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Amandine Collin was born in 1991 and graduated in biomedicine (research focus) in 2014 from the Université catholique de Louvain. After completing an additional diploma in management in ICHEC Brussels School of Management, she joined the Pole of Pneumology of the Institute of Experimental and Clinical Research. There, she worked as a PhD student on mucosal immunity in cystic fibrosis, more particularly on immunoglobulin A secretion and epithelial differentiation on multiple cystic fibrosis models.

Dans la mucoviscidose, des infections pulmonaires récurrentes suggèrent une immunité défectueuse. L'épithélium et la sécrétion d'immunoglobuline A (IgA) représentent des lignes de défense majeures des muqueuses. L'objectif de cette thèse est d'évaluer l'immunité IgA et la différenciation de l'épithélium respiratoire dans la mucoviscidose.
Nous avons observé une augmentation de la production d'IgA et d'expression bronchique de pIgR dans les poumons de patients, due à une réponse immunitaire Th17 contre l'infection. En revanche, en absence d'infection, les cellules issues de ces patients et les expériences murines montrent une régulation négative de pIgR en présence de mutations F508del de CFTR, associée avec la réponse des protéines dépliées. Nous avons aussi démontré une altération de la différenciation et de l'intégrité de l'épithélium respiratoire chez les patients atteints de mucoviscidose.
En conclusion, cette thèse révèle dans la mucoviscidose une interaction complexe entre les cellules épithéliales dédifférenciées et l'infection (à Pseudomonas aeruginosa), qui relient CFTR, l'activation de la réponse des protéines dépliées, une réponse immunitaire (Th17) et la sécrétion d'IgA dans le poumon.

*In cystic fibrosis (CF), recurrent pulmonary infections suggest lung impaired mucosal immunity. The epithelium and the secretion of immunoglobulin A (IgA) by polymeric immunoglobulin receptor (pIgR) are major lines of mucosal defence. Therefore, this thesis aimed at assessing airway epithelial function (IgA secretion) and differentiation in CF.*
*We observed an enhanced bronchoepithelial pIgR and IgA production in the CF lung, indicating upregulated humoral mucosal immunity due to Th17 host immune response against infection. By contrast, in the absence of infection, cell and animal experiments displayed a negative regulation of pIgR expression upon CFTR F508del mutation, associated with unfolded protein response. We also showed a ciliated cell defect and several signatures of impairment of the airway epithelium integrity, including epithelial-to-mesenchymal transition, in CF lung and cell.*
*In conclusion, this thesis reveals in CF a complex interplay between dedifferentiated epithelial cells and (Pseudomonas aeruginosa) infection, which connects CFTR dysfunction, unfolded protein response activation, and a (Th17) host immune response to IgA secretion in the lung.*

2021 Amandine Collin Impaired airway IgA immunity and epithelial differentiation in cystic fibrosis

UCLouvain

IREC Institut de Recherche Expérimentale et Clinique

# Impaired airway immunoglobulin A immunity and epithelial differentiation in cystic fibrosis

**Amandine Collin**

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Thèse présentée en vue de l'obtention du grade
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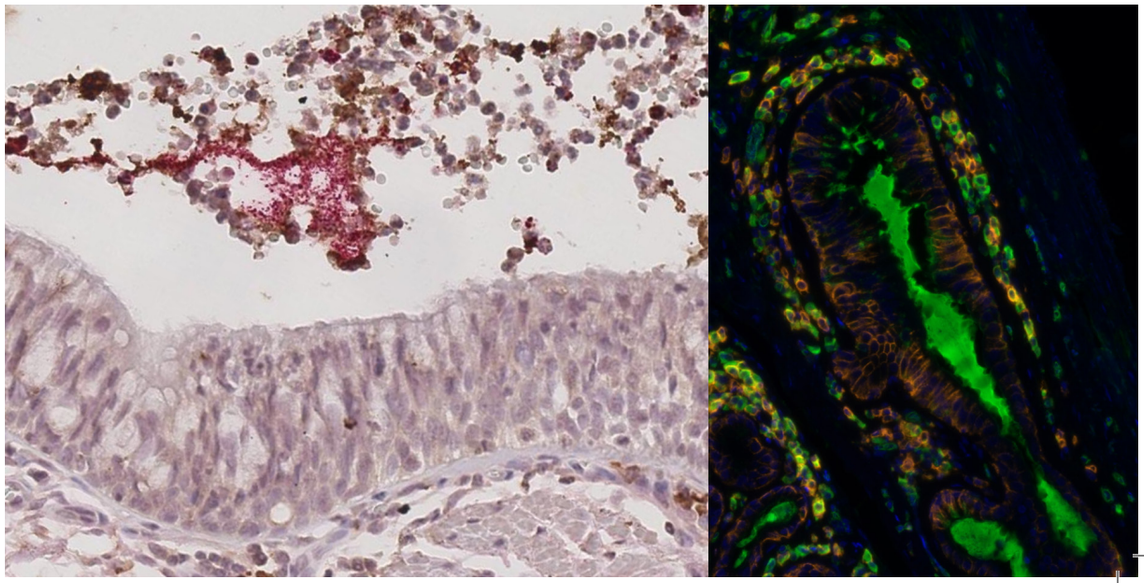

UNIVERSITÉ CATHOLIQUE DE LOUVAIN

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INSTITUT DE RECHERCHE CLINIQUE ET EXPÉRIMENTALE (IREC),

PÔLE PNEUMOLOGIE, ORL ET DERMATOLOGIE

# IMPAIRED AIRWAY IMMUNOGLOBULIN A IMMUNITY AND EPITHELIAL DIFFERENTIATION IN CYSTIC FIBROSIS

**Amandine COLLIN**

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je le remercie pour sa patience infinie et pour tout le travail qu'il effectue pour nous, notre maison, notre futur.

# CONTENTS

# LIST OF ABBREVIATIONS

- ALI Air-liquid interface
- APRIL A proliferation inducing ligand
- ASL Airway surface liquid
- BAFF B cell activating factor
- BAL Bronchoalveolar lavage
- BEBM Bronchial epithelial basal medium
- BSA Bovine serum albumin
- cAMP Cyclic AMP
- CD Cluster of differentiation
- CF Cystic fibrosis
- CFTR Cystic fibrosis transmembrane conductance regulator
- COPD Chronic obstructive pulmonary disease
- d-Ig Dimeric immunoglobulin
- DMEM Dulbecco's modified Eagle's medium
- DMSO Dimethyl sulfoxide
- eIF2α Eukaryotic initiation factor 2α
- ELISA Enzyme-linked immunosorbent assay

- EMT Epithelial-to-mesenchymal transition
- ENaC Epithelial Sodium ($Na^+$) Channel
- ER Endoplasmic reticulum
- FcαR Immunoglobulin A Fc receptor
- FEV1 Forced expiratory volume in one second
- FOX Forkhead box
- GAPDH Glyceraldehyde 3-phosphate dehydrogenase
- HBEC Human bronchial epithelial cells
- HRP Horseradish peroxidase
- IFN-γ Interferon-γ
- Ig Immunoglobulin
- IL Interleukin
- J Joining
- KO Knockout
- MALT Mucosa-associated lymphoid tissues
- MCIDAS Multiciliate differentiation and DNA synthesis associated cell cycle protein
- MUC Mucin
- MRSA Methicillin-resistant *Staphylococcus aureus*

- Myb — Myb proto-oncogene
- NE — Neutrophil elastase
- p63 — Tumour protein 63
- *Pa* — *Pseudomonas aeruginosa*
- PBS — Phosphate-buffered saline
- pIgR — Polymeric immunoglobulin receptor
- RBM — Reticular basement membrane
- RFX — Regulatory factor X
- RORγt — RAR-related orphan receptor γt
- RPMI — Roswell Park Memorial Institute
- SC — Secretory component
- SDS — Sodium dodecyl sulfate
- S-Ig — Secretory immunoglobulin
- SPDEF — SAM pointed domain containing ETS transcription factor
- STAT — Signal transducer and activator of transcription
- TBS — Tris-buffered saline
- TGFβ — Transforming growth factor β
- Th — T-helper
- TLR — Toll-like receptor

- TNF Tumour necrosis factor
- UPR Unfolded protein response
- vPAO1 Virulent *Pseudomonas aeruginosa* PAO1 laboratory strain
- XBP-1 X-box binding protein 1

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# CHAPTER 1: INTRODUCTION AND GOALS OF THE THESIS

## 1 Introduction

### *1.1 Cystic fibrosis*

Cystic fibrosis (CF) is the most common life-limiting autosomal recessive disorder in Caucasian population. This disease was identified in 1938 by Dorothy Andersen who described fibrosis of the pancreas in 49 patients, later associated with lung infection and salt loss during the 1948's heat wave in New York (1, 2). Patients with CF develop a multisystemic disease (3), caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, discovered in 1989 (4-6).

#### 1.1.1 Epidemiology

CF affects around 85,000 individuals worldwide (7). In Belgium, in 2018, 1320 CF patients were included in the Belgian CF Registry – in the knowledge that more than 90% of patients are registered (8, 9). Incidence varies according to race and ethnicity (3) and is around 1 in 3000 live births in people of northern European ancestry. Belgium is the fifth country with the greatest incidence worldwide (10) where it affects 1/2850 births (11). Moreover, the carrier frequency is estimated at one in 25 people (12). During the last decades, improving diagnostic methods, establishing new-born screening (in Belgium in 2019 (13)) and better clinical outcome have impacted the epidemiology of CF. It is suggested that the prevalence is increasing because of the improved survival (3). Indeed, this survival improvement is noticeable in CF population. CF was for a long time considered as a paediatric concern. However, in 2018, in Belgium, 64.3% of patients were adults, versus 38.4% in 2000 (8, 9).

### 1.1.2 Pathophysiology

CF is caused by mutations in *CFTR* gene, which is expressed by multiple cell types, notably by epithelial cells. Therefore, CFTR protein is present in multiple organs which explains the systemic nature of the disease.

#### 1.1.2.1 Cystic fibrosis transmembrane regulator

*CFTR* is located in the long arm of human chromosome 7 (and in chromosome 6, in mouse (14)) (15). This gene encodes an anion channel, member of ATP-binding cassette family. The produced 1480 amino acids protein is characterised by two nucleotide-binding domains, able of binding and hydrolysing ATP, and two membrane-spanning domains that give form to the pore through the plasma membrane (16). Moreover, contrary to other members of ATP-binding cassette family, it carries an additional regulatory domain. The latter is a highly charged random structure, which makes accessible nine consensus sequences for phosphorylation by protein kinase A. Phosphorylation of this region is a key event for channel opening (17).

CFTR is expressed at the apical surface of all epithelia. It has been localised in the epithelial ciliated cells of the nasal mucosa (18), trachea (19), lung, exocrine pancreas, intestines, sweat glands, kidney (20), salivary glands (21), testis and uterus (22) as well as in serous cells in submucosal glands (23) and in ionocytes, rare cells which have recently been discovered and highly express CFTR (24, 25). Moreover, CFTR has more recently shown to be expressed in other cell types, including immune cells (26), smooth muscle cells (27), neural cells (28) or heart cells (29). In addition to this presence in mature organs, CFTR is expressed during development, including in the lungs (29-31). The pattern can differ from adult organs and, in the lung, the highest expression is seen during the second trimester of gestation (30, 31).

CFTR protein was firstly purified by Bear *et al*. (32) and is responsible for anion conductance, more particularly chloride (33), bicarbonate (34), thiocyanate (35) and glutathione (36). Channel opening is regulated by protein kinase A, which is able to phosphorylate the consensus sequences in the regulatory domain, through cyclic AMP (cAMP) (37). Similarly to other members of the ATP-binding cassette protein family, ATP hydrolysis by nucleotide-binding domains is required (38).

In addition, CFTR was described to regulate other ion channels and transporters. Interestingly, Epithelial $Na^+$ Channel (ENaC) activity is shown to be downregulated by CFTR (23) and is implicated in the current paradigm of CF physiopathology.

#### 1.1.2.2 From the gene defect …

Currently, over 2000 mutations in this gene have been described. Around 3% involve large rearrangements of CFTR, 1% affects the promoter region, 13% seem to be neutral variants and the effect of the remaining 7% is unclear (39). The majority of mutations are missense (39%) while 16% are frameshift, 11% are splicing, 9% are nonsense, and 2% implicates in frame insertion or deletion (39). Around 360 are known to be “disease-causing” (40). CF disease is highly heterogeneous in severity and in progression, and genotype only partly contributes to this variability. To date, CFTR mutations are divided into seven groups according to the functional consequence on the protein (7).

| Mutation class | Mechanism of dysfunction | Representative mutations | Notes |
|---|---|---|---|
| I | Reduced channel number by major disruptions of the CFTR gene – insertions, deletions and premature termination codons | G542X, R553X | Usually associated to more severe phenotypes |
| II | Misfolding mutations that destabilise the CFTR protein and that promote to premature degradation by the proteasome, disrupting its normal localisation to the plasma membrane | F508del | F508del is the most common mutation |
| III | Reduced function by disordered regulation and gating, which causes diminished ATP binding and hydrolysis | G551D | G551D is the most prevalent example of patients of the class III (approximately 4 to 5% on at least one allele) (41) |
| IV | Defective chloride conductance | R117H, R334W, R347P | |
| V | Aberrant splicing that reduces or eliminates full-length and stable mRNA transcripts | 3849-10kbC→T, A455E | |
| VI | Mutations that reduce surface stability and accelerate the turn-over | Q1412X, 432delTC 4279insA | |
| VII | No mRNA transcription | dele2,3 (21 kb), 1717-1G→A | (Pharmacologically) unrescuable mutations |

**Table 1. CFTR mutations classification.**
Adapted from (7, 42, 43).

The classification of CFTR alleles into mutation classes facilitates the understanding of the cellular defect. Nevertheless, it is now clear that many mutations lead to more than one feature (3). Member of class II mutations, F508del (deletion of phenylalanine at the position 508) accounts for about two-thirds of mutated alleles in northern European and North American populations (11) and results in destabilised protein folding due to altered nucleotide-binding domain 1 stability, leading to degradation by the proteasome. In addition, the F508del protein is subject to altered gating (class III) and cell surface residence time (class VI) (44). In the 2017 Belgian CF Registry data, 83.8% of patients carried at least one copy of the mutation (8, 9). Although CFTR mutation frequencies vary according to population, no other mutation is found in more than 5% of CF patients (11).

Class I-III – and VII (7) – mutations are usually associated with a more severe disease, compared with mutations associated with a residual function of CFTR (11, 42).

Nevertheless, patients with a same CFTR mutation (e.g. F508del homozygous patients) may display very different clinical phenotypes, suggesting the existence of modifier genes and/or environmental cofactors (11, 12). On the genetic side, it is estimated that 50% of phenotypic differences in pulmonary disease could be attributed to modifier genes (45). Transforming growth factor β1 (TGF-β1) was the first described. Polymorphisms, more particularly codon 10 CC, lead to a higher production of TGF-β1, associated with a more severe pulmonary disease (46). TGF-β1 polymorphisms would be responsible of 15-20% of variability in the severity of pulmonary disease (45). A second modifier gene, mannose-binding lectin-2, is a protein from collectin family which selectively links D-mannose and N-acetyl-D-glucosamine on the bacteria surface and triggers innate immunity (complement system) to eliminate these bacteria. Polymorphisms in this gene lead to a defective protein and to a significant increase in the risk of infection by *Pseudomonas aeruginosa* (*Pa*). Together with TGF-β1 polymorphism, they could be associated

with a worsening of respiratory function (47). In addition to modifier genes, exposure to some environmental factors has been shown to influence disease progression. These factors include tobacco consumption and second-hand smoke, pollutants, climate and exposure to microorganisms. Social factors, such as access to specialised care, adherence to treatment and variation in clinical practice, may also impact the clinical patients' phenotype or course (3, 11).

#### 1.1.2.3 … To the disease

Different hypotheses were postulated in order to explain the CF physiopathology. It remains possible that aspects of these theories could coexist (11).

The "low-volume hypothesis" is the traditional way of explaining airway mucosal obstruction. Due to the loss of ability to inhibit ENaC activity, a defective CFTR protein leads to increased sodium influx, resulting in airway surface liquid (ASL) dehydration and decreased periciliary volume. This ultimately causes compressed cilia and affects mucociliary clearance. In addition, dehydrated mucus forms niches of local hypoxia which may aggravate the risk of infection (11). The contribution of ENaC to CF pathophysiology is supported by the fact that a mouse model characterised by excessive functioning of ENaC displays a CF-like phenotype (48).

The "high-salt hypothesis" allows to explain transepithelial potential difference measured in the disease. Defective CFTR prevents chloride to follow sodium influx, and leads to chloride retain in the ASL (more negatively charged), as noted in the CF sweat gland epithelium (16). Therefore, sodium chloride in the ASL is highly concentrated (11) and could inactivate innate antimicrobial peptides, such as β-defensin-1 (49), potentially predisposing to bacterial infections with pathogens such as *Pa* (50).

A more recent hypothesis suggests that loss bicarbonate conductance is associated with reduced pH. As high salt concentration, decreased pH may impair antimicrobial molecules activity (51). Moreover, bicarbonate release is thought to be necessary for proper mucin expansion and thus participates in adequate viscoelastic properties of mucus (52). CFTR defect leads to defective mucin conformation and viscous secretion with abnormal adherence properties (53).

Since CFTR is also expressed in non-epithelial cell types, additional implications of the protein are progressively described. Of note, impaired innate defence associated with a CFTR defect in neutrophils (54) or macrophages (55)) has been recently reported, as well as impaired excitability of neurons (56).

### 1.1.3 Clinical expression

Regardless of the implicated hypothesis, CFTR dysfunction leads to a plethora of manifestations in the organs where it is expressed ( Figure 1). Lungs, pancreas, gastrointestinal tract, vas deferens and sweat glands could be affected, although airway disease remains the leading cause of morbidity and mortality (3).

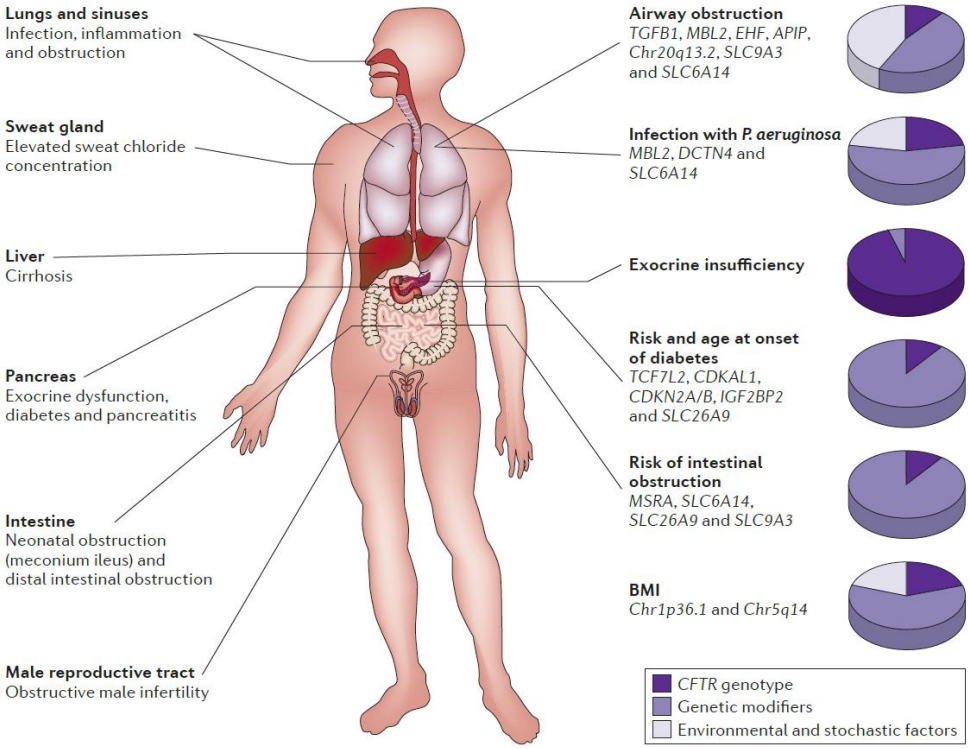


**Figure 1. CF is a multisystemic disease.**
CF affects multiple organs (bold) through different symptoms (on the left). Although CFTR genotype is mainly responsible for exocrine insufficiency of the pancreas, genetic modifiers (including some listed) and to a lesser extend environmental and stochastic factors have an important impact on phenotype (on the right) (45).

#### 1.1.3.1 Gastrointestinal disease

About 90% of CF patients suffer from exocrine pancreatic insufficiency. Because CFTR is expressed in ductal epithelium, its dysfunction can provoke decreased volume and acidity of pancreatic secretion that are associated with obstruction and premature activation (by lower pH) of digestive proenzymes in pancreatic ducts (42). Therefore, pancreatic secretion of lipase, proteases and amylase drops, leading to fat maldigestion and steatorrhea. This manifestation is the

most reliable to CFTR genotype. When it is developed during the first decade of life, patients carry at least one class I-III mutation (57).

Mucus dehydration and dysregulated pH are also harmful in the intestine. Patients display an abnormal milieu where dysmotility, lumen acidity and antibiotic use will lead to infection and inflammation. Meconium ileus, obstruction of the terminal ileum by abnormally viscous meconium, affects around 20% of CF neonates (42). Furthermore, intestinal inflammation, mucous viscosity and slow transit can lead later to obstruction, called distal intestinal obstructive syndrome (58). Thus, patients develop colonic dysbiosis and are at risk to bacterial pullulation and *Clostridium difficile* infection (58). Obstruction manifestations are associated with signs of inflammation, as observed in intestinal biopsies and in CFTR knockout (KO) mouse model (59).

CFTR is also expressed in biliary duct. Therefore, CF patients may suffer from obstructive liver disease, ranging from hepatic steatosis in 25-70% (57) to biliary cirrhosis with portal hypertension in 4.1% of cases in Belgium in 2018 (8).

Of note, gastrointestinal cancers incidence is increased in CF adult patients, particularly after transplantation (58). Chronic inflammation, caused by CF disease, might explain pancreatic cancer initiation. Another possible mechanism relates the CFTR loss to epithelial-to-mesenchymal transition (EMT), known as an process in tumour metastasis (60).

#### 1.1.3.2 Endocrine secretion dysfunctions

Obstruction of pancreatic ducts with thickened secretions will progressively cause fibrosis of the pancreas. Thus, Langerhans islets are gradually damaged and insulin production is impacted (57). Insulin insufficiency will lead to CF-related diabetes mellitus in 18.7% of patients in 2018 in Belgium (8). It will participate to

lung disease and infection due to the reduction of body mass and the favourable environment for bacteria (57).

CF-related bone disease is secondary to multiple factors, including vitamin D and vitamin K (fat-soluble) deficiency, but also low caloric intake, malnutrition, glucocorticoid use and low physical activity (58, 61). Chronic systemic inflammation and lung infection will also favour bone resorption in CF patients and osteoblasts with F508del mutation shows defect in maturation. Despite normal in prepubertal well-nourished CF children, low bone mineral density is observed in around 20% of young adults (61). In Belgium in 2018, 34.9% of patients suffered from osteopenia and 8.7% from osteoporosis (8).

#### 1.1.3.3 Reproduction

Men with CF display normal sexual potency and spermatogenesis. However, 98% are infertile. This infertility is caused by atretic or absent vas deferens and enlarged or absent seminal vesicles (62). Women with CF exhibit normal reproductive function, although cervical mucus is dehydrated (43).

#### 1.1.3.4 Respiratory disease

CF patients can display upper airways and lung manifestations. In upper airways, a variety of conditions have been described (42). Almost all patients will develop chronic rhinosinusitis and nasal inflammation will promote the appearance of nasal polyps. However, sinus symptoms are thought to be underestimated (63). Together with the lung, respiratory symptoms are the major cause of morbidity (3) although lung disease is responsible for the mortality of at least 67% of CF patients in 2017, in Europe (9).

CF lung disease is mainly a muco-obstructive disorder characterised by mucus plugging, chronic neutrophilic inflammation, and recurrent infection of the

airways. Despite interpersonal variations, in the most severely affected patients, it appears during early life and results in progressive structural lung damage, including bronchiectasis (abnormal enlargement of the airways) and destruction of the lung parenchyma (64, 65).

As CFTR is expressed in the airways *in utero*, developmental defects have been considered. Data shows structural tracheal abnormalities in mouse, pig and rat models of CF shortly after birth. Studies on CF foetuses demonstrated dilatation of tracheal submucosal gland ducts, lack of microvilli on airway epithelial cells, and slight inflammation (66). In addition, in CF infants, before 2 weeks of age, narrowed trachea have been noted and a study reports tracheomalacia in 15% of CF children cohort. These features suggest a possible congenital defect (67), although CF lung affection is mainly studied during lifetime.

The lungs of children with CF quickly become infected and inflamed. Indeed, Sly *et al.* reported pulmonary inflammation, including detectable neutrophil elastase (NE) in 30% of CF infants at 3 months of age, structural lung disease in 80% and lung infection in 20%, although the majority of infants were asymptomatic (68). However, the so-called "chicken-and-egg" question about which event comes first in the CF lung disease (inflammation or bacterial infection) remains a source of debate (69). Autopsies of CF children show mucopurulent plugs with severe inflammation in submucosa and epithelial alterations in the lung (even in patients under 4 months). These events are shown to be related to infection and chronicity of infection (70, 71). Similarly, comparable inflammatory cells profile, interleukin (IL-)8 concentration and NE activity were measured in bronchoalveolar lavage (BAL) from CF infants without infection and control subjects. These parameters were closely related with the pathogen presence in the lower airways and disappeared when antibiotics successfully eradicated the bacteria (72-74). In addition, studies in a CF pig model demonstrated no inflammation in foetuses and shortly after birth, while inflammation appears with first bacterial infection (75). Conversely, other

studies demonstrated increased concentration of cytokines and neutrophilic inflammation in BAL of young infants, without the presence of detectable pathogens (76, 77) and recent data related to CF sterile inflammation (in CF animal models and CF samples) showed the potential responsibility of hypoxic injury, resulting in necrosis, IL-1α release and sterile neutrophilic inflammation (64).

Increased concentration of pro-inflammatory factors (IL-1α, IL-1β, TNF-α, IL-6) is also measured in CF adults in sputum (78, 79) and increased concentration of IL-1β, TNF-α, IL-6, IL-4 and IL-8 are shown in BAL (80, 81). Conversely, the anti-inflammatory IL-10 is downregulated in CF patients (81). IL-1 receptor antagonist protein or soluble TNF-α receptor have been shown to be relatively similar to the amount measured in healthy subjects (81-83). This create an imbalance between pro- and anti-inflammatory cytokines, the latter not enabling to counteract the inflammatory environment (83).

A hallmark of CF lung disease is the neutrophil infiltration. Typically, in CF BAL, neutrophils are the more prominent cell population (versus macrophages in healthy subjects) and increased NE activity is observed (80, 81). Activated neutrophils are recruited in the airways and secrete diverse compounds such as reactive oxygen species, proinflammatory molecules (IL-8 and proteases – NE and cathepsin G). As pro- and anti-inflammatory cytokines imbalance, anti-protease defences (e.g. α1-antitrypsin) and antioxidant molecules (e.g. glutathione) are overwhelmed by proteases and reactive oxygen species, respectively (69, 84). Despite their important role in infection management, large recruitment of neutrophils (further increased by IL-8 release) which discharge large quantity of inflammatory mediators is mainly responsible for damages in CF lung tissues and progressive decline of lung function (85). Indeed, NE is able to cleave structural proteins, further favours mucus viscosity (64), cleaves opsonins and receptors responsible for phagocytosis (83). Together with other proteases (e.g. increased matrix metalloproteinases-8 and -9 concentrations in CF BAL (86)) and reactive

oxygen species also derived from neutrophils, endogenous and bacterial elastase damages the airway epithelium and its structure, which leads to bronchiectasis and bronchomalacia (85), and inflammation mediated by neutrophils was shown to be correlated with a lower airway function in BAL as soon as in the infancy (87). Although the debate about the first event happening in CF disease (inflammation or infection) remains, hyperresponsiveness of CF airway epithelial cells and blood neutrophils were observed *in vitro* after lipopolysaccharide stimulation (88).

Besides the local inflammation, increased inflammatory markers were also measured in the blood, supporting the systemic effects observed in patients with CF (cachexia, hyperglobulinaemia and osteopenia) (83).

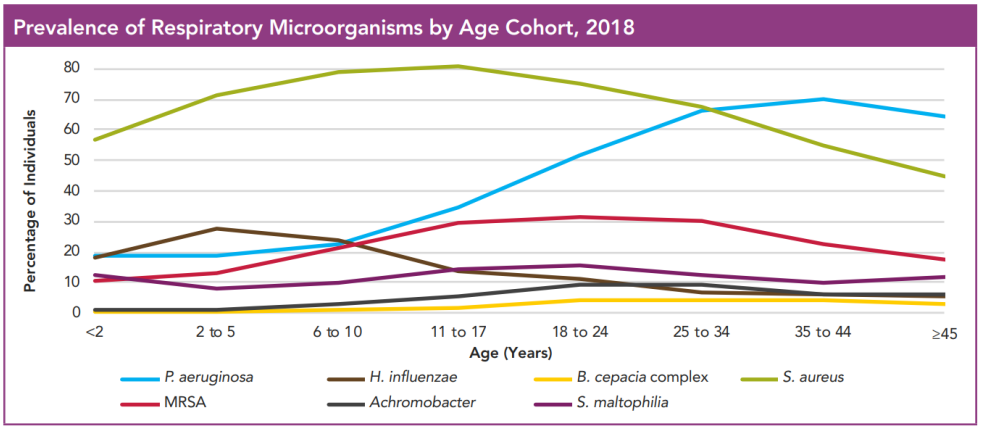


**Figure 2. Pathogen prevalence in CF patients as function of the age in 2018, in United States of America (89).**

In CF airways, defective immune responses (viscous mucus, defective immune cells and antimicrobial molecules, and decreased mucociliary clearance due to CFTR mutations) result in a predisposition to acute and, progressively, chronic lung infection to opportunistic pathogens (69). Figure 2 shows the most frequently found pathogens according to the age of the patients. Typically, infections will be first due to respiratory viruses, *Haemophilus influenzae* and *Staphylococcus aureus*,

prevailing at the beginning of lifetime. These will be replaced with age by more harmful and resistant pathogens, such as *Pa* and other Gram-negative bacteria.

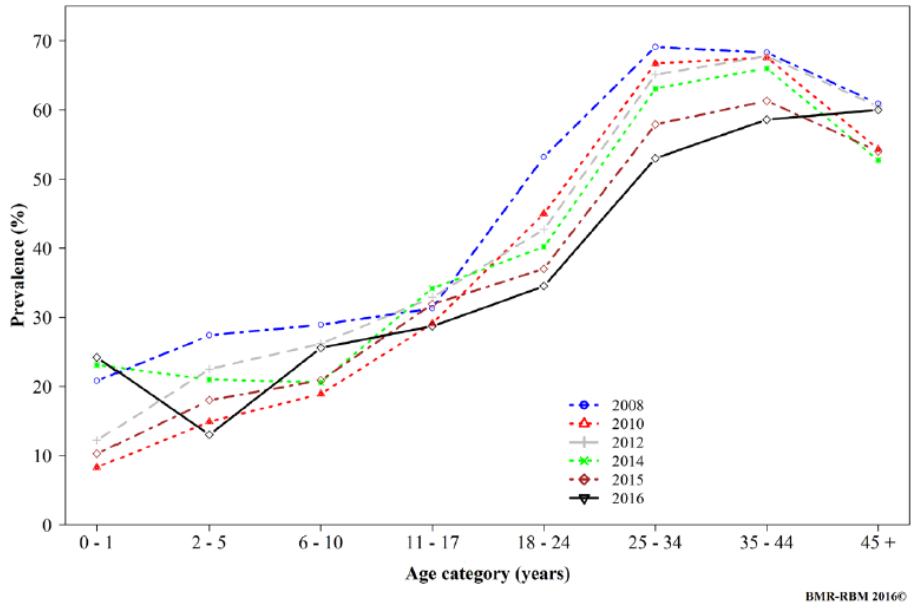


**Figure 3. Prevalence of *Pseudomonas aeruginosa* infection in CF patients as function of the age from 2008 to 2016, in Belgium (8).**

*Pa* is the bacterium that most commonly infects CF adult lung and is associated with a worsening of lung function and vital prognosis (90, 91). Figure 3 shows the prevalence of *Pa* infection in CF patients in Belgium according to the age and the year. This bacterium is an ubiquitous Gram-negative aerobe/facultative anaerobe with a high adaptive capacity. In addition to defect of innate immunity in CF patients, *Pa* secretes different virulent factors (pyocyanin, proteases, exotoxins, etc.) which impairs neutrophils function and survival. However, CF lung microenvironment is stressful for *Pa*: anaerobic environment due to mucus viscosity, immune response, competition between bacteria, and aggressive antibiotics treatment. These stresses lead to mutations and selection of *Pa* strains that better suit for the CF airway (69).

