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Cystic fibrosis: a comprehensive review

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Abstract With almost 100 000 people affected worldwide, cystic fibrosis (CF) represents one of the most fatal inherited conditions found in Caucasian individuals, being clinically characterized by a progressive pulmonary dysfunction, pancreatic insufficiency, and male infertility. Alterations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein has been found to be the sole responsible for the disease, with over 2000 defects being identified since 1989. Here we present, at a basic descriptive level, the current understanding of the clinical and genetic traits of CF gene modifications, the challenges associated with the early diagnosis and management strategies but also new emerging therapies that can improve the individual's life expectancy by enabling patient-specific treatment.

Keywords: cystic fibrosis, CFTR mutations, gene therapy, neonatal screening

Introduction

Cystic fibrosis (CF) is a complex, progressive, monogenic condition, with varying incidence rates that reach as high as 1 affected individual in 2000 to 3000 live births. Despite its long history, the disease was firstly described in 1938 as a clinical syndrome, but it was not until 1989 when gene cloning made possible the discovery of a link between the aberrant *CFTR* gene expression and the diverse clinical manifestations of the condition (Coakley and Boucher, 2007). However, despite the fact that only a low number of individuals display the long-established clinical manifestations of CF, the rate of carriers for this genetic mutation is remarkably prominent in the population worldwide (Cutting, 2015) and recent research indicated that even carriers for this mutation that exhibit no clinical symptoms could present a risk for various subclinical physiological affections which can be further aggravated by stress or other environmental stimuli (Wang et al., 2000; Cohn et al., 2005). Moreover, even though one of the most common causes of mortality associated with CF is represented by recurrent respiratory infections that lead to early obstructive lung disease and respiratory failure, other organs such as the pancreas, liver, intestine, bones, sinuses, and the male reproductive tract are prone to be affected (Rey et al., 2019), making CF a multisystem illness that can alter any organ with an epithelial origin (Coakley and Boucher, 2007).

However, regardless of the massive advances made in the understanding of the genetic and molecular disease mechanisms and clinical progress in diagnosis, unfortunately CF still remains an incurable affection, that when kept under control through an organised lifestyle, individuals can often reach adulthood with the average prognosticated age of survival of 43 for females and 48 for males (Keogh et al., 2018). However, the demanding and often tiresome routine that patients must maintain throughout their life in order to keep their infliction under control can often leave undesirable effects on both their physical, as well as their mental wellbeing (Jaques et al., 2020). With this in mind, the ongoing research focused on therapy improvement that could be directly connected with several important changes in patient care such as nutritional supplementation, airway clearance, long-term antimicrobial treatment (Shteinberg et al., 2021) and very recently the development of small molecule agents named CFTR (Cystic fibrosis transmembrane conductance regulator) modulators that can potentiate the function of the affected protein or restore the low levels of protein found on the surface of the cells (Patel et al., 2020).

In this paper we will review the genetic and cellular mechanisms of CF, its pathophysiology and diagnosis methods, followed by management of disease and future approaches for treatment and a potential cure for this condition.

Molecular biology of cystic fibrosis

1. CFTR structure and functions

Recognized previously as an autosomal recessive disorder, the responsible gene for CF was first identified in 1989 on the long arm of the 7th chromosome as an encoder for the CFTR protein (Boucher, 2004). CFTR, a member of the adenine-binding cassette transporter (ATPase family), is a transmembrane protein found on the apical surface of exocrine epithelial cells and functions as a chloride (Cl^-) channel (Nissim-Rafinia et al., 2007), even though there is also evidence that it may act as a transporter for glutathione and bicarbonate (HCO_3^-) (Molina and Hunt, 2017). Moreover, aside Cl^- and HCO_3^- ion transport, through epithelial sodium channel (ENaC) inhibition, CFTR can also lower the transport of sodium (Na) (Molina and Hunt, 2017). In terms of protein structure, CFTR comprises 1480 amino acids with a molecular weight of approximatively 170 kDa, and five domains which include two membrane-spanning domains (MSD1 and MSD2), each containing six transmembrane segments (TM1 - TM12) which form the channel, two nucleotide-binding domains (NBD1 and NBD2), capable of ATP hydrolysis and one regulatory domain (R), which presents a large number of phosphorylation sites (Cant et al., 2014) (Fig. 1).

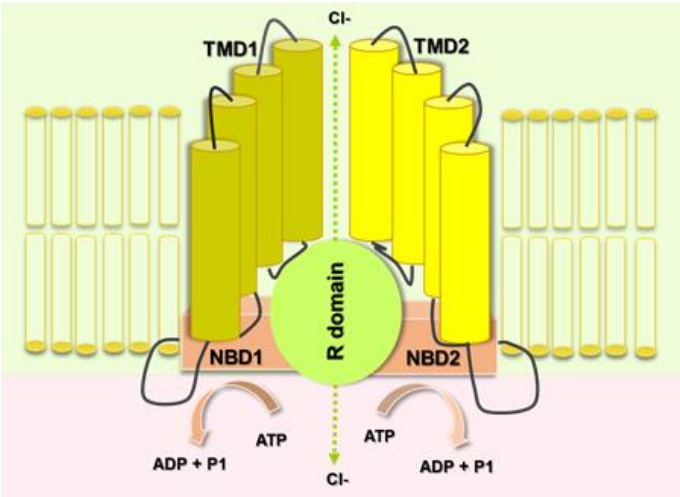

Fig. 1. The structure of the cystic fibrosis transmembrane conductance regulator (CFTR).

Chloride channel activation is attributed to two vital processes - the cyclic adenosine monophosphate (cAMP) dependent phosphorylation of sites from the regulatory domain by protein kinase A (PKA) and the hydrolysis of ATP by the NBDs (Liu et al., 2017). Any disturbances in this process caused by various alterations in the *CFTR* gene can induce a specific symptomology in terms of impairment of the ion transportation, changes in the mucous characteristics and inflammatory alterations (Rey et al., 2019). Even though the severity of the disruption can vary by mutation type and can affect the degree of the infliction, phenotypic variations can be found even amongst individuals with the same gene mutation (Liu et al., 2017), therefore suggesting that the CFTR channel fulfils a function more complex and intricate than it was thought.

