1. Pathogenesis of CF

Authors: Angela Koutsokera, Elizabeth Tullis, Marc Chanson, Alain Sauty, Michael A. Morris

1. INTRODUCTION

- Cystic fibrosis (CF) is an autosomal recessive genetic disorder (i.e. affected individuals carry mutations on both copies of the gene; carriers of a single mutation are asymptomatic).
- CF results from mutations of a single gene, the *CF transmembrane conductance regulator (CFTR)* gene, situated on the long arm of chromosome 7. The *CFTR* gene encodes a transmembrane chloride (Cl^-) channel which regulates water and ion transport across the surface of epithelial cells.
- The terms "mutation" and "polymorphism" are often incorrectly used to indicate "disease-causing" and "neutral/benign" respectively, a practice that is misleading and should be avoided when possible. Formally, "mutation" simply indicates variation from the reference sequence without any indication of functional consequence or disease association, whereas "polymorphism" designates a variant with an allelic frequency over 1% in the general population. **The neutral term "variant" is now preferred in reporting laboratory results, accompanied by comments concerning the eventual association with disease.** This convention will be followed in this document.
- More than 2000 *CFTR* variants have been identified at different frequencies among different populations, but not all of them are CF-causing. For some variants the phenotypic repercussions are not clear and, in general, **the link between genotype and clinical phenotype can be variable and unpredictable**.
  - The spectrum of CF clinical phenotypes is wide and characterized by varying degrees of organ involvement, disease severity and progression rate.
  - The term CFTR-related disease (CFTR-RD) is used for clinical entities associated with *CFTR* dysfunction which do not fulfil the diagnostic criteria for CF.
- The genetic laboratory should report on relevant identified variants and also whether they are:
  - CF-causing variants, predicted to be associated with CFTR residual function or not
  - Variants of varying clinical consequences (VVCC)
  - Variants of unknown significance (VUS)

  Note: Variants which are likely to have no clinical relevance should generally not be reported.
- Clinical severity depends on the residual CFTR activity. Modifier genes, such as *TGF-β* or Mannose Binding Lectin-2 (MBL2), may play a role in the clinical phenotype but cannot currently be used to make clinical predictions.

2. VARIANT NOMENCLATURE (TABLE 1)

- Two nomenclature systems are in use
  - The traditional nomenclature system and
  - The HGVS (Human Genome Variation Society) nomenclature

- The traditional system is commonly used and is generally easier to understand. It is used in this document for ease of reading.
- However, traditional nomenclature is imprecise and non-standardized and has led to errors of testing or interpretation. All formal documents communicating genotypes (laboratory reports, clinical summaries and publications) should include HGVS coding of the variant, plus the traditional names if desired.

Table 1: Examples of variant nomenclature (Adapted from[1])

***CFTR* testing result**	**Traditional nomenclature**	**HGVS nomenclature**
No variant detected	Normal	c.[=];[=] *i.e. "=" to the reference sequence*
Heterozygote	F508del/normal	c.[1521_1523delCTT];[=] *"[ ]" indicate the two alleles*
One variant found in a probable compound heterozygote	F508del/unknown	c.[1521_1523delCTT];[?]
Compound heterozygote	F508del/621+1G>T	c. [489+1G>T];[1521_1523delCTT] *in numerical order*
Two variants, phase unknown	F508del and L138P	c.413T>C(;)1521_1523delCTT, p.L138P(;)F508del *amino acid changes (p.) can optionally be included*
Heterozygote with two variants on one allele	R117H-T5/normal	c.[350G>A;1210-12T[5]];[=]
Intron 8 polyT variants*	T5/T7	c.1210-12T[5];[7]

*See **Section 7** of this chapter, "The particular case of IVS8 T5"

3. CFTR VARIANTS

- *CFTR* variants may be classified into four categories, according to their potential to cause disease **(Table 2, Figure 1)**

  a) **CF-causing variants**
  b) **Variants of varying clinical consequences (VVCC)**
  c) **Variants of unknown significance (VUS)**
  d) **Variants of no clinical consequences**

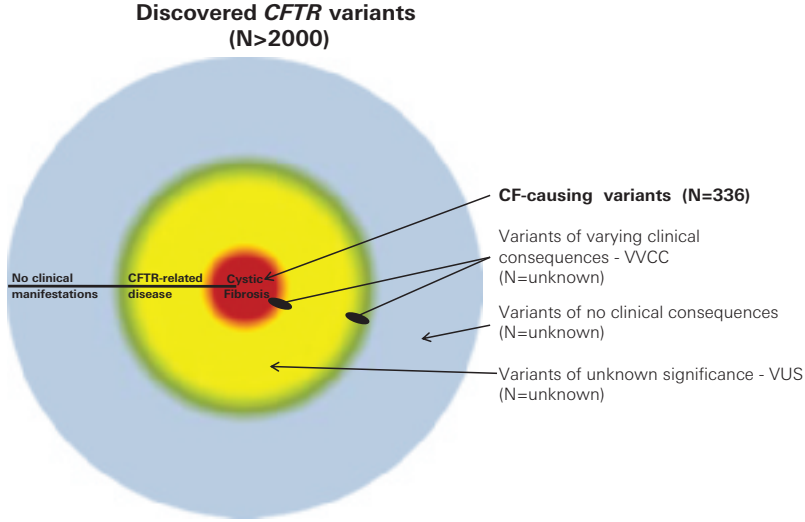
- It must be kept in mind that interpretation of clinically relevant genomic variation is particularly challenging. This is highlighted by the study of Sosnay et al. which assessed the disease liability of variants by studying 39.966 CF patients. In this population, 159 *CFTR* variants had an allele frequency of ≥0.1% and only 127 (80%) met both clinical and functional

