Structure-Function Relationships of CFTR in Health and Disease: The Pancreas Story

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1. Introduction

In 1938, Dr. Dorothy Anderson published a paper describing the characteristic of Cystic Fibrosis (CF) in the pancreas; the term of "cystic fibrosis" refers to the autopsy findings of fibrosis with cyst in the pancreas of children who died early in life with this disease. In 1949 Anderson also discovered that CF is a genetic condition. More recently this was described to be caused by mutations in the CF transmembrane conductance regulator (CFTR) (83). Other than the pancreas, CF also affects the lungs, liver, kidneys, male reproductive and gastrointestinal tract (4, 82). The disease leads to shortened life expectancy most often due to respiratory failure resulting from airway obstruction, bacterial infections and inflammation (9, 98).

CFTR, a member of the ATP-binding cassette (ABC) transporter protein family, is the cAMP-dependent Cl channel at the apical membranes of most epithelial cells (75). Mutations of CFTR gene cause CF, which is the most common fatal autosomal recessive disorder with a disease frequency of 1 in 2,500 live births and a carrier rate of approximately 5% in Caucasian population (33, 114). The disease is characterized by a malfunction of exocrine tissues due to dysregulation of an epithelial chloride (Cl) channel. The major clinical features include chronic pulmonary disease, pancreatic exocrine insufficiency, intestinal disease (especially constipation) and an increase in the concentration of sweat chloride (87). In the lungs, airways become colonized with bacteria and repeated pulmonary infections ensue. The recurrent infections and inflammation result in submucosal gland hypertrophy and excessive mucus secretion. The impaired mucociliary clearance and plugging of small airways cause progressive bronchiectasis and ultimately lead to respiratory failure (100). Following the lung, the pancreas is the most affected organ in CF. It has been documented that most of the CF patients have pancreatic exocrine insufficiency, which leads to maldigestion and potentially malnutrition (117). In this context, malabsorption of fat and fat-soluble vitamins are the most common nutritional deficient seen in this disease.

In this chapter, we will focus on how decreased CFTR function leads to protein plugging of the ducts and pancreatic atrophy. We will also shed light on the latest animal models to better understand the CF pancreatic disease and its relationship to chronic pulmonary disease and intestinal disease.

2. CF Animal Models

Mice, rats, pigs, and ferrets for CF research

Since the discovery of the CF gene many animal models have been generated to mimic the CF symptoms in human patients. The earliest models were in mice with ΔF508 mutation -*cftr* mutation (16, 89, 96, 116). CF mouse models have made significant contributions toward our understanding of the disease and the development of therapies (96). Different CF mouse models have been developed, such as the exon 10 knockout (KO)

model, the ΔF508 model (16, 105), and the G551D model (22). However, significant limitations have been acknowledged in translating the information gained from CF mice to the humans. For example, unlike human CF patients, CF mice show neither pulmonary pathophysiology nor obvious pancreatic pathology or liver problems (88). Recently established CFTR KO rats recapitulate several features of human CF disease; however, they do not develop spontaneous lung infections (96). CFTR KO ferrets, and CFTR KO and ΔF508 pigs generated by nuclear transfer have shown a similar pathology to that observed in human CF patients (101), including lung, pancreatic and liver phenotypes that were not often found in CF mice. However, neither pigs nor ferrets are convenient laboratory species. Both CF ferrets and CF pigs suffer from meconium ileus, which causes these animals to die within a few days after birth; therefore, they are associated with high maintenance cost and require special animal handling skills (45). These factors have limited the applicability of CF pigs and ferrets almost exclusively to the labs originally producing these animals and a few groups closely associated with them (**Table 1**). An ideal animal CF model would mimic the characteristics of human CF patients including the pancreatic insufficiency, but not require exceptional expertise or resources.

Rabbits for CF research

There are anatomical, genetic, and biochemical similarities between rabbit and human (44), making the rabbit a potentially more relevant model for biochemical, molecular and physiological characterization of CF pathology and for the development of CF therapies than mice. As shown in **Table 2**, the amino acid sequence of rabbit CFTR shares about 93% identity to that in human (**Table 2**). Rabbit also has a chromosome arrangement that is similar to humans: 44 chromosomes in rabbit vs. 46 in human; both rabbit and human CFTR genes are present on chromosome 7. Compared to other large animals, such as the pig and ferret, a rabbit is a standard lab animal species that can be easily housed in most research institutes and is relatively economically affordable. Though rabbits airways have some anatomic and physiologic features similarly found in humans, the main concern associated with using rabbits for CF research is the absence of airway submucosal glands (SMGs) in rabbits (115). Since CFTR is abundantly expressed in SMGs of human airways, it has been hypothesized that dysfunction of SMGs initiates CF-like lung disease, leading to mucus accumulation observed in CF patients, as well as in CF pigs and ferrets, both of which contain SMGs in their airways. On the other hand, the absence the glands from mice airways has been cited as one of the explanations

Table 1. Characteristics of cystic fibrosis and key clinical consequences noted in animal models. With emphasis on the rabbit model the table summarizes the differences of phenotypes of CF in all known animal models. In addition, we showed the type of laxatives that will help overcome the gut impaction. The pancreas defect was also shown in the different models. * represents the cost of the animal model on a 1 to 5 scale.

	Knock-out	DF508 KI	Pancreas	Lung, liver Phenotypes	Meconium ileus	Laxatives	Cost	Special Care
CF Mice	Yes	Yes	Yes/mild	?	Yes	Golytely	*	No
CF Pigs	Yes	Yes	Yes/severe	Yes	Yes, 100% death <48hrs	Surgery, or gut-corrected	*****	Yes
CF Ferrets	Yes	No	Yes/severe	Yes	Yes, 75% death <72hrs	gut-corrected	****	Yes
CF Rats	Yes	No?	No	?	Yes	Golytely	*	No
CF Zebrafish	Yes	?	Yes/Severe	?	?	?	*	No
CF Rabbits	Yes	Yes	Yes/Severe	Yes	Yes	Golytely	**	No

for lack of lung disease in CFTR KO mice. Therefore, it has been predicted that CFTR deficient/defective rabbits are unlikely to display mucociliary defects and spontaneous lung infections associated with CF. However, our preliminary data reveals that CF rabbits have a similar airway pathology to that in human CF patients (**Tables 1 & 2**). Although CFTR^{-/-} KO rabbits eventually develop distal intestinal obstruction, meconium ileus is rarely observed in the animal within the first month after birth because rabbit has a large functional cecum. To some extent, our work challenges the traditional view on the importance of SMGs in CF pathology, suggesting that SMGs may not be a critical player for the development of CF lung disease. In support of this view, overexpression of βENaC in mice can produce mucus obstruction in the small, non-glandular airways (57), which are thought to be the site of disease initiation in cystic fibrosis neonates (124). In CF pigs, the mucus appears to arise from goblet cells in the surface of the epithelium of the airways (99). Mucus accumulation in CF ferret airways is associated with variable levels of goblet and mucus cell hyperplasia in the surface airway epithelium and submucosal glands (101). More recent data indicated that defective goblet cell exocytosis in CFTR KO mice contributes to CF-associated disease in the intestine (55). In fact, though SMGs in airways might be a primary site for CF pathogenesis, a critical role for mucus-producing goblet cells in CF airway pathology has not been excluded. Indeed, our preliminary data revealed that the goblet cells, which exist in rabbit airways, maybe the primary contributor to mucus accumulation in the airways of CFTR KO rabbits (**Table 1 & 2**).

