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Cystic fibrosis genetics: from molecular understanding to clinical application

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Abstract

The availability of the human genome sequence and tools for interrogating individual genomes provide an unprecedented opportunity to apply genetics to medicine. Mendelian conditions, which are caused by dysfunction of a single gene, offer powerful examples that illustrate how genetics can provide insights into disease. Cystic fibrosis, one of the more common lethalautosomal recessive Mendelian disorders, is presented here as an example. Recent progress in elucidating disease mechanism and causes of phenotypic variation, as well as in the development of treatments, demonstrates that genetics continues to play an important part in cystic fibrosis research 25 years after the d iscove1y of the disease-causing gene.

Cystic fibrosis (OMIM 219700) is a life-limiting autosomal recessive disorder that affects 70,000 individuals worldwide. The condition affects primarily those of European descent, although cystic fibrosis has been reported in all races and ethnicities. Abnormally viscous secretions in the airways of the lungs and in the ducts of the pancreas in individuals with cystic fibrosis cause obstructions that lead to inflammation, tissue damage and destruction of both organ systems (FIG 1). Other organ systems containing epithelia -such as the sweat gland, biliary duct of the liver, the male reproductive tract and the intestine -are also affected. Loss of pancreatic exocrine function results in malnutrition and poor growth, which leads to death in the first decade of life for most untreated individuals. Replacement of pancreatic enzymes and intensive therapy guided by multidisciplinary teams have revolutionized the treatment of cystic fibrosis, resulting in progressive improvements in survival to a median predicted age of 37years for children born with cystic fibrosis today[1]. Obstructive lung disease is currently the primary cause of morbidity and is responsible for 80% of mortality[2].

Twenty-five years ago, a variant (p.Phe508del; also known as F508del in legacy nomenclature) in the cystic fibrosis transmembrane conductance regulator *(CFTR)* gene was found to be the most common cause of cystic fibrosis[3-5]. Demonstration that CFTR functions as a chloride channel regulated by cyclic AMP (cAMP)-dependent phosphorylation [6] was consistent with the ion transport disturbances documented in cystic fibrosis tissuesM Key insights into cystic fibrosis pathophysiology were derived from the

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study of CFTR mutants[9], correlation ofCFTR dysfunction with the cellular manifestations of cystic fibrosis[10] and elucidation of protein partners involved in biogenesis and membrane functionu Identification of disease-causing variants in *CFTR* contributed a tool for both the diagnosis of cystic fibrosis and the identification of cystic fibrosis carriers[12], demonstrated the degree to which CFTR dysfunction correlates with clinical features[1] and revealed that CFTR dysfunction can create phenotypes other than cystic fibrosis[14] Over the past 5 years, there has been remarkable progress in the development of small-molecule therapy targeting CFTR bearing select disease-causing variants[15,16]

The purpose of this Review is to highlight advances over the past decade in our understanding and treatment of cystic fibrosis that were informed by genetics. Given the breadth of the cystic fibrosis field, not all of the important contributions and publications relevant to the topic can be included. Examples have been chosen to illustrate that genetics continues to have a role in the research of Mendelian disorders long after the causative variants and the responsible gene have been discovered. This Review covers new insights into the processing defect caused by the F508del variant, advances in stem cell technology that can enable testing of therapeutics for a wide range of *CFTR* genotypes and the development of new animal models that are informing our understanding of organ pathology in cystic fibrosis. I also summarize progress in parsing genetic and nongenetic contributions to variability in cystic fibrosis and in the identification of modifier loci. The final section describes efforts to determine the molecular and phenotypic consequences of the majority of cystic fibrosiscausing variants and to develop molecular treatments for every defect in *CFTR.*

Insights into disease mechanism

Molecular basis of CFI'R dysfunction. Almost 2,000 variants have been reported to the Cystic Fibrosis Mutation Database, one of the first and most successful locus-specific databases. Among these variants, 40% are predicted to cause substitution of a single amino acid, 36% are expected to alter RNA processing (including nonsense, frameshift and mis-splicing variants), 3% involve large rearra ngem ents of *CFTR,* and 1% affects promoter regions; 14% seem to be neutral variants, and the effect of the remaining 6% is unclear. Diseasecausing variants can affect the quantity and/or function of CFTR at the cell membrane (FIG 2). Historically, *CFTR* variants have been grouped into five (and sometimes six) functional classes[9] The class system provides a useful framework for understanding the primary defect at the cellular level. However, binning of variants into one class is problematic, as multiple processes can be affected by a single variant. For example, F508del causes aberrant folding ofCFTR and subsequent degradation of the majority of the synthesized protein [17] The minor fraction of F508del-CFTR that is trafficked to the cell membrane has severely reduced membrane residency and aberrant chloride channel function[18]. Furthermore, the three-nucleotide deletion responsible for the F508del variant also causes a synonymous change in the triplet that encodes isoleucine at codon 507 (ATC-7ATT). The change alters the structure of the F508del-CFTR mRNA, which leads to a reduction in translation efficiency[19]. Thus, F508del could be assigned to at least three classes. Various missense variants also cause defective processing and alter chloride channel function of CFTR[20,21]. Appreciating the diversity of effects caused by a *CFTR* variant is important in the design of molecular treatments for cystic fibrosis (see below).

Disease-causing variants provide a reservoir of naturally occurring deleterious amino acid deletions and substitutions that have proved to be informative for dissecting the tertiary structure of CFTR. CFTR is composed of three major motifs: domains that interact with ATP, termed nucleotide-binding domain 1 (NBDl) and NBD2; regions that anchor the protein in the membrane known as membrane-spanning domain 1 (MSD 1) and MSD2; and an area containing numerous sites for phosphorylation called the regulatory domain (also known as the R domain) (FIG 2). It had been recognized for some time that deletion of phenylalanine at codon 508 (F508) causes instability of NBD 1, but how this localized structural defect causes m isfolding of the entire protein was poorly understood[22]. Furthermore, it was known that disease-causing missense variants outside the NBD 1 domain - notably, a cluster in the fourth cytosolic loop (CL4) within MSD2 -also cause misfolding of the protein[224]. Modelling based on the atomic structure of related proteins[25,26] and cysteinecrosslinking experiments[27] revealed an interaction between the NBDs and MSDs of CFTR. Notably, F508 occurs at an interface between NBDl and CL4, and seems to be capable of forming hydrogen bonds with arginine at codon 1070 (R1070)[26]. Restoration of NBD 1 assembly using suppressor mutations produces only partial recovery of CFTR processing, which indicates that the F508del variant also affects interactions elsewhere in the full-length protein [28]. Intriguingly, introduction of the disease-associated p.Arg1070Trp (legacy R1070W)[29] variant in CL4 and correction of NBD 1 misfolding using synthetic suppressor mutations could restore processing to F508del-CFTR [28,30,31]. These findings lay the groundwork for structure-based selection of molecules that correct the processing defects in CFTR caused by disease-causing variants[32].