Table 2: Common *CFTR* variants classified according to their disease-causing potential (adapted from[2])

Clinical phenotype	Examples of variants	
	Consistent effects	**Inconsistent effects***
CF-causing	F508del G542X, G551D, R553X, 1717-1G>A, W1282X, 3905insT, N1303K	L206W T5(TG13) D1152H R117H
CFTR-related disease	T5(TG12), V562I, L997F	L206W T5(TG13) D1152H T5(TG11) R117H
No clinical consequences	I148T, V470M, F508C, S1235R	T5(TG11) R117H

* Note that some variants (last column) can have very inconsistent effects; for example:
- D1152H can be CF-causing or associated with CFTR-RD
- R117H can be either associated with CF, CFTR-related disease or have no clinical consequences

Figure 1: CFTR variants discovered (until August 2018) and vtheir clinical significance. Of note that some variants have very inconsistent effects and the overlap of cycles can be even greater that the one depicted here, as for example in the case of R117H which can be found in CF, CFTR-related disease or in patients with no clinical manifestations.



criteria consistent with disease. For the remaining variants, assessment of disease penetrance (i.e. proportion of people with a given genotype who exhibited symptoms of CF) led to their characterization as either neutral or indeterminate.

- It is recommended to study the literature for reliable data on the implication of specific CF variants; information can also be found in the following sites:
  https://cftr.iurc.montp.inserm.fr/cftr/
  http://www.genet.sickkids.on.ca/app
  http://www.cftr2.org/index.php
  http://www.umd.be/CFTR/W_CFTR/gene.html

4. CF-CAUSING VARIANTS

- For a variant to be considered CF-causing, one of the criteria presented in **Table 3** must be fulfilled.

Table 3: Criteria for a variant to be considered CF-causing

Variant severely affects CFTR synthesis or function by changing the amino-acid sequence
Variant introduces a premature termination signal (insertion, deletion or nonsense variants)
Variant alters the 'invariant' nucleotides of intron splice sites
Variant predicted to modify the protein sequence, identified as the only change on one allele in a CF patient, after complete gene sequencing.

- **The classes of CF-causing variants:** Not all CF variants affect CF function to the same degree. The effect of a 'CF-causing variant' on the CFTR protein has been used to categorize a variant in one of six classes (**Figure 2, Table 4**).
  - Taking into account the new drugs targeting the molecular defect in CF, the classic classes of CF-causing variants are expected to evolve. Recently two modified classifications of the traditional class I CF-causing variants have been proposed: a) to include in Class I only those variants associated with "no synthesis of the CFTR protein" and to create a new Class for variants associated with "no mRNA transcription" (traditionally also included in class I), b) to divide Class I variants into Class IA (no mRNA) and Class IB (no CFTR protein).
  - Moreover, it has been recognized that a single 'CF-causing variant' may lead to **combinational defects of the CFTR channel**. For example, F508del causes a combination of class II, III and VI defects, this being the rationale behind the use of combinational therapies for homozygous F508del patients. More specifically, F508del impairs conformational maturation (folding defect) resulting in premature degradation and decreased transport of the protein to the apical cell membrane (class II). The CFTR protein which escapes degradation and reaches the apical cell membrane also exhibits a channel gating defect (class III) and a stability defect resulting in increased turnover (class IV) **(Figure 3)**.