However, other pathogens may typically infect the CF lung:

- Methicillin-sensitive *Staphylococcus aureus* usually appears early in lifetime (in the first decade of life) while the methicillin-resistant *Staphylococcus aureus* (MRSA) infection mainly occurs later ( Figure 2). This human commensal pathogen commonly found in healthy subjects is more prevalent in CF patients. Indeed, this bacterium is able to grow easily in anaerobic environment, thus CF airways may represent a compatible environment. However, it remains difficult to differentiate carriers without any clinical manifestations from infected patients and, while *Pa* prevalence is currently decreasing, *Staphylococcus aureus* is more and more prevalent. Different studies suggest that *Staphylococcus aureus* worsens pulmonary disease when it appears early and is persistent (92). Association between initial *Staphylococcus aureus* colonisation and early *Pa* colonisation significantly alters the prognosis of patients with CF, even more than early *Pa* infection alone. This could be due to its virulence factors and the capacity to escape antibiotic treatments and immune response (93). Persistent MRSA infection leads to greater lung damage, more important lung function decline as well as earlier and more frequent death than infection with methicillin-sensitive bacteria (92).

- *Haemophilus influenzae* acquisition occasionally follows *Staphylococcus aureus* in CF patient lung and has unclear consequences (93).

- *Achromobacter xylosoxidans* (93)

- *Stenotrophomonas maltophilia* was assumed to be harmless before evidence suggests its participation in CF lung damage (93).

- *Burkholderia cepacia* complex infection was related to rapid deterioration, and poorer prognosis after lung transplantation, as well as adverse outcomes even though the patient did not undergo transplantation (3).

- Fungi, including *Aspergillus fumigatus,* are also increasingly recognised as a matter of concern since they may promote more rapid decline in pulmonary function (93) and with an increased rate of pulmonary exacerbations (3).

- Nontuberculous mycobacteria prevalence has increased since 1990s in CF patients and *Mycobacterium avium* complex and *Mycobacterium abscessus* complex are the most isolated species. Their consequence on lung disease remains difficult to decipher and may vary according to the species and the time of acquisition. However, *Mycobacterium abscessus* complex seems responsible for a decreased forced expiratory volume in one second (FEV1) and a higher transplantation requirement (94).

- Viral infection is also a common cause of exacerbations in patients with CF (3).

### 1.1.4 Diagnosis

In western countries, diagnosis is increasingly helped by new-born screening, although it was only introduced in Belgium in 2019. The median age at diagnosis is six months (8). However, more than 10% of the patients are diagnosed during adolescence or adult life (8, 95). One of the reasons could be the high variability of the CF phenotype (genotype-dependant or independent).

#### 1.1.4.1 New-born screening

New-born screening in Belgium consists in measurement of immunoreactive trypsinogen in blood spots taken from new-born children. A very high immunoreactive trypsinogen (> percentile 99) concentration suggests pancreatic injury consistent with (but not specific for) CF (95). This marker is increased even in infants with so-called less severe mutations (as class IV or V mutations) that are associated with pancreatic sufficiency (11). However, this test alone does not confirm the diagnosis of CF. Genetic testing is then performed for the 12 more frequent mutations in Belgium. In case of the presence of one or two CF-causing mutations, a sweat test is then realised to confirm the diagnosis. In the absence of CF mutations and if immunoreactive trypsinogen measurement was above percentile 99.9, a second test is performed at day 21. If the second immunoreactive trypsinogen measurement is above percentile 99, a sweat test is also proposed to the patient. Even though new-born screening has to be carefully interpreted, it increases early diagnosis and follow-up, which is known to improve outcomes. In countries where it was implemented since decades, it is associated with a decreased CF incidence (95).

#### 1.1.4.2 Sweat test

Sweat test was described for the first time in 1959 by Gibson and Cooke (3). In 2019, it was still the most reliable and available diagnostic test for CF (95). Indeed, more than 95% of patients presenting CF symptoms may be diagnosed with a sweat test. Healthy patients produce hypotonic sweat after CFTR reabsorbed chloride in the sweat duct. In CF, as CFTR is defective, chloride reabsorption does not happen (95). The test measures chloride concentration in collected sweat after stimulation by pilocarpine (a muscarinic receptor agonist) (3, 95). Chloride concentration in sweat will be above 60 mmol/L for most CF patients. However, intermediate values

(between 30 mmol/L and 60 mmol/L) should lead to further investigations. To confirm CF, a second measurement has to be done at another time point (95).

#### 1.1.4.3 Genetic testing

An increasing number of CFTR mutations are described as "CF-causing" (352, in January 2020) (39, 40). In practise, CF patient's samples are first tested for a standard CFTR mutations panel, which includes the most common disease-causing mutations. This panel permits to detect 80-85% of all mutations in the population, knowing that around 70% of mutations are F508del. If this test fails to detect two CFTR mutations and patient displays symptoms or sweat chloride concentration above 60 mmol/L, a sequencing of the *CFTR* gene can be performed by qualified laboratories (95).

#### 1.1.4.4 Nasal potential difference and intestinal current measurements

Nasal potential difference and intestinal current measurements are used to measure CFTR function, like sweat test. Nevertheless, they are less widely available and expect a larger expertise. They are particularly useful to confirm the diagnosis when sweat test values are intermediate and that two disease-causing CFTR mutations are not identified.

Nasal potential difference consists in *in vivo* measurement of the transepithelial potential. Patients with CF display a more negative nasal potential (related to increased sodium influx). Moreover, an increased change in potential is measured upon blockage of the sodium channel by amiloride and little or no change upon CFTR stimulation (95).

Intestinal current measurements consist in *ex vivo* measurements of the CFTR function. Rectal biopsy allows to analyse the sample in an Ussing chamber.

Intestinal chloride transport is impaired and absorptive processes are normal or increased in CF. Intestinal current measurement measures responses to carbachol and histamine of the tissue. In patients, absent chloride efflux does not mask anymore the potassium efflux, contrary to healthy people. Thus, current inversion occurs (95).

### 1.1.5 Treatment

Since *CFTR* gene discovery, more and more studies tried to develop compounds able to correct CFTR molecular defect. Until now, despite promising results showed by modulators, symptomatic treatments are still needed to slow down disease progression (7). Figure 4 shows how mostly symptomatic treatments improved survival of patients.

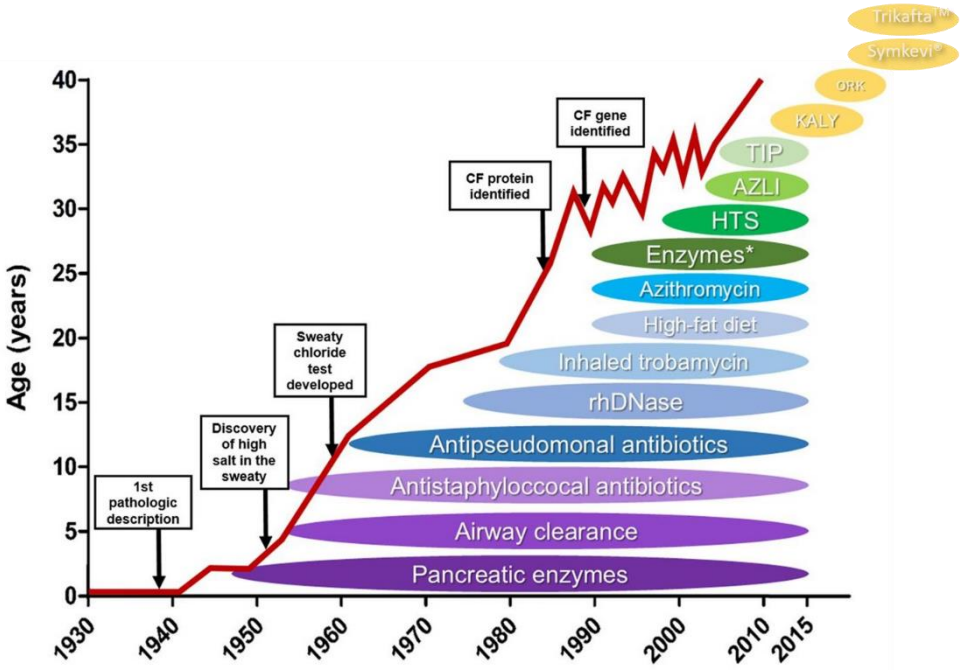


**Figure 4. Evolution of CF treatments and survival in function of time.**
HTS, high throughput screening; AZLI, aztreonam for inhalation solution; TIP, tobramycin inhalation solution; KALY, Kalydeco™; ORK, Orkambi™; *enteric-coated pancreatic enzymes (Adapted from (10)).

#### 1.1.5.1 Symptomatic treatments

Symptomatic treatments considerably improve survival of patients with CF and quality of life since fifties. Recombinant pancreatic enzymes were the first implemented. Since then, pancreatic insufficiency is controllable by improving the digestion thanks to pancreatic enzymes substitution and associated with an optimal diet (43).

Management of lung disease represents a great part of CF treatments. Strategies are developed to improve proper hydration of the ASL and mucociliary clearance, to prevent and control the infections and to reduce the excessive inflammatory response (7).

Improvements of airway hydration was first performed with hypertonic saline. It is still used today with mannitol by altering osmolarity of ASL. It promotes mucociliary clearance and FEV1, but to a lesser extent than recombinant human DNase (43). Recombinant human DNase-1 was shown to decrease sputum viscosity and reduce the lung function decline. However, 30% of patients seem to be non-responders (7).

Preventing bacterial lung infection stays the primary aim for CF lung treatment. Pathogens may be transmitted either by direct patient-to-patient contact or from numerous environmental reservoirs. For these reasons, hygienic measures and separation between infected versus non-infected patients to limit cross infection are important. Moreover, inhaled and systemic antibiotics has increased life expectancy of patients with CF (7). As mentioned before, CF children become initially infected with *Staphylococcus aureus* or *Haemophilus influenzae*. Prophylactic antistaphylococcal therapy lowers the rate of *Staphylococcus aureus* cultures, cough, and the rate of admissions during the observation period. However, continuous antistaphylococcal therapy was associated with a higher rate of *Pa*

acquisition, especially in the first six years of life (92). With age, *Staphylococcus aureus* infection rate decreases and *Pa* infection increases. *Pa* becomes highly difficult to eradicate, thanks to its biofilm production and the development of mutated strains, increasing resistance to antibiotics. An European consensus on antibiotic therapy against *Pa* has advocated aggressive approaches with repeated courses of high-dose antibiotics (96). Inhalation route is sometimes used to prevent form adverse effects and to reach high concentrations in the airways. Frequent change of antibiotic allows to prevent from resistance (43).

A balanced inflammation has to be maintained in CF patients. Indeed, on one hand, chronic neutrophilic inflammation is responsible for lung damage but, on the other hand, CF inflammatory response is thought to prevent infection from invasiveness. Consequently, medications responsible for slight decreased inflammation and increase of antiprotease and antioxidant activity are considered. Steroids showed serious side-effects and even severe safety concerns. Until now, Ibuprofen is used to manage inflammation in CF children since oral high-dose was associated with a reduced rate of decline of lung function (7).

#### 1.1.5.2 Targeted treatments

Since the discovery of *CFTR* gene, correcting the defect at genetic level was attempted. The gene therapy allows transient expression of functional CFTR on the cell surface, in cell cultures and in mice (3). However, in human, administration of the functional gene through adenoviral vector did not show clinical benefit and was associated with immune response at repeated dose (97). Less immunogenic adeno-associated virus showed first prolonged CFTR expressions, but a longer-term trial did not show benefits (98). Lentiviral vectors displayed high transfection efficiency and prolonged CFTR expression but are still under investigation (99). Likewise, liposomes were considered because of their potential to overcome limitations of viral vectors. Indeed, they are less immunogenic and therefore better suited for repeated

doses treatment. However, these advantages are related with a lower level of transfection efficiency (3).

In parallel, research explored how to correct CFTR protein defect, accordingly with the type of dysfunction (Table 1). To date, three drugs are available after demonstrating clinical efficacy in CF patients. Ivacaftor (VX-770) – a potentiator – is able to activate CFTR. It showed positive results in the treatment of CF patients with the G551D mutation in terms of improvements in lung function and in body mass index, reduction of the number of pulmonary exacerbations and improvement of quality of life (41), including long-term benefits like decreased risk of *Pa* infection, reduced decline of FEV1, better nutritional status, etc. (7). In addition to G551D mutation, eight other class III mutations can be treated by this molecule (100). Ivacaftor was furthermore tested in patients with residual CFTR function (101). Despite variable response, it was approved for adult patients. However, only 5% of CF patients are concerned by these mutations (7). For this reason, researchers are preferentially looking for a treatment for the most common mutation, F508del. Lumacaftor (VX-809) restores CFTR folding and processing *in vitro*. However, it did not reveal effective in clinical trials. Indeed, F508del-CFTR suffers from improper folding as well as defective conductance. Therefore, Ivacaftor-Lumacaftor combination (Orkambi™) was used and showed an improvement of FEV1 of about 3% in patients homozygous for F508del, whereas no significant benefit was observed in heterozygous patients (102, 103). Despite limited short-term benefits, it has been shown to improve long-term survival (104). Recently, Tezacaftor (VX-661), a new corrector, was approved and showed, in combination with Ivacaftor as potentiator (Symkevi®), better results than its predecessor in patients with two F508del mutations as well as heterozygous for F508del and one residual function mutation (105, 106). Finally, Elexacaftor is a new corrector, that interacts at a different site on the CFTR protein than Tezacaftor and further facilitates the cellular trafficking of F508del-CFTR to the cell surface when

it is used with Tezacaftor (107). The association of Ivacaftor-Tezacaftor-Elexacaftor (Trikafta™/Kaftrio®) showed impressive results (+14% of FEV1) and is approved by the U.S. Food and Drug Administration and the European Medicines Agency for patients carrying at least one F5508del mutation (108, 109).

Despite these promising advances, access to new drugs is not guarantee since treatments are expensive (311,000 US$ per person per year for Ivacaftor and 250,000 US$ per person per year for combination of Ivacaftor and Lumacaftor) and has to be administered all along the patient's life. Consequently, some health authorities slow down to approve reimbursement (7).

### *1.2 Respiratory epithelium*

As mentioned above, CF patients display chronic inflammation and chronic infections of the airway (3) and lung disease remains mainly responsible for the morbidity and the mortality of CF patients (9). Therefore, epithelium lining the airways is particularly exposed to inflammatory environment and pathogens and multiple aggressions are further responsible for remodelling.

Respiratory epithelium surface is about 100 m² (110) and is constantly in contact with inhaled particles: bacteria, viruses, toxins, particles (toxic or not), etc. Thus, airway epithelium constitutes a physical barrier and has developed protective mechanisms preventing invasions of foreign particles (111, 112).

#### 1.2.1 Structure of the airway epithelium

Human conducting airways consist of a pseudostratified epithelium. It includes ciliated cells and smaller numbers of basal cells as well as secretory club cells and goblet cells (Figure 5). Rare cell populations include ionocytes, neuroendocrine cells and tuft cells (24, 111, 113).

Ciliated cells are the most abundant cell type in the bronchial epithelium and account for over 50% of airway epithelial cells. They are characterised by their approximately 300 apical, motile cilia per cell (111, 112), which are composed by dyneins and motor proteins responsible for coordinated directional beating (113). Energy is provided by the multiple mitochondria right below the apical surface (111). Basal and club cells are described as the progenitors of ciliated cells. Briefly, differentiation occurs with coordinated absence of Notch signalling and expression of geminin coiled-coil domain containing protein. The latter activates multiciliate differentiation and DNA synthesis associated cell cycle protein (MCIDAS), which takes part in centriole amplification and organisation of apical microtubules required for cilia formation and activates a transcriptional network. It includes forkhead box (FOX) J1, regulatory factor X (RFX) and MYB proto-oncogene (MYB), which stimulates the transcription and the assembly of ciliary proteins (113).

Goblet cells are characterised by an electron lucent granules localised in the apical pole of the cell and account for 5-15% of epithelial cells of the large airways (111, 112). They are responsible for the secretion of mucins. Mucins (MUC) are large, mostly polymeric, highly glycosylated proteins. Fourteen different mucins are expressed in the airways, but the more abundant are MUC5AC and MUC5B. Moreover, MUC5AC is preferably secreted along the conducting airways. Airway goblet cell differentiation is allowed by Notch signalling which activates the expression of SAM pointed domain containing ETS transcription factor (SPDEF). Alone, it is responsible for the regulation of a transcriptional network required for differentiation as well as the synthesis and the packaging of mucins (113). As for FOXA3, it may participate in the regulation of genes responsible for mucus production and goblet cell differentiation in the chronic lung diseases (114).

Basal cells are located below the surface epithelial cell layer (113) and are connected to the reticular basement membrane (RBM) by hemi-desmosomes (111).

Their number decreases with the airway diameter and they act as progenitors in large airways. They are characterised by nuclear expression of tumour protein 63 (p63) (112).

Club cells are recognised by electron dense secretory granules (111), which contain characteristic club cell secretory protein 16, but also mucins (113) and surfactant proteins (111). In addition, club cells, which probably originate from basal cells, may give rise to goblet or ciliated cells (113), thus acting as facultative progenitors in small airways (112).

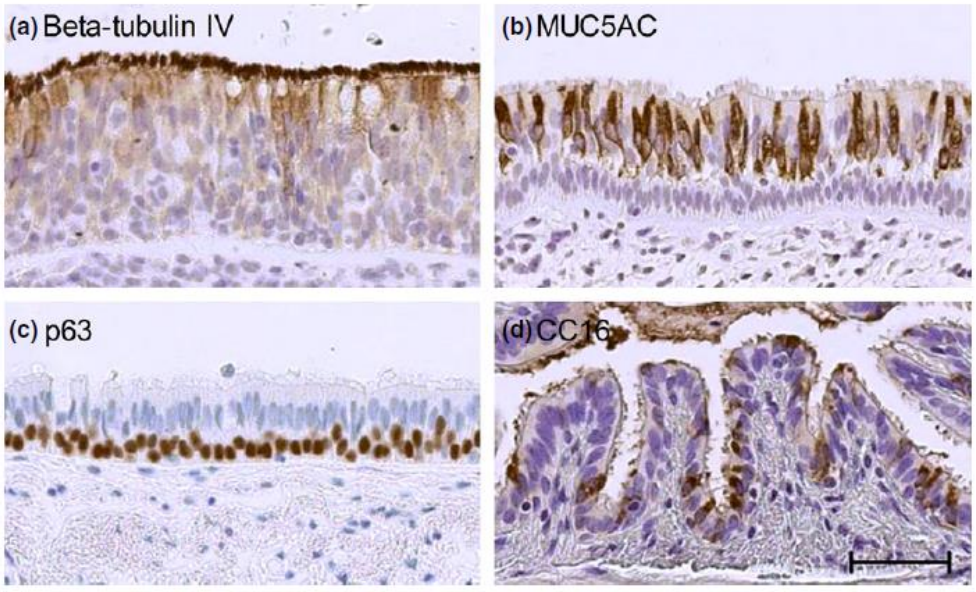


**Figure 5. Human bronchial pseudostratified epithelium is composed by four main cell types.**
(a) β-tubulin IV immunostaining (indicating ciliated cells) in a large airway. (b) MUC5AC immunostaining (indicating goblet cells) in a large airway. (c) p63 immunostaining (indicating basal cells) in a large airway. (d) Clara cell secretory protein 16 (CC16) immunostaining (indicating club cells) in a small airway. Scale bar, 50 μm (112).

Ionocytes, neuroendocrine cells, and tuft cells are found in low numbers.

Ionocytes, first identified in *Xenopus* and zebrafish skin, were recently discovered in mouse trachea and nose as well as in human bronchi, and account for 0.5 to 1.5 % of the airway epithelial cells (24). Their specification depends on Notch signalling and FOXI1 expression (25). Pulmonary ionocytes express high levels of CFTR: in mouse trachea, 54.4% of the total *CFTR* transcripts are produced by ionocytes while this cell type only represents few percent (24). In addition, CFTR activity (and not only its expression) has been shown to relate to the density of ionocytes (25). Therefore, these cells may be responsible for fluid regulation at the epithelial surface and contribute to the proper viscosity of mucus in the airways (24).

Neuroendocrine cells are innervated airway epithelial cells and were suggested to have a neuroimmunomodulatory function, linking inhaled stimuli with neurogenic and immune responses (115).

Tuft cells, also known as brush cells, are chemosensory cells endowed with apical microvilli. Their function remains poorly understood, although a role in type-2 immunity was attributed to this cell type in the gut (116).

### 1.2.2 Roles of the airway epithelium

Pseudostratified epithelium forms an interface, a physical barrier between lumen of the bronchi (external environment) and the inside of the body. Therefore, it is the first line of defence in case of aggressions (by pathogens, allergens, pollutants, etc.) and different mechanisms may contribute to prevent invasion.

#### 1.2.2.1 A physical barrier

Apical junction complexes include tight junctions and adherens junctions and are essential for the structural integrity of the epithelium (112).

Tight junctions establish an apical network around epithelial cells (Figure 6). They are involved in the maintenance of the non-permeability of the epithelium and in the regulation of paracellular ionic movements. They divide the apical from the basolateral pole of the cells and therefore are essential for epithelial polarity (112). These junctions are composed by three main types of transmembrane proteins (claudin, occludin and junctional adhesion molecules) and zonula occludens (ZO) proteins in the cytoplasm (111), which interact with the cytoskeleton (112).

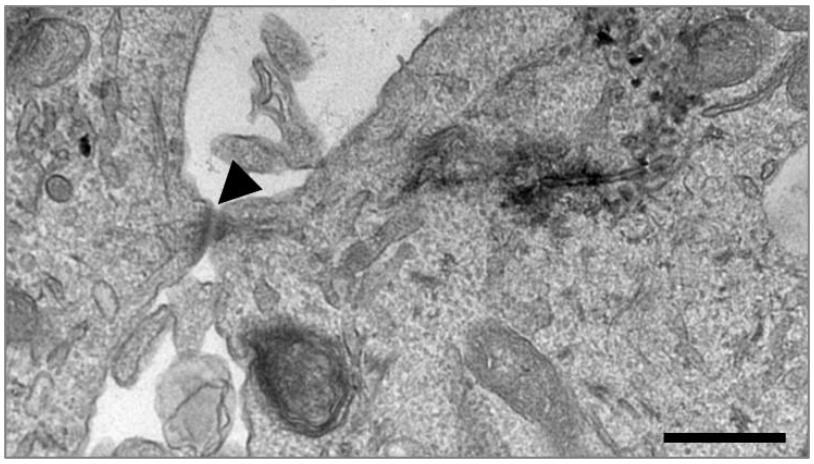

**Figure 6. Tight junction (arrow) observed by transmission electronic microscopy between two airway epithelial cells cultured in air-liquid interface.** Scale bar, 500 nm.

Claudin family members allow claudin-claudin interactions in the tight junction and the control of ion permeability (117). The role of occludin remains enigmatic. Loss of function studies showed no difference in tight junction morphology (118, 119). However, in 16HBE1o- (a control bronchial cell line) and in primary mouse tracheal epithelial cells, when occludin expression is reduced by oxidative stress, airway barrier function is disturbed (120). Junctional adhesion molecules and coxsackie adenovirus receptor are immunoglobulin-like proteins, located the most basolaterally in tight junctions (117). These transmembrane proteins are thought to induce immune responses in case of pathogens invasion

(117). Finally, ZO proteins account for three: ZO-1, ZO-2 and ZO-3. They contained a multitude of domains able of protein-protein interactions and are thought to cluster and stabilise tight junctions in the apical membrane (117). In the absence of ZO-1/ZO-2 *in vitro*, cell polarity did not seem affected, but permeability is highly increased (121, 122).

Adherens junctions take part in initiation and maintenance of cell to cell adhesion and are located just below tight junctions. They are mainly composed by E-cadherin and α-/β-catenin (117). E-cadherin extracellular domains allow the adhesion between adjacent cells through a calcium-dependant liaison. α-catenin and β-catenin link E-cadherin to microtubule network and actin cytoskeleton. Moreover, E-cadherin has a regulatory influence on cell proliferation and differentiation. It prevents epithelial growth factor receptor and β-catenin activation by retaining these proteins into the junctions. These proteins are known to favour cell proliferation and de-/trans-differentiation (111).

Besides apical junction complexes, hemidesmosomes enable basal cells to anchor and gap junctions allow intercellular communications (112).

#### 1.2.2.2 Mucociliary clearance

While junctions allow cells to prevent pathogens invasion, mucociliary clearance enable airways to clear inhaled particles (microbes, pollutant particles, etc.) out and represents another protective mechanism. It is allowed by the coordinated action of multiple cell types: cells lining the submucosal glands, goblet cells and ciliated cells. Normal clearance requires coordinated mucus secretion, NaCl and water transports and cilia beating (112).

Mucus secretion (by goblet cells and submucosal glands) establishes a protective layer able to efficiently trap inhaled particles. Mucus is composed by more than 200 different proteins, but the major component is mucins (111). As mentioned

before, 14 different types of mucins are expressed into the airways, but the most abundant are membrane associated-mucins MUC1, MUC4, MUC16 and MUC20, gel-forming MUC5AC and MUC5B, as well as non-gel forming MUC7. These high molecular weight glycoproteins are characterised by at least one large region of repeating sequences enriched in serine and threonine, which are the sites of O-linked glycosylation. Thus, carbohydrates represent 70-80% of mucins molecular weight (123, 124). Gel forming MUC5AC and MUC5B are produced mostly by goblet cells and mucous cells from submucosal glands, respectively. Mucins expression are stimulated by environment. For example, MUC5AC is upregulated by viruses, smoke component, cytokines (through IL-13) (125). Their production is complex since high glycosylation, dimerization by disulphide bonds and further polymerisation (123). Then, mucins are highly condensed into secretory granules. Studies on mice and piglets demonstrated that low pH (6.2), regulated by bicarbonate, and high concentration of calcium could be responsible for this impressive packaging capacity. When mucins are secreted, they are rapidly dissolved and expanded through increased pH and calcium chelation – acidity and calcium being associated with mucus higher viscosity (53, 126). Highly glycosylated mucins enable mucus to entrap the foreign particles.

This mucus layer is floating above a less viscous layer, called the periciliary liquid. This less viscous layer provides a suitable environment for cilia beating. Active ionic transports (mostly sodium and chloride) are responsible for maintaining the periciliary liquid at an adequate height (approximately 7 µm – around the height of a cilia) and for adequate hydration of the mucus layer. Membrane-bound mucins take part in the physical properties of the periciliary liquid. Moreover, periciliary liquid prevents mucus layer to adhere to cell surface and thus facilitates mucus expulsion by cilia beating (111, 125).

As mentioned in the "1.2.1 Structure of the airway epithelium" section, ciliated cells are the most abundant cell type in the bronchial epithelium and their numerous

motile cilia per cell allow to propel up the mucus, in association with air flow and cough (111, 112).

Finally, mucus layer also contains many other substances secreted by airway epithelial cells. These molecules are antimicrobial, antiviral and/or pro-/anti-inflammatory and are able to link mucins, which is suggested to behave as a "scaffold" to present and to organise these proteins (111).

#### 1.2.2.3 Antimicrobial role

Airway epithelium can secrete a multitude of proteins which constitute host innate immune defence against pathogens. These substances include lysozyme, lactoferrin, proteases, protease inhibitors, reactive oxygen species, and polymeric immunoglobulins (111), representing a chemical barrier (112).

Multiple peptides are produced by epithelial cells or immune cells and secreted in order to aggregate, trap and kill pathogens. These peptides are divided into different sub-categories according to their composition (111, 127).

Lysozyme is one of the most abundant antibacterial protein in the airway. While neutrophils are the major source of lysozyme, lung epithelial cells and other immune cells also produce it. When it is secreted, it can damage bacteria through a cleavage of the bacterial wall peptidoglycans or by a non-enzymatic mechanism. Lysozyme is constitutively expressed and, in addition to antibacterial activity, has shown effect on fungi (127).

Secretory leucocyte protease inhibitor is produced by a large number of immune cells as well as goblet and club cells of the epithelium. In addition to constitutive expression, it is upregulated by lipopolysaccharide, NE and pro-inflammatory cytokines (tumour necrosis factor α (TNF-α) or IL-1β). This protein interacts with heparan-sulphate from bacteria, inhibits the growth of fungi and have

some antiviral activities, as demonstrated on human immunodeficiency virus. Besides its antimicrobial activities, secretory leucocyte protease inhibitor carries an anti-NE domain, which potentially acts on other serine proteases (127). Thus, it prevents from proteases adverse effects (127).

Elafin is a serine antiprotease and shares an almost identical protease inhibitory domain as secretory leucocyte protease inhibitor, despite a less broad spectrum. Nevertheless, elafin represents a significant part of the anti-elastase activity measured in BAL in healthy people (127).

Defensins are antimicrobial proteins, particularly resistant to proteolysis. If α-defensins are mainly produced by neutrophils, bronchial epithelial cells are responsible for production of β-defensins. These differs from α-defensins by a different conformation of their disulphide bonds. In the respiratory tract, β-defensin-1 to β-defensin-4 are the most prevalent. β-defensin-1 is constitutively expressed while the others are activated by microbes or cytokines (TNF-α or IL-1β). β-defensins provide a protection against a wide range of bacteria, virus and fungi (127).

As defensins, cathelicidin provides a wide bactericidal activity as well as anti-virus and anti-fungi. It is produced by immune cells, bronchial epithelial cells or keratinocytes and, in addition to a constitutive production, its expression is stimulated by TNF-α, IL-17A, interferon γ (IFN-γ), vitamin D or pathogens. Cathelicidin has been shown to provide strengthening to epithelial barrier through the upregulation of the expression of claudins or occludins (proteins forming the tight junctions) (127).

Moreover, some of these antimicrobial peptides (secretory leucocyte protease inhibitor, elafin, α1-antitrypsin), produced by the epithelium, also mitigate the activity of proteases released by pathogens as well as recruited immune cells and participate to protease/antiprotease balance. Proteases (serine proteases, matrix metalloproteases, cysteine cathepsins or proteases from bacteria) regulate various

physiological events (tissue remodelling, precursor processing, mucin expression,…) and high concentration of active proteases can lead to lung damages (127).

### 1.2.3 The respiratory epithelium in CF

The lungs of children with CF quickly become infected and inflamed. As detailed in the "1.1.3.4 Respiratory disease" section, neutrophil infiltration, bacterial infection and subsequent hyperinflammation – appearing in the first months of life (70) – will lead to an imbalance between pro- and anti-inflammatory cytokines (83), protease and anti-protease defences, as well as oxidant and antioxidant molecules (69). Indeed, endogenous and bacterial proteases and reactive oxygen species are implicated in respiratory epithelium damage, which leads to bronchiectasis and bronchomalacia (85) and a worsening of lung function (87).

#### 1.2.3.1 Remodelling

First studies related to pulmonary changes in CF autopsies revealed epithelium metaplasia, inflammatory infiltrates, bronchiectasis and mucopurulent plugs in patients from two to 27 years (70, 128). Commonly, it was associated with loss of cilia, goblet cells hyperplasia (70) and bronchial gland enlargement (128, 129).