Tissue culture. Assessment of the functional consequence of variants requires the appropriate cellular context. For CFTR, primary epithelial cells are the most relevant system; however, these cells are short-lived and require accessioning from internal organs such as the lung . Furthermore, availability and compliance substantially limit acquisition of primary cells from individuals with rare *CFTR* genotypes. Recent advances in stem cell biology have provided new methods for generating well-differentiated cell lines from individuals with cystic fibrosis. Human embryonic stem cells and intestinal stem cells from individuals with cystic fibrosis have been coaxed into differentiating into secretory epithelial cells that manifest defects in CFTR-mediated chloride transport[34,35]. Small-molecule correctors for the F508del variant were efficacious in restoring CFTR function in both cell types, which showed the utility of these systems for evaluating therapeutics[32,34]. Genetic reversion of the F508del variants to wild type using CRISPR-Cas9 editing has been achieved in intestinal organoids[36]. Conversion of well-differentiated epithelial cells from human ectocervix and trachea into 'conditionally reprogrammed' cells using Rho kinase inhibitor offers an alternative approach to derive individual-specific cell types[37].These methods provide new tissue models for examining function and dysfunction of CFTR from individuals with specific *CFTR* genotypes.

Animal models. One of the most useful tools in the investigation of genetic disorders is animal models. Cystic fibrosis is unique among human genetic disorders in that five animal models (mouse[38], rat[39], ferret[40], pig[41] and zebrafish[42]) have been created. Although these animal models have not replicated human cystic fibrosis precisely, their differences have proved to be highly instructive for understanding disease mechanisms at the organ-system level (TABLE 1). The *Cftr* gene in mice has been extensively manipulated to derive lines that do not express CFTR and lines that express CFTR bearing variants equivalent to those observed in humans (for example, F508del and p.Gly551Asp (legacy G551D)) [4]. Even though airway epithelial cells display ion transport abnormalities that are consistent with loss of CFTR function, overt lung disease is not evident in newborn or young mice with cystic fibrosis[44]. The absence of lung disease similar to that seen in humans with cystic fibrosis has been ascribed to the presence of alternative pathways for chloride transport in mouse epithelial cells[45]. This observation suggests that ion channels other than CFTR might be exploited to recover chloride transport in cystic fibrosis cells. Indeed, a suitable candidate may have already been identified in *TMEM16A* (also known as *ANOl),* which encodes a calcium-activated chloride channel first identified in mouse airways[46]. Despite the phenotypic differences between mice and humans, the study of cystic fibrosis mouse models has provided invaluable insights into the role of other ion channels in the development of lung disease, the biology ofintestinal obstruction and the evaluation of candidate modifiers[47], and such models also provide an *in vivo* platform for testing therapeutics[43].

To create animal models that are more likely to recapitulate disease mechanisms operating in the lung, investigators have selected species on the basis of specific anatomical features. Of particular importance are the number and distribution of airway submucosal glands — a site of high CFTR expression - in humans[48]. The lungs of pigs and ferrets represent reasonable approximations of human lung architecture, and successful *CFTR* knockouts have been achieved in both species using homologous recombination[40,49]. The porcine model of cystic fibrosis develops lung disease comparable to that observed in humans, albeit at an earlier stage of life[50]. Consequently, the cystic fibrosis pig provides an opportunity to address the sequence of events in early-stage lung disease and to evaluate therapeutics in new-born animals. One of the key issues in the early stages of human cystic fibrosis is the genesis of inflammation in the lungs. Some studies suggest that infection of the airways trigger inflammation, whereas others demonstrate the presence of an inflammatory response in the absence oflung infection[51]. This distinction is important, as treatment strategy of each scenario is markedly different. In the lungs of cystic fibrosis pigs, inflammatory markers are not elevated until polymicrobial infection ensues[50]. Furthermore, pigs with cystic fibrosis exhibit an inability to eradicate bacterial pathogens, which may be partly due to abnormalities in the pH of the airway surface liquid caused by reduced CFTR-dependent bicarbonate transport [52]. Reduction in the size of the large airways in newborn pigs with cystic fibrosis has been associated with air trapping, a defect noted in human infants with cystic fibrosis[53]. Congenital anomalies in tracheal rings in mice, pigs and humans with cystic fibrosis (TABLE 1) support the concept that defects in the development of the pulmonary tree contribute to early-stage lung disease in cystic fibrosis. Finally, ion transport studies in newborn cystic fibrosis pigs have questioned the longstanding concept that sodium absorption is increased in the airway epithelia[54]. The cystic fibrosis pig model provides compelling evidence that loss of chloride and bicarbonate transport, maldevelopment of the airways and infection are the important drivers of early-stage lung disease in cystic fibrosis.

Box 1

Twin studies for estimating heritability

A twin study represents a naturally balanced, age-matched design that provides a powerful method for determining the contribution of genetics too trait. This opprooch capitalizes on the different rotes of variant shoring among monozygotic twins (who ore presumed to be 100%identical except for *de novo* mutations), and dizygotic twins and siblings(who shore −50% oftheir variants). Environmental exposures ore similar (but not identical) *in utero* and continue to be similar os twins grow in the some household. Thus, by comparing the degree of similarity among mono zygotic twin pairs to di zygotic twin pairs, *on* estimate *con* be obtained ofthe degree to which trait variance *con* be attributed to genetic variation (that is, heritability)[117]. Heritability measures generated in this manner range from 0 to 1. Estimates approaching 1.0 indicate strong genetic influence, whereas those near zero exclude a prominent role for genetic variation. The effect of shored environment *can* be estimated by comparing trait variance among twin pairs living together to those living apart. finally, within-pair variance for monozygotic twins provides a measure of unique environmental and stochastic components, as the genetic variance is, by definition, almost zero between mono zygotic twins. As the number of twin pairs aviilable for study of a Mendelian disorder isgenerally small, only crude estimates of genetic control of trait variance can be obtained. Nevertheless, the approach outlined above is widely applicable, as family-based studies *can* be used to estimate genetic control of traits that constitute any Mendelion disorder.