Figure 2: Classes of CF-causing variants

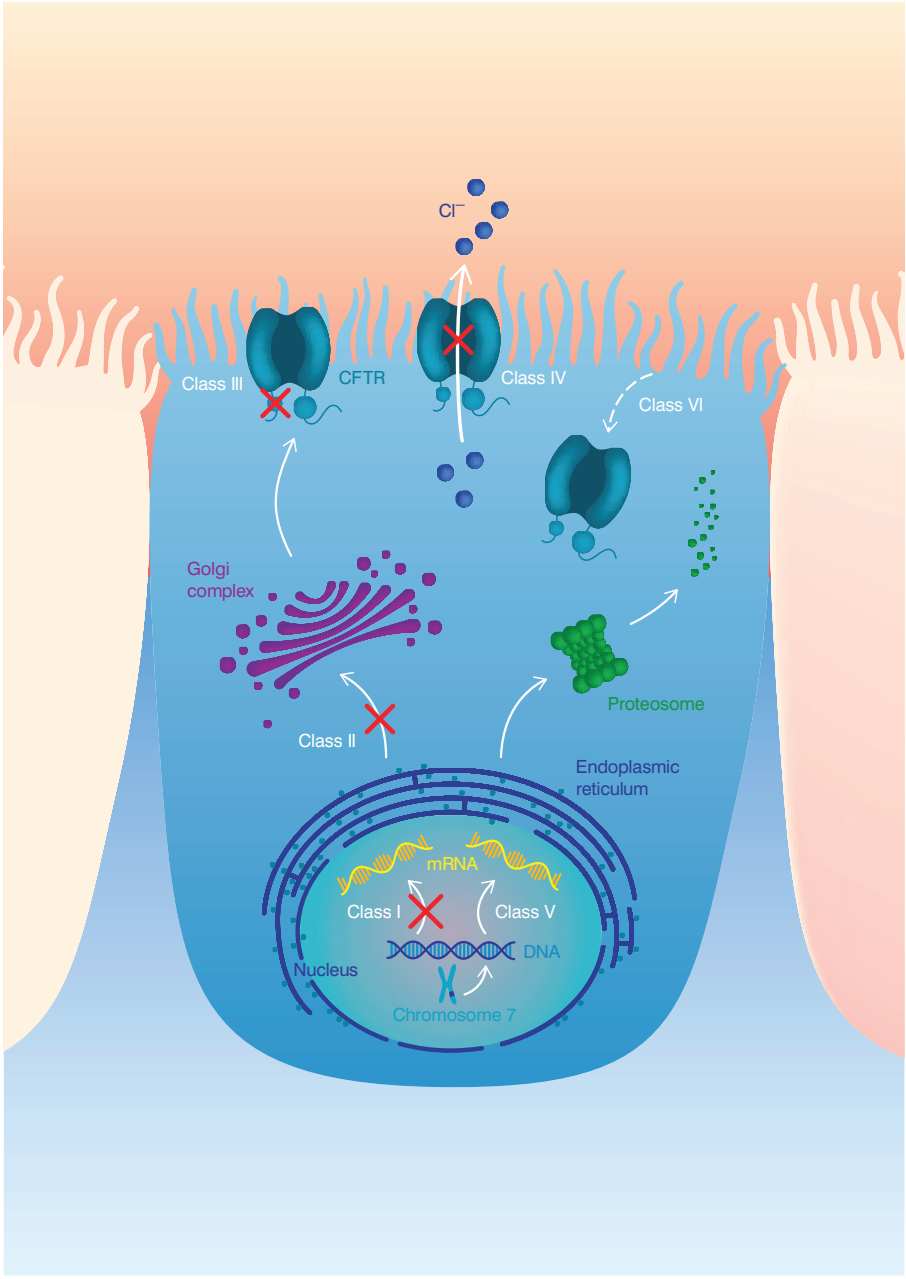


Table 4: Classes of CF-causing variants

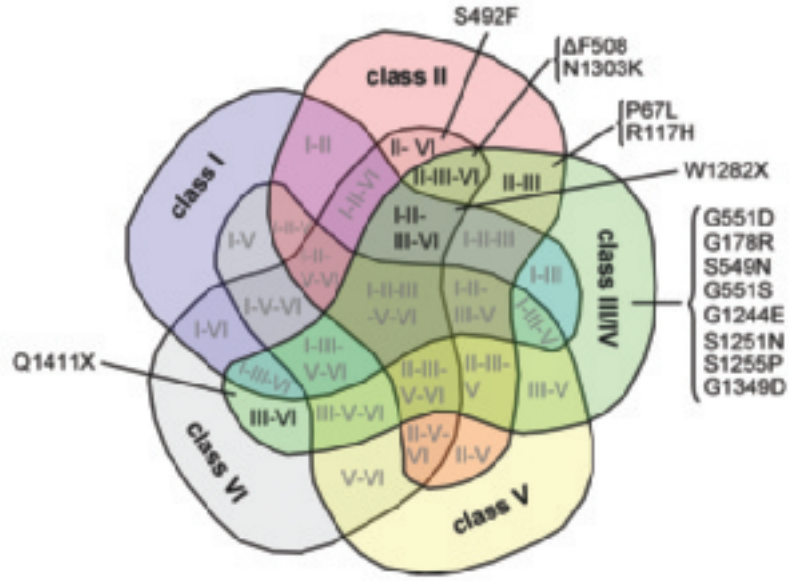
	Class I	Class II	Class III*[a]	Class IV*[a]	Class V	Class VI
Definition	Production defect: unstable, truncated or no CFTR synthesis	Processing defect: defective maturation and premature CFTR degradation	Gating defect: defective regulation of the opening of the Cl- channel	Conductance defect: defective permeation of anions through the channel	Quantitative defect: reduced amounts of functional CFTR	Stability defect: abnormally high CFTR turnover
Examples*[b]	E60X, 621+1G>T, 711+1G>T, G542X, R553X, 1717-1G>A, 1898+1G>A, 2183delAA>G, 3120+1G>A, W1282X	1078delT, I507del, **F508del***[c], S549N, S549R, 1677delTA, 3659delC, N1303K	G178R, S549N, S549R, G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P	G85E, R117H, R334W, R347P, R347H, D1152H	IVS8 T5, A455E, 2789+5G>A, 3849+10kbC>T	4171delC, Q1412X

*[a] The CFTR potentiator Ivacaftor is being used therapeutically for Class III variants and also for R117H (class IV variant). It has been mostly studied for the class III variant G551D. In 2017, the United States Food and Drug Administration (FDA) approved ivacaftor also for patients who have at least one of 23 residual function CFTR variants (i.e. variants which result in partially functioning CFTR). For more details see **Chapter *"CFTR modifiers"***.