2. CFTR mutation classification

To date, the CFTR Mutation Database comprise over 2000 known mutations (***Table 1***), though many of the effects on CFTR protein function caused by the presence of these alterations have not been extensively studied (Castellani et al., 2008). According to the effect caused by a specific DNA alteration on the expression of a certain gene, mutations can be categorised as missense (single amino substitution), frameshift (insertions or deletions), nonsense/stop (early termination codon) plus splicing mutations – mutations that affect mRNA processing and can cause incorrect intron splicing (Farinha et al., 2018). In this context, the *CFTR* gene mutations detected worldwide consists of 38.74 % missense, 16.25% frameshift, 10.93% splicing and 8.41% nonsense alterations (Rommens, 2021). Although no preferable hot spots for mutations have been found along the length of the *CFTR* gene, various studies suggested that certain CFTR amino acids (e.g., R117, R347, I506, and S549) and specific nucleotides (e.g., 460, 1058) present a higher affinity for mutation (Nissim-Rafinia, 2007). Moreover, it was observed that the MSD1 and NDB1 domains found in the first bisection of the protein, are more prone to mutations in comparison to the R domain, therefore suggesting the hypothesis that each domain possesses a different functional role (Nissim-Rafinia, 2007). For example, Kidd et al. (2004) demonstrated that the NBD domains of the CFTR protein possesses different role by observing an enhanced ATPase activity after the heterodimerization of both domains in comparison to one at the time. Moreover, another ABC protein (MRP1) exhibited similar results in terms of affinity binding and hydrolysis of the two NBDs, which were very distinct (Hou et al., 2003).

Table 1. Count of CFTR mutation type

Type of mutation	*Number*	*Frequency (%)*
missense	815	38.74
frameshift	342	16.25
sequence variations	269	12.79
splicing	230	10.93
nonsense	177	8.41
large insertion/deletion	59	2.80
frame insertion/deletion	43	2.04
promoter	17	0.81
unknown	152	7.22

Although all of the mutations attributed to CF affect the secretion of Cl^- in the epithelia where the CFTR gene is expressed, they can lead to different cellular phenotypes accountable for the wide clinical heterogeneity of CF (Farinha et al., 2018). By now it is clear that most of the CFTR alterations are associated with the classical clinical presentation where the affected individuals carry two severe *CFTR* mutations, thus presenting the traditional

phenotype in early age (usually below 1 year) associated with pancreatic insufficiency (PI), poor weight gain and elevated sweat chloride. However, only few mutations were identified as being responsible for an atypical phenotype, where individuals were diagnosed after the age of 10 and presented a better nutritional status, a mildly elevated sweat chloride and approximatively 60-70% had pancreatic sufficiency (PS) (Farinha et al., 2018).

In order to create a conceptual framework for these mutations, several classifications of the *CFTR* alterations have been proposed, most of them being based on the functional defects presented at protein level (De Boeck and Amaral, 2016). Initially when first implemented, the original classification grouped mutations into four classes, but as time progressed, another 3 groups have been added, leading to the most recent classification which described 7 major groups. However, this classification raised some recent concern due to the oversimplification of the mutational impact or the lack of categorically classification for the more recent *CFTR* mutations (Ferec and Cutting, 2012). Nevertheless, this classification may be of use for the initial therapeutic approach for some individuals, as several new strategies with the purpose of targeting CF symptoms caused by a specific class of *CFTR* mutations have been employed. Table 2 comprises the present acknowledged seven types of mutations.

Class I mutations are usually caused by nonsense, frameshift, or splicing alterations, having as a consequence the premature termination of codons (PTCs) and a truncated CFTR protein (Chen et al., 2021). PTCs can significantly reduce mutant mRNAs' half-lives through the nonsense-mediated mRNA decay (NMD) pathway but also via alteration of the pre-mRNA splicing pattern, thus leading to a small amount of non-functional CFTR protein (Nissim-Rafinia, 2007).

Table 2. *CFTR* mutation classification and the potential therapeutic strategies

class	I	II	III	IV	V	VI	VII
***CFTR* mutation**	non-functional CFTR protein	no CFTR traffic	defective channel regulation	reduced channel conductance	reduced CFTR synthesis	reduced CFTR stability	non-functional mRNA
type of mutation	nonsense; frameshift; canonical splice	missense; amino acid deletion	missense; amino acid change	missense; amino acid change	splicing defect; missense	missense; amino acid change	none
specific mutation examples	Gly542X Trp128X Arg553X 621 + 1G→T	Phe508del Asn1303Lys Ile507del Ser549Asn	Gly551Asp Gly178Arg Gly551Ser Ser549Asn	Arg117His Arg347Pro Arg117Cys Arg334Trp	3849 + 10kbC→T 2789 + 5G→A 3120 + 1G→A 5T	4326delTC Gln1412C 4279insA	1717 – 1G→A
potential therapy	synthesis rescuing	traffic rescuing	channel activity restoration	channel activity restoration	correct splicing	stability promotion	none

Moreover, genotype-phenotype studies demonstrated that the premature termination of codons is usually associated with the severe form of CF (Nissim-Rafinia, 2007). A potential therapeutic strategy for PTCs has been suggested and it involves the read-through of the nonsense codon, therefore allowing the full-length synthesis of the protein.

Class II mutations are associated with an impaired processing and transport of the CFTR protein which is mostly degraded in the proteasome (De Boeck, 2020). After the translation process of the CFTR protein, the resulting product goes through a glycosylation process and folding that takes place in the endoplasmic reticulum (ER) and Golgi apparatus, thus allowing the transportation of protein to the apical membrane of the cell. However, when a class II mutation takes place, this process becomes impaired and the processed protein will be abnormally degraded (Nissim-Rafinia, 2007). This class included alteration characterised by deletion of amino acids residues including one of the most well-known CF specific mutations, *i.e.*, F508del or missense mutations, which is in general associated with several clinical phenotypes and PI (Farinha et al., 2018).

Class III mutations are associated with a reduced activity of the Cl^- channel despite exhibiting adequate levels of ATP. Moreover, a wide range of mutations can modifiy the NBD-ATP binding regions, whereas some mutant variants can retain a certain degree of sensitivity to nucleoid binding (Chen et al., 2021).

Class IV mutations causes a diminished rate of the ion flow and duration of the channel opening even though the chloride currents are still generated in response to cAMP stimulation (Chen et al., 2021). Thus, both mutation classes III and IV can be associated with the production of the CFTR protein which can be processed, transported and inserted into the apical surface of cells but displays a dysfunctional conductance (Nissim-Rafinia, 2007).