Mammalian models of cystic fibrosis have also provided insights into disease processes in other affected organ systems. Pancreatic exocrine dysfunction is closely correlated with the development of neonatal intestinal obstruction in humans with cystic fibrosis. However, animal models exhibit minimal (mouse) to severe (pig) pancreatic exocrine disease com pared with humans, yet the incidence of intestinal obstruction is higher in all mammalian models than in humans (TABLE 1). Recovery of intestinal expression of CFTR prevents obstruction in cystic fibrosis mice, pigs and ferrets[40,55,56].These two observations suggest that loss of CFTR function in the intestine rather than pancreatic exocrine dysfunction is the primary cause of intestinal obstruction in cystic fibrosis. Diabetes mellitus is an age-dependent complication that affects 40% of individuals with cystic fibrosis by 35 years of age[2] this disorder is also closely correlated with pancreatic exocrine dysfunction. Destruction of the exocrine pancreas has been proposed to stress the endocrine pancreas, which leads to loss of insulin-secreting cells[57]. However, features of diabetes occur before the development of severe pancreatic exocrine disease in ferrets[58] and in the absence of substantial loss of insulin-producing cells in cystic fibrosis pigs[59]. Both observations suggest that cystic fibrosisrelated diabetes is the result of an intrinsic defect in the endocrine pancreas caused by loss of CFTR function.

Variation in disease severity

The relative contribution of genetic modifiers. Individuals with cystic fibrosis show a high degree of variability in disease severity, complications and survival. It was initially postulated that a substantial fraction of phenotypic variability would be explained by allelic heterogeneity in the dysfunctional gene[60]. *CFTR* genotype correlates well with pancreatic exocrine disease severity and modestly with sweat chloride concentration[61,62]. However, it has been difficult to detect a relationship between lung function and *CFTR* genotype[61,63], with a few notable exceptions[64]. Analyses of families with affected twins (BOX 1) have quantified the degree to which variables beyond *CFTR* — such as genetic modifiers, environmental factors and/or stochasticity -influence variability in lung disease severity (FIG 1). Affected monozygotic twin pairs exhibit greater similarity for lung function than affected dizygotic twin and sibling pairs (siblings were used as a proxy for dizygotic twins)[65,66]. By comparing clinical measures of affected twin pairs when they lived together to the same measures after they moved apart, 50% of the difference in lung function measures could be attributed to genetic modifiers. The remaining variation was due to environmental exposures, primarily those unique to each individual, and to stochastic factors[67]. Together, these family-based studies demonstrate that genetic modifiers have considerable influence on lung function variation in cystic fibrosis.

The contribution of genetic modifiers to four other traits that are relevant to survival in cystic fibrosis has been estimated (FIG 1). Chronic colonization of the lungs with the bacterial pathogen *Pseudomonas aeruginosa* is a feature of advancing lung disease in cystic fibrosis and is associated with reduced survival[68]. Establishment of chronic *P aeruginosa* infection and age at establishment are highly influenced by genetic factors[69]. Poor growth is a hallmark of cystic fibrosis owing to pancreatic exocrine disease and deficiency of insulin-like growth factor 1. Although replacement of pancreatic digestive enzymes has improved nutritional status of individuals with cystic fibrosis, those with extremely low body mass index (BMI) remain challenging to treat. Genetic control of BMI independent of *CFTR* seems to be substantial, as estimated heritability ranges from 0.54 to 0.8 (REFS 70,71). Affected-twin analysis also revealed that genetic modifiers are primarily responsible for the age at onset of diabetes (heritability: l.O; confidence interval: 0.42-1.0)n. Diabetes mellitus is associated with more rapid decline in lung function in individuals with cystic fibrosis, and medical management of glucose levels improves surviva17 . Finally, obstruction of the small intestine (a condition known as meconium ileus) complicates the management of 15% of newborns with cystic fibrosis. Strain-specific differences in the rate of intestinal obstruction in cystic fibrosis mice first showed that this trait could be modified by genes other than *Cftr*[74]. Analysis of twins with cystic fibrosis demonstrated that genetic modifiers have the predominant role in the development of intestinal obstruction (heritability: l.0) [75].

Finding variants that modify cysticfibrosis. Extensive understanding of cystic fibrosis pathophysiology presents an opportunity to interrogate candidate genes as potential modifiers. In the lungs, loss of CFTR leads to exuberant inflammation, neutrophil recruitment, tissue damage and eventual replacement with fibrotic connective tissue. At least SO genes encoding proteins that participate in these cellular and tissue functions have been investigated as candidate modifiers of lung disease severity in cystic fibrosis[76]. As the results of candidate modifier gene studies have been extensively reviewed elsewhere[76,80], this Review highlights the insights gained from the study of one candidate gene that illustrates the potential and challenges in dissecting the complex interactions that modify disease severity.

A key element of lung disease in cystic fibrosis is the response to recurrent tissue injury. Transforming growth factor beta 1 *(TGFBl)* was an intriguing candidate for a modifier of cystic fibrosis lung disease, as it is involved in tissue repair and extracellular matrix production. Furthermore, variants in *TGFBl* have been associated with risk of asthma and chronic obstructive pulmonary disease (COPD) -two conditions that have features in common with cystic fibrosis lung disease. Two variants that increase levels ofTGFBl were correlated with severe lung disease as determined by airway flow measurements[81]. However, the direction of effect differs between cystic fibrosis and COPD; the same alleles show a deleterious consequence in one disorder but a protective effect in the other. It has been speculated that the presence of functional CFTR may invert the clinical consequences of increased *TGFBl* expression in individuals with COPD[81] Gene-gene and gene-environment studies using *TGFBl* variants have begun to address the issue of context. An obvious first place to look for gene-gene interaction is between *TGFBl* modifier variants and *CFTR* disease-causing variants. Drumm, Knowles and colleagues established *TGFBl* as a modifier primarily in individuals who are homozygous for the common cystic fibrosis-causing variant F508del[81]. A subsequent study demonstrated that the alternative alleles of the same *TGFBl* variants were associated with less severe lung disease, but the effect was limited to individuals with *CFTR* genotypes other than F508del homozygosity[82]. Interaction has been reported between *TGFBl* and mannose-binding lectin 2 *(MBL2),* which is another genetic modifier of cystic fibrosis . Variants associated with increased *TGFBl* expression amplify the deleterious effects of MEL deficiency upon lung infection and airway deterioration[84]. Environmental context is also important, as variants in *TGFBl* exacerbate the pernicious effects of exposure to second-hand smoke in patients with cystic fibrosis[85]. These observations indicate that deducing the clinical effect of a modifier variant greatly depends on the context in which it occurs.