Goblet cell hyperplasia – an increased number of goblet cells – in CF was further demonstrated by Burgel *et al.* in lung transplants (130), despite some contradictory results showing no difference in MUC5AC volume of epithelial staining in a smaller non-transplanted CF population (131). In addition to goblet cells, basal cell hyperplasia was described (70, 129), with evidence of proliferative zones (132).

Early studies also described squamous metaplasia in the lung of CF children (70) and adults (129). It consists in a benign non-cancerous change of surface epithelial cells to a squamous morphology. This feature was also observed in the tracheal epithelium from CF children (133).

Thickened RBM was observed in CF lung tissue and correlated with TGF-β1 concentration in BAL, but not with inflammation (134).

Degradation of structural components of the extracellular matrix were reported. It includes elastin, collagen and glycosaminoglycans of which fragments or metabolites were found in endobronchial biopsies or BAL. Protease/anti-protease imbalance, associated with inflammation and infection, is thought to be the primary cause of this event (66).

#### 1.2.3.2 Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is a process by which a polarised epithelium de-differentiate into mesenchymal cells (135). Therefore, cells loose polarity markers (such as ZO-1 or E-cadherin), and develop migratory capacity, associated with mesenchymal characteristics. This includes a spindle shape, the expression of vimentin and the secretion of fibronectin (an extracellular matrix protein) (136, 137). EMT is a physiological process during development and repair (136). De-differentiated/mesenchymal cells can spread and cover the lesion after an aggression, before re-differentiate in a mucociliary epithelium. Under chronic inflammation and repeated aggressions, EMT persists and remodelling occurs (138).

In CF, the presence of EMT remains elusive in CF lung while it is present in other chronic lung diseases (139). The expression of EMT markers was increased in IB3 cells, a cell line derived from a CF bronchial epithelium, compared with the C38

control cell line. EMT was reversible when TGF-β activity was inhibited (140). This cytokine is known to promote this process (141).

#### 1.2.3.3 Unfolded protein response

The endoplasmic reticulum (ER) plays an important role in the cell since it is the site of secreted and membrane proteins synthesis. The epithelium produces multiple secreted and transmembrane proteins, as antimicrobial molecules, cytokines, pattern recognition receptors, etc. However, protein homeostasis can be disrupted in CF. Indeed, in patients carrying class II mutations (such as F508del), CFTR folding, which takes place into the ER, is defective and could lead to the accumulation of misfolded proteins and ER stress. The unfolded protein response (UPR) is a signalling pathway that detects misfolding and restores protein homeostasis. Three different ER transmembrane sensors (protein kinase R-like ER kinase, activating transcription factor 6 and inositol-requiring enzyme 1) are bound to the binding immunoglobulin protein, an ER chaperone protein. Since this chaperone has a higher affinity to the hydrophobic regions of the misfolded proteins, it dissociates from the sensors and this triggers the downstream UPR pathways. The UPR signalling is complex and results in increasing the ER folding capacity and decreasing the ER folding load. Therefore, it allows on one hand to upregulate the expression of chaperones and to expand the size of the ER, and on the other hand to stop protein translation and to promote the degradation of misfolded proteins through ER-associated degradation. In case of persistent UPR activation, pro-apoptotic pathways leading to cell death are initiated (142). UPR signalling is further described in Figure 7.

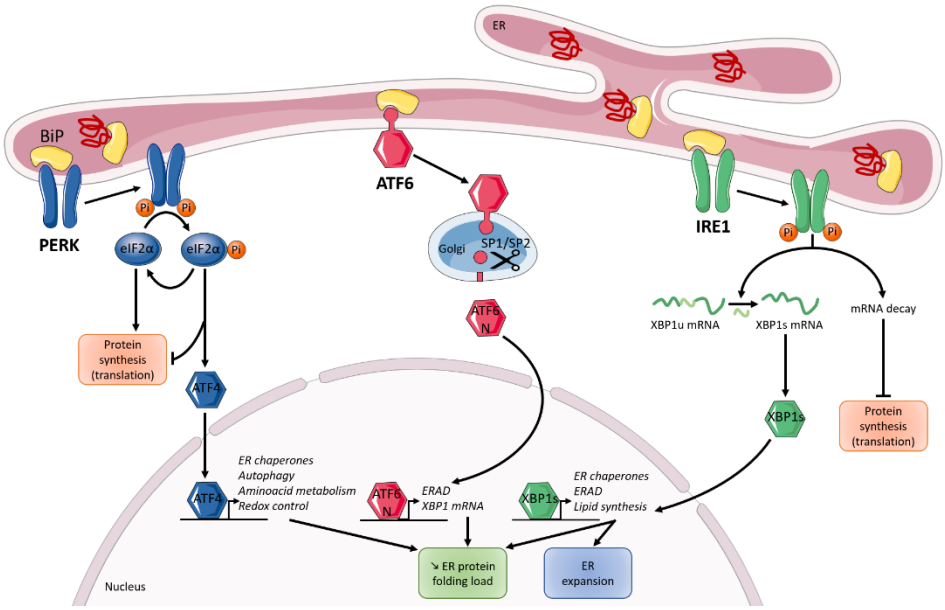


**Figure 7. UPR signalling.**
The accumulation of unfolded protein in the ER lumen results in the dissociation of BiP from the three UPR sensors PERK, ATF6, and IRE1 and their activation. PERK dimerizes, and autophosphorylates, which activates its cytosolic kinase domain. PERK phosphorylates eIF2α. This allows the inhibition of protein synthesis and triggers ATF4, a transcription factor that induces the expression of multiple proteins, further leading to a decreased ER folding load. Activation of ATF6 leads to its translocation to the Golgi where it is cleaved by S1P and S2P into an active transcription factor which results in the transcription of XBP1 mRNA and ERAD proteins. Activation of IRE1 results from its dimerization and autophosphorylation. IRE1 contains an endoribonuclease domain which processes XBP1u mRNA. XBP1s mRNA is translated into an active transcription factor, that targets genes responsible for decreased ER protein folding load and for lipid synthesis, permitting ER expansion. UPR is a complex pathway that interacts with other, including redox control, inflammation, apoptosis, which are not mentioned here (142-144). UPR, unfolded protein response; ER, endoplasmic reticulum; BiP, binding immunoglobulin protein; PERK, protein kinase R-like ER kinase; ATF, activating transcription factor; SP1/SP2, site 1 and site 2 proteases; XBP1u, unspliced X-box binding protein 1; XBP1s, spliced X-box binding protein 1; ERAD, ER-associated degradation.

Although other CFTR-expressing cell types may develop UPR, this response was particularly addressed in bronchial epithelial cells. In freshly isolated CF human bronchial cells, X-box binding protein 1 (XBP-1) splicing, following inositol-requiring enzyme 1 activation, and some of its targeted genes were upregulated. *In*

*vitro* spliced XBP-1 overexpression was associated with ER expansion (145). CF bronchial cells displayed UPR activation at 6-11 days in air-liquid interface culture associated with a pro-inflammatory state, while UPR was not detected in 30-40 days-old cultures (145, 146). Increased XBP-1 splicing and hyperinflammation state were also measured in different cell lines carrying at last one copy of F508del CFTR mutation, as well as in peripheral blood mononuclear cells and lung tissues from end-stage CF patients (147, 148). Chronic infection and inflammation may also drive ER stress and UPR activation. Mucopurulent secretions from CF patients applied on control epithelial cells caused XBP-1-dependant expansion of the ER (145). *In vivo*, *Pa* infection also induced lung inflammation and XBP-1 splicing in mice (149).

### *1.3 IgA mucosal immunity*

The different mucosae through the body represent around 400 $m^2$ (100 $m^2$ of alveolar surface, excluding bronchia) (150, 151). As mentioned in the "1.2 Respiratory epithelium" section, this surface is mostly covered by a pseudostratified epithelium which is exposed to external agents (infectious, molecules, diet, etc.) (150). To deal with it, numbers of defence mechanisms are deployed to counteract aggressions by external agents such as physical (epithelium as barrier), chemical (antimicrobial molecules, lysozyme, proteases and antiproteinases…) and immunological mechanisms.

Immunoglobulin (Ig) A is the most abundant Ig in the serum, after IgG. At mucosal sites, it represents at least 70% of Ig produced in mammals. Indeed, IgA is mostly released in the different lumens of the body, including the digestive tract (3 g per day in intestine) and the airways (152).

### 1.3.1 Structure of IgA

Monomeric IgA is a 160 kDa protein (151) and two subclasses of IgA exist in humans, namely IgA1 and IgA2. Moreover, two allotypic variants of IgA2, IgA2m(1) and IgA2m(2) are characterised, more predominant in Caucasian and African population respectively. IgA2n, a third possible allotype, was described, but never isolated (153). One notable difference between IgA1 and IgA2 is the 13-amino acid deletion in the hinge region in IgA2. Therefore, IgA2 is more resistant to proteinases and is mostly observed in secretions, compared to serum. Conversely, IgA1 is the most produced subclass: it represents around 90% of IgA in serum, but this percentage decreases in secretions (154).

In addition, IgA can polymerise. IgA produced by bone marrow plasma cells is predominantly monomeric and present in serum (88%) while the mucosal, secreted form of IgA is mostly polymeric (80%) (110, 151, 154). While trimeric and tetrameric IgA exist, polymeric IgA is mostly found as a dimeric form. Supplementary 18 C-terminal amino acids typically found in Ig able to polymerise allow the link between two IgA and a joining (J) chain. J chain is a 15-kDa polypeptide, which is not critical for polymerisation but it is necessary for binding to the polymeric immunoglobulin receptor (pIgR)/secretory component (SC), as discussed in "Polymeric immunoglobulin receptor-mediated IgA transport" section (151, 154). Figure 8 shows an illustration of the different forms of IgA.

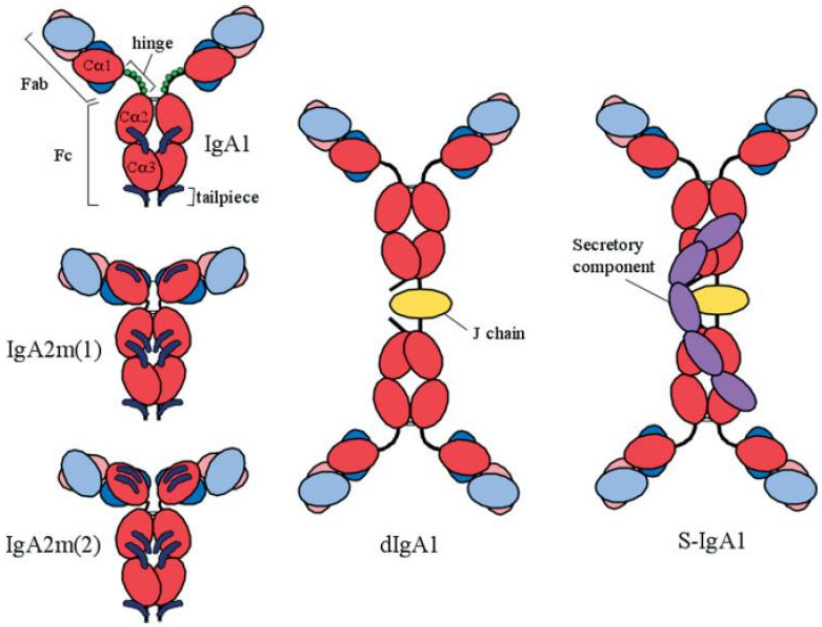


**Figure 8. Schematic representation of IgA1, IgA2m(1), IgA2m(2), d-IgA and S-IgA.**
IgA, immunoglobulin A; d-IgA, dimeric IgA; S-IgA, secretory IgA; J, joining (154).

### 1.3.2 Production of IgA

Mucosal IgA is produced in the mucosa-associated lymphoid tissues (MALT), which function independently from the systemic immune system (154). MALT are lymphoid tissues related to surface and glandular epithelium and are characterised by follicles, where B-cells are preferentially observed, and comprise secondary germinal centres after antigenic stimulation. They are similar to lymph nodes, except they lack afferent lymphatics (155). The most studied MALT is Peyer's patches, major components of gut-associated lymphoid tissues, but such lymphoid tissues were described in bronchia, as BALT. MALT share some specific features such as a large predominance of IgA-producing cells. However, airway mucosa differs from digestive mucosa by the apparent absence of MALT in the adult

respiratory tract (156), except in pathological condition (157). Isolated lymphoid follicles, interpreted as BALT, were found in children and teenagers up to 20 years with no relation with history of atopy or respiratory disease (157-159). They are composed by localised lymphocytes aggregates, with high endothelial venule to facilitate the recirculation of lymphocytes between the blood and the lymph (155) and the encounter between lymphocyte and its cognate antigen (160).

Classically, after their cognate antigen recognition, conventional B cells (B2 cells in mice) are responsible for the production of antigen-specific IgA antibodies which undergo somatic hypermutation that leads to affinity maturation (161). In contrast, B1 cells can generate polyreactive IgA with lower affinity through class switch recombination regulated in a T cell-independent pathway, as shown in the gut (156, 161). Therefore, B1 cells are considered as an "innate-like" population of B cells (156). In mice, these different B cell populations have been studied for decades while human B1-like cells have recently been described as CD20+CD27+CD38$^{low/int}$CD43+ since this cell population achieve key functional criteria characteristic of mouse B1 cells (162, 163).

Class switch recombination to the IgA isotype is triggered by TGF-β (164), associated with retinoic acid and IL-21 (165), as well as IL-10 (166). Those cytokines are produced by T cells and dendritic cells (165, 166). In addition, BAFF and APRIL promote human IgA1 and IgA2 class switching, respectively (164, 167). As observed in mice, in the absence of transmembrane activator and calcium-modulating cyclophilin-ligand interactor (TACI, a shared BAFF and APRIL receptor), decreased serum IgA concentration and decreased IgA response to T-cell independent stimulus have been shown (168).

The IgA+ effector B cells have to migrate to the effector sites, including the mucosal lamina propria, via lymph and blood (164). B cells must adhere to endothelium and transmigrate across it thanks to chemokines and adhesion

molecules. Homing to the airways might depend on α4β1 integrin and C-C motif chemokine receptor 10, expressed on B cells, and vascular cell adhesion protein 1 and C-C motif chemokine ligand 28, expressed in the respiratory tract venules. Molecules expressed at the surface of B cells and the respiratory tract venules differ depending the destination tissues (169). Terminal differentiation into plasma cells occurs in the effector sites under the action of B-cell activating factor, a proliferation inducing ligand, IL-6 and IL-10, released by dendritic cells as well as intestinal or airway epithelial cells (164).

### 1.3.3 Polymeric immunoglobulin receptor-mediated IgA transport

Polymeric (mainly dimeric, d-) IgA, produced by plasma cells in the mucosa, must be transported through the epithelium in order to reach the cavities of the body. pIgR, expressed at the epithelial surface, allows transepithelial transport of d-IgA (called transcytosis) and release of secretory (S-)IgA in the lumen, including the airways (152).

#### 1.3.3.1 Expression of pIgR

pIgR is a transmembrane protein expressed in all the epithelial cells. *PIGR* gene is localised in the chromosome 1 (in human, as well as in mouse) and its regulation was extensively studied in the intestinal epithelial cells (Figure 9). Basal level of pIgR mRNA is sustained by upstream stimulatory factor-1 and -2, in cooperation with activator protein 2 by linking the E-box of the promoter region (150, 152, 170). However, multiple factors could stimulate its transcription: cytokines, microbes, retinoic acid or hormones (171). Cytokines include IFN-γ, TNF-α, IL-1 and IL-4. IFN-γ through the activation of signal transducer and activator of transcription (STAT) 1 induces the expression of IFN regulatory factor 1, which binds a site on exon 1 of *PIGR* gene (172). IL-4, in cooperation with IFN-γ, upregulates pIgR mRNA production via STAT6 (173, 174). TNF-α stimulates pIgR

transcription by initiating the degradation of inhibitor of nuclear factor κB, the release of RelA subunit and subsequent *de novo* synthesis of RelB (175). In addition, TNF-α induces the expression of pIgR activator IFN regulatory factor 1, through nuclear factor κB activation (176) and both classical and alternative nuclear factor κB pathway can lead to pIgR expression (177). More recently, TGF-β has been shown to downregulate *PIGR* expression (178), contrary to IL-17 upregulation of pIgR protein expression (179). Interestingly, pIgR expression can be stimulated by a variety of T cell lineages (T-helper (Th)1, Th2 and Th17) or inflammation (TNF-α and IL-1) (171). Some microbial products can also directly induce pIgR expression. Lipopolysaccharide through Toll-like receptor (TLR)-4 and double-strand RNA through TLR-3 have been associated with increased transcription (180). Finally, steroidal hormones have been shown to have an effect (activation or inhibition) on pIgR expression in reproductive human organs (152).

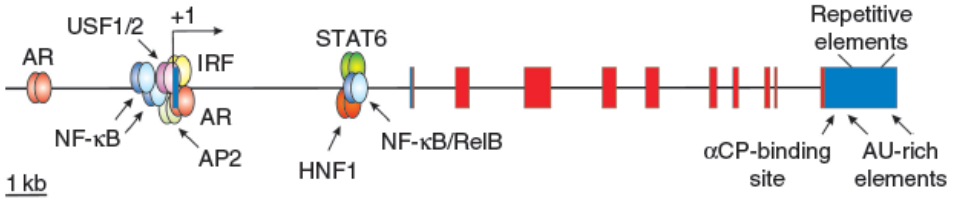


**Figure 9. *PIGR* gene and regulatory elements.**
*PIGR* is composed by 11 exons, including untranslated regions (blue boxes) and the coding region (red boxes), and is regulated by different transcription factors, that are represented on their possible binding sites. AR, androgen receptor; AP, activator protein; αCP, cytoplasmic poly(C)-binding protein α; IRF, interferon regulatory factor; HNF, hepatocyte nuclear factor; NF-κB, nuclear factor-κB; STAT, signal transducer and activator of transcription; USF, upstream stimulatory factor (152).

#### 1.3.3.2 pIgR protein

pIgR is translated into the ER as a 90-100 kDa precursor. It is then sent to the Golgi apparatus where it is highly glycosylated, until reaching 100-120 kDa

(151). Thus, glycosylation represents until 25% of its molecular weight (181). pIgR is constituted by 5 extracellular domains (from D1 to D5), one 23-amino acids transmembrane helix and one cytoplasmic C-terminal tail of 103 amino acids (Figure 10). Interestingly, extracellular domains are considered as "Ig-like" since these 100-110 amino acids domains have similarities to the variable regions of Ig (182). D1, D4 and D5 domains of pIgR are highly conserved among species (181). pIgR selectively transport polymeric Ig and some domains are crucial for its binding properties: D1 is necessary and sufficient for d-IgA (and d-IgM) non-covalent binding (151) and d-IgA is able to link D5 via a disulphide bridge (152). Joining (J) chain (Figure 8), a 15-kDa peptide, is not necessary for dimer formation, but is essential for pIgR binding of d-IgA or pentameric IgM. Ig polymerise though heavy chain terminal extensions (or tailpieces) and a J chain, and form disulphide bonds with J chain to stabilise the polymer. In J chain-deficient mice, d-IgA was measured in breast milk, nasal and intestinal secretions, while it was not associated with pIgR transport (183).

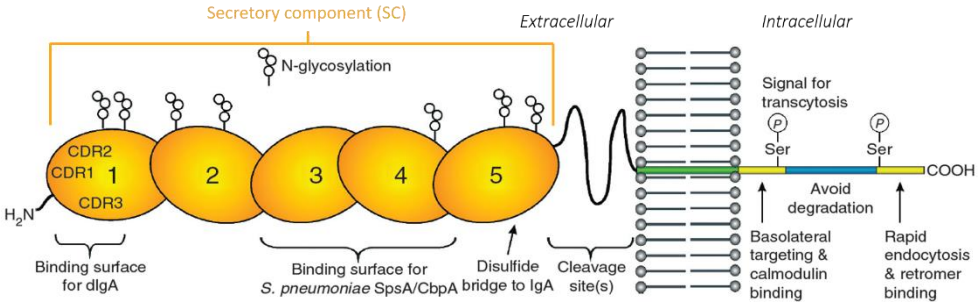


**Figure 10. Structure of the polymeric immunoglobulin receptor.**
pIgR is a transmembrane protein (green box), composed by five extracellular "Ig-like" domains (orange circles, from D1 to D5) also called secretory component, a transmembrane helix (green line) and an intracellular domain, which allows transcytosis signalling (yellow lines) (Adapted from (152)).

#### 1.3.3.3 Trafficking of pIgR

Mature pIgR is trafficked to the basolateral pole of polarised and differentiated epithelial cells, where it is able to bind d-IgA thanks to the J-chain of the later (170, 184).

Transcytosis is illustrated in Figure 11 and begins by the endocytosis of pIgR/d-IgA complex via a clathrin-coated pit, which requires two essential tyrosine residues (668 and 734) in the cytoplasmic tail (185). Into the cell, pIgR is trafficked through the basolateral early endosomes associated with Rab5 GTPase and early endosome antigen 1 effector protein, where a minor part can be recycled to the basolateral membrane. The majority of pIgR/d-IgA complex is then sent in an actin- and microtubulin-dependent manner to Rab17-positive common recycling endosome, a perinuclear compartment, where most of the endocytosed proteins are sorted. For example, transferrin receptor is recycled to the basolateral pole while the bulk of pIgR is shuttled into the apical recycling endosome. This compartment contains the proteins Rab11 and Rab25. Release of the d-IgA into the lumen follows the fusion of the apical recycling endosome with the apical plasma membrane (152, 182).

Transcytosis is further regulated by the different GTPases associated with the endosomes, by phosphorylation of the serine 664 and 726 (186, 187) and, in rat hepatocytes, by ligand binding (188).

During the routing or at the apical pole, the complex is cleaved by a still undefined leupeptin-sensitive endopeptidase (189). This protein is not cell-specific since ectopic expression of pIgR in different cell types leads to its cleavage, although the polarisation of the cell increases the cleavage capacity, which is more efficient at apical than basolateral pole (152). It results in the release of the extracellular part of pIgR – called SC (Figure 10) – bound to IgA, which forms S-IgA (Figure 8 –

right). In addition, transcytosis may substantially occur without the binding of d-IgA and produces free SC (190). According to Marshall *et al.*, it may represent 60% of SC found in secretions (191), while half of pIgR is recycled to the basolateral pole if d-IgA is absent (192, 193).

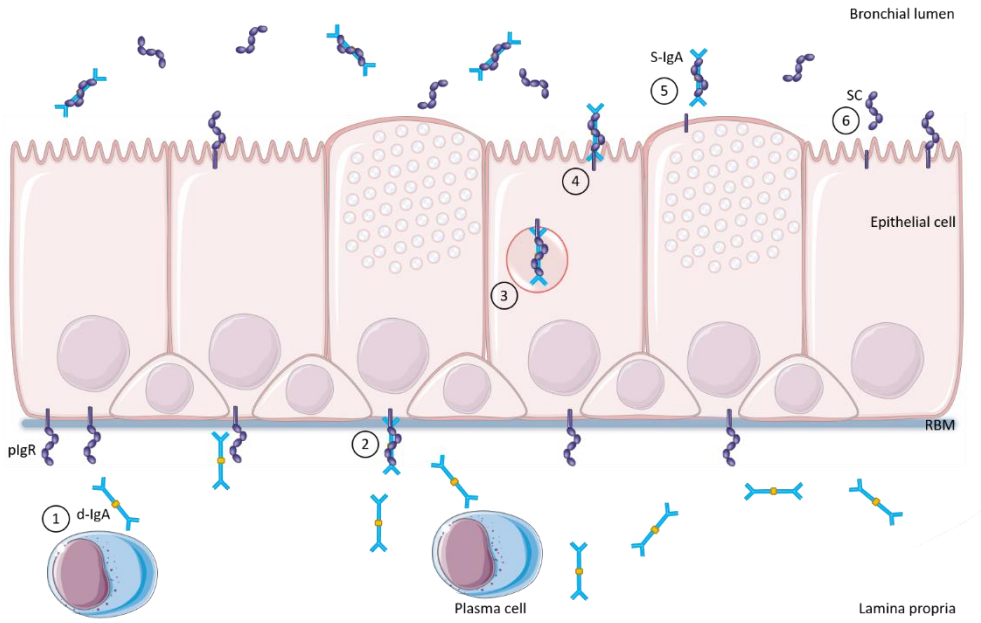


**Figure 11. Transcytosis of IgA.**
Dimeric IgA is produced by plasma cells (1) and can bind pIgR (2), expressed at the basolateral pole of the epithelium. The pIgR-d-IgA complex is then sent to the apical pole (3-4), where pIgR is cleaved and S-IgA is released (5). Transcytosis can also occur without the binding of S-IgA and provide free SC (6).

### 1.3.4 Roles of S-IgA and SC

Into the lumen, IgA antibodies are able to neutralise pathogens and toxins through so-called immune exclusion (194). S-IgA agglutinates bacteria and particles to prevent their attachment to the epithelium. This phenomenon prevents microbes attaching potential targets (195) and thereby represents a frontline defence mechanism, achieving immunity without the need of an inflammatory counterpart.

For instance, S-IgA prevents mannose-specific lectins of *Escherichia coli* attachment *in vitro* by agglutinating the bacteria (196). Furthermore, *in vivo* experiments have shown immune exclusion of *Enterococcus faecum* (197) and *Salmonella enterica typhimurium* (198). Such interference was also suggested *in vivo* with rotavirus (150) as well as *in vitro* with influenza virus (199).

In addition, *in vitro* studies suggest the ability of specific IgA to neutralise viruses into the cell, during transcytosis. This has been shown with influenza virus (200), measles virus (201), human immunodeficiency virus (202), and rotavirus (203). *In vitro* study demonstrated that IgA can bind soluble antigens in the lamina propria and that the immune complex can be transported into the lumen via a pIgR-dependant mechanism (150). Figure 12 schematises the main S-IgA roles.

S-IgA does not activate the classical complement pathway and may even inhibit it (204). However, it is able to initiate lectin-dependant activation through mannan binding lectin (154). Moreover, S-IgA could bind the IgA Fc receptor (FcαR) expressed by myeloid cells (neutrophils, monocytes, macrophages, dendritic cells) and promotes phagocytosis, antibody-dependent cell-mediated cytotoxicity, cytokine secretion, superoxide generation, degranulation, antigen presentation and calcium mobilisation (154, 205). Of note, S-IgA is also more efficient than serum IgA to induce degranulation and superoxide production by eosinophils (152).

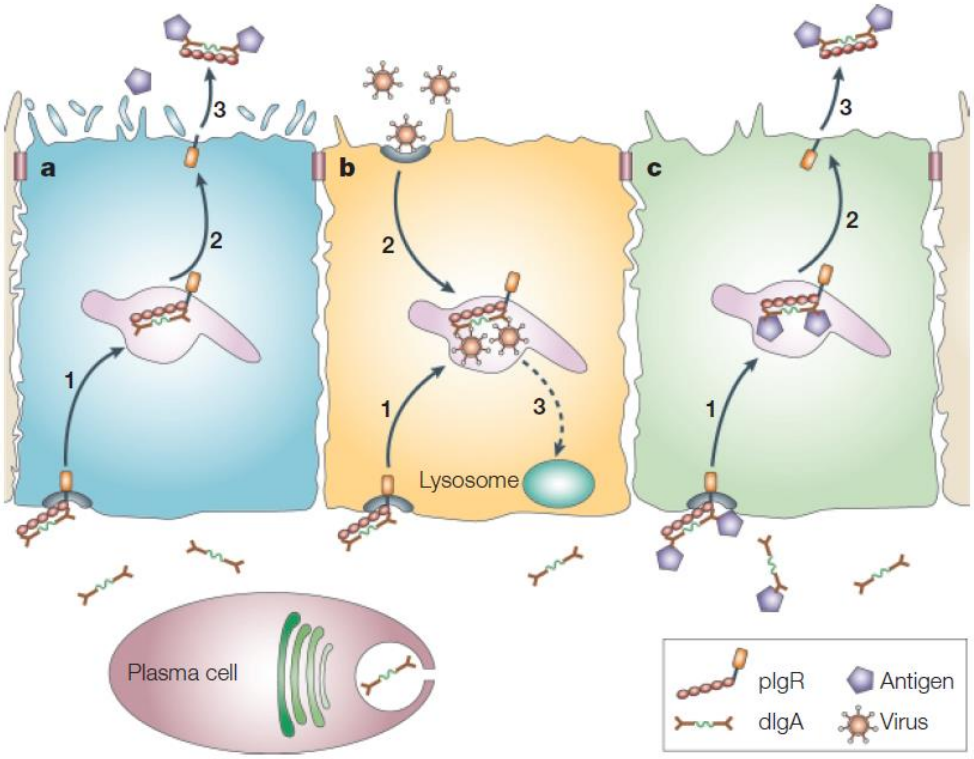


**Figure 12. Immune neutralisation by S-IgA and pIgR.**
(a) d-IgA is transported by pIgR to form S-IgA, which can bind and neutralise a luminal antigen. (b) Intracellular viruses can be neutralised during the routing and possibly sent to the lysosomes for degradation. (c) Antigens in the lamina propria can be trapped by d-IgA and secreted through pIgR-mediated IgA transcytosis. S-IgA, secretory immunoglobulin A; pIgR, polymeric immunoglobulin receptor; d-IgA, dimeric immunoglobulin A.

SC also has its proper roles. First, it stabilises S-IgA and protects it against proteolytic degradation, including by NE, *in vitro* and *in vivo* in the intestinal tract and oral cavity (152). An *in vitro* study also suggests that SC enhances effective function of IgA, notably by increasing neutralisation capacity against influenza virus (206). Then, it regulates the proper localisation of S-IgA by linking the mucus and forming an interface between the lumen and the epithelium, as shown in a mouse model (207). Free SC has its proper antimicrobial properties: it inactivates toxin A

from *Clostridium difficile* (208, 209) and decreases *Escherichia coli* attachment on the epithelium (210), as well as *Streptococcus pneumoniae* SpsA surface protein binding (Figure 10) (211). In addition, SC has anti-inflammatory properties since it binds neutrophil chemoattractant IL-8 and other chemokines, and inhibits their activity (212). Massive glycosylation of SC is critical for nonspecific roles of SC and S-IgA and loss or change in carbohydrates is associated with defective SC (183). Indeed, treatment of SC with galactosidase or neuraminidase leads to disrupted protective interaction with toxin A from *Clostridium difficile* and with *Escherichia coli*, showing the importance of galactose and sialic acid for SC roles. However, loss of fucose did not affect interaction with toxin A and with *Escherichia coli* (209).

pIgR KO mice were generated and highlighted some other roles of S-Ig. These mice display a decreased concentration of IgA in bile, intestines and faeces (213), while no change in saliva suggests an alternative transport or diffusion of IgA through the epithelium (213, 214). Alternative transports include asialoglycoprotein receptor-mediated endocytosis and FcαR/CD89-mediated binding (213). Interestingly, IgA from intestines of pIgR KO mice seems degraded compared to wild-type mice, confirming the protective role of SC in S-IgA (214). However, d-IgA concentration in the circulation is dramatically increased and the massive deposition of IgA immune complexes can lead to IgA nephropathy (213, 214). Surprisingly, the number of IgA+ plasma cells is higher in the lamina propria of the intestines from pIgR KO mice, suggesting a possible contribution of pIgR to mucosal B-cell homeostasis (199). Increased IgG antibodies to *Escherichia coli* in the serum of pIgR KO mice further indicate a possible role in mucosal homeostasis (214). Furthermore, whether no spontaneous pathology was described in the young mice (probably due to redundancy of defence mechanisms and compensation with IgG), the absence of pIgR and/or S-IgA compromises the ability to control the inflammation and cure of *Escherichia coli* colitis (215), as well as decreases the protection against influenza virus after intranasal immunisation (216, 217). More

recently, one study has shown that pIgR KO mice display progressive lung inflammation while aging, a process associated with bacterial invasion and the development a chronic obstructive pulmonary disease (COPD)-like remodelling with emphysema. This inflammation worsens with the administration of non-typeable *Haemophilus influenzae*, while S-IgA administration decreases the inflammatory response, in line with the ant-inflammatory role of S-IgA. In addition, germ-free housing prevented the inflammation and the subsequent remodelling, suggesting an important role of pIgR and/or S-IgA in the maintenance of lung homeostasis (218). In line with this study, Reikvam *et al.* reported altered microbiota in the faeces and caecum of pIgR KO mice, compared with wild-type (215), although initial studies demonstrated the same clearance capacity of *Citrobacter rodentium* or *Salmonella typhimurium*, and no difference in intestinal microbiota composition (152). Altogether, these data highlight the role of pIgR in mucosal homeostasis through the regulation of the microbiota and epithelial inflammation, thereby protecting from chronic inflammatory disease at mucosal barriers.