ΔF508 mutation

Mutations of the gene encoding CFTR lead to CF, and more than 2023 CF mutations (disease related or not) have been identified (http://www.genletsickkids.on.ca/cftr/) in the CFTR gene. The most common mutation in CF is the deletion of the phenylalanine residue at position 508 (ΔF508) (13), which is in the first nucleotide binding domain of CFTR. The ΔF508 mutation is present in more than 90% CF patients. A critical issue in CF disease is the inability of ΔF508CFTR to achieve the native, folded state required for its export from the endoplasmic reticulum (ER) and traffic to the cell surface. Instead, ΔF508 protein is exclusively retained in the ER and degraded by the ubiquitin-proteasome system (13, 43, 113). A therapeutic strategy aimed at facilitating ΔF508 folding and trafficking is highly desirable for treatment of the disease because ΔF508 mutant has a substantial CFTR Cl current if it reaches the cell surface (23). A new drug, Orkambi, that combines a CF corrector (Lumacaftor, which acts as a chaperone for a correct protein folding which increases the number of CFTR proteins to the cell surface) and potentiator (Ivacaftor, increases the activity of the CFTR (conductance) at the cell surface) recently has received breakthrough

Table 2. CFTR related characteristics among species.

	No. of amino acid	Identity to human (%)	CFTR locus (Ch*)	Days of gestation	Avg. litter size	Sexual maturation	Avg. life expectancy (years)	# of chromosome
Human	1480	100	7	280	1	10-16 yrs	~78	46
Mouse	1476	78	6	21	6	6-8 wks	2	40
Rat	1476	78	4	22	6	8 wks	2-3	42
Pig	1482	92	18	114	10	6-8 months	10-15	38
Ferret	1484	91	?	42	8	4-6 months	8-10	40
Zebrafish	1485	55	18	3	185	3 months	3-5	25
Rabbit	1481	93	7	30	8	4-6 months	8-10	44

therapy designation to treat CF patients with ΔF508CFTR. However, this drug only improves lung function assayed by forced expiratory volume in 1 second (FEV1) by 2.6-4%. Therefore, further research on ΔF508 mutation is needed to develop a better drug to treat CF patients.

3. CFTR Deficiency in CF

The *Cftr* gene encodes the CFTR protein, a member of the ABC transporter superfamily, which is the cAMP-dependent Cl channel at the apical membranes of most epithelial cells, making it unique among members of this protein family (75). The CFTR protein migrates to the surface of cells that line the pancreatic duct, airways, gastrointestinal tract, biliary tract, part of the male reproductive tract and cells that are part of sweat glands (50, 75, 79). CFTR forms a pore or channel that allows ions, including chloride and bicarbonate, to move from one side of the cell membrane to the other (**Figure 1**) (58, 83). Channel activation is mediated by cycles of regulatory (R) domain phosphorylation by PKA/PKC, ATP-binding to the nucleotide-binding

domains, and ATP hydrolysis (**Figure 1**). Demonstration that CFTR functions as a chloride channel regulated by cyclic AMP (cAMP)-dependent phosphorylation is consistent with the ion transport disturbances documented in cystic fibrosis tissues (for review, see(20)). These disturbances in ions change the concentration of molecules in the fluid within the ducts or organs (117).

4. CFTR Protein Structure

The CFTR protein is comprised of 1480 amino acids organized into 5 functional domains (87, 114). As other ABC transporters, CFTR has two membrane-spanning domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2) and one regulatory domain (R) (**Figure 1**). For more insights regarding the structure of CFTR see the review by Patrick and Thomas (74). The two TMDs, each composed of 6 transmembrane segments, form the CFTR channel pore, and the two NBDs interact with nucleotides to regulate channel activity opening and closing of the TMDs (**Figure 1**) (74, 114). The R domain, through
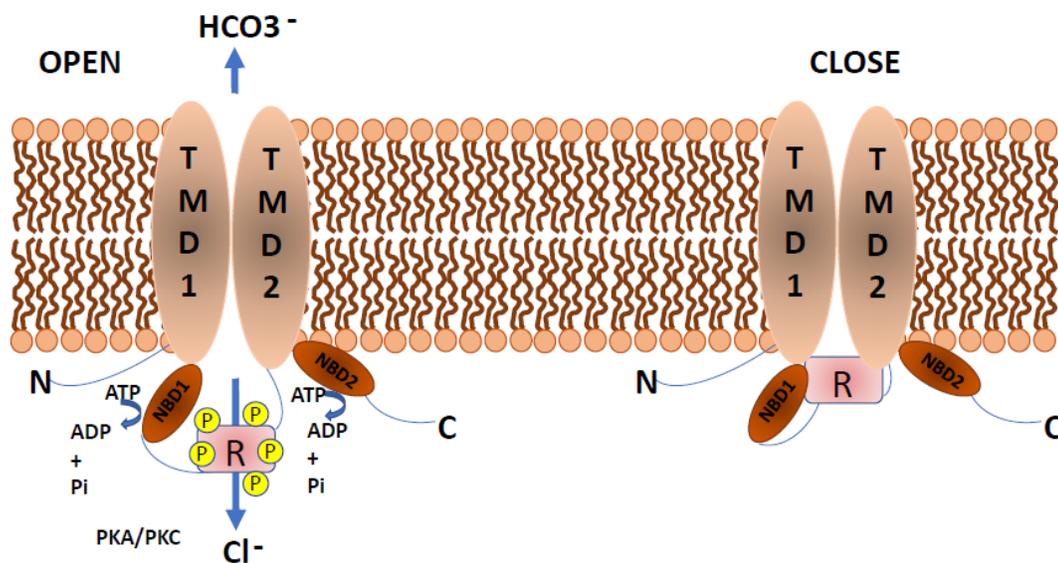

Figure 1. Activation of the CFTR channel. The cystic fibrosis transmembrane conductance regulator (CFTR) protein channel is a member of the ABC transporter superfamily. It acts in apical part of the epithelial cells as a plasma-membrane, cyclic AMP-activated chloride and bicarbonate anion. CFTR has two membrane-spanning domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 channel and NBD2) and one regulatory domain (R). CFTR is a key regulator for cell surface water-salt homeostasis of the apical membranes of epithelial cells in multiple organs including the pancreas that produces alkaline fluid in pancreatic ducts. The open status of the CFTR is initiated by ATP binding at the NBD domains. The activation of the channel is dependent on phosphorylation by cyclic AMP-dependent protein kinase (PKA) at multiple sites in the R domain. The magnitude of response to PKA is amplified by phosphorylation of CFTR by protein kinase C (PKC).