Class V mutations cover promoter alterations that diminish the transcriptional process and substitution of the amino acids causing an inefficient maturation of the CFTR protein. However, splicing mutations represents one of the most common alterations for this class; therefore, the levels of correctly spliced mRNA end up being affected by either a partial exon skipping or intron inclusion. These mutations represent approximatively 50% of CFTR known mutations and include alterations

that are quite frequent in the general population. Moreover, certain mutations and polymorphisms can affect the splicing pattern by disrupting exonic splicing motifs (Nissim-Rafinia, 2007). Importantly, the mutations from this class can lead to different levels of correctly spliced transcripts among distinct individuals and among different organs of the same individual (Nissim-Rafinia and Kerem, 2002; Nissim-Rafinia et al., 2004). In addition, it was found that the CFTR expression can be attributed to the levels of correctly spliced transcripts, namely higher levels were associated with a milder pathology, while lower levels led to a more severe expression (Kerem, 1997). The therapeutic strategies for this mutation category aim to enhance the levels of correctly spliced mRNA and to up-regulate the *CFTR* expression by either the use of antisense oligonucleotides capable of inhibiting the cryptic splicing or by finding small molecules that could increase the levels of the correctly spliced transcripts (Nissim-Rafinia, 2007).

Class VI mutations lead to a significant instability of plasma membrane, due to the premature recycling of an unstable CFTR protein from the apical membrane that will be degraded in lysosomes (De Boeck, 2020).

Class VII mutations has been introduced in order to classify mutations where the CFTR mRNA is not even produced and no pharmacological approach could be identified (De Boeck and Amaral, 2016).

Clinical sympthomatology of cystic fibrosis

Caused by a dysfunctional chloride and/or other ions such sodium and bicarbonate transportation, CF is characterised by the presence of a thick, viscous secretion in the lungs, liver, intestine, pancreas, and the reproductive tract. The progression of the affection varies significantly and can begin from a few months to decades after birth (Fig. 2), with many individuals manifesting mild or non-traditional symptoms, making it even harder for clinicians to diagnose the conditions based on only a few atypical CF signs and symptoms. In the following section we will present the pathophysiology associated with the main organs affected by CF.

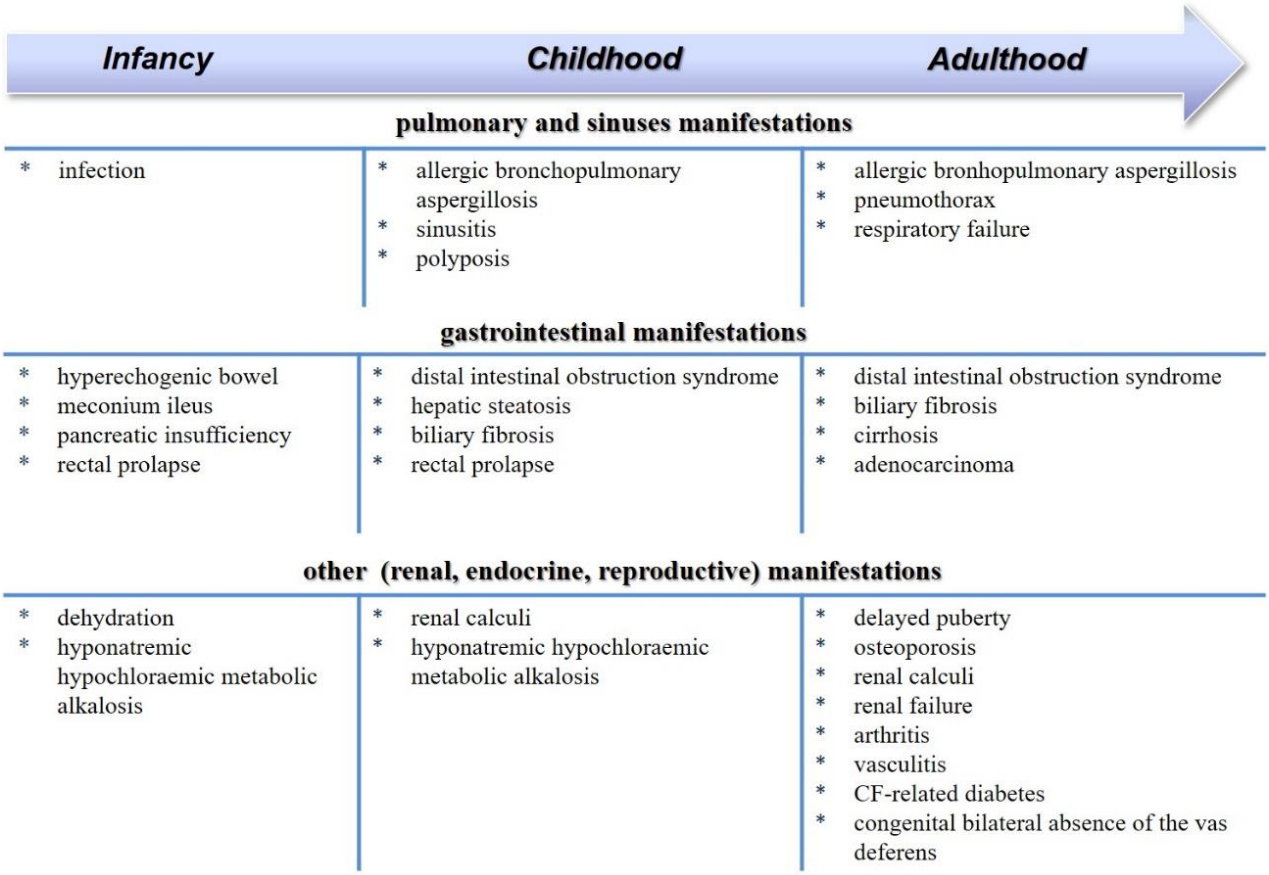

Fig. 2. The clinical manifestations of CF from infancy to adulthood

1. Pathophysiology of the lung disease

Generally, in the lung of healthy individuals, CFTR can be detected in the superficial epithelium and on the apical surface of the ciliated cells found in the gland ducts. However, in CF, the submucosal glands and distal airways are generally blocked with thick secretions, making the normal mucocilliary clearance impossible, therefore leading to an impaired airway protection against various bacterial agents (Horsley, 2015). As the disease progresses, chronic infections caused by either *Staphylococcus aureus* or *Pseudomonas aeruginosa* often take place and are usually identified with the help of