To identify novel modifiers, research groups have combined patient populations to achieve sufficient power in genome-wide methods. Formation of a North American Cystic Fibrosis Gene Modifier Consortium — composed of investigators at the University of North Carolina, USA; the Hospital for Sick Kids in Toronto, Canada; and Johns Hopkins University in Baltimore, Maryland, USA - facilitated both association and linkage studies on 3,500 individuals. Although the three sites used different study designs, each agreed to use the same measure of lung disease severity, thereby enabling an analysis of unrelated subjects recruited by the centres in North Carolina and Toronto, as well as replication in related subjects participating in the Johns Hopkins study. In a genome-wide association study (GWAS), a significant region was detected between two genes on chromosome 11: *EHF,* which encodes an epithelial transcription factor, and *APIP,* which encodes an inhibitor ofapoptosis[86]. The known functions ofEHFand APIP suggest biologically plausible roles in modifying lung function in cystic fibrosis[86]. To search for rare variants that modify lung function, linkage analysis was carried out on 486 affected sibling pairs. A locus on chromosome 20ql3.2 harbouring only 4 genes was identified[86]. The identification of each locus demonstrates the feasibility of genome-wide approaches for uncovering new pathways that modify disease severity in cystic fibrosis.

Searching for genetic modifiers of other cystic fibrosis traits has provided mechanistic insights on several fronts. First, risk variants for other diseases operate as modifiers of similar conditions in patients with cystic fibrosis. Variants in four genes that confer risk for type 2 diabetes mellitus on the general population *(TCF7L2, CDKALl, CDKN2A/B* and *IGF2BP2)* modify age at onset of diabetes in cystic fibrosiif7(FIG 1). The Z allele of*SERYINA,* which causes al-an ti trypsin deficiency and confers risk for emphysema and liver disease, modifies risk of cirrhotic liver disease in cystic fibrosis[88] Second, modifiers exhibit pleiotropic effects on the cystic fibrosis phenotype. Variants in *SLC26A9* - which encodes a chloride and bicarbonate channel that interacts with CFTR -modify risk for neonatal intestinal obstruction and diabetes8iw. Furthermore, solute carriers associated with risk of neonatal intestinal obstruction also modify lung disease severity in young patients with cystic fibrosis *(SLC9A3* and *SLC6A14)* and age at first infection with *P. aeruginosa (SLC6A14)*[90]. Third, informing GWASs with knowledge ofCFTR function increases the yield of significant associations. This approach, termed hypothesis-driven GWASs, revealed that proteins residing in the same cellular location as CFTR are enriched for modifiers of neonatal intestinal obstructionw. Fourth, exome sequencing can find rare modifier variants. Variants in *DCTN4* - which encodes a dynactin protein involved in autophagy- are associated with age at onset of chronic infection with *P. aeruginosa*[91]. The identification of *DCTN4* as a modifier may provide a mechanistic link to defective autophagy in cystic fibrosis cells[92]. Conversely, loss-of-function variants in *CFTR* have been linked to a variety of other conditions (BOX 2). Together, these findings illustrate that the complex mechanisms underlying trait modification in cystic fibrosis can be informed by genetics, especially if approaches beyond standard association and linkage are undertaken.

Molecular diagnosis and therapy

Screening, diagnosis and functional annotation of **CFTR** *variants.* For almost 2 decades, a panel of 23 of the most common variants - vetted by an expert committee of the American College of Medical Genetics — has been used to diagnose cystic fibrosis and to screen for carriers and affected newborns . Expansion of the panel to increase sensitivity remained challenging, and the disease liability for most of the remaining *CFTR* variants was not known[94]. The Clinical and Functional Translation of *CFTR* (CFTR2) project was initiated in 2010 to increase the number of annotated *CFTR* variants. Clinical and *CFTR* genotype data collected on 39,696 individuals enrolled in cystic fibrosis patient registries and clinics in North America and Europe were used to determine the penetrance of variants for cystic fibrosiszo. To widely and rapidly disseminate results, features associated with each variant are available on a public website (see CFTR2). Content is tailored to educate patients, family and the public about the clinical implications that can and cannot be inferred from *CFTR* genotype. Inclusion of 127*CFTR* variants annotated as disease-causing in screening assays increased the sensitivity for detection of cystic fibrosis alleles in white European individuals from 85% to 95%20_ Although this improvement seems modest, the recessive nature of cystic fibrosis requires ascertaining variant status in two *CFTR* genes. Thus, at

95% sensitivity, only 0.25% of individuals with cystic fibrosis will have neither diseasecausing variant identified. Translation of these findings should improve the accuracy of screening programmes, and will aid diagnosis and treatment, as 99.75% of individuals with cystic fibrosis should carry at least one disease-causing *CFTR* variant. Owing to population differences in the frequency of CFTR variants, in particular the F508del variant, the sensitivity of screening is lower in non-white individuals. Inclusion of affected individuals from South America, Africa, the Middle East and East Asia in the current phase ofCFTR2 recruitment will provide a more complete inventory of variants found in non-white populations. As of late 2014, CFTR2 has obtained data on73,000 individuals, thereby exceeding the estimated number of individuals with cystic fibrosis worldwide (70,000). Given the completeness of the ascertainment, the sensitivity of screening in all populations will be improved, substantially in some cases, once variant annotation is completed.

Molecular therapyfor cysticfibrosis. The most exciting development in cystic fibrosis research since the identification of *CFTR* has been the successful implementation of therapy that augments the function of mutant CFTR. Increasing the chloride channel activityofmutant forms ofCFTR was shown to be a viable therapeutic approach two decades ago[95]. Development of compounds that selectively activated CFTR at doses that could be achieved *in vivo* proved to be difficult, and attention was focused on gene replacement methods for the next decade. However, the search for a molecular correction ofCFTR has not been abandoned. A unique partnership between a biotech company and the US Cystic Fibrosis Foundation initiated empirical screens for small molecules that target CFTR mutants. A promising compound termed ivacaftor (also known as VX-770 and Kalydeco (Vertex Pharmaceuticals)) increased chloride transport of primary airway cells bearing the G551D variant up to 50% of wild-type level[96]. Significant but more modest increases in G551D-CFTR activity were observed in immortalized cell lines (10-30% of wild-type CFTR)[97,98]. The increase in CFTR function observed in cell lines achieved levels that would be expected to produce a clinical response in lung function (BOX 3). Indeed, Phase Ill clinical trials over 4-week and 48-week intervals demonstrated that ivacaftor improved lung function (by 10% on average) and reduced sweat chloride concentration (to an average concentration below the diagnostic threshold of 60 mM) in individuals with cystic fibrosis carrying the G551D variant [15,99] (FIG 3a). Assessment of *in vivo* response to ivacaftor using sweat volume measures in 5 patients estimated recovery of 1.6-7.7% of wild-type CFTR function, while estimates derived from sweat chloride concentration and nasal potential difference measurements in 39 individuals indicated recovery of 35-40% of normal CFTR function[100,101].