interactions with the N-terminal cytosolic region of TMD1, also controls the channel activity (12, 64, 114). NBDs are responsible for the binding and hydrolysis of the ATP, which causes a conformational change in the TMDs leading to the transport of substrates across cell membranes (95). CFTR mutations can occur in any of the five protein domains. However, many mutations occur in NBD1, including the ΔF508 mutation. The location of the CFTR mutations can affect the formation or function of the CFTR protein (**Figure 2, Table 3**) (114). In **Table 3** we summarized the role of not only the major domains described for CFTR but also the connecting sequences which include: N-terminal, intracellular loops (ICL), extracellular loops (ECLs), transmembrane helixes 1 through 12, C-terminal domain. The N-terminal domain was shown to be involved in the folding and the trafficking of the CFTR protein through protein-protein interactions (e.g. syntaxin
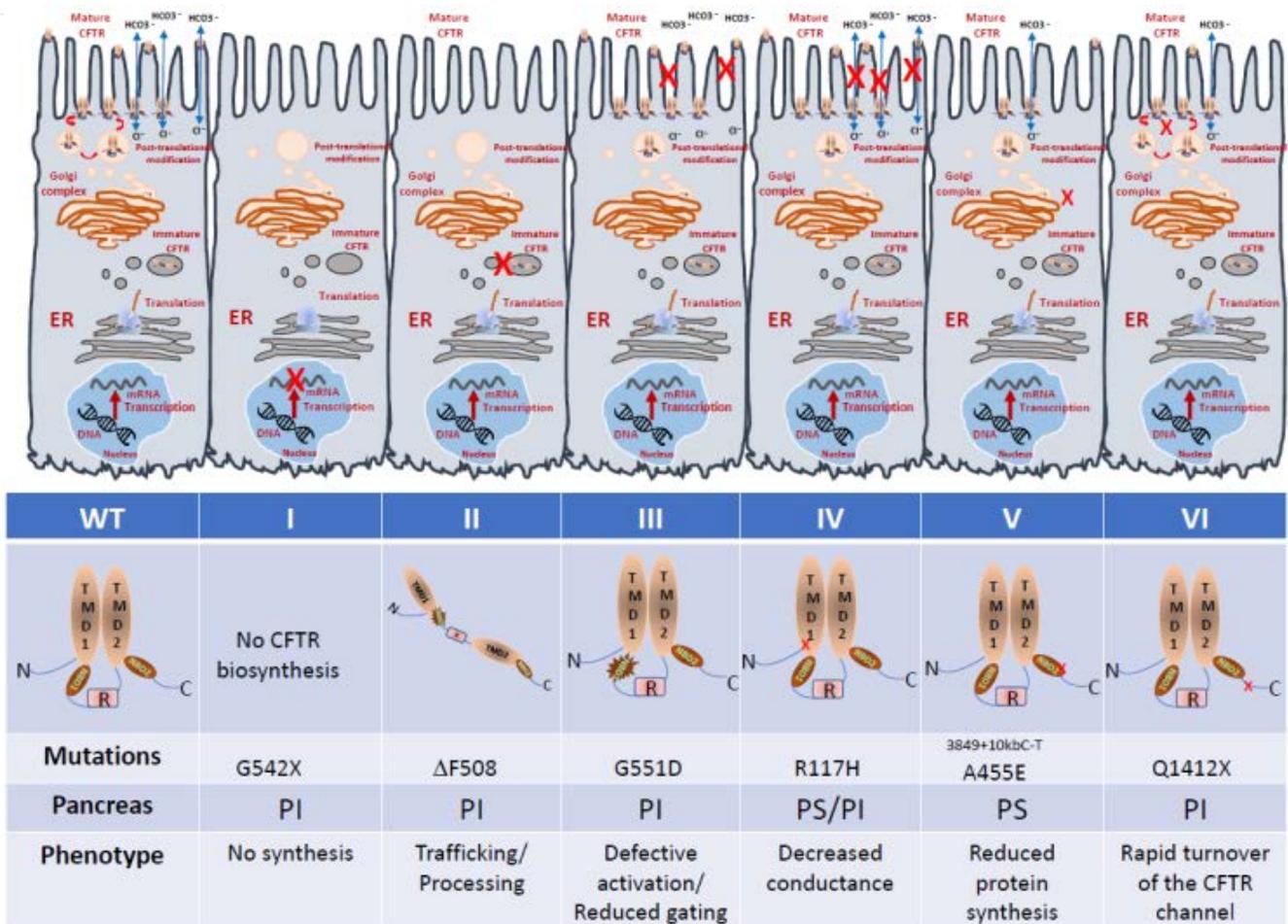

	WT	I	II	III	IV	V	VI
Mutations		G542X	ΔF508	G551D	R117H	3849+10kbC-T A455E	Q1412X
Pancreas		PI	PI	PI	PS/PI	PS	PI
Phenotype		No synthesis	Trafficking/ Processing	Defective activation/ Reduced gating	Decreased conductance	Reduced protein synthesis	Rapid turnover of the CFTR channel

Figure 2. Classes of CFTR mutations. **Class I** mutations lead to no protein synthesis, which includes mutations that includes premature stop codons and nonsense mutations. **Class II** mutation include the most frequent mutation of CF disease, ΔF508, which lead to trafficking, improper folding, and processing defects of the CFTR protein. This class is the primary target in the CF research and the main target by the pharmaceuticals companies. **Class III** mutations affect the ATP binding at one of the 2 binding sites in the NBDs. The CFTR protein reaches the cell surface but the mutations render the CFTR channel nonfunctional which impairs the opening of the channel. **Class IV** mutations also involve CFTR protein reach the cell surface but with reduced ion passage through the channel because of the structural defect caused by the mutations in the CFTR channel. **Class V** mutations affect the amount of CFTR protein that reaches the cell surface because of a splicing problems or inefficient trafficking. **Class VI** mutations lead to a rapid turnover of the CFTR channel at the cell surface. Examples of CFTR mutations regarding their pancreatic defects, PI or PS.
The mutations mentioned in the table are representative of each class, but we have to keep in mind, according to the new classification some mutations are classified in more than one class which will result of having more than one defect.

Table 3. Domains structure of CFTR. A detailed description of the CFTR domains consist of a n-terminal, 6 intracellular loops (ICL), 6 extracellular loops (ECL), 12 transmembrane (TM) arranged into TMD1 and TMD2 consist of 6 TM for each domain, NBD1 and NBD2, and the c-terminal domain. The specified aa for each domain, their known function, positive charge residues, some mutations causing CF, and their effects on the pancreas are as well described.

PI: pancreas insufficient, PS: pancreas sufficient.