radiographic evidence of bronchiectasis (Chen et al., 2021). The precise mechanism by which infection occurs is still very unclear and very debatable and one hypothesis suggests that the inhaled bacterial agents cannot be cleared properly or that the initial infection may follow an insult to the lung. However, despite the nature of the stimuli that initiate the infection, the lungs of CF individuals often become colonised at an early age with bacteria, and as time progresses these infections become persistent and induce a florid inflammatory reaction that results in the recruitment and activation of neutrophils, event which will stimulate the production of mucin. Further on, due to the accumulation of the bacterial products, cellular debris and DNA polymers, the mucus becomes even more viscous and harder to expel. Moreover, the persistent neutrophil inflammatory response leads to the release of elastase and matrix metalloproteinases which in turn cause the proteolysis and chondrolysis of the bronchial cartilaginous support, with consequent bronchiectasis. This will result in filled up airways with purulent secretions and a never-ending cycle of infection, chronic inflammatory activity, and progressive destruction of the endobronchial structure (Horsely, et al., 2015). The symptoms associated with a time progress include acute aggravation of coughing fits, tachypnea, dyspnoea, increased sputum production, anorexia, weight loss and malaise. Moreover, these acute manifestations are associated with the transient loss of lung function that may be restored with treatment but more than often it progresses towards permanent loss of lung function over time (Chen et al., 2021).

2. Pathophysiology of the digestive system disease

Approximatively 85% of CF new-born babies present PI, with the additional 20% - 25% manifesting this condition only after the first year of life (Chen et al., 2021). Generally, in healthy individuals, CFTR is present in high levels on the pancreatic duct epithelium, being involved in the production of a high-volume of bicarbonate-rich secretion, which is responsible for the solubility of the enzymes derived from the acinar structures and their subsequent flushing into the duodenum. However, in the presence of CFTR with an impaired function, the volume of fluid and bicarbonate secreted is diminished, resulting in a secretion with an increased viscosity (Dondos and Westaby, 2007). Post-mortem studies (Imrie et al., 1979) and a murine *in vivo* model [Durie et al., 2004] suggested that the injury to the pancreas is secondary to duct obstruction by the high viscosity secretion, and it is likely that in most cases the pancreatic injury starts *in utero* (Imrie et al., 1979), where the initial blockage of the duct resulted in the progressive replacement of acinar structure with fatty and fibrotic tissue. The endocrine tissue is almost always preserved into infancy and the islet of Langerhans ends up being embedded into the areas replaced by the fatty tissue and fibrotic stroma (Dondos and Westaby, 2007). Moreover, various studies suggested other possible factors that could influence the evolution of pancreatic injury in CF, such as the inflammatory response (De Lisle et al., 2001). Data reported in literature indicates that the failure of duodenal bicarbonate secretion initiates a pancreatic acinar stress response associated with the activation of various inflammatory pathways (De Lisle et al., 2001). The presence of a persistent inflammatory reaction could further damage the pancreas and might explain the late progression from PS to PI in some CF individuals.

Common symptoms of PI include steatorrhea, a condition recognised through its specific symptoms such as very frequent, bulky and foul-smelling stools that may have an oily aspect, coupled with improper weight gain due to a defective malabsorption of fat and proteins (Chen et al., 2021). In addition, infants with untreated PI may present edema, hypoproteinaemia, electrolyte loss and anaemia due to the malabsorption of micro- and macro-nutrients. Moreover, some individuals may present manifestations caused by deficiencies of fat-soluble vitamins such as vitamin A, D, E and K (Chen et al., 2021).

Another CF-associated digestive system disorder present in about 10% to 20% of new-born babies is meconium ileus characterised by the obstruction of the bowel by meconium which can be either simple or complex (Derichs et al., 2010). The simple form occurs in the proximal area to the blockage and involves the dilatation of the small bowel, while the more complex condition results in further complications such as volvulus, tissue death, atresia or even intestine rupture (Chen et al., 2021). Affecting almost 44% to 50% of people with a well-known diagnostic of meconium ileus (Colombo et al., 2011), distal intestinal obstruction syndrome is characterised by the accumulation of viscous matter within the bowel lumen of the terminal ileum and caecum, causing either incomplete or complete blockage.

3. Pathophysiology of the urogenital tract disease

In males, the functional CFTR can be normally identified staring from 18 weeks *in utero* in various structures such as the epididymis, vas deferens, seminal vesicles and the ejaculatory ducts, reason why individuals with CF present these structures either atretic or obstructed or in some cases did not form at birth. Obstructive azoospermia due to the inherited bilateral absence of the vas deferens is found in over 95 % of male individuals with CF (Popli and Steward, 2007). Moreover, these individuals present atrophic or absent seminal vesicles caused mainly by dehydrated secretion that results in a blockage in the genital tract *in utero* (Filburn et al., 2016). In CF female individuals, the cervix, endometrium, and fallopian tubes express CFTR but from an anatomical point of view they are normal. However, the prospective fertility of females with CF is yet to be determined since they choose to not procreate, but studies have reported success rates of around two-thirds of women with CF who choose to conceive without the help of assisted reproductive techniques (Edenborough, 2015).

4. Cystic fibrosis-related diabetes

Cystic fibrosis-related diabetes (CFRD) is a very common complication that touch approximately 35% of the CF individuals (Olesen et al., 2020). CFRD is a direct consequence of *CFTR* gene mutation, so there is no coincidence that type 1 or type 2 diabetes have a very different pathophysiology than CFRD (***Table 3***), with only a few common manifestations such as the disappearance of islet cells' function and varying degrees of insulin resistance leading to a deleterious tolerance to glucose (Hart et al., 2018; Moran et al., 2018).

In terms of clinical features, due to the hidden nature of the condition, the classical characteristics of diabetes are observed in only about one-third of individuals diagnosed with CF, and in many cases the symptoms are recognised solely due to the CF affection alone (Hodder, 2007). The average age that CFRD becomes apparent is around 18-21 years old (Koch et al., 2001), with a female preponderance over male (17.1% vs 12%) (Marshall et al., 2005).

Table 3. Comparison of CFRD with type 1 and type 2 diabetes

	Age of onset	*Epidemiology*	*Body constitution*	*Symptoms*	*Insulin secretion*	*Diet (calorie)*	*Treatment*
Cystic fibrosis-related diabetes	18-21 years	5-10%	thin	uncommon	↓↓	high	insulin
Type 1 diabetes	<20 years	1%	normal	common	↓↓↓	variable	insulin
Type 2 diabetes	>40 years	5-7%	obese	common	↓	restrictive	insulin, OHAs

OHAs-oral hypoglycaemic agents

5. Other conditions associated with cystic fibrosis

Adult individuals with CF present a reduction in the bone mass when compared to healthy people of same age, sex and body mass index (BMI) (Nishiyama et al., 2018; Putman et al., 2019), due to the low vitamin D and K absorption, poor nutrition, physical inactivity and glucocorticoid treatment. Moreover, low bone mass was associated with severe lung disease, and it was present in approximatively 50% of patients with CF who were waiting for a lung transplant (Cairoli et al., 2019). Another 20-80% of people who suffer from CF present clinical manifestations of autoimmune diseases such as arthropathy and vasculitis. In addition, increasing evidence suggests that both young children and adults suffering from CF and with an additional risk factor such as diabetes can also present endothelial dysfunction (Eising et al., 2018; Vizzardi et al., 2019).