### 1.3.5 IgA mucosal immunity in CF

Regarding its role in infection and inflammation, pIgR expression has already been studied in other chronic lung diseases. pIgR expression was shown to be decreased in airway epithelial cells in chronic obstructive pulmonary disease COPD (178, 219) and in upper airway diseases, as allergic rhinitis and chronic rhinosinusitis with nasal polyps (220). Despite no study on pIgR expression in CF, different studies showed increased IgA concentrations in serum of CF patients, compared with controls (221), more particularly in patients with *Pa* infection (222) and with a more severe disease (223). This increase was also suggested in sputum or in BAL of CF patients (224). Marshall *et al.* demonstrated an increased concentration of free SC in CF sputum (191). Specific anti-*Pa* IgA has also been shown to be upregulated in serum when patients suffer from infection (222, 225, 226) or poor

pulmonary function (227), but also in sputum (228) and nasal secretion (170). However, other researches showed decreased IgA secretion in CF saliva (229) and in the gastric luminal perfusate (230). Decreased free SC concentration in sputum and in saliva from CF patients were also reported (231).

## 2 Goals of the thesis

CF is increasingly considered as a mucosal immunodeficiency syndrome. Multiple defaults of innate immunity mechanisms (defective antimicrobial molecules, neutrophils, transport of antioxidants, etc.) were shown to be related at least indirectly to CFTR mutations (69, 232). However, data about IgA immunity remained controversial and its transport by pIgR was not yet addressed. In addition, changes in the airway epithelium, where pIgR-mediated transcytosis occurs, were suggested for decades in patients with CF (70), but detailed phenotypic studies of lung tissues and/or primary CF epithelium were largely lacking.

Therefore, this project aims to assess airway epithelial function and differentiation in CF:

(i) by determining epithelial pIgR and IgA immunity through a multimodal approach (including human lung tissue, sputum, primary human bronchial epithelial cell cultures (HBEC) and mouse model) and addressing the possible mechanisms (CFTR-dependant or not) responsible for pIgR dysregulation,

(ii) by characterising cellular populations and EMT of the airway epithelium from patients with CF.

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# CHAPTER 2: LUNG IMMUNOGLOBULIN A IMMUNITY

## DYSREGULATION IN CYSTIC FIBROSIS

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## 1 Abstract

Background: In CF, recurrent infections suggest impaired mucosal immunity but whether production of S-IgA is impaired remains elusive. S-IgA is generated following pIgR-mediated transepithelial transport of d-IgA and represents a major defence through neutralisation of inhaled pathogens like *Pa*.

Methods: Human lung tissue (n = 74), human sputum (n = 118), primary HBEC (cultured in air-liquid interface, ALI) (n = 19) and mouse lung tissue and bronchoalveolar lavage were studied for pIgR expression, IgA secretion and regulation.

Results: Increased epithelial pIgR immunostaining was observed in CF lung explants, associated with more IgA-producing plasma cells, sputum, and serum IgA, especially *Pa*-specific IgA. In contrast, pIgR and IgA transport were downregulated in F508del mice, CFTR-inhibited HBEC, and CF HBEC. Moreover, the UPR due to F508del mutation, inhibited IgA transport in Calu-3 cells. Conversely, pIgR

expression and IgA secretion were strongly upregulated following *Pa* lung infection in control and F508del mice, through an inflammatory host response involving IL-17.

Conclusions: A complex regulation of IgA secretion occurs in the CF lung, UPR induced by CFTR mutation/dysfunction inhibiting d-IgA transcytosis, and *Pa* infection unexpectedly unleashing this secretory defence mechanism.

## 2 Introduction

CF is a lethal genetic autosomal recessive disease, caused by mutations in the CFTR gene (1), which encodes for an anion channel expressed in epithelial cells. Whereas all epithelia may be affected, lung disease is mainly responsible for the morbidity and mortality of patients with CF (2). It is characterised by sticky mucus, chronic neutrophilic inflammation, recurrent opportunistic infections, and progressive formation of bronchiectasis. *Pa* is the most common bacteria found in the CF adult lung and is associated with a worsening of lung function and vital prognosis (3, 4). The susceptibility to opportunistic *Pa* infection relates to impaired mucosal immunity underlying a permissive environment in CF airways. In the normal lung, the airway epithelium and immune resident cells ensure frontline defence against pathogens through mechanical and immune barrier functions. In addition to mucociliary clearance, the epithelium can sense and respond to pathogens, and is able to secrete several antimicrobial molecules and cytokines/chemokines (TNF-α, IL-8/CXCL8 and IL-1β) in order to recruit or activate innate immune cells and, if required, to support activation of adaptative immunity. In CF, multiple aspects of those mechanisms are impaired including mucociliary clearance or production of antimicrobial proteins (5). In addition, some studies suggest an imbalance between Th1 and Th2 responses, patients with CF with chronic *Pa* infection displaying a Th2-dominated response, while Th1 response

against *Pa* was related to a better outcome (6, 7). This immune response is accompanied by increased *Pa*-specific IgG antibodies in the serum from infected patients with CF (8), as well as by an increased local production of *Pa*-specific IgA (9, 10).

IgA exerts antimicrobial, antiviral, as well as anti-inflammatory activities. Polymeric – mainly d- – IgA, produced by subepithelial plasma cells, is transported into secretions by pIgR-mediated transcytosis. After binding of d-IgA, the pIgR/d-IgA complex is sent to the apical pole where the extracellular part of the receptor – called SC – is cleaved, releasing SC bound to IgA to form S-IgA, as well as free SC (11). At the epithelial surface, S-IgA may neutralise antigens and pathogens through so-called immune exclusion. In addition, SC itself may contribute to these anti-microbial and anti-inflammatory functions (12, 13), particularly by inhibiting the activity of pro-inflammatory molecules such as IL-8/CXCL8, and by protecting S-IgA from proteolytic degradation (14, 15). pIgR expression has been studied in several chronic respiratory diseases (16-18), where it is downregulated, while no data is available in CF. Previous studies reported increased IgA concentrations in serum (19) or BAL of patients with CF (20), as well as increased free SC in CF sputum (21). Conversely, decreased IgA secretion was described in CF saliva and in gastric luminal fluid (22, 23).

This study was designed to assess epithelial pIgR and IgA immunity in the CF lung through a multimodal approach including lung tissue, sputum, serum and primary epithelial cell cultures, as well as mice harbouring the F508del CFTR mutation (F508del mice) and CFTR inhibition in primary epithelial cell cultures. We demonstrate that pIgR/SC expression and IgA production are increased in the human CF lung. While CFTR dysfunction *per se* leads to downregulation of pIgR expression and IgA transcytosis, through activation of ER stress, chronic lung *Pa* infection is associated with an upregulation of pIgR-mediated IgA secretion through a host inflammatory response involving IL-17.

# 3 Methods

## *3.1 Patients*

Lung tissue were obtained from 74 patients, which included explants obtained from 36 patients with end-stage CF disease compared with lung tissue from 38 control patients undergoing lung surgery for a solitary lung tumour (n = 32) or unused lung donors (n = 6). Sputum and serum were obtained from 118 subjects, with 67 patients with CF at stable state compared with 51 age-matched control subjects with no clinical evidence of lung disease and normal lung function. 25 patients were enrolled for cell culture, which included seven control subjects (undergoing lung resection surgery for a solitary lung tumour) and 18 patients with severe CF (undergoing lung resection surgery for transplantation) (Table 2). Patients with CF were classified according to their state of infection by *Pa* as follows; 1) chronic infection, when having more than 50% of months, when samples had been taken, positive in a 12-months period, 2) intermittent infection, when 50% or less months, when samples had been taken, positive in a 12-months period, 3) free of infection, when there was no growth of *Pa* in the last 12 months, and 4) never infected, when *Pa* was never isolated in culture. Among *Pa*-never infected patients, eight out of ten were infected by other bacteria (such as *Staphylococcus aureus*, *Achromobacter*, or *Burkholderia* species) and two patients were infected by *Aspergillus fumigatus*.

| | | Controls | Patients with CF | All | P-value |
|---|---|---|---|---|---|
| **Lung tissue series** | Subjects, n | 38 | 36 | 74 | |
| | Sex, F/M | 21/17 | 20/16 | 41/33 | |
| | Age, years (mean ± SD) | 58 ± 14 | 31 ± 9 | 45 ± 18 | *p < 0.0001* |
| | Smoking history, never/former/current | 34/3/1 | 33/3/0 | 67/6/1 | |
| | Genotype, F508del homozygous/F508del heterozygous/other mutations | NA | 22/10/4 | NA | |
| | $FEV_1$, % predicted (n = 68) (mean ± SD) | 94 ± 18 | 24 ± 8 | 60 ± 38 | *p < 0.0001* |
| **Sputum and serum series** | Subjects, n | 51 | 67 | 118 | |
| | Sex, F/M | 35/16 | 36/31 | 71/47 | |
| | Age, years (mean ± SD) | 31 ± 10 | 34 ± 11 | 33 ± 11 | *p = 0.0666* |
| | Smoking history, never/former/current | 51/0/0 | 66/1/0 | 117/1/0 | |
| | BMI, kg/m² (mean ± SD) | 23 ± 5 | 22 ± 3 | 22 ± 4 | *p = 0.1009* |
| | Genotype, F508del homozygous/F508del heterozygous/other mutations | NA | 36/24/7 | NA | |
| | $FEV_1$, % predicted (mean ± SD) | 100 ± 9 | 67 ± 23 | 81 ± 25 | *p < 0.0001* |
| | VC, % predicted (mean ± SD) | 102 ± 10 | 87 ± 19 | 93 ± 17 | *p < 0.0001* |
| | *Pa* infection status, C/I/F/N (3) | NA | 33/12/12/10 | NA | |
| | Inhaled corticosteroids, yes/no (n = 48) | NA | 41/7 | NA | |

| | | | | | |
|---|---|---|---|---|---|
| **Human-derived epithelial cells series** | Subjects, n | 7 | 18 | 25 | |
| | Sex, F/M | 1/6 | 9/9 | 10/15 | |
| | Age, years (mean ± SD) | 62 ± 8 | 35 ± 12 | 43 ± 16 | *p < 0.0001* |
| | Smoking history, never/former/current | 3/1/3 | 17/1/0 | 20/2/3 | |
| | Genotype, F508del homozygous/F508del heterozygous/other mutations | NA | 9/7/2 | NA | |
| | $FEV_1$, % predicted (n = 16) (mean ± SD) | 97 ± 14 | 31 ± 20 | 48 ± 34 | *p < 0.0001* |
| | Subjects, n (pIgR expression) | 6 | 8 | 14 | |
| | Subjects, n (UPR activation) | 8 | 10 | 18 | |
| | Subjects, n (*Pa* supernatant stimulation) | 0 | 5 | 5 | |
| | Subjects, n (IL-17 stimulation) | 0 | 6 | 6 | |

**Table 2. Patient characteristics for epithelial pIgR and IgA immunity study.**
CF, cystic fibrosis; F, female; M, male; SD, standard deviation; FEV1, forced expiratory volume in the first second; NA, not applicable; BMI, body mass index; VC, vital capacity; Pa, Pseudomonas aeruginosa; C, chronically infected by Pa; I, intermittently infected by Pa; F, free of Pa infection; N = never infected by Pa; pIgR, polymeric immunoglobulin receptor; UPR, unfolded protein response. N is specified when data are missing.

### *3.2 Immunohistochemistry for human and mouse pIgR, and human IgA*

Lung samples were fixed in 4% formaldehyde and embedded in paraffin wax. Paraffin lung sections (5 μm-thick) were deparaffinised in toluene and rehydrated through a graded series from methanol to water. Except for human pIgR/SC, sections underwent antigen retrieval treatment using cooker pressure

treatment during 5 min at a pressure of 15 psi. Endogenous peroxidase, biotin, and streptavidin sites were inactivated by hydrogen peroxide, biotin, and avidin solutions or Bloxall (Vector Laboratories Inc., USA), as appropriate. Nonspecific protein binding sites were blocked with 5% bovine serum albumin or horse serum (Bio-Rad, USA) in tris-buffered saline (TBS) for 1 h, according to the secondary antibody. Rabbit polyclonal anti-human pIgR (1 µg/mL; home-made Lp877), mouse anti-human IgA (4 µg/mL; Thermo Fisher Scientific, Germany), or goat anti-mouse pIgR (R&D Systems, UK) were incubated overnight at 4°C. After washes with TBS 0.1% Tween 20, sections were incubated with goat anti-rabbit IgG (1:3000; Sigma-Aldrich, USA) – followed by streptavidin-horseradish peroxidase (tbs) amplification –, horse anti-mouse Ig (ImmPRESSTM Reagent) or mouse anti-goat IgG (1:100; Sigma-Aldrich), respectively. Revelation was performed by using 3,3'-Diaminobenzidine (Sigma-Aldrich). Sections were counterstained with Hematoxylin (Sigma-Aldrich) and coverslipped. Slides were scanned using Leica SCN400 (Leica, UK) before selecting the ten preserved fields. Quantification was done with Leica software and expressed as percentage of positive area for pIgR expression and for IgA immunostaining as the number of IgA positive cells per $mm^2$.

### *3.3 Dual immunohistochemistry for IgA and cluster of differentiation (CD)138, and for CD3 and RAR-related orphan receptor (ROR)γt*

As previously, unstained paraffin sections were deparaffinised, rehydrated, and subjected to antigen retrieval. Endogenous peroxidase activity was blocked by incubation in Bloxall (Vector lab, USA) for 15 min and 0.3% hydrogen peroxide in TBS 5% goat serum (Abcam) for 30 min respectively. Each section was subjected to two sequential stainings, each including a blocking with TBS 5% goat serum followed by primary antibody incubation and corresponding secondary HRP-conjugated polymer antibody (Invitrogen). Mouse anti-human IgA (Thermo Fisher

Scientific) and rabbit anti-CD138 (Acris, Germany), or rabbit anti-CD3 (Cell Signaling, USA) and mouse anti-RORγt (Sigma) were successively used as primary antibody. Each HRP-conjugated polymer mediated the covalent binding of a different fluorophore using tyramide signal amplification, as previously described (24). Finally, sections were counterstained with Hoechst (Thermo Fisher Scientific) and mounted with fluorescence mounting medium (Dako, USA). As negative controls, we used corresponding control isotypes diluted at the same concentration as related primary antibody. Images were acquired with Panoramic P250 Flash III slide scanner (3DHistech, Hungary). Quantification of the CD3 and RORγt co-staining was performed with Author software (Visiopharm®) and was expressed as the number of positive cells per µm².

### *3.4 Basal cell isolation*

The bronchial section was digested by pronase E from *Streptomyces griseus* 1 mg/mL (Sigma-Aldrich) in Roswell Park Memorial Institute (RPMI) medium (Lonza, Belgium) supplemented with 200 U/mL penicillin and 200 µg/mL streptomycin (Lonza) overnight at 4°C. Cells were labelled with anti-CD151 conjugated with phycoerythrin (Clone 14A2.H1, BD Biosciences) and anti-CD142 conjugated with allophycocyanin (Clone HTF1, Miltenyi Biotec, Netherlands) antibodies after blocking with FcR Blocking Reagent (Miltenyi Biotec) and dead cells discrimination by Fixable Viability Stain 450 (BD Biosciences, USA). Cell sorting was performed with a FACSAria cell sorter (BD Biosciences). Basal cells ($CD151^+/CD142^+$) were selected and seeded in a 75-cm² cell culture flask in bronchial epithelial basal medium (BEBM) (Lonza), supplemented with 200 U/mL penicillin and 200 µg/mL streptomycin (Lonza) (as well as Primocin™ 100 µg/mL (InvitroGen, France) for CF cells), bovine serum albumin 1.5 mg/mL (Sigma-Aldrich) and retinoic acid 100 µM (Sigma-Aldrich). Cells were cultured at 37°C and

5% $CO_2$ to reconstitute a primary bronchial epithelium. When reaching 90% confluency, cells were trypsinised and 100,000 cells were seeded in 0.33 $cm^2$ insert with 0.4 µm polyester membrane pore (Corning, USA) in submerged condition for 10-15 days. Then ALI condition was applied for two weeks in BEBM:Dulbecco's Modified Eagle Medium (DMEM) (Lonza) 1:1 supplemented as pure BEBM.

### *3.5 In vitro epithelial assays*

Primary human bronchial epithelial cells (HBEC) were treated after culture for two weeks in ALI condition with 25 µL of CFTR inhibitors (GlyH-101 or PPQ-102) (CF Foundation) (1, 5, 10, 20 µM), or 25 µL of *Pa* supernatant, at the apical pole for 48 h. Basal media, apical washes, and cell lysates (for western blot or reverse transcription and real-time polymerase chain reaction (RT-qPCR)) were then harvested. HBEC cultures were also treated for 48 h with increasing doses of IL-17 (10, 20, 40 ng/mL). Basal media, apical washes, and cell lysates (for western blot or RT-qPCR) were then collected and harvested.

For the transcytosis assay of d-IgA, after two weeks in ALI condition, 1 mg/mL d-IgA was added at basolateral pole for 72 h, and S-IgA was measured by sandwich Enzyme Linked Immunosorbent Assays in the apical wash (300 µL phosphate-buffered saline, PBS).

### *3.6* Pa *supernatant preparation and stimulation*

*Pa* mucoid clinical strain was processed according to Massion and colleagues to obtain *Pa* supernatant (25). Briefly, *Pa* mucoid clinical strain was seeded in Trypticone Soy Broth medium (BD, USA) and incubated during 72 h, at 37°C. Then, bacteria culture was centrifuged at 10,000 g during 50 min, at 4°C. Supernatant was filtered (0.2 µm) and stored at -80°C. After two weeks in ALI

condition, ALI-HBEC were stimulated with 25 µL of *Pa* supernatant at apical pole for 48 h. Sterile PBS was used as control condition. Basal media, apical washes, and cell lysates (for western blot or RT-qPCR) were then harvested.

### *3.7 Calu-3 cell culture*

Calu-3 cells (passages 10-15) were cultured at 37°C and 5% $CO_2$ in a RPMI medium (Lonza), supplemented with 10% foetal bovine serum, 200 U/mL penicillin and 200 µg/mL streptomycin (Lonza). Cells were maintained in a 75-cm² cell culture flask and split when they reached 90% confluence. For UPR activation, Calu-3 cells were stimulated with thapsigargin (Sigma-Aldrich) (0.2, 0.5, 1, 2 µM) or tunicamycin (Sigma-Aldrich) (2, 5, 10, 20 µg/mL), diluted in dimethyl sulfoxide (DMSO), during 6 h or 24 h. DMSO was used as control condition. Media and cell lysates (for western blot or RT-qPCR) were then harvested. For transcytosis assay of d-IgA, Calu-3 cells (50,000 per insert) were seeded in 0.33 cm² insert with 0.4 µm polyester membrane pore (Corning). After they reached confluency, they were stimulated with 10 µg/mL tunicamycin and 1 mg/mL d-IgA was added at basolateral pole for 48 h. DMSO was used as control condition. Basal media and apical washes or media were then collected. Calu-3 cell cultures were also treated for 48 h with increasing doses of IL-17 (5, 10, 20 ng/mL). Media and cells lysates were then collected and harvested.

### *3.8 Sputum and serum collection, and processing*

Sputum from patients with CF was obtained during a physiotherapy session while sputum from healthy subjects was induced by using the method of Pizzichini and colleagues, with minor modifications (26). Sputum induction was achieved with an aerosol of hypertonic saline generated by the NE-U17 ultrasonic nebulizer

(Omron). Healthy subjects equipped with a nose clip, inhaled increasing concentrations of saline (3, 4, and 5%) for 7 min each through a mouthpiece. We then asked subjects to rinse their mouth or swallow to minimise contamination with saliva. Finally, we invited them to cough sputum into a sterile container. Collected sputum was diluted 1:10 (w:v) in PBS with 10 U/mL DNase (Pulmozyme 2500 U/2.5 mL – Roche, USA) and incubated under agitation, during 30 min, at 37°C. Then, it was filtered with 48 µm pore nylon filter (Prosep, Belgium) and centrifuged at 450 g, during 5 min. Neutrophil elastase activity was measured in supernatant. Remaining supernatant was treated with PMSF Protease Inhibitor 2% v:v in order to inactivate elastase and was stored at -80°C. Cells were processed for cytospin (fixed in methanol and staining by Diff-Quick method (Kwik-Diff™ Stains, Thermo Fisher Scientific) and epithelial contamination was assessed. The blood was centrifuged at 650 g for 20 minutes, the serum was collected, and stored at -80 °C.

### *3.9 F508del mice and model of* Pa *lung infection with* Pa*-coated beads*

Mice (FVB/129) homozygous for the F508del mutation (F508del mice, kindly received from Dr T. Leal, UCLouvain Brussels, Belgium) and wild-type littermates (WT mice) were housed in a specific pathogen free animal facility, in ventilated cages. 15 F508del mice and 15 WT received no treatment (eight females and seven males in each group). In parallel, nine F508del (eight females and one male) and six WT (four females and two males) mice were instilled with agarose beads, coated with a clinical strain of *Pa* (isolated from a patient with CF). Sterile beads were used to instil seven F508del (four females and three males) and eight WT (three females and five males), as control. Beads were prepared according to Martin and colleagues (27). Dissolved agarose (coloured with Indian ink) (Type XII, Sigma-Aldrich®) was mixed with mineral oil (Sigma-Aldrich®) at 50°C, and with around 109 colony-forming units of *Pa* in culture. This mix was cooled down with ice while

mixing for 20 min. After rinsing by centrifugations at 4°C, beads were sieved twice to obtain a homogenous solution of 300 to 400 µm beads, a size considered optimal to penetrate the lower airways, but not the alveoli. In order to control the bacterial load, beads and *Pa* culture were spread on agar-agar and counted after incubation during 24 h at 37°C. A volume of 40 µL of beads solution at 5% in PBS (sterile beads or *Pa*-coated) was instilled in mouse trachea after anaesthesia with Ketamine 15 mg/mL (Nimatek® 100 mg/mL Eurovet, Netherlands) and Xylazine 2 mg/mL (Rompun® 2%, Bayer, GmbH D24106, Germany) at 100µL/10g body weight. Eventual respiratory stress could appear after instillation and therefore mice were closely monitored and received oxygen supplementation if needed. After 15 days, mice were sacrificed, BAL was performed, and lungs were collected for immunochemistry, RT-qPCR and western blot, as well as blood to obtain serum.

### *3.10 Western blot for pIgR/SC and (phospho-)eukaryotic initiation factor 2α ((P-)eIF2α)*

Cells were lysed with Laemmli's Blue. After boiling for 5 min, lysates were separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and proteins were transferred to a nitrocellulose membrane. After blocking with TBS 5% bovine serum albumin, the membrane was incubated with rabbit anti-pIgR (1:6000; homemade Lp877), rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:3000; Sigma-Aldrich), mouse anti-eIF2α (1:2000; Cell Signaling), rabbit anti-P-eIF2α (1:1000; Cell Signaling), and mouse anti-β-actin (1:1000; Sigma-Aldrich) overnight at 4°C, then with goat anti-rabbit IgG (1:2000; Cell Signaling) or anti-mouse IgG (1:5000; Sigma-Aldrich) for 1 h at room temperature. Detection of immunoreactivity was performed with the ECL™ Prime Western Blotting Detection chemiluminescence reagent (Amersham™ ECL, GE Healthcare, UK) blot and quantification was performed by using Quantity One software.

### *3.11 Enzyme Linked Immunosorbent Assays for SC, S-IgA, S-IgM, specific IgA to* Pa *and IL-17*

SC, S-Ig, and total Ig concentrations were measured in HBEC apical washes, sputum supernatants or serums, in plates coated with anti-SC (1:2000; Ch606), anti-IgA (1:1000; ACP17) or anti-IgM (Sigma-Aldrich) as capture antibody. After washes, samples and standard incubated 2 h, at 37°C, and detection was performed with biotinylated anti-SC (1:4000; Ch606) – followed by Streptavidin-HRP amplification – or HRP-linked anti-IgA (1:5000; Sigma-Aldrich) or anti-IgM (1:5000; Sigma-Aldrich).

In sputum supernatants, specific IgA to *Pa* were determined by coating 2.25 µg of antigens from serotypes 001-015 (Statens Serum Institute, Denmark) and detection was performed with the antibody mentioned above, and IL-17 was measured by Duoset (R&D Systems).

In mouse BAL and serum, concentrations of mouse total IgA, as well as (only in BAL) mouse S-IgA and mouse SC, were measured. Plates were coated with anti-mouse IgA (1:1000; Sigma-Aldrich) or anti-mouse SC (1:200; R&D Systems). After blocking, samples and standard (IgA κ myeloma (Sigma-Aldrich) or pure BAL) were loaded and incubated 2 h, at 37°C. Immunodetection was carried out by using biotinylated anti-mouse IgA (SYnAbs, Belgium) and Streptavidin-HRP amplification. For mouse SC, samples and standard (R&D Systems) were coated in plates and incubated overnight at 4°C. Mouse SC was captured with goat anti-mouse SC (R&D Systems) and detected by biotinylated anti-goat IgG (Sigma-Aldrich) and Streptavidin-HRP amplification.

### *3.12 RT-qPCR*

After extraction, RNA was reverse-transcribed with RevertAid H minus Reverse transcriptase kit (Thermo Fisher Scientific, UK) with 0.3 µg of random hexamer, 20 U of RNase inhibitor, and 1 mM of each dNTP following the manufacturer's protocol in a thermocycler (Applied Biosystems). For the expression measurement, the reaction mix contained 2.5 µL of complementary desoxyribonucleic acid diluted 10-fold, 200 nM of each primer (Table 3), and 2x iTaq UniverSybr Green® Supermix (Bio-Rad) in a final volume of 20 µL. The cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. To control the specificity of the amplification products, a melting curve analysis was performed. Copy number was calculated from the standard curve. Expression levels of target genes were normalised to the geometric mean of the values for the housekeeping genes (ribosomal protein S13, S18 and L27 in human lung tissue and primary HBEC, S18 and hypoxanthine-guanine phosphoribosyltransferase 1 in Calu-3, and TATA-box binding protein, hypoxanthine-guanine phosphoribosyltransferase 1 and tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta in mouse tissue).

| **Human** | | |
|---|---|---|
| **Gene** | **Forward primer sequence 5'→ 3'** | **Reverse Primer sequence 5'→ 3'** |
| *PIGR* | CTC TCT GGA GGA CCA CCG T | CAG CCG TGA CAT TCC CTG |
| *XBP-1$_s$* | ATG GAT GCC CTG GTT GCT GAA GA | TGC ACC TGC TGC GGA CTC A |
| *XBP-1$_u$* | AGC ACT CAG ACT ACG TGC ACC TCT | CCA GAA TGC CCA ACA GGA TAT CAG |

| | | |
|---|---|---|
| *IL17* | TGG AAT CTC CAC CGC AAT GA | GCT GGA TGG GGA CAG AGT TC |
| *HPRT1* | TGA CAC TGG CAA AAC AAT GCA | GGT CCT TTT CAC CAG CAA GCT |
| *RPS13* | TCG GCT TTA CCC TAT CGA CGC AG | ACG TAC TTG TGC AAC ACC ATG TGA |
| *RPS18* | TGT GGG CCG AAG ATA TGC T | TGA TCA CAC GTT CCA CCT CAT |
| *RPL27* | TGG TAG GGC CGG GTG GTT GC | ACT TTG CGG GGG TAG CGG TC |
| **Mouse** | | |
| **Gene** | **Forward primer sequence 5'→ 3'** | **Reverse primer sequence 5'→ 3'** |
| *Pigr* | CTG TGC CCG AAA CTG GAT CA | GTG GAG ACC CCT GAA AAG ACA |
| *Xbp-1$_s$* | CTG AGT CCG AAT CAG GTG CAG | GTC CAT GGG AAG ATG TTC TGG |
| *Xbp-1$_u$* | CAG CAC TCA GAC TAT GTG CA | GTC CAT GGG AAG ATG TTC TGG |
| *Il17* | AGT CCA GGG AGA GCT TCA TCT | GGT CTT CAT TGC GGT GGA GAG T |
| *Tbp* | CTA TCA CTC CTG CCA CAC C | ATG ACT GCA GCA AAT CGC TTG |
| *Hprt* | AGT CCC AGC GTC GTG ATT AG | TGA TGG CCT CCC ATC TCC TT |
| *Ywhaz* | TGC GTG ACA TCT GCA ACG AT | GAC TGG TCC ACA ATT CCT TTC T |

**Table 3. List of primers for epithelial pIgR and IgA immunity study.**
pIgR, polymeric immunoglobulin receptor; XBP-$1_s$, spliced X-box binding protein 1; XBP-$1_u$, unspliced X-box binding protein 1; IL17, interleukin 17 ; HPRT1, hypoxanthine-guanine phosphoribosyltransferase 1; RPS13, ribosomal protein S13; RPS18, ribosomal protein S18; RPL27 ribosomal protein L27; TBP, TATA-box binding protein; YWHAZ, tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta.

### *3.13 Transmission electron microscopy*

ALI-HBEC were fixed overnight at 4°C in 2.5% glutaraldehyde BEBM:DMEM 1:1 (Lonza), supplemented as mentioned before, washed in cacodylate buffer (pH 7.4) and postfixed twice in 1% osmium tetroxide (and 1.5% ferrocyanide) for 1 h. Then, cells were stained with 1% uracyl acetate for 1 h at room temperature, dehydrated in ethanol and embedded in epoxy resin (Agar 100 resin; Agar Scientific, UK). Quantification of the ER area was performed with ImageJ software. Results were expressed as the percentage of ER area.

### *3.14 Statistical analysis*

GraphPad Prism 8.0.2 software (GraphPad Inc., USA) and SPSS (IBM) were used for statistical analysis. Normality of the data distribution was first assessed. Comparisons between two groups were performed using an unpaired Student's t test, except when data distribution were not normal. In such case, comparisons between two groups were performed using the Mann-Whitney U-test, whereas comparisons between more than two groups were performed using the Kruskal-Wallis test followed by a Dunn's post-hoc test or Friedman test in case of paired values. Comparisons were considered as significant if p-value was under 0.05.

### *3.15 Ethics statement*

All patients received information and signed a written consent to the study protocol, which was approved by our local clinical ethical committee (Ref. protocol "CLARA" 2005/22SEP/149 – update 29/11/2016, S51577 S52174 S55877) and CPP Ile de France II #1072 for lung tissue samples, Ref. 2015/09Jan/014 for sputum samples). Murine experiments were approved by our local Faculty Ethical committee (Ref. 2018/UCL/MD/04).

# 4 Results

## *4.1 Upregulation of epithelial pIgR and IgA expression in the human CF lung*

pIgR expression was increased in the bronchial epithelium from patients with CF, compared with controls, in both large and small airways (Figure 13a-c) while no significant difference was observed at the mRNA level in lung homogenates (Figure 13d).