Domains	Amino acids	Functions	Positive charge residues	Mutations causing CF	References	Pancreas
N-terminal	1 to 81	Folding and/or trafficking Protein Interaction			(28, 65)	PI
TM1	82 to 103	Regulation of pore function; Pore lining	K95		(39, 111, 112, 120)	PS/PI
ECL1	104 to 117	Stability of the CFTR ion pore		R117C/H/L/P	(34, 42, 49, 111)	PS/PI
TM2	118 to 138	CFTR pore lining	R134		(52, 111, 112, 120)	PS/PI
ICL1	139 to 194	Pore opening		E193K, I148T	(11, 18, 56)	PS/PI
TM3	195 to 215	ND		Q207 form a bond with the mutation V232D	(103)	
ECL2	216 to 220				(34)	
TM4	221 to 241	Loss of function of pore		V232D	(26, 73, 103)	PS/PI
ICL2	242 to 307	Protein folding, Processing in the ER			(7, 56)	
TM5	308 to 328	Anion binding			(112)	
ECL3	329	ND				
TM6	330 to 350	Pore lining; anion selectivity	R334, K335, R347		(112, 120)	PS/PI
ICl2.5	351 to 432	ND				
NBD1	433 to 586	Hydrolyzation of ATP, Channel opening, regulation of the sodium ion channel		Delta(F508); G551D; G542X	(90, 119)	PI
R	587 to 859	Phosphorylation sites for PKA/PKC		D648V, E664X, E656X and 2108delA	(3)	PI
TM7	860 to 870	ND				

Region	Residue range	Function		Mutations	References	
ECL4	881 to 911	Glycosylation at N894 and N900		Q890X, K892C	(10, 29, 42)	
TM8	912 to 932	ND		S912L	(29)	
ICL3	933 to 990	Conductance		S945L, H949Y, G970R	(29, 94)	PS/PI
TM9	991 to 1011	ND				
ECL5	1012	ND				
TM10	1013 to 1034	Processing	R1030			PI
ICl4	1035 to 1102			L1065P, R1070Q, Q1071P	(29)	
TM11	1103 to 1123	Pore lining; anion selectivity			(39, 111, 112, 120)	PS/PI
ECL6	1124 to 1128	ND				
TM12	1129 to 1150	Pore lining; anion selectivity		M1137V, M1137R, I11139V and deltaM1140	(107, 112, 120)	PS/PI
ICL5	1151 to 1218	Conductance		D1152H, D1154G, W1204X	(29, 107)	PS/PI
NBD2	1219 to 1386	Maturation, gating		N1303K, G1349D, G1244E, S1251N, S1255P, and G1349D	(107)	PI
C-terminal	1387 to 1480	ND				

1A) and mutations in this domain reduced the function of the channel (28, 65). Some transmembrane (TM) helices have been studied more than the others. For instance, the results from many studies suggested that transmembrane segments,TM1, TM2, TM6, TM11 and TM12 form the pore lining and regulate the pore function by selecting the anions (111, 112, 120), whereas TM5 plays a role in the anion binding (112). Also, V232D mutation in TM4 leads to a loss of function because it forms a bond with Q207 in TM3 which does not occur in the WT form of CFTR (73, 103). The mutation V232D is not the only mutation that involves a change from a neutral/hydrophobic residue to a polar or charged residue, causing CF. Therien et al, studied more than 31 mutations in TMD1 including all 6 TM helices and concluded that CFTR mutations in the TMs lead to a loss of function through the formation of membrane-buried interhelical hydrogen bonds (103). Extracellular Loops (ECLs) 1 through 6 represent about 4% of the CFTR protein, whereas 77% is in the cytoplasm and the rest, 19%, includes TM1 to 12. CF-associated mutations in the ECLs have been shown to affect channel gating (34), and interactions with extracellular anions (54, 121). For example, mutations D110H/E and R117C/H/L/P in ECL1 are associated with CF disease. These mutations affect the stability of the CFTR ion pore, resulting in reduced conductance of CFTR (34, 42). Q890X and K892C mutations in ECL4 have been reported to affect channel gating and

extracellular anion interaction (10, 29, 42). ECL4 is the only extracellular loop that contains N-linked glycosylation sites (N894 and N900) (10). Many studies have been performed on the function of CFTR's intracytoplasmic loops (ICLs) including their roles in regulating inter-molecular interactions as well as CFTR interactions with other proteins. E193K and I148T mutations in ICL1 have been reported to affect the pore opening (11, 18). Recent studies showed that ICL 2/3-NBD2 interface and ICL1/4-NBD1 interface have a role in protein folding and processing in the ER (7, 56). In addition, S945L, H949Y and G970R mutations in ICL3, L1065P, R1070Q and Q1071P mutations in ICL4, and D1152H, D1154G and W1204X mutations in ICL5 have been shown to affect the conductance of CFTR (29, 94, 107),. As for the rest of the domains, ATP-binding events occurring in both NBDs allow the hydrolysis of intracellular ATP to ADP (6). This event allows the conformational changes, and that change in structure allows the CFTR channel to transition from an opened to closed state, thus controlling the gating kinetics of the channel (6). NBD1 was studied more than NBD2 due to the presence of frequent ΔF508 deletion of NBD1 in the CF patients. The ΔF508 is present in more than 70% of the CF patients, resulting in destabilization of the CFTR protein (6, 90, 119). Also, mutations ΔF508 and G551D modify the interactions between NBDs and NBDs–ICLs (6). Other well-studied NBD1 mutations such as G542X and G551D result in channel-gating problems (6, 90, 109, 119). Similarly, NBD2 mutations like N1303K, p.Ile1234_Arg1239del, G1244E, S1251N, S1255P, and G1349D are CF disease related, and are considered to be gating mutations (109). The R domain, along with NBDs, control the channel activity of CFTR. The activation of the channel is dependent on the phosphorylation by cyclic AMP-dependent protein kinase (PKA) (93). To date, more than 15 phosphorylation sites in the R domain have been attributed to PKA phosphorylation, which contribute in varying proportions to the response to activation of the CFTR channel (93). It has been reported that CFTR also can be phosphorylated by several other protein kinases including protein kinase C (PKC), casein kinase II, cyclic GMP activated protein kinase, and Src kinase (93). The R domain has multiple phosphorylation sites for PKC, which modulate PKA-induced domain-domain interactions (92).

Many CF-related mutations like D648V, E664X, E656X and 2108delA in the R domain disrupt the normal function of the R domain, e.g. the transport of HCO_3^- in secretory epithelia and in CF (3, 14). The same authors showed that mutants reported to be associated with CF with pancreatic insufficiency do not support HCO_3^- transport, and those associated with pancreatic sufficiency show reduced HCO_3^- transport (14).

5. Classification of CF Patients

Traditional classification
As mentioned above, more than 2023 CF mutations have been identified (http://www.genletsickkids.on.ca/cftr/) in the CFTR gene. These mutations are categorized based on the dysfunctions of CFTR at different levels of the maturation and function of the CFTR channels (**Figure 2**). Traditionally, these dysfunctions were divided into 6 groups based on function (Classes III, IV, and VI) and processing (Classes I, II, and V) of the CFTR (**Figure 2**) (51, 58, 108). The class I mutations includes a nonsense, frame-shift, or splicing mutations which prevent CFTR biosynthesis by introducing a premature termination codon (PTC). The most common mutation in this class is the G542X (**Figure 2**).

The class II mutations include a missense mutation which causes misfolding of the CFTR to lead to its degradation in the ER by quality-control machinery, resulting in the absence of functional protein at the cell surface. The most important mutation in this class is the ΔF508 presents in more than 90% of CF patients (**Figure 2**).

The class III mutations include a missense mutation which lead to a non-functioning CFTR at the cell surface resulting in unstable and reduced channel gating characterized by a lower open probability. The most common mutation representing this class is the G551D (**Figure 2**).