Furthermore, due the time-consuming and tiring management of the condition, depressive episodes and anxious feelings are more than often encountered in individuals with CF, with a prevalence rate of 10-19% in males to 22-23% in females (Quittner et al., 2014).

MODELS OF CYSTIC FIBROSIS DISEASE

1. In vitro models

The early pioneering in vitro research regarding the function and regulation of CFTR was conducted on various epithelial cell lines capable of expressing high levels of CFTR, with many of these cells being mainly isolated from carcinogenic tissue, lungs that were donated and later on were artificially immortalized or common airway cell cultures who were virally transduced in order to express high levels of a mutant CFTR protein. However, the use of cell lines that overexpress the CFTR protein could be questionable in terms of faithfulness of CF cell physiology (Molenda et al., 2014). For example, cell lines isolated from cancerous tissue represent a poor choice in the study of understanding how the disease can affect the individual's metabolism due to disruptions in the cell cycle dynamics and the Warburg effect (Jones and Thompson, 2009; Cairns et al., 2011; Phan et al., 2014). Furthermore, the in vitro study of endoplasmic reticulum stress in cell lines modified to overexpress CFTR could lead to the possibility of triggering unwanted effects such as endoplasmic reticulum stress. In order to overcome these impediments and be able to study the normal function of the *CFTR* gene, new cell culture methods that allow a favourable amplification of human primary cells have been developed (Chapman et al., 2010; Liu et al., 2012; Suprynowicz et al., 2012; Reynolds et al., 2016). One such technique is represented by conditional programming which allows the doubling of cell population for multiple times, which under the right cell culture conditions, can differentiate into mature mucocilliary cells that possess electrophysiological traits similar to those in primary cultures in a lower passage (Reynolds et al., 2016; Mou et al., 2016). By using this conditional reprograming strategy, a vast number of human primary-like cell cultures can be differentiated back to their almost-original primary cell states with a wide range of applicability for *in vitro* testing. Moreover, compared to the induced pluripotent stem cells, the conditionally reprogramed cells do not need further treatment with various growth factors in order to

propagate and do not require transfection with various exogenous genes (Molina and Hunt, 2017).

However, for individuals with rare CF genotypes, the preferred method of treatment needs to be specific for the certain individual and it is based on the genotype of both the pathogenic organism and the patient. Therefore, by using unique cell models that share similarities with specific patients, a more directed form of treatment for individuals with infrequent mutations can be developed (Mou et al., 2015; Dekkers et al., 2016). In this context, systems based on primary human airway cells harvested from CF individuals such as lung-on-a-chip, recellularized tracheal scaffolds, induced pluripotent stem cell-derived organoids, have been developed (Ikpa et al., 2014). Dekkers et al. (2016) collected rectal samples from individuals with uncharacterised genotypes such as G1249R/F508del and F508del/R347P and used them to create intestinal organoids with the purpose of investigating the effects of the newly developed CFTR modulators. The results obtained showed a positive correlation with the clinical outcomes, especially in terms of forced expiratory volume in 1 s (FEV_1), airway resistance at a flow of 0.5 L/s (Raw 0.5), sweat chloride levels and nasal potential difference.

Although the field of CF-specific disease models has been crowned with success, the future therapies that will be developed most probably will be challenged by the various and numerous rare mutants found in CF-individuals; therefore, the use of patient-derived organoids can represent an effective strategy to improve patient outcome.

2. In vivo models

The early ‘90s marked the introduction of the very first CF mouse model and ever since various strategies were used to develop strains that can mimic the pathophysiology of individuals with CF (Fisher et al., 2011), including one knock out mouse model that had as a primary target ENaC instead of CFTR (Mall et al., 2004). However, the developed murine CF models do not mirror the human disease to the perfection mainly due to the anatomical and physiological differences in organs (lung and pancreas) between mice and humans. E.g., mice with mutated CFTR can present an impaired chloride transport but the resulting pathology can differ from human CF models. On the other hand, mice with critical CFTR mutations may display gastrointestinal manifestations similar to those found in patients with CF, however, if not fed a special diet; these mice end up dying of intestinal obstruction soon after birth therefore making the use of CF mice model unsuitable as a human CF model. Moreover, the recurrent bacterial infections, chronic inflammatory activity, thick mucus accumulation and tissue remodelling have not been observed within the lungs of mice with CF, and a possible explanation for this phenomenon may be represented by the lack of the ciliated epithelium and submucosal glands. Another possible explanation could be represented by the presence of other chloride channel Ca-activated as ICACCS, which could make up for the CFTR dysfunction (Chen et al., 2021). In addition, the long-term investigation of the pathological effects of human CF disease by CF mice models is impossible due to the short lifespan of mice. In order to overcome some limitation imposed by mice models such as a short lifespan and inability to acquire spontaneous lung infections, CF ferret models were developed. In comparison to the mouse model, the ferret model presented submucosal glands and similar cell types as the human airway (Sun et al., 2010) but also blockage in the gastrointestinal area and pancreatic architectural distortions similar to humans with CF (Sun et al., 2014; Lavelle et al., 2016). However, a considerable effort is needed to produce enough CFTR knockout ferrets in order to ensure that some animals may survive infancy and reach puberty so that they can be used as CF models (Chen et al., 2021). In addition, it was observed that the features of the pulmonary infection of CFTR knockout ferrets at the beginning and end of life are similar to the pulmonary manifestations displayed in patients with CF, therefore highlighting the potential of this animal model (Chen et al., 2021). However, the animal that mostly resembles the human lung and gastrointestinal tract is the swine (Welsh et al., 2009; Meyerhold et al., 2020), making pigs one the most important species for CF investigations. In 2008, Roger et al. produced a *CFTR* gene knockout and F508del pig models using a recombinant adeno-associated virus (AAV mediated) method and it was observed that in the knockout model the absence of the CFTR protein with a normal function caused similar phenotypic effects in the lungs, gastrointestinal tract, and pancreas as those found in CF-individuals. Moreover, a significant difference between mice and pig models is the ability of the later to acidify the airway in a similar manner to how human airways are acidified in CF individuals. This phenomenon contributes to the bacterial colonization of the airways, which is further aided by various alterations in the biochemical and electrostatic interactions of bacteria with the secreted glycoproteins when the airway surface liquid pH is acidified (Tang et al., 2016). These important findings regarding the susceptibility of the CF airways to recurrent and chronic bacterial infections would have not been discovered without a more accurate human model such as the pig.