*[b] For convenience, many of the variants present in typical first-level screening panels are indicated.

*[c] F508del (c.1521_1523del: the deletion of three nucleotides removing the codon for phenylalanine at position 508) is the most common CF-causing variant worldwide. It affects the structure of CFTR in the nucleotide-binding domain-1 preventing conformational maturation (folding defect) which results in premature degradation and decreased transport of the protein to the apical cell membrane (mainly class II defect). Interestingly, the CFTR protein which escapes degradation and reaches the apical cell membrane also exhibits a channel gating defect (class III) and a stability defect resulting in increased turnover (class IV).

Figure 3: Refined classification of CF variants accounting for complex phenotypes of major CFTR according to variant class. Venn diagram indicating all combinations of mutation classes with examples (republished with permission of the American Society for Cell biology, from Veit et al. 2016 Ref[3], permission conveyed through Copyright Clearance Center, Inc). *Note:* ΔF508 refers to F508del.



5. VARIANTS ASSOCIATED WITH CFTR-RELATED DISEASE

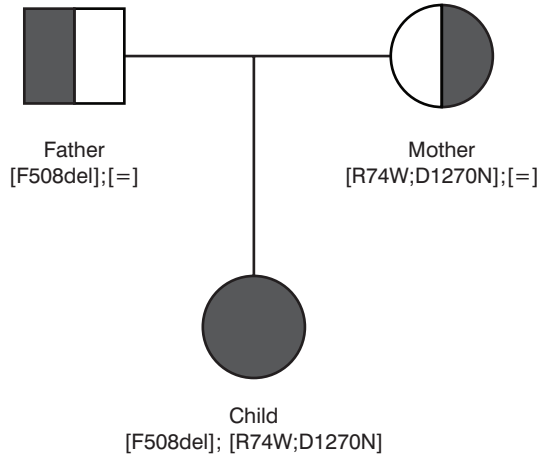
- Several clinical entities associated with CFTR dysfunction do not fulfil the diagnostic criteria for CF. These are commonly but not always, single-organ disease phenotypes and are called CFTR-related disease or disorder (CFTR-RD) **(see also Chapter "*Diagnosis in adults*")**.
- The best-recognized forms of CFTR-RD are:
  - congenital bilateral absence of the vas deferens (CBAVD)
  - acute recurrent or chronic pancreatitis
  - diffuse bronchiectasis
- It is not possible to predict clinical manifestations based uniquely on genotype data. As shown in **Table 2**, some variants have varying clinical consequences and can be associated with CFTR-RD, CF or no disease.

6. COMPLEX ALLELES

- As CF is an autosomal recessive disease, patients must have disease-causing variants on both copies (alleles) of their *CFTR* gene (one inherited from the mother, one from the father). Typically, there is only one disease-causing variant per allele.

- Some rare patients have not two but three or more variants. For example a patient affected with CF may have received one mutated allele from the father and two or three from the mother, in "*cis"* on a *complex allele* **(Figure 5).**

Figure 5: In this example case, the girl affected with CF has received one mutated allele from each parent: F508del from her father and a complex allele with two variants from her mother.

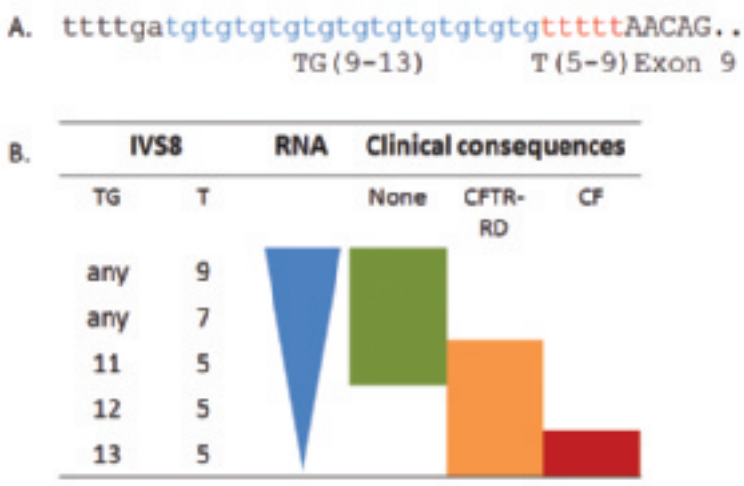


- One consequence of the existence of complex alleles is that it is not always sufficient to find two *CFTR* variants to confirm a diagnosis: ideally it should be proven, by studying the parents, that the variants are on separate alleles (in "*trans*") but in some cases it can be probabilistic (e.g. F508del and R117H are essentially certain to be in trans).
- When the two variants are on the same parental *CFTR* allele (in "*cis*") and not on different alleles (in "*trans*"), the diagnosis of CF cannot be established by this genetic test result. In such a case, it is necessary to search for another variant, located in *trans*, for example by sequencing the entire gene.