The class IV mutations include missense mutation which leads to a reduced CFTR channel conductance. The decrease in conductance is caused by an abnormal the conformation of the pore resulting in disruption of the ion flow. The most common mutation in this class is the R117H (**Figure 2**).

The class V mutations introduce splicing or promoter defects in the CFTR gene, resulting in a reduced amount of CFTR protein at the cell membrane caused by reduced protein synthesis. Those mutations affect the gene expression, but do not change the conformation of the channel. The most representative mutations in this class are the 3849+10kbC-T and A455E (**Figure 2**).

The class VI mutations include missense mutation which lead to a decrease in the CFTR stability. These mutations result in an accelerated turnover of CFTR protein at the cell membrane and reduced apical cell surface expression (108). The most representative mutation in this class is Q1412X (**Figure 2**).

Though more than 2023 mutations/variants have reported for CFTR, whether each of these can cause channel dysfunction and disease is largely unknown. However, studies to predict the functional consequences and clinical outcome of individual patients carrying these mutations are being conducted (24, 97). These interpretations of such studies have been challenged by the general lack of correlation between the genotype and the clinical severity (24) (**Table 1**).

New classification
The lack of correlation between the genotype and the phenotype of the CF patients led to a new classification based on the severity and the clinical symptoms of the CF patients. Recently, Dupuis et al, studied meconium ileus (MI) in CF patients, and reported that only a subset of patients with CF develop MI (24). Furthermore, MI demonstrates notable heritability. Although studies have shown that non-CFTR genes contribute to susceptibility, the CFTR genotype itself affects the occurrence of this complication; only patients with the more severe CFTR variants are at risk for MI (24). It was hypothesized that the susceptibility to MI is influenced by specific CFTR genotypes, and that the prevalence of MI can be used to discriminate among severe CFTR mutations (24). The pleiotropic molecular defects of a single mutation in the CFTR has limited the drug therapy effects for some mutants which have been categorized as class I, II, or II/IV (108). The authors proposed a modification of the traditional class I–VI CF mutations classification. This expanded classification of the major mechanistic categories (87, 114, 122) accommodates the unusually complex, combinatorial molecular/cellular phenotypes of CF alleles. The new classification consists of 31 possible classes of mutations, including the original classes I, II, III/IV, V, and VI, as well as their 26 combinations (108). For example, according to the expanded classification, G551D will be designated as a class III mutation as before (114), while ΔF508 will be classified as class II–III–VI, W1282X as class I–II–III–VI, P67L as class II–III, Q1412X as class III–VI and R117H as class II–III/IV, reflecting the composite defects in mutant CFTR biology (108). More evidence supporting the new classification came from a study by Vertex Pharmaceuticals where they tested 54 missense mutations and found that 24 of them have both processing and gating defects (106).

6. CFTR Function and Its Role in Pancreas

Cystic fibrosis and exocrine pancreas
As mentioned above, CFTR is predominantly expressed on the apical membrane of epithelial cells in the small pancreatic ducts. CFTR acts as a selective ion channel involved in chloride, bicarbonate (HCO_3^-), water transport across the apical membranes of epithelial cells in multiple organs including the pancreas that produces alkaline fluid in pancreatic ducts (30, 70, 117). HCO_3^- is an important ion in the pancreatic juice. It also facilitates solubilization of the digestive enzymes and mucins (52). Indeed, aberrant HCO_3^- transport has a crucial role in human diseases (79, 80). In his review, Quinton proposed that in CF patients, the HCO_3^- is required to form normal mucus. His explanation is that once granule is released, HCO_3^- sequesters Ca^{2+} and H^+ ions away from the mucin anions to form a complex with them. Therefore, lack of secreted HCO_3^- in CF patients impairs Ca^{2+} removal, prevents normal mucin expansion, and promotes stasis of mucus in the ducts or on the luminal surfaces of affected organs (79). In addition, reduced secretion of HCO_3^- and chloride (Cl^-) leads to a more acidic and viscous luminal content (39). MUC6 is one of the pancreatic mucins expressed by 13 weeks of gestation and shows a very similar distribution to that of CFTR. In addition, MUC6 mucin is the main constituent of the complexes that form in small ducts and cause obstruction (81). Therefore, CF patients carrying mutations in the *CFTR* gene, showed a

lower pH, low flow of secretions and high protein concentration in the pancreas duct secretions, which lead to precipitates in the duct lumina that obstruction and injury (30, 117). Meyerholz and his colleagues showed in the CF pig model that the changes (obstruction) could be detected in gestations as early as week 17 (59). They showed that the site of obstruction ranged from the distal jejunum to the proximal spiral colon, similar to that reported in humans with meconium ileus (59, 63). The obstruction in the acini and ducts lead to dilatation which causes epithelial injury and destruction, inflammation, fibrosis and fatty infiltration (30, 41, 59). Tucker and colleagues reported that acinar plugs developed before mucous metaplasia and found that early acinar plugs are composed of zymogen granules and were distinct from mucus in pancreatic tissue of cystic fibrosis patients (104). These findings then indicate that zymogen material from the acinar cell, not mucus, may become inspissated in the acinus in early cystic fibrosis, and that subsequent mucous metaplasia occurs as the obstruction and exocrine atrophy progress (104).

CFTR and Hyperinflammation

As mentioned above airways of the lungs become colonized with bacteria and repeated pulmonary infections ensue. The recurrent infections and inflammation result in submucosal gland hypertrophy and excessive mucus secretion. The impaired mucociliary clearance and plugging of small airways cause progressive bronchiectasis and ultimately lead to respiratory failure (100). Many studies have been done to explain the cause of the hyperinflammation in the CF patients. Many have suggested that the balance between Th1, Th2, and Th 17 could play a role in the CF disease (31). It has been established that Th17 is known to be a key player in autoimmune diseases (40). In addition, the authors showed that Th17 is regulated by miR-183C via inhibition of Foxo1(40). For a long time, it has been thought that CFTR mutations in epithelia cells have an indirect effect on the immune system which causes the inflammation in the lungs, because of the colonization of bacteria in the lungs like P. aeruginosa, Burkholderia cenocepacia, and Mycobacterium abscessus which causes the infections in the CF patients (31). Recently, emerging evidence points the problem to be

directly affecting the immune cells. Since it has been shown that CFTR is expressed in lymphocyte T cells (like Th2, Th17, and Tregs) and macrophages, so CFTR mutations causing CF may have a direct impact on these cells. Grumlli et al summarized in his review, that CFTR mutations have a direct effect on the T cells function which results in an enhanced Th2 response, a reduced Treg population and elevated Th17 response which translate by an increase of neutrophils and recruitments by IL-17 to the lung which leads to the destruction of alveoli in the lungs of CF patients (31). In addition to the lymphocytes and neutrophils, macrophages also has been shown to participate to the lung decay found in CF patients through activation of MMp12 (31). Understanding better the mechanisms behind the infections and the immune response could lead to a better drug therapy targeted to each patient depending on the severity of the CF disease.