Recently, with the help of CRISPR/Cas9 method, CFTR knockout and F508del mutated CF rabbit models were generated (Xu et al., 2016). Rabbits are considered to be ideal animals that can mimic the CF affected human lung, due to their airway anatomy and inflammatory response which are similar to humans. Preliminary *in vivo* studies using CF rabbits to model human CF indicate that CF rabbit models will most likely represent the future in the *in vivo* study for human CF disease.

DIAGNOSIS OF CYSTIC FIBROSIS

Over the years the approach of diagnosis of CF has evolved with occurring advances in CF pathophysiology and genetics. When CF was firstly identified in the late '30s it was mainly perceived as a gastrointestinal pathology, therefore its diagnosis was purely constructed on the individual's phenotypic characteristics and presence of PI (Filbrun et al., 2016).

However, a major break-through in CF diagnosis was made when in children affected by this condition, the risk of developing hyponatremic dehydration was observed, therefore leading to the use of sweat chloride concentration measurement (sweat test) as a prime investigation for CF (Filburn et al., 2016).

Furthermore, the identification of the *CFTR* gene permitted an even more complex form of diagnostic, i.e., the genetic identification of the affection which led to the implementation of newborn screening (NSB) as an early possibility of diagnosis (Filburn et al., 2016). However, various regions do not have access to a newborn screening program; thus, the diagnostic criteria is based either on suggestive clinical features (***Table 4***) coupled with family history or on the evidence of CFTR dysfunction coupled with family history or either on the detection of two specific *CFTR* mutations.

Table 4. Clinical manifestations of cystic fibrosis necessary for a firm diagnosis

Organ	*CF clinical manifestation*
lungs	recurrent bacterial infections; bronchiectasis; pneumothorax; haemoptysis; respiratory failure
upper airways	chronic sinusitis; nasal polyps
pancreas	pancreatitis; pancreatic insufficiency
liver	neonatal jaundice; liver disease; fatty liver; cirrhosis; biliary calculi
intestine	meconium ileus; distal intestinal obstruction syndrome; malnutrition; dyslipidaemia
kidneys	urinary tract calculi
reproductive tract	male – congenital bilateral absence of the vas deferens; azoospermia; infertility female – cervical mucus abnormality
endocrine;	cystic fibrosis-related diabetes
sweat glands and skin	hypochloremic metabolic alkalosis; dehydration; aquagenic palmoplantar keratoderma

In order to establish a firm CF diagnosis for genotypes of differing clinical consequences such as NM_000249.3:350G>A(Arg117His), the sweat chloride investigation or the advanced electrophysiological evaluation of the individual is required (Shteinberg et al., 2021). The sweat test was introduced in the late '50s, when thresholds for normal and elevated sweat chloride levels were first established; therefore if the concentration of chloride surpass the 60 mmol/L limit, the diagnosis for CF was confirmed, while concentrations between 40-60 mmol/L would only raise suspicion and under 40 mmol/L were considered normal (Chen et al., 2021). However, sweat chloride increases with age so the sweat threshold for an infant is lower in comparison to an elder child or an adult (Filbrun et al., 2016). Moreover, individuals with unusual genotypes may present a normal sweat chloride concentration requiring additional testing in order to confirm a diagnosis.

Data found in the US Cystic Fibrosis Patient Registry indicates that the minimal age of diagnosis is around 6 months old, but the average age is generally around 3 years old, suggesting that most than often individuals are diagnosed in later childhood or even as adults (Filburn et al., 2016). This finding combined with various data indicating the relevance of a firm diagnosis in the first years of life led to the implementation of newborn screening programs for CF. However, early endeavours at finding a satisfactory biomarker were unsuccessful mainly due to the low specificity and sensitivity but also to the difficulty in obtaining proper specimens. After a pancreatic proenzyme called immunoreactive trypsinogen (IRT) was found in elevated levels in infants with CF, a new biomarker for CF with high sensitivity and unchallenging measurement was identified (Molina and Hunt, 2017). The newborn screening (NBS) panels includes *CFTR* mutations most commonly found in the local population and the IRT levels can vary from one year to another due to changes in ambient temperature or other cumulative factors. Moreover, despite its high sensitivity, the neonatal IRT test is not specific, mainly due to the fact that some infants without CF may often present transient high levels of IRT in the very first days after being born, or after a preterm birth, or due to perinatal stress. In addition, African-American ancestry could also represent a contributor for elevated IRT levels (Giusti et al., 2008). As consequence to this limitation, an additional step in the screening process was incorporated in order to increase its specificity (Wagner et al., 2012). In most countries, the additional step requires a DNA evaluation on the dried blood spot, while in others the IRT test is repeated 2-4 weeks later, at which time the new-borns affected by CF will still present high levels of IRT when compared to non-CF infants whose levels would have return to normal. Compared to the first approach, the later avoids detecting large number of carriers but requires the presence of the infant at the

testing centre after a couple of weeks and most importantly it does not present the same high specificity as the IRT/DNA approach (Grosse et al., 2004). Altogether, the benefits of NBS could be seen very visible on the life of individuals with CF who have been diagnosed in early infancy compared to later adults. A study conducted in 1984, by the Wisconsin Cystic Fibrosis Neonatal Screening Project, showed that individuals diagnosed through NBS presented a more favourable nutritional outcome, diminshed pulmonary complications and an ameliorated cognitive outcome due to the early correction of vitamin E deficiency (Farrell et al., 2001; Koscik et al., 2004; Lai et al., 2005).