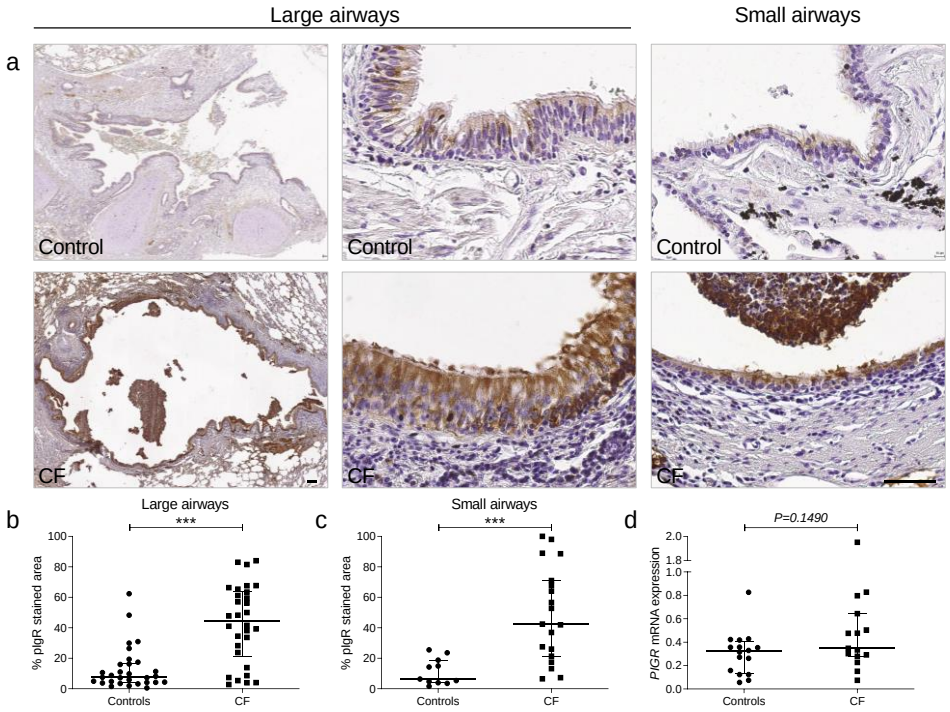


**Figure 13. pIgR expression is increased in airway epithelium from control and patients with CF at the protein level.**
(a) pIgR immunostaining in a large airway and in a small airway from one representative control and one representative patient with CF. (b) pIgR expression in large airways from 30 patients with CF, as compared with 30 controls (****p<0.0001*, Mann-Whitney test). (c) pIgR expression in small airways from 19 patients with CF, as compared with 11 controls (****p=0.0003*, Mann-Whitney test). (d) *PIGR* mRNA expression (corrected for housekeeping genes) in lung homogenates from 15 patients with CF, compared with 16 controls (*p=0.1490*, Mann-Whitney test). Bars indicate median and interquartile ranges. pIgR, polymeric immunoglobulin receptor; CF, cystic fibrosis. Scale bar, 100 µm.

As an increased number of lymphoid aggregates was confirmed in our CF lungs, compared with controls (Figure 14), IgA immunostaining was carried out on lung tissue, and confirmed a large increase in the number of IgA+ cells in CF lungs in subepithelial areas (Figure 15a) and in the surrounding submucosal glands (Figure 15b). A dual immunostaining for IgA and CD138 indicated that >70% of IgA+ cells consisted of IgA+ plasma cells (Figure 15c).

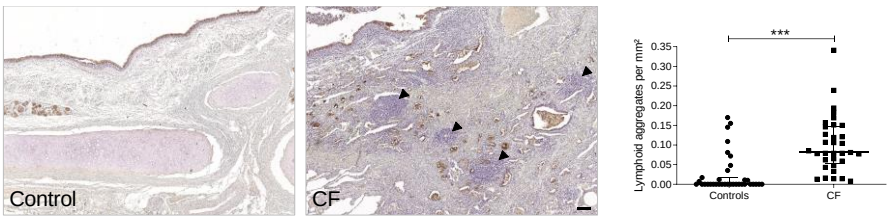


**Figure 14. CF patients display peribronchial lymphoid follicles.**
Subepithelial area from one representative control and from one representative CF patient (lymphoid aggregates showed by arrows) and number of lymphoid aggregates per mm² of lung tissue from 33 CF patients, compared with 35 controls (****p<0.0001*, Mann-Whitney test). Bars indicate median and interquartile ranges. CF, cystic fibrosis. Scale bar, 100 µm.

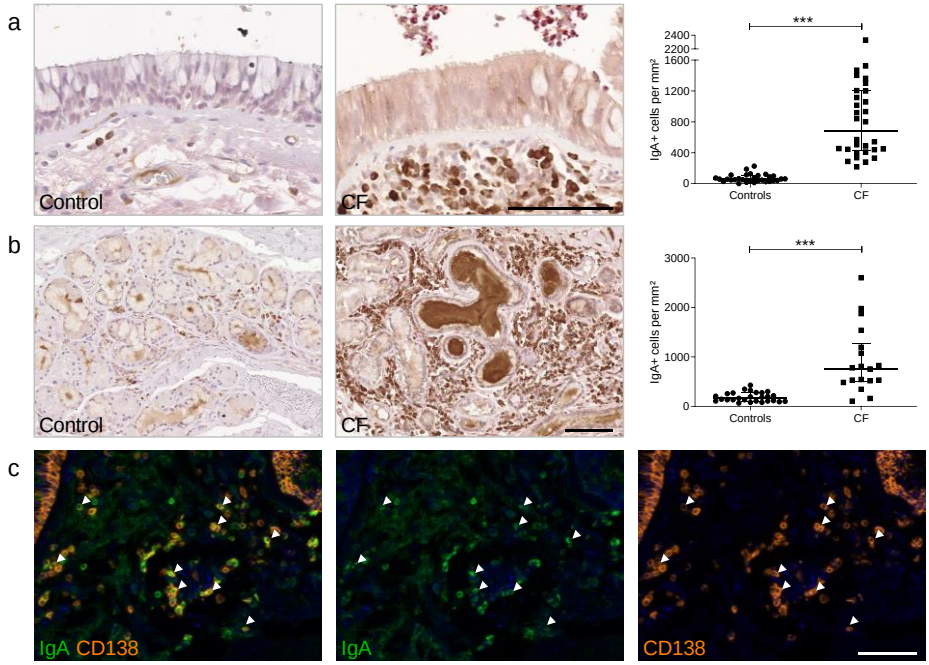


**Figure 15. Patients with CF display increased IgA+ cells number.**
(a) IgA staining in the subepithelial area from one representative control and one representative patient with CF and number of IgA+ cells in the subepithelial area from 30 patients with CF, as compared with 30 controls *(***p<0.0001*, Mann-Whitney test). (b) IgA staining in the glandular area from one representative control and from one representative patient with CF and number of IgA+ cells in the glandular area from 18 patients with CF, as compared with 27 controls *(***p<0.0001*, Mann-Whitney test). (c) Identification of plasma cells (CD138+ cells (orange)) producing IgA (green; arrows) in the subepithelial area from one patient with CF. Bars indicate median and interquartile ranges. IgA, immunoglobulin A. Scale bar, 100 µm.

### *4.2 Upregulation of total and* Pa*-specific IgA in human CF sputum*

Despite raised NE activity in sputum from patients with CF (Figure 16), which is able to cleave IgA and SC (28), the concentration of IgA was increased in sputum and serum from patients with CF, compared with controls, especially in chronically *Pa* infected patients (Figure 17a, d). Never *Pa* infected patients were nevertheless infected with other bacteria (such as *Staphylococcus aureus*, *Achromobacter*, or *Burkholderia* species) or *Aspergillus fumigatus* (in eight and two out of ten patients, respectively).

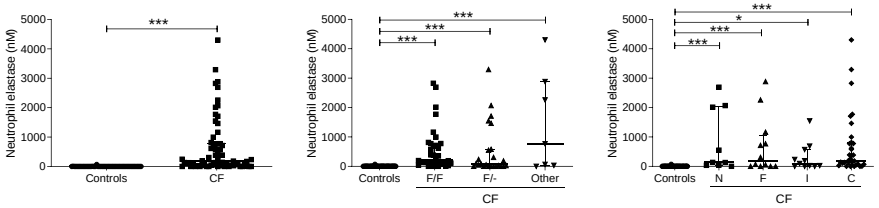


**Figure 16. NE is increased in airways from CF patients.**
NE concentration in sputum from 51 controls and 65 CF patients (****p<0.0001*, unpaired Student's t-test), distributed following their genotype (****p<0.001*, Kruskal-Wallis test followed by Dunn's test) or distributed following Leeds criteria (****p<0.001*, **p<0.05*, Kruskal-Wallis test followed by Dunn's test). NE, neutrophil elastase; *Pa*, *Pseudomonas aeruginosa*; CF, cystic fibrosis; ΔF, F508del mutation; C, chronically infected by *Pa*; I, intermittently infected by *Pa*; F, free of *Pa* infection; N, never infected with *Pa*.

A similar increase was observed for IgM (Figure 18a), also secreted following pIgR-mediated transport. In addition, a trend for increased S-IgA and no change for SC (Figure 17b-c) were noticed. Of note, higher concentrations of IgA and SC were measured in sputum from F508del homozygous patients when compared with patients without this mutation (Figure 18b-e).

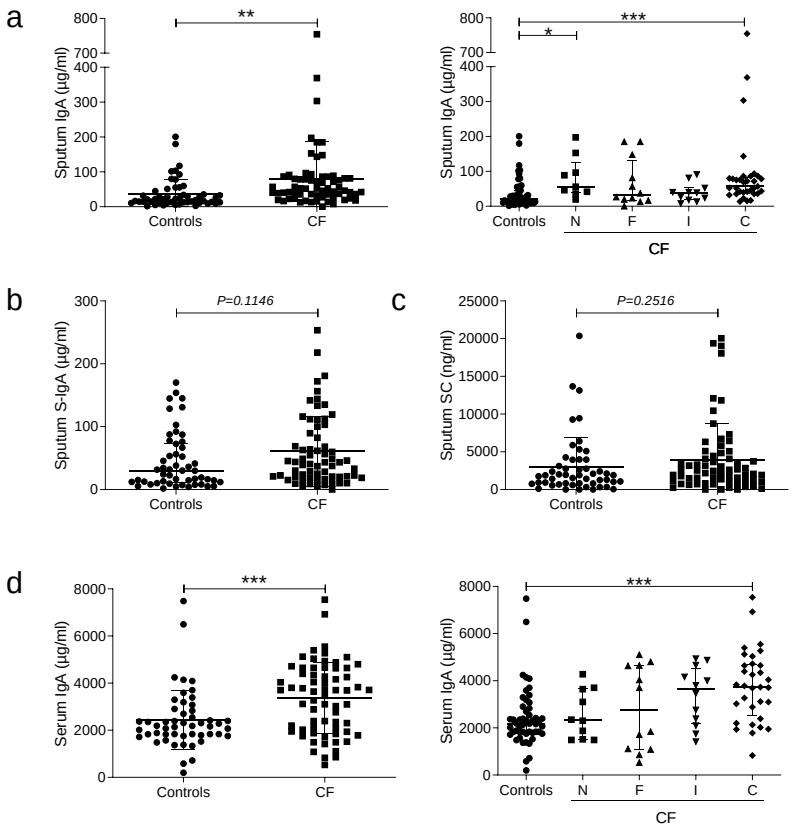


**Figure 17. IgA concentration is increased in sputum and serum from patients with CF.**

(a) IgA concentration in sputum from 51 controls and 65 patients with CF *(**p=0.0092*, unpaired Student's t-test), distributed following Leeds criteria (****p< 0.001*, ***p< 0.01*, Kruskal-Wallis test followed by Dunn's test). (b) S-IgA concentration in sputum from 51 controls and 65 patients with CF (*p=0.1146*, unpaired Student's t-test). (c) SC concentration in sputum from 51 controls and 65 patients with CF (*p=0.2516*, unpaired Student's t-test). (d) IgA concentration in serum from 51 controls and 66 patients with CF (****p=0.0006*, unpaired Student's t-test), distributed following Leeds criteria (****p<0.001*, Kruskal-Wallis test followed by Dunn's test). Bars indicate mean and standard deviation or median and interquartile ranges (Leeds distribution). IgA, immunoglobulin A; *Pa*, *Pseudomonas aeruginosa*; CF, cystic fibrosis; S-IgA, secretory immunoglobulin A; SC, secretory component; C, chronically infected by *Pa*; I, intermittently infected by *Pa*; F, free of *Pa* infection; N, never infected with *Pa*.

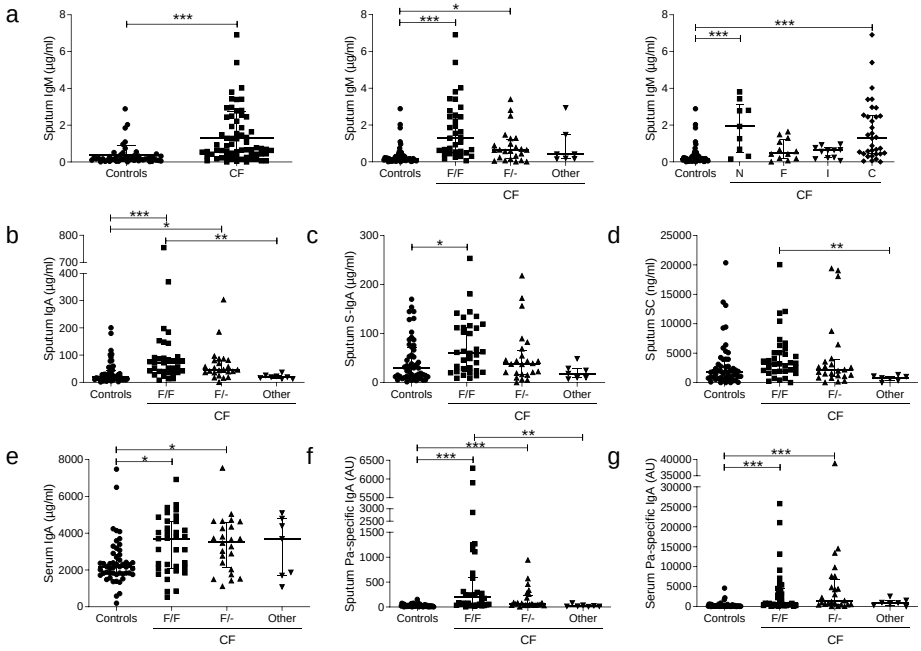


**Figure 18. Ig and *Pa*-specific Ig concentrations are increased in airways and serum from CF patients.**

(a) IgM concentration in sputum from 51 controls and 65 CF patients (****p<0.0001*, unpaired Student's t-test), distributed following their genotype (*****p<0.001, *p<0.05*, Kruskal-Wallis test followed by Dunn's test) or distributed following Leeds criteria (*****p<0.001*, Kruskal-Wallis test followed by Dunn's test). (b) IgA, (c) S-IgA and (d) SC concentration in sputum from 51 controls and 65 CF patients, distributed following their genotype (*****p<0.001, **p<0.01, *p<0.05*, Kruskal-Wallis test followed by Dunn's test). (e) IgA concentration in serum from 51 controls and 66 CF patients, distributed following their genotype (**p<0.05*, Kruskal-Wallis test followed by Dunn's test). (f) *Pa*-specific IgA concentration in sputum from 51 controls and 65 CF patients, distributed following their genotype (*****p<0.001, **p<0.01*, Kruskal-Wallis test followed by Dunn's test). (g) *Pa*-specific IgA concentration in serum from 51 controls and 66 CF patients, distributed following their genotype (*****p<0.001, **p<0.01*, Kruskal-Wallis test followed by Dunn's test). Bars indicate mean and standard deviation or median and interquartile ranges (genotype and Leeds distribution). Ig, immunoglobulin; *Pa*, *Pseudomonas aeruginosa*; CF, cystic fibrosis; S-IgA, secretory immunoglobulin A; SC, secretory component; ΔF, F508del mutation; C, chronically infected by *Pa*; I, intermittently infected by *Pa*; F, free of *Pa* infection; N, never infected with *Pa*.

In addition, a *Pa*-specific IgA response was observed in sputum and serum from patients with CF, chronically or intermittently infected with *Pa* (Figure 19), and in patients carrying at least one F508del mutation (Figure 18f-g).

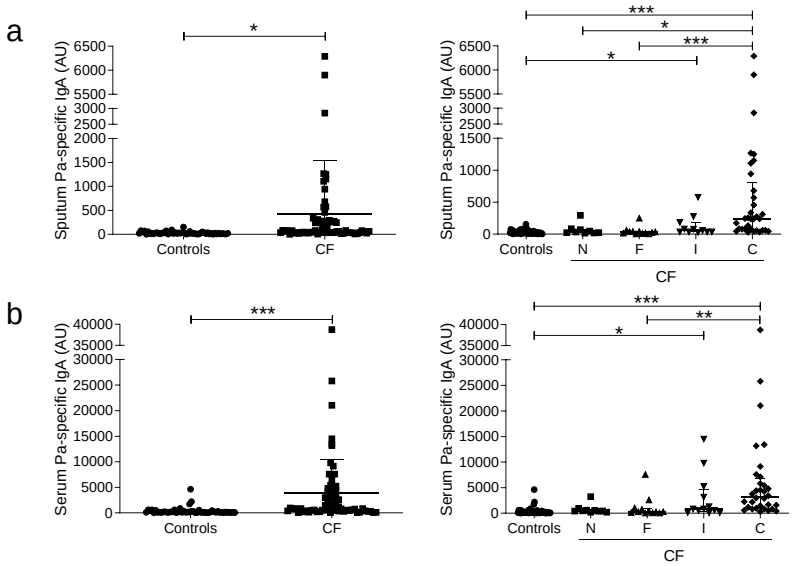


**Figure 19. *Pa*-specific Ig concentrations are increased in airways and serum from CF patients.**

(a) *Pa*-specific IgA concentration in sputum from 51 controls and 65 patients with CF (**p=0.0107*, unpaired Student's t-test), distributed following Leeds criteria (****p<0.001*, **p<0.05*, Kruskal-Wallis test followed by Dunn's test). (b) *Pa*-specific IgA concentration in serum from 51 controls and 66 patients with CF (****p=0.0003*, unpaired Student's t-test), distributed following Leeds criteria *(***p<0.001, **p<0.01, *p<0.05*, Kruskal-Wallis test followed by Dunn's test). Bars indicate mean and standard deviation or median and interquartile ranges (Leeds distribution). Ig, immunoglobulin; *Pa*, *Pseudomonas aeruginosa*; CF, cystic fibrosis; ΔF, F508del mutation; C, chronically infected by *Pa*; I, intermittently infected by *Pa*; F, free of *Pa* infection; N, never infected with *Pa*.

### *4.3 Downregulation of pIgR expression following CFTR dysfunction in the human bronchial epithelium*

In order to avoid the major risk of infection of primary CF HBEC, airway basal cells were sorted (by using CD142 and CD151 staining), and cultured in ALI

for two weeks to reconstitute *in vitro* a differentiated CF bronchial epithelium (29). In contrast to tissue data, a reduced pIgR expression was observed in CF HBEC, compared with the epithelium derived from control lungs both at protein (Figure 20a) and mRNA level (Figure 20b). In addition, the CF-derived epithelium displayed strong decreases in SC and S-IgA release in the apical washes (Figure 20c-d). To confirm this negative link between CFTR dysfunction and pIgR expression, control HBEC were treated at 2 weeks in ALI culture with increasing concentrations of two CFTR inhibitors (GlyH-101 and PPQ-102). After 48 h of treatment, a dose-dependent decrease in pIgR/SC production and SC release in apical washes was observed (Figure 20e-f).

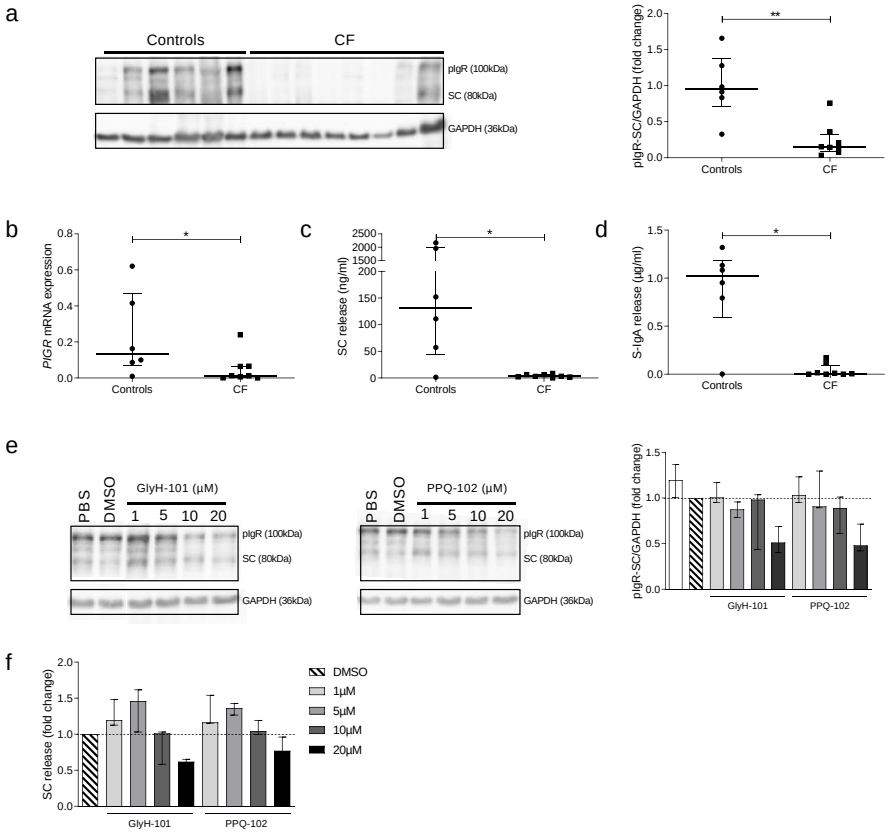


**Figure 20. pIgR expression and SC/S-IgA release are downregulated in ALI-HBEC derived from patients with CF and in control ALI-HBEC upon CFTR inhibition.**

(a) Representative western blot for pIgR in CF ALI-HBEC, compared with control ALI-HBEC and its pIgR-SC/GAPDH expression quantification in eight CF ALI-HBEC, compared with six control ALI-HBEC (***p=0.0027*, Mann-Whitney test). (b) *PIGR* mRNA expression (corrected for housekeeping genes) in eight CF ALI-HBEC, compared with six control ALI-HBEC (**p=0.0426*, Mann-Whitney test). (c) SC release in apical washes of eight CF ALI-HBEC, compared with six control ALI-HBEC (**p=0.0293*, Mann-Whitney test). (d) After transcytosis assay, S-IgA release in apical washes of eight CF ALI-HBEC, compared with six control ALI-HBEC (**p=0.0127*, Mann-Whitney test). (e) Representative western blot for pIgR in control ALI-HBEC, treated 48 h with increasing concentrations of GlyH-101 and PPQ-102 or DMSO as control and its pIgR-SC/GAPDH expression quantification in three control ALI-HBEC. (f) Concentration of SC released in apical washes of three control ALI-HBEC, treated 48 h with increasing concentrations of GlyH-101 and PPQ-102 or DMSO as control. Bars indicate median and interquartile ranges. pIgR, polymeric immunoglobulin receptor; SC, secretory component; S-IgA, secretory immunoglobulin A; ALI-HBEC, human bronchial epithelial cells in air-liquid interface; CF, cystic fibrosis.

### *4.4 Downregulation of pIgR expression and IgA secretion following CFTR dysfunction in F508del mice*

In order to confirm pIgR downregulation in "sterile" environment upon CFTR dysfunction *in vivo*, we studied pIgR expression in F508del mice. A decreased pIgR expression was observed, as compared with WT mice (Figure 21a), as well as a lower concentration of total IgA in BAL (Figure 21b). As in human lung, S-IgA in BAL (Figure 21c) and *Pigr* mRNA expression in lung homogenates were not significantly changed (Figure 21d).

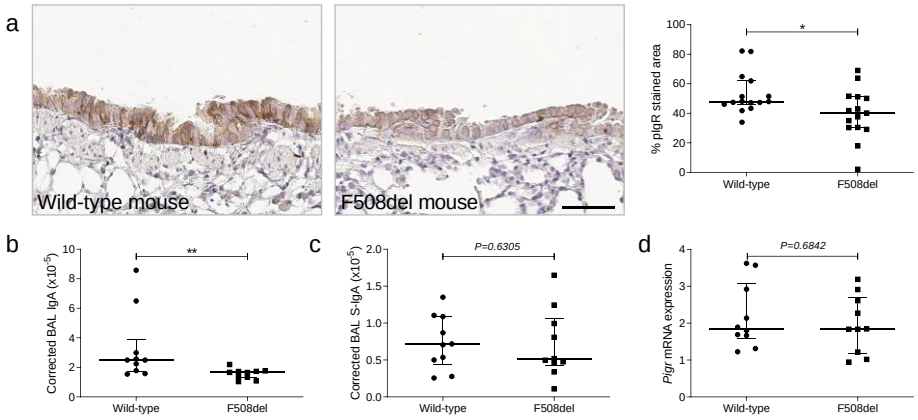


**Figure 21. pIgR expression and IgA secretion are decreased in airways from F508del mice.**

(a) pIgR immunostaining in an airway from one representative WT mouse and from one representative F508del mouse and quantification in airways from 15 F508del mice, as compared with 15 WT mice (**p=0.0344*, Mann-Whitney test). (b) IgA concentration, normalised for total protein content, in BAL from ten F508del mice, compared with ten WT mice (***p=0.0058*, Mann-Whitney test). (c) S-IgA concentration, normalised for total protein content in BAL from ten F508del mice, compared with ten WT mice (*p=0.6305*, Mann-Whitney test). (d) *Pigr* mRNA expression (corrected for housekeeping genes) in lung homogenates from ten F508del mice, compared with ten WT mice (*p=0.6842*, Mann-Whitney test). Bars indicate median and interquartile ranges. pIgR, polymeric immunoglobulin receptor; IgA, immunoglobulin A; WT, wild-type; S-IgA, secretory immunoglobulin A; BAL, bronchoalveolar lavage. Scale bar, 50 µm.

### *4.5 UPR is activated in CF-derived HBEC and F508del mice, and affects SC secretion*

To explore the mechanism of pIgR downregulation observed upon CFTR dysfunction, in both human CF HBEC and F508del mice, we hypothesised that the ER stress and UPR could be involved. Indeed, F508del mutation has been associated with an accumulation of misfolded proteins in the ER, resulting in ER stress and UPR activation (30). UPR activation assessed by the mRNA ratio of active, spliced/unspliced, XBP-1 was confirmed in F508del mouse lungs (Figure 22a), which received no treatment. A trend was also observed in the reconstituted epithelium from patients with CF (most of them harbouring at least one copy of the F508del mutation) as compared with controls (Figure 22b). In addition, ER stress was also evidenced by transmission electron microscopy showing dilated ER cavities in CF, while this feature was absent in control HBEC (Figure 22c). Therefore, we stimulated UPR in Calu-3 cells (by thapsigargin or tunicamycin), an epithelial cell line which stably expresses pIgR at high level, to evaluate whether this regulates pIgR expression. UPR activation leading to phosphorylation of eIF2α (Figure 22d-e), increased spliced/unspliced *XBP1* mRNA ratio (Figure 22f) and dose-dependently decreased pIgR protein expression (Figure 22g) and SC release (Figure 22h). In addition, d-IgA transcytosis was impaired in Calu-3 cells treated with tunicamycin (Figure 22i). These data indicate that UPR activation *per se* (or that follows ER stress) could recapitulate the downregulation of pIgR expression and IgA transport observed in CF cell cultures and in CF mouse lungs.

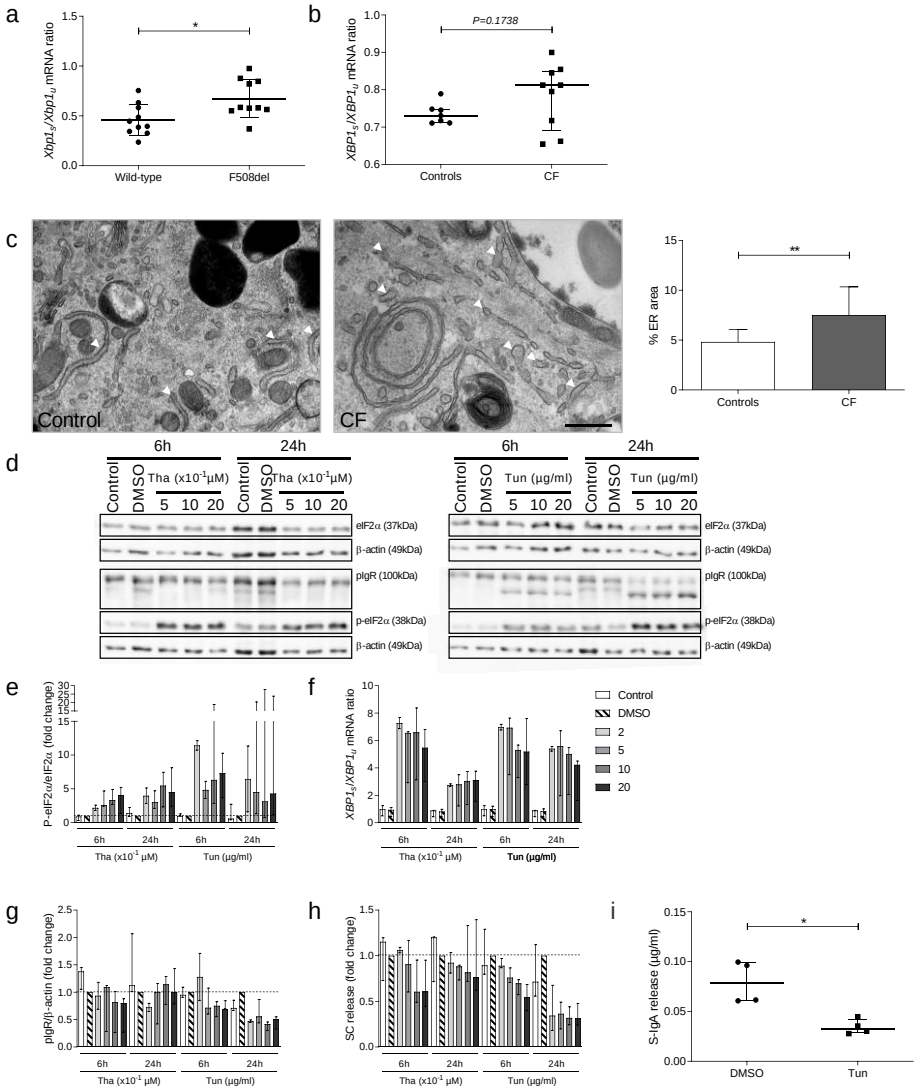


**Figure 22. UPR is activated in CF-derived human HBEC and in lungs from F508del mice at baseline and it downregulates SC secretion in Calu-3 cells.**
(a) *Xbp1s/Xbp1u* mRNA ratio (corrected for housekeeping genes) in lung from ten F508del mice receiving no treatment, compared with ten WT mice (*$p=0.0133$, Mann-Whitney test). (b) *XBP1s/XBP1u* mRNA ratio (corrected for housekeeping genes) in HBEC (cultured in sterile condition) from nine patients with CF, compared with seven controls ($p=0.1738$, Mann-Whitney test). (c) Transmission electron microscopy in HBEC (cultured in sterile condition) from one control and from one patient with CF and quantification of ER (arrows) surface in one control and from one patient with CF (**$p=0.0094$, unpaired Student's t-test). (d) Representative western blot for eIF2α, P-eIF2α and pIgR in Calu-3 cells treated with

increasing concentrations of thapsigargin, tunicamycin or DMSO as control condition. (e) Quantification of P-eIF2α/eIF2α protein expression in Calu-3 cells after treatment with increasing concentrations of thapsigargin and tunicamycin, compared with DMSO as control condition (n = 3). (f) *XBP1s/XBP1u* mRNA ratio (corrected for housekeeping genes) in Calu-3 cells treated increasing concentrations of thapsigargin and tunicamycin or DMSO as control condition (n = 3). (g) Quantification of pIgR/β-actin expression in Calu-3 cells after treatment with increasing concentrations of thapsigargin and tunicamycin, compared with DMSO as control condition (n = 3). (h) SC secretion upon treatment with increasing concentrations of thapsigargin and tunicamycin, compared with DMSO as control condition (n = 3). (i) After transcytosis assay, S-IgA release upon thapsigargin treatment (**p=0.0286*, Mann-Whitney test) (n = 4). Bars indicate median and interquartile ranges, except for (c) (mean and standard deviation). UPR, unfolded protein response; XBP1s, spliced X-box binding protein 1; XBP1u, unspliced X-box binding protein 1; eIF2α, eukaryotic initiation factor 2 α; P-eIF2α, phospho-eukaryotic initiation factor 2 α; pIgR, polymeric immunoglobulin receptor; SC, secretory component; S-IgA, secretory immunoglobulin A; Tha, thapsigargin; Tun, tunicamycin. Scale bar, 500 nm.