Cystic fibrosis and endocrine pancreas

CF is also recognized to affect the endocrine pancreas. There is a correlation between glucose abnormalities, morbidity and mortality in CF patients (67, 69). Glucose abnormalities include cystic fibrosis-related diabetes (CFRD) and impaired glucose tolerance (IGT). CFRD is one complication in the CF patients occurring in more than 40 % of adults and 25% of adolescents, which is preceded by episodes of impaired glucose tolerance (19, 61, 67, 91). Both reduced insulin secretion and insulin resistance are observed in CFRD (36, 62, 66, 78). CFRD has characteristics of both type I and II diabetes and does not belong to either one of the diabetes classes. It is characterized by the loss of functional β cell mass and varying degrees of insulin resistance (**Figure 3**) (30, 62, 66). Mutations in CFTR that lead to both a decrease in islet cell mass and dysfunction in the β cell are the cause of CFRD (17, 67, 86). It is believed that cross-talk between pancreatic exocrine and endocrine components can also contribute to the CFDR (**Figure 3**). Destruction of pancreatic exocrine tissue caused by a decrease in the islet cell mass evolves to dysfunctional endocrine β cells (5). β cell dysfunction may be caused by increased oxidative and endoplasmic reticulum (ER) stress which are associated with CFRD (27,

77, 84, 118). It is very well documented that glucose deprivation leads to ER stress (118). Some CFTR mutations (Like ΔF508) can cause the accumulation of unfolded proteins in the ER, triggering an evolutionarily conserved response, termed the unfolded protein response (UPR) (2). In addition, aberrant Ca^{2+} regulation in the ER lumen causes protein unfolding and rapid degradation of mutated CFTR proteins may contributes to the complex multi-organ CF pathology (48). It is known that ER stress triggers β cell death typically by apoptosis when protein misfolding is persistent or excessive (76). As Harding at al stated, the special sensitivity of insulin-producing cells to a mutation (like CFTR mutations) that affects a signaling protein responsive to ER stress may also be relevant to the development of more common forms of human diabetes mellitus. The major abnormality in most patients with CFRD is resistance to the action of insulin. However, glucose intolerance develops only after β cell decompensation renders the endocrine pancreas unable to keep up with the demand imposed by IR (37). Because of their high rates of protein synthesis, β-cells are particularly susceptible to ER stress, which may trigger CFRD (67). The expression of CFTR was reported to be required in β-cells for glucose-induced secretion. Therefore, CFTR plays a significant role in the normal function of pancreatic β-cells (85). Ntimbane and his colleagues have summarized the factors leading to an abnormal glucose homeostasis in CF patients: (a) impairment of β-cell function with progressive fibrosis of islets of Langerhans with resultant distortion, ischaemia, cell death and a decrease in islet numbers; (b) impairment of other islet cell functions; (c) impairment of the insulinotropic gut hormone secretin; (d) changes in insulin sensitivity; and (e) altered insulin clearance rate (67). The exact cause and mechanism of CFRD are still largely unknown. The most probable cause of CFRD is a combination of many events and unlikely to be attributable to one defect. The clinical effects and disease states associated with CF patients with CFRD include: chronic pancreatic inflammation, dysfunction of the immune system, oxidative stress, impaired insulin production and secretion, variable state of IR and altered entero–insular axis hormones (30, 67).
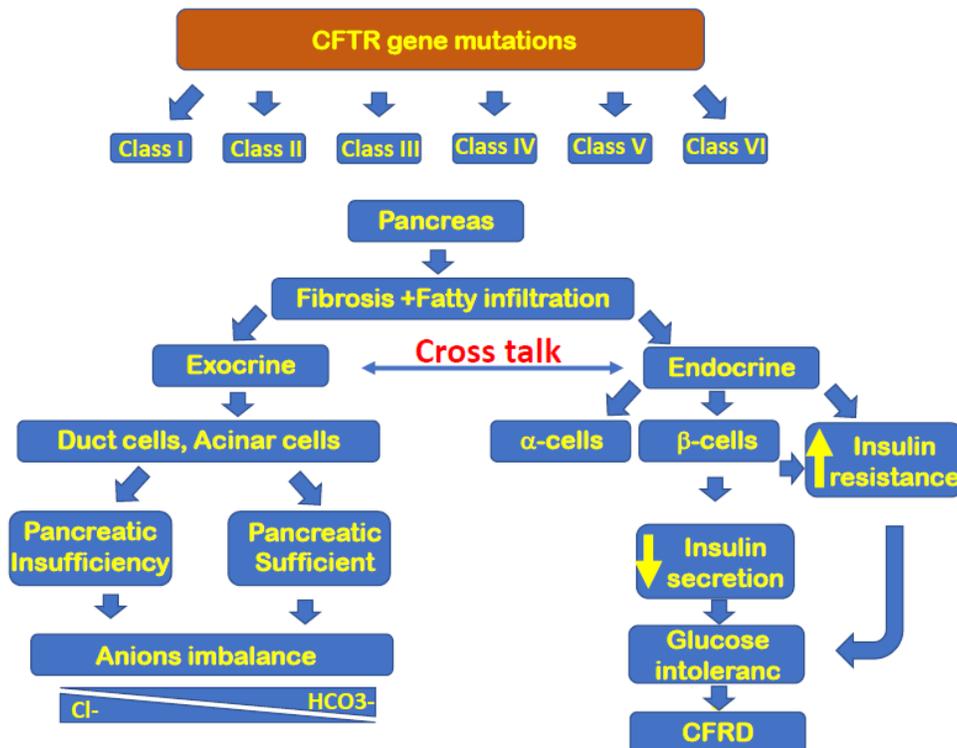

Figure 3. CF and pancreas defects. Schematic representation of the cross talk between exocrine and endocrine of the pancreas. Defects in the exocrine pancreas leads to PI and PS, causing the anions imbalance. Defects in the endocrine pancreas leads to CFRD. As summarized in this diagram, many factors, defects and the crosstalk between endocrine and exocrine leads to the phenotypes described in the CF patients including PI, PS, and CFRD.

Insulin secretion and CFTR

All the proposed causes mentioned above are supported by evidence to explain the pathogenesis of impaired insulin secretion in CFRD. An additional cause not mentioned above is the expression and direct effect of CFTR on insulin secretion in the β-cells (26, 32, 47, 102). The expression of CFTR has been reported in cultured β-cells derived from mice (MIN6) or rat (RINm5F) (26, 32, 68). But the earliest report came in 2007 from studies by Boom and his colleagues showing the expression of CFTR protein in rat islet cells and the significantly higher level in non-beta than in beta- cell populations (8). They also showed by immunohistochemistry studies that CFTR expression also occurs in glucagon-secreting alpha-cells (8). Guo and his colleagues demonstrated that glucose-induced whole-cell currents, membrane depolarization, electrical bursts or action potentials, Ca^{2+} oscillations and insulin secretion in β-cells are dependent on CFTR, indicating an essential role of CFTR in the regulation of insulin secretion (32). Their studies showed that specific inhibitors of CFTR (GlyH-101 and CFTRinh-172) blocked a CFTR Cl^- gating needed for insulin secretion in primary β-cells and ΔF508-CFTR mutant mouse islets (32). In addition, Edlund and her colleagues detected small CFTR conductance in both human and mouse beta-cells. The augmentation of insulin secretion by activation of CFTR by cAMP (forskolin or GLP-1) in the presence of glucose was significantly inhibited by the specific CFTR inhibitors. They also demonstrated reduced cAMP-dependent exocytosis upon CFTR-inhibition, concomitant with fewer docked insulin granules (26). These reports and others from the patients with CFTR mutations showed insufficiency of secreted insulin. However, these studies did not describe the molecular mechanism that cause a decrease in insulin secretion. In addition, to the role of CFTR in regulating insulin secretion and exocytosis after glucose-induced membrane depolarization leading to insulin secretion, the study also demonstrated that CFTR molecules act upstream of the chloride channel Anoctamin 1 (ANO1; TMEM16A) in the regulation of cAMP- and glucose-stimulated insulin secretion (26). Thus the impaired insulin secretion seen in patients with CF would be caused by the lack of glucose-induced Cl^- efflux through both CFTR Cl^- channels and ANO1 due to a decreased membrane depolarization (26, 32). In summary these study showed that CFTR is an important regulator of pancreatic β-cell insulin secretion, exocytosis, and membrane depolarization, and is induced by glucose via elevation of cytosolic Ca^{2+} concentration (32, 47, 58).

Marunaka in his recent review summarized new studies connecting the role of CFTR and ANO1 in insulin secretion (58). Briefly, it has been established that intracellular Cl^- concentration ($[Cl^-]$) is a very useful marker of channel activity. In these recent studies, the authors showed that $[Cl^-]$ measured using N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) is about 100 mM under the basal condition in RINm5F β cell line, and application of CFTRinh-172 (an inhibitor of the CFTR channel) increased $[Cl^-]$ about 26 mM (32). This means that CFTR indeed mediates Cl^- efflux under basal condition, which may maintain a relatively depolarized membrane potential in the β-cells at rest, and the electrochemical potential of Cl^- in the intracellular space is larger than that in the extracellular space (32, 58). This was confirmed by showing that membrane potentials of pancreatic β cells expressing wild-type CFTR $Cl-$ channels are −61~−67 mV [70], but hyperpolarized to −75 mV when using CFTRinh-172 or ΔF508 (32). Thus, CFTR has an important role in determining the resting membrane potential of the β-cells by acting as a Cl^- channel to maintain the membrane depolarization (32). The same study also showed that ΔF508 CFTR Cl^- channel decreases membrane depolarization induced by glucose and increases $[Ca^{2+}]$ due to the activation of voltage-dependent Ca^{2+} channels (32, 58). In his review, Marunaka concludes that the loss of CFTR function leads to insulin insufficiency which is caused by the higher intracellular Cl^- electrochemical potential in pancreatic β cells. "In general, Cl^- uptake into the intracellular space is mediated via active Cl^- transporting systems, such as Na^+-Cl^- cotransporter (NCC) and/or NKCC, driven by the Na^+,K^+-ATPase-generated Na^+ chemical potential difference between the intracellular and extracellular spaces: the intracellular Na^+ chemical potential < the extracellular Na^+ chemical potential. Therefore, if we could increase the $[Cl^-]$ by elevating the NCC- and/or NKCC-mediated Cl^- uptake, the insufficiency of insulin secretion would be improved via membrane depolarization due to an

elevation of Cl− efflux from pancreatic β cells of ΔF508 CFTR-expressing CF patients.

Recent studies contradict the findings by Guo et al, by presenting some evidence using the ferret's pancreas that β-cells do not express CFTR (102). The authors showed that CFTR RNA is expressed in exocrine and not in endocrine cell types of islets and pancreas. They used a different approach, smFISH, to show the expression of CFTR. WT and CFTR-KO neonatal ferret pancreas were used to perform *CFTR* and *INS* dual smFISH. As expected, the *INS* was present in both genotypes, but *CFTR* was present in the WT pancreas (102). More importantly, *CFTR* RNA was not co-expressed in *INS* (β-cell), GCG (α-cell), PPY (PP cell), or SST (δ-cell) expressing cells but was expressed in KRT7-expressing ductal cells in the WT pancreas. Similarly, same findings were shown in dissociated cells from isolated adult ferret and human islets (102). These findings contradict the previous findings by Guo et al, and demonstrate that exocrine-derived duct cells associated with isolated islets express the highest levels of CFTR and support a mechanism by which CFTR dependent duct/islet crosstalk might influence β-cell insulin secretion (102).

Although there is evidence that supported each of these contradicting studies, a clear resolution to the question of whether CFTR directly or indirectly functions within the β-cells or other islet cell types to support insulin secretion needs further clarifications.

Role of glucose transporters (GLUTs) in CF and their impact in the pancreas

As mentioned above, glucose abnormalities in CF include CFRD and impaired glucose tolerance. The relationship between CFTR and the causes of CFRD is still not very well established. Studies are also emerging regarding the implication of GLUT transporters in developing the diabetic state in CF patients (53). In studies regarding obesity and diabetes, it was reported that CFTR was significantly decreased, while GLUT5 and Villin were increased in the jejunum (53). It is known that CFTR Cl− channel provide the major route for Cl− exit across the apical membrane in normal murine intestine and disturbance in the anion exchange and recycling of K^+ is thought to be one of the causes of diabetes (53).

Recently it was reported that GLUT4 and apical insulin are expressed in normal human airway epithelial cells (60). The authors also showed that cells expressing F508del-CFTR have impaired glucose uptake, elevating action on the trans epithelial resistance, and diminishing action on paracellular flux of small molecules after insulin stimulation (60). In a different study, GLUT4 subfractionation demonstrated that, despite insulin stimulation, the GLUT-4 content of intracellular-associated subfraction was significantly higher in CF subjects compared with controls, corresponding to significantly lower GLUT-4 content in cell surface-associated subfraction (35). These findings are consistent with the abnormal subcellular localization of GLUT-4. Impairment of GLUT-4 translocation in CF correlated with higher TNF-α levels in all CF subjects than in controls (35). CF patients that have CFRD with a decreased insulin secretion could be explained by the elevation of TNF-α and impaired translocation of GLUT-4. In addition, the results indicate that the function of CFTR Cl− channels is required for insulin to stimulate glucose uptake, elevate the transepithelial resistance, and diminish the paracellular flux of small molecules in airway epithelial cells (58).

7. Pancreatic Defect and CFTR

In general, the exocrine pancreatic disease and its progression correlates well with the genetic factors of the CF patients (1, 49). CF patients have been divided into two classes, pancreatic insufficient (PI) and pancreatic sufficient (PS) (1, 46, 49). Approximately 85% of patients with CF have PI which is categorized as the "severe" CF phenotype, the rest of 15% are PS patients and thus "mild" CF phenotype. The exocrine pancreas in PI patients no longer secretes the required digestive enzymes. Therefore, CF patients often require an oral pancreatic enzyme supplement each meal (117). Pancreatic damage from CF can be detected in utero in subjects with PI and show obstruction of the small ducts and acini which will lead to the destruction of the pancreas with only a few islets or ducts left in a sea of adipose tissue (30). In contrast, PS-CF patients do have pancreatic damage, as measured by the high levels of serum immunoreactive trypsinogen (IRT), but retain normal digestion due to a sufficient endogenous function of exocrine

pancreatic ducts (30, 117). As we have mentioned above, CF patients are classified into six traditional classes from I to VI based on their *CFTR* mutations. The exocrine pancreatic phenotypes PI and PS are directly linked to genotype (1, 46, 49, 114).

Wilschanski and his colleagues describe that CF patients homozygous or compound heterozygous for severe alleles belonging to classes I, II, III, or VI confer PI. Whereas a mild class IV or V allele sustains pancreatic function in a dominant fashion even if the second mutation is severe and falls into PS (117). They go further and explained that this observation appears plausible because all known mild alleles belong to class IV or V, all of which are (or predicted to be) associated with some residual chloride channel activity at the epithelial apical membranes (117). On the other hand, in a recent review by Gibson-Corley and his colleagues, they described the PI CF patients to be in the classes I, II, III, IV and VI because they have mutations that render CFTR to be absent or non-functional (30). The remainder of the patients belonging to class V or mild class IV considered PS, due to less severe *CFTR* mutations (30).

In both reviews, it was mentioned that this classification of the PI and PS do not entirely fit into the six classes of CFTR mutations. Those who are considered PS still show pancreatic destruction as the serum level of IRT is elevated but will not require enzyme replacement for normal digestion (30). Some class I mutations with the stop codon at the end of the gene are CF patients with PS (117). In addition, a small portion (~3%) of CF patients with a severe mutation on both alleles are considered PS at diagnosis, but eventually transition from PS to PI (25, 117). Another example, G85E a missense mutation and few other mutations do show variable pancreatic phenotypes (117). PS CF patients are more susceptible to developing pancreatitis than the PI patients (25). It is known that pancreatic ducts have an essential part in CF and chronic pancreatitis, and only PS patients develop pancreatitis, suggesting that partially impaired the function of pancreatic ducts is retained in PS patients (38). Druno and colleagues showed that there is a strong correlation between genotype and phenotype in patients with CF and pancreatitis (25). They showed from a CF cohort study of about 1000 patients followed over a period of 30 years that PS CF patients carry at least one mild mutant allele and are at a significant risk of developing pancreatitis. Symptoms of pancreatitis may precede the diagnosis of CF. Pancreatitis is associated with an otherwise mild CF phenotype (25). A larger CF cohort study of about 10071 patients had reported that out of 331 patients with PS, 34 cases had pancreatitis, where the occurrence of pancreatitis among patients with PI was 15 cases out of 2971 patients (21). More evidence goes toward the correlation of PS CF patients and the development of pancreatitis, with a novel pancreatic insufficiency prevalence score where they divided the patients into 3 groups: severe, moderate-severe, and mild, with the mild mutations more susceptible to the risk of developing pancreatitis (71, 117). The new classification of CFTR mutations into 31 new classes reflects the composite defects in mutant CFTR biology (108). We will need to take into considerations the complexity of the disease and the severity of the CFTR genotype and their relationship with risk of pancreatitis. In a very recent study, for example, three sibling patients with a novel missense mutation, the R248G in exon 6 of the *CFTR* gene, present a recurrent acute pancreatitis (110). A similar missense mutation, R248T, has been previously reported as a mild CFTR-RD mutation that is not associated with pancreatitis. The R248G mutation may alter the normal function of CFTR more than the R248T mutation based on the clinical phenotypes of the three patients (110). As the authors conclude, future structure-functional studies on the CFTR protein can provide further insight into the impact of the R248G mutation at the molecular level.

8. CFTR Mutations and Pancreatitis

It is very well established by now that CFTR is a key protein in the pancreatic duct, which regulates the exchange of anions between the luminal surface and the cytoplasm of the duct cells. As mentioned, in CF patients the pancreas is one of the first organs to fail because mutations in CFTR play a critical role in pancreatic pathophysiology. The large number of mutations known to date in CFTR lead scientists to tackle this complex disease with so many symptoms from a different angle, i.e.,to make a specific correlation between CFTR mutations with certain symptoms such as

pancreatitis in its both forms chronic and acute to determine the severity of the disease. In an Austrian cohort study of 133 pancreatitis patients, the frequency of CFTR mutations was 11.2% (123). In patients classified as 'idiopathic definitive chronic pancreatitis,' the frequency of mutations was 12.7%, whereas patients with 'acute pancreatitis' or 'possible chronic pancreatitis', had a frequency of CFTR mutations of 10% and 9.1%, respectively (123). The authors concluded, the frequency of CFTR mutations is highest in patients with definitive chronic pancreatitis and may, therefore, be regarded as a risk factor for the development of chronic pancreatitis (123). Another large Canadian CF cohort study of 2481 subjects with PS-CF (with and without pancreatitis) showed some correlation between the severity of CFTR genotype and the risk of pancreatitis (71). They showed that patients carrying mild mutations are more likely to develop pancreatitis than those who had moderate -severe mutations (71). Therefore, patients with mild mutations had 71% increase in the risk of developing pancreatitis at any given time than those with moderate-severe mutations (71). Thus, approximately 20% of PS-CF patients develop pancreatitis (71). Coffey at al, in their review summarized the complexity of the correlation between the CFTR mutations and the development of pancreatitis by categorizing the mutations into four groups based on the clinical status of the patients: (i) CF-causing mutations, (ii) mutations associated with CFTR-related disease, (iii) mutations with no known clinical consequence, and (iv) mutations with unknown clinical relevance (15). Also, Ooi and colleagues found that certain diseases that resemble CF at an organ-specific level (e.g. pancreatitis) are also strongly associated with mutations in the CFTR gene (15, 72) In conclusion, pancreatitis in the CF patients and the relationship with multiple mutations of CFTR are very complex due to the multiple levels of the disease symptoms, which are different from mutation to another due to the extended classification of those different mutations into the 27 different classes according to the new classification.

9. Conclusion

In CF patients the pancreas is one of the first organs to fail because mutations in CFTR have a critical role in pancreatic pathophysiology. CFTR is the key regulator of the pancreatic duct that regulates the anion exchange between the luminal surface and the cytoplasm of the duct cells. The large number of CFTR mutations are leading scientists to approach an understanding of their functional impact from a different angle with the goal of making a specific correlation between CFTR mutations and certain symptoms such as PI and PS. The lack of correlation between the genotype and the phenotype of the CF patients has led to a new classification (31 possible classes of mutations) based on the severity and the clinical symptoms of the CF patients. The pleiotropic molecular defects of a single mutation in CFTR has limited the effects of drug therapy for some mutants which have been categorized as class I, II, or II/IV (108). The expanded classification of the major mechanistic categories will accommodate the unusually complex, combinatorial molecular/ cellular phenotypes of CF alleles. In addition to the new proposed classification, one more level of complexity is to categorize the mutations into four groups based on the clinical status of the patients: (i) CF-causing mutations, (ii) mutations associated with CFTR-related disease, (iii) mutations with no known clinical consequence, and (iv) mutations with unknown clinical relevance (15). All the recent discoveries and the new hypothesis will help shed light on the complex CF disease from a new perspective, which will help develop a new combined therapy to rectify the mutation or mutations at different levels of CFTR defects.

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11. References

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