Durham, NC), both yielding disappointing results in CF trials (Hirsh et al., 2006; Donaldson & Boucher, 2007). Excessive blocking of ENaC may cause severe harm, via undesirable accumulation of fluid in the lungs, i.e. pulmonary oedema (Althaus et al., 2011). Instead, compounds which normalize ASL homeostasis are needed through physiological regulation of ENaC. If achieved independently of CFTR, such ENaC normalization would have the virtue of correcting Na^+ ion transport in CF patients bearing any *CFTR* mutation. Despite detailed knowledge on how several ENaC regulators control both channel numbers at the cell surface and its open probability (Butterworth et al., 2009), many aspects of ENaC biogenesis, trafficking, and regulation remain obscure.

A recent study, performing a large-scale siRNA screen in combination with live cell microscopy in human airway epithelial cells and screening over 6000 genes identified over 1500 candidates, evenly divided between ENaC channel inhibitors and activators (Almaca et al., 2013). Detailed investigation showed that inhibition of DGKι, a protein involved in PiP2 metabolism, downgrades ENaC activity, leading to normalization of both Na^+ and fluid absorption in CF airways to non-CF levels in primary human lung cells from CF patients (Almaca et al., 2013). DGKι thus seems a promising new drug candidate for CF.

Secondly, amongst possible alternative Cl^- channels, anoctamins 1 and 6 (ANO1; ANO6) stand out as key candidates to potentially bypass lack of CFTR in CF [reviewed in (Kunzelmann et al., 2011)]. ANO1 (TMEM16A) is the much sought after Ca^{2+}-activated Cl^- channel (CaCC) present in many CF affected epithelial cells (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008), and which has been shown to transport HCO_3^- as well as Cl^- after physiological stimulation (Jung et al., 2013). ANO6 (TMEM16F) was recently identified as an essential component of the outwardly rectifying Cl^- channel (ORCC), which is also involved in epithelial anion transport (Martins et al., 2011). Notably, knockout of ANO1 in mouse airways leads to a CF-like phenotype, reflecting the importance of anoctamins for airway anion secretion (Rock et al., 2009; Kunzelmann et al., 2012). The demonstration that transient ANO1 currents ATP-activated through luminal P2Y2 purinergic receptors in CF airways can be further pharmacologically potentiated (twice of that observed in non-CF airways) suggests that these channels may be a valid target for the treatment of CF (Tian et al., 2013).

Thirdly, increasing of electrical driving of luminal Cl^- secretion by stimulation of the basolateral Ca^{2+}-activated K^+ channel SK4 by the benzimidazol compound 1-EBIO (Roth et al., 2011) or activation of cAMP-regulated K^+ channels (KvLQT1) by agonists of the cAMP pathway, such as ß-adrenergic compounds (Amaral & Kunzelmann, 2007) and blockers of phosphodiesterase (PDE) like amrinone or milrinone (Amaral & Kunzelmann, 2007) are all potential approaches to be explored in by-passing therapies for CF.

6.2. Gene & cell therapies

To date, gene therapy has failed to demonstrate a clinical benefit for CF. Notwithstanding, much knowledge has been gained from the preclinical and clinical studies that were performed. This includes key information about endpoints for efficacy assessment (Griesenbach et al., 2008, 2012) and the basic biology of the epithelium that is essential for a better understanding of the CF pathophysiology. Most recent efforts have been carried out by the UK CF Gene Therapy Consortium [reviewed in: (Armstrong et al., 2014)]. The results of the large multidose (12 administrations at monthly intervals) gene therapy clinical trial using a lipid vector will be available in the second half of 2014.

One major problem of single-dose gene therapy achieved by vectors that insert its CFTR transgene into the hosting cell (e.g. lentiviruses) is that cells lose expression of the "intruding" gene over time, lasting only for 4–6 weeks. This is mostly due to the usage of a "shorter version" of the *CFTR* gene (called complementary DNA, or cDNA) which is only ~6 kb instead of the long (~190 kb), full genomic CFTR gene. The absence of intercalating regions (introns) in such "shorter gene versions" leads to silencing of the genes by complex mechanisms, still not fully elucidated. Thus, one plausible alternative is to use larger CFTR constructions which, however, due to their large size cannot be inserted into conventional vectors. Human artificial chromosomes (HACs) containing half of the CFTR genomic sequence were already inserted into mammalian cells by suicidal bacteria and shown to maintain CFTR expression over 50 cell generations (Laner et al., 2005; Rocchi et al., 2010). Although with many barriers to overcome, such an approach may still hold promise of gene therapy success for CF.