After a positive NBS test, CF can be either confirmed or ruled out, depending on the results obtained from the combination of the sweat test and/or genetic evaluation. However, in some cases these investigations can offer inconclusive results, therefore the terms CFTR-related metabolic syndrome or inconclusive diagnosis were introduced to describe the situation when an infant is tested IRT positive but does not meet all the required diagnostic criteria for CF and most importantly seems healthy (Ren et al., 2015; Munck et al., 2015). One of the most common reasons for this situation is represented by the presence of one or more *CFTR* modifications of different clinical consequences, therefore newborns affected by this condition will either exhibit a sweat chloride concentration under 30 mmol/L and two CFTR variants or either a sweat chloride concentration in the range between 30-60 mmol/L and none or one CFTR mutations (Shteinberg et al., 2021). The majority of children with this syndrome will most likely not display a symptomatic manifestation of CF in childhood but in later years they may exhibit various clinical manifestations of the disease. Thus, a close long-term monitoring by clinicians is recommended.

However, the sufficient function of CFTR in the sweat gland may not always be enough to prevent the development of CF clinical manifestation in other organs such as the lungs, thus other diagnostic investigations that target the respiratory and the gastrointestinal tract have been implemented (Shteinberg et al., 2021). However, these tests posse a challenge mainly due to the fact that they are not available for use in clinics worldwide, and most importantly they require a high degree of expertise in order to be performed properly.

New therapies and future perspectives

1. CFTR modulators

Early therapeutic approaches for CF were mainly focussed on mucus clearance and nutritional state improvement but also on the management of the recurring infections (Flume et al., 2009). Nebulised therapies in combination with chest physiotherapy were used in order to augment mucin clearance through mucus mobilisation, while inhaled antibiotics were used to manage chronic infections and prevent further complications. However, these therapies targeted only the symptoms and consequences of CFTR impairment, ignoring the defective gene responsible for CF (Donaldson et al., 2007) (Fig. 3 summarises the newly employed strategies for CF treatment). More recently, a new therapeutic approach which involves the use of small molecules agents that promotes CFTR function or restores the diminished protein levels at the apical cell' surface, called CFTR modulators, have been developed. The first CFTR modulator that entered a clinical trial was ivacaftor, an oral drug that acts as an enhancer and was used in the monotherapy for mutations within cell surface protein. This type of CFTR modulators targets alterations such as conductance mutations, gating mutations or mutations resulting in low concentrations of CFTR. Crucial clinical trials on individuals with gating mutations showed that the oral drug ivacaftor led to significant improvements in several particular important clinical metrics namely the FEV_1, sweat chloride concentration, exacerbation rates and BMI (Ramsey et al., 2011). Moreover, there is evidence that ivacaftor exhibits a positive effect on other non-G551D gating mutations (Yu et al., 2012), results which were subsequently confirmed by several clinical trials (DeBoeck et al., 2014). However, the most common *CFTR* mutation – F508del which causes a premature degradation of the CFTR protein before it reaches the cell surface due to its impaired folding process, cannot be tackled by ivacaftor only. Therefore, in order to restore the function of the protein, the required treatment involves the correction of protein folding and trafficking, coupled with the prevention of post-translational degradation and cell expression improvement. In this context, another type of CFTR modulators called correctors were developed, and amongst them lumacaftor was the first CFTR corrector with a proven efficacy in F508del-homozygous individuals (Van Goor et al., 2006; Van Goor et al., 2009). In addition, an alternative CFTR corrector to lumacaftor called tezacaftor was developed, and the results obtained in several clinical trials showed that when coupled with ivacaftor, this CFTR corrector was capable of enhancing spirometry and reducing the sweat chloride levels and exacerbation risks (Taylor-Cousar et al., 2017). Furthermore, in F508del homozygotes and heterozygotes individuals with a concomitant residual function alteration, the combination of tezacaftor with ivacaftor has been proven as effective (Rowe et al., 2017). The development of combinations consisting of three different drugs that includes correctors with a distinct mechanism of action represented a major break-through in CF treatment. The two-correctors combination, each with its own mechanism of action, was able to restore the F508del CFTR function to very high levels, both in cell culture studies and in clinical trials, presenting a remarkable influence both on F508del homozygotes (Heijerman et al., 2019) and heterozygotes with an insignificant function mutation (Middleton et al., 2019).

2. Read-through agents

Affecting approximately 10% of individuals with CF, premature stop alteration causes shorten unstable mRNA molecules and implicitly the lack of a full-length CFTR protein; therefore new therapeutic approaches had to be developed since these mutations are not responsive to either known CFTR correctors. Read-through agents are molecules with ribosomal binding that allow the translation of a full-length functional protein (Rowe and Clancy, 2009). An example of said agents that gained a particular interest is Ataluren, a read-through molecule reported to be efficient in individuals affected by the nonsense mutation Duchenne muscular dystrophy (nmDMS) and is at the moment used to treat specific individuals with this condition (Mercuri et al., 2020). However, late phase clinical trials in individuals with CF that resulted from nonsense mutations did not show significant differences in FEV_1 or in sweat Cl concentrations, when comparing the Ataluren theraphy to the placebo treatment (Abidi et al., 2017; Aslam et al., 2017). Another read-through agent known as ELX-02 demonstrated its efficiency in individuals with nonsense mutations, entering Phase II of clinical trials with G542X nonsense mutations patients (Crawford, et al., 2020).

3. Gene and mRNA-based therapies

With the discovery of the *CFTR* gene over more than 3 decades ago, gene therapy for individuals affected by CF has been an attractive, if somewhat a distant aim. In contrast to the approaches described above, gene manipulation possesses the potential to treat CF individuals with any type of mutation through the introduction of correct copies of *CFTR* DNA into the affected epithelial cells (Burney and Davies, 2012). However, this approach requires two main components namely a copy of the normal CFTR gene with the necessary regulatory constructs and a transfer agent capable of transfection (Jaques et al., 2020). Even so, this therapeutic approach does come with a big challenge that needs to be overcome – how to deliver the genetic material to the targeted cells. Over the years, the delivery of the *CFTR* gene into the epithelial cells has been done with the help of numerous viral vectors but with no success due to the lung cell turnover and the elicited immune response which hindered this effort (Karda et al., 2016; Donnelley and Parsons, 2018). The previous viral approaches were focused on adenoviruses, adeno-associated viruses and Sendai virus, but due to the dysfunctional transduction of the *CFTR* gene, they have been proved inefficient (Griesenbach et al., 2010). A class of viruses that look more promising is represented by lentiviruses due to their enhanced transducing abilities (Jaques et al., 2020), reduced immunogenicity and easiness in the administration of repeated dosing (Griesenbach et al., 2012). However, lentiviruses possess a critical limitation in the absence of surface proteins capable of recognising receptors on the epithelial cells in the airways. To sidestep this, the UK CF Gene Therapy Consortium used the F and HN envelope proteins from a Sendai virus and developed a simian immunodeficiency virus (SIV) (Mordali et al., 2017).