### *4.6 Upregulation of pIgR expression and (S-)IgA production upon Pa infection*

In order to reconcile the apparent discrepancy between upregulated pIgR expression in human CF lungs and its downregulation upon CFTR dysfunction in HBEC and in F508del mice, we reasoned that the negative CFTR-pIgR link could be overcome during lung infection with *Pa*. We observed that WT mice instilled with *Pa*-coated beads displayed increased pIgR expression in the airway epithelium compared with sterile beads (Figure 23a). In addition, a strong upregulation of IgA and S-IgA concentrations was observed in BAL upon *Pa* infection both in WT and F508del mice (Figure 23b-c), associated with an increase in *PIGR* mRNA expression (Figure 23d).

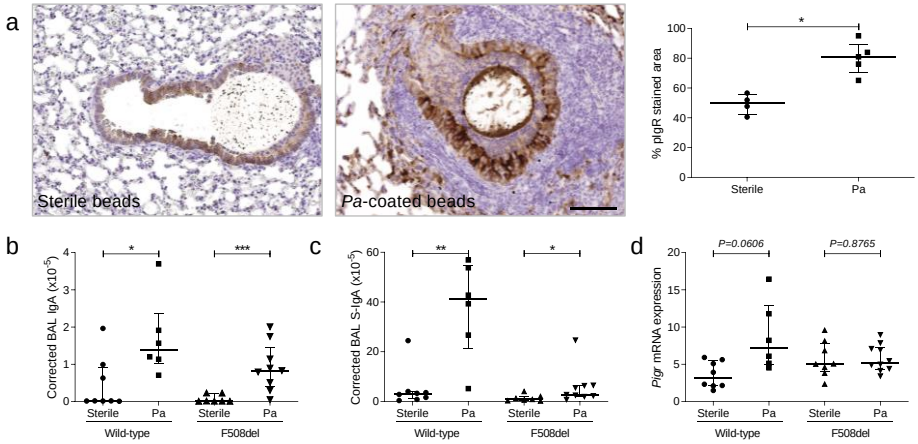


**Figure 23. pIgR expression and (S-)IgA secretion are upregulated in lungs from mice infected with *Pa*.**

(a) pIgR immunostaining in an airway from one representative WT mouse instilled with sterile beads or *Pa*-coated beads and quantification (in the airway epithelium surrounding the beads) in five WT mice instilled with *Pa*-coated beads, compared with four WT mice instilled with sterile beads (*$p=0.0159$, Mann-Whitney test). (b) IgA concentration, normalised for the total protein content, in BAL from six WT mice instilled with *Pa*-coated beads, compared with eight WT mice instilled with sterile beads (*$p=0.0200$, Mann-Whitney test) and from nine F508del mice instilled with *Pa*-coated beads, compared with seven F508del mice instilled with sterile beads (***$p=0.0007$, Mann-Whitney test). (c) S-IgA concentration, normalised for the total protein content, in BAL from six WT mice instilled with *Pa*-coated beads, compared with eight WT mice instilled with sterile beads (**$p=0.0013$, Mann-Whitney test) and from nine F508del mice instilled with *Pa*-coated beads, compared with seven F508del mice instilled with sterile beads (*$p=0.0164$, Mann-Whitney test). (d) *Pigr* mRNA expression (corrected for housekeeping genes) in lungs from six WT mice instilled with *Pa*-coated beads, compared with eight WT mice instilled with sterile beads (*$p=0.0134$, Mann-Whitney test). Bars indicate median and interquartile ranges. pIgR, polymeric immunoglobulin receptor; *Pa*, *Pseudomonas aeruginosa*; S-IgA, secretory immunoglobulin A; SC, secretory component; BAL, bronchoalveolar lavage. Scale bar, 100 µm.

### *4.7* Pa *infection upregulates pIgR expression through an indirect mechanism involving IL-17*

To address the mechanisms by which *Pa* could upregulate pIgR expression and IgA secretion, CF HBEC were incubated with the supernatant of a *Pa* clinical strain culture. Only marginal and not significant changes in *PIGR* mRNA expression,

pIgR protein, SC release and d-IgA transcytosis were observed in CF-derived cells treated for 48h with *Pa* supernatant (Figure 24).

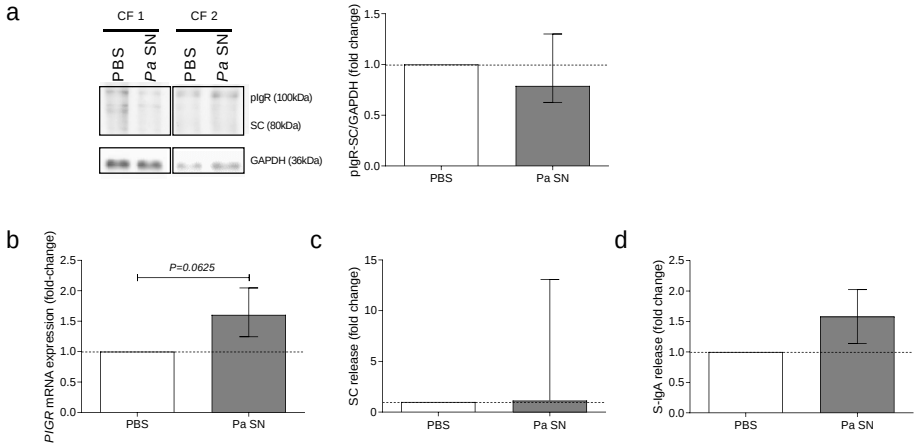


**Figure 24. Regulation of pIgR expression and SC/S-IgA release in CF-derived HBEC following stimulation with *Pa* supernatant.**
(a) Representative western blot for pIgR in ALI-HBEC from CF patient treated with *Pa* SN or PBS as control condition and its pIgR-SC/GAPDH expression quantification in ALI-HBEC from five CF patients treated with *Pa* SN or PBS (*p=0.8125*, Wilcoxon test). (b) *PIGR* mRNA expression (corrected for housekeeping genes) in ALI-HBEC from five CF patients treated with *Pa* SN or PBS as control condition (*p=0.0625*, Wilcoxon test). (c) SC release in apical washes of ALI-HBEC from five CF patients treated with *Pa* SN or PBS (*p=0.6250*, Wilcoxon test). (d) After transcytosis of d-IgA, S-IgA concentration in apical washes from two CF ALI-HBEC treated with *Pa* SN or PBS (*p=0.5000*, Wilcoxon test). Bars indicate median and interquartile ranges. pIgR, polymeric immunoglobulin receptor; SC, secretory component; S-IgA, secretory immunoglobulin A; CF, cystic fibrosis; HBEC, human bronchial epithelial cells; *Pa*, *Pseudomonas aeruginosa*; ALI, air-liquid interface; SN, supernatant; PBS, phosphate-buffered saline.

An indirect mechanism was then explored by looking at the inflammatory response induced by *Pa* infection. In particular, we evaluated IL-17 as it is reportedly increased upon *Pa* infection, and it is able to upregulate *PIGR* mRNA expression in intestinal cells (31, 32). An induction of *Il17* mRNA expression was noticed in the lungs from mice instilled with *Pa*-coated beads, compared with mice instilled with sterile beads (Figure 25a).

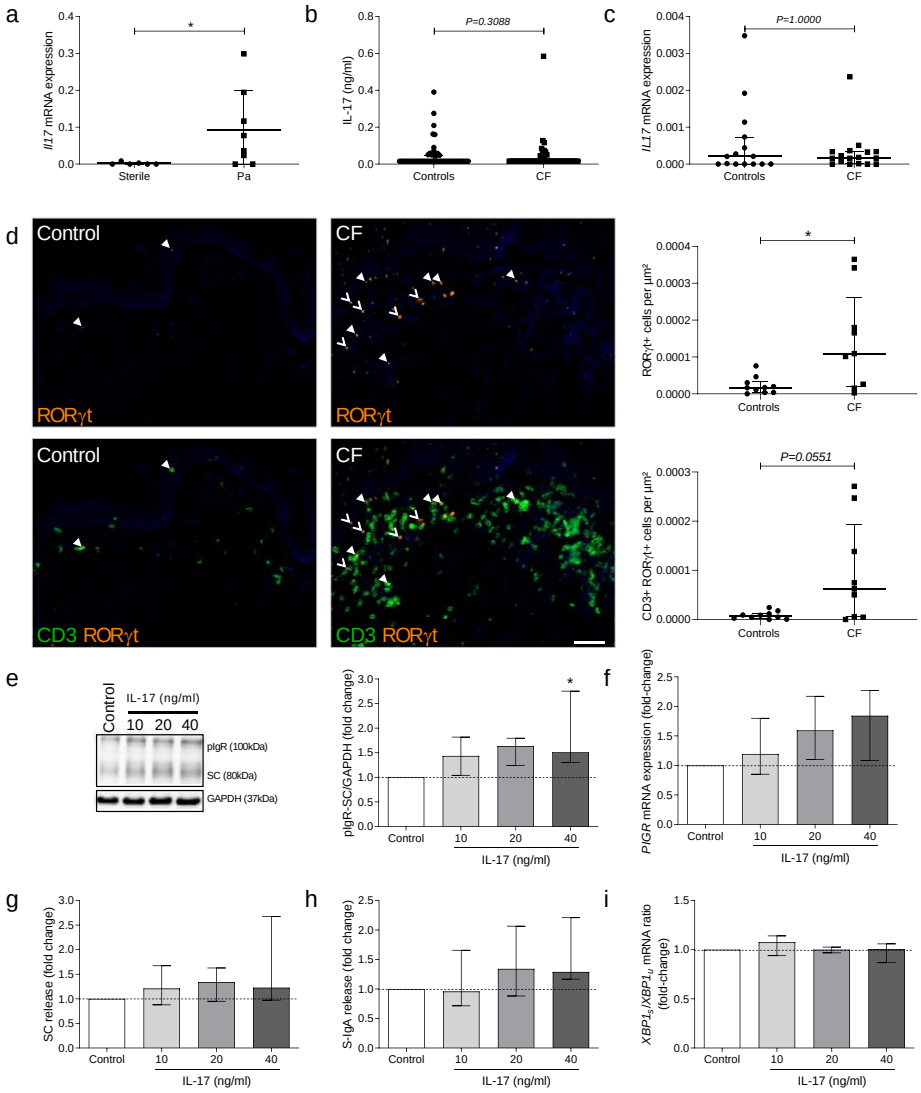


**Figure 25. IL-17 is increased in lungs from mice infected with *Pa* and upregulates pIgR expression in ALI-HBEC derived from patients with CF.**
(a) *Il17* mRNA expression (corrected for housekeeping genes) in lungs from six WT mice instilled with *Pa*-coated beads, compared with eight WT mice instilled with sterile beads (*$p=0.0373$, Mann-Whitney test). (b) IL-17 concentration in sputum from 65 patients with CF, compared with 51 controls ($p=0.3088$, Mann-Whitney test). (c) *IL17* mRNA expression (corrected for housekeeping genes) in lung homogenates from 15 patients with CF, compared with 16 controls ($p=1.0000$, Mann-Whitney test). (d) RORγt staining (and CD3) in the subepithelial area from one representative control and one representative patient with CF, and number of RORγt+ cells (filled and no-filled arrows), including CD3+ RORγt+ cells

(filled arrows), in the subepithelial area from 9 patients with CF, as compared with 10 controls *(*p=0.0279, p=0.0551,* Mann-Whitney test). (e) Representative western blot for pIgR in CF ALI-HBEC, treated with increasing concentrations of IL-17 and its pIgR-SC/GAPDH expression quantification in six CF ALI-HBEC, treated with increasing concentrations of IL-17 (**p=0.0437*, Friedman test). (f) *PIGR* mRNA expression (corrected for housekeeping genes) in five CF ALI-HBEC, treated with increasing concentrations of IL-17. (g) SC release by six CF ALI-HBEC, treated with increasing concentrations of IL-17. (h) SC release by six CF ALI-HBEC, treated with increasing concentrations of IL-17. (i) *XBP1s/XBP1u* mRNA ratio (corrected for housekeeping genes) in five CF ALI-HBEC, treated with increasing concentrations of IL-17. Bars indicate median and interquartile ranges. IL-17, interleukin 17; *Pa*, *Pseudomonas aeruginosa*; pIgR, polymeric immunoglobulin receptor; CF, cystic fibrosis; ROR, RAR-related orphan receptor; CD, cluster of differentiation; SC, secretory component; S-IgA, secretory immunoglobulin A; XBP1s/u, spliced/unspliced X-box binding protein 1. Scale bar, 50 µm.

While IL-17A upregulation was not recapitulated in sputum or lung tissue from patients with CF (Figure 25b-c), immune cells producing IL-17 (RORγt+ cells, including CD3+ RORγt+ Th17 cells (33, 34)) were increased in subepithelial areas of patients with CF, compared with controls (Figure 25d), as well as in total lung tissue (Figure 26). When CF-derived cells were stimulated by increasing concentrations of IL-17A, we observed a dose-dependent increase in pIgR expression (Figure 25e), associated with a trend to increased *PIGR* mRNA expression, SC release and d-IgA transcytosis (Figure 25f-h), which did not influence UPR activation through XBP-1 splicing (Figure 25i).

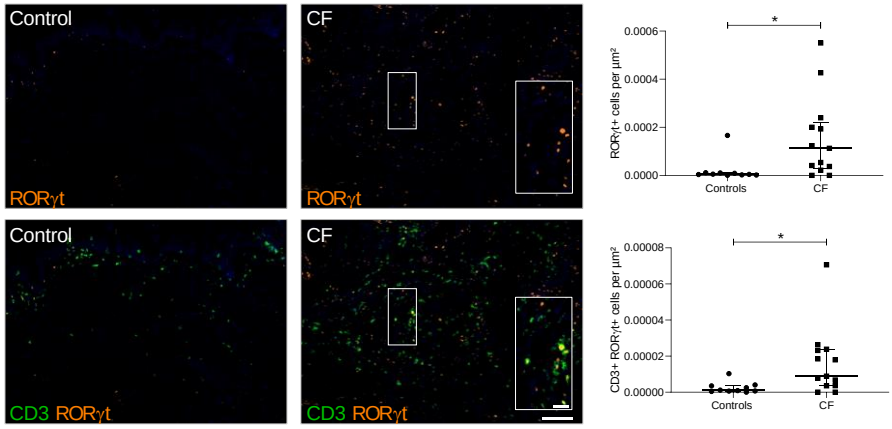


**Figure 26. IL-17-producing immune cells are increased in CF.**
RORγt staining (and CD3) in the total lung tissue from one representative control and one representative CF patient, and number of RORγt+ cells, including CD3+ RORγt+ cells, in the total lung tissue from 13 CF patients, as compared with 10 controls *(*p=0.0147, *p=0.0147,* Mann-Whitney test). Bars indicate median and interquartile ranges. CF, cystic fibrosis; ROR, RAR-related orphan receptor; CD, cluster of differentiation. Scale bar, 100 µm.

Upregulated *PIGR* mRNA was also measured in Calu-3 cells stimulated by IL-17A (Figure 27a-c). As shown in CF-derived HBEC, no influence on UPR activation through XBP-1 splicing was observed upon IL-17A treatment (Figure 27d). These data suggest that *Pa* infection could stimulate pIgR expression and S-IgA production in the CF lung by activating an inflammatory host response that involves IL-17.

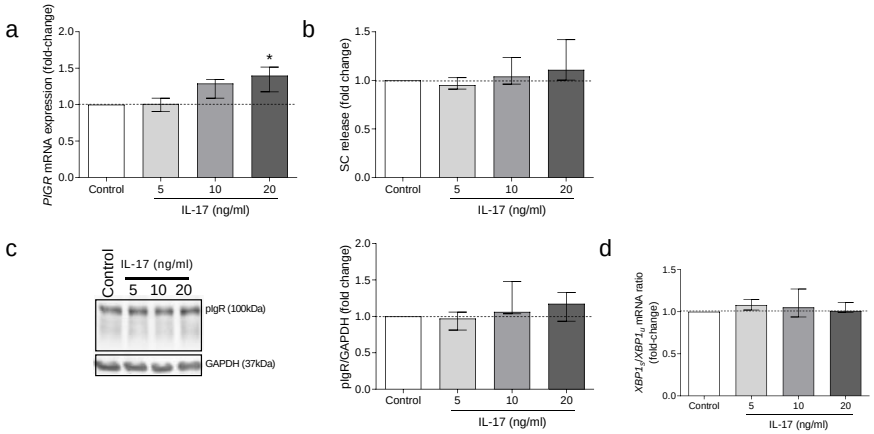


**Figure 27. IL-17 upregulates pIgR mRNA expression in Calu-3 cells.**
(a) *PIGR* mRNA expression (corrected for housekeeping genes) in Calu-3 cells treated with increasing concentrations of IL-17 (n = 5). (b) SC release by Calu-3 cells treated with increasing concentrations of IL-17 (n = 5). (c) Representative western blot for pIgR in Calu-3 cells treated with increasing concentrations of IL-17 and pIgR/GAPDH expression in Calu-3 cells treated with increasing concentrations of IL-17 (n = 5). (d) *XBP1s/XBP1u* mRNA ratio (corrected for housekeeping genes) in Calu-3 cells treated with increasing concentrations of IL-17 (n = 5). Bars indicate median and interquartile ranges. IL-17, interleukin 17; pIgR, polymeric immunoglobulin receptor; SC, secretory component; XBP1s/u, spliced/unspliced X-box binding protein 1.

# 5 Discussion

This study shows that epithelial pIgR expression, IgA production, and IgA+ B cells are all upregulated in the CF lung, sputum and serum. This IgA response includes specific antibodies to *Pa* which represents a major opportunistic bacterium in this disease. Secondly, experiments in airway epithelial basal/stem cell-derived cultures from CF lungs and in F508del mice revealed a constitutive downregulation of pIgR/SC production and d-IgA transcytosis, as recapitulated by adding CFTR inhibitors in control cells. This negative CFTR-pIgR pathway involved UPR activation following the ER stress observed in CF. Finally, an *in vivo* model of chronic lung infection by *Pa*-coated microbeads enabled to identify that infection

could restore pIgR expression and IgA production in the lungs from F508del mice, through an IL-17 inflammatory host response (Figure 28).

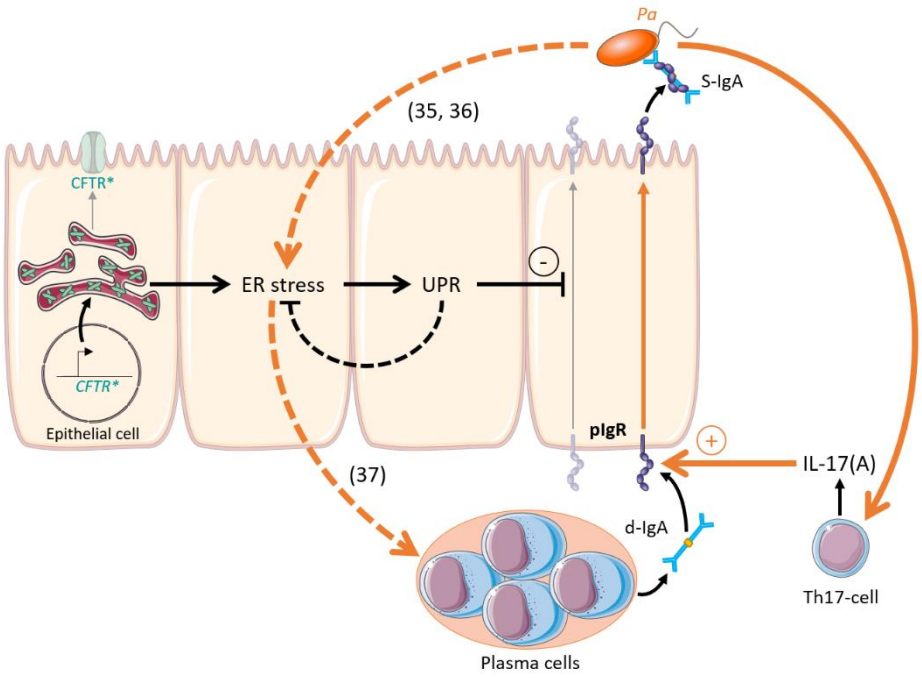


**Figure 28. Model of pIgR regulation in CF lung.**
First, accumulation of misfolded F508del-CFTR (CFTR*) is responsible for ER stress. Consequently, activation of UPR is able to downregulate SC and S-IgA secretion. Second, *Pa* infection modulates this pathway (orange arrows). *Pa* drives host IL-17 response which stimulates *PIGR* expression and further increases ER stress and UPR activation (35, 36). In addition, if ER stress caused by *Pa* infection becomes insufficiently counterbalanced by UPR activation, it may contribute to IgA upregulation, as previously shown (37). CFTR, cystic fibrosis transmembrane regulator; UPR, unfolded protein response; SC, secretory component; S-IgA, secretory immunoglobulin A; d-IgA, dimeric immunoglobulin A; *Pa*, *Pseudomonas aeruginosa*; IL-17, interleukin 17; pIgR, polymeric immunoglobulin receptor.

In contrast to previous data in COPD or chronic rhinosinusitis, this study shows that bronchial pIgR expression is upregulated in CF (16-18, 38). Numbers of IgA+ B cells are also increased in this disease, as previously observed in COPD (39). Whereas decreased SC concentration in saliva from patients with CF was previously

reported (40), Marshall *et al.* observed an increased concentration and altered glycosylation of SC in CF sputum (21). Our study could reconcile the apparent discrepancy between previous studies regarding IgA (20-23), by suggesting that IgA upregulation may selectively occur in the lungs (but not in the digestive tract) as those are the target of acquired bacterial infection (e.g. *Pa*) in CF. This increased IgA response is particularly noticed in patients with severe disease, and includes the production of serum/systemic and respiratory/secretory *Pa*-specific IgA (9, 41-45). Our study robustly confirms increased IgA levels in CF airways, as well as increased serum IgA, and that this response includes a specific reactivity to *Pa* at both levels.

A major finding of this study is that epithelial pIgR expression and IgA transport are disrupted in primary cultures of HBEC from patients with CF, as well as in the lung from F508del mice. In human lung homogenates, *Pigr* gene expression was not affected, maybe due to the large amount of non-pIgR expressing cell types into the lung. On the contrary, the use of CF-derived HBEC showed impaired pIgR expression when cultivating in the absence of inflammatory stimulus and allows to distinguish slight difference (notably for *PIGR* mRNA levels). *In vitro* experiments using selective CFTR inhibitors could confirm that CFTR dysfunction induces pIgR protein downregulation and defective pIgR protein expression is also demonstrated in non-infected F508del mice. F508del mutation, which was also carried by most of our patients with CF from whom cells were derived, is associated with improper folding and accumulation of the CFTR protein in the ER. Therefore, we hypothesised and confirmed that ER stress and subsequent UPR activation could represent a mechanism involved in pIgR downregulation, in line with previous reports in short-term cultures of CF-derived cells and in a CF cell line (30, 36). We could also demonstrate UPR activation through XBP-1 splicing in the lungs from F508del mice even in the absence of infection. Furthermore, our data show that UPR activation in epithelial cells induces a downregulation of pIgR/SC production as well as of d-IgA transcytosis. In addition, it has been shown that UPR activation in CF not only fails

to resolve the ER stress but also sensitises the inflammatory response to Toll-like receptor stimulation (30). Interestingly, pIgR downregulation (among several other proteins) was also linked to UPR activation in the context of ulcerative colitis (46), whereas another recent study showed that mice with a restricted deletion of XBP-1 in the intestinal epithelium (thus enable to develop UPR to counteract ER stress in these cells) display T cell-independent accumulation of IgA+ plasma cells and increased IgA secretion, which mediates protection against spontaneous enteritis (37). In line with the latter, our data further show that UPR activation itself disrupts epithelial pIgR expression and that lungs from patients with CF (and presumably increased ER stress) display increased numbers of mucosal IgA+ plasma cells. Taken together, our study and these data globally indicate that ER stress and UPR pathway represent key regulatory checkpoints of IgA production at mucosal surfaces.

Infection with *Pa*, the most common bacteria in adult patients with CF (3, 47), was studied as a plausible mechanism that could underlie the upregulation of IgA in the human (infected) CF lung, and which is absent in cell cultures and CFTR-mutated mice. The model of chronic *Pa* lung infection favours the retention of the bacteria in the airways and reproduces some features of the CF lung disease such as strong neutrophilic inflammation and peribronchial lymphoid aggregates (48, 49). Only a slight increased inflammatory score in FVB F508del mice was demonstrated, in contrast with C57BL/6J CFTR-KO mice showing some lymphoid aggregates (50, 51). When compared with the same genetic background, CFTR-KO and F508del mice display the same phenotype and, until now, no diseased lung phenotype related to CFTR misfolding has been observed in F508del mice (52). F508del mice (on FVB/129 background) classically display a reduction in body weight, CF nasal electrophysiological profile and tendency to intestinal mucus obstruction, associated with normal survival (51, 53). Variabilities between murine models are mostly attributed to the strain background (53). We show that *Pa* infection overcomes the (mutated) CFTR-driven inhibitory pathway on the pIgR, thereby unleashing IgA

production in the CF lung. This phenomenon occurs independently from UPR regulation, as *Pa* infection further increases ER stress and UPR activation (35, 36), and activates the host inflammatory response that includes the release of IL-17. This cytokine is able to stimulate pIgR expression in airway epithelial cells as previously shown in intestinal epithelial cells (31). Interestingly, Th17-mediated responses (through IL-17A production) were shown to upregulate pIgR expression in the airway epithelium and IgA/M concentrations in BAL from mice (54). Other inflammatory cytokines such as IL-1β and TNF-α, which are also upregulated upon *Pa* infection and able to stimulate pIgR transcription in airway cell lines (55, 56), could also be implicated. Similarly, the observation of increased levels of IgA in sputum from never infected patients (Figure 17a) suggests that other opportunistic bacteria (e.g., *Staphylococcus aureus*, *Burkholderia cepacia*) could drive similar mechanisms as those evidenced with *Pa*. However, increased concentration of IL-1β and TNF-α as well as IL-8, and neutrophilic inflammation has been demonstrated in BAL of young infants, without the presence of detectable pathogens, suggesting "sterile inflammation" (57, 58). But these statements remain controversial (59, 60). We could not assess this in our patients with CF cohort (for lung tissues and sputum) since these adult patients (although few were never infected by *Pa*) were carriers of other pathogens. Recent studies related to CF sterile inflammation (in CF animal models and CF samples) showed the potential responsibility of hypoxic injury, resulting in necrosis, IL-1α release and sterile neutrophilic inflammation (61). The possible induction of IL-1β and activation of NF-κB by IL-1α might be responsible of pIgR expression early in patients with CF, even before infection detection. As mentioned before, despite showing no CF-like lung phenotype, F508del mice develop a minor but spontaneous inflammation in the lungs. However, we could notice that pIgR expression was still affected in this model although the decrease was less marked than in cellular model. This could be due to isolation of cells from inflammatory microenvironment.

The preserved IgA response to pathogens was already suggested by increased *Pa*-specific IgA antibodies in saliva and nasal secretions from patients with CF (9), as well as by increased serum specific IgG correlated to the presence of *Pa* in the sinuses (62). Such *Pa* sinusitis may precede intermittent lung infection, suggesting that paranasal sinuses may represent a bacterial reservoir able to re-contaminate the patient after lung eradication (63). *Pa*-specific IgA in saliva was also assessed as a predictor of *Pa* chronic infection (64, 65). Similarly, we observed in our study that a local specific IgA response to *Pa* is consistently observed in chronically infected patients, both in serum and sputum secretions. Whether those IgA assays could provide the added value to IgG antibodies and bacteriology for the diagnosis of *Pa* (chronic) infection should be further studied in larger and multicentric clinical cohorts.

One limitation of our study first relates to the end-stage status of patients with CF recruited for tissue samples (lung explants). It is however remarkable that sputum data from a large spectrum of disease severity retrieved consistent findings, indicating that S-IgA upregulation encompasses the full spectrum of adult patients with CF. Second, we encountered difficulties to observe differences in IL-17 levels (and did not observe changes in *PIGR* expression despite SC alterations) in lung homogenates. While increased number of IL-17-producing cells were shown in CF, we could not confirm increased IL-17 expression or secretion possibly as a result of the dilution of the signal by the nature of the sample (tissue homogenate). As mentioned above, while pIgR mRNA changes were observed in isolated broncho-epithelial cells, no difference was measured in lung homogenates, possibly due to a similar dilution of the signal. In addition, this study did not directly assess whether IgA plays a protective role in the lung from patients. It was reported that overglycosylation of SC in CF reduces its capacity to bind and neutralise IL-8/CXCL8, hence favouring neutrophilic inflammation (21). It is further possible that accumulated S-IgA in the bronchial lumen (Figure 15b), following increased

production and impaired mucociliary clearance, could form immune complexes that – instead of being eliminated to achieve immune exclusion – activate FcαRI-bearing phagocytes (66). Thus, the exact role of upregulated lung IgA responses during CF should be further investigated. Finally, pIgR dysregulation in the absence of lung infection was assessed in F508del patients but the state of IgA secretion (through pIgR) remains unclear in non-F508del patients and could be further addressed in a dedicated study of such patients even though these mutations are rarer and most patients suffer from early infection. It would be interesting to also address this point in CFTR-KO mice, compared to F508del mice.

In conclusion, this study in CF reveals a complex interplay between epithelial cells, B cells, and bacteria (*Pa*), which connects CFTR dysfunction, UPR activation, and a (Th17) host innate immune response to IgA secretion in the lung.

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# CHAPTER 3: LOSS OF CILIATED CELLS AND ALTERED AIRWAY EPITHELIAL INTEGRITY IN CYSTIC FIBROSIS

Adapted from a manuscript submitted for publication in *Journal of Cystic Fibrosis*.

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## 1 Abstract

Background: In CF, the respiratory epithelium is the target tissue of both the genetic abnormality of the disease and of external aggressions, notably by pathogens (*Pa*). A detailed characterisation of the CF bronchial epithelium is however lacking, as most previous studies focused on the nasal epithelium or on cell lines. This study aimed to characterise the abnormal phenotype and EMT in CF bronchial epithelium and to evaluate in CF cell cultures whether abnormalities persist ex vivo.

Methods: Explant lung tissues (n = 44) were assessed for bronchial epithelial cell phenotyping by immunostaining. HBEC were derived from basal cells isolated from CF patients or control donors and cultured in ALI for 2, 4 or 6 weeks.

Results: Enhanced MUC5AC and decreased β-tubulin expression were observed in CF airways reflecting a decreased ciliated/goblet cell ratio, associated with increased number of vimentin-positive cells, indicating EMT process. These features were recapitulated *in vitro*, in CF-derived reconstituted epithelium. However, they were not induced by CFTR inhibition or *Pa* infection, and most

abnormalities tended to disappear in long-term culture (6 weeks) except for increased fibronectin release, an EMT marker.

Conclusions: This study provides new insights into airway epithelial changes in CF, which are imprinted through an acquired mechanism that we could not relate to CFTR function.

## 2 Introduction

The respiratory epithelium is the first barrier against inhaled pathogens and covers the airways from the nose to the terminal respiratory bronchioles (1). It is composed by four main cell types (ciliated cells, goblet cells, basal cells and club cells) and acts as a chemical, immunological as well as physical barrier. Physical properties are permitted by apical junction complexes that are composed by tight and adherens junctions. Tight junctions are composed by occludins (ZO-1 among other), and adherens junctions are formed by E-cadherin, α- and β-catenins. Apical junctions complexes interact with the cytoskeleton and play a critical role in epithelial polarisation and permeability (2).

CF is one of the most common genetic disease in Caucasian population, caused by mutations on the CFTR gene. Lung disease in CF is characterised by recurrent opportunistic infections (e.g. to *Pa*), associated with chronic inflammation (3). The airway epithelium, intrinsically altered through the genetic defect in CFTR function, is therefore particularly exposed and its integrity is susceptible to be further affected by pathogens or other external agents (2).