Another approach which is becomingly increasingly attractive is the genomic correction of a mutation in the patient's own cells (Murphy & Atala, 2013). The classical approach of *CFTR* gene targeting to correct a mutation by Small Fragment Homologous Replacement (SFHR) exhibited very low efficiency: ~4% (Colosimo et al., 2001). However, more recently it was shown that it is possible to edit the genome of cells at much higher efficiencies using Transcription Activator-Like Effector Nuclease (TALEN) or Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/CRISPR-associated (Cas) systems) technologies (Gaj et al., 2013; Mali et al., 2013) in parallel to differentiate human embryonic pluripotent stem cells (hESCs), into a fully differentiated airway epithelium [reviewed in: (Siller et al., 2013)]. Moreover, although historically hESCs embryonic stem cells were thought to be the most likely workable source of pluripotent stem cells, Nobel-prize winner Yamanaka has shown the feasibility of generating induced pluripotent cells (iPS) from adult fibroblast cultures (Takahashi & Yamanaka, 2006). This breakthrough has opened new avenues for generation of stem cells and their differentiation into any tissue including lung epithelium for therapeutic applications such as CF [reviewed in: (Moodley et al., 2013)].

So, combination of these approaches where somatic cells can be obtained from CF patients, corrected in vitro by TALLEN/CRISPRs to correct genomic CFTR and then direct differentiation to generate a functional airway epithelium to be administered to the patient with CF appears today as a potentially feasible approach. In the context of CF, a proof of concept study has already demonstrated the feasibility of this approach in epithelial organoids (Schwank et al., 2013). Indeed, the CRISPR/Cas9 genome editing system was used to correct the CFTR *locus* by homologous recombination in cultured intestinal stem cells of patients (Schwank et al., 2013).

7. Impact of new treatments on the clinical course of CF, clinical practice and clinical decision making — perspective from the CF clinic

The potential for CFTR modulator therapy and other strategies that attack the basic CF defect has been a tremendous boost to the CF community and generally very positive for those giving and receiving care. Despite this, there are challenges beyond those posed by the complexity of CFTR dysfunction.

7.1. Natural history of CF

The natural history of CF has changed and an indication to support this is the reduction in the rates of lung function decline from historical rates of 2–3% per year loss of FEV_1% predicted to ~0.5% per year (Que et al., 2006). Consequently, the number of adults with CF is rapidly increasing. As discussed earlier, the need for 'new' trial outcome measures has been clear as studies using traditional trial endpoints (e.g. change in lung function) demand vast numbers of patients to achieve sufficient power.

It is now important to see if improved short-term outcomes of CFTR modulators in the setting of clinical trials are maintained in the "real world" setting over longer time periods and ultimately whether such therapies change the natural history of the disease. Furthermore, as many children less than five years of age have structural changes of bronchiectasis (Mott et al., 2012; Sly et al., 2013), it is important that

trials be performed in children under the age of six (the lower age limit in most CF clinical trials which have included children) (Stick & Sly, 2011). Short-term studies are ongoing, examining the effect of ivacaftor in young children and the results are eagerly awaited.

Over the past 20 years, a number of effective therapies have moved from clinical trials to the clinic including dornase-alpha, tobramycin solution inhalation and hypertonic saline and macrolide antibiotics amongst many others (O'Sullivan & Freedman, 2009; www.cff.org/research/drugdevelopmentpipeline). These may well not only have contributed to the decrease in the rate of lung function decline but also have impacted on trial design, outcome measures and numbers required to study (Liou et al., 2010). In addition, the burden of daily treatments has increased and it is well known that adherence to complex regimens of CF therapies is poor in some and inadequate in many (Abbott et al., 1994; Sawicki et al., 2009; Sawicki & Tiddens, 2012). Thus studies of strategies which promote adherence are important as it may be necessary for combinations of CFTR modulators to be prescribed for many years as has been the case in the treatment of another chronic disease, HIV infection (Pennings, 2013).

7.2. CFTR modulators

The promise of life-changing therapies for CF was raised following the discovery of the *CFTR* gene in 1989 (Riordan et al., 1989). With the advent of CFTR modulators and especially as the phase 3 trials were launched (Opar, 2011), the enthusiasm to participate has led to very rapid recruitment for studies (www.CFTR.2.org, 2014). At times when trial participation was limited, disappointment has occurred in the CF community, amongst CF teams and even beyond as has been seen by the volatility of share prices of companies investing in novel compounds. In recent years, social media has also provided a conduit for communication about CF care in general between patients and families and discussion of current and ongoing, as well as future clinical trials and provides challenges in limiting discussion about the impact of therapy on individuals actively participating in studies (Foulkes, 2011).