Box 2

CFTR in other diseases

Male infertility

The concept that dysfunction of cysticfibrosistronsmembrone conductonce regulator (CFTR) could create disorders other than cysticfibrosis was first illustrated by obstructive mole infertility due to congenital bilaterol obsence of the vas deferens (CBAVD; OMIM 277180)[118] The onotomicol features of CBAVD ore identical to those seen in moles with cysticfibrosis, and some individuals with CBAVD hove subtle features of cysticfibrosis, such os mildly elevated sweat chloride concentration or minimol oirwoydiseose[119] However, a fraction of moles with CBAVD manifest no evidence of cysticfibrosis in detailed studies ofthe lungs, pancreas and S1Neot glond [120]. Furthermore, the distribution of *CFTR* variants differs between CBAVD and cystic fibrosis, and a much higher fraction of variants isassociated with residual function occurring in CBAVD [121]. Thus, CBAVD is port of the cystic fibrosis spectrum caused by CFTR dysfunction (FIG. 1), but it is also viewed as clinically distinct, particularly in moles with features limited to the vos deferens.

Pancreatitis

Loss-of-function variants in *CFTR* have also been linked too variety of conditions collectively termed 'CFTR-opothies', asreviewed by others[14,122]. Discovery of a pothologicol role for CFTR has proved to be particularly instructive for the study of poncreotitis. Poncreotitis is a known complication of cysticfibrosis, primarily occuring in individuals with preserved pancreatic exocrine function[123] Poncreotitis in the general population is a heterogeneous disorder with heritable and idiopathic sporadic forms[124]. A subset of heritable forms of recurrent acute and chronicponcreotitis can be attributed to CFTR dysfunction[124] As with CBAVD, the distribution of *CFTR* variants differs from that of cystic fibrosis. Recent evidence suggeststhat cells expressing CFTR bearing variants associated with poncreotitis, but not with cystic fibrosis, manifest defective bicarbonate transport, while chloride channel function is preserved[125]. This concept aligns well with the role of CFTR as an important mediator of bicarbonate transport in the poncreoticducts [126].

Manifestations in cystic fibrosis carriers

CFTR variants also act as risk alleles for multigenic disorders in the general population. Idiopathic disseminated bronchiectasis is a relatively rare pulmonary airway disease that manifests features similar to those observed in the lungs of individuals with cystic fibrosis. Several studies have shown a higher frequency of deleterious *CFTR* variants in individuals with bronchiectosisthon in control subjects". Bronchiectosisthot is complicated by infection with non-tuberculous mycobocterio or with the fungus *Aspergillu.s fumigatus* has also been associated with *on* increased frequency of *CFTR* variants[127,128] Chronic rhinosinusitis (CRS) is an aetiologically heterogeneous condition affecting ~15% of the general population in the United States. CRS is a common complication in individuals with cysticfibrosis. Genotyping of subjects meeting rigorous criteria for CRS revealed *on* excess of carriers of a single deleterious *CFTR* variant compared to disease-free controls[129]. In these studies, the entire coding region of CFTRwosexomined to exclude a second deleteriousvoriont. Support for the concept that presence of a single loss-of-function variant in *CFTR* predisposes to CRS was derived from the observation that the obligate heterozygous carriers of the deleterious *CFTR* variant (that is, the parents of individuals with cystic fibrosis) hod a threefold increase in prevalence of CRS[130]

Assessing whether poncreotitis, bronchiectosis or sinusitis *con* be attributed to CFTR dysfunction in a heterozygouscysticfibrosiscorrier requires detailed phenotyping to exclude other conditions, including mild forms of cystic fibrosis [131]. This challenge will become porticulorlypertinent oswe enter into *on* age where CFTR dysfunction *con* be treated *ot* the molecular level. Furthermore, associating *CFTR* variants with common multigenic disorders hos substantial implications, os there ore *on* estimated 20 million heterozygous carriers of cystic fibrosis in the world. Many carriers of deleterious *CFTR* variants ore becoming owore oftheir status os population testing iswidespread in the United States and is becoming more common in Europe. However, the penetronce of most *CFTR* voriontsfor the traits discussed above is not known. Establishing the penetronce of variants in diseose-ossocioted genesis a considerable and important challenge[132]

Box 3

Reversing cystic fibrosis: how much CFTR is needed?

Effective treatment of cystic fibrosis *at* the molecular level requires restoration of cysticfibrosistronsmembrone conductance regulator (CFTR) function in affected tissues. Genotype ond phenotype correlation revealed that organ systems hove different requirements for CFTR function [13] Splice sitevoriontsthot reduce but do not eliminate production of some CFTR mRNA hove been porticulorly informative in this regord [133]. Improvement in lung function meosuresond otherfeotures of cysticfibrosis (for example, S1Neot chloride concentration) occurs *at* CFTR mRNA levels above 5% of normal[134,135]. Splice sitevorionts that allow CFTRtronscript levels to reach 10-25% of normal levels hove been found in individuals that do not hove cystic fibrosis lung diseose[136,137] As the residual RNA transcript isoffull length, it hos been assumed that the quantity of transcribed CFTR protein will correspond to the level of remaining full-length CFTR transcripts. A lower boundary of 10%of normal levels for relieving pulmonary disease is supported by cell mixing experiments, which indicate that oirwoy epithelial ion transport is normalized when 6-10% of cells hove corrected CFTR function [138] Rescue of other key functions of the respirotoryepithelio may require higher levels of CFTR function. Restoration of mucus transport required −25% of cellsto be corrected[139]. These estimates represent overages; variation in genetic ond non-genetic modifiers islikely to broaden the range of CFTR correction required *at* the individual level.