Autopsy studies showed early pulmonary changes in CF patients such as bronchiectasis, squamous metaplasia or goblet cells hyperplasia (4-6). More recently, different studies focused on epithelial specification in CF bronchial biopsies or lung explants, with increased proliferative zones and basal cell

hyperplasia (7, 8), glandular hyperplasia (8, 9), and increased mucins-expressing cells (8, 10). Some of these features were reproduced in vitro in the upper airway epithelium. Of note, basal cell hyperplasia observed in cultured nasal epithelial cells isolated from CF patients could be reproduced by adding inflammatory mediators in control cultures (11). Ciliated cells, whose number and function are decreased in COPD (1), seem also decreased in CF-derived nasal cultures (11).

EMT is a phenomenon by which a polarised epithelium de-differentiate into mesenchymal cells. This physiological process during development and repair involves loss of polarity/epithelial markers (such as ZO-1 or E-cadherin), enhanced migratory capacity and acquisition of mesenchymal features like spindle shape, the expression of vimentin and the secretion of extracellular matrix proteins like fibronectin. After aggression, EMT allows mesenchymal cells to migrate on the lesion. Once cells form a layer, they can re-differentiate into a mucociliary epithelium. However, upon chronic inflammation, persisting EMT may lead to remodelling (12). Different studies showed that remodelling occurs in CF, but the presence of EMT remains elusive. Only one study assessed the expression of EMT markers (fibronectin and N-cadherin), increased in IB3 cells, a cell line derived from a CF bronchial epithelium, compared with the C38 control cell line (13). Concordantly with previous data showing that TGF-β signalling promotes EMT, these features were reversible upon TGF-β inhibition in IB3 cells (13). In addition, TGF-β expression is increased in CF and associated with a thickening of the RBM (14) like in other chronic obstructive diseases (15). Finally, it remains unclear to which extent airway epithelial changes are directly related to CFTR dysfunction or to secondary alterations following repeated infections or chronic inflammation.

This study aimed to characterise cellular populations and EMT in the airway epithelium from a large cohort of CF patients, and to evaluate whether these changes could be recapitulated *in vitro*, in the reconstituted bronchial epithelium.

# 3 Methods

Supplemental data are available in the chapter 5.

## *3.1 Patients*

Lung tissue were obtained from 44 patients, which included explants obtained from 20 patients with end-stage CF disease, compared with lung tissue from 24 control patients undergoing lung surgery for a solitary lung tumour (n = 20) or unused lung donors (n = 4). 21 patients were enrolled for cell culture, which included 8 control subjects and 13 patients with severe (Table 4).

| | | **Controls** | **Patients with CF** | **All** | **P-value** |
|---|---|---|---|---|---|
| Lung tissue series | Subjects, n | 24 | 20 | 44 | |
| | Sex, F/M | 13/11 | 10/10 | 23/21 | |
| | Age, years (mean ± SD) | 58 ± 13 | 31 ± 9 | 46 ± 17 | *p < 0.0001* |
| | Smoking history, never/former/current | 19/2/3 | 17/1/2 | 36/3/5 | |
| | Genotype, F508del homozygous/F508del heterozygous/other mutations | NA | 12/4/2 | NA | |
| | $FEV_1$, % predicted (n = 39) (mean ± SD) | 95 ± 17 | 26 ± 6 | 61 ± 37 | *p < 0.0001* |
| HBEC | Subjects, n | 8 | 13 | 21 | |
| | Sex, F/M (n = 20) | 1/7 | 5/7 | 6/14 | |
| | Age, years (mean ± SD) | 59 ± 11 | 31 ± 11 | 42 ± 18 | *p < 0.0001* |
| | Smoking history, never/former/current | 2/1/5 | 11/1/1 | 13/2/6 | |

| | | | | | |
|---|---|---|---|---|---|
| | Genotype, F508del homozygous/F508del heterozygous/other mutations | NA | 8/3/2 | NA | |
| | $FEV_1$, % predicted (n = 20) (mean ± SD) | 97 ± 14 | 31 ± 11 | 48 ± 34 | *p < 0.0001* |
| | Subjects, n (short-term culture) | 8 | 13 | 21 | |
| | Subjects, n (long-term culture) | 5 | 7 | 12 | |
| | Subjects, n (CFTR inhibition) | 3 | NA | 3 | |
| | Subjects, n (vPAO1 infection) | 6 | NA | 6 | |

**Table 4. Patient characteristics for characterisation of cellular populations and EMT in the airway epithelium.**

CF, cystic fibrosis; F, female; M, male; SD, standard deviation; $FEV_1$, forced expiratory volume in the first second; NA, not applicable; vPAO1, virulent PAO1. N is specified in the left column when data are missing.

### *3.2 Immunohistochemistry for β-tubulin, MUC5AC, p63 and vimentin*

Lung samples were fixed in 4% formaldehyde and embedded in paraffin wax. Paraffin lung were immunostained for β-tubulin (Sigma-Aldrich, Saint-Louis, MO, USA), MUC5AC (OriGene Technologies Inc., Rockville, MD, USA), p63 (Dako, Carpinteria, CA, USA) and vimentin (Dako). As negative controls, we used corresponding control isotypes diluted at the same concentration as related primary antibody. Images were acquired with a SCN400 slide scanner (Leica Biosystems, Newcastle, United Kingdom). Quantification of the staining relative area or the number of stained cells was performed with TissueIA software (Leica) on intact tissue sections, using the same threshold, which was adjusted using negative control.

Results were expressed as the percentage of stained area or percentage of stained nuclei for nuclear staining. Quantification of the epithelial shedding and epithelium thickening were manually performed with Author software (Visiopharm®) and were expressed as the percentage of concerned perimeter (epithelial shedding length/total bronchial perimeter and epithelium thickening > 100 µm length/total bronchial perimeter). 100 µm is equivalent to 200 % of the mean of control epithelium height (51.63 µm). Quantification of the reticular basement membrane thickening was manually performed with ImageJ software and was expressed as the mean of reticular basement membrane height in µm

### *3.3 Basal cell isolation and primary culture of human bronchial epithelial cells*

Digested cells from bronchus were sorted as described (16). Basal cells ($CD151^+/CD142^+$) were selected and cultured as non-sorted HBEC (17). ALI condition was applied for 2, 4 or 6 weeks. Basal media, apical washes and cell lysates were then harvested for further analyses. At each timepoint, the transepithelial electrical resistance (TEER) was determined using a Millicell-ERS Volt-Ohm Meter (Merk Millipore, Darmstadt, Germany). Measure of a blank resistance (of the semipermeable membrane only) was subtracted to each measure and then each measure was expressed in $\Omega/mm^2$.

After culture for 2 weeks in ALI condition, primary HBEC were stimulated with 25 µl of 10 µM CFTR inhibitors (CFTRinh-172, GlyH-101 or PPQ-102) (CF Foundation) at apical pole and in the basal medium for 48 h (n = 3) or with 25 µl of virulent *Pseudomonas aeruginosa* PAO1 laboratory strain at the apical pole for 4h. After virulent PAO1 exposure, cells were rinsed with sterile PBS and incubated for 30 min with PBS gentamycin 50 µg/ml (Sigma-Aldrich), followed by incubation in ALI condition for additional 48 h (18). Sterile PBS was used as control condition.

Doses used (in CFTR inhibitors and virulent PAO1 stimulations) did not affect cell viability, according to lactate dehydrogenase assay. Basal media, apical washes and cell lysates (for western blot or RT-qPCR) were then harvested.

### *3.4 Virulent PAO1 preparation*

Virulent PAO1 was prepared as described (18).

### *3.5 Western blot for fibronectin, vimentin, E-cadherin, occludin, β-tubulin and GAPDH*

Cells were analysed for fibronectin, vimentin, E-cadherin, β-tubulin and occludin by western blot and quantification (normalised with GAPDH was performed by using Quantity One software (Bio-Rad, Hercules, USA).

### *3.6 ELISA for fibronectin*

HBEC basal medium and fibronectin standard (Sigma) were coated in plates with bicarbonate buffer overnight, at 4°C. After blocking with PBS BSA 1%, detection was performed with a first incubation with mouse anti-fibronectin (BD Pharmingen), followed by a second incubation with HRP-linked anti-mouse IgG (Sigma), for 1 h each. Revelation was performed with 3,3',5,5'-tetramethylbenzidine and stopped with $H_2SO_4$ 1.8 M.

### *3.7 RT-qPCR of* MCIDAS, MYB, RFX2, FOXJ1, DNAI1, DNAI2, TJP1 *and* VIM

RNA was extracted by the phenol-chloroform method. After reverse-transcribed, the expression levels were quantified by RT-qPCR. To control the specificity of the amplification products, a melting curve analysis was performed. Samples were run and the copy number was calculated from the standard curve. Data analysis was performed using Bio-Rad CFX software (Bio-Rad). Expression levels of target genes (Table 5) were normalised to the geometric mean of the values for the 3 housekeeping genes (*RPS13*, *RPS18* and *RPL27*).

| **Gene** | **Forward primer sequence 5'→ 3'** | **Reverse Primer sequence 5'→ 3'** |
|---|---|---|
| *MCIDAS* | GAC GCG CTT GTT GAG AAT AA | CAC GTT CCG CTC CTT GAG |
| *MYB* | TAC TGC CTG GAC GAA CTG ATA A | CTG GCT GGC TGG CTT TTG AA |
| *RFX2* | CCG AAC TCC AGA CCT GAG CAT | TGA ACT CTG GGG AGG GAG AT |
| *FOXJ1* | CCT GGC AGA ATT CAA TCC G | GCG TAC TGG GGG TCA AT |
| *DNAI1* | GAG TTG ACC GAT GCG GAG TT | GGT TCC CAA CCT GGG TGT AG |
| *DNAI2* | GCG ATT CAT ACA TCT GGG AC | CAG CAG GCT ATC TGT CCA T |

| | | |
|---|---|---|
| *TJP1* | GTG GTT CTT CGA GAA GCT GGA | TGC AGG CGA ATA ATG CCA GA |
| *VIM* | CGG GAG AAA TTG CAG GAG GA | AAG GTC AAG ACG TGC CAG AG |
| *RPS13* | TCG GCT TTA CCC TAT CGA CGC AG | ACG TAC TTG TGC AAC ACC ATG TGA |
| *RPS18* | TGT GGG CCG AAG ATA TGC T | TGA TCA CAC GTT CCA CCT CAT |
| *RPL27* | TGG TAG GGC CGG GTG GTT GC | ACT TTG CGG GGG TAG CGG TC |

**Table 5. List of primers for characterisation of cellular populations and EMT in the airway epithelium.**
MCIDAS, multiciliate differentiation and DNA synthesis associated cell cycle protein; RFX2, regulatory factor X2; FoxJ1, forkhead box J1; DNAI, dynein axonemal intermediate chain; TJP1, tight junction protein 1; VIM, vimentin; RPS13, ribosomal protein S13; RPS18, ribosomal protein S18; RPL27, ribosomal protein L27.

### *3.8 Statistical analysis*

GraphPad Prism 8.0.2 software (GraphPad Inc., San Diego, CA, USA) and SPSS (IBM) were used for statistical analysis. Normality of the distributions were first assessed. Comparisons between two groups were performed using a Mann-Whitney U-test (non-parametric data). Multiple group comparisons of paired, non-parametric data were performed with Friedman test. Comparisons were considered as significant if p-value was under 0.05.

### *3.9 Ethics statement*

All patients received information and signed a written consent to the study protocol, which was approved by the local clinical Ethical committee (Ref. protocol "CLARA" 2005/22SEP/149 – update 29/11/2016 (UCLouvain) and Ref. S51577, S52174, S55877 (KULeuven)).

## 4 Results

### *4.1 Altered airway epithelial specification in CF lungs*

The main epithelium cell types were first assessed by immunostaining on lung tissues from CF patients and controls. We observed increased MUC5AC staining – goblet cells – in the bronchial epithelium from CF patients, compared with controls (Figure 29a-b) and a decreased β-tubulin staining – ciliated cells (Figure 29c-d). In contrast, p63-positive basal cells were not affected (Figure 29e-f). This altered specification defect was associated with EMT-related dedifferentiation, as evidenced by an increased number of vimentin-positive spindle-shaped cells in bronchial epithelium from CF patients, compared with controls (Figure 29g-h). This was associated with a thickening of the basement membrane, a trend of increased epithelial shedding and epithelium height (Figure 30).

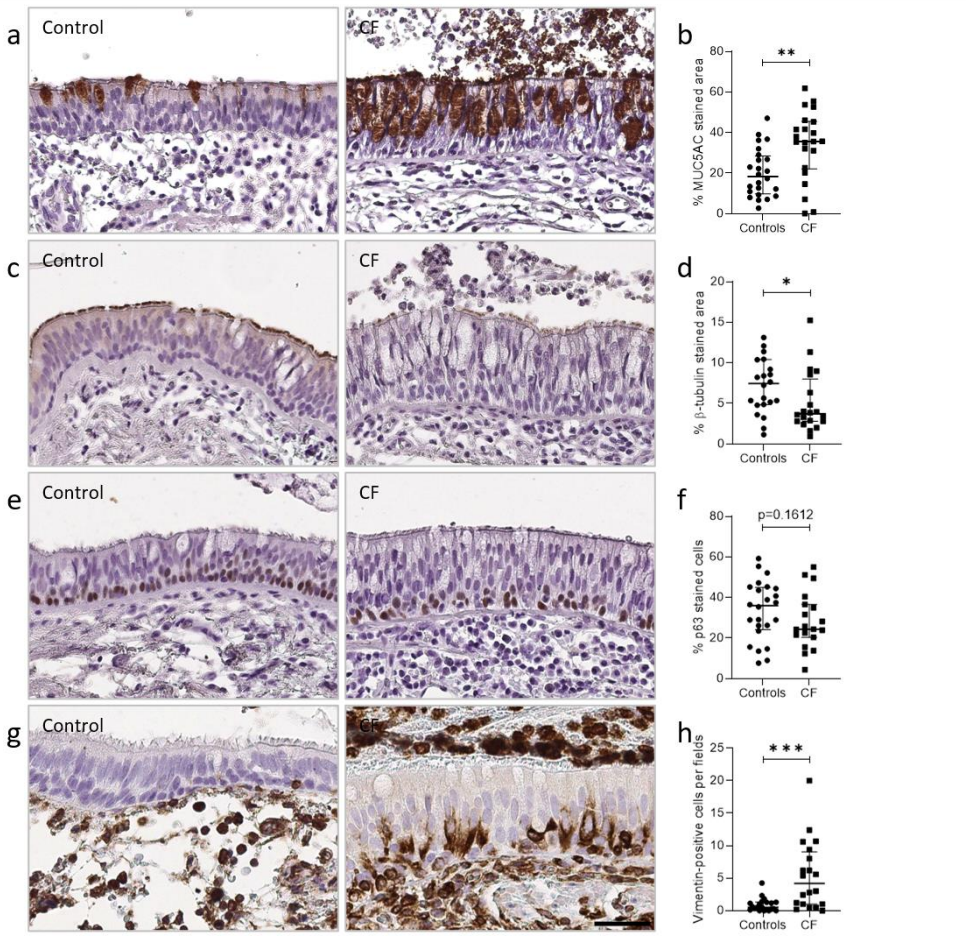


**Figure 29. Airway epithelial differentiation is altered in the lung from patients with severe CF.**

(a) Epithelial MUC5AC immunostaining in a large airway from one representative control and one representative CF patient. (b) MUC5AC staining quantification in the bronchial epithelium from 20 CF patients, as compared with 24 controls (*$p = 0.0040$). (c) Epithelial β-tubulin IV immunostaining in a large airway from one representative control and one representative CF patient. (d) β-tubulin IV staining quantification in the bronchial epithelium from 20 CF patients, as compared with 22 controls (*$p = 0.0251$). (e) Epithelial p63 immunostaining in a large airway from one representative control and one representative CF patient. (f) Percentage of p63-positive cells in the bronchial epithelium from 19 CF patients, as compared with 24 controls ($p = 0.1612$). (g) Epithelial vimentin immunostaining in a large airway from one representative control and one representative CF patient. (h) Number of vimentin-positive cells per field in the bronchial epithelium from 20 CF patients, as compared with 21 controls (***$p = 0.0005$). P-values were determined by Mann-Whitney test. Bars indicate median and interquartile ranges. CF, cystic fibrosis; MUC5AC, mucin 5AC. Scale bar, 50 µm.

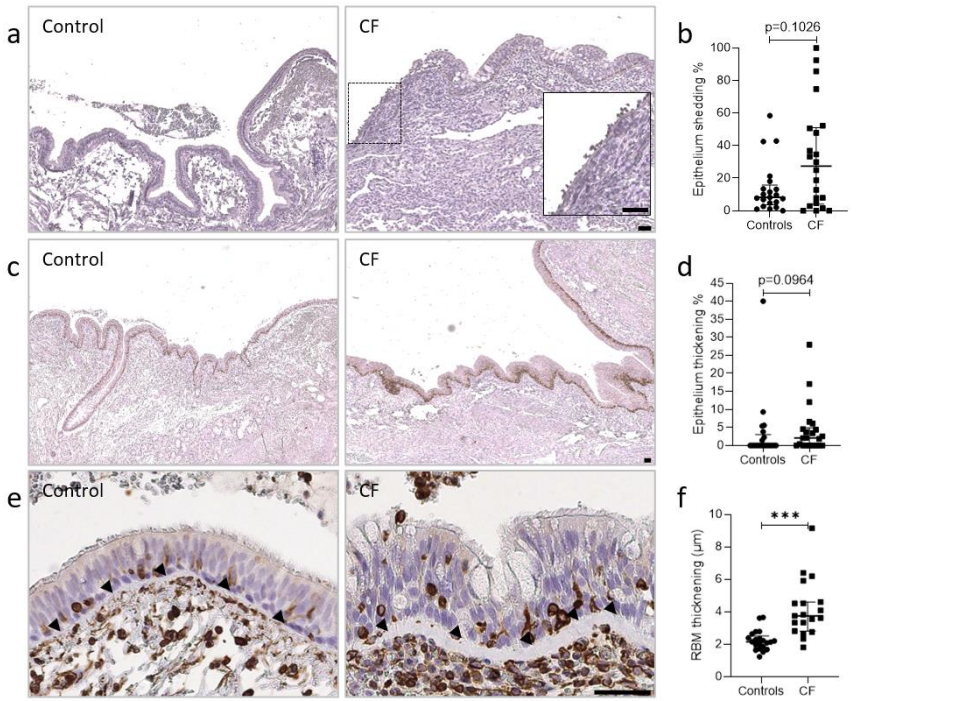


**Figure 30. Airway epithelial morphology is altered in the lung from patients with severe CF.**

(a) Epithelial shedding (box) in a large airway from one representative control and one representative CF patient. (b) Percentage of bronchial perimeter with epithelial shedding from 20 CF patients, as compared with 24 controls ($p = 0.1026$). (c) Epithelium thickening in a large airway from one representative control and one representative CF patient. (d) Percentage of epithelium thickening in the bronchial epithelium from 20 CF patients, as compared with 24 controls ($p = 0.0964$). (e) RBM (arrows) thickening in a large airway from one representative control and one representative CF patient. (f) RBM thickening mean in the bronchial epithelium from 20 CF patients, as compared with 24 controls (***$p = 0.0001$). P-values were determined by Mann-Whitney test. Bars indicate median and interquartile ranges. CF, cystic fibrosis; RBM, reticular basement membrane. Scale bar, 50 µm.

### *4.2 Altered airway epithelial differentiation in cultured CF cells*

We used ALI cultures derived from FACS-sorted basal cells of CF and control patients, enabling us to study the lower airway epithelium from CF patients whilst avoiding culture infections (19). We evaluated whether alterations in the (multi)ciliated lineage, the junctional properties and EMT features were maintained

*ex vivo*. As goblet cells hyperplasia has already been widely studied (9, 10), we focused our work on ciliated cells. After 2 weeks in ALI culture, CF-derived epithelium displayed decreased β-tubulin protein expression, as compared with controls ( Figure 31a-b).

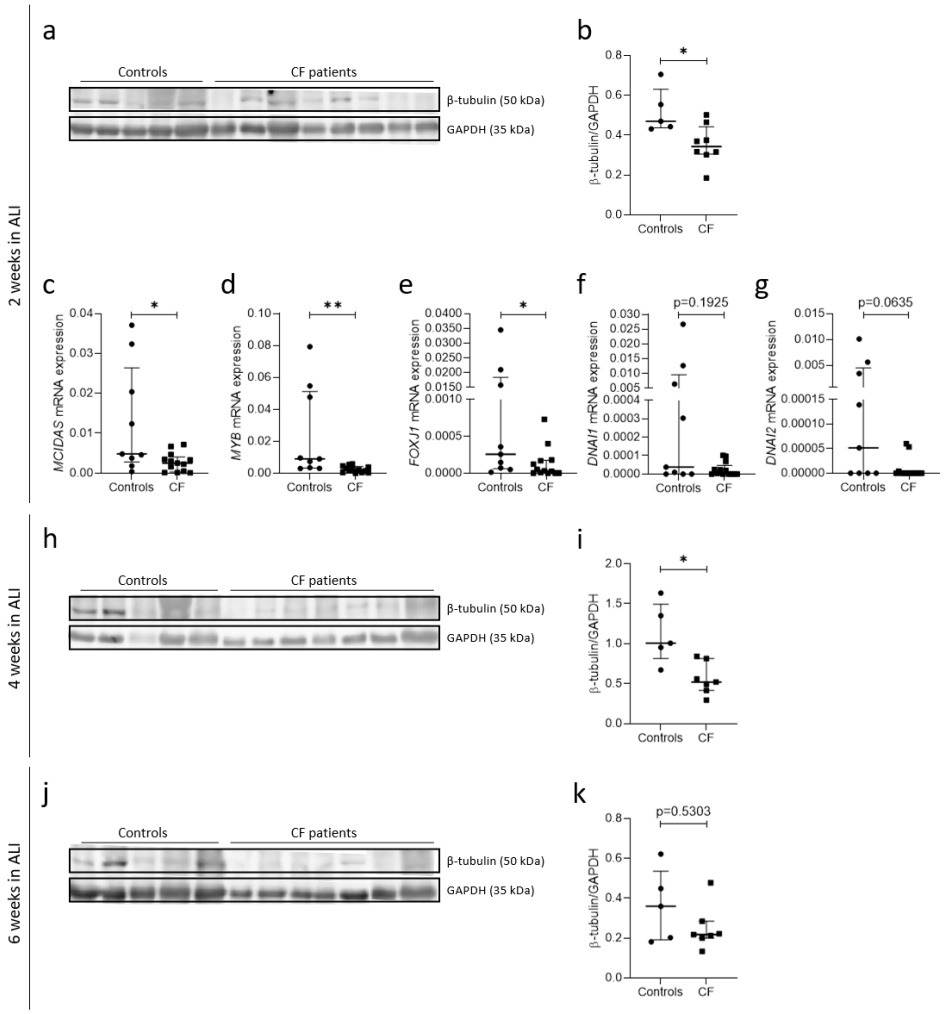


**Figure 31. Epithelial specification is altered in the cultured CF epithelium.**
(a) Representative western blot for ciliated cells marker β-tubulin in CF HBEC, compared with control HBEC in ALI culture for 2 weeks. (b) β-tubulin protein expression in 8 CF HBEC, compared with 5 control HBEC in ALI culture for 2 weeks (*$p = 0.0295$). (c) *MCIDAS* (*$p = 0.0304$), (d) *MYB* (**$p = 0.0019$), (e) *FOXJ1* (*$p = 0.0343$), (f) *DNAI1* ($p = 0.1925$), (g) *DNAI2* ($p = 0.0635$) mRNA expression (corrected for housekeeping genes) in 13 CF HBEC, compared with 9 control HBEC in ALI culture for 2 weeks. (h) Representative

western blot for ciliated marker β-tubulin in CF HBEC, compared with control HBEC in ALI culture for 4 weeks. (i) β-tubulin protein expression in 7 CF HBEC, compared with 5 control HBEC in ALI culture for 4 weeks (*$p = 0.0101$). (j) Representative western blot for ciliated marker β-tubulin in CF HBEC, compared with control HBEC in ALI culture for 6 weeks. (k) β-tubulin protein expression in 7 CF HBEC, compared with 5 control HBEC in ALI culture for 6 weeks ($p = 0.5301$). P-values were determined by Mann-Whitney test. Bars indicate median and interquartile ranges. CF, cystic fibrosis; HBEC, human bronchial epithelial cells; ALI, air-liquid interface; MCIDAS, multiciliate differentiation and DNA synthesis associated cell cycle protein; FoxJ1, forkhead box J1; DNAI, dynein axonemal intermediate chain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

In addition, expression of genes related to multiciliated cell lineage (*MCIDAS*, *MYB* and *FOXJ1*) were affected in reconstituted CF epithelium, compared with control cells ( Figure 31c-e). Gene expression of the dynein complex of respiratory cilia (*DNAI1* and *DNAI2*) also tended to decrease in CF cells, compared with control cells ( Figure 31f-g). As observed at 2 weeks in ALI culture, β-tubulin expression was decreased until 4 weeks in bronchial cells derived from CF patients, compared with controls ( Figure 31h-i). This decrease disappeared after 6 weeks of ALI culture ( Figure 31j-k).

In order to further assess epithelial integrity, junctional proteins and EMT markers were investigated. After 2 weeks in ALI culture, whereas expression of junctional proteins and *TJP1* gene were maintained, we observed a decreased TEER in CF-derived epithelium compared to control culture (Figure 32a-e). E-cadherin and occludin expression, as well as barrier function (through TEER), were reduced in cells after 4 weeks (Figure 32f-i) and restored at 6 weeks in ALI culture (Figure 32j-m).

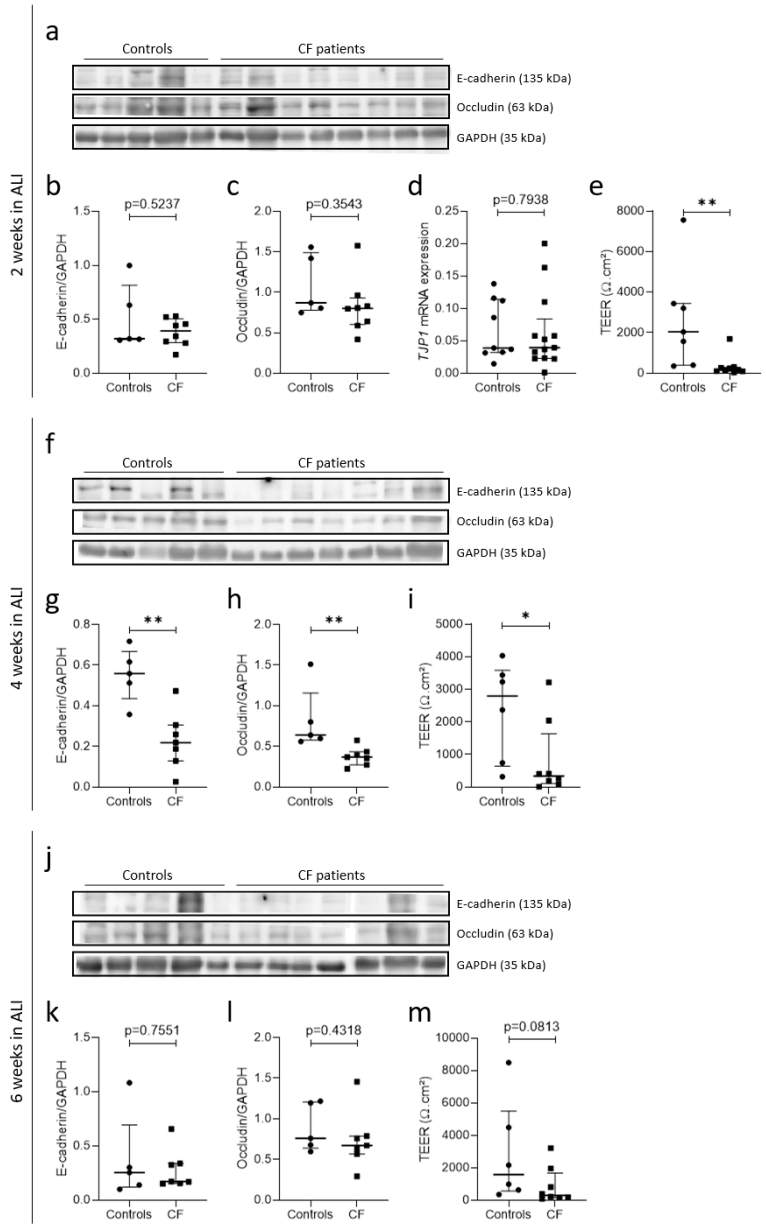


**Figure 32. Junctional properties are altered in the cultured CF epithelium.**
(a) Representative western blot for junction proteins in CF HBEC, compared with control HBEC in ALI culture for 2 weeks. (b) E-cadherin (*p = 0.5237*) and (c) occludin (*p = 0.3543*) protein expression in 8 CF HBEC, compared with 5 control HBEC in ALI culture for 2 weeks. (d) TJP1 (*p = 0.7938*) mRNA expression (corrected for housekeeping genes) in 13 CF HBEC, compared with 9 control HBEC in ALI culture for 2 weeks. (e) TEER in 8 CF

HBEC, compared with 7 control HBEC in ALI culture for 2 weeks (**$p = 0.0012$). (f) Representative western blot for junction proteins in CF HBEC, compared with control HBEC in ALI culture for 4 weeks. (g) E-cadherin (**$p = 0.0016$) and (h) occludin (**$p = 0.0051$) protein expression in 7 CF HBEC, compared with 5 control HBEC in ALI culture for 4 weeks. (i) TEER in 8 CF HBEC, compared with 7 control HBEC in ALI culture for 4 weeks (*$p = 0.0293$). (j) Representative western blot for junction proteins in CF HBEC, compared with control HBEC in ALI culture for 6 weeks. (k) E-cadherin ($p = 0.7551$) and (l) occludin ($p = 0.4318$) protein expression in 7 CF HBEC, compared with 5 control HBEC in ALI culture for 6 weeks. (m) TEER in 8 CF HBEC, compared with 7 control HBEC in ALI culture for 6 weeks ($p = 0.0813$). P-values were determined by Mann-Whitney test. Bars indicate median and interquartile ranges. CF, cystic fibrosis; HBEC, human bronchial epithelial cells; ALI, air-liquid interface; TJP1, tight junction protein 1; TEER, transepithelial electrical resistance; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Concerning EMT, increased fibronectin release and *VIM* mRNA expression were measured at 2 weeks in ALI culture (Figure 33a-e). Fibronectin expression and release tend to be higher at 4 weeks with a significant difference persisting at 6 weeks in ALI culture (Figure 33f-h, j-l), while increased vimentin seen at mRNA level at 2 weeks was not found at protein level at 4 and 6 weeks in ALI culture (Figure 33i, m).