Apart from viral vectors, non-viral ones have attracted a great interest, namely a DNA plasmid expressing the *CFTR* gene complexed with cationic liposome (GL67A), which became one of the first non-viral gene therapeutic strategy to enter a clinical trial with the purpose of detecting any functional mutations in the gene (Jaques et al., 2020). However, the end results were disappointing, especially when compared to other new drugs which showed either similar or better clinical outcomes (Ramsey et al., 2011; Davies et al., 2013; De Boeck et al., 2014; McKone et al., 2014; Moss, et al., 2015; Elborn et al., 2016; Ratjen et al., 2017; Konstan e al., 2017; Taylor-Cousar et al., 2017; Rowe et al., 2017; Middleton et al., 2019; Heijerman et al., 2019).

The presence of thick mucus in the airways of CF individuals, which acts as a barrier for the exogenous molecules, represents one of the main challenges that need to be overcome for the successful delivery of DNA to the epithelial cell (Lai et al., 2009). In order to address this problem, DNA has been compressed into small and dense structures called nanoparticles (NP) (Suk et al., 2011; Lababidi et al., 2019) capable of penetrating the mucus mesh due to their small size. Furthermore, the penetration can be enhanced by coating the NPs with electrostatically neutral molecules that can reduce the electrostatic interactions with the mucus or with mucolytics, molecules that cleave the mucus fibres (Ong et al., 2019). By using this therapeutic approach, the amount of DNA that reaches the targeted cells is enhanced, therefore increasing the efficacy and viability of the gene therapy (Velino et al., 2019).

In terms of mRNA-based therapies, a new approach is represented by MRT5005, designed for the restoration of the CFTR function through the delivery of correct copies of *CFTR*-encoded mRNA through a nebuliser directly into the lung epithelial cells.

4. CRISPR/Cas9 gene editing

CRISPR/Cas9 gene editing represents a novel approach that possesses the potential for *CFTR g*ene mutations correction (Marangi and Pistritto, 2018). Cas9 is a nuclease used in DNA editing by complexing with a guide RNA (gRNA) that is specific for the targeted DNA, followed by the localization of the targeted DNA sequence and the introduction of a double-strand break (DSB) at the specific site. This sequence of events is then succeeded by the activation of the DNA DSB repair processes known as nonhomologous end-joint (NHEJ) and homology-direct repair (HDR) (Hsu et al., 2014). Donor DNA can then be provided and used to repair the DSB, resulting in transgenic DNA (Hille and Charpentier, 2016).

In a study the CRISPR/Cas9 technology has been used to correct *CFTR* gene alteration within the induced pluripotent stem cells.(Suzuki et al, 2016). With the identification of stem cell niches within the lungs, the harvesting of these cells and the correction of the CFTR gene mutations in individuals with CF was possible, before reinserting them back into their environmental niches (Marangi and Pistritto, 2018). Another method uses viral and non-viral platforms for CRISPR/Cas9 delivery to the pulmonary epithelial cells, although transfection issues could represent a major challenge that needs to be overcome.

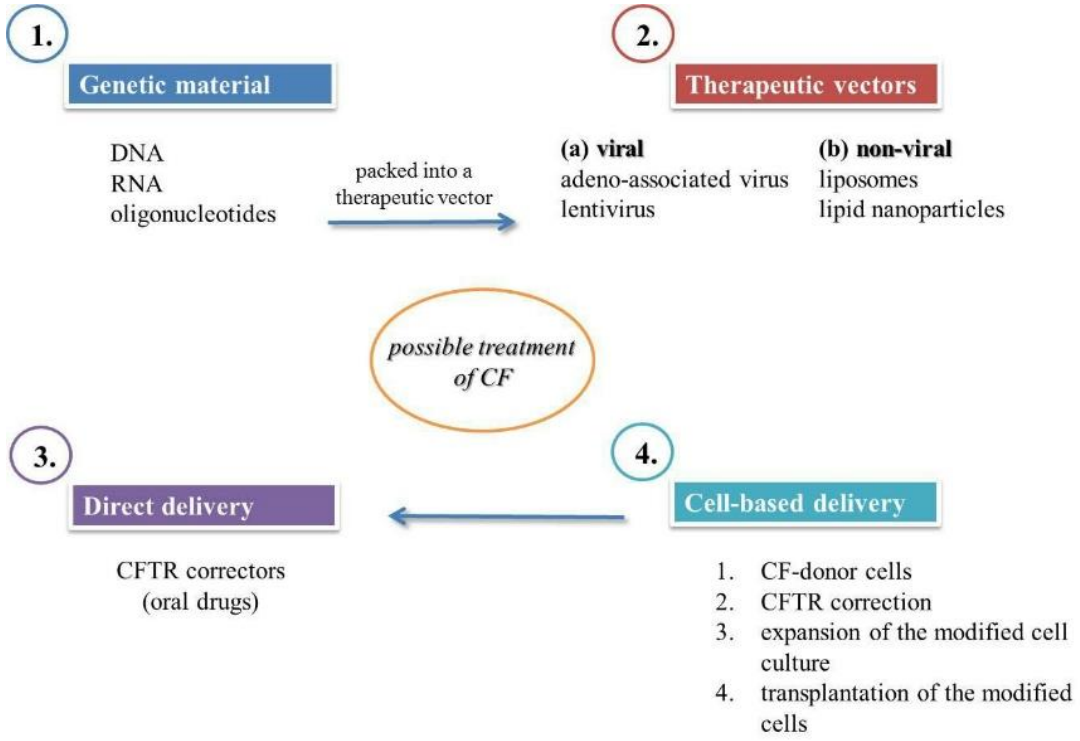

Fig. 3. The new therapeutic approaches for CF

CONCLUSIONS

Even though cystic fibrosis is currently seen as a persistent disease that greatly shortness life expectancy of individuals, the average survival age has increased remarkably in the last 50 years and in the present, it exceeds 40 years of age. Owing to advances in diagnosis, CF is no longer recognised solely as affecting only young children, but now it is recognised as a disease that affects both young and adults. At present, more than half of the CF individuals are over 60 years old age, indicating that the more advanced active treatment played an important role in prognostic improvement, increase of life quality and lifespan prolongation. A vast number of potential therapeutic approaches that target the altered *CFTR* gene or protein have been evaluated and still continue to be developed. However, further work is still needed but now a real prospect for new therapies directed towards the underlying defect of CF, exists.

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