The successful clinical deployment of ivacaftor encouraged the search for compounds that can correct other defects in CFTR. The prevalence of the F508del variant has attracted intense interest in reversing the folding defect caused by this variant. Screening of small molecules followed by chemical modification of active compounds led to the formulation oflumacaftor (also known as VX-809), which increased chloride transport of primary airway cells bearing F508del-CFTR to 14% of wild-type levels[102] A clinical trial oflumacaftor produced a dose-dependent improvement in CFTR function measured in the sweat gland of patients carrying the F508del variant [1]. However, CFTR function was not augmented in nasal epithelia, and lung function measures were not improved [1] (FIG 3a). As ivacaftor confers wild-type levels of open probability on F508del-CFTR, albeit as a result of a different pattern of channel gating[96] clinical trials combining ivacaftor with the corrector lumacaftor have been undertaken. This approach follows the reasonable logic that a potentiator can increase the activation of'corrected' F508del-CFTR, thus amplifying the effect achieved by each compound alone[102] (FIG 3b). A Phase II clinical trial demonstrated that the combination oflumacaftor and ivacaftor improved measures of lung function and sweat chloride concentration in individuals homozygous for the F508del variant [16] This encouraging result has been followed by the announcement that 2 Phase III clinical trials of combined lumicaftor and ivacaftor involving 1,100 F508 homozygotes over a 24-week period documented improvement in lung function (See Vertex press release). Although the changes in lung function measures were modest, there were concurrent improvements in secondary end points, providing encouraging evidence of clinical efficacy. However, two groups have recently reported that ivacaftor exposure for 48 hours diminishes the correction of F508del-CFTR conferred by lumacaftor in primary and immortalized cells[104,105] Reduction in the quantity of 'corrected' F508del-CFTR due to ivacaftor may explain the modest responses observed in the clinical trials (FIG 3b). Thus, compounds selected for combinatorial therapy will have to be carefully screened for undesirable interactions. With a panel of 'correctors' and 'potentiators' in hand, screening can proceed for combinations that act cooperatively[32,105] (FIG 3b). Furthermore, the available drugs could be used to screen for novel compounds that interact synergistically to further recover function of F508del-CFTR [106] (FIG 3b).

Although F508del and G551D account for a large fraction of cystic fibrosis alleles, 7% of patients with cystic fibrosis carry neither variant. To extend available therapy to as many individuals as possible, variants that permit translation of the CFTR protein can be evaluated for response to clinically approved corrector and potentiator compounds. However, as hundreds of translatable variants have been found in *CFTR,* it will be challenging to perform clinical efficacy studies of all variants, and many of these variants are carried by only a few affected individuals. Therefore, a new approach is required to extend approved efficacious treatments to individuals with rare variants in *CFTR*. It has been proposed to group *CFTR* variants into theratypes according to their effect on the CFTR protein and in response to corrector and potentiator compounds. Previously unclassified variants can be provisionally assigned to theratypes on the basis of their effect on CFTR quantity and function in cell-based studies. Response of CFTR bearing the unclassified variant to the profile of compounds that define the theratype would confirm that the assignment is appropriate (FIG 3c). Measures of *in vivo* CFTR function can then be used to verify clinical response and to justify ongoing treatment of individual patients [107] To this end, 9 missense variants were shown to cause a defect in activation (that is, gating) of CFTR and reduction in chloride transport ranging from 0% to 9.7% of normal levels[108] As noted for the GSS 1D variant, ivacaftor treatment ofimmortalized cells expressing CFTR bearing each of these variants increased chloride transport from 21% to 1S7% of normal levels[108], thereby predicting that clinical response should occur in individuals carrying these variants (FIG 3c). Subsequently, a Phase III clinical trial of 39 individuals demonstrated that ivacaftor was efficacious for 8 of the 9 variants, leading to rapid approval by the US Food and Drug Administration (NDA 203188). The four individuals carrying one variant did not respond sufficiently to warrant approval for ivacaftor treatment. The rapid expansion of small-molecule therapy for cystic fibrosis, from cell-based studies to clinical application, provides a new paradigm for drug development for genetic disorders and an excellent example of the promise of personalized medicine.

To treat all patients with cystic fibrosis, it will be necessary to address variants that prevent or severely decrease production of the CFTR protein through alterations in RNA processing. Nonsense and frameshift variants that introduce a premature termination codon (PTC) pose several hurdles that must be cleared to achieve therapeutic quantities of the protein. Most PTC variants invoke RNA degradation through the nonsense-mediated RNA decay (NMD) pathway[109]. Counteracting NMD to stabilize mRNA *in vivo* is challenging owing to the possibility of off-target effectsno. Synthesis of a full-length protein requires readthrough of the PTC, and some success in suppressing nonsense variants in *CFTR* using compounds derived from aminoglycosides has been achieved [111] (FIG 3d). Clinical trials of PTC suppressors have documented modest recovery of CFTR function in the nasal epithelia, although improvement in lung function has not been reported [112]·m. Even when readthrough is successful, the incorporation of a non-native amino acid at the location of the nonsense variant could affect protein processing and function[109].Thus, augmenting the function ofCFTR with ivacaftor following PTC suppression seems to be a viable approach to exceed the therapeutic threshold (FIG 3d). Alternatively, strategies such as induction of the unfolded protein response could be used to attenuate NMD, thereby stabilizing transcripts with PTCs for possible readthrough [114] Variants that cause aberrant RNA splicing pose a different set of challenges. On the one hand, suppression of variants that activate cryptic splice sites, such as c.3717 + 12191 *C→7*T (legacy 3849 + 10kbC-7T), can substantially increase the amount of normally spliced RNA transcript and protein (FIG 3d). On the other hand, variants that alter canonical nucleotides in splice sites are proving difficult to treat, although manipulation of splicing factors (for example, U1 small nuclear RNA) and the splicing process (for example, *trans-splicing)* shows some promise [115] The remaining variants are rearrangements, which require replacement of one or more exons or the entire coding sequence of *CFTR.* Transfer of DNA to epithelial cells has been extensively explored as a therapeutic approach for cystic fibrosis, but efficient delivery to airway cells *in vivo* remains problematic[116]. Finally, although molecular treatment is at hand, variation in response[15,35] indicates that underlying individual differences will have to be addressed to achieve the goal of attaining a normal lifespan for all patients with cystic fibrosis.

Conclusions

The discovery of *CFTR* 25 years ago was a triumph for genetics and a potent demonstration of its ability to deliver the molecular culprit in a Mendelian disorder. Cystic fibrosis is now positioned to reap the dividends of personalized medicine as variantspecific therapy is deployed, and a growing understanding of the genetic and environmental modifiers of cystic fibrosis enables targeting of individual risk factors. The development of new genetic models of cystic fibrosis in pigs, ferrets, rats and zebrafish provides opportunities to investigate pathophysiology and to explore therapies at the earliest stages of disease. Newborn and population screening enables prospective management of affected individuals from birth, and genomic variation will provide information on the trajectories that individual patients are likely to follow. Genetics has played and will continue to play a key part in achieving a normal lifespan for individuals with cystic fibrosis.