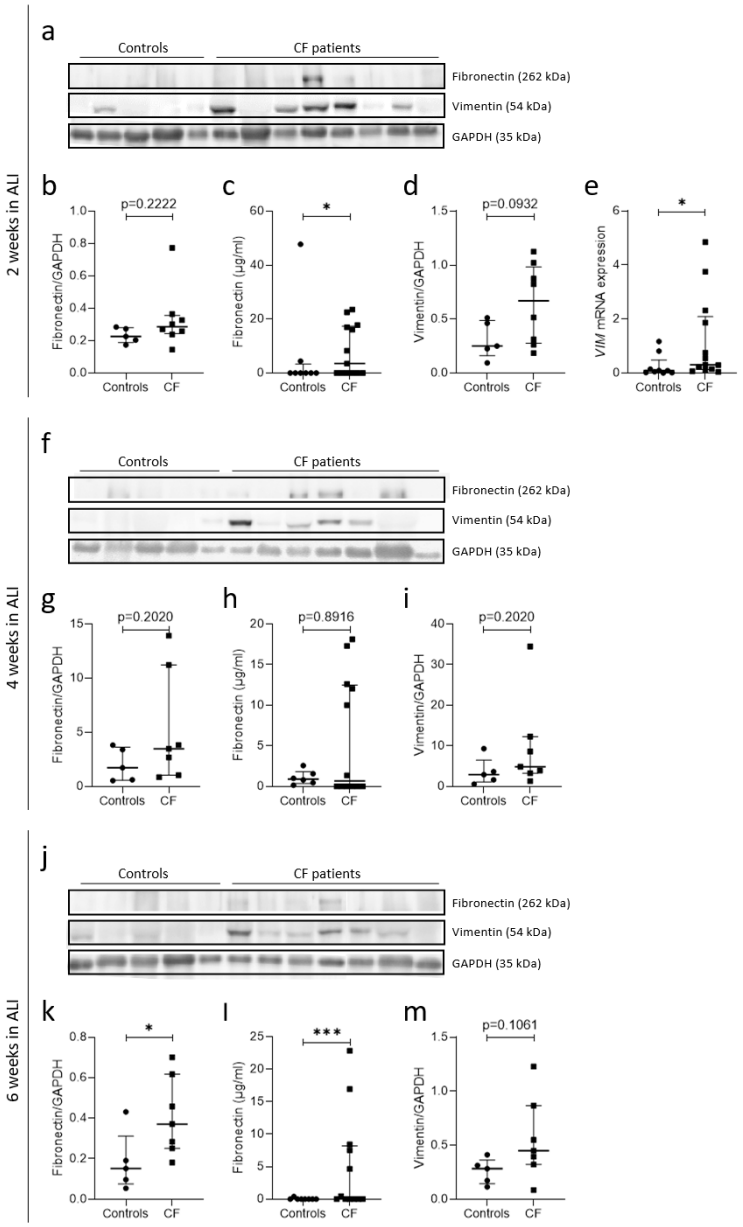


**Figure 33. Epithelial dedifferentiation is observed in the cultured CF epithelium.**

(a) Representative western blot for EMT markers in CF HBEC, compared with control HBEC in ALI culture for 2 weeks. (b) Fibronectin expression in 8 CF HBEC, compared with 5 control HBEC (*p = 0.2222*) and (c) fibronectin release in basolateral medium of 13 CF ALI-HBEC, compared with 8 control HBEC in ALI culture for 2 weeks (**p = 0.0198*). (d)

Vimentin expression in 8 CF HBEC, compared with 5 control HBEC ($p = 0.0932$) and (e) *VIM* mRNA expression (corrected for housekeeping genes) in 13 CF HBEC, compared with 9 control HBEC in ALI culture for 2 weeks (*$p = 0.0304$). (f) Representative western blot for EMT markers in CF HBEC, compared with control HBEC in ALI culture for 4 weeks. (g) Fibronectin in 7 CF HBEC, compared with 5 control HBEC ($p = 0.2020$) and (h) fibronectin release in basolateral medium of 8 CF ALI-HBEC, compared with 6 control HBEC in ALI culture for 4 weeks ($p = 0.8916$). (i) Vimentin expression in 7 CF HBEC, compared with 5 control HBEC in ALI culture for 4 weeks ($p = 0.2020$). (j) Representative western blot for EMT markers in CF HBEC, compared with control HBEC in ALI culture for 6 weeks. (k) Fibronectin expression in 7 CF HBEC, compared with 5 control HBEC (*$p = 0.0480$) and (l) fibronectin release in basolateral medium of 8 CF ALI-HBEC, compared with 6 control HBEC in ALI culture for 6 weeks (***$p = 0.0032$). (m) Vimentin expression in 7 CF HBEC, compared with 5 control HBEC in ALI culture for 6 weeks ($p = 0.1061$). P-values were determined by Mann-Whitney test. Bars indicate median and interquartile ranges. CF, cystic fibrosis; EMT, epithelia-to-mesenchymal transition; HBEC, human bronchial epithelial cells; ALI, air-liquid interface; VIM, vimentin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

### *4.3 Airway epithelial differentiation and EMT are not directly related to CFTR dysfunction or* Pa *infection*

As some aspects of EMT-related dedifferentiation were maintained *in vitro*, we wondered whether these defects could be reproduced upon CFTR inhibition in control cultures. We measured no difference in the expression of genes related to multiciliated cell lineage and of *DNAI1*, while CFTR function was repressed in control HBEC by three different inhibitors. Moreover, we could not reproduce EMT features observed in CF-derived HBEC (Figure 34a-f).

Finally, we assessed the ability of virulent PAO1, a *Pseudomonas aeruginosa* laboratory strain, to drive EMT-related dedifferentiation. We observed no difference in the expression of genes related to multiciliated cell phenotype in HBEC exposed for 48 h to virulent PAO1. Despite a trend for increased fibronectin release, *VIM* mRNA levels did not differ after virulent PAO1 exposure (Figure 34g-l).

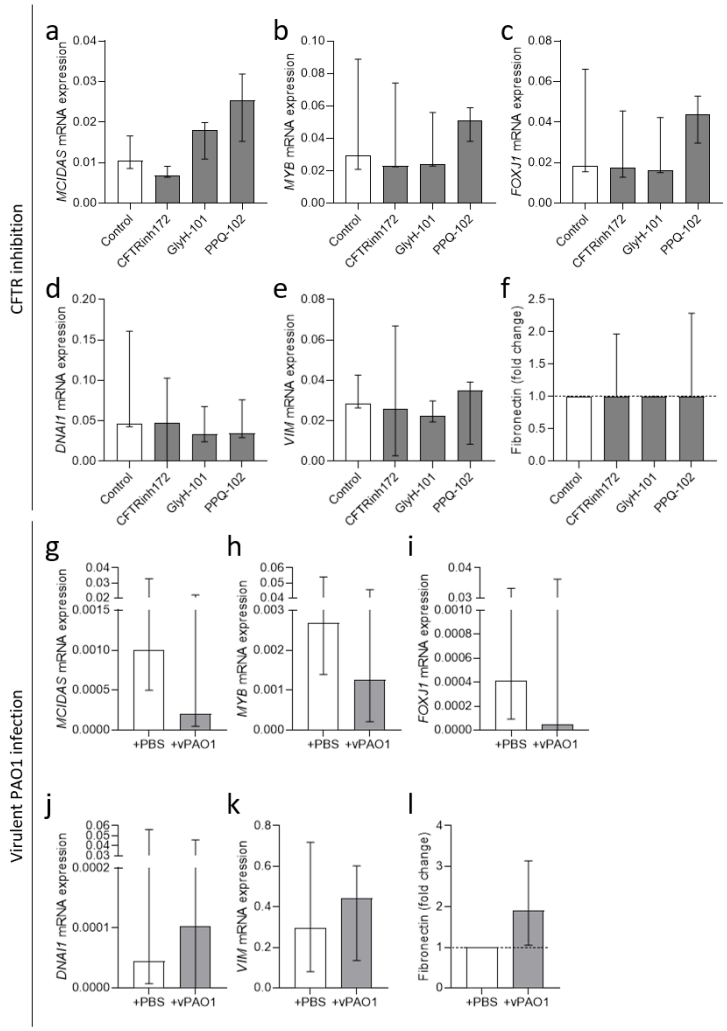


**Figure 34. Airway epithelial differentiation and EMT are not related to CFTR dysfunction or *Pa* infection.**

(a) *MCIDAS*, (b) *MYB*, (c) *FOXJ1*, (d) *DNAI1* and (e) *VIM* mRNA expression (corrected for housekeeping genes) in 3 control ALI-HBEC, treated during 48 h with 10 µM of CFTRinh172, GlyH-101 or PPQ-102. (f) Fibronectin release in basolateral medium of 3 control ALI-HBEC, treated during 48 h with 10 µM of CFTRinh172, GlyH-101 or PPQ-102. (g) *MCIDAS*, (h) *MYB*, (i) *FOXJ1*, (j) *DNAI1* and (k) *VIM* mRNA expression (corrected for housekeeping genes) in 5 control ALI-HBEC, stimulated during 48 h with vPAO1. (l) Fibronectin release in basolateral medium of 6 control ALI-HBEC, stimulated during 48 h with vPAO1 (*p = 0.0932*). P-values were determined by Wilcoxon test. Bars indicate median and interquartile ranges. CFTR, cystic fibrosis transmembrane regulator; HBEC, human bronchial epithelial cells; ALI, air-liquid interface; MCIDAS, multiciliate differentiation and

DNA synthesis associated cell cycle protein; FoxJ1, forkhead box J1; DNAI, dynein axonemal intermediate chain; vPAO1, virulent PAO1; VIM, vimentin; CF, cystic fibrosis.

## 5 Discussion

This study provides new evidence that epithelial cell differentiation is altered in the bronchi of a large cohort of CF patients. Increased MUC5AC and decreased β-tubulin expression are observed in CF lower airways, associated with increased number of vimentin-positive cells. In addition, a methodology enables to reconstitute the airway epithelium from CF lungs following basal cell isolation where these features can be recapitulated *ex vivo*, indicating an 'imprinting' of the CF epithelium for several indices of impaired integrity. This is not reproduced upon CFTR inhibition or virulent PAO1 infection on HBEC, suggesting that the underlying mechanism is independent from CFTR dysfunction as well as from *Pseudomonas aeruginosa* infection alone. Interestingly, the altered specification for ciliated cells, higher permeability as well as mesenchymal phenotype that were observed at 2 and/or 4 weeks in CF cultures as compared with controls, are no longer observed at 6 weeks, except for fibronectin secretion. This indicates that these epithelial changes are not genetically imprinted.

Pulmonary epithelial changes in CF are suggested for decades (4), but systematic phenotypic studies in lung tissues or *in vitro* models using primary culture lack. We first confirm, in a large cohort of patients, a goblet cells hyperplasia witnessed by an increased MUC5AC expression in CF epithelium (4-6). This was also demonstrated by Burgel *et al.* on lung explants (10), although no difference in MUC5AC volume of epithelial staining was observed in a smaller CF population (9). In addition to goblet cells, basal cells hyperplasia and squamous metaplasia have been described (4, 20). In our cohort however, we observe no difference in p63+ basal cells proportion between CF and control bronchi, in line with Dupuit *et al.* study, describing no basal cells hyperplasia in nasal polyps (21). However, in our

study, only intact epithelium is analysed to avoid bias caused by the incidence of the cutting. Proliferative areas have been already described in CF lung (7), associated with surface epithelium shedding (21).

Decreased β-tubulin expression suggests cilia and/or ciliated cells defect. Loss of cilia was noticed in autopsies from young CF patients (4). We confirm these data both in lung tissues and in primary HBEC, but this alteration is maintained in CF epithelial culture after 2 and 4 weeks in ALI only. This loss of specific CF phenotype at 6 weeks could relate on an epigenetic mechanism, that can be modulated by the *in vivo* environment, rather than on a direct link to CFTR dysfunction or infection, as *Pa* stimulation and CFTR inhibition do not influence the expression of the studied proteins. At 2 weeks of ALI culture, decreased expression of genes related to ciliated lineage (*MCIDAS*, *MYB* and *FOXJ1*) suggests that the ciliated cell defect is imprinted, since it is also observed in the epithelium derived from basal/stem cells and cultured *in vitro* up to 4 weeks, as previously shown in CF nasal epithelial cells (11). Conversely, no difference in ciliogenesis was measured in coculture of CF nasal epithelium and fibroblasts (22) and during regeneration of CF nasal polyps grafted in rat trachea (23). This discrepancy could be related to the use of different models. Altered ciliated cells generation is a feature that has been demonstrated in asthma (24) and in COPD, which shares other characteristics with CF like goblet cells hyperplasia and squamous metaplasia (25). In this last study, the authors observed changes in cell types proportion related partly with TGF-β. This cytokine is known to be increased in COPD (15) and in lung tissue of CF patients (14) and is largely implicated into EMT process.

During EMT, a well-differentiated polarised epithelium de-differentiates into mesenchymal cells, characterised among others by their spindle shape, vimentin expression and fibronectin secretion (12). In our study, altered cell specification is associated with increased vimentin-positive cells, suggesting EMT-related remodelling in CF lung. Upregulated vimentin expression is maintained when

progenitor/stem basal cells differentiated *in vitro*, although only increased fibronectin secretion remains up to 6 weeks of ALI culture in CF cells, suggesting as the most persistent feature. If EMT process has been suggested in CF cell lines in the literature, it is the first time that it is shown in lung tissue or on primary HBEC. Indeed, previous studies focused on CF nasal epithelium (23) and a CF cell line (13). We demonstrate that EMT markers and ciliated cells defect disappear after 6 weeks of ALI culture and could not be induced in control HBEC by CFTR inhibition. This supports that CF bronchial cells are intrinsically abnormal, although not arguing for a CFTR direct consequence. Similar memory of EMT features at first and reversibility over time were shown in COPD-derived HBEC (15, 26). In mechanistic studies, chronic inflammation was associated with epigenetic changes able to activate EMT features (27). In addition, it remains possible that the *in vitro* environment (e.g. retinoic acid or other supplements to the culture medium) could be responsible for epithelial changes by affecting epigenetic modifications (28) and thereby suppress the CF epithelial changes following long-term culture, almost completely abolished in our study at 6 weeks.

Finally, *Pa* infection alone could not reproduce *in vitro* epithelial abnormalities. In line with this, only an indirect effect was demonstrated through activated alveolar macrophages cell lines by *Pseudomonas* (29).

One limitation of our study relates to the end-stage status of CF patients recruited for tissue samples (lung explants), while ethical issues preclude the access to early-disease tissue. In addition, an animal model of CF lung disease could help to further study the molecular mechanism of the changes acquired in the airway epithelium of CF patients, but currently mouse models mostly fail to reproduce intrinsic, CFTR-related lung disease (30).

In conclusion, we show for the first time a ciliated cell defect and several signatures of impairment of the airway epithelium integrity, including EMT, in a

large cohort of CF patients. Cultures of bronchial basal cells re-differentiated *in vitro* highlight that this phenotype is imprinted in the CF lung, likely through an epigenetic mechanism that is reversible (in long-term culture) and that we could not relate to CFTR dysfunction.

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# CHAPTER 4: CONCLUSIONS AND PERSPECTIVES

## 1 Conclusions

This thesis aimed to address airway epithelial biology in CF, and more particularly epithelial pIgR and IgA immunity.

We first demonstrated that epithelial pIgR expression, IgA secretion, and IgA+ B cells are increased in the CF lung, sputum and serum. A specific IgA response to *Pa* was also shown in infected patients. However, CFTR defect in bronchial epithelial basal/stem cell-derived cultures and in F508del mice was associated with decreased production of pIgR and IgA transcytosis, and UPR activation due to endoplasmic reticulum stress in CF was shown to be related to pIgR defect. IL-17, produced by Th17 and other immune cells and increased upon *Pa* infection, could upregulate pIgR expression. Second, we demonstrated a ciliated cell defect and impaired airway epithelium integrity, that includes EMT, in CF. This phenotype is imprinted in bronchial basal cells re-differentiated *in vitro*. We associated the persistence of those features to a CFTR-independent reversible mechanism (in long-term culture), possibly of epigenetic nature.

Chronic inflammatory lung diseases were shown to share epithelial defects such as altered airway surface liquid volume or composition, altered mucins production, defective mucociliary clearance, or increased inflammation (1). Previous studies demonstrated decreased pIgR expression and IgA secretion in patients with COPD (2, 3) and with chronic rhinosinusitis with nasal polyps (4), as well as decreased pIgR expression in patients with asthma (5). In COPD, pIgR degradation by neutrophil-derived serine proteinases was demonstrated (6). Epithelial de-differentiation was also suggested as a potential source of pIgR downregulation in chronic rhinosinusitis with nasal polyps (7), as well as in COPD (3, 8). Another study

could associate local S-IgA deficiency in the airways with localised bacterial colonisation, inflammation and airway wall remodelling (9).

A well-known driver of EMT is TGF-β1. In addition to its role in remodelling, this regulatory factor represents a major signalling component of developmental and inflammatory pathways in the lung (8). Airway epithelium of COPD patients display an increase expression of TGF-β, which has been shown to downregulate *PIGR* mRNA expression in HBEC (8). Alike COPD, TGF-β is upregulated in lung tissue and BAL of CF patients (10, 11). Polymorphisms in *TGFB1* gene leading to an upregulated production of TGF-β are associated with a more severe pulmonary disease in patients (12). In our study, we observed increased NE activity in CF sputum and EMT features in CF lung and CF-derived bronchial cell cultures, while pIgR and IgA secretion were upregulated in patients. An additional role of epithelial de-differentiation on pIgR downregulation in CF cells, which was not addressed in our study, remains possible.

Discrepancies between observations in samples from CF patients and those in isolated cells led us to consider which additional factor(s) could intervene *in vivo* in order to overcome UPR, NE and EMT effects. One potential factor – absent from cell culture – was infection (and more particularly *Pa* infection). COPD patients suffer from infectious exacerbations and *Pa* is isolated from a small proportion (5-10%) of COPD sputum during exacerbations (13). In previous studies about IgA immunity in COPD, patients were not discriminated according to their bacterial colonisation (notably *Pa*) and consequences of *Pa* infection were not addressed. In addition, differences are noticeable between *Pa* infection in COPD and in CF: only a small proportion of isolates are mucoid and COPD patients generally clear *Pa*, contrary to CF patients (14). In CF, evidence showed that a more pronounced inflammation, due to prolonged *Pa* infection (notably through IL-17), which drives an increased pIgR mediated IgA secretion in CF, including *Pa*-specific response.

Regarding the observation that S-IgA deficiency associated with bacterial presence (through 16S rRNA detection) and airway remodelling were localised in COPD (9), we could hypothesise that such differences could appear along the CF respiratory tract and that the state of epithelial differentiation and pIgR expression might not be homogenous, while IgA overall secretion remains increased.

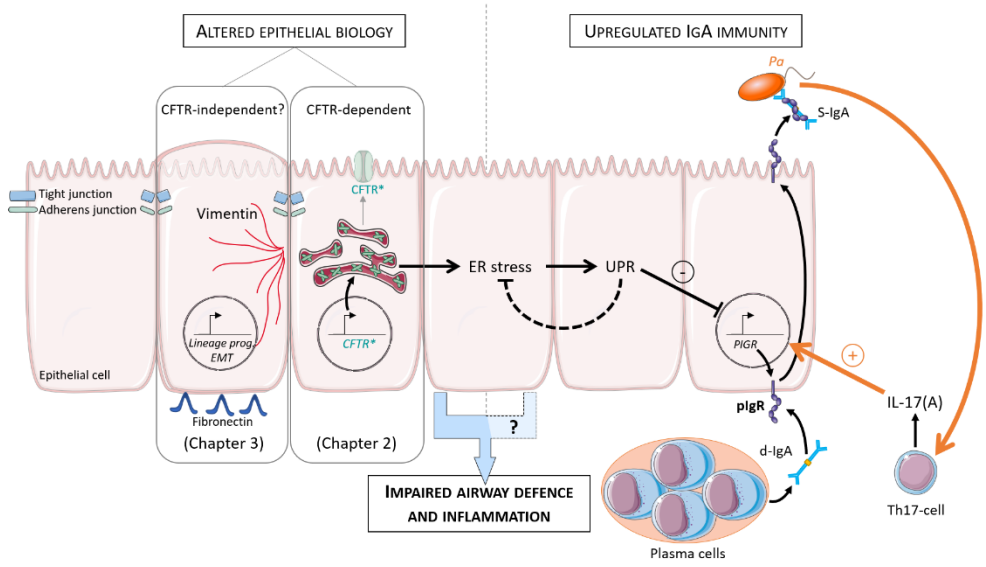


**Figure 35. Model of epithelial alterations and pIgR regulation in CF lung.**
CF airway displays altered epithelium: ER stress (CFTR-dependent) (chapter 2), defective ciliated cell lineage programming and EMT features that could not be related to CFTR (chapter 3). Following ER stress, activation of UPR is able to downregulate SC and S-IgA secretion. Second, *Pa* infection modulates this pathway (orange arrows). *Pa* drives host IL-17 response which stimulates *PIGR* expression. CF, cystic fibrosis; ER, endoplasmic reticulum; UPR, unfolded protein response; EMT, epithelial to mesenchymal transition; pIgR, polymeric immunoglobulin receptor; SC, secretory component; d-IgA, dimeric immunoglobulin; S-IgA, secretory immunoglobulin; Th, T-helper; *Pa*, *Pseudomonas aeruginosa*; prog, programming.

CF displays important defaults of innate immunity mechanisms. Defective antimicrobial molecules, neutrophils, transport of antioxidants, etc. were shown to

be related at least indirectly by CFTR mutations (15, 16). This thesis highlights that CF airway epithelium exhibits alterations (ciliated defect, EMT features, decreased epithelial integrity and ER stress), directly or indirectly linked with CFTR, while pIgR and IgA transport, a mucosal defence mechanism, are upregulated in CF patients and may be related to (*Pa*) chronic infection and following Th17 inflammation (Figure 35).

## 2 Limitations

One main limitation of this work first relates to the end-stage status of patients with CF recruited for lung explants and the infection status for lung explants and sputum. However, the accessibility to samples remains difficult since ethical issues exclude the access to early-disease tissue. Moreover, concerning the infection status of expectorating patients, the proportion of CF children able to expectorate spontaneously is low (< 10% at 6 years of age increasing to ~40% at 10 years in (17)) and bacterial infection is present at very early stage.

In our study, we highlight the potential effect of UPR on pIgR expression, in mice and patients carrying F508del mutation. However, we could not confirm this role in mouse models or patients with other mutation classes. Comparing F508del mice to CFTR KO mice would have been very interesting. Unfortunately, to our knowledge, no null mouse model (on FVB/129 background) exists and could be compared with our strain, avoiding the background genetic variabilities. Similarly, comparing CF patients with F508del mutations to more patients with non-F508del mutations (or any class II CFTR mutations) could be interesting to clarify the effect of UPR on IgA secretion system. This would imply another, larger (multicentric) study. Indeed, in Belgium, 60% of patients with CF are adults and 85.5% carry at least one F508del mutation (18) and the number of eligible patients in a single centre is too low to draw firm conclusions. In addition, pharmacological inhibition of CFTR

function in healthy cells was shown to downregulate (at a lesser extend) pIgR expression and SC secretion while no folding defect was present. Mechanisms other than UPR should be addressed to explain pIgR downregulation in patients with no class II mutation and in CFTR-inhibited healthy cells.

Finally, in the chapter 3, an extensive analysis of the generation of ciliated cells was performed at 2 weeks in ALI culture but we could not repeat it at 4 weeks, when the terminal differentiation usually occurs. In addition, an animal model of CF lung disease could help to further study the molecular mechanism of the changes acquired in the airway epithelium of CF patients, but currently available models mostly fail to reproduce intrinsic, CFTR-related lung disease (19). Indeed, we could not relate pharmacological inhibition of CFTR function to ciliogenesis defect and upregulation of EMT markers. However, CFTR is expressed *in utero* in trachea and lung from CF patients, tracheal abnormalities were actually demonstrated in CF animal models and new-born. Therefore, in order to elucidate a potential role of CFTR in respiratory epithelium differentiation, we might in the future prefer a prolonged functional inhibition (more than 72h and/or during the first weeks of the differentiation) or a CFTR silencing, although differentiation defects cere lost in long-term culture, suggesting a CFTR-independent mechanism.

# 3 Perspectives

## *3.1 Clinical diagnosis of CF lung infection by* Pa

First, specific IgG antibodies in serum and microbiological culture are currently used for the diagnosis of *Pa* infection in patients with CF. However, *Pa* presence in the sinuses was suggested to precede lung infection and to sustain potential re-infection. Furthermore, *Pa* upper airways infection leads to less

inflammation and less important IgG response as in the lung (20, 21). Different studies showed an increased *Pa*-specific IgA in nasal secretions and saliva of CF patients with chronic *Pa* infection (22, 23), but the latter failed to reproduce a correlation between specific IgA in saliva and new or recurrent *Pa* lung infection in another cohort (21). Therefore, the potential interest of specific IgA measurements in secretions (saliva or sputum) in *Pa* infection diagnosis should be further studied in larger and multicentric clinical cohorts.

### *3.2 IL-17, a key regulator of IgA immunity?*

In the chapter 2, we relate the increased pIgR expression to *Pa* infection and an IL-17 mediated response. This cytokine is indeed increased in chronically infected CF patients and in chronically infected mice late after the onset (24). IL-17 was implicated in autoimmune disease but has shown importance in managing microbiota or infection. Disruption of IL-17 receptors in the intestinal epithelium resulted in segmented filamentous bacteria dysbiosis due to reduced expression of α-defensins, *Pigr*, and *Nox1* (25). In the lung, IL-17 and IL-17 receptor A KO mice had a higher risk to be colonised after *Pa* AA43 isolate instillation, compared with WT mice, and developed a less extended inflammation (24). Altogether, these data suggest that IL-17 could have crucial role in managing bacterial colonisation in intestines and in the lung. While pIgR expression was shown to be implicated in the intestines of IL-17 receptors KO mice, it remains unclear whether it is disrupted in IL-17 KO mice. Studying *Pa* chronic infection in IL-17 KO mice in the context of CF and the potential benefits of S-IgA instillation could confirm the role of this pathway on pIgR expression and highlight the role of pIgR in the management of infection.

In addition, other factors could be implicated in pIgR modulation, related to medication or response to *Pa* infection.

Although we could not demonstrate difference in sputum IgA between inhaled corticosteroids (ICS)-treated and ICS-naive patients (data not shown) and that no difference of pIgR expression could be seen between ICS-treated and ICS-naive COPD patients (26), glucocorticoids have been shown to induce *PIGR* expression in human breast and colon cancer cell-lines and in rat liver and intestine (27). On the other hand, a study demonstrated that broad-spectrum antibiotic treatment was shown to downregulate local IgA immunity in mice and human patients in intensive care unit, while IgA contributed to early defence against *Pa* (28). Therefore, analysing more deeply antibiotics therapy and *Pa* resistance in our cohort might possibly give new information to further explain pIgR/IgA modulation in CF patients.

In our study, stimulation of HBEC with *Pa* exoproducts did not show significant upregulated *PIGR* expression, although a trend could be noticed. In our experiments, we used *Pa* supernatant from stationary-phase culture, as previously shown to contain molecules which recapitulate effect observed under *Pa* infection, such as increased IL-8 and vascular endothelial growth factor production by epithelial cells (29, 30) and this technic was used in multiple studies (31-34). However, others advise to collect bacterial supernatant from exponential-phase culture to avoid molecules degradation and possible contamination with products from dead bacteria (35-39), while Adam *et al.* reported that exoproducts were mostly produced and secreted from live bacteria, rather than being released from dead bacteria (31). Exoproducts from *Pa* culturing until exponential phase or stationary phase could be measured and compared, and repeating the HBEC stimulation with *Pa* supernatant from exponential phase culture could be performed if supernatants composition were significantly different.

*Pa* is a Gram-negative bacterium, thus expressing lipopolysaccharide, and TLR-4 stimulation by lipopolysaccharide was associated with increased *PIGR* expression in intestinal epithelial cells (40). While virulent PAO1 strain infection of

HBEC did not shown significant effect on pIgR regulation (data not shown), lipopolysaccharide could be tested on these cells. Interestingly, TLR-4 agonist, such as monophosphoryl lipid A, is used as vaccine adjuvant (notably against hepatitis B) and is shown to promote host immunity (41). In an *in vivo* study, TLR-4 agonistic antibody UT12 was shown to improve the immune response mediated by the neutrophils against *Pa* infection (42). Therefore, it would be interesting to analyse how epithelial cells could respond to this molecule, notably whether pIgR expression and IgA transport are upregulated.

### *3.3 Pathophysiological role of upregulated S-IgA in the CF lung*

The increased presence of pIgR and S-IgA were shown in the CF lung; however, the functionality of S-IgA was not addressed. Marshall *et al*. demonstrated that defective glycosylation of SC in CF patients impairs the ability of SC to neutralise IL-8/CXCL8 and favours neutrophilic inflammation (43). Moreover, proper glycosylation was shown to be important for proper antimicrobial roles (nonspecific attachment of bacteria) (44) and proper localisation of S-IgA by linking the mucus and forming an interface between the lumen and the epithelium, as shown in a mouse model (45). Future research could address the ability of IgA/S-IgA/SC from patients with CF to play their role in microbial homeostasis and in forming interface to protect the epithelium. The latter could also be affected by sticky mucus produced in the airways. In addition, accumulation of S-IgA in the bronchial lumen, following impaired mucociliary clearance, could lead to IgA immune complexes that – instead of being eliminated to achieve immune exclusion – activate FcαRI-bearing phagocytes and induce a more pronounced inflammation (46). Furthermore, the contribution of S-IgA immunity against *Pa* in CF could be address by applying the model of *Pa* chronic infection in pIgR KO and IgA KO mice, since this model – by preventing the clearance of the bacteria – provides features observed in CF patients (even in non-CF mice) (29).

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# CHAPTER 5: SUPPLEMENTARY METHODS

## 1 Loss of ciliated cells and altered airway epithelial integrity in CF

### *1.1 Basal cell isolation and primary culture of HBEC*

HBEC were obtained from a piece of large, cartilaginous bronchus tissue resected following lung transplantation or lobectomy. Bronchus section was digested by pronase E (Sigma-Aldrich). Cells were labelled with anti-CD151-PE (Clone 14A2.H1, BD Biosciences, San Jose, CA, USA) and anti-CD142-APC (Clone HTF1, Miltenyi Biotec, Leiden, Netherlands) antibodies after blocking with FcR Blocking Reagent (Miltenyi Biotec). Fixable Viability Stain 450 (BD Biosciences) was added to discriminate dead cells. Cell sorting was performed with a FACSAria cell sorter (BD Biosciences). Dead cells and doublets were excluded from the cell population. Only basal cells ($CD151^+/CD142^+$) were selected and seeded in cell culture flask in Bronchial Epithelial Basal Medium (BEBM; Lonza, Verviers, Belgium), supplemented with 200 U/ml penicillin and 200 µg/ml streptomycin (Lonza) (as well as Primocin™ 100 µg/ml (InvitroGen, Toulouse, France) for CF cells), bovine serum albumin 1.5 mg/ml and retinoic acid 100 µM (Sigma-Aldrich). When cells reached around 90% confluence, they were trypsinised and 100,000 cells were seeded in 0.33-$cm^2$ insert. They stayed in submerged condition for 10-15 days. ALI condition was applied for 2, 4 or 6 weeks in BEBM:DMEM (Lonza), supplemented as pure BEBM, to allow differentiation into a pseudostratified, mucociliary airway epithelium.

### *1.2 Virulent PAO1 preparation*

A single laboratory strain PAO1 colony grew overnight in Luria-Bertani broth medium (BD, Sparks, MD, USA) with agitation at 37°C. The PAO1 suspension was further diluted in this medium and grew again until mid-log phase. The bacterial suspension was centrifuged at 3,000 g, for 15 min, at room temperature, and the pellet was washed with PBS. Then, the bacterial pellet was diluted to adjust the optical density and obtain the approximate desired inoculate.

### *1.3 Western blot for fibronectin, vimentin, E-cadherin, occludin, β-tubulin and GAPDH*

Cells were lysed with Laemmli Blue. After boiling during 5 min, lysates were separated by 12% SDS-polyacrylamide gel. Then, proteins were transferred to nitrocellulose membrane. After blocking with tris buffered saline supplemented with bovine serum albumin 5%, membrane incubated with mouse anti-fibronectin (BD Pharmingen), mouse anti-vimentin (Dako), mouse anti-E-cadherin (Dako), mouse anti-β-tubulin (Sigma-Aldrich), rabbit anti-occludin (Merck Millipore) and rabbit anti-GAPDH (Sigma-Aldrich) overnight at 4°C, then with goat anti-rabbit IgG (Cell Signaling, Leiden, The Netherlands) or anti-mouse IgG (Sigma-Aldrich) during 1 h, at room temperature. Detection of immunoreactivity was performed with the chemiluminescence reagent (Amersham™ ECL, GE Healthcare, Buckinghamshire, UK) and quantification was performed by using Quantity One software (Bio-Rad, Hercules, USA).

# CHAPTER 6: BIBLIOGRAPHY

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