7.3. New dilemmas

Several clinical dilemmas have emerged over the past two years as ivacaftor has been prescribed in the clinic. In many, the impact on health and quality of life is dramatic. The access of ivacaftor on a "Named Patient Program" prior to registration and or remuneration has allowed patients with advanced disease access to the drug, e.g. patients on the active lung transplant waiting list. What should the patient do and what should the CF team advise if the patient improves but remains in respiratory failure and a donor becomes available (Barry et al., 2014)? Secondly, the improved health in young women after commencing on ivacaftor may lead some to consider "am I well enough to have a baby?" Will the woman's health deteriorate on cessation and what will be the impact on a potential pregnancy? Thirdly, what should the clinicians' advise be when confronted with a patient who wishes to stop other maintenance treatments for CF as they are time consuming and now perceived (by the patient) to be unnecessary as the patient feels so well. Finally, consultations in the clinic have become very focused on CFTR modulators, their study and 'what is my CF gene'? Having a systematic approach to address questions as they arise in the clinic and where required access to extended *CFTR* gene testing should be considered.

To date, one CFTR modulator, ivacaftor, has been proven to be effective in children over six years of age and adults and the results of other studies are awaited. Industry is currently working on the development of many further CFTR modulators and these may come to clinical trial in the next several years. Regulatory approval of ivacaftor was provided by the FDA and the EMA over the past 18 months and funding approved in many jurisdictions. The cost of ivacaftor is not publically disclosed in most countries; however it is known to be extremely high compared with other therapies in CF and has been subject of some discussion in the medical literature (Bush & Simmonds, 2012; O'Sullivan et al., 2013; Cohen & Raftery, 2014). As the prevalence of the CF mutation, G551D, varies from country to country, the financial impact on the CF health budget varies. In Ireland, 14% of patients have this mutation, whereas in The Netherlands and Belgium it is rare (De Boeck et al., 2014). In high prevalence countries, it is possible that the cost of ivacaftor could exceed the current total budget for CF care and has and will continue to be contentious where there are so many competing demands on the limited health budgets (Bush & Simmonds, 2012; Cohen & Raftery, 2014). In February 2014, the FDA extended approval for ivacaftor to include another eight CFTR-gating mutations and it is estimated that this will allow an additional 150 patients in USA access to this therapy (investors.vrtx.com/releasedetail.cfm?Releaseid=827435). It is unclear how this will impact on care in the future as more CFTR modulators become available. As the readership of P&T would well appreciate the development and study of new drugs requires enormous investment and is a high risk venture with many compounds progressing to phase 3 trials not proceeding to regulatory approval (Simon, 2008; Garazzino et al., 2013). To support this investment, the NIH has recently launched a programme (Accelerating Medicine Partnership; AMP) which aims to bring together government, not-for-profit organisations and industry to accelerate drug discovery by enhancing current pathways (Anonymous, 2014).

8. Conclusions

Since the discovery of the *CFTR* gene, the understanding of the complex biology of CFTR protein function has advanced significantly and allowed prospects of developments of therapies specifically designed to address the basic defects of CF. Several CFTR modulator therapies including 'potentiators', premature termination codon 'read-through' therapies and 'correctors' that underwent extensive in vitro evaluation are at present in late phase clinical trials. Ivacaftor, the first of these therapies, has demonstrated 'proof-of-principle' for CFTR modulators and is now licenced for global use in patients with the G551D *CFTR* mutation. Ongoing clinical trials are examining the safety and efficacy of first generation combination corrector/potentiator drugs for the most common *CFTR* mutation, F508del and results are eagerly awaited. Other CFTR modulator drugs have been identified and are currently being evaluated in pre-clinical studies and early phase clinical trials. Recent improvements in clinical care have resulted in improvement in outcomes for people with CF including reduced rates of lung function decline. Therefore determination of benefit of new therapies has led to intense investigation of clinical trial end-points such as lung clearance index. Whilst there are significant challenges to deliver these new therapies, there is much excitement in the CF community for therapies which have the potential to alter the natural history of CF. To obtain our ultimate goal of full CFTR correction in all patients, we need to continue basic biology research efforts.

Conflicts of interest

Scott Bell has participated and been supported to attend Investigator Meetings for Vertex Pharmaceuticals, has been a member of a Writing Group for manuscript preparation (combination ivacaftor/lumacaftor phase 2 study) and has been a site PI for a number of Vertex-sponsored trials. He has been supported to attend and to speak at Symposia (Gilead).

Kris De Boeck has served on advisory boards for Vertex, Ablynx, Aptalis, Galapagos, Gilead, Pharmaxis and PTC. She has been the principal investigator for studies initiated by Vertex, Gilead, Pharmaxis and PTC.

Margarida Amarail has served as a consultant to Vertex and Galapagos, has been supported to attend and to speak at Symposia

(Novartis, Gilead and Vertex) and to participate in an educational grant programme by Facilitate Ltd.

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