Inflammation and infection in cystic fibrosis

Chief Editor:
Prof. Dr. Carsten Schwarz

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Prof. Dr. Isabelle Sermet-Gaudelus
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Introduction

Foreword from ECFS president

The European Cystic Fibrosis Society (ECFS) has a proud track record of supporting our clinical and research community with concise and timely published books, each focussing on a specific area of CF. The initiative began in 2012 with 'Healthcare issues and challenges in adolescents with cystic fibrosis' followed by 'Living with Cystic Fibrosis' (2015), 'The early cystic fibrosis years' (2018) and 'Optimising pharmaceutical care' (2020).

The advances of the last few years in the treatment of cystic fibrosis are leading many to enjoy healthier and longer lives. However, despite the impacts CFTR modulators have on lung function, exacerbation rates and quality of life, most studies show that chronic infection and inflammation remain a clinical challenge. This area was therefore chosen as the topic for our latest book in the series, in which fundamental scientific and clinical experts have come together to highlight advances and remaining areas in need of research.

The book takes the reader through basic science of inflammation, cellular biology and the role of CFTR. I found it particularly refreshing to see chapters devoted to macrophages, T cells and smooth muscle cells, in addition to valuable updates on respiratory epithelial cells and neutrophils. Animal models useful for studies of inflammation, the clinical development of anti-inflammatory therapies and the effects of CFTR modulators then lead into the second half of the book with more of an infection focus. People with CF acquire a multitude of pathogens, the clinical impacts of which may differ, as do the treatments. There is a strong focus on detection, an area of increasing clinical challenge as so many people no longer expectorate sputum.

The ECFS is hugely appreciative of all the authors contributing to the book, and wish particularly to thank Carsten Schwarz, Isabelle Sermet-Gaudelus, Dorota Sands and Daniel Peckham for their leadership as the editorial team. We are sure this book will prove an invaluable learning and reference resource and are very pleased to offer it to our members.

Jane C. Davies
ECFS President

Chapter 1
Inflammation and CFTR dysfunction

Authors
David P. Nichols, Michael W. Konstan, Thomas J. Kelly

Introduction
Inflammation is an important pathological feature of many systemic diseases and contributes to morbidity such as pain, tissue damage, bone loss, airway and vascular remodeling. In cystic fibrosis (CF), inflammation is an early manifestation that persists not only as a functional host response but also as an excessive, dysregulated, and injurious feature of CF [1]. In several cell types and organs, inflammation has been directly linked to the primary cellular abnormality in CF; dysfunctional or absent cystic fibrosis transmembrane conductance regulator (CFTR) protein. Inflammation in CF is also a response to other disease complications, particularly chronic mucostasis and bacterial infection in the airway. Related abnormalities in the sinuses and gastrointestinal tract similarly promote inflammation, which may further impair localized upper airway and intestinal clearance, thus worsening dysbiosis in a positive feedback cycle [2-4].

Inflammation at mucosal surfaces in CF has been described as having a chronic acute phenotype, reflecting a dominance of neutrophils and pro-inflammatory cytokines and chemokines. This pattern is more typical of a transient, acute inflammatory host response that progresses to a state of resolution, typified by macrophages and regulatory signaling. However, the homeostatic transition to resolution of inflammation is impaired in CF, probably due to both the direct and indirect consequences of CFTR dysfunction. A persistent, neutrophil-dominated environment results in free proteases (e.g. neutrophil elastase) and other components that may contribute to tissue damage. In addition, leukocytes frequently necrose to release intracellular contents such as neutrophil-derived DNA into the local mucosal environment [5, 6]. Free DNA in the airways increases viscosity of mucous secretions. This manifestation of dysregulated airway inflammation is targeted by inhaled dornase alfa, a recombinant form of DNase that cleaves DNA into smaller fragments and reduces the viscosity of airway secretions [7]. Dornase alfa was the first therapy developed specifically for pwCF [8, 9].

Therapies directly focused on reducing or altering the host inflammatory response in CF have been part of drug development efforts for decades [1]. There have been successes, even though it is more complicated to pharmacologically mitigate inflammation in a condition also characterized by chronic bacterial infection and risk of immunocompromise. High-dose ibuprofen was one of the earliest anti-inflammatory approaches, and was shown to be effective in children with CF [10-13]. Chronic macrolide treatment with azithromycin, which appears to reduce inflammation through pleotropic yet uncertain effects, is another therapy proven effective in pwCF, primarily in those with *P. aeruginosa* airway infection [14-18]. Long-term use of systemic corticosteroids was effective at reducing pulmonary disease but often resulted in intolerable side effects and is not commonly used today [19].

Clinical trials and/or real-world clinical use of dornase alfa [9], high-dose ibuprofen [9, 12, 13], and chronic azithromycin [17] have all demonstrated that chronic treatment slows lung function decline in CF. This underscores an important role for inflammation in the pathophysiology of CF lung disease. However, the mechanism(s) by which anti-inflammatory therapies protect against loss of lung function are uncertain. Correcting the underlying defect in CF through the use of CFTR modulators is expected to decrease the downstream effects of absent or abnormal CFTR in the CF lung, including airway obstruction, infection and inflammation. Thus, one would expect that benefits would include slowing the progression of lung disease, as measured by loss of lung function over time. Real-world clinical evidence has shown slower loss of lung function over time with both ivacaftor and elexacaftor/tezacaftor/ivacaftor (ETI) [20, 21]. It is difficult to discern how much of this effect is due to targeting inflammation, either directly or indirectly. However, there are data indicating that these modulators improve the inflammatory state [22-27].

1.1 The airway environment

Therapeutically targeting inflammation in CF requires an understanding of the relationship between CFTR function and the inflammatory response.

The first obvious relationship between CFTR function and inflammation is the impact of impaired CFTR activity on the lung environment. Mutations in *CFTR* lead to impaired ion transport and fluid regulation in the airways resulting in poor mucociliary clearance, obstructed airways and progressively chronic bacterial infections. Chronic infection certainly provides a persistent inflammatory stimulus that can account for CF inflammatory responses, but multiple studies conducted early in life indicate that chronic infection is not required to observe elevated inflammatory markers in the airways [28, 29]. Studies in very young children find increased inflammation even when infection cannot be identified, which may relate to altered mucin properties and localized hypoxic conditions in the airway [30, 31]. Studies in older children and adults with CF similarly find altered mucins likely interacting with chronic infection and inflammation in the airway [31, 32]. Likewise, animal studies have shown that CF-absent or -deficient models challenged with bacteria in the airways display more aggressive or prolonged inflammatory responses than paired non-CF controls [33]. Non-microbial factors also contribute to the CF inflammatory response.

The altered airway mucus environment has been shown to contribute to CF airway inflammation. The most direct study of this phenomenon involves the development of the βENaC overexpressing mouse. This mouse model presents the phenotype of sodium hyperabsorption characteristic of CF airways, resulting in impaired mucociliary clearance and mucus accumulation. These mice have normally functioning CFTR but display chronic airway inflammation in the absence of detectable bacterial infection, strongly suggesting that impaired mucociliary clearance is an important factor of the inflammatory response [34-36]. The CF ferret model also displays poor airway clearance. Chronically treating the CF ferrets with antibiotics from birth to suppress airway infections revealed that these ferrets still displayed baseline inflammation [37].

1.2 Endoplasmic reticulum stress response

Though infection and poor mucociliary clearance are fundamentally linked with the CF airway inflammatory phenotype, evidence also suggests an inherent effect of lost CFTR function on epithelial inflammatory signaling pathways. Murine studies demonstrate increased airway inflammation after bacterial challenge in CF mice of multiple *CFTR* mutations compared to control animals of the same genetic background [38-43]. Unlike the βENaC overexpressing mice, CFTR-deficient mice do not exhibit overt mucus accumulation or airway obstruction, suggesting that there are intrinsic cellular factors related to CFTR function that influence inflammatory responses. Evidence from various immortalized and primary cell models identified elevated inflammatory signaling in CF cells, again suggesting more inherent intracellular factors are important, although contradictory results have been published with cell models [38-43].

The first mechanism proposed to link mutations in *CFTR* to inflammation focused on the misfolding of the CFTR protein in response to the F508del mutation. The hypothesis was that endoplasmic reticulum (ER) stress from the accumulation of misfolded F508del *CFTR* triggers the unfolded protein response (UPR) and elevates inflammatory signaling. Expression of F508del *CFTR* in immortalized Chinese hamster ovary (CHO) cells was sufficient to stimulate NF-kB activity and inflammatory cytokine production [38-43]. Though the ER stress response is certainly a pro-inflammatory stimulus, similar inflammatory responses are seen in CF models of genotypes that do not result in CFTR misfolding. Markers of ER stress are also seen in mouse models lacking any *CFTR* expression at all (i.e. *CFTR*-null) suggesting that the UPR response is not required to link CFTR to altered intracellular signaling [44].
Oxidative stress pathways also significantly contribute to CF inflammation, particularly through nuclear factor E2–related factor-2 (Nrf2) signaling. Reduced Nrf2 activation and nuclear localization in CF cells increases production of reactive oxygen species (ROS) production [45]. Interventions targeting Nrf2 activation with triterpenoid compounds effectively reversed inflammatory signaling in preclinical CF models [46]. A recent study found that CFTR modulator therapy restored Nrf2 signaling [47].

1.3 Calcium signaling

Calcium signaling in epithelial cells may be an important link between the airway surface condition and ER stress response in CF epithelial cells. The hypothesis arises from observations that components of supernatant from CF mucopurulent material (SMM) initiate ER calcium expansion, resulting in ER stress and subsequent UPR signaling, leading to the inflammatory phenotype [38, 40, 48, 49]. This hypothesis combines the impact of CFTR dysfunction influencing calcium regulation in CF cells and how the local environment triggers this pathway. Current studies are investigating components of the ER stress response, such as IRE1, as potential anti-inflammatory therapies.

1.4 Other pathways influencing CF inflammatory signaling

Other factors and pathways have been identified that appear to influence CF inflammatory responses, though the direct link to CFTR function is not always clear. Multiple lipid pathways have been shown to be involved in inflammation signaling or in the resolution of inflammation, and lipid imbalances exist in the serum of people with CF (pwCF). More recent studies identified elevated arachidonic acid (AA) levels coupled with deficiencies in docosahexaenoic acid (DHA) production. DHA has known anti-inflammatory pathways, and its deficiency is a reasonable contributor to airway inflammation in CF. Mouse and cell studies show potential efficacy of DHA

supplementation to controlling some aspects of the inflammatory response [50-53]. Human clinical trials testing DHA supplementation have not clearly shown benefit, unfortunately.

The cholesterol pathway has also been implicated in CF inflammatory signaling. In CF epithelial cells, cholesterol abnormally accumulates in the perinuclear region in late endosomes/lysosomes [54, 55]. Consequently, *de novo* cholesterol synthesis is upregulated. This has also been observed *in vivo* with a 2-fold increase in synthesis rates seen in the lungs and livers of CF mice [55]. Inhibiting the elevated cholesterol synthesis with statins reduced inflammatory signaling in CF cell models and could represent another avenue of anti-inflammatory therapy [56].

The IFN-γ/STAT1 pathway was influenced by statin exposure in CF cells, and impaired STAT1 activation was restored by inhibiting cholesterol synthesis, likely through a mechanism involving RhoA GTPase [56]. Additional work found that restoring STAT1 signaling in primary CF bronchial epithelial cells was important to anti-viral responses, underscoring the potential significance of this pathway [57].

Two mechanisms have been proposed to explain the accumulation of cholesterol in CF cells. The first mechanism is related to the ER stress pathway described above. CHO cells exogenously expressing F508del *CFTR* exhibited cholesterol accumulation while cells expressing a non-folding mutation such as G551D did not [58]. A second mechanism involves altered microtubule regulation in CF cells. Key to these changes is reduced tubulin acetylation, which can be corrected by inhibiting the tubulin deacetylase cytoplasmic histone deacetylase 6 (HDAC6). Inhibition of HDAC6 restores microtubule acetylation and endosomal transport resulting in the normal distribution of cholesterol and normalization of inflammatory signaling pathways in CF cell models [44]. Depletion of HDAC6 expression in CF mice has multiple effects including better growth and eliminating CF-like inflammatory responses to airway bacterial challenge [59]. Microtubule function can certainly influence multiple cellular functions and may impact other inflammatory cell types.

The resolution of inflammation appears to be impaired in CF. Two lipid mediators that are known to promote the resolution of the inflammatory response, lipoxin A4 and resolvin D1, have been shown to be deficient in CF airways. Lipoxin A4 levels were found specifically to be deficient in CF airway fluid. CF mouse studies testing administration of lipoxin A4 found improved inflammatory resolution, identifying this pathway as a potential therapeutic target [60, 61]. Other CF mouse studies found that resolvin D1, a DHA-derived lipid that is also important to dampening inflammatory responses, also shows efficacy in controlling aggressive inflammation [62-64]. These data demonstrate therapeutic potential for supplementing or altering the status of lipid mediators, although human trials have not yet proven clinical benefit [65]. Modulating lipid balance using the small molecule compound fenretinide, an inhibitor of *de novo* ceramide synthesis, has been shown to have anti-inflammatory properties and to impact lipid balance in CF models [66-69]. Clinical studies are examining the efficacy of this treatment.

1.5 CFTR and inflammation in non-epithelial cells

Most studies of CFTR function and inflammation have focused on epithelial cell biology and cytokine production. Recent studies, however, have examined the impact of impaired CFTR activity on leukocytes. A clear example of non-epithelial involvement in the CF inflammatory responses is seen in a study where *CFTR* expression was conditionally knocked-out of myeloid cells in mice. These myeloid-specific *CFTR* knockout mice displayed CF-like inflammatory responses in response to bacterial challenge [70]. More specifically, accumulating evidence indicates that macrophage function is adversely affected in response to *CFTR* mutation and may improve with CFTR modulator treatment [71-73]. Toll-like receptor (TLR) 4 trafficking may be impaired in CF macrophages, which could increase pro-inflammatory cytokine production [74]. The CFTR/TLR4 pathway has also been shown to regulate anti-bacterial responses of macrophages [75].

CFTR has also been implicated in neutrophil function. Identified abnormalities in neutrophil degranulation improved with CFTR modulator drugs [24]. Lymphocyte function, and especially T cells, have also been reported as abnormal in CF. *CFTR* expression can be identified in CD4+ T cells, and F508del *CFTR* expression leads to reduced expression of the anti-inflammatory cytokine IL 10 [76]. Most CF studies focus on regulatory T-lymphocytes (Treg) cells and TH17 cells. Treg cells are suppressed in CF in both mouse models and in human clinical studies. This is relevant since Tregs work to suppress, among other T-lymphocytes, Th17 cell responses [77, 78]. The imbalance between Tregs and Th17 cells results in excessive IL-17 production in CF airways, contributing to neutrophil recruitment [79, 80]. CFTR has a functional role in several cell types beyond the epithelium, suggesting that therapies rescuing CFTR function may have broad effects on host response.

1.6 Conclusion

Chronic lung inflammation is a hallmark of CF, leading to lung damage that remains an important pathologic feature of this disease. Thus, much effort has been expended over the years to develop anti-inflammatory therapies to decrease inflammation in the CF lung, with the aim of slowing or halting disease progression. Although some approaches show promise, a better understanding is sorely needed of how abnormal or absent CFTR causes an excessive inflammatory response to infection, but also promotes inflammation even in the absence of infection. In this opening chapter, we discuss the airway environment and several of the mechanistic pathways that influence inflammation, including many with direct links to CFTR. The following chapters explore these mechanisms in greater detail and shed further light on therapeutic targets and approaches to combat inflammation.

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Chapter 2
Airway epithelial cells for cystic fibrosis studies

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Introduction
The respiratory system consists of organs and tissues that allow the movement of air between the lungs and the environment, enabling the gas exchange of oxygen (O_2) inwards by carbon dioxide (CO_2) outwards. This gas exchange also contributes to the maintenance of the acid-base balance in the blood. Other non-vital functions of the respiratory system include odor sensing and phonation [1-3].

Functionally, the respiratory system can be divided into two main regions: the conducting zone (upper airway tract) and the respiratory zone (lower airway tract) (Figure 1). The conducting zone includes all the structures that provide a passage for air to go in and out of the lungs, such as the nasal cavity, mouth, pharynx, larynx, trachea, bronchi (large airways), and the bronchioles (small airways). In the upper airways, the incoming air is warmed, humidified, filtered and cleaned before gas exchange in the respiratory zone, which includes the terminal bronchioles and alveoli [1-3].

Since the air contains microorganisms and debris that can be pathogenic, the respiratory system has a robust defense system. A mucous layer and beating cilia constitute the potent mucociliary clearance (MCC) mechanism that traps and removes particles and microorganisms from the lungs. In addition, the airways have a rich lymphatic system, allowing a local influx of lymphatic fluid that helps absorb and remove damaged tissue (e.g., due to inflammation) [2-4]. MCC abnormalities, whether related to impaired fluid secretion, ciliary dysfunction or disruption of epithelial cells lining the respiratory tract, contribute to lung disease, including chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (CF) [5].

COPD is a heterogenous group of diseases, all associated with chronic systemic inflammation affecting primarily the lungs. This disease is characterized by chronic overproduction and hypersecretion of mucus by goblet cells and decreased MCC due to poor ciliary function, leading to airway obstruction [6].

Asthma is an airway disease caused by chronic inflammation of the lower respiratory tract. It is characterized by over-responsiveness of the lungs to environmental allergens, viral and bacterial respiratory infections, leading to mucus hypersecretion and bronchoconstriction [7, 8].

In contrast to COPD and asthma, CF is a genetic recessive disorder characterized by variants in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Dysfunctional CFTR and abnormal ion transport impair MCC, leading to chronic airway infection and inflammation [9].

2.1 The airway epithelium and cell types

The airway tract is lined by epithelium, a primary tissue made up of epithelial cells [10, 11]. When classified by shape, epithelial cells can be classified as squamous when they are flat (Figure 2A), cuboidal when their width, height, and depth are the same (Figure 2B), and columnar when they are tall and slender (Figure 2C). Epithelial cells can be organized into one layer of cells (called a simple epithelium) (Figure 2A-C) or into multiple cell layers (called a stratified epithelium) (Figure 2D-F). They can also be organized in a pseudostratified epithelium, in which there is only one cell layer, however, the cells have different sizes, having the appearance of a stratified epithelium (Figure 2G). A transitional epithelium is when the organization of the epithelial cells allows them to flatten and slide over one another (Figure 2H). This is only present in stretching organs like the bladder [12].

Epithelial cells are polarized, which means they are spatial-temporally compartmentalized, with apical, basal, and lateral regions which are functionally and structurally different (Figure 3). The apical side faces the lumen area or the external environment, and can have specialized structures such as microvilli, cilia, and stereocilia, that confer different functions to the cells. For example, the intestine and kidney microvilli are associated with fluid absorption, while in the lungs, cilia movement contributes to MCC. The basal side of epithelial cells faces the internal environment, and is where the cell is anchored to the extracellular matrix supporting the epithelial tissue. The lateral side is where adjacent cells are attached by adherent proteins; it is also where epithelial cells communicate with each other through tight and gap junctions (Figure 3) [13-15].

The upper airway tract is lined with a pseudostratified polarized columnar epithelium mainly composed of progenitor basal cells, ciliated cells and goblet cells (Figure 4). As the epithelium nears the gas exchange region, it becomes thinner, and the cellular composition changes, with increased expression of club secretory cells and decreased expression of ciliated cells in the bronchioles (Figure 4). Near the alveoli, the epithelium is simple and composed of two types of pneumocytes. These alveolar epithelial (AT) cells are either squamous shaped type I AT (ATI) or cuboidal-shaped type II AT (ATII) (Figure 4). The endothelium of the surrounding capillaries, together with the alveolar epithelium, forms the respiratory membrane, where gas exchange occurs by simple diffusion [2, 3, 14].

The different respiratory cell types and their characteristics, and molecular markers are summarized in Figure 4. Basal cells are adult stem cells underlying the basal lamina of airways and their principal role is to maintain and repair the tissue [16]. Basal cells represent 6 to 30% of airway epithelium, depending on the location in the respiratory tract [17]. They are responsible for attaching the columnar epithelium to the basement membrane zone as well as interacting with immune cells and neurons [18]. Basal cells are also important to promote regeneration after injury, due to their ability to self-renew and to differentiate into specialized cells such as ciliated and secretory cells. These specialized cells go on to form the pseudostratified airway epithelium (Figure 2G). Basal cells can be identified by several cellular markers, including tumor protein 63 (TP63), cytoskeletal protein keratin 5 (KRT5) and nerve growth factor receptor (NGFR) [19].

Multi-ciliated cells are responsible for MCC by providing a mechanism for trapping and expelling the pathogens and chemicals from the airways [20]. The rhythmic beating of cilia, essential for MCC, is achieved by the presence of abundant mitochondria at the apical region of ciliated cells, to ensure the availability of ATP to fuel ciliary motion [21].

Goblet cells are responsible for synthesis and secretion of gel-forming polymeric glycoproteins called mucins [22]. The mucins are the major constituents of the mucus which protects the airways by capturing pathogens and inhaled particles and expelling them through MCC. The mucus also contains water, ions, proteins and other macromolecules with anti-microbial, anti-protease and antioxidant activity, which are also important for local defense of the airways [23, 24]. Mucin-producing cells highly express the mucins MUC5AC and MUC5B, specific markers of goblet cells [24].

Club cells are non-ciliated epithelial cells that can differentiate into more specialized ciliated or goblet cells, depending on the environment. These cells play a role in airway repair after injury, in which they can dedifferentiate back to basal cells. Club cells secrete anti-inflammatory and immunomodulatory proteins, particularly club cell secretory protein CC-10/16, also known as SCGB1A1 [10, 25].

ATI cells, comprising 95% of the alveolar surface area, are responsible for the gas exchange process [26]. ATII cells produce pulmonary surfactant, which reduces surface tension during respiration, preventing the collapse of the alveoli [26].

The pseudostratified epithelium also contains rare cell types such as tuft/brush cells, pulmonary neuroendocrine cells (PNECs) and pulmonary ionocytes. These rare cell types represent about 1% of the airways (Figure 4) [10, 11].

The brush or tuft cells are rare chemosensory epithelial cells containing long and thick microvilli on their apical side. Their chemosensory function derives from their ability to secrete different biological mediators in response to diverse stimulus, such as bacterial signaling molecules [27].

PNECs are rare airway epithelial cells sharing both neurosensory and endocrine functions due to their ability to sense the environmental stimulus [28].

Ionocytes are the most recently discovered rare cell type, and their function is associated with ion transport, fluid and pH regulation. This cell type expresses high levels of CFTR protein and transcription factors FOXI1 and ASCL3 [29].

In summary, from the upper to the lower airway the epithelium lining changes in organization and cell content according to its function.

2.2 Airway epithelial cell models for respiratory research

Currently, several human-derived cell models are used for respiratory disease research, including primary cells from different regions of the respiratory tract and immortalized cell lines generated from primary cells.

2.2.1 Human primary cells

One of the most physiologically relevant models for respiratory disease studies are primary human airway epithelial (pHAE) cells. Those include human nasal epithelial (HNE) cells obtained from nasal brushing and human bronchial epithelial (HBE) cells obtained from explanted lungs. The first step involves the isolation of basal cells, which expand when cultured in well-defined media, increasing the cell population. When cultured under airway-liquid interface (ALI) conditions, these cells have the ability to polarize and to differentiate into specialized cell types forming the pseudostratified epithelium (Figure 2G) with mucociliary differentiation.

The formation of the pseudostratified epithelium with functional cilia beating and mucus secretion reproduce the key features of *in vivo* airways [30, 31].

Recently, 3D-models called organoids have emerged. Organoids are self-organizing 3D-structures that can be generated both from basal cells and ALI-differentiated cultures [31, 32]. Airway organoids are used to model different airway diseases [32], allowing a better understanding of the organ complexity.

However, primary cells have limited expansion and lose the capacity to differentiate after a few passages (typically 4). Furthermore, usage of primary cells for research requires both hospital ethical approval along with patient informed consent.