7. THE PARTICULAR CASE OF INTRON VARIANT SEQUENCE 8 "IVS8 T5"

- This variant concerns a region inside the *CFTR* gene which controls mRNA splicing, and indirectly controls the amount of functional CFTR protein produced.
- The *CFTR* locus in humans has a variable number (9–13) of TG repeats followed by a polythymidine tract (poly T tract) of 5, 7, or 9 Ts in intron 8 **(Figure 6)**.
- Shorter polyT tracts are associated with lower levels of correctly-spliced mRNA, and lower quantities of functional CFTR protein in the cell.
  - T9 and T7 tracts have no pathological consequences as the amount of mRNA results in sufficient CFTR function, whereas

Figure 6: The particular case of "IVS8 T5" A) The TGmTn variant sequence in intron 8 ("IVS8") of the *CFTR* gene. The example would be traditionally described as "IVS8 T5(TG11)". B) Consequences of the TGmTn variants. Shorter T tracts are associated with defective splicing of RNA, leading to a reduced quantity of CFTR protein; Longer TG tracts aggravate this effect and modulate the severity of the T5 effect. T5, in the presence of another CF-causing variant in trans, may result in CF (e.g. F508del/5T). The combination of 5T in cis with R117H is considered a CF-causing variant (although it can result in a sweat Cl^- test <60mmol/l).



- T5 tracts result in more significant reduction in CFTR function which
  - can have no consequence OR
  - can result in CFTR-RD OR even
  - in the presence of another CF-causing variant in trans, they can be associated with CF. Usually there is residual *CFTR* function, e.g. pancreatic sufficient CF.

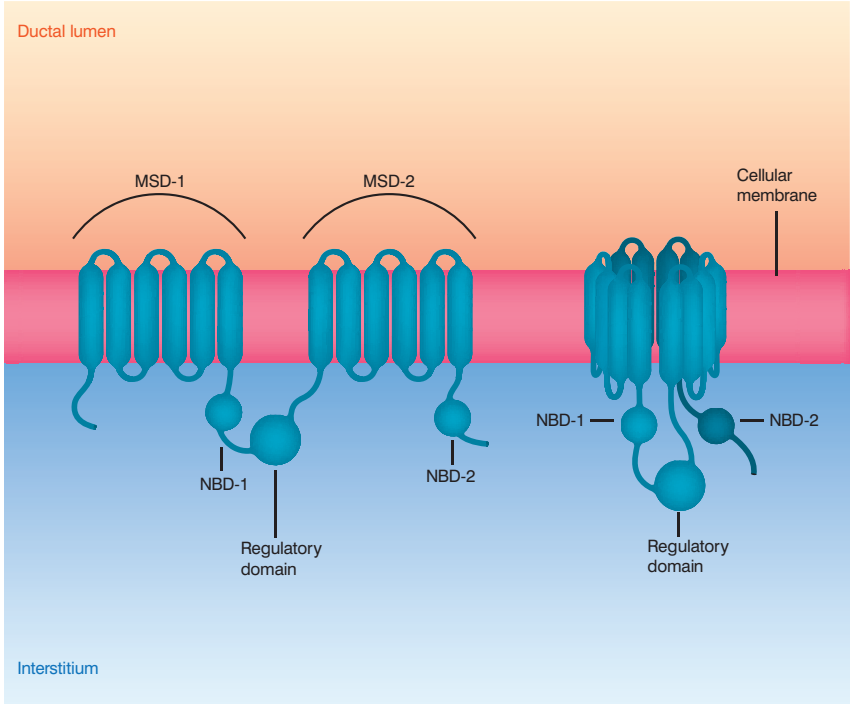
- The number of TG repeats immediately adjacent to 5T correlates with exon 9 skipping and abnormal splicing. Transcripts bearing more TG repeats increase the penetrance of 5T.

8. STRUCTURE OF NORMAL CFTR

- CFTR is a member of the ABC (ATP-Binding Cassette) proteins. It is comprised of 1480 amino-acids and it is expressed in the apical cell membranes of epithelial cells.
- CFTR structure (**Figure 7**)
  - Two regions of six membrane-spanning domains (MSD-1 and MSD-2): they form the Cl^-/HCO_3^- channel.
  - Two nucleotide-binding domains (NBD-1, NBD-2): they mediate ATP hydrolysis hence regulating the opening and closing of the channel.

- R domain: its phosphorylation allows the opening of the channel.
- The PDZ domain at the C-terminus region: is required for the polarization of CFTR to the apical plasma membrane.

Figure 7: Structure of the normal CFTR

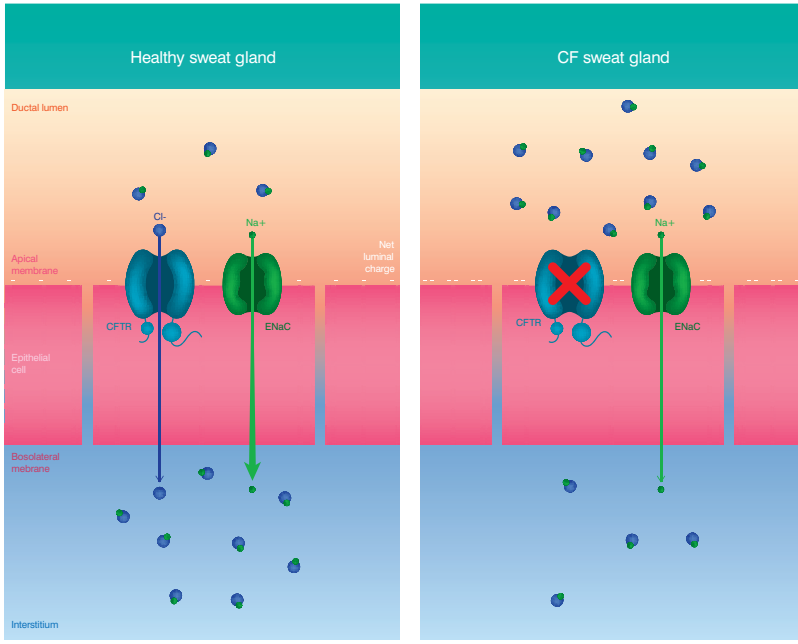


9. FUNCTION OF CFTR

- CFTR is present at the apical membrane of the epithelial cells. It is expressed throughout the body and has a role in the function of many organs.
- The main function of CFTR is that of an **apical epithelial Cl^- channel (Figures 8-10); however its role is multifaceted** and extends beyond Cl^- transport.
- **CFTR regulates/influences**
  - HCO_3^- transport (e.g. CFTR secretes HCO_3^- but also regulates the function of SLC26A anion transporters such as Cl^-/HCO_3^- exchanger): this mechanism plays an important role on fluid transport not only in the pancreatic ducts and the gastrointestinal tract but also in the lung.
  - The pH of airway surface liquid
  - Water transport: fluid transport is driven by active Cl^- transport (in the lungs) or by HCO_3^- transport (in the pancreas, duodenum).

- Epithelial sodium channels (ENaC): It consists of four subunits (two alpha, one beta and one gamma). ENaC regulation by CFTR is not fully elucidated, but appears to play major role in CF. **When the NBD-1 region of CFTR is active, it has an inhibitory effect on ENaC.**
- Other chloride channels [e.g. SLC26A9, TMEM16A, CaCC which is a Ca^{2+} regulated Cl^- conductance, ORCC (Outward Rectifying Cl^- Channels) which is a cAMP regulated Cl^- conductance]
- Potassium channels (e.g. ROMK+)
- The secretion of other anions (e.g. glutathione, thiocyanate)
- The production of mucins
- CFTR regulates the sweat Cl^- absorption and the β-adrenergic sweat secretion (but not the cholinergic sweat secretion) **(Figure 9)**.

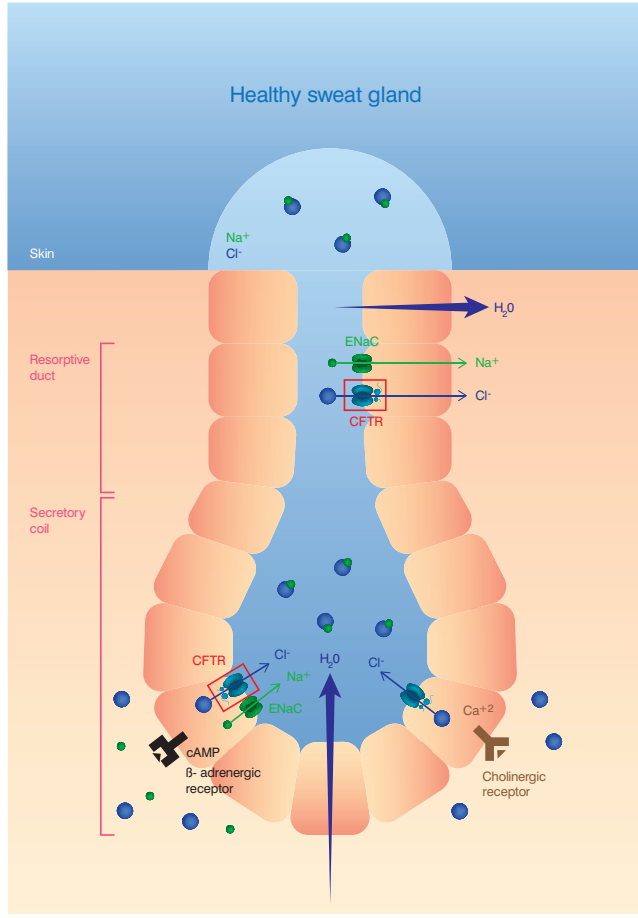
Figure 8: CFTR-regulated sweat chloride absorption in a) a healthy sweat duct, b) a CF sweat duct



10. DYSFUNCTION OF CFTR

- **In the sweat glands (Figures 8 and 9):**
  - **Sweat Cl^- absorption:** Abnormal CFTR → decreased reabsorption of Cl^- from the ductular lumen into the interstitium, while there is an excessive Na^+ transport in the interstitium through other channels → the ductal lumen becomes more negative than the interstitium (elevated transepithelial potential difference) →

Figure 9: CFTR regulates the sweat Cl^- absorption and the β-adrenergic sweat secretion (but not the cholinergic sweat secretion).

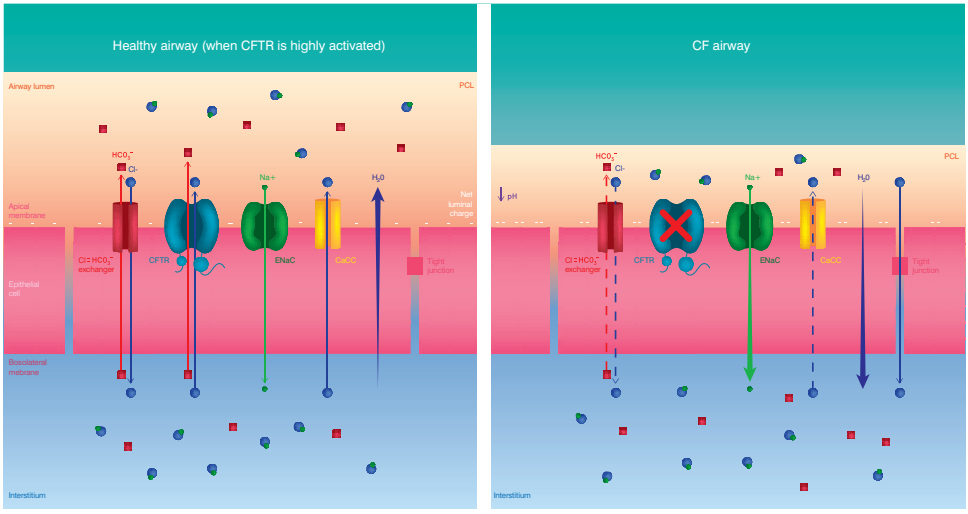


decreased total NaCl flux into the interstitium → high concentration of NaCl in the sweat of CF patients and increased transepithelial potential difference (phenomena used for diagnostic purposes).

- **Sweat secretion (Figure 9):** Abnormal CFTR → reduced β-adrenergic sweat production → reduced volume of sweat in CF (phenomenon used for diagnostic purposes).

- Note: during the sweat Cl^- test, application of pilocarpine stimulates the cholinergic sweat production (which is unaffected by CFTR). This leads to the production of sweat, used for the subsequent measurement of the Cl^- concentration (which is affected by CFTR). During

Figure 10: Function of CFTR in the airway epithelium of a) healthy airway and b) CF airway. In the healthy lung the direction of Cl^- movement through CFTR depends on its level of activation: at a low level of activation CFTR absorbs Cl^- in the interstitium whereas at a high level of activation CFTR secretes Cl^- in the airway lumen. In CF, the currently favored model emphasizes the hyperactivation of ENaC, the activation of non-CFTR Cl^- pathways and the decreased secretion of HCO_3^- leading to low airway surface liquid pH.



the β-adrenergic sweat secretion test the mean maximal β-adrenergically stimulated sweat rate (which is affected by CFTR) is measured **(see Chapter "*Diagnosis in adults*")**.

- **In the lungs (Figure 10):**
  - The airway surface liquid (ASL) contains two aqueous layers:
    1. Mucus layer containing gel-forming mucins
    2. Periciliary layer (PCL): which provides the microenvironment of beating cilia; it lubricates their movement and prevents mucus adhesion.
  - **The low-volume model** is currently the favoured model**:** it postulates that the normal ASL has salt levels similar to those of plasma. This model emphasizes the hyperactivation of ENaC in the absence of CFTR, the activation of non-CFTR Cl^- pathways and the decreased secretion of HCO3-.
    - Abnormal CFTR → hyperactivation of ENaC → Na^+ hyperabsorption into the interstitium → increased transepithelial potential difference (airway lumen more negative than interstitium) → relative increase of Cl^- permeability into the interstitium through non-CFTR pathways → net increase in the absorption of NaCl → osmotic increase

of water absorption into the interstitium → dehydration, depletion of volume of the airway-surface fluid, viscous and adherent mucous → abnormal mucociliary clearance → inflammation, bacterial infection → tissue destruction.

– Abnormal CFTR → abolition of HCO_3^- secretion through CFTR and dysfunction of Cl^-/HCO_3^- exchanger → decreased secretion of HCO_3^- in the airway lumen → decreased airway pH → decreased activity of antimicrobial peptides and further hyperactivation of ENaC → inflammation, bacterial infection → tissue destruction.

- **In the pancreas:**
  - Abnormal CFTR → reduced HCO_3^- secretion from the pancreatic duct cells into the lumen → pancreatic enzyme precipitation, mucus accumulation → reduction of pancreatic enzyme delivery to the duodenum → pancreas destruction (exocrine and endocrine pancreatic insufficiency).

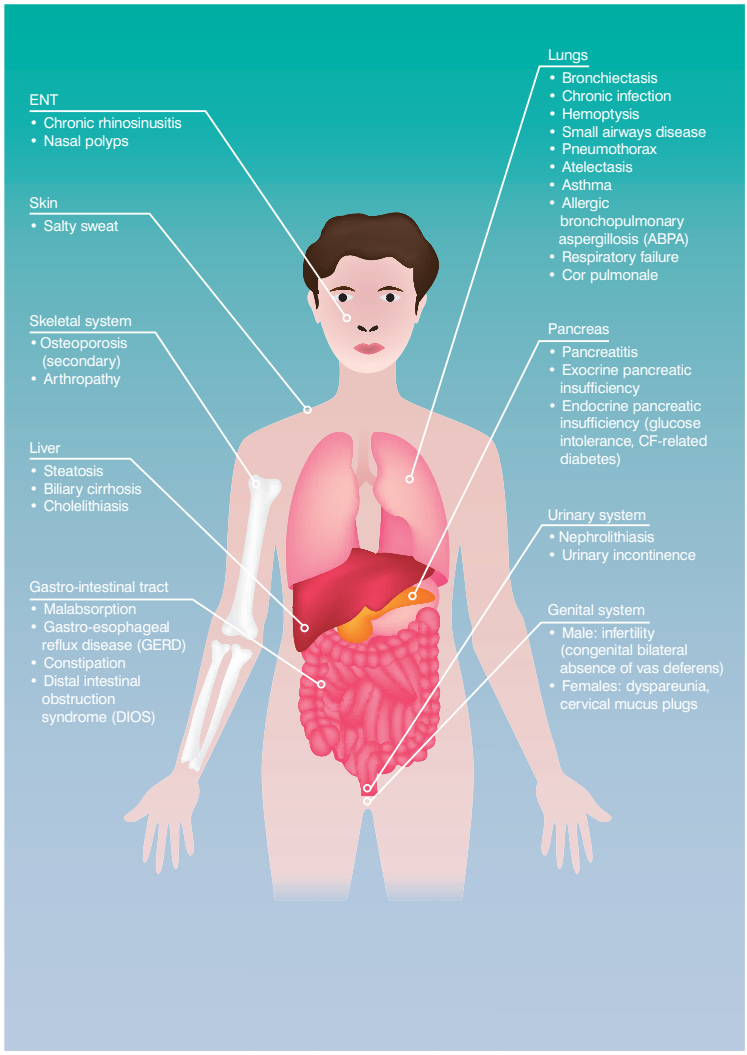
11. MECHANISMS IMPLICATED IN CF

- **As described above, the 2 main mechanisms are** a) the abnormal NaCl and fluid transport across the epithelial surface leading to the production of mucus with abnormal properties (mucus plugging) and b) decreased pH of the airway-surface liquid leading to antimicrobial activity impairment.
- **Additional mechanisms:**
  - Defective mucin unfolding: mucins are glycoproteins which are the main component of mucous. They are packed in the granulae of goblet cells with hydrogen ions and Ca^{+2} and unfold upon release on the mucosal surface. Bicarbonate secreted through CFTR contributes to the unfolding of mucins by increasing the pH and by chelating Ca^{+2}. This mechanism is impaired in CF leading to defective mucin unfolding and mucous plugging.
  - Concomitant host-defense defects resulting in increased levels of IL-8, IL-6. TNFα and LTB4, reduced levels of anti-inflammatory cytokines.
  - Oxidant/antioxidant imbalance.
  - Animal studies suggest that infants with CF may have congenital airway defects, contributing to disease progression (e.g. air trapping due to a structural airway abnormalities).
  - Impaired airway surfactant function.
- The diversity of mechanisms and of effects of CFTR defects contribute to a vicious cycle and sustained disease/complexity of treatment.

12. THE SPECTRUM OF CLINICAL MANIFESTATIONS OF CF

- CFTR is expressed in epithelia throughout the body and its absence or malfunction may lead to a large spectrum of clinical manifestations **(Figure 11).**
- *CFTR* genotype and CF phenotype associations are often discordant, and for that reason the genotype cannot predict the clinical course of individual patients.
- The clinical phenotype (type and number of affected organs, severity of the disease, age of onset) depends on genetic CFTR and non CFTR modifiers, environmental factors and treatment.

Figure 11: Organs affected by CFTR dysfunction and manifestations of CF



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