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Glossary

Pancreatic exocrine	Pertaining to the portion of the pancreas that produces digestive enzymes that are combined with alkaline secretions from the pancreatic ducts and secreted into the intestine to aid digestion.
Locus-specific databases	Collections of DNA variants that have been reported in disease-associated genes.
CRISPR-Cas9 editing	A method that uses an RNA guide and *a* DNA-binding protein to cleave DNA at *a* specific location to create sequence-specific changes via homologous recombination with *a* donor template.
Intestinal organoids	Epithelial "mini-guts" grown *in vitro* from biopsies of the rectal mucosa or from stem cells from *a* single individual.
Airway submucosal glands	Mucus-secretingglands found in the connective tissue that provide fluid for hydrating the surface of the airway epithelial cells and enabling ciliary function.
Airway surface	Iiquid Fluid interface between the air and the cells in the lungs that confers protection from infection and facilitates removal of foreign particles.
Tracheal rings	Incomplete rings of highly elastic cartilage found in the anterior two-thirds of the tracheal wall.
Endocrine pancreas	Portion of the pancreas that produces hormones (insulin and glucagon) that are essential for glucose homeostasis.
Pseudomonas aeruginosa	Widely distributed gram-negative bacteria that show *a* predilection for acute and chronic infection of the Iungs of individuals with cystic fibrosis.
Meconium ileus	Obstruction of the gut that usually develops *in ubero* in the ileum of the small intestine and that is highly suggestive of cystic fibrosis.
Airway flow measurements	Series of standardized tests assessing the rate and volume of air that can be inhaled and exhaled: they are used to determine the degree of disease in the lungs in individuals with cysticfibrosis.

Vas deferens	A tubular structure that conveys sperm from the testis to the urethra of the penis.
Disseminated bronchiectasis	Pennanentdilation of the airwa}'S (bronchi) throughout the lungs.
Phase III clinical trials	The third of four phases of evaluating *a* drug in affected subjects that confirms its safety and efficacy.
Nasal potential difference	Measurement of voltage across nasal epithelia th at represents the transport of ions and that. under specific conditions. can assess the function of cystic fibrosis tran smembran *e* conductance regulator (CFTR) ***in vivo.***
Open probability	A measure of the average fraction of time that *a* channel is open.
Phase II clinical trial	The second of four phases of evaluating *a* drug in affected subjects that establishes the efficacy of *a* drug compared to *a* placebo.
Theratypes	A recently invented tenn used to classify disease-associated DNA variants according to the molecular-based treatment to which they respond.

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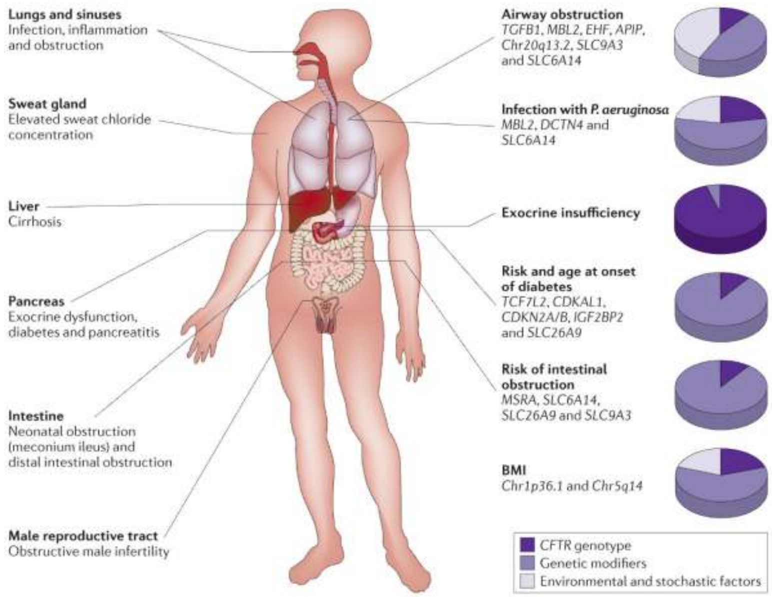

Figure 1. Cardinal features of cystic fibrosis and relative contribution of genetic modifiers to variation in select cystic fibrosis traits

A diagnosis of cystic fibrosis is based on the presence of clinical findings shown on the left, along with an elevated sweat chloride concentration (>60mM). The degree of organ system dysfunction varies considerably among affected individuals. Genetic modifiers and non-genetic factors both contribute to airwayobstruction and infection with *Pseudomonas aeruginosa* -two traits that define lung disease in cystic fibrosis. Cystic fibrosis transmembrane conductance regulator *(CFTR)* genotype is the primarydeterm inant of the degree of pancreatic exocrine dysfunction. The presence of *CFTR* variants associated with severe pancreatic exocrine dysfunction is essentially a pre-requisite for the development of diabetes and intestinal obstruction. In the setting of severe endocrine dysfunction, genetic modifiers determine when, and if, diabetes occurs and whether neonatal intestinal obstruction occurs. Genetic variation plays the predominant part in nutritional status as assessed by body mass index (BMI)[70].

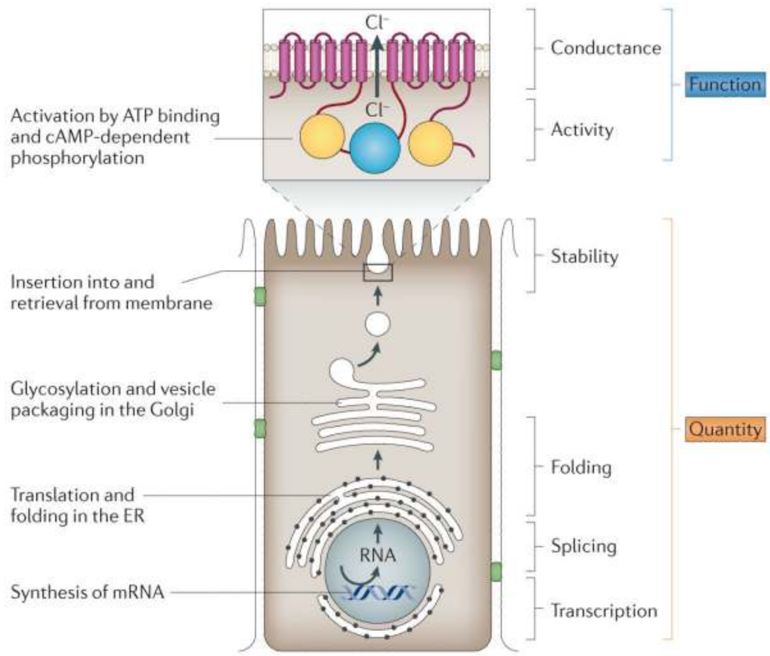

Figure 2. Molecular consequences of variants in CFTR

Thedegree to which epithelial ion transport is altered in an individual with cystic fibrosis is determined by the effect of each disease-causing variant on the quantity and the function of cystic fibrosis transmembrane conductance regulator (CFTR). The key steps of CFTR biogenesis in an epithelial cell are depicted. The membrane-spanning domains of CFTR are shown as red boxes, the two nucleotide-binding domains as yellow circles, and the regulatory domain as a blue circle. The quantity of CFTRprotein in the apical cell membrane is a product of the amount of RNA transcribed, the efficiencyof RNA splicing, the fraction of protein correctly folded and the stability of the protein in the membrane. The level and/or content of *CFTR* transcripts can be affected by disease-causing variants in the promoter (for example, c.-234T→A (also known as -102T→A in legacy nomenclature)) [140] and splice sites (for example, c. 3717+12191 C-→T (legacy 3849+10 kb C→1))[141], or by variants that introduce a premature termination codon (PTC) and that lead to RNA decay (for example, p.Gly542X; (legacy G542X)[142]. The processing of CFTR can be altered by variants that cause aberrant folding of the protein, leading to degradation (for example, p.PheS08del (legacy F508del))[18], or by variants that cause reduced membrane stability as a result of increased rates of endocytosis (for example, p.Asn287Tyr (legacy N287Y))[143]. The function of C FTR is dependent on activity of the ion channel and on the efficiency of conductance of ions through the channel. Disease-causing variants cause reduction in activity(for example, p.Gly551Asp (legacy G551D)[144] or changes in the conduct ion proper!ies of the chloride channel (for example, p.Arg334Trp (legacyR334W))[144]. cAMP,cyclicAMP; ER, endoplasmic reticulum.

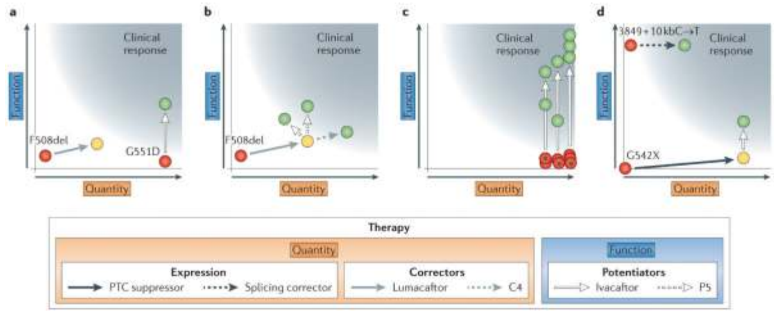

Figure 3. Molecular treatments for cystic fibrosis

The effects of various therapies (shown as arrows) on the quantity and/or function of mutant cystic fibrosis transmembrane conductance regulator (C FTR; FIG. 2) are shown. At least 10% of 'normal' CFTR function is required for 'clinical response' in lung function (grey region). The indistinct border of the clinical response accommodates various estimates of the amount of C FTR ion transport that have to be achieved and the probability that the product of quantity and function may generate a nonlinear function in certain circumstances. Red circles indicate the approximate product of quantity and function of CFTR bearing the indicated variant. Yellow circles indicate a shift in the product of quantity and function that does not achieve a clinical response, and green circles indicate a shift that does produce a clinical response. a I Single-drug strategies for G551D (also known as p. Gly551Asp) and F508del (also known as p.Phe508del) are shown. CFTR bearing the G551Dvariant is found at normal levels in the cell membrane but cannot be activated. The potent iator ivacaftor increases the activity of G551D-C FTR, thereby increasing chloride transport in airway epithelia to a level that produces a clinical response in the lungs. The common variant F508del causes a defect in protein folding, which greatlyd iminishes CFTR quantity at the plasma membrane and reduces both membrane residency and channel activity. Application of lumacaftor to cells increases the quantity and, to a lesser degree, the chloride transport of F508del-CFTR, but not to a level that produces a clinical response in the lungs. b ICombinatorial strategies for F508del are shown. In cell-based studies, chronic administration of ivacaftor counteracts the increase in CFTR stability conferred by lumacaftor[119,120]. Use of other potentiators that do not antagonize the effect oflumacaftor, such as the investigational compound P5, could provide higher therapeutic benefit[120]. An alternative approach is to combine correctors that affect different stages of CFTR folding, such as lumacaftor and C4 (REF. 32). c ITheratype strategy for rare variants is shown. Red circles with asterisks ind icatevar iants that permit product ion of normal or near-normal quantity of CFTR but that alter the activity of the C FTR chloride channel. As this functional defect is similar to that caused by G551D, each variant is tested for response to ivacaftor in cell-based studies. Recovery of C FTR function that exceeds 10% of levels seen in normal individuals indicates that a clinical response is expected, thereby justifying clinical trials. dIStrategies for nonsense and splice site variants are shown. The splice sitevariant 3849+ 10kbC→T (also known as c.3717+12191C→1) activates a cryptic splice donor site, causing reduction of the full-length *CFTR* transcript to ~8% of normal levels. Suppression of the cryptic splice site can increase CFTR protein levels above 10%of normal[145]. The nonsense variant G542X (also known as p.Gly542X) introduces a premature termination codon (PTC) that causes severe reduction in mRNA levels and an absence of the CFTRprotein. Use of

PTC suppressors increases transcript and protein levels, leading to a modest recovery of CFTR function that falls short of a din ical response. Combining ivacaftor with a PTC suppressor produces a 2. 5--4-fold increase in function of G542X-CFTR that could be sufficient to produce a clinical response in lung function[146].

Table 1

Phenotype features in humans and animal models with mutated *CFTR*

Features*	Human	Pig	Ferret	Mouse	Rat	Zebrafish
Aberrant chloride transport	↑	↑	↑	↑	↑	↑
Intestinal obstruction	↑	↑↑	↑↑	↑↑‡	↑↑‡	–
Growth disturbance	↑	↑↑	↑↑	↑↑	↑	–
Maldevelopment of trachea	↑	↑	↑	↑	↑	–
Pancreatic exocrine dysfunction	↑	↑↑	↑	–	–	–
Obstructive lung disease	↑	↑	↑	–	–	–
Liver dysfunction	↑	↑↑	↑	–	–	–
Diabetes mellitus	↑	↑	↑	–	–	–
Anomalous vas deferens	↑	↑	↑	–	↑	–

CFTR, cystic fibrosis transmembrane conductance regulator.

*Data are summarized from reviews[43,48] and primary publications[39,42,58,59,147].

‡Intestinal obstruction occurs after the neonatal period In murine models around the time of weaning.