2.2.2 Immortalized cell lines

One way to overcome primary cell senescence is to use immortalized cell lines. These *in vitro* models can be generated from primary cells by lentiviral transfection of oncogenes and/or by the use of the human telomerase reverse transcriptase (hTERT) protein, a telomerase activator. Cancer cells have overcome senescence, for example, by introducing mutations in tumor suppressor genes. Immortalized cell lines are useful *in vitro* models for several reasons. First, they proliferate indefinitely, providing an unlimited supply of material. Second, since all cells come from the same initial cell, there is no genetic and phenotypic variation (isogenic cells), which guarantees consistent and reproducible results. Third, they are relatively easy to handle and cost-effective compared to primary cells. However, some disadvantages exist. These cells do not always recapitulate the physiological characteristics of the tissue of origin, as they have been transformed to proliferate indefinitely. Some immortalized cell lines lose the ability to differentiate or polarize.

Below, we describe the most important immortalized cell lines available to use in CF airway research.

16HBE14o- cells were isolated from the bronchial epithelium of a one-year-old male heart-lung patient and immortalized with the origin-of-replication defective SV40 plasmid (pSVori-) [33]. This cell line maintains the characteristic features of normal differentiated bronchial epithelial cells. When grown in liquid-covered culture (LCC) conditions, the cells form electrically tight and polarized cell layers with microvilli, but no cilia, and can be used in drug absorption or transport studies [34-36]. However, with increased passage, these cells start to lose their ability to polarize. 16HBE14o- cells are widely used in CF research, as they express high levels of CFTR mRNA and protein.

Another cell line widely used in CF studies is the **CFBE41o– cell line** that was created from the bronchial epithelial cells of a CF patient homozygous for the F508del *CFTR* mutation [37]. CFBE41o– cells were transduced to stably express wildtype and F508del *CFTR*. This process, however, generates lines with variable numbers of integrated sequences expressing exogenous *CFTR* at high levels [38, 39]. These cells can polarize and form tight junctions under LCC conditions. They are mainly used to study CFTR function and its response to small molecules [40-42].

The **BCi-NS1.1 cell line** was generated by immortalizing basal cells from a 42-year old Hispanic male healthy nonsmoking individual using retrovirus-mediated expression of hTERT [43]. These basal cells can be grown in ALI conditions, generating an electrically tight, pseudostratified differentiated epithelial monolayer. Over the period of 30 days in ALI these cells differentiate into ciliated, club and goblet cells, as well as ionocytes, while also keeping a population of basal cells [43].

The same investigators generated the **cell line hSABCi-NS1.1** using the same technique to immortalize primary epithelial cells from the small airways of a 50-year-old African American male, healthy nonsmoking individual [44]. Compared to the BCi-NS1.1 cells, hSABCi-NS1.1 cells have lower values of transepithelial electrical resistance (TEER) when cultured in ALI, as well as lower expression of basal, club and ciliated cell genes, with increased expression of small airway-associated genes [44]. Both BCi-NS1.1 and hSABCi-NS1.1 emerge as very promising *in vitro* models for studies of cell differentiation, as well as of the human air–blood barrier. The limiting factor in using these cells is the long 30-day culture duration.

Induced pluripotent stem (iPS) cells constitute a powerful cell model as they originate from an individual somatic cell and can generate any cell type if specific biological factors are provided in the media. As they are patient-specific, they can be used in personalized medicine approaches (e.g., prediction of drug efficacy). However, the lack of standardized protocols for iPS cell production and characterization, along with the laborious procedures required for their culture, hinders the wide application of these cell models [45].

2.2.3 Lung-on-a-chip

Standard cell culture techniques fail to represent the complex multifaceted interactions that take place in the respiratory system. Lung-on-a-chip technology combines microfabrication techniques with modern tissue engineering, to create a new *in vitro* model that can mimic the complicated mechanical and biochemical behaviors of a human living breathing lung. This technology involves a microfluidic device for continuous delivery of nutrients and removal of waste products that is divided horizontally by a thin, porous membrane, lined on one side by epithelial lung cells in interface with air, and lined on the other side by endothelial cells in interface with media, mimicking the capillary interface of the human lung. Two vertical side chambers undergo cyclic suction that makes the sheet of cells stretch and relax rhythmically, just like lung cells during breathing. This model has great potential to be used in drug development, disease modeling, and personalized medicine. It could also replace animals in scientific research, as it can mimic human responses. However, more optimization and standardization need to occur for this to become a reality. This model recapitulates the dynamic environment and mechanical forces present in the lungs. Cells from epithelia, endothelia and the immune system can be added to this microdevice. The biggest disadvantages of this system, preventing its widespread use, are its high level of specialization and complexity, as well as the equipment, techniques and high costs. Optimized standardized protocols for this system are necessary for its broader use in laboratories all over the world. Nevertheless, it has great potential for the future of human health research [46-48]. Co-cultures are a good example of taking the next step in *in vitro* research. This method involves the use of different cells such as epithelial, endothelial and immune cells, to create a more physiologically relevant model of the airways [49]. However, co-culture protocols are relatively complex and time-consuming which can hinder their broad application in research. Furthermore, data obtained from such studies should be carefully interpreted because this technique involves multiple variables that can influence data reliability [49].

2.3 Conclusion

In summary, there are multiple cell lines that can be used in airway research. Depending on the research goal, cells from either the upper or lower airway can be chosen. There is also the option to choose cell lines that can polarize and differentiate, all without having to use specialized and complex cell culture techniques as those used in primary cell lines. Nowadays, the variety of cell lines available for research is vast, and new models are emerging.

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Figure 1
Airway epithelium.

Composition of the pseudostratified epithelium of the large airway, small airway and alveoli of the human lung. The large airway is composed mainly of basal, ciliated and goblet cells, whereas the small airway is composed mainly of club cells. The alveoli are mainly composed of ATI and ATII cells. Adapted from [10].

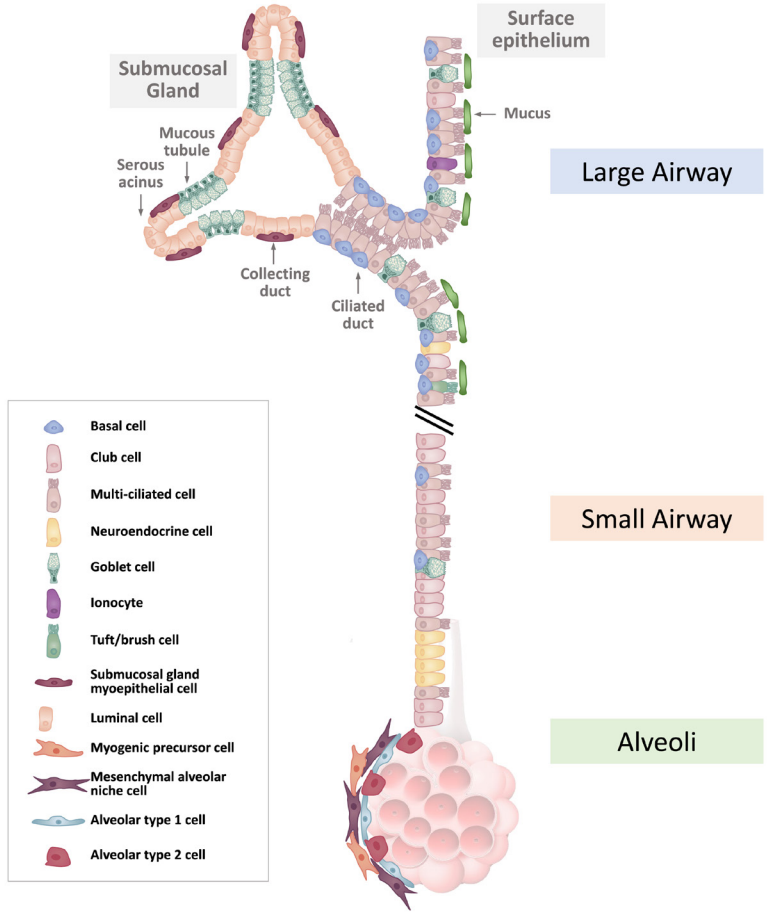

Figure 2
Epithelial cell classification according to their shape and organization.

A. Simple squamous epithelium,
B. Simple cuboidal epithelium,
C. Simples columnar epithelium,
D. Stratified squamous epithelium,
E. Stratified cuboidal epithelium,
F. Stratified columnar epithelium,
G. Pseudostratified columnar epithelium,
H. Transitional epithelium.

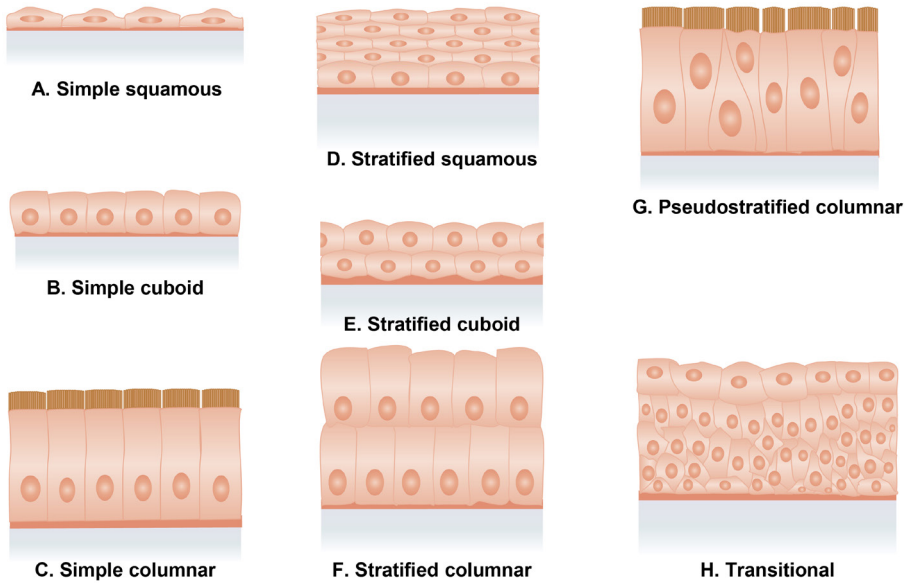

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Figure 3
Schematic representation of a polarized epithelial cell.

The apical side faces the lumen area or the external environment. The basal side is the region that faces the internal environment, and the lateral side is where adjacent cells are connected by adherent, tight and gap junctions. Adapted from [50].

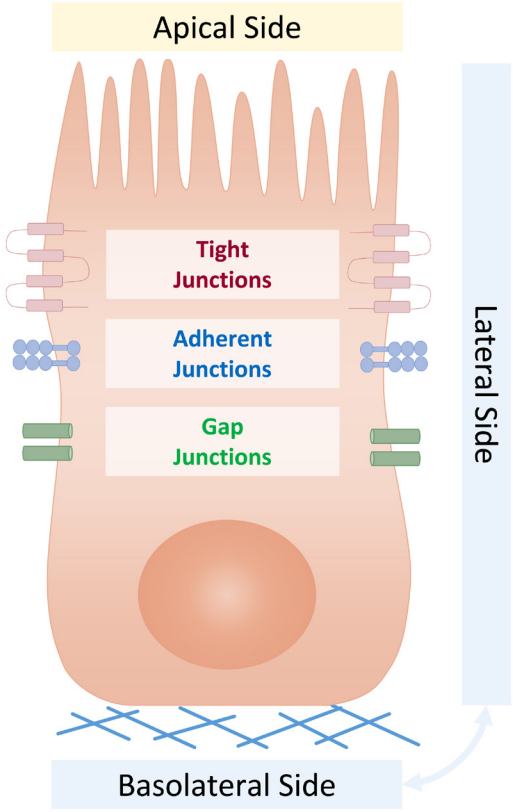

Figure 4
Respiratory cell types and characteristics [10,20,26,32,51].

Cell type	Role	Cellular marker	Localization	Progeny
Basal 	Regeneration and repair of airways	TP63 KRT5 KRT14	Proximal and distal airways	
Ciliated 	Mucus clearance	CDHR3 DNAI1 FOXJ1	Proximal and distal airways	-
Goblet 	Mucin secretion	SPDEF TFF3 MUC5AC	Proximal and distal airways	-
Club 	Precursor of ciliated and secretory cells	SCGB1A1	Proximal and distal airways	
Tuft/brush 	Immune response regulator	POU2F3	Proximal and distal airways	
Neuroendocrine	Neuropeptides secretion	CHGA CGRP	Proximal and distal airways	
Ionocytes 	Unknown/ Ion transport / pH regulation	FOXI1 CFTR ASCL3	Proximal and distal airways	-
Alveolar epithelial cells type 1 	Gas exchange	SPC	Alveoli	
Alveolar epithelial cells type 2 	Pulmonary surfactant production	HOPX PDPN AGER	Alveoli	

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Chapter 3
Macrophages in cystic fibrosis

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Introduction

Immune dysfunction is a hallmark of cystic fibrosis (CF) pathophysiology. Host defense and immune defects impair infection clearance and inflammation resolution, contributing to CF pathogenesis. The role of macrophages and monocytes in CF has been investigated since 1982, when the first reports of altered monocyte and macrophage activation emerged, outlining metabolic hyperactivity and increased production of pro-inflammatory cytokines, elastase, and tissue-damaging mediators such as matrix metalloproteinases. Nearly half a century later, there remains much to learn about the role of these cells in CF pathogenesis. Novel technologies enable the interrogation of macrophage and monocyte phenotypes and function at unprecedented resolution. Thus, our understanding of the essential role of monocytes and macrophages as drivers and modulators of pathogenic innate and adaptive immunity in CF continues to evolve. Here, we review the fundamentals of macrophage and monocyte biology in the context of CF pathogenesis, outline areas of ongoing interest and controversy, and look into the future for emerging fields of study that aim to leverage the contributions of these cells as a target for therapeutic intervention to delay or mitigate CF lung disease.

3.1 Macrophages: key contributors to lung disease

3.1.1 Role of macrophages in host defense and inflammation

Macrophages are present throughout the human body and populate nearly every tissue [1]. The 19th century Nobel laureate Elie Metchnikoff described macrophages as the “phagocytic” component of the immune system [2], combating infections directly through bacteria internalization and degradation. Macrophages are the first line of tissue immune surveillance through their unique capability to recognize pathogens. In the presence of microorganisms, a vast array of pathogen recognition receptors (e.g. complement receptors, Toll-like receptors [TLR], RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors [3]) activates a regulated inflammatory response that ensures microbial clearance and promotes inflammation resolution [4, 5]. In addition, macrophages continuously patrol and scan tissues for perturbations in their environment, responding to minute disruptions in pH, oxygen level, and metabolite concentrations, among others, to promote a return to tissue homeostasis [6]. Functionally, macrophages remove dying or dysfunctional cells, products of tissue damage, and cellular waste. In addition, macrophage molecular signatures demonstrate their plasticity in response to the surrounding environment [7-9] to perform unique tissue-specific tasks, such as removing phospholipid surfactant components in the alveoli or recycling iron in the spleen.

3.1.2 Advances in macrophage biology: role of tissue-resident and circulating subpopulations

Tissue-resident macrophages differ in respect of ontogeny, location, and function [10]. For example, alveolar and interstitial macrophages in the lungs originate during the embryonic and fetal waves of hematopoiesis and retain self-renewal capability [10]. However, over the life span of an organism, in response to aging and various insults (e.g., infection damage, pollution, etc.), damaged/senescent macrophages are replaced by circulating classical monocytes (defined as CD115+CD14+CD16- in humans [11]) in a CC-chemokine receptor 2 (CCR2)-dependent manner [12-24]. Influenced by the microenvironment, recruited monocytes undergo extensive reprogramming, and their gene profile becomes similar to tissue-resident macrophages, forming a complex mixture of embryonic- and monocyte-derived cells [10, 25, 26]. Meanwhile, circulating non-classical monocyte populations (CD115+CD14lowCD16+ in humans) patrol blood vessel walls of peripheral organs [27], replenish resident macrophages in a steady state, and help repair damaged tissues [22-28].

3.2 Advances in macrophage biology trained immunity

Until recently, innate immune cells were not thought to retain the "memory" of previous microbial encounters. However, it is now established that initial exposures to microorganisms, microbial products, or vaccines shape the epigenome of monocytes/macrophages through a biological process named trained immunity [29]. Long-term "memory" is ensured by the retention of epigenetic information in hematopoietic stem cells that give rise to the myeloid lineage [30]. This "learning" ability of innate immune cells from the first encounter with a microorganism constitutes an encoded ancestral host defense mechanism to address recurrent infections. It shapes the strength and duration of a future encounter with pathogens, ensuring a much faster, efficient, and less noxious response for the host [31-33]. However, an emerging line of investigation also shows that maladaptive trained immunity can lead to pathogenic responses, contributing to the severity of cardiovascular, autoinflammatory, and chronic inflammatory diseases [29, 30, 34].

3.2.1 Macrophages as therapeutic targets in inflammatory disease

Many inflammatory acute and chronic diseases are characterized by alterations in macrophage/monocyte abundance, heterogeneity, and function [35, 36]. Evidence suggests that macrophages are major drivers of exaggerated inflammation and pathogenesis in inflammatory diseases [37, 38]. Macrophage dysfunction is associated with pulmonary conditions (including CF, chronic obstructive pulmonary disease, asthma, pulmonary fibrosis [39-43]), severe infections (e.g., Covid-19 [44]), atherosclerosis, autoimmune disorders (e.g., rheumatoid arthritis [45], non-alcoholic steatohepatitis [46], Crohn's disease [47]) and metabolic diseases. The literature also extensively describes how tumor-associated macrophages create an immunosuppressive tumor microenvironment and contribute to cancer pathogenesis, by producing cytokines and growth factors and triggering the inhibitory immune checkpoint proteins released in T cells [48]. Based on the broad range of clinical conditions and pathogenic processes modulated by macrophages, therapeutic interventions targeting these cells may become the next frontier in the treatment of inflammatory disease. It remains unknown whether therapeutic interventions targeting pathogenic macrophage populations would be sufficient to prevent or ameliorate the pathogenesis of complex diseases with multiple pathogenic cell types. Further, macrophage heterogeneity creates challenges in the development and targeting of therapeutics aimed at specific subsets of cells. A lack of drug specificity that can distinguish pathogenic macrophages from their functional counterparts may render drugs ineffective or cause adverse effects. Understanding the biology of different macrophage subpopulations participating in the progression of diseases, identifying unique markers, and developing subset-targeted nanotechnologies for delivery will help refine therapeutic approaches that are safe and precise [44, 49-53].

3.3 Macrophages and monocytes in CF lung disease

3.3.1 CFTR expression on macrophages

Macrophages express low levels of cystic fibrosis transmembrane conductance regulator (CFTR) on their plasma membrane and cellular vesicles involved in the clearance of intracellular bacteria (e.g. autophagosomes) [54], and contain a low number of CFTR mRNA transcripts in their nuclei and cytoplasm (Figure 1) [55-57]. While overall CFTR transcript abundance is low, CF macrophages and monocytes harbor extensive abnormalities in antigen presentation, pro-inflammatory signaling, phagocytic activity, and cell survival transcriptional programs [57-59].

3.3.2 Transcriptional and functional transformation of monocytes and macrophages upon recruitment to CF airways

Macrophage numbers are elevated in the airways of fetuses and young children with CF before pathogen acquisition [60, 61]. This suggests that non-infectious lung inflammation occurs even in the early stages of CF. Increased numbers of pro-inflammatory monocytes and macrophages disrupt inflammation resolution, promote pathogen colonization, foster pathogenic remodeling in CF airways [23, 60, 62], and drive lung tissue damage [63]. Upon transmigration from the circulation, monocytes recruited into the lungs acquire a unique CF tissue-resident phenotype, suggesting that the lung environment (e.g., interaction with pathogens, pro-inflammatory milieu) modulates the phenotype and function of CF monocyte/macrophages [57]. For example, transmigrated CF macrophages display altered expression of immune receptors (e.g., TLR4, TLR5, CD11b, and MHC class II) and scavenger receptors involved in phagocytosis [23, 64]. Single-cell RNA sequencing studies allow detailed characterization of monocyte/macrophage subpopulations and their CF-specific gene expression profiles [65], including transcriptional transformations after exposure to pathogens such as *Burkholderia cepacia* [66] or *Mycobacterium abscessus* [67]. Epigenetic changes associated with these infections or muco-obstructive disease can also alter macrophage phenotypes and activity, contributing to defective function that exacerbates both infection and a dysregulated inflammatory response [68].

3.3.3 Metabolic dysfunction and metabolic reprogramming in CF macrophages

Macrophages from humans with CF and animal models exhibit numerous metabolic abnormalities. Mitochondrial dysfunction has also been reported in CF macrophages after infection [69, 70]. CF-associated defects in the balance of mitochondrial membrane potential, sustainability of available ATP, succinate, NADPH, protein kinase C, and calcium release contribute to impaired bacterial clearance in CF [58, 71]. Hosts require efficient phagocytosis, autophagy, and oxidative antimicrobial activity to clear extracellular and intracellular pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *B. cenocepacia*, and *M. abscessus* [70, 72-74]. While epigenomic alterations contribute to impaired autophagy and bacterial clearing, other metabolic abnormalities in CF may further disrupt bacterial clearance, including impaired management of

iron metabolism through ferroportin and heme oxygenase-1 dysfunction [53, 72, 75, 76]. Adequate macrophage function is essential to mitigate the intensity and duration of the exaggerated CF inflammatory response.

Metabolic reprogramming of CF macrophages has been implicated via the IRE1α-XBP1 pathway, which can contribute to the exacerbated inflammatory response related to ER stress, contributing to macrophage polarization toward a pro-inflammatory phenotype followed by altered metabolic activity [77]. The transcriptional and functional diversity of CF macrophage polarization actively participates in the inflammatory environment [78]. For example, the activation of the IRE1α-XBP1 pathway can be enhanced by the production of TNF-α [79] and microRNAs [80, 81]. This could represent a new direction for anti-inflammatory therapeutic development in CF. Further, epigenetic reprogramming of airway macrophages contributes to muco-obstructive lung disease [68]. In these studies, mucostasis induces polarization of macrophages toward an inflammatory phenotype that exacerbates pro-inflammatory phenotypes and enhances tissue damage.

3.3.4 Macrophage-specific phagocytic defects in CF

Defective autophagy, autophagosome formation, and pathogen-induced signaling defects in CF result in ineffective phagocytosis and phagolysosomal formation. Several mechanisms [58, 82] have been proposed as the underlying cause of these defects, including abnormal phagocytic priming by TLR4 [83], abnormal calcium conduction through TRPV2 [84], or direct pathogen recognition defects in NRF2 [85], Fcγ receptors, MARCO, CD36, Dectin-1 or Scavenger receptor A [86, 87]. The advent of CFTR modulators has provided more insight into these mechanisms of host defense in CF [88].

3.3.5 Mechanisms of excessive inflammatory signaling and impaired inflammation resolution in CF macrophages

Uncontrolled activation of PPARγ and PTEN-PI3K-AKT immune signaling, autophagy, the unfolded protein response, and inflammasome activation contribute to hyperinflammatory responses to environmental conditions in CF airways, including dehydrated mucus, presence of inflammatory mediators, and pathogen colonization [64, 76, 89, 90]. Macrophages isolated from *Cftr*$^{-/-}$ mice have low basal levels of PPARγ expression and attenuated lipopolysaccharide (LPS)-driven induction of PPARδ and LXRα [91]. In addition, in response to TLR-MyD88 signaling, CF macrophages display low activation of the PI3K/AKT signaling [92], which plays a key role in regulating immune function and in down-regulating levels of microRNAs that amplify TLR4 signaling (e.g. miR-155, let7e, miR-125b, and miR199a-5p) in macrophages.

Functionally, CF macrophages have a hyper-inflammatory phenotype. Studies have demonstrated that *in vitro* cultures of macrophages from peripheral blood of CF patients have an exaggerated inflammatory response to several inflammatory mediators, such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) abundant in CF airways [92-95]. Consistent with *in vitro* human data, macrophages from CF mice, rats, ferrets, and pigs have also been shown to be pro-inflammatory with increased production of pro-inflammatory mediators, such as interleukin (IL)-8, IL-6, reactive oxygen species (ROS), and lipid metabolites [92, 93, 95-99]. Further, studies in which bone marrow chimeras were made by transplanting wild-type and CF mice with either wild-type or CF bone marrow demonstrated that monocytes and macrophages exacerbate hyper-inflammation in CF lungs through defective CFTR function [100-102]. These data were corroborated in a *P. aeruginosa* infection model using myeloid-specific *cftr* knock-out and knock-in mouse models [96, 102].

3.4 Insights from human studies of macrophage distribution and function in CF

The past decade has seen an accelerated expansion of physiologic and molecular knowledge of macrophage function and how it correlates in clinical models of CF pulmonary and extrapulmonary disease [64, 65, 90, 103]. While these models may closely reflect specific aspects of CF pathogenesis, the fundamental roles of macrophages and monocytes in human CF disease are not fully understood. Emerging areas of interest in human-based research are noted below.

3.4.1 Airway macrophage distribution is influenced by age and disease state

The airways of children with CF may exhibit early signs of tissue damage and inflammatory cell recruitment [64]. Despite the presence of modest inflammation, children harbor an increased number of dysfunctional alveolar macrophages, with lower percentages of recruited monocytes, macrophages, and neutrophils, as shown in observational cohorts such as the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) [60, 104, 105]. During teenage years and into adulthood, as respiratory pathogen acquisition and exacerbation frequency increase, reports of macrophage-to-neutrophil ratios vary, likely representing a wide variability between those with residual CFTR function and milder clinical phenotypes and those with more severe and established disease [106, 107]. In adults, the distribution of airway monocytes and macrophages is also variable; however, adults often exhibit increased numbers of recruited monocytes and macrophage subpopulations that may represent the beginning of an exaggerated inflammatory response [57, 108]. Immune cells from respiratory secretions may be obtained from different sites, and therefore are likely to reflect microenvironment influences from microbial pathogens, microbiome, local inflammation and tissue injury, and pH/solute transport balance. While pro-inflammatory monocytes and heat-shock-activated macrophages may be detected in sputum samples [57], bronchoalveolar lavage fluid (BALF) may exhibit a predominance of alveolar macrophage subpopulations [108].

3.4.2 Macrophages as drivers or effectors of CF lung disease

The knowledge gap between transcriptional and proteomic profiles of individuals at different stages of disease and therapy, and the mechanism by which these profiles are associated with clinical outcomes in CF, raises the question of how monocytes and macrophages participate in CF lung disease pathogenesis. A specific question is whether monocytes and macrophages are initiators or effectors of pro-inflammatory signaling and impaired host defense that promote tissue injury and foster disease progression. For example, neutrophils are well-established contributors to inflammation, tissue damage, and CF disease in CF airways. While the number of neutrophils is proportionally larger in some CF airway samples, macrophages and monocytes are also enriched in the airways of stable children and adults with CF [57, 106, 108-110]. In the first single-cell RNA sequencing characterization of sputum from the airways of adults with CF, neutrophils accounted for 64% of all CF airway immune cells, while monocytes and macrophages only accounted for approximately 20% [57]. Yet, monocytes and macrophages exhibited a much higher number of differentially expressed genes than neutrophils, underscoring critical deficits in transcriptional programs associated with phagocytic activity, antigen presentation, cell survival, and exaggerated inflammatory responses [57]. While transcriptional differences do not prove causality, these impaired host de-

fense and inflammatory programs are consistent with previously reported monocyte and macrophage functional defects contributing to CF lung disease. Recently, another single-cell RNA sequencing study of BALF immune cells demonstrated that monocyte and macrophage cell populations also vary in CF airways according to the site of sampling, and uncovered several alveolar macrophage clusters with abnormal transcriptional programs that may contribute to CF lung disease [108]. These findings are consistent with previously reported observations [60, 111] and underscore the importance of considering age, sex, disease state, and area of sampling when interpreting the functional role and contribution of cell populations to CF pathogenesis

3.4.3 Impact of CFTR modulators on monocyte and macrophage function

Recent studies have investigated macrophage function in human primary monocytes from individuals treated with the potentiator ivacaftor alone or in combination with correctors lumacaftor and tezacaftor [65, 112-116]. These studies highlighted the broad impact of CFTR modulators on transcriptomic and proteomic profiles of blood mononuclear cells. Yet, these studies did not establish a relationship between transcriptomic or proteomic profiles and long-term clinical outcomes reported in clinical trials of these drugs [117-120]. More recently, these studies were extended to elexacaftor/tezacaftor/ivacaftor (ETI), a highly effective modulator therapy now available for pwCF with at least one F508del mutation [121]. These studies demonstrated that ETI treatment improves both important clinical outcomes and the antimicrobial function of monocytes/macrophages [122, 123]. One of these studies also reported that changes in macrophage CFTR function after ETI therapy had a strong correlation with improvements in percent predicted forced expiratory volume in one second ($ppFEV_1$), body mass index (BMI), and bacterial clearance in patients [123], suggesting that the effect of ETI on macrophages may predict the clinical response to ETI treatment in patients.

3.5 Future studies of importance

The significance of innate immunity in the pathogenesis of CF and for future therapeutic development becomes important in the era of CFTR modulator therapy which is expected to extend life expectancy of individuals with CF. Below, we discuss line of investigations that will need to be implemented.

3.5.1 Role of sex in monocyte and macrophage function

In the era of highly effective modulator therapy, variability in treatment response between men and women raises questions about the role of sex in immune contributions to CF disease [124-126]. Despite increased interest in this field, few data are available on the role of sex on monocyte and macrophage function. For example, women with CF experience increased rates of exacerbation and lung function decline than men, which may be partially attributed to monocyte and macrophage dysregulation [125-128]. *In vitro* studies have demonstrated that sex hormones modulate the inflammatory and antigen presentation functions of macrophages [107, 129, 130]. Therefore, it is reasonable to think that sex hormone fluctuations may also consistently modulate human monocytes and macrophages *in vivo*.

3.5.2 Role of monocytes and macrophages in extrapulmonary manifestations in CF

Pancreatic disease, diabetes, gastrointestinal disease, bone metabolism, liver disease, and cardiovascular issues are increasingly common complications of CF, as patients live longer in the age of modulators [131-133]. Hyper-inflammation and the presence of immune cell infiltrates, including macrophages, are observed in these extrapulmonary organs affected by CF [134-140]. Diabetes has been associated with dysfunctional macrophage autophagy [141], which would be consistent with defective CF macrophages [142]. Macrophage dysfunction in aging or chronic disease has been a focus outside of CF, and has also become a new research direction in CF [133]. Aging introduces the concepts of cellular senescence [86] and how this impacts macrophage function. Since many of the attributes of aging may or may not be related to deficient CFTR, but to chronic disease, therapeutics aimed at supporting immunity and susceptibility and management of infections may be beneficial [102, 143]. Future research should investigate how pulmonary and extra-pulmonary CF manifestations are linked to inflammation and organ remodeling due to the dynamic shift in the composition of resident and monocyte-derived macrophage populations.

3.5.3 Targeting monocytes and macrophages to impact CF disease severity and progression

Preclinical studies of CF suggest that therapeutic approaches to immunomodulate monocytes/macrophages can help improve lung inflammation [110], host defense mechanisms [82], and tissue remodeling [144]. Monocytes primed to express high levels of heme-oxygenase 1, an anti-inflammatory and antioxidant mediator, decreased pathological lung neutrophilic inflammation and IL-17 airway concentration without compromising the host defense against *P. aeruginosa* [51, 53, 92]. Moreover, treatment with recombinant resolvin (Rv) D1, a specialized pro-resolving lipid mediator, has considerable beneficial effects on CF macrophages, inducing pathways that favor the resolution of the pro-inflammatory response and increase phagocytic activity, thus improving morbidity in a CF mouse model of *P. aeruginosa* infection [145]. Drugs that modulate the IL-1 pathway and the downstream activation of NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) [146, 147], and therapeutic interventions that act to establish macrophage's autophagy flux during bacterial infections [148-150], are also effective in immunomodulating monocytes/macrophages, and improving the host response to bacterial challenges in pre-clinical models of CF. Future studies must investigate whether these observations in animal models can be confirmed in human studies.

3.6 Conclusions

CFTR-deficient macrophages exhibit a dysfunctional response to the challenging conditions of CF airways (Figure 2). This abnormal immune response is multifactorial, with epigenetic, transcriptional, metabolic, and functional contributions. The complex and wide-reaching abnormalities in CF macrophages result in abnormal innate and adaptive immune regulatory functions that foster excessive inflammation, delay resolution, and drive clinical manifestations. CFTR modulator therapy shows promise to mitigate the role of macrophages in CF lung disease. We look forward to future studies that elucidate the role of sex and aging in macrophage-driven CF pathogenesis and whether targeting macrophage subsets with immunomodulatory drugs to mitigate excessive inflammation delays the development of CF lung disease.

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Figure 1

ScRNAseq reveals an airway immune cell repertoire shift from alveolar macrophages to recruited monocytes and polymorphonuclear neutrophils in CF.

Uniform Manifold Approximation and Projection (UMAP) visualization of 20,095 sputum cells from nine pwCF and five controls. Each dot represents a single cell, and cells are labelled by **(A)** cell type, **(B)** disease status, and **(C)** *CFTR* expression. Note the overall low *CFTR* expression in airway immune cells.

Abbreviations: Mo=monocyte; MoMΦ=monocyte-derived macrophage; alvMΦ=alveolar macrophage; cDC: classical dendritic cell, pDC: plasmacytoid dendritic cell; B: B-lymphocyte; T & NK: T-lymphocytes and NK-cells; PMN: polymorphonuclear neutrophil.

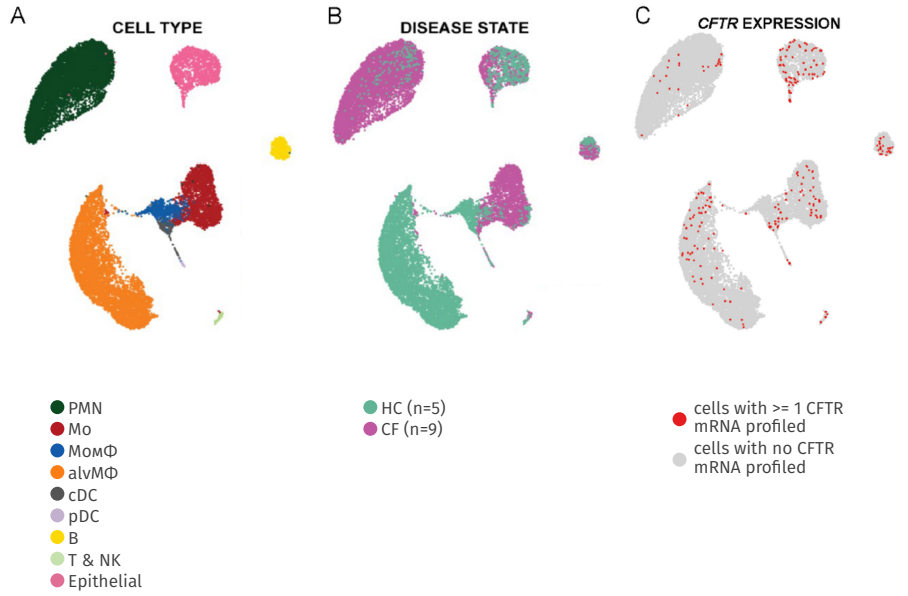
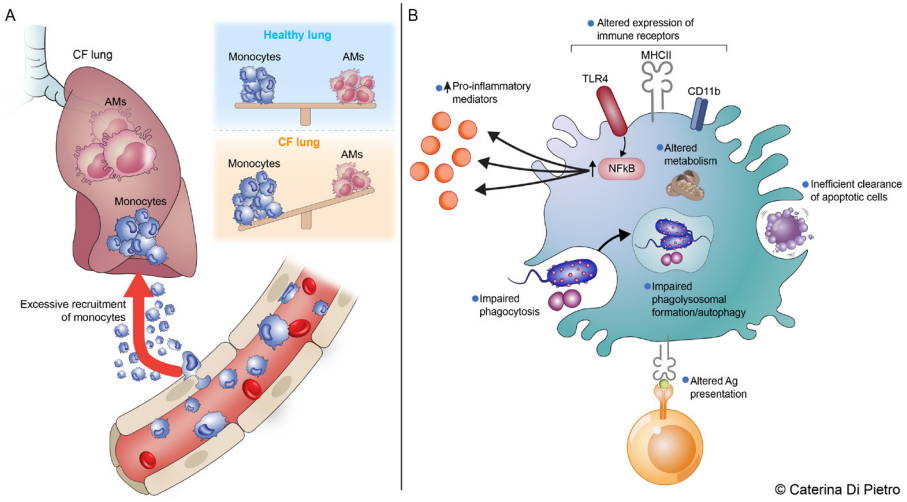


Figure 2

Pathways of macrophage and monocyte dysregulation in CF lung disease.

The macrophage/monocyte populations have an altered distribution in CF lungs **(A)** and display dysregulation of many vital pathways required for a normal immune response to pathogens and maintaining lung tissue homeostasis **(B)**.



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Chapter 4

Regulation of immune protection and pathology by CD4+ T cells in people with cystic fibrosis

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Introduction

People with cystic fibrosis (CF) suffer from chronic respiratory infections and inflammation caused by bacterial, viral, or fungal pathogens, leading to progressive impairment of lung function. CD4+ T helper cells, as well as CD8+ T cells, innate gamma/delta (γ/δ) T cells and natural killer (NK). T cells orchestrate adaptive immune responses and therefore play an essential role in both host defense against pathogens as well as immune pathology, such as chronic inflammation or allergy. In this chapter we focus on the role of CD4+ T cells in lung pathology of people with CF (pwCF) with CF, including the role of specific pathogens known to play a role in disease exacerbation and loss of lung function.

4.1 CD4+ T cells are central orchestrators of adaptative immune responses

CD4+ T cells express T cell receptors (TCRs) with a single specificity enabling selective recognition of pathogen-derived peptides (antigenic epitopes) presented by antigen-presenting cells on human leukocyte antigen (HLA) molecules. Following antigen recognition, CD4+ T cells differentiate into various effector subsets for an immune response optimized for the particular pathogen. In the thymus, differentiation occurs into mainly two distinct lineages. Firstly, conventional Th cells (Tcons) are essential for protective immune responses against pathogens, although insufficient or dysregulated Tcon responses can lead to immune pathologies such as allergies, autoimmunity, or inflammatory diseases. The second major Th cell lineage are the "regulatory" T cells (Tregs). Their main function is to control or suppress inappropriate immune responses against host and harmless antigens or to limit immune responses against pathogens to prevent immune pathologies. Tregs are characterized by expression of the transcription factor forkhead box protein P3 (Foxp3) and a specific epigenetic and transcriptional gene expression profile. Tregs and Tcons can be divided, according to their phenotype, into naive cells that have not been exposed to their antigen *in vivo* and antigen-experienced "memory" T cells.

Memory Tcons can further be divided into several Th subgroups according to their functional properties, e.g., their ability to produce specific effector cytokines: interferon (IFN)γ (Th1), interleukin (IL)-4, IL-5, IL-13 (Th2), IL-17, IL-22 (Th17, Th22), IL-21 (T follicular helper cells, TFH). These effector programs, which also include different migration and homing capacities, are centrally regulated by so-called master transcription factors that determine the overall effector program of the Th subset, such as T-bet for Th1, GATA-3 (Th2), ROR-gt (Th17) or BCL-6 (TFH). Although this functional classification has been helpful in the past, recent data revealed a variable degree of plasticity of T cell phenotypes, which is an active area of research.

Several lines of evidence suggest that both exaggerated inflammatory CD4+ T subsets and a deficiency in the number and function of Tregs may contribute to chronic airway inflammation in pwCF.

4.2 Alterations of regulatory T cells

Although Tregs are central for immune tolerance, few studies have investigated alterations of Tregs in pwCF. Reduced frequencies of peripheral Tregs were identified in patients with allergic bronchopulmonary aspergillosis (ABPA) [1], as well as in children with CF [2] which correlated with reduced lung function [2]. Hector *et al.* quantified Tregs in peripheral blood and airway samples from a larger number of children with CF (n>50) and showed that absolute numbers and percentages of CD4+CD25+Foxp3+ Tregs were significantly decreased in peripheral blood and also in bronchoalveolar lavage fluid (BALF) compared to controls [3]. The decreased number of peripheral Treg correlated with loss of lung function [3], as previously shown [2]. However, no differences in the frequencies of peripheral Tregs were observed between adults with CF and control adults [4]. Such variability may be due to the different ages of the study participants (see below) and/or technical differences to quantitate Tregs.

In addition, chronic *Pseudomonas aeruginosa* infection has been linked to a particular decline in Treg cells numbers, as compared to pwCF who never tested positive for *P. aeruginosa* [3, 5]. Colonization of pwCF with other pathogens, such as *Staphylococcus aureus, Haemophilus influenzae, Aspergillus fumigatus*, or *Candida albicans* had no effect on the Treg frequencies [3]. Furthermore, a link between *P. aeruginosa* infection and reduced Treg numbers was confirmed in a *Cftr*$^{-/-}$ mouse model of acute *P. aeruginosa* pneumonia [3]. In addition to Treg numbers, the suppressive capacity of polyclonal Tregs from pwCF was also impaired, when tested in *in vitro* suppression assays [3, 6]. However, the mechanisms underlying the reduced Treg numbers and function and how they may be influenced by *P. aeruginosa* remain poorly understood. CFTR modulator therapy had no effect on Treg frequencies in pwCF [5]. In *Cftr*$^{-/-}$ mice, dysfunctional indoleamine 2,3-dioxygenase-1 (IDO) activity has been suggested that was associated with reduced Treg cell activities and inflammatory responses [7]. Furthermore, decreased levels of 1a,25-dihydroxyvitamin D3, the biologically active form of vitamin D, were reported in pwCF, and *in vitro* treatment of CD4+ T cells with 1a,25-dihydroxyvitamin D3 resulted in an increase of transforming growth factor beta (TGF-β)-expressing Tregs [1]. Thus, enhancement of Treg function might be a novel approach to control chronic inflammation in pwCF.

The reduction in Treg numbers was more pronounced in older children with CF and affected memory, but not naive, Tregs [3]. This is partially in line with our own recent data, showing a marked increase of Tregs with a naive-like phenotype in pwCF [6]. The age-dependent differences in Treg decline suggest that Treg impairment in CF is acquired and not inherent to CF Tregs themselves [8]. Consistently, pwCF do not have clinical manifestations of general Treg dysfunction such as systemic autoimmunity, intestinal or skin inflammation, as observed in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) who carry a functionally impaired Foxp3 gene [9, 10]. Accordingly, we observed no differences in the frequencies or suppressive capacity of *A. fumigatus*-reactive Tregs in pwCF [6]. This supports the concept that Treg alterations in CF might be due to other mechanisms like an enhanced Treg turnover or exhaustion [3], which might be further enhanced by infections such as *P. aeruginosa*, but may not affect all Treg specificities to the same degree. It will be important to further disentangle the mechanisms underlying Treg alterations and the affected target specificities in pwCF in future studies, in order to develop targeted therapeutic strategies.

4.3 Increased Th2 responses in people with CF

The best documented evidence for a role of T cells in the pathogenesis of CF exists for Th2 mediated allergic inflammation in the context of fungal sensitization [11]. Although Th2 cells can exhibit many host-protective functions such as maintenance of barrier defense and regulation of tissue regeneration, excessive and chronic activation of the type 2 cells contributes to reduced pathogen defense and fibrosis [12]. In particular, fungal pathogens have been identified as triggers of allergic Th2 reactions in pwCF [1, 13-19]. In particular, ABPA, a detrimental hypersensitivity reaction against the ubiquitous airborne fungus *A. fumigatus*, is a frequent severe allergic complication, affecting approximately 5-15% of pwCF [20]. In addition to ABPA, sensitization to *A. fumigatus* is common in pwCF and recent data show that 40% of pwCF display a Th2 response against the fungus that is virtually absent in healthy individuals [6]. This suggests that altered host-pathogen interaction in pwCF contribute to the development of Th2 responses, such as more intense or prolonged fungal exposure, fungal filamentation and release of additional antigens and/ or a local lung tissue barrier defect. In healthy individuals, *A. fumigatus* generates a mainly Treg response. This suggests that in healthy individuals, *A. fumigatus* is a major tolerogen rather than an immunogen [21], similar to harmless plant pollen or house dust mite [13] (and reviewed in [21]).

The reasons why some pwCF develop ABPA, while others do not, are still not understood. Furthermore, Th2 responses are focused on *A. fumigatus* but not on other environmental fungi. Recently, cross-reactivity of *A. fumigatus*-reactive Th2 cells to other common filamentous fungi, such as *Scedosporium apiospermum* has been identified, in particular in patients with ongoing or a history of ABPA [6]. This suggests that the development of multi-fungal cross-reactive Th2-cells might be a potential risk factor for ABPA with several environmental fungal species contributing to the development and/or exacerbation of ABPA [6]. As mentioned before, *A. fumigatus-specific* Treg responses are not impaired in pwCF [13, 15]. However, like for other hypersensitivities, Th2 responses against *A. fumigatus* are focused on distinct allergenic proteins [6, 13, 22] and many of these proteins are secreted by the fungus. Comparison of Treg versus Th2 specificity revealed that Th2 cells only develop against *A. fumigatus* proteins that are not protected by specific Tregs. This indicates that antigen specific escape of Th2 effector cells from Treg-mediated tolerance is an important mechanism for allergy development [13].

Besides *A. fumigatus*, *P. aeruginosa* has also been shown to increase Th2 responses in pwCF [23-25]. *P. aeruginosa*-infected patients had significantly higher levels of Th2 cells and Th2-related cytokines in BALF compared to non-infected patients [23], which negatively correlated with lung function parameters [23]. However, it remains unclear whether *P. aeruginosa* itself triggers Th2 responses in pwCF or whether rather a pre-existing Th2-type cytokine milieu predisposes for *P. aeruginosa* colonization [25]. *S. aureus*, which is emerging as an important player in allergic inflammation [26], is frequently found in CF lungs and Th2 responses against certain secreted *S. aureus* proteins are elevated in pwCF [27].

Understanding the conditions which control Th2 cell development and maintenance in the CF lung and the characterization of their specific target antigens will be important to develop therapeutic or preventive immune modulation, targeting immune pathology without compromising protective immunity.

4.4 Th17 cells in CF

Human and murine studies suggest that pwCF display an increased Th17 immune response in the lung [14, 25, 28]. Increased levels of Th17-derived cytokines can be identified in lungs, bronchial mucosa, lung draining lymph nodes, sputum and BALF of pwCF [25, 28-31]. IL-17A production has been shown to be mainly attributable to CD4+ T helper cells in CF lung tissue, but also to several innate-like lymphocytes [28, 31, 32]. In experimental systems, Th17 has been described to mediate protection against fungi and different bacterial microbes. In humans, genetic or treatment-induced deficiencies of the Th17 system lead to increased colonization with *C. albicans* and to a lesser degree with *S. aureus*. This suggests that these two commensal microbes are major Th17 targets [33, 34]. We recently showed that among a panel of 30 different members of the human mycobiome, *C. albicans* was the only fungal species driving dominant Th17 responses in humans [14]. However, *C. albicans*-reactive T cell responses also induce a population of Th17 cells that is cross-reactive against the airborne fungus *A. fumigatus*, and these cross-reactive cells are strongly expanded in people with asthma, COPD and CF [14]. *A. fumigatus*-reactive Th17 cells were detected in lungs of pwCF [14, 28] where they may contribute to local tissue inflammation. Furthermore, increased *A. fumigatus*-reactive Th17 responses were identified in pwCF with untreated acute ABPA, but not in patients who were treated with anti-fungal treatment and corticosteroids or in patients with a history of ABPA [14]. In contrast, no difference in the Th2 compartment was observed between these patient groups, indicating that the selective increase of cross-reactive Th17 cells might be an initiator or exaggerator of acute ABPA [14], as has been shown in a murine lung allergy model [35]. In addition, increased Th17 responses have been linked to chronic *P. aeruginosa* infection, and specific Th17 cells against *P. aeruginosa* can be detected in peripheral blood of pwCF [25, 36]. However, a Th17-skewed immune response has been suggested to precede the colonization with *P. aeruginosa* [25], indicating that additional microbes may contribute to the expansion of Th17 cells in pwCF.

Th17 cells are known to contribute to recruitment, activation, and migration of neutrophils to sites of inflammation. Accordingly, a significant correlation of IL-17A and neutrophil counts in BALF was observed in pwCF [25, 31, 37]. Furthermore, increased IL-17A levels in BALF [25] and IL-17A secreting T cells in blood [4] negatively correlated with lung function parameters, as well as computed tomography (CT)-based assessment of lung tissue destruction [25]. Overall, this suggests that Th17 cells might play a central role for promoting pathogenic inflammation in CF and that inhibition of IL-17A might be a therapeutic strategy to reduce tissue destruction in pwCF [32]. Indeed, ablation of IL-17A in mouse models of CF resulted in reduced neutrophilic airway inflammation and structural lung damage [32, 38-40]. However, the role of Th17 cells for protection against lung colonizing pathogens and/or chronic inflammation, as observed in pwCF, is still not fully understood. For example, in murine models of acute pulmonary *P. aeruginosa* infection there is evidence that Th17 cells might play an important role in vaccine-induced protection [41]. Furthermore, the reasons for a Th17-skewed immune response in pwCF remain unknown. Further investigation is required to determine whether the Th17-skewed response reflects a genetic or environmental bias to preferentially prime Th17 responses, or whether it is rather a consequence of multiple infections with Th17-inducing pathogens.

4.5 Conclusion

Emerging data suggest that exaggerated Th2 and Th17 responses against lung microbes are a key feature of immune pathology in the CF lung. Key questions for the future are 1) what antigens are targeted by the pathogenic T cells, 2) which signals contribute to the pathogenic T cell phenotype development, 3) how to stratify patients according to the underlying specific immune pathology and 4) how the immune pathology can be specifically targeted therapeutically either via neutralization of the contributing cytokines or ideally via the direct elimination of the pathogenic microbe-specific T cells.

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Inflammation and infection in cystic fibrosis
Chapter 4 - Regulation of immune protection and pathology by CD4+ T cells in people with CF

Chapter 5
Airway smooth muscle cells in cystic fibrosis pathophysiology

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Introduction
This chapter covers the basics about smooth muscle, its role in the airways and how changes in airway smooth muscle can contribute to the cystic fibrosis (CF) disease phenotype. We also discuss how these changes are interconnected with other changes associated with CF, namely the impact of both cystic fibrosis transmembrane conductance regulator (CFTR) absence and the inflammatory environment.

5.1 Airway smooth muscle

Smooth muscle (SM) is a type of tissue present in the walls of hollow and passage organs, such as the intestine, the stomach, the uterus, arteries and veins of the cardiovascular system, and in the tracts of the urinary, reproductive, and respiratory systems. In SM, fibers group in branching bundles, which do not run parallel and ordered as in skeletal muscle, allowing SM cells to contract much strongly than striated muscle [1].

SM cells are in general 3-10 µm thick and 20-200 µm long. The cytoplasm is homogenously eosinophilic and consists mainly of myofilaments. In SM cells, nuclei are located in the center and the cell membrane forms small invaginations into the cytoplasm that are functionally similar to T-tubules formed in the skeletal musculature. SM cells anchor to the connective tissue by a basal lamina. Their cytoplasm contains large amounts of actin and myosin and a calcium-containing sarcoplasmic reticulum (SR), which participates in contraction regulation. Figure 1 presents the different cell types at the bronchi [1].

The main function of SM is contraction and its function in each organ system is quite specific – in the airway, the main function of SM is to regulate bronchiole diameter.

SM contracts in response to Ca^{2+} levels (see Figure 2) [2]. Intracellular calcium (Ca^{2+}) effluxes from stores in the SR in response to several stimuli. Upon release from the SR, Ca^{2+} binds to and activates calmodulin, which activates myosin light chain (MLC) kinase (MLCK) which in turn phosphorylates a regulatory light chain in myosin. Phosphorylation of MLC induces a conformational change which activates myosin ATPase activity and promotes the interaction between myosin and actin. This interaction triggers cross-bridge cycling, generating tension which is proportional to the amount of Ca^{2+} in the cell. SM contraction is further enhanced due to the presence of connexins that allow intercellular communication and Ca^{2+} flow to neighbor cells.

Pathological activation of SM cells in the airway is associated with respiratory diseases, especially with asthma – where hypercontractility of SM leads to airway narrowing and obstruction. A large proportion of individuals with CF present signs and symptoms like those of asthma,

such as cough and wheezing, as well as airway hyper-responsiveness to histamine or methacholine [3, 4] and some degree of airway obstruction [5]. This suggests that airway SM (ASM) plays a role in CF pathophysiology. However, few studies have addressed what has been coined "cystic fibrosis asthma" – a term that is used to describe people with CF (pwCF) who experience episodes of acute airway obstruction, who have family history of asthma or evidence of atopy [6].

5.2 Increased ASM mass associated with CF

Early studies assessing the dimensions of the airways in pwCF found an increased area of both the inner wall area and SM in lungs obtained from pwCF compared to samples from people with chronic obstructive pulmonary disease (COPD). These changes probably contribute to the severity of airflow obstruction and to the bronchial hyperresponsiveness which is characteristic of pwCF [7]. Later studies used bronchoscopy and design-based stereology to assess if the previously observed increased ASM content associated with CF was due to hyperplasia (increased ASM cell proliferation) or to hypertrophy (an increase in the structural and contractile machinery in individual cells). Although a small number of subjects were analyzed, an increase in ASM mean cell size was not detected, indicating hyperplasia without hypertrophy. This suggested that an accumulation of ASM cells is a possible mechanism contributing to airway narrowing and bronchial hyperresponsiveness in CF [8]. ASM hyperplasia was also observed in another study in school-age children with CF, suggesting that airway remodeling associated with CF occurs early in life [9]. However, this pediatric study also found that the increased ASM cell number was related to an increase in ASM cell size, suggesting that hyperplasia and hypertrophy can both play a part in the increased mass of ASM associated with CF. ASM changes observed in this study were similar between children with CF and children with bronchiectasis, suggesting that the abnormalities may not depend directly on CFTR [9]. Increased neutrophil levels are characteristic of both CF and bronchiectasis, therefore it is possible that the inflammatory environment is a key player in the changes in mass and function of ASM in CF.

5.3 *CFTR* expression and function in ASM cells

Although CFTR is primarily expressed in epithelial cells (in the airways and other organs), there is evidence that it is also expressed in several types of non-epithelial cells including lymphocytes, endothelial cells and cardiac myocytes [10-14]. *CFTR* expression has also been reported in SM cells, including tracheal smooth muscle, rat and mouse aortic and ileal smooth muscle, as well as in the bronchi of individuals with CF [10-12, 15-19].

Given its expression in ASM cells, it is plausible that the CFTR chloride (Cl^-) channel has a relevant role. Considering that Cl^- transport affects SM membrane potential and thus Ca^{2+} signaling, the absence of CFTR could potentially affect properties of contraction, relaxation and signaling. Primary cultures of human ASM cells obtained from the main or lobar bronchi of pwCF had a decreased Ca^{2+} release in response to histamine. In non-CF cells, a similar result was obtained upon CFTR inhibition (chemical or antisense oligonucleotide-mediated knockdown). These results suggest that CFTR in ASM cells modulates the release of Ca^{2+} in response to contractile agents, and its absence may explain the asthma diathesis observed in pwCF [20]. Similar results were obtained in a porcine model where the absence of CFTR resulted in ASM hypercontractility due to increased muscle tone, prolonged activation of the actomyosin network and deficient Ca^{2+} reuptake to the SR. This later defect was attributed to the absence of CFTR in the SR membrane, and thus to the lack of a Cl^- current that contributes to the driving force for Ca^{2+} inward flow [21]. Although this hypothesis is logical, there is no definitive proof of CFTR expression and function in the SR membrane – especially due to the lack of any obvious similarity of the biophysical properties of the SR membrane Cl^- conductance and CFTR [22].

Recent data using the CF pig model identified a transcriptional signature characteristic of CF ASM with changes in muscle contraction-related genes, ontologies, and pathways. Interestingly, proline-rich tyrosine kinase 2 (PYK) stood out as a relevant player in ASM physiology, being over-phosphorylated in CF, and therefore a target to decrease SM contraction [23].

5.4 Signaling and inflammation

Inflammation has a central role in CF lung pathology and disease progression. In CF airways, the inflammatory environment is characterized by a large number of neutrophils and increased concentrations of pro-inflammatory mediators, such as TNF-α, interleukin (IL)-1β, IL-6, IL-18, IL-17, IL-33, GM-CSF, G-CSF, and HMGB-1 [24]. Many of these mediators have the potential to increase contractile signaling in ASM.

In the presence of increased concentrations of inflammatory mediators, ASM becomes hypercontractile. Although ASM contractile properties are not different between pwCF and controls, the response in pwCF is significantly altered in the presence of IL-13, evidencing an impairment of β-adrenergic-induced relaxation, in association with increased MLCK expression which further contributes to the hypercontractile phenotype [25]. The IL-13 effect occurs through mediation of the Ca^{2+} response to stimulation with histamine. IL-13 promotes activation of c-jun N-terminal kinase and extracellular MAPKs and it also upregulates CD38, which leads to Ca^{2+} release in response to agonist stimulation [26].

IL-8 was also described to induce greater contraction in ASM cells from pwCF, which was apparently associated with increased levels of the contractile protein MLC_{20}. Similar to IL-13, the hypercontractile response to IL-8 suggests that the changes in ASM observed in pwCF are at least partially mediated by the inflammatory environment [27].

IL-17 is markedly increased and further promotes inflammatory signaling. Once chronic infection is established (particularly with *Pseudomonas aeruginosa*), IL-17 further promotes neutrophil influx. IL-17 has also been shown to promote airway hyperresponsiveness associated with ASM contraction. In addition, human bronchi pretreated with IL-17 exhibit increased contraction in response to methacholine [28].

Besides the contractility effect, the pro-inflammatory environment can also affect ASM cell migration and proliferation. IL-13 has been shown to inhibit ASM proliferation [26], but Th17 cytokines have been shown to increase ASM proliferation [29]. This latter study [29], in combination with the observation that IL-17 is increased in pwCF, could explain the observed increase in ASM mass in pwCF.

Although the relation between inflammation and ASM dysregulation in CF is a complex process, bacterial infection appears to be one of the main triggers, as it stimulates IL-8 secretion by epithelial cells leading to neutrophil recruitment, which then further release TNF-α and IL-8. This large increase in IL-8 induces greater ASM contraction, in part due also to increased MLC

expression and phosphorylation. Further IL-8 production may occur also due to activation of EGFR mediated by TLR signaling. Furthermore, the Th2 proallergic phenotype of CF T-helper cells is characterized by high concentrations of IL-4 and IL-13, which induce production of IgE, and altogether lead to airway hyperresponsiveness. Finally, IL-17, also raised in pwCF, increases ASM contraction and proliferation. IL-17 is also a key contributor to the asthma symptoms present in pwCF.

5.5 Conclusion

In summary, although the relation between ASM and CF has not been explored in detail, ASM is affected in CF. There is an increase in ASM mass (due to either hypertrophy or hyperplasia). CF is associated with ASM hypercontractility which can be attributed to either 1) ASM response to the characteristically inflammatory CF environment, or 2) the lack of CFTR (an aspect needing further clarification, despite the confirmed expression of *CFTR* in ASM).

Figure 1
Schematic representation of the cellular organization of the intralobar bronchi.

The main cell types of the surface epithelium are shown, together with the basal lamina and the airway smooth muscle cells.

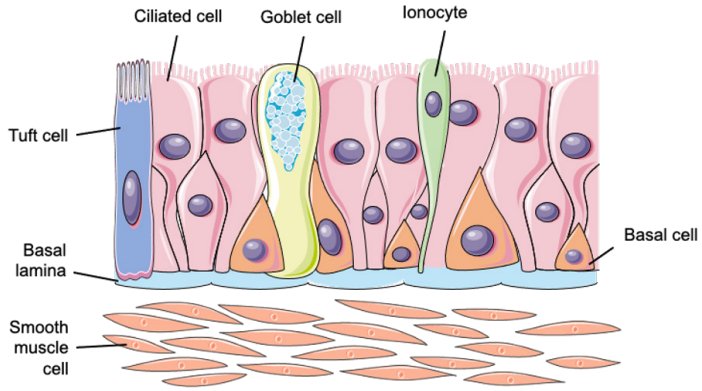
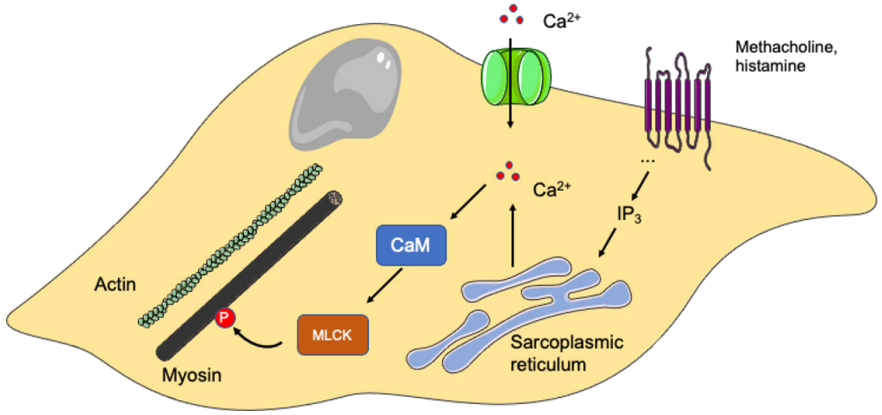


Figure 2
Overview of the signalling pathways driving airway smooth muscle contraction.

Cyclic cross-bridging of actin and myosin generate the contractile force and depends on phosphorylation of myosin. This occurs in response to an elevation of cytosolic Ca^{2+}, which activates calmodulin (caM) and consequently myosin light chain kinase (MLCK). Increase of cytosolic Ca^{2+} occurs mainly in response to G protein-coupled receptor activation by agonists such as methacholine and histamine, which leads to an increase in IP3 that promotes Ca^{2+} release from the sarcoplasmic reticulum.



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Inflammation and infection in cystic fibrosis
Chapter 5 - Airway smooth muscle cells in cystic fibrosis pathophysiology

Chapter 6

Lipid mediators in cystic fibrosis: from pathophysiological roles to therapeutic opportunities

Authors

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Introduction

Acute inflammation is a primordial response meant to protect against injuries or infections. The ideal and most common outcome is resolution, which eliminates the noxious stimuli and allows tissue return to homeostasis. Celsus' cardinal signs of inflammation *rubor* (redness), *tumor* (swelling), *calor* (heat), and *dolor* (pain) are macroscopic manifestations of cellular and molecular processes that occur in the microscopic scale. Polymorphonuclear neutrophils (PMN) are among the first leukocyte responders that swarm and accumulate in inflamed tissues. Monocytes enter the inflammatory site as a second wave and differentiate into macrophages that start resolution by clearing microbes, cellular debris, and apoptotic leukocytes through non-phlogistic phagocytosis. On the contrary, failure to resolve inflammation leads to further damage to the host.

Non-resolving inflammation is a hallmark and a contributor to morbidity and mortality in cystic fibrosis (CF). Inflammation in CF starts early after birth, affects almost every organ, and is strictly intertwined with the cystic fibrosis transmembrane conductance regulator (CFTR) basic defect [1]. Evidence indicates that absence or defects in CFTR result in excessive responses of cells involved in inflammation, such as leukocytes [2], platelets [3, 4], and endothelial cells [5]. Our group reported that CFTR loss-of-function triggers enhanced platelet activation, PMN recruitment, and impairment of pro-resolving mechanisms, such as PMN apoptosis, and production of selected specialized pro-resolving lipid mediators (SPM).

CF endothelial cells exhibit impaired barrier function, enhanced interleukin (IL)-8 production, reduced nitric oxide release, and increased vesiculation [5, 6]. An airway microfluidic chip with CF bronchial epithelial cells interfaced with endothelial cells demonstrated increased mucous production, ciliary beating and cytokine release, as well as enhanced PMN migration [7], thus mirroring *in vivo* hallmarks of airway inflammation in patients.

6.1 Lipid mediators in inflammation

Lipids are commonly associated with two basic biological functions: as building blocks of cells and as an energy source. However, there is also a third fundamental function of lipids, where slight modifications of lipid chemical structures generate signaling and regulatory mediators that are bioactive at relative low concentrations *in vivo*. Omega-6 and omega-3, the two main families of polyunsaturated fatty acids (PUFA) which are bio precursors of chemical mediators, have a prominent immuno-biological role and value as pharmacological therapeutic targets.

The biosynthesis of omega-6 PUFA and omega-3 proceeds through subsequential and alternating regiospecific desaturation and elongation reactions of linoleic acid (LA, 18:2n-6) or α-linolenic acid (ALA, 18:3n-3) (Figure 1). The term oxylipins refers to PUFA-derived compound produced by non-enzymatic or enzymatic oxidation by cyclooxygenases (COX), lipoxygenases (LO), and cytochrome P450 [8]. Octadecanoids are oxylipins derived from LA, ALA or other C18 PUFA. Eicosanoids are derived from C20 fatty acids such as arachidonic acid (AA) or eicosapentaenoic acid (EPA). Docosanoids are derived from C22 PUFA like docosapentaenoic acids (DPAs, 22:5n-3 or 22:5n-6), and docosahexaenoic acid (DHA, 22:6n-3).

Oxylipins can act near the production site, in an autocrine or paracrine manner, or have endocrine functions, but unlike other chemical mediators (hormones, cytokines, and growth factors) have a very short half-life (in the order of seconds) *in vivo*.

Seminal work of Ulf Svante von Euler at the Karolinska Institute of Sweden in 1935 led to the identification of eicosanoids, which he named "prostaglandins" (PG), from seminal gland extracts [9].

In the early 1960s, the structural elucidation of PG by Bergstrom, Samuelsson and colleagues, identified the omega-6 AA as a precursor of series 2-PG and series 4 leukotrienes (LT) [10]. Since these early works, a vast repertoire of chemical mediators biosynthesized from PUFA has been identified. AA is normally present in cell membranes as phosphatidylserine (PS) or phosphatidylinositol (PI) ester. In response to various stimuli (cytokines, growth factors, bacterial toxins), cells activate phospholipase A2 (PLA2), which in turn liberates AA.

Further metabolism of AA via COX leads to the formation of 2-series PG and thromboxanes (Figure 2) that are key mediators of vasodilation, vascular leakage, edema formation, pain, and blood cell activation (e.g., leukocyte, platelet) during inflammation. Moreover, thromboxanes are endowed with many homeostatic functions in the cardiovascular, renal, gastro-intestinal, and genito-urinary system (Figure 1) [11]. For this reason, classical anti-inflammatory pharmacological approaches that block these eicosanoids can have serious side effects (e.g., thrombosis, bleeding, gastric ulcer, renal failure) [12].

Leukotrienes are a different class of eicosanoids generated from AA by 5-LO, a dioxygenase that introduces hydroperoxyl groups into the PUFA backbone (Figure 2). This class includes LTB4 and cysteinyl-LT, i.e., LTC_4, LTD_4, and LTE_4 that are among the most potent mediators of immune cell chemotaxis and activation in inflammatory loci and have pivotal roles in bronchoconstriction, vasodilation, and allergic responses (Figure 1) [13]. For more detailed coverage, readers are directed to the following excellent papers [9, 14-17].

6.2 Lipid mediators in resolution of inflammation

One of the most significant strides in our comprehension of inflammation and immunity was the discovery that resolution of inflammation is not a passive process, due to disappearance of igniting agents (e.g., infectious microbes) and/or dilution of pro-inflammatory mediators. Inflammation resolves when noxious agents are actively removed, dead cells cleared, and destroyed tissues repaired [18]. Serhan and colleagues demonstrated that the resolution of inflammation is an active process initiated by the production of "proresolving molecules" (or immunoresolvents). To mathematically define cellular and biochemical components of active resolution, as well as to establish roles of proresolving mediators, quantitative resolution indices have been introduced in experimental settings [19]. Applying these indices into clinics could help monitor disease progression or response to therapies.

Among proresolving molecules are the proteins annexin A1 and melanocortins (linked to the corticosteroid pathway) and the lipid molecules derived from omega-3 and omega-6 PUFA lipoxins, resolvins, protectins, and maresins, collectively named as "specialized proresolving lipid mediators".

SPM stop excessive PMN infiltration and activation, counter pro-inflammatory signals, enhance the active clearance of pathogens and death cells by macrophages, protect organ from loss of function, and stimulate tissue regeneration thus accelerating the resolution of inflammation (Figure 3).

Using a system lipidomics-informatics approach to self-resolving inflammation, pioneering research from the lab of Dr. Serhan led to the discovery of SPM in inflammatory exudates during resolution [19]. SPM act locally and on different cell types by transiently binding to multiple receptors. Notably, SPM biosynthesis is inhibited by pro-inflammatory mediators, for instance PGE_2, generated during the onset of the inflammatory response [20], indicating that the beginning of inflammation programs its end (Figure 3). The main biosynthetic pathways and members of SPM families are shown in Figure 4.

LXA_4 and B_4, the first SPMs identified [21], arise from the transcellular metabolism of AA including PMN 5-LO and platelet 12-LO [22, 23]. In a second LX biosynthetic pathway, AA is converted into LX by the systemic activity of 15-LO in epithelial cells or macrophages and 5-LO in leukocytes [24, 25]. A third pathway produces isomers of LX 15-epi-LXA4 and B4 [26].

The four members of the E Rv system (RvE1, RvE2, RvE3 and 18S-Rv1) identified are derived from EPA via COX-2 and 5-LO transcellular biosynthesis [27] (Figure 4).

n-3 DPA is the second most abundant omega-3 PUFA in human tissues and is an intermediate in the biosynthetic route from EPA to DHA. n-3 DPA is also converted into a wide number of SPM, including RvD n-3 DPA, MaR n-3 DPA, PD n-3 DPA, and 13-series Rv (Rv); these SPM have a unique OH group in the C13 position of the PUFA chain, in contrast to other SPM [28-31] (Figure 4).

D-series resolvins (RvD) include six distinct molecules (RvD1-6) differing in the number, position, and chirality of their hydroxyl residues, and by position and isomerism of their C=C bonds [32]. RvD can carry a C17 hydroxyl group in the *S* (when derived via LO-initiated biosynthesis) or in *R* configuration (from acetylated COX-2-initiated routes). Aspirin and N-acetyl sphingosine acetylate COX-2 and raise 17*R*- RvD levels (e.g., 17R-*R*vD1) [33, 34].

Protectins are SPM of DHA produced by PMN, macrophages, and eosinophils, following enzymatic activity of 15-LO (PD1 or neuroprotectin when produced in neural system) or aspirin-acetylated COX-2 (AT-PD1) and subsequent enzymatic epoxidation and hydrolysis. PD1 isomerization leads to the production of PDX, a less-characterized PD [35].

Maresins (MaR1 and MaR2) are a third family of SPMs produced from DHA at sites of inflammation via 12-LO enzymatic activity in macrophages [36, 37], probably during the resolution of inflammation.

In addition, recent works uncovered additional SPM containing sulfido-conjugates derived from glutathione conjugation to DHA. These cystei-

nyl-SPM include resolvin conjugates in tissue regeneration (MCTR) and protectin conjugates in tissue regeneration (PCTR). cys-SPM also have tissue-protective activities, in addition to their pro-resolution role [38-40].

SPM regulate inflammation and resolution by activating different cells' G-protein-coupled receptors (GPCR) (recent reviewed in [41, 42]), which transmit signals quickly and activate intracellular pathways to promote resolution. Several SPM receptors have been identified to date, primarily using library screening, labeling ligands for specific binding, integrated with GPCR-β-arrestin systems, and functional cellular responses that include gain and loss of function in prototypical pharmacology approaches (Figure 5).

Readers interested in cell- and tissue/organ-specific SPM bioactions are directed to more recent reviews [15, 41].

6.3 Lipid mediators in cystic fibrosis

6.3.1 Lipid unbalances in CF

In 1929-30 George Burr and Mildred Burr conclusively established that PUFA are nutritionally essential for the growth and functions of almost every organ of mammals, settling a long-standing debate at the time [43, 44]. A vast literature demonstrates deficits or disequilibrium of PUFA in tissues from pwCF. Several studies reported a significant imbalance of omega-6:omega-3, with AA and decreased DHA levels in CF [45-47]. The main cause of these lipid abnormalities is fat malabsorption due to pancreatic insufficiency. However, the increased expression and activity of PLA2 associated with CFTR dysfunction represents another possible mechanism of elevated AA in CF [48]. Clinical studies aimed at restoring lipid imbalances in pwCF using dietary omega-3 were inconclusive, however these studies suffered from a lack of standardized dose and regimen, use of non-pharmaceutical-grade lipid supplements, or poor compliance of study participants. A recent randomized controlled trial on intravenous omega-3 PUFA treatment of Covid-19 met its primary endpoint of changes in inflammatory biomarkers for leukocyte counts and lipid metabolites, highlighting the important immunoresolving actions of omega-3 PUFA [49].

6.3.2 Lipid-derived chemical mediators in CF

It is now clear, that in addition to these lipid deficit and omega-6:omega-3 imbalances, the ability to produce endogenous SPM and to resolve inflammation is defective in pwCF, while the production of pro-inflammatory PG and LT is higher. Initial work by Konstan *et al.* found 5-15 times higher concentrations of LTB_4, TXB_2, PGE_2, and $PGF_{2\alpha}$ in bronchoalveolar lavage fluid (BALF) from pwCF compared to healthy subjects, and in sufficient amounts to recruit and activate PMN into the airways [50]. This has been confirmed recently using more advanced lipidomics approaches [51]. A significant decrease in LXA_4 and LXA_4:LTB_4 ratio in BAL [52, 53] and of RvD1 in sputum from pwCF were demonstrated [54, 55]. Results from our group demonstrate that CFTR dysfunction directly alters 12-LO activity in platelets required for LXA_4 biosynthesis [4] and subsequent studies demonstrated lower expression of 15-LO in CF cells [53, 56]. Collectively, these results indicate that defective SPM biosynthesis in CF is mechanistically linked to the CFTR basic defect through cellular mechanisms that remain unclear. On the contrary, the higher production of LTB_4 could reflect the enhanced expression and activity of PLA2 or 5-LO. A second possible explanation could be that the higher amount of AA released in CF cells is preferentially converted by 5-LO into LTB_4 rather than SPM. Along this line, Freedman and coworkers provided an elegant demonstration that 5-LO cellular localization is crucial to dictate LT or SPM biosynthesis in macrophages [57]. Finally, results from our group demonstrate that the ability to transduce SPM proresolving signals through GPCR is impaired in CF. In their studies, Simiele *et al.* and Pierdomenico *et al.* demonstrated a reduced promoter activity and negative epigenetic regulation of LXA_4 and RvD1 receptor ALX by the microRNA miR-1841b in cells from pwCF [58, 59], opening an intriguing line of investigation on the mechanisms the impaired resolution of inflammation in CF.

6.3.3 Pro-resolving lipid mediators in CF

SPM are endowed with exquisite protective actions to control excessive inflammation and tissue damage, and to enhance an active return to homeostasis. Wild type and *Cftr* knockout mice with chronic *P. aeruginosa* infection are a gold standard preclinical system for testing new anti-inflammatory agents for CF. Using this system, we demonstrated that RvD1 stops further PMN airway infiltration, reduces bacterial load, and prevents lung damage, survival, and weight recovery [55]. RvD1 also lowers level of several cytokines and chemokines that are increased in CF airways and stimulates clearance of *P. aeruginosa* through phagocytosis [55]. In acute *P. aeruginosa* in mice, a LXA_4 stable analog reduced PMN recruitment and bacterial load in the short term [52].
LXA_4 and RvD1 also restore airway surface hydration activating CFTR-independent Cl^- efflux and inhibiting Na^+ reabsorption in CF bronchial epithelia [60-63], which is particularly relevant for people with *CFTR* mutations who do not respond to CFTR modulator therapies. RvD1 and LXA_4 also dampen cytokine production and epithelial damage induced by bacterial infection [60, 63, 64], upregulating the expression of genes that promote cell survival, such as tumor protein 63 [55].

SPM counteract excessive inflammation during viral infections or viral and bacterial coinfections. For example, RvD1 and RvD2 shut down cytokine storm with human macrophages from pwCF and stimulate bacterial clearance [65]. 17R-RvD1 is protective during influenza A and pneumococcal pneumonia, and PD1 has direct antiviral activity on influenza virus. RvE1 reduces inflammation caused by herpes simplex virus. In light of the impact that respiratory viruses have on the CF population, these proresolving functions of SPM may be useful in controlling disease severity and perhaps long-term symptoms.

SPM regulate inflammatory responses in the circulation. They counter LTB_4-induced PMN adhesion and migration [66-68] and vascular permeability in lungs [69]. Further, SPM stop endothelial cells-leukocyte interactions activating nitric oxide release [70, 71] and reduce neutrophil extracellular traps that contribute to thrombosis and lung injury in CF [72, 73]. RvE1 has the unique property of reducing platelet:leukocyte interactions [74] and platelet aggregation and activation [75], which are enhanced in pwCF.

6.3 Conclusion

Acute inflammation is a protective host response, and its ideal outcome is complete resolution. Non-resolving inflammation characterizes CF and contributes to its morbidity and mortality. Lipid mediators play essential roles in inflammation and resolution, an active process where SPM are biosynthesized from omega-3 and omega-6. SPM include lipoxins, resolvins, maresins, and protectins. SPM biosynthesis and GPCR expression are defective in CF, leading to impaired resolution of inflammation. SPM act at multiple levels on cells and mechanisms involved in the pathophysiology of CF airway inflammation. Preclinical and clinical studies

suggest that SPM can activate resolution of infection and inflammation, thus opening the door for SPM-based human resolution pharmacology for individuals with CF.

Inflammation and infection in cystic fibrosis
Chapter 6 - Lipid mediators in CF: from pathophysiological roles to therapeutic opportunities

Table 1
List of mediators, their G-protein coupled receptors and biological action.

Mediator	Target GCPR	Biological action
TXA_2	TP	• Contraction and proliferation of vascular smooth muscle cells • Platelet aggregation • Bronchoconstriction
$PGF_{2\alpha}$	FP	• Bronchoconstriction • Contraction of the uterus
PGI_2	IP	• Release of vascular smooth muscle cells • Inhibition of platelet aggregation • Bronchodilation • Increased secretion of mucus in the gastrointestinal tract • Increased renin secretion (hypertension) • Hyperalgesia
PGD_2	DP1 DP2	• Bronchoconstriction • Chemotaxis of eosinophils and Th2 lymphocytes • Inhibition of platelet aggregation • Increased renin secretion
PGE_2	EP1 EP2 EP3 EP4	• Vasodilation and vasoconstriction • Increase or inhibition of platelet aggregation • Angiogenesis • Immune response inhibition • Fever and hyperalgesia • Angioedema • Increased gastrointestinal mucus secretion • Bronchodilation • Remodeling of bone tissue • Increased renal blood flow (diuresis) • Birth induction (high dose inhibition)
LTB_4	BLT1 BLT2	• Smooth muscle contraction • Chemotaxis of neutrophils
LTC_4, LTD_4, LTE_4	cysLT1 cysLT2	• Bronchoconstriction • Contraction of airway smooth muscles cells

Figure 1

Biosynthesis of omega-3 and omega-6 PUFAs.

The biosynthesis of longer-chain omega-3 and omega-6 PUFAs proceeds via a series of alternating position specific desaturation and elongation steps from ALA and LA, respectively. See [15] for further details.

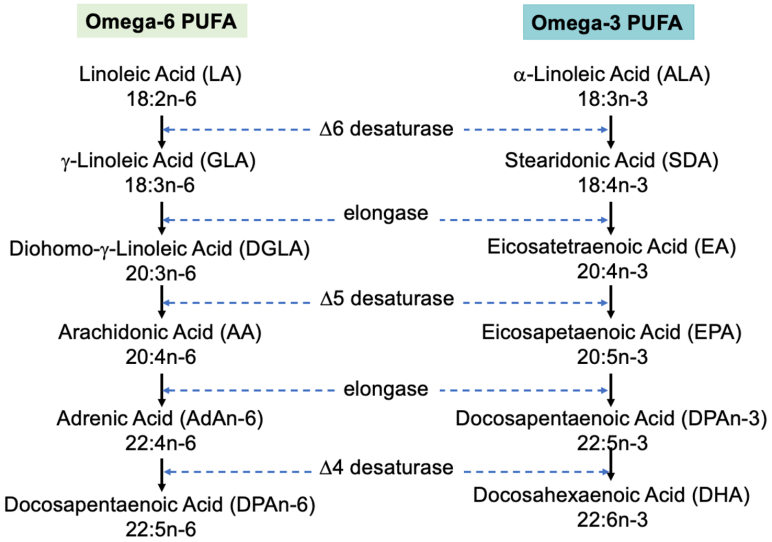


Figure 2

Biosynthesis of prostaglandins and leukotrienes from arachidonic acid (AA) via cyclooxygenase (COX)-1 and 2 and 5-lipoxygenase (5-LO) and subsequent enzymes.

For further details see [14]. 5-HpETE, 5-hydroperoxyeicosatetraenoic acid.

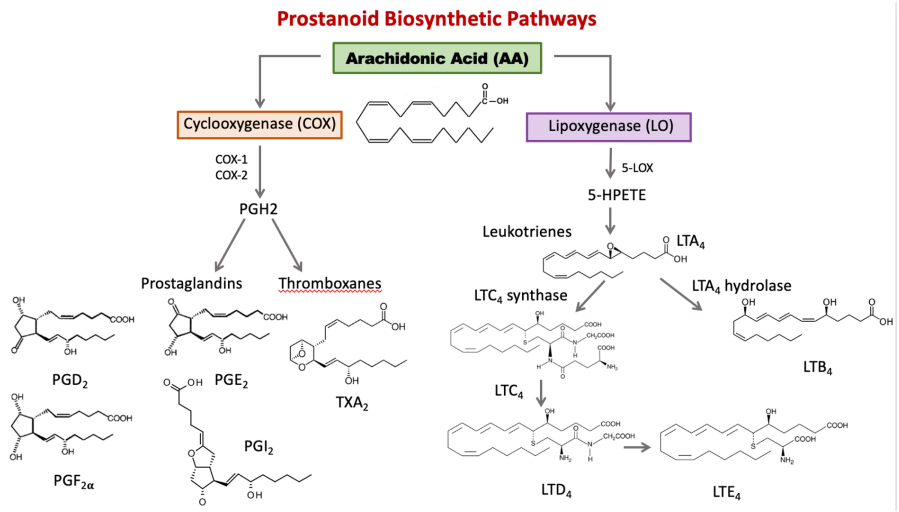


Figure 3

Schematic illustration of the acute inflammatory response with the temporal position of lipid mediators in this tissue protective response.

Injuries or infections ignite the acute inflammatory response that is normally a host protective mechanism. The first event in acute inflammation is oedema formation, followed by infiltration of polymorphonuclear neutrophils (PMN), and then monocyte and macrophages that clear PMN, dead cells, debris, and bacteria leading to resolution, which is essential for ensuring host protection and sparing from tissue damage. Prostaglandins and leukotrienes are local mediators of edema, swelling, leukocyte recruitment, and pain. A lipid mediator class switch introduces the active phase of resolution leading to the biosynthesis of specialized proresolving mediators (SPM). SPM controls resolution by actively ceasing excessive leukocyte recruitment and activation, reducing vascular permeability and oedema, regulating leukocyte differentiation and actions, and enhancing efferocytosis (i.e., non phlogistic phagocytosis of dead cells), microbial killing, and tissue repair.

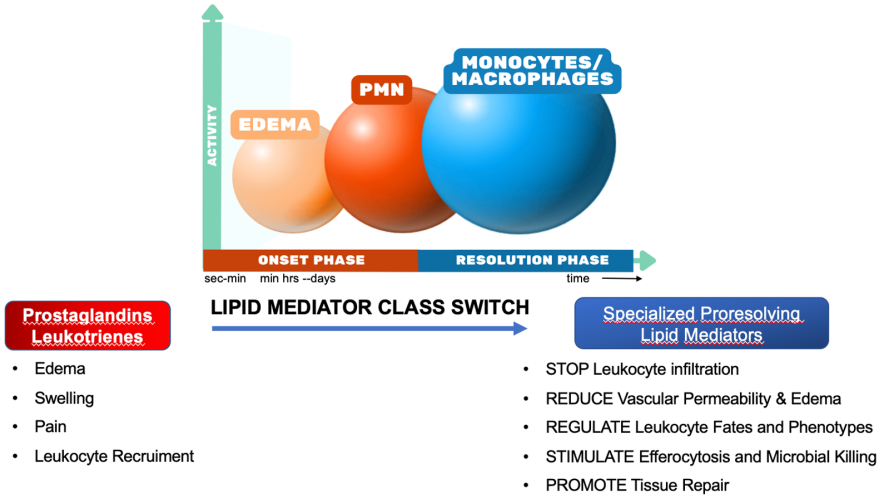


Figure 4

Biosynthesis of specialized pro-resolving lipid mediators (SPM).

SPM are biosynthesized from arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPAn-3), and docosahexaenoic acid (DHA) through cyclooxygenase (COX)-2 and different lipoxygenases (LO)

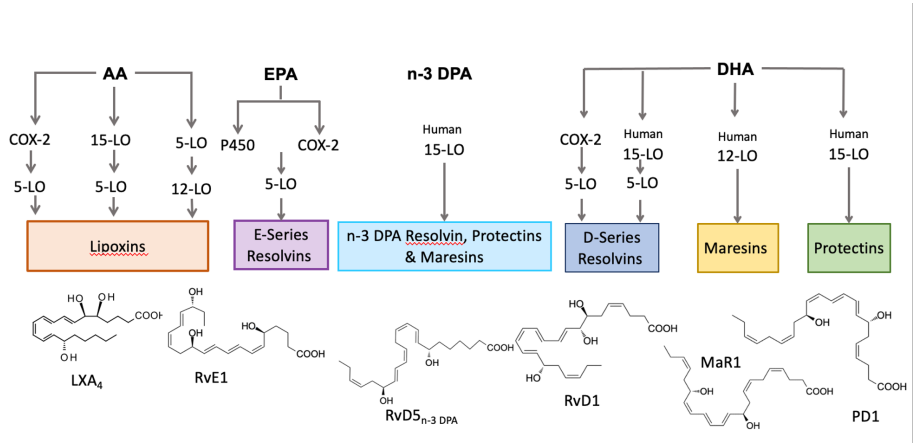
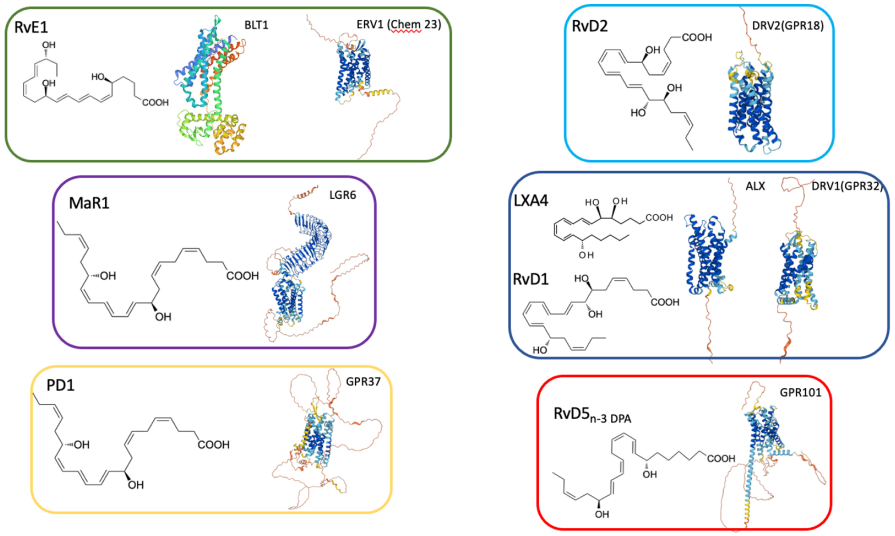


Figure 5
Structure of identified GPCR of SPM.

In addition to binding as an agonist ERV1/ChemR23, RvE1 also is an antagonist of BLT1 receptor. Molecular graphics and analyses of GPCR were performed with UCSF ChimeraX, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from National Institutes of Health R01-GM129325 and the Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious Diseases.



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Chapter 6 - Lipid mediators in CF: from pathophysiological roles to therapeutic opportunities

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Inflammation and infection in cystic fibrosis
Chapter 6 - Lipid mediators in CF: from pathophysiological roles to therapeutic opportunities

Chapter 7
Anti-inflammatory therapeutic approaches

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Introduction

Recognition of the role of inflammation in cystic fibrosis (CF) lung disease has prompted studies on anti-inflammatory drugs with the hope to prevent or slow lung deterioration. Inflammation in CF is complex and the exact pathophysiological mechanisms linking inflammation to lung anatomical modifications remain poorly understood [1]. Muco-obstruction is an important trigger of pro-inflammatory pathways, since the mucus plug both generates a hypoxic environment and provides a reservoir for bacteria [2]. Moreover, airway inflammation is excessive and sustained relative to the infectious stimuli, reinforced by an imbalance between pro-inflammatory and pro-resolution mediators, and propagated by neutrophils, macrophages and T-lymphocyte [1]. Anti-inflammatory therapies addressing these mechanisms include well known anti-inflammatory molecules or repurposed drugs, according to the pathways targeted.

7.1 Traditionnal anti-inflammatory therapies in CF

7.1.1 Glucocorticoids

In CF patients with severe disease or during exacerbations, short-term administration of oral steroids had no benefits [3, 4]. In contrast, children with milder lung disease [5-7], particularly those colonized with *Pseudomonas aeruginosa*, had improvements in clinical parameters and inflammatory markers [7]. At higher doses, serious adverse effects prompted the early termination of one trial [7] and whereas the beneficial effects on lung function were not sustained, growth retardation persisted years after the termination of treatment [8]. For inhaled corticosteroids, short-term studies suggested little improvement in either lung function or inflammation [9-11]. Higher doses seemed more effective but had an increased frequency of adverse effects [12]. Large retrospective studies reported a decrease in forced expiratory volume in the first second (FEV_1) decline, with greater benefits in 6-12 year-olds [13, 14]. Overall, although steroids slow lung disease progression, this comes at a significant cost, with serious adverse effects that must be closely monitored.

7.1.2 Ibuprofen

High-dose ibuprofen inhibits both the cyclooxygenase (COX) and lipoxygenase (LOX) pathways [15]. Pharmacokinetic studies stressed the need for individual adjustments in CF patients to achieve efficient plasma concentrations (50-100 µg/mL) [16]. Several trials report slower FEV_1 decline, improved body mass index (BMI) and fewer exacerbations [17-19], the effects being more pronounced in patients who were younger [17] and with less severe disease [20]. Recently, ibuprofen has been linked to improved long-term survival, but only in children with mild disease [21]. Despite being well tolerated and beneficial, the narrow therapeutic window, requiring individually titrated pharmacokinetics, could partly explain why ibuprofen remains infrequently prescribed.

7.1.3 Azithromycin

The immunomodulatory properties of azithromycin are not fully elucidated. With treatment duration less than six months, several studies report improvements in lung function and/or exacerbations, independently of *P. aeruginosa* colonization [22-25]. These beneficial effects, however, rapidly recede when treatment is ended [22]. Importantly, long-term administration improved lung function in both children and adults [26-28] and gains could be greater in *P. aeruginosa* infected patients [26, 27]. When treated with azithromycin from diagnosis, infants with CF had fewer pulmonary exacerbations, weight gain, and improved FEV_1, however bronchiectasis at 36 months of treatment remained comparable to placebo group [28]. Overall, the pulmonary benefits of azithromycin appear to be greater in *P. aeruginosa* infected patients treated for less than 12 months compared to non-colonized patients [28]. Chronic administration to obtain an anti-inflammatory effect must be balanced with the risk of bacterial resistance emergence.

7.2 Targeting neutrophils

7.2.1 Antiproteases

CF inflammation is characterized by a persistent neutrophil infiltration and a release of harmful serine proteases such as neutrophil elastase (NE). Endogenous inhibitors, such as alpha1-antitrypsin (AAT) and secretory leukocyte protease inhibitor (SLPI), are rapidly overwhelmed. Antiprotease therapies in development include aerosolized purified human AAT, recombinant AAT and recombinant SLPI. Inhaled AAT and SLPI decreased NE activity, but lung function remained unchanged [29-33]. Several synthetic NE inhibitors are in clinical or preclinical trials. AZD9668 improved inflammation biomarkers although clinical outcome was unchanged [34]. POL6014 aerosols showed good results in a phase I study [35] and a phase II study is underway in CF patients (NCT03748199). Antagonists for CXCR2, a chemokine receptor on neutrophils, are expected to decrease NE activity but SB-656933, a reversible CXCR2 antagonist, failed to improve lung function [36]. Another strategy is inhibiting serine proteases before their activation. Brensocatib, a dipeptidyl peptidase 1 inhibitor, successfully reduced exacerbation rate when tested in non-CF bronchiectasis [37] and a phase II study is underway in adults with CF (NCT05090904).

7.2.2 Antioxidants

Neutrophils also release large amounts of oxidants that can overwhelm antioxidant defenses and participate in lung tissue damage. Reduced glutathione (GSH), a major antioxidant, is decreased in CF airways and can be increased locally with inhaled GSH supplementation. GSH or GSH-precursors such as N-acetylcysteine (NAC) have been shown to suppress proinflammatory oxidants [91]. They have been greatly studied whether inhaled or per os, but only a few studies reported an improvement in FEV_1 [38-41], and when present, FEV_1 change was not always sustained throughout the duration of the study [41] or was only observed in a sub-group of patients [38]. Other antioxidants are also significantly lower in CF patients such as b-carotene. Supplementation has been associated with less pulmonary exacerbations [42, 43] although FEV_1 remained unchanged [43]. Combinations of different antioxidants do not further improve clinical outcomes [44, 45].

7.3 Targeting lipid mediators: leukotrienes, eicosanoids, ceramides

As described in Chapter 6, an imbalance in fatty acids in CF has been reported, with high omega-6 poly unsaturated fatty acid (PUFA) arachidonic acid (AA) and low omega-3 PUFA docosahexaenoic acid (DHA). Catabolism of AA gives rise to a series of pro-inflammatory second messengers, including prostaglandins and leukotrienes. Omega-3 PUFA such as DHA and eicosapentaenoic acid (EPA) on the other hand, lead to pro-resolving mediators. Lipid imbalance in CF also includes increased ceramides, another family of lipid mediators produced from sphingolipids.

7.3.1 Restoring the AA/DHA ratio

Omega-3 supplementation, with DHA and EPA, has indeed been reported to normalize the AA/DHA plasmatic ratio in nearly all studies [46-50]. However, studies were conducted on relatively small cohorts, only a few led to an improvement in lung function especially with short-term administration [48, 50, 51] or a decrease in inflammatory markers [50, 52, 53], and the two were rarely correlated. Compared to pediatric cohorts, adult cohorts reported a better gain in FEV_1 [48, 50, 51].

7.3.2 Leukotriene antagonists

Lipid mediators produced by the 5-lipoxygenase (LOX) generate LTA4, which is then converted to LTB4 by the LTA4 hydrolase. To date, leukotriene antagonists target either LTB4 or LTA4 hydrolase. Initial studies with the LTB4 antagonist amelubant reported no adverse effects [54], however a large clinical trial was prematurely stopped due to serious pulmonary exacerbations and respiratory signs [55]. More recently, Elborn *et al.* [56, 57] showed more promising results concerning safety, tolerance and efficacy with another leukotriene antagonist, acebilustat. This LTA4 hydrolase inhibitor reduced NE activity and neutrophil count. Although lung function was unaffected, pulmonary exacerbations tended to decrease in patients with better baseline lung function and in patients receiving concomitant CF transmembrane conductance regulator (CFTR) modulator therapy [56, 57].

7.3.3 Fenretinide

Fenretinide, or LAU-7b, is an orally available synthetic retinoid that has pleiotropic effects that are only partially understood. In mouse and cell models, fenretinide normalized AA/DHA, increased ceramide levels, reduced IL-8 release, and authors suggest benefits when combined with CFTR modulators [58-60]. In a phase I trial (NCT02141958) in adults with CF, ascending doses of LAU-7b demonstrated good safety and tolerability. A phase II study in 166 adults has been completed (NCT03265288) with full results pending.

7.3.4 Ceramides

Shingomyelinases hydrolyse sphingomyeline to ceramides. Repurposing of the antidepressant amitriptyline, a sphingomyelinase inhibitor, resulted in lower total ceramide levels and better lung function [61, 62]. Recent *in vitro* studies also showed promising results with myriocin, a sphingolipid synthesis inhibitor [63].

7.3.5 Pro-resolution

Lenabasum, a synthetic endocannabinoid-mimetic, binds to the cannabinoid type 2 (CB2) receptor on activated immune cells and fibroblasts. CB2 activation can help trigger the production of pro-resolving lipid mediators while reducing pro-inflammatory mediators [64]. A phase II, placebo-controlled study in adults with CF in 2016 assessed safety and tolerability, first at a low dose then at a higher dose. Although FEV_1 remained stable throughout the study, fewer exacerbations were reported, especially with the highest dose. Sputum neutrophils, and inflammatory mediators were also improved [65].

7.4 CFTR modulators

Studies *in vitro* show that pre-treatment with ivacaftor, ivacaftor/lumacaftor or ivacaftor/tezacaftor reduced cytokine production upon different stimuli (*P. aeruginosa*, lipopolysaccharide) [66-68]. Transcriptomic studies on whole blood cells from CF patients revealed differentially expressed genes in pre-ivacaftor/lumacaftor versus post-drug profiles that highlight pathways related to immunity and inflammation [69]. A possible anti-inflammatory effect of the triple therapy through lipid mediator signaling has also been suggested, with a modification in ceramide levels [70, 71]. Rowe *et al.* reported no significant changes after 6 months of ivacaftor treatment in any sputum markers of inflammation, including NE activity, despite improved airway obstruction [72]. In contrast, another study reported that ivacaftor reduced NE, IL-8, IL-1β during the first years of treatment [73]. In a larger adult cohort, IL-1β and IL-6 in bronchoalveolar lavage fluid (BALF) significantly decreased after 8-12 weeks of ivacaftor, and pulmonary function improved [74]. Ivacaftor/lumacaftor, ivacaftor/tezacaftor and the triple combination showed similar improvements in cytokine decrease [75, 76].

7.5 Novel targets

7.5.1 Cytokines

Several therapeutic strategies target the increased IL-8 cytokine levels, such as anti-IL-8 antibodies, IL-8 decoys, SCH527123 (navarixin) a CXCR1/2 antagonist that inhibits IL-8 binding and miR-17 overexpression, a microRNA that targets IL-8 [77-79]. Other directions focus on IL-17 for which monoclonal antibody therapies have already been developed and approved for other inflammatory diseases. Roscovitine, an orally available, selective cyclin-dependent kinase inhibitor, could regulate Th17 signaling [80]. In a 4-week trial in adults with CF chronically infected with *P. aeruginosa*, roscovitine was relatively well-tolerated, but no changes in inflammation markers, lung function or clinical outcome were observed [81].

7.5.2 Matrix metalloproteinases

Along with tissue remodeling and degradation of extracellular matrix, matrix metalloproteinases (MMPs) can release cytokines and chemokines. Several MMPs have been reported to be elevated in CF [82]. Andecaliximab (or GS-5745) is a monoclonal antibody that binds and inhibits MMP9 activity and has shown good tolerance in other inflammatory diseases [83]. Part 1 of a trial in CF adults to evaluate the effect of GS-5745 on FEV_1 has been completed (NCT02759562) but part 2 was discontinued due to a lack of participants.

7.6 Conclusion

Once bronchiectasis has occurred in CF lungs, the structural changes may imply persistent inflammation. Further studies are required to understand whether early introduction of CFTR modulators will avoid lung inflammation. Targeted anti-inflammatory therapies require a fine tuning between deleterious inflammation and preserving physiological inflammatory host defense to avoid serious adverse effects. Many novel therapeutic approaches are under evaluation, and it is important to identify the most appropriate patient population in terms of age, severity, infection, and co-therapies.

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Chapter 7 - Anti-inflammatory therapeutic approaches

Table 1
Principal clinical trials with traditional anti-inflammatory therapies.

	Drug regime	Study duration	Patients (severity of lung disease)	Inflammation markers	Lung function (FEV1)	Exacerbations	Ref
Oral corticosteroids	Prednisone (2 mg/kg/48 h)	4 years	45 children (mild to moderate)	ESR and IgG: ↓	↑	↓	[5]
	Prednisolone (2 mg/kg/day then 1 mg/kg/48 h)	12 weeks	24 children (mild)	IgG, IL1a, sIL-2R: ↓	↑ FEV_1	na	[6]
	Prednisone (1 or 2 mg/kg) *	4 years	285 children (mild to moderate)	IgG: ↓	↑ FEV_1 at 1 mg/kg ↑↑FEV_1 if PA	ns	[7]
	Prednisone (1 or 2 mg/kg/48 h)	na	224 children (mild to moderate)	na	ns	na	[8]
	Prednisone (2 mg/kg)	5 days	24 adults (acute exacerbation)	ns	ns	na	[3]
	Prednisone (2 mg/kg)	7 days	53 adults and children (acute exacerbation)	na	ns	ns	[4]
Inhaled corticosteroids	Beclomethasone	16 weeks	26 adults (chronic infections)	ns	ns	na	[9]
	Beclomethasone (1500 µg/day)	30 days	46 children (FEV_1 ≤55%)	na	↑FEV_1.	na	[12]
	Fluticasone (500 mg/day)	3 weeks	26 adults (chronic PA)	ns	ns	ns	[10]
	na	6 months	2978 children	na	↓ decline rate	na	[13]
	na	2 years	852 children (mild)	na	↓ decline rate ↓↓ in <12-year-olds	na	[14]
	adjusted to 50-100 µg/mL	4 years	85 adults and children (FEV_1>60%)	na	↓ decline rate, ↓↓ in <13-year-olds	ns	[17]
	adjusted to 50-100 µg/mL	2 years	142 children (FEV_1>60%)	na	ns	↓	[18]
Ibuprofen	na	2 years	17 175 children and adults (FEV_1>60%)	na	↓ decline rate	na	[19]
	adjusted to 50-100 µg/mL	28 days	72 children and adults (FEV_1>40%)	IL-6: ↓	ns	ns	[20]
Azithromycin	250 -500 mg 3 times/week	24 weeks	185 adults and children (chronic PA, FEV_1 >30%)	NE: ↓	↑ FEV_1	↓	[22]
	250 -500 mg 3 times/week	12 months	82 children (FEV_1 >40%)	na	ns	↓	[84]
	250 mg/day versus 1.2 g/week	6 months	208 children and adults (FEV_1>90%)	CRP: ↓	↑ FEV_1	↓	[23]
	500-1250 mg/week	8 weeks	38 children and adults (chronic PA, FEV_1 30-80%)	CRP, IL-8: ↓	ns	na	[85]
	250-500 mg 3 times/week	24 weeks	260 children (mild to moderate, no PA)	neutrophils, SAA, CRP, calprotectin: ↓	↑ FEV_1	↓	[25], [24]
	na	3 years	4715 adults and children	na	↓ decline rate if PA+	ns	[26]
	na	7 years	2055 children and adults	na	↓ decline rate	↓	[27]
	10 mg/kg 3 times/week	3 years	130 infants from diagnosis to 3 years	NE and IL-8: ↓	↑ FEV_1	↓	[28]

Abbreviations: CRP=C-reactive protein; ESR=erythrocyte sedimentation rate; FEV1=forced expiratory volume in one second; Ig=immunoglobulin; na=not assessed; NE=neutrophil elastase; ns=not significant; PA=*P. aeruginosa*; SAA= serum amyloid A

Table 2

Principal clinical trials targeting proteases and oxidants.

	Drug regime	Study duration	Patients (severity of lung disease)	Inflammation markers	Lung function (FEV1)	Exacerbations	Ref
Antiproteases	Purified AAT (1.5-3 mg/kg BID)	1 week	12 adults	NE: ↓	na	na	[31]
	Recombinant SLPI (50-100 mg BID)	1 week	20 adults (mild)	NE, neutrophils, IL-8: ↓	na	na	[29], [30]
	Recombinant AAT (500, 250, and 125 mg BID)	8-10 weeks	39 male adults	ns	na	na	[33]
	AAT (25 mg /day)	4 weeks	52 children and adults (FEV_1>25%)	NE, IL-8, IL-1b, TNF-α, neutrophils: ↓	ns	na	[32]
	AZD9668 (60 mg BID)	4 weeks	56 adults (moderate)	IL-6: ↓	ns	ns	[34]
	POL6014 (lonodelestat) 20-960 mg	24 h	74 adults (mild)	NE: ↓	na	na	[35]
GSH and NAC	Aerosolized GSH (66 mg/kg 4 times/day)	8 weeks	19 children	na	ns	na	[86]
	NAC (per os) (600-1000 mg NAC 3x/day)	4 weeks	18 children and adults (mild to moderate)	neutrophils, IL-8, NE: ↓	ns	na	[87]
	nebulized GSH (646 mg /12h)	6 months	153 children and adults (mild to moderate)	ns	↑ FEV_1 only at 3 months	ns	[41]
	GSH (per os) 65 mg/kg 3 times/day	6 months	44 children	calprotectin: ↓	↑ FEV_1	na	[40]
	Inhaled GSH (10 mg/kg/12h)	12 months	105 children and adults	ns	↑ FEV_1 if moderate lung disease	ns	[38]
	NAC (per os) (900 mg 3x/day)	24 weeks	70 children and adults (mild to moderate)	ns	Stable versus decrease in placebo	ns	[39]
	L-glutathione (per os) 65 mg/kg/day	24 weeks	60 children (mild)	ns	ns	ns	[88]
Vitamins	β-carotene (0.5 mg/kg) & Vit E (110-450 IU)	16 months	33 children and adults	NE/α1-PI complex: ↓	na	na	[45]
	β-carotene (1 then 10 mg/kg/day)	24 weeks	38 children and adults	na	na	↓	[42]
	β-carotene (1 then 10 mg/kg/day)	6 months	24 children and adults	na	ns	↓	[43]
	Vit E & Vit A versus Vit E, Vit A, Vit C, Se and β-carotene (25 mg)	12 weeks	46 children	na	ns	ns	[44]

Abbreviations: AAT=alpha one antitrypsin; α1-PI=alpha one proteinase inhibitor; FEV1=forced expiratory volume in one second; GSH=glutathione; Ig=immunoglobulin; IL=interleukin; na=not assessed; NAC=N-acetylcysteine; NE=neutrophil elastase; ns=not significant; SLPI=secretory leukocyte protease inhibitor; TNF-α=tumor necrosis factor alpha; Vit=vitamin.

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Table 3

Principal clinical trials targeting lipid mediators.

	Drug regime	Study duration	Patients (severity of lung disease)	Inflammation markers	Lung function (FEV1)	Exacerbations	Ref
Omega 3 supplementation	EPA (2.7 g/day)	6 weeks	16 adults (PA colonization)	neutrophil chemotaxis to LTB4: ↓	↑ FEV_1	na	[51]
	n-3 ethyl ester versus n-6 fatty acids	18 weeks	14 children	DHA: ↑, LTB4:↓	ns	na	[46]
	3.2 g EPA and 2.2g DHA vs olive oil ethyl esters	6 weeks	25 children (pancreatic insufficient)	AA/DHA ↓	na	na	[47]
	EPA and DHA (1.3% caloric intake)	8 months	50 adults	AA, IgG: ↓ linoleic acid: ↑	↑ FEV_1	↓	[48]
	algal oil (50 mg/kg/day of DHA)	6 months	20 children and adults	AA/DHA ↓	ns	na	[49]
	Omega-3 PUFA (390-1170 mg/day)	1 year	17 children and adults	LTB4/LTB5↓	na	na	[52]
	n-3, n-6 PUFA or saturated FA (50 mg/kg/day)	3 months	35 children and adults (severe, pancreatic insufficient)	ESR, IL-8: ↓ with n-3 ESR, AA: ↑ with n-6	ns	ns	[53]
	Seaweed DHA (50 mg/kg/day)	48 weeks	96 children and adults	ns	ns	ns	[89]
Leukotriene inhibitors	Amelubant (BIIL284 BS) 75-150 mg /day	196 days	420 children and adults (FEV_1 25- 85%)	na*	na*	↑↑*	[55]
	Acebilustat (CTX 4430) 50-100 mg/day	15 days	17 patients (mild to moderate)	NE: ↓ neutrophils: ↓ (100 mg group)	ns	Ns	[90]
	Acebilustat (CTX 4430) 50-100 mg/day	48 weeks	200 adults (FEV_1 >50%)	ns	ns	↓↓If FEV_1 >75 and/or CFTR modulators	[56], [57]
Amitriptyline	Amitriptyline (25-75 mg/day)	84 days	23 adults (chronic infection)	ceramide: ↓	↑ FEV_1 (25 mg group)	na	[61]
	Amitriptyline (25 mg/day)	28 days	40 adults (moderate)	ceramide: ↓	↑ FEV_1	na	[62]
CB2	Lenabasum 1 or 5 mg/day then 20 mg twice a day	16 weeks	74 adults (mild to moderate)	NE, neutrophil count, IL-8: ↓	ns	ns	[65]

* trial prematurely stopped due to serious adverse effects; several parameters not completely assessed)

Abbreviations: AA=arachidonic acid; DHA=docosahexaenoic acid; EPA=eicosapentaenoic acid; ERS=erythrocyte sedimentation rate; FEV1=forced expiratory volume in one second; Ig=immunoglobulin; IL=interleukin; LT=leukotriene; na=not assessed; NE=neutrophil elastase; ns=not significant; PA=*P. aeruginosa*; PUFA=polyunsaturated fatty acid.

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Chapter 8
CFTR modulators and inflammation

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Introduction

Cystic fibrosis (CF) is one of the most common, life limiting autosomal recessive diseases to affect people of European descent. It is caused by mutations in the cystic fibrosis conductance regulator (*CFTR*) gene. To date, over 2000 mutations have been identified, of which <400 mutations are known to cause CF. The defective gene results in abnormalities in the production and function of CFTR protein, an anion channel responsible for the transport of chloride and bicarbonate ions and a regulator of other epithelial transport proteins, including epithelial sodium channels (ENaC).

The multisystem nature of CF reflects the expression of *CFTR* in a wide variety of cells and tissues. Dysfunction of this essential protein results in a debilitating disease with clinical manifestations including chronic upper and lower airway infections, bronchiectasis, pancreatic insufficiency and intestinal malabsorption, and male infertility.

CF exhibits features of autoinflammation with infiltration of neutrophils and macrophages at target sites [1-3]. This exaggerated innate immune-driven inflammatory response is a critical driver of disease progression and is accentuated by the presence of acute and chronic infection [1, 2].

CFTR modulators are oral treatments which augment CFTR function by either improving CFTR intracellular trafficking with correctors or by increasing CFTR open probability with potentiators. The first CFTR modulator, a potentiator called ivacaftor, gained marketing authorization from EMA in 2012. Three combinations of correctors and potentiators are currently available: lumacaftor/ivacaftor, tezacaftor/ivacaftor and elexacaftor/tezacaftor/ivacaftor (ETI). Over 80% of people with CF (pwCF) are eligible for at least one CFTR modulator. Evidence is gathering on the impact of CFTR modulators on CF inflammation. In this chapter, we review the impact of CFTR modulators on local and systemic inflammation in CF.

8.1 The basic defect and inflammation

Mutations in the *CFTR* gene result in defective CFTR-mediated airway epithelial chloride and bicarbonate transport, with upregulation of ENaC-mediated airway epithelial sodium transport. These changes alter fluid and electrolyte homeostasis and result in dehydrated and acidified airway surface liquid (ASL), altered mucus viscoelasticity and defective mucociliary clearance. The recent introduction of the highly effective CFTR modulator combination ETI is associated with a significant improvement in lung function, reduced sputum expectoration and decrease in the frequency of pulmonary exacerbations. Clinical response to these small molecules appears to reflect the level of CFTR correction

and improvement in sweat chloride [4-7]. These drugs are likely to directly downregulate inflammation by improving CFTR function, increasing ASL hydration, normalization of mucus and reducing pulmonary exacerbations [8].

CF airways have increased sodium transport across apical ENaC with a corresponding increase in basolateral Na/K ATPase activity [3, 9]. Aberration Na^+ transport has been shown to accentuate the NLRP3 inflammasome, potentially through an increase in potassium efflux, a known trigger of NLRP3 activation [3, 10]. Overexpression of βENaC in mice results in CF-like lung disease, with ASL dehydration, inflammation and airway mucous obstruction [11-13]. In humans, genetic variants in the β- and γ- ENaC chains, with functional abnormalities in ENaC, have been associated with bronchiectasis and CF-like symptoms [14]. In contrast, mutations associated with hypomorphic ENaC activity appear to reduce disease progression in patients homozygous for F508del [15]. *In vitro* overexpression of βENaC in bronchial epithelial cell lines is associated with an increase in pro-inflammatory cytokine secretion, in the absence of CFTR dysfunction [3]. Similarly, in human bronchial epithelial CF cell lines, lipopolysaccharide (LPS)-induced NLRP3 inflammasome activity can be downregulated by S18, a SPLUNC1-derived ENaC antagonist [3]. These results suggest that dysregulated, ENaC-dependent signaling may drive exaggerated inflammatory responses in CF. It therefore follows that partial correction of CFTR function, as a result of CFTR modulator therapy, will positively impact on inflammation, by indirectly downregulating ENaC [16, 17]. However, to our knowledge, this ENaC-related effect of CFTR modulators on inflammation has not yet been reported in any *in vivo* or *in vitro* model. Other examples of triggers driving inflammation in CF include hypoxia, aberrations in mitochondrial metabolism, endoplasmic reticulum (ER) and oxidative stress [18-20].

8.2 Airway inflammation

Airway inflammation in pwCF is characterized by neutrophilic inflammation, persistent activation of nuclear factor-κB, increased concentrations of neutrophil elastase (NE) and elevated levels of pro-inflammatory cytokines, such as interleukin (IL)-8, and chemokines [21, 22]. Dysregulation of channel function is also associated with the intracellular accumulation of misfolded proteins and ER stress, with activation of the IRE1α-XBP1 pathway that regulates a subset of unfolded protein response genes [19, 23]. There is also growing evidence of a heightened inflammasome signaling in CF airways, with IL-1α, I-1β, and IL-18 being elevated in the sputum of pwCF compared to controls [24]. There is varying data on the impact of CFTR modulators on CF-related airway inflammation. In the small 2014 GOAL study (n=14), inflammatory markers in induced or spontaneously expectorated sputum did not decrease after 6 months of ivacaftor treatment [25]. Even after adding further patients to this cohort, reaching a total of 31 subjects, there were no significant changes in airway microbial communities or measures of inflammation following ivacaftor treatment [26].

In contrast, Hisert *et al.* found a significant decrease in sputum inflammatory markers in repeated assessments in 12 pwCF carrying a G551D mutation and treated with ivacaftor [27]. Already during the first week of ivacaftor treatment, NE, IL-1β and IL-8 significantly declined, and levels of these inflammatory markers continued to decline over 2 years of treatment. The decline in IL-1ß levels was confirmed by Nissen *et al*, in spontaneously expectorated sputum from 15 pwCF [28]. These findings accord well with results from non-invasively sampled upper airway epithelial lining fluid. Sequential measurement of nasal lavage fluid following ivacaftor therapy in patients with a G551D mutation showed a significant reduction in IL-1β and IL-6 levels, with parallel improvements in sweat test and pulmonary function [29]. A more recent study reported significant reductions in sputum IL-1β and IL-8 levels after one and three months of ETI therapy, respectively [30]. Improvements in systemic inflammation appear to mirror changes in lung inflammation following modulator therapy [31, 32]. Thus, restoring CFTR function *in vivo* has been shown to downregulate airway inflammation in CF. The observed differences between studies could be due to variations in sample size, collection techniques such as expectorated sputum versus induced sputum, ability to collect high quality samples as a direct result of treatment, sputum processing, patient age and prevalence of *P. aeruginosa* status [26]. An inverse relationship between microbial community richness in sputum and inflammatory markers in blood have been observed post-ivacaftor therapy [33]. Treatment with ivacaftor has also been associated with a significant reduction in serum calprotectin and granulocyte colony stimulating factor (G-CSF) and increases in calreticulin, changes which correlate with improved lung function [34].

Some discrepancies between responses to different CFTR modulators have been described. In a small cohort of preschool children, ivacaftor but not lumacaftor/ivacaftor was associated with reduced lower airway pulmonary inflammation [35]. A similar discrepancy in inflammatory markers between drug combinations has been described in CF monocytes [36]. Furthermore, short term treatment with ivacaftor/lumacaftor has also been reported to reduce sputum IL-1β concentration in F508del homozygous patients [37].

In vitro, ivacaftor/lumacaftor reduces IL-8 transcripts and p38 MAPK phosphorylation in fully differentiated primary bronchial epithelial cells following *P. aeruginosa* exposure. This CFTR-dependent mechanism is likely to contribute to the downregulation of inflammation in CF [38].

An anti-inflammatory response to CFTR modulators has also been demonstrated in animal models. How this equates to humans is difficult to interpret. However, the administration of ivacaftor in a humanized G551D rat model is associated with reduced neutrophil influx and lower airway concentrations of tumor necrosis factor (TNF)-α, IL-1α, and IL-6 [39]. This anti-inflammatory response was noticeably dampened down when ivacaftor was administered after lipopolysaccharide (LPS) stimulation.

8.3 Monocytes and neutrophils in CF and inflammation

Serum monocytes from pwCF exhibit an enhanced NLRP3-inflammasome signature with increased IL-18, IL-1β, caspase-1 activity and ASC speck release [3, 36, 40]. This exaggerated pro-inflammatory response can be downregulated differentially by CFTR modulators. One of the first studies to demonstrate potent systemic anti-inflammatory properties of CFTR modulators *in vivo* showed that a three-month treatment with tezacaftor/ivacaftor and lumacaftor/ivacaftor significantly decreased serum IL-18 and TNF-α levels [36]. Only tezacaftor/ivacaftor resulted in reduced IL-1β. When peripheral blood mononuclear cells were stimulated with LPS/ATP, tezacaftor/ivacaftor and lumacaftor/ivacaftor treatments resulted in a significant decrease in IL-18, TNF-α, caspase-1, and an increase in IL-10 [36]. A significant reduction in IL-1β and pro-IL-1β mRNA was only seen with tezacaftor/ivacaftor treatment. Similar results have been reported with ETI using a similar protocol [41]. In this study, P2X7R overexpression was proposed as a mechanism promoting inflammasome activation in CF. ETI therapy decreased NLRP3 ex-

pression in CF monocytes, and reduced inflammasome activation and IL-1β secretion [41].

The increased longevity of CF neutrophils is due to decreased apoptosis, which can be reversed by ivacaftor in patients carrying the G551D mutation [42]. Delayed neutrophil apoptosis results in increased neutrophil extracellular traps formation which is likely to accentuate inflammation [42]. One study reported no demonstrable effect of ETI on *in vitro* neutrophil inflammasome driven responses [24].

In F508del/F508del monocyte-derived macrophages, lumacaftor corrected phagocytosis of *P. aeruginosa*, but this restored phagocytosis was attenuated when ivacaftor was added. In contrast, ivacaftor alone and lumacaftor/ivacaftor reduce the monocyte-derived macrophage secretion of pro-inflammatory cytokines including IL-6, IL-8, TNF-α, interferon (IFN)-γ, and granulocyte-macrophage CSF, in response to *P. aeruginosa* [43].

8.4 CF pancreas and inflammation

CF is associated with exocrine and endocrine pancreatic dysfunction. Studies in children have shown improvement in both fecal elastase and diabetic status after CFTR modulator therapy [44]. Intriguingly, CF is associated with intra- and inter-islet inflammation with elevated IL-1β immunoreactivity in CF patients with and without diabetes [45, 46]. Increased IL-1β secretion has also been reported in other tissues including the lung and monocytes, with levels falling after CFTR modulator therapy [3, 36, 41]. Furthermore, IL-1β inhibits beta-cell function in both type 1 and 2 diabetes; this may explain one of the mechanisms resulting in improved glucose excursion in children post CFTR modulators. By down regulating local and systemic inflammation, CFTR modulators may play an important role in improved pancreatic function, especially in early life [45, 46].

8.5 CF gut and inflammation

The pathogenesis of gastrointestinal inflammation is likely to be multifactorial and related to a milieu of CFTR dysfunction, pancreatic insufficiency and malabsorption, CF-related diabetes, hyperacidity, gut dysbiosis, diet and exposure to drugs such as antibiotics. The gut may also modulate inflammation systemically and impact other organs such as the lungs through the gut/lung axis [47].

One of the commonest markers of systemic and intestinal inflammation is fecal calprotectin (FCP). This marker can be significantly elevated in pwCF compared to controls [34, 48-50]. FCP levels are increased in pancreatic insufficient individuals and levels correlate with severe CF phenotype, chronic *P. aeruginosa* colonization, CF-related diabetes and frequency of pulmonary exacerbations [51]. In contrast, FCP levels may fall following intravenous antibiotics, suggesting a link between bowel and lung disease [51, 52]. When obtained during clinical investigation of unexplained vomiting or for exclusion of coeliac disease, duodenal biopsy samples of pwCF (aged 2-23 years) have shown high levels of expression of ICAM-1, IL-2 receptor, IL-2, and IFN-γ, consistent with persistent local activation of the immune system in the absence of mucosal injury [53].

Several small studies have investigated the impact of ivacaftor on gut inflammation, with differing conclusions. For example, FCP was decreased with ivacaftor [54] and with lumacaftor/ivacaftor [54, 55], but another study found no significant change in gut inflammation after partial restoration of CFTR function with ivacaftor [56]. These varying results may reflect small population size and natural variation in levels of gut inflammatory markers. However, with implementation of ETI recently significant declines of FCP have been found in larger cohorts of pwCF from the US [57] as well as from Ireland and the UK [58] after 1 and 6 months of triple CFTR-modulator therapy. In parallel, in the latter study, a significant and marked decline of M2-pyruvate kinase (M2-PK) was found, which as a marker of cell proliferation is found elevated in inflammation and in malignant processes.

8.6 Conclusion

There is growing evidence that CFTR modulators downregulate local and systemic inflammation in CF. The effect can differ between modulator therapies and reflects a combination of improved lung and gastrointestinal physiology, reduced pulmonary infections and alteration/normalization in the innate inflammatory response. Up to now, correcting CFTR and sodium transport is likely to be the most effective way to tackle the exaggerated inflammatory response, through normalization of the underlying mechanism rather than inhibiting selected pathways which may increase the risk of infection. Further large scale studies are needed to characterize the anti-inflammatory properties of the next generation of CFTR modulators and to see if they are able to normalize inflammation.

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Chapter 9

Animal models to study inflammation and infection in cystic fibrosis

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Introduction

Cystic fibrosis (CF) is a multifaceted autosomal recessive genetic chloride (Cl^-) channelopathy, caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, and presents clinically as a multisystem disorder [1, 2]. To date, more than 2,000 *CFTR* mutations have been identified, most resulting in defective Cl^- transport and/or protein misfolding. These mutations are assigned to different classes depending on whether they involve defective *CFTR* (Classes I, II and V) or dysregulated Cl^- secretion (Classes III, IV, VI). The commonest and most clinically severe is F508del (ΔF508), a Class II mutation caused by a deletion of phenylalanine at position 508, present in one or both alleles in approximately 90% of people with CF (pwCF) [3].

PwCF have significantly increased morbidity and decreased life expectancy, primarily due to pulmonary disease, which is characterized by frequent exacerbations and chronic inflammation. The pathogenesis of CF lung disease is explained in part by loss of airway surface liquid (ASL) hydration, resulting in a reduction in periciliary height and a decrease in mucociliary clearance, leading to mucostasis and airway obstruction. The ensuing clinical course is hallmarked by persistent neutrophil recruitment [4, 5], with free neutrophil elastase activity detectable in airway samples of children with CF as young as 3 months old [6]. Despite the abundance of neutrophils however, the build-up of mucus secretions provides an ideal milieu for bacterial growth and colonization, initially *Haemophilus influenzae* and *Staphylococcus aureus* in infants, and later *Burkholderia cenocepacia* and *Pseudomonas aeruginosa*. In addition to neutrophils, a range of immune cells including lymphocytes, monocytes/macrophages and dendritic cells all express *CFTR* and are directly affected by altered *CFTR* expression or CFTR function, impairing their ability to resolve infections and inflammation [7].

Extra-pulmonary clinical manifestations of CF include impairment of the gastrointestinal tract, pancreatic insufficiency, liver disease and impaired fertility. In light of the range of clinical indications and immune cell expression profile of CFTR, researchers have explored animals to facilitate CF-related research studies [8, 9]. Animal models of CF have served to further our understanding of the mechanisms of disease progression and pathology and facilitated development of certain therapeutics. Over time, genetic technology improved and transformed the field, expanding research to include large animal species that have similar pathological outcomes to humans in terms of CF, including pigs and sheep [10, 11]. The *CFTR* knockout pig model and a pig with the F508del mutation were developed in 2008. The pig model has a number of research benefits including long life span for evaluation of CF disease progression and efficacy of long-term therapeutics. Smaller animals of CF currently used in research include mice [12],

rats [13], ferrets [14] and rabbits [15], with other non-mammalian models including zebrafish [16], and more recently, *Drosophila* [17], a popular insect model for studying human disease.

Over the last decade, CF care has been transformed by the development of small molecule drugs targeting the underlying CFTR protein defect. These compounds are known as potentiators and correctors, which function to improve the folding and/or trafficking of the mutated CFTR protein to the plasma membrane. The potentiator currently in clinical use to specifically target Gly551Asp (G551D) *CFTR* mutations is ivacaftor (VX770) [18]. The most recent and most effective CFTR modulator therapy is composed of three drugs: elexacaftor (VX-445), tezacaftor (VX-661) and ivacaftor. This triple therapy is known as ETI. Treatment with ETI is currently approved for patients either homozygous or heterozygous for the F508del *CFTR* mutation. This chapter provides an update on current CF animal models for the study of inflammation and infection of the airways, and highlights research exploring the impact of CFTR modulator therapy.

9.1 Mouse models to study CF pathology

The *CFTR* knockout (*Cftr*^{tm1UNC}) mouse was first proposed as a model of CF in 1992 [19] and was quickly followed by the generation of *Cftr*^{tm1CAM}, *Cftr*^{tm1HSC}, *Cftr*^{tm1BAY} and *Cftr*^{tm3BAY} [20-23]. Models with specific CF-causing mutations were also introduced, yielding class II F508del (*Cftr*^{tm1EUR}, *Cftr*^{tm1KTH} and *Cftr*^{tm2CAM}) and G480C (*Cftr*^{tm2HGU}) mutations [24-27], the class III G551D mutation (CFTR^{tm1G551D}) [28] and the G542X stop mutation [29] (Table 1). To further mimic human *CFTR* mutations, CF mice have been generated by transgenic expression of human *CFTR* on a mouse *CFTR* knockout background [12]. Of interest, treatment with G418, an aminoglycoside, increased read-through of the G542X mutation yielding functional CFTR protein production, making this an important murine model for further investigation of nonsense mutations [29].

The nasal epithelium of most mouse models of CF, including *Cftr*^{tm1EUR}, *Cftr*^{tm2HGU} and *Cftr*^{tm1G551D}, mirrors the abnormal electrophysiological profile seen in humans with significant hyperpolarization of nasal potential difference compared to non-CF mice [26, 28, 30-32]. In contrast however, none of the *CFTR* mouse models developed spontaneous lung inflammation without challenge. No lower airway epithelial abnormalities were reported for the knockout models *Cftr*^{tm1UNC}, *Cftr*^{tm1CAM}, *Cftr*^{tm1BAY} or *Cftr*^{tm1HGU} or in models carrying F508del or G551D alleles such as *Cftr*^{tm1KTH}, *Cftr*^{tm1EUR} or *Cftr*^{tm1G551D} [19, 21, 22, 26-28, 30]. This lack of airway abnormalities was thought to be due to the expression of a non-CFTR calcium-activated Cl^- channel (CACC) in mouse tissues [33]. A further mouse model of importance is the epithelial sodium (Na^+) channel (ENaC) model, involving overexpression of the β-subunit of ENaC (β-ENaC), leading to accelerated Na^+ absorption and a CF-like pulmonary clinical phenotype involving airway mucus obstruction and chronic airway inflammation, offering a related model for the study of CF lung disease [34].

9.1.1 Infection and inflammation therapeutic studies in mouse models of CF

As murine models of CF do not display chronic airway bacterial infection, experimentally-induced chronic bacterial infection models have evolved. Embedding bacterial cells including *P. aeruginosa* in agarose beads is widely employed, and successfully explored the impact of mucus on persistence of *P. aeruginosa* infection and the efficacy of antimicrobial therapies [35]. In turn, *Pseudomonas* lipopolysaccharide (LPS) is a driver of inflammation, triggering increased interleukin-1β (IL-1β) levels via NLRP3 inflammasome activation. NLRP3 inhibition *in vivo* with MCC950 inhibited IL-1β and improved *P. aeruginosa* clearance in the lungs of CF mice [36].

The cellular response of mouse CFTR to human therapeutic modulators has been explored. A recent study investigated the effect of tezacaftor and ivacaftor on mouse F508del CFTR channel conductance and reported improved CFTR-mediated Cl^- conductance in F508del macrophages, which impacted lysosomal activities for improved *B. cenocepacia* clearance [37]. Furthermore, an additional trigger of inflammation includes fatty acid abnormalities, involving alterations in the arachidonic acid (AA) and docosahexaenoic acid (DHA) ratio. DHA is normally metabolized to yield anti-inflammatory resolvins and protectins. The DHA:AA ratio in CF is imbalanced in favor of AA, contributing to the increase in pulmonary inflammation. Of major importance, airway function was significantly improved in mice homozygous for the F508del *CFTR* mutation upon treatment with ETI [38], and further improved by addition of LAU-7b, an oral formulation of fenretinide, previously shown in a clinical trial to normalize lipid imbalances in CF (NCT02141958). This indicates that mice treated with LAU-7b plus ETI have significantly better pulmonary function than mice treated with ETI alone [38]. This suggests that a potential clinical benefit from using ETI in combination with LAU-7b, further improving lung function in pwCF.

9.2 Pig models to study CF pathology

The pig shares 92% *CFTR* sequence homology with human *CFTR*, with anatomical similarities between the human and porcine lung [39]. The presence of submucosal glands and goblet cells makes the porcine model of CF airways disease a credible representation of the human CF phenotype, which can be assessed by computed tomography, as in humans [40]. The first CF pig was generated in 2008, resulting in two models: the null allele and the heterozygous F508del mutation [10]. Similar to people with CF [41], cAMP-stimulated Cl^- transport was diminished in nasal and tracheal transepithelial airways of CF piglets with the *Cftr*$^{-/-}$ mutation [42]. In 2011, *Cftr*$^{F508del/F508del}$ homozygous pigs were generated [43]. Restoration of CFTR activity *in vivo* using an adenovirus-based vector expressing human *CFTR* re-established transepithelial Cl^- current, increased (ASL) pH and decreased mucus viscosity [44].

9.2.1 Airway infection and inflammation in pig models of CF

The CF pig has provided an opportunity to study the inflammatory responses of CF airways at birth. In initial studies, the early transcriptional response to *S. aureus* challenge in CF pigs was explored, and induction of apoptotic genes (i.e. *CASP3*, *BCL10*, and *FASR*) in CF pigs was found to differ from non-CF pigs. The authors concluded that a loss of CFTR reduced the early transcriptional host defense response in CF piglets, compared with their non-CF counterparts [45]. Single-cell RNA-sequencing data indicated that a lack of *CFTR* minimally affected the airway transcriptome of newborn CF pigs, suggesting that infection and inflammation drive transcriptomic alterations in progressive CF [46]. In corroboration, chronic bacterial infections of the airways by *Staphylococcus*, *Streptococcus*, and *Enterococcus* have been reported in CF pigs [47, 48]. Moreover, reduced extracellular antiviral defenses have been documented against Sendai virus in CF pigs, although this effect was linked to differences in ASL pH [49].

When *Cftr*$^{-/-}$ pigs are born, they have no airway inflammation or infection, yet structural abnormalities exist, including tracheal ring irregularities, narrowed proximal airways and increased baseline airway resistance, which contribute to

obstruction [50]. Subsequently, CFTR was identified in the sarcoplasmic reticulum of porcine airway smooth muscle (ASM) cells, regulating ASM force generation. Of interest, pharmacological induced airway narrowing in healthy samples was impacted by ivacaftor, indicating that CFTR potentiators may reduce airway narrowing in people with CF with specific *CFTR* mutations [51].

9.3 Ferret and rat models of CF

The similarity in lung cell biology and anatomy between ferrets and humans makes the ferret CF model an ideal candidate for CF research. Similar to porcine CFTR, ferret CFTR also has a 92% amino acid identity with human CFTR (hCFTR) [52], with comparable submucosal gland expression of *CFTR* within the serous tubules. Moreover, CFTR pharmacologic and bioelectric functionality in ferret epithelia is similar to that of human epithelia. The ferret's short gestation period and time to reach adolescence (4-6 months) make it a cost-effective model to maintain. Development of this CF model utilized recombinant adenovirus gene targeting of the *CFTR* gene to introduce partial disruption of exon 10 or complete deletion of the *CFTR* gene [14], thereby generating $Cftr^{+/-}$ breeding pairs for the production of CFTR-deficient ferrets [53]. More recently, a ferret model homozygous for the G551D mutation was generated [54]. Even on antibiotics, CF ferrets are susceptible to bacterial lung infections (*Streptococcus*, *Staphylococcus*, *Enterococcus*, or *Escherichia*). Consistent with CF individuals, the $Cftr^{-/-}$ ferret presents with dysfunction of the proximal airways including impaired submucosal gland fluid secretion. Pulmonary disease progresses and is associated with mucus obstruction, impaired clearance and air trapping [55].

The rat CFTR amino acid sequence possesses 76% identity with hCFTR [56], with CF models including *Cftr* knockout [13, 57], F508del rat strains [13, 58] and a strain with the *Cftr* G542X nonsense mutation using CRISPR/Cas9 gene editing [59]. A further model included the humanized CF rat model expressing hCFTR with the G551D mutation [60]. These rat models exhibit a CF bioelectric profile in nasal epithelium, with increased mucus in nasal [57] and tracheal airways recorded [57, 58]. As the animals age, they develop airway submucosal gland hypertrophy, increased mucus viscosity, and exhibit deficits in mucociliary transport [61], with reduced ability to clear bacterial infections of *P. aeruginosa* compared to young knockout rats, in which mucociliary transport defects had not yet developed [33]. Components of the mucus that change as the rat ages, and that contribute to the viscosity of the mucus, included increased Muc5b concentration [62].

9.3.1 Therapeutics development using the ferret and rat models of CF

CF pathology in the ferret model has numerous similarities to humans, particularly neonates. The appearance of lung infections both at the start and end of the $Cftr^{-/-}$ ferret life span mirrors human disease, highlighting the potential benefits of this model. The development of the G551D specific mutant has established an exemplary animal model for CF. This model was used to assess whether *in utero* developmental abnormalities could be rescued by ivacaftor, the first study of its kind. $Cftr^{G551D/G551D}$ mice developed fewer pathologies in the pancreas, intestine and male reproductive tract. Of major interest, $Cftr^{G551D/G551D}$ ferrets appear to have sufficient *CFTR* expression to maintain normal lung clearance and innate immunity while on ivacaftor treatment. This model was then used to assess withdrawal of the modulator and subsequent progression of airway disease. $Cftr^{G551D/KO}$ ferrets who discontinued ivacaftor developed lung disease with mucus obstruction in the airways and submucosal glands. Quantitative proteomics of ferret bronchoalveolar lavage fluid (BALF) prior to, and one month after ivacaftor discontinuation, demonstrated significant enrichment of proteins associated with neutrophil degranulation [54].

The impact of ivacaftor has also been assessed in the hCFTR rat expressing G551D. These rats had reduced cytokine levels (including tumor necrosis factor [TNF]-α, IL-6 and IL-1α) after seven days of ivacaftor treatment. However, this anti-inflammatory effect of ivacaftor was absent in rats who also received LPS inflammatory stimulus [64]. This indicates that rescuing CFTR activity may not be sufficient to resolve established inflammation in the lungs of pwCF.

The G542X rat model may be valuable to assess how novel therapies can overcome premature termination codons in the CFTR transcript resulting from *CFTR* nonsense mutations [59].

9.4 Conclusion

Continued improvement of animal models will deepen our understanding of CF pathology and will be invaluable tools to test new or repurposed compounds and therapies for CF (Table 1). A recent study showed that *in utero* administration of CFTR modulators protects the animals from developmental abnormalities associated with CF [54], suggesting that early intervention could improve CF outcomes. Such studies offer a new approach to the use of existing models to understand the role of CFTR in CF pathophysiology and inform future therapies.

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Table 1
Studies that have employed CFTR modulators in animal models of CF.

Ref	Study year	Animal model	Mutation	Treatment	Outcome
[38]	2022	Mouse	F508del	Triple modulator (32 mg/kg ivacaftor, 21 mg/kg tezacaftor, 42 mg/kg elexacaftor) in combination with LAU-7b (25 mg/kg)	↓ long-chain ceramides ↓ airway hyperplasia and immune cell infiltration
[51]	2016	Pig	*Cftr*-/-	Ivacaftor (10 mM)	↑ transepithelial current in wildtype porcine airway epithelial cells only
[54]	2019	Ferret	*Cftr* G551D/G551D *Cftr* G551D/KO	Ivacaftor (20 mg/kg)	Improved pancreatic exocrine function & glucose tolerance. ↓ mucus accumulation and bacterial infections in the lung
[63]	2021	Rat	*hCftr* G551D	Ivacaftor (30 mg/kg)	↓ inflammatory cytokines No impact on lipopolysaccharide (LPS) response

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Inflammation and infection in cystic fibrosis
Chapter 9 - Animal models to study inflammation and infection in cystic fibrosis

Chapter 10
Epidemiology of airway infection in cystic fibrosis

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Introduction

This chapter provides a general overview of the major pathogens involved in cystic fibrosis (CF), while subsequent chapters delve deeper into specific pathogens. People with CF (pwCF) develop typically recurrent infections during their lifetime. The microbiome changes over the lifetime and is therefore age-dependent. Bronchopulmonary exacerbations are usually caused by bacteria such as *Pseudomonas aeruginosa* or *Staphylococcus aureus* [1-3].

10.1 Bacteria

In children with CF, *S. aureus* and *Haemophilus influenzae* are typically detected. However, in older pwCF, *P. aeruginosa* is more commonly detected, followed by *S. aureus*. Other rare bacteria (with a prevalence less than 20% in adult pwCF) are also detected.

Within the rare bacteria group, *Stenotrophomonas maltophilia, Achromobacter* spp. and *Burkholderia* spp. are the main bacteria that cause chronic colonization as well as acute bronchopulmonary infections [4-7]. Interestingly, the epidemiology of diagnosed bacterial infection has changed notably over the last two decades, especially for *P. aeruginosa*. As per the 2022 CF Foundation Patient Registry Annual Report, the percentage of individuals with a positive culture for *P. aeruginosa* has declined. The largest decline was observed in pwCF younger than 18 years, for whom the percentage with a positive *P. aeruginosa* culture declined from 47.0% in 1997 to 16.8% in 2021 [1]. Reasons for this decline might be multifactorial. Beside early antibiotic eradication treatment of incident infections, implementation of CFTR modulator therapy since 2012 may also play a role [8]. More scientific knowledge about the microbiome in pwCF has been gained over the last years by using methods such as 16S ribosomal ribonucleic acid (rRNA) gene sequencing of respiratory tract specimens. It helped to characterize the polymicrobial nature of lower airway infections in CF. These studies revealed that the core microbiota included *Streptococcus, Prevotella, Rothia, Veillonella*, and *Actinomyces*. However, it was observed that when common CF pathogens such as *Pseudomonas, Burkholderia, Stenotrophomonas*, or *Achromobacter* were present in the lungs, these microorganisms tended to dominate the microbial community within individuals [9-11]. A major research question in microbiology for CF is whether the current highly effective CFTR modulator therapy will impact the microbiome, even in individuals with chronic colonization of these pathogens.

10.2 Fungi

Over the last few decades, multiple studies have investigated fungal colonization and infection in the airways of pwCF. While *Aspergillus fumigatus* and *Candida albicans* remain the most common fungal species detected in pwCF, recent research has shown that the range of fungal species associated with CF has significantly diversified [12-21]. Thus, other significant fungal pathogens in CF have been recognized as potentially causing severe, frequently difficult to treat fungal infections. These pathogens include some *Scedosporium* species (*S. boyidii, S. apiospermum, S. aurantiacum, S. minutisporium*) and the closely related species *Lomentospora prolificans*. In addition, very rare fungal species such as *Exophiala dermatitidis, Arxula adeninivorans* and *Trichosporon mycotoxinivorans* have been described causing severe fungal pneumonia in pwCF [22-27].

Invasive fungal infections are often diagnosed late, resulting in delayed and inadequate treatment. PwCF also commonly experience allergic bronchopulmonary aspergillosis (ABPA) and bronchitis caused by fungal infections [28-32].

The diagnosis and treatment of *A. fumigatus*-related conditions remain a challenge in CF due to overlapping features of disease, and absence of clinical guidelines for *A. fumigatus*-related conditions outside of ABPA.

As *Aspergillus* spp. are the filamentous fungus detected most often in CF, the impact of this species is of high importance. A German study demonstrated a significant lower forced expiratory volume in one second (FEV_1) in pwCF where *Aspergillus* spp. could be detected in respiratory samples in a meaningful number. In particular, pwCF without chronic *P. aeruginosa* infection had a significant lower FEV_1 and higher number of pulmonary exacerbations in the case of one or more positive *Aspergillus* spp. cultures [13]. A big question in the era of highly effective CFTR modulator therapy is whether the prevalence of pulmonary fungal diseases in CF is decreasing or not. According to the ECFS patient registry (ECFSPR), the percentage of pwCF with ABPA was comparable over the period 2018 to 2020 (4.75% in 2018, 5.12% in 2019 and 4.55% in 2020) [33]. Despite the increasing number of studies focusing on fungal pulmonary diseases in CF, an international survey revealed that huge heterogeneity still exists in terms of the availability of diagnostic tools, as well as diagnostic and therapeutic guidelines [34].

10.3 Viruses

Since the SARS-CoV-2 pandemic started in 2020, viral infections have received increased attention and concern about their threat to pwCF [35-38]. The most commonly identified viruses in pwCF are usually respiratory syncytial virus (RSV), human rhinovirus, influenza types A and B, and parainfluenza virus. However, many other viruses, including human metapneumovirus, picornavirus, coronavirus, and coxsackie/echo virus, have also been identified in pwCF [39-47]. Regarding SARS-CoV-2 infection, initial studies from Europe have shown that CF-related diabetes, impaired lung function, and lung transplantation are risk factors for severe SARS-CoV-2 infection, and therefore require special attention [37, 38, 48].

Prevention measures including vaccination are recommended, as viral infections in CF might increase the risk of pulmonary exacerbations in both children and adults with CF [45, 47] and can also increase inflammation leading to tissue damage and longer duration of intravenous therapy [49, 50].

10.4 Conclusion

In summary, bacterial infections are typically the primary focus during exacerbations in pwCF, but fungal and viral infections are also important differential diagnoses. As pwCF treated with highly effective CFTR modulator therapy produce less sputum, future studies will need to investigate whether other biomarkers, such as serological biomarkers, are useful, or whether bronchoscopy with bronchoalveolar lavage may be a good alternative for obtaining high-quality samples.

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Chapter 11
Microbiome and CFTR modulator therapy

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Introduction

Cystic fibrosis (CF) arises due to mutations in the CF transmembrane conductance regulator (*CFTR*) gene which acts as a cAMP-regulated channel, enabling the transport of anions, including chloride and bicarbonate, across the apical membrane of epithelial cells [1]. Abnormal CFTR protein in the lung leads to impaired ion and fluid homeostasis, with dehydration and acidification of the epithelial surface. The resultant build-up of thick, sticky mucus provides a favorable environment for airway colonization by a variety of microbes. This polymicrobial community leads to the pulmonary infections that drive the morbidity and mortality associated with CF [2].

Our understanding about the polymicrobial nature of CF airway infections has expanded with the development of culture-independent molecular approaches, enabling better characterization of both upper and lower airway infections [3-7]. The CF microbiome is often highly personalized and changes with age, different disease stages and with different CF genotypes [8-10].

In the last decade, the introduction of CFTR modulator therapies has transformed the CF treatment landscape [11]. These small molecule drugs enhance or even restore the dysfunctional CFTR protein product arising from certain CF-causing mutations. These agents have been classified into five main groups based on their effects on abnormal CFTR protein [1].

To date, one potentiator (ivacaftor) and three correctors (lumacaftor, tezacaftor and elexacaftor) have been advanced to the clinic, enabling variant-specific corrective therapy for nearly 90% of people with CF (pwCF) aged 12 years and older in the United States and Europe [12].

This new era raises the question of whether the introduction of CFTR modulator therapies will enable eradication of chronic airway pathogens. Alternatively, might CFTR modulator therapies alter the impact of colonizing pathogens, such that treatment aims will shift?

11.1 Microbial ecology

The concept of the airway microbiome emerged after culture-independent studies dispelled the long-held paradigm that the human lung was sterile [13]. The term microbiome is defined as the genomic composition of a specific ecological niche (e.g. gut, lung, soil, water) [14]. This definition includes the microbe identification as well as their gene repertoire. On the other hand, microbiota refers only to the community of microorganisms themselves. Although the terms microbiome and microbiota encompass all the microbes present in a given ecological niche, including bacteria, fungi, archaea and viruses, most current research focuses on the bacterial kingdom due to the methodology used.

Several culture-independent methods can be used to quantify the microbiome based on nucleic acid (DNA and RNA) content. One of the most commonly used is 16S rRNA gene sequencing. The 16S rRNA-encoding gene comprises nine constant regions and nine hypervariable regions (V1–V9) which can be used to characterize the bacteriome efficiently, following PCR-amplification of the gene [15]. The fungome and archaeome are also accessible using other marker elements, such as the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA (rRNA) [16] or specific primers that amplify the archaeal 16S region [17]. The only way to reliably assess the whole microbiome is to perform metagenomic or metatranscriptomic analyses where the whole nucleic acid content is sequenced [18], offering an unbiased measure of the abundance of each taxon and their gene repertoire.

In the microbiome field, operational taxonomic units (OTUs) are defined as single entity microbial sequences sharing similarities (mostly 97% identity) [19]. OTUs have been employed mostly to limit the impact of sequencing errors on the number of taxa found in a niche. The definition of taxonomic units and the abundance of each taxon is the principal metric used in the microbiome field but two other metrics, α-diversity and β-diversity, are used to simplify the high-dimension data associated with microbiome analysis into a single metric [20].

Alpha diversity describes the ecological diversity within a certain niche, with the Shannon diversity index being the most commonly used metric [21]. This index accounts for several parameters such as the number of taxa (richness) and the distribution (evenness and dominance) of each taxon. While richness and evenness are included in the same equation for α-diversity, it is sometimes informative to evaluate those parameters independently [21]. Beta diversity is a measure of the degree of difference in membership or structure between two or more microbiota (e.g. two different niches), with many equations and indices existing to measure β-diversity.

In health, the upper airways are in general dominated by *Streptococcus, Neisseria, Prevotella, Rothia* and *Haemophilus* while the lower airways represent a sub-selection of those microbiotas and are dominated also by *Streptococcus, Prevotella* and *Veillonella* [13].

In CF the respiratory microbiome evolves in the first years of life. In the early stages of disease, the microbiome is rich and diverse, and is highly similar to the oropharyngeal microbiota seen in health. By early adulthood however, the CF microbiome becomes dominated by specific CF-associated pathogens [22, 23]. Such pathogens include *Pseudomonas aeruginosa, Staphylococcus aureus, Achromobacter* spp., *Burkholderia* spp. or *Stenotrophomonas maltophilia*. This shift, also called dysbiosis, is not fully understood. Current work suggests that impaired airway clearance creates a hostile environment which, along with selective challenges encountered in the lower airways (e.g. host immune response, antimicrobial exposure, physiochemical properties of viscid mucus), drives a change in the composition of the airway microbiota [23]. Although causality is poorly understood, most studies reveal that dominance of a CF pathogen within the microbiota is linked to decreased microbial diversity, poorer lung function, higher degrees of inflammation, higher rates of exacerbation and greater antibiotic use [9, 24-29].

11.2 Impact of CFTR modulators on alpha diversity

In this overview, we discuss α-diversity, based on studies using culture-independent methods (e.g., metagenomics or culturomics) (Table 1).

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Studies examining the effect of ivacaftor, the first approved CFTR modulator therapy, suggest an initial positive effect on microbial diversity. One of the main effects observed has been the increase in α-diversity or in at least one of the components of α-diversity such as richness or evenness. A study of 14 pwCF in the UK showed an increase in richness and a trend for improved α-diversity after one year of treatment [30]. Similar results were observed using extended culture from the same group [31]. Other studies investigating the impact of ivacaftor in the first 6 months of treatment showed a non-significant trend towards an increase in α-diversity [32-35]. A study with a longer treatment period showed an increase in richness and diversity in the first year, with a decline in microbiome diversity from year two [36]. These results suggest that the positive impact of CFTR modulation via ivacaftor on α-diversity is not sustained.

Studies investigating the impact of the combination CFTR modulator therapies on microbial diversity have reported mixed results for improvements in α-diversity and richness. The lumacaftor/ivacaftor combination was reported to increase Shannon diversity of the airway microbiome after only 8 to 16 weeks of treatment [37] and treatment with combination elexacaftor/tezacaftor/ivacaftor (ETI) showed a significant increase in Shannon diversity index and Pielou evenness [38]. However, another study of lumacaftor/ivacaftor failed to show a significant increase in α-diversity [39]. The difference between studies could be explained by the small cohort size and heterogeneity. Even though the age of the patients is quite similar, the microbial diversity is different, and it seems that pwCF with a higher initial α-diversity showed a better improvement after CFTR modulation. One hypothesis for this finding may be that improved mucociliary clearance with CFTR modulator therapy affects the early microbiome dysbiosis observed in CF, but is ineffective at later stages when CF pathogens have dominated the microbiome and developed resistant biofilms. Only two studies have observed a decrease in the total bacterial load after treatment [36, 37] and as seen for α-diversity, this effect was not observable during the second year [36]. Of note, several studies did not replicate this finding [31, 33, 34, 37, 38].

11.3 Impact of CFTR modulators on CF-specific pathogens

Alpha diversity is mostly influenced by the establishment of a dominant pathogen in the CF microbiome. Therefore, a major focus of CFTR modulator studies has been the impact on key CF pathogens such as *P. aeruginosa*. No significant changes in the molecular detection of *P. aeruginosa* have been observed either by 16S rRNA relative abundance or qPCR detection but trends for reduction have been observed [30, 31, 33, 34, 37]. The drop in *P. aeruginosa* abundance seems to be limited to the first year of treatment, with increased abundance observed thereafter [36]. It is interesting to note that after treatment with ivacaftor the relative abundance and absolute numbers of commensals, and especially anaerobes, increases [30, 31, 37]. PwCF treated with lumacaftor/ivacaftor combination therapy did not have reductions in the relative abundance or absolute quantities of pathogens such as *P. aeruginosa* [37, 39]. Similar results were observed with ETI [38]. The authors of this study used an interesting index summing the abundance of all “CF pathogens” and “anaerobes”. A log ratio of pathogens/anaerobes showed a significant decrease in the first year of treatment [38].

Promisingly, most studies showed a decrease in the abundance of the CF pathogens after treatment, to some extent. The lack of significance is most likely due to the small sample size of each

cohort and the prevalence of the CF pathogens in those cohorts. Using the aforementioned log ratio of pathogens/anaerobes may be useful to see the impact on a global microbiome scale, despite the heterogeneity in CF pathogens in smaller cohort sizes. An interesting hypothesis requiring further study is whether CFTR modulator therapies are not eradicating CF pathogens but rather modifying host physiology, altering the lung ecological niche for bacteria and promoting commensal growth. In addition, the use of CFTR modulators in early life could limit alterations of lung mucus and prevent initial colonization by CF-specific pathogens. This remains an important direction for future research.

11.4 Limitations, remaining questions and future directions

While recent studies have demonstrated promising evidence of the effect of CFTR modulators on the microbiome of pwCF, many aspects require further research to completely understand their impact.

The data currently available are mostly from small cohorts that are heterogeneous in terms of CFTR modulator therapy, with contrasting results and limited understanding of long-term effects. Therefore, large scale prospective longitudinal studies are required to understand the impact of CFTR modulator therapies on the CF airway microbiology. An example is the PROMISE study to examine the clinical effectiveness of ETI [40].

Improved culture-independent techniques allow easier and more accurate identification of bacterial members of the microbial community. However, the impact of fungi and viruses are yet to be understood. To better understand how new treatments affect the microbiome, it is essential to study all kinds of microbes and their roles in the polymicrobial community. The gene repertoire and expression should also be a major focus in the field. Therefore, extending the analytical methods to transcriptome and resistome could provide valuable information about the underlying mechanisms in microbial dysbiosis during treatment.

Surveillance and antimicrobial treatment choice have been traditionally based upon culture-dependent analysis of lower serial respiratory tract samples, usually expectorated sputum, but also bronchoalveolar lavage (BAL). However, adults treated with CFTR modulators are often unable to expectorate sputum [7]. This provides an opportunity to reconsider sampling methods in the context of CFTR modulator therapies and introduce potential alternatives (e.g. exhaled breath condensate) [41].

Despite several studies investigating the direct and indirect antimicrobial effects of CFTR modulator therapies, it remains necessary to understand the entire picture. Hence, it is important to examine their impact on polymicrobial community dynamics, to use novel applications of sequencing methodologies (e.g. metagenomics, metatranscriptomics), and to optimize infection diagnosis and management.

11.5 Conclusion

The introduction of CFTR modulator therapies has changed the landscape of CF, with the exciting promise of improvement in all aspects of CF care. However, it is important to note that our understanding about some of these aspects and the long-term effects is limited. This is especially relevant for the impact of CFTR modulator therapies on the CF microbiome. The recent studies present interesting but sometimes contradictory results.

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Ivacaftor has been found to increase microbial richness in the first year of its implementation, but this benefit then wanes. The same pattern is recognizable with the Shannon diversity index. Ivacaftor may have the potential to decrease *P. aeruginosa* load, but this finding is not consistent across all studies. Lumacaftor/ivacaftor and ETI seem to significantly increase the Shannon diversity index, but this finding is limited by the relatively small number of studies carried out whose data cover a year at the most.

Nonetheless, it is reasonable to conclude that CFTR modulators have a promising effect on the microbiome, especially during the first year of treatment. However, several aspects, importantly including the long-term effects, must be tackled in the near future. A number of encouraging attempts to define the findings more clearly are ongoing. The remaining questions in the field of CF lung infection provide exciting research opportunities. Moreover, new therapies have appeared on the horizon that may affect the microbiome differently, such as ETX001, a promising TMEM16A potentiator or BI 1265162, an epithelial sodium channel inhibitor [42]. Undoubtedly, achieving success will require a collaborative effort from researchers, clinicians, industry and pwCF to further discoveries in this field.

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Table 1

Effect of CFTR modulators on the airway microbiota.

Author	Study location (number pwCF)	Mean age (years)	Treatment and duration	Method	Observation
Sosinski *et al.*, 2021	USA (n=24)	32.5	ETI 3 to 6 months (202 days ± 108)	V4 amplicon PCR	Microbial richness: No significant change Shannon diversity index: ↑ *P. aeruginosa* load: ↓ Note: Peilou evenness ↑ and log ratio of pathogens/anaerobes ↓
Neerincx *et al.*, 2021	Netherlands (n=20)	25.3	Lum/iva 3 to 12 months	V4–V5 PCR & metagenomics	Alpha diversity: No significant change *P. aeruginosa* load: ↓ after 6 months but not significant Note: before and after microbiota results from 15 people with CF only
Graeber *et al.*, 2021	Germany (n=15)	20.5	Lum/iva 8-16 weeks	V4 amplicon PCR	Shannon diversity index: ↑ Total bacterial load: ↓
	USA (n=14)	27	Iva 6 months	V1-V2 PCR	Shannon diversity index: No significant change Total bacterial load: No significant change *P. aeruginosa* load: ↓ and *Prevotella* load ↑
Einarsson *et al.*, 2021	Ireland (n=14)	26	Iva 1 year	V4 amplicon PCR	Microbial richness: ↑ Alpha diversity: ↑ but not significant Note: ↑ bacterial load in anaerobes
Durfey *et al.*, 2021	USA/Ireland (n=13)	34.5	Iva Up to 2.5 years	Culture	*P. aeruginosa*: No eradication with combination of CFTR modulation and antibiotic therapy
Harris *et al.*, 2020	USA (n=31)	27	Iva 6 months	V1-V2 PCR	Microbial richness: No significant change Shannon diversity index: No significant change Note: Younger patient more responsive in the microbiome
Hahn *et al.*, 2020	USA (n=17; 5 on CFTR versus 12 without)	Between 2-6 years	Iva (n=3) Lum/iva (n=2) 1 year	V4 amplicon PCR (cross-sectional-exclude)	Alpha diversity: ↑ comparing children with CF not receiving CFTR modulators and children with CF receiving CFTR modulators. Note: The age and treatment are not given precisely
Ronan *et al.*, 2018	Ireland (n=14)	21.6	Iva 1 year	V4 amplicon PCR	Microbial richness, Shannon diversity index: ↑ *P. aeruginosa* load ↓ and *Streptococcus* species, *Rothia species, Haemophilus* species, and *Prevotella* species: relative proportion ↑
Peleg *et al.*, 2018	Australia (n=20)	32	Iva 4 weeks	V1–V3 PCR	Microbial richness: No significant change Shannon diversity index: No significant change *P. aeruginosa* load: No significant change
Hisert *et al.*, 2017	USA (n=12)	29.5	Iva 32 months (Up to Day 975)	N/A	Microbial richness: ↑ in 1st year, then ↓ Shannon diversity index: ↑ in 1st year, then ↓ Total bacterial load: ↓ *P. aeruginosa*: no eradication; after 1st year densities rebounded
Heltshe *et al.*, 2015	USA (n=151)	21.1	Iva 6 months	Culture	*P. aeruginosa*: eradication (29%) or remaining uninfected (88%)
Bernarde *et al.*, 2015	France (n=3)	12	Iva 13 months	V3–V4 (PCR)	Microbial richness: No significant change Shannon diversity index: No significant change *P. aeruginosa* load: No significant change

Abbreviation: ETI=elexacaftor, tezacaftor, ivacaftor; PCR=polymerase chain reaction; V1-V4=hypervariable regions

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Chapter 12
Detecting bacteria

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Introduction
Cystic fibrosis (CF) lung disease is characterized by chronic airway infections with intermittent pulmonary exacerbations due to a broad spectrum of different microorganisms (bacteria, mycobacteria, fungi and viruses). The microbiological processing of CF samples requires special laboratory protocols as well as experienced microbiologists. For decades, CF airway microbiology has been based on bacterial and fungal culture methods, focusing on the most prevalent microbial pathogens such as *Staphylococcus aureus, Haemophilus influenzae, Pseudomonas aeruginosa,* other non-fermentative gram-negative bacteria, in particular *Burkholderia cepacia* complex (Bcc), *Achromobacter* spp. and *Stenotrophomonas maltophilia*, in addition to nontuberculous mycobacteria (*Mycobacterium abscessus, M. avium* complex) (see Chapter 18) and *Aspergillus fumigatus* (see Chapters 22-24). Emerging and much more rarely recognized pathogens include *Inquilinus limosus, Pandoraea* spp., *Exophiala dermatitidis* and members of the *Scedosporium apiospermum* species complex. Moreover, CF airway pathogens are characterized by pathoadaptive alterations leading to phenotypic variants and morphotypes such as auxotrophic and/or slow-growing variants, small colony variants (SCVs) and mucoid *P. aeruginosa* [1].

More recently, non-culture-based methods have shown that CF airway infection is even more complex and may involve anaerobes or even *Streptococci* that are not covered by standard cultivation procedures (see Chapter 12).

Continuous follow-up of microbial colonization has been an approved standard of care in CF patient management [2]. Cultures of the respiratory tract can detect new pathogens, guide therapy, monitor the success of eradication therapies, may differentiate transient versus persistent colonization or infection, and may also guide hygienic measures. The diagnostic value of anti-*P. aeruginosa* antibodies as a predictor of early *P. aeruginosa* infection is still under debate and is probably best in young non-expectorating children. At some CF centers, the current practice is to determine *P. aeruginosa* antibodies at least once a year (in *P. aeruginosa* negative individuals). Serology is performed always in combination with culture and no clinical decisions are based on *P. aeruginosa* serology alone [3].

Clinical microbiology laboratories need to adhere to consensus microbiology practices often specified in national guidelines [4-8]. Laboratories processing CF respiratory tract specimens must use a standardized set of selective and chromogenic culture media along with prolonged incubation times. CF pathogens should be identified to the species level using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) or molecular methods (e.g. 16S rDNA Sequencing) [6, 7].

12.1 Microscopic examination of CF respiratory secretions

Microbiological guidelines recommend gram stain of sputum to assess quality based on the ratio of leucocytes to squamous epithelial cells (a ratio of >5 indicates acceptable quality). Due to an insufficient positive / negative predictive value regarding the presence of CF pathogens, microscopic examination is not recommended for CF respiratory secretions for routine microbiology (with the exception of bronchoalveolar lavage fluid) [6, 7].

12.2 Homogenization

Due to the viscous nature of CF respiratory secretions prior to microbiological processing, a homogenization step is necessary to reduce viscosity and to homogenize the unequal distribution of CF pathogens, in particular when quantitative culture is performed. Initial homogenization of sputum samples improves bacterial and fungal recovery rate [9]. For homogenization, the addition of 0.1% solution of dithiothreitol (DTT) is typically recommended (alternatively N-acetyl L-cysteine may be used). DTT has no inhibitory effect on the recovery of target organisms such as *H. influenzae, S. pneumoniae, M. catarrhalis* or *P. aeruginosa* [10].

12.3 Microbiological culture of CF respiratory secretions

For CF microbiology all types of respiratory tract specimens (throat swab/cough swab, sputum, induced sputum or bronchoalveolar lavage) are obtained at least quarterly and during exacerbations. Expectorated sputum is easy to obtain and thus the specimen of choice. Isolation of a CF pathogen from sputum is likely to represent the infecting organism from the lower airways. For children who cannot expectorate, oropharyngeal cough swabs, induced sputum (secretion following inhalation of 5% hypertonic saline solution), or bronchoalveolar lavage fluid are recommended. Cough swabs are obtained by asking the patient to cough onto a swab placed in, but not touching, the posterior pharynx. Of note, pwCF on CFTR modulator therapy often become non-productive for sputum. In this situation, oropharyngeal samples or, if possible, induced sputum should be obtained. Invasive sampling procedures such as bronchoscopy or lung aspirates are indicated in certain circumstances. No consensus statement exists for how to best implement sinonasal samples into the clinical care of children with CF. Bronchoalveolar lavage fluid specimens must be quantitatively cultured.

Laboratories need to apply media that allow supportively and/or selectively the culture of the most prevalent CF pathogens such as *H. influenzae, S. aureus, P. aeruginosa,* Bcc and fungi (Table 1) [11].

Surveillance cultures (oropharyngeal/cough swabs, sputum, induced sputum), intended to detect initial *P. aeruginosa* acquisition, should be performed regularly, at least four to six times per year. In clinically stable individuals with chronic *P. aeruginosa* infection, respiratory tract cultures are recommended at least quarterly (every 3 months) during routine clinic visits. Chronic *P. aeruginosa* infection is defined by positivity of more than 50% of respiratory samples collected during the past 12 months (at least four samples are requested) and/or significantly raised anti-pseudomonal antibodies (modified Leeds criteria) [12].

Table 1 describes the recommended culture media for routine microbiological investigation in CF (laboratories are strongly encouraged to apply these media as a minimal requirement) [6, 7]. *P. aeruginosa* may be recovered from both nonselective agar (chocolate agar /blood agar) and selective agar for gram-negative organisms such as MacConkey agar. Therefore, the use of a selective agar specific for *P. aeruginosa* (e.g. *Pseudomonas* isolation agar, cetrimid agar) is not required. However, the use of these selective media may assist in identification, particularly due to improved pigmentation [6, 7, 13].

Chromogenic agar media may facilitate a selective and more sensitive growth of *S. aureus* as well as the identification of *S. aureus* SCVs. It is important to use special culture conditions for the isolation of *S. aureus* SCVs, because these phenotypes are associated with chronic or persistent infections, are more resistant to antibiotics. For CF microbiology it is recommended to report on mucoid phenotypes (*P. aeruginosa*) and SCVs. Among agar media, mannitol salt agar and chromogenic *S. aureus* agar are effective in the recovery of SCV and wild-type strains. SCVs are detected visually on laboratory agar plates and exhibit slower growth rates due to metabolic defects (e.g., thymidine biosynthesis deficiency [13]), and they therefore require special susceptibility testing. However, there are no standardized methods for detection and susceptibility testing.

Bcc selective media should have a high sensitivity and specificity. Commercially available Bcc selective agar show differences in the recovery of different species of the Bcc, the selectivity and the recovery of non-Bcc organisms. Thus no specific manufacturer can be recommended [6, 14].

12.4 Extended cultivation procedures

There is insufficient evidence to currently recommend extended cultivation procedures for routine CF microbiology, but they may improve recovery of CF pathogens.

Quantitative culture using serial sputum dilutions and whole agar plating offers the possibility to report individual species counts, prevents overgrowth of slow-growing phenotypes and may allow efficient separation and hence recognition of different CF pathogens and/or their phenotypic variants.

Extended incubation (14 days) of Bcc selective agar is recommended as an expedient culture method for the isolation of rapidly growing non-tuberculous mycobacterium (NTM, *Mycobacterium abscessus*) [15].

12.5 Bacterial identification

Accurate bacterial identification is important both for implementing an appropriate treatment regimen and for epidemiological purposes. The pros and cons of different methods are discussed below.

12.5.1 MALDI-TOF-MS analysis

MALDI-TOF MS identifies isolates by applying a laser-absorbing matrix to single colonies on a target plate. The colonies are ionized by a pulsed laser beam within the instrument. The mass and charge of these peptides generates a genus/species-specific spectrum. MALDI-TOF MS may also be used for direct testing from certain clinical samples or following formic acid extraction for improved profiling. It is a rapid and cost-effective method and although there is variation

between the databases of the two most frequently used platforms (Bruker and bioMérieux VITEK®-MS), both are reliable for species-level identification of common CF-related organisms such as *P. aeruginosa* and *S. aureus*, if the microbe is present above the limit of detection. Formic acid extraction is required for mucoid *P. aeruginosa* and specialized extraction methods are recommended for NTM such as *M. abscessus*. Species-level confirmation by molecular methods is recommended for the Bcc and to differentiate between the numerous closely related species of the *Achromobacter* and *Pandoraea genera*. In-house databases may be created to increase the reliability of standard databases [16]. The detection of specific biomarker peaks may also be used to identify particular strains and lineages [17].

12.5.2 Molecular methods

Accurate species-level identification (known as speciation) of certain bacterial organisms is not currently possible using standard MALDI-TOF databases alone. Molecular methods, such as the sequencing of housekeeping genes, are required for confirmation. For example, *recA* gene sequencing has been shown to be a useful means of speciation of the *B. cepacia complex*, although it is not sufficient to differentiate between *B. contaminans, B. lata* and *B. aenigmatica* which belong to the closely related "taxon K" group [18]. Similarly, *nrdA* may be used for speciation of the *Achromobacter* genus [19] and *gyrB* for *Pandoraea* species [20].

12.6 16S rRNA gene sequencing

16S rRNA gene sequencing may also be used for genera that are not adequately represented on MALDI-TOF databases, and for which there is limited available information about appropriate housekeeping genes. Examples include *Sphingomonas* spp., *Sphingobacterium* spp. and *Bordetella* spp.16S rRNA gene sequencing has been particularly useful for direct testing on clinical sample extracts and for revealing the diversity of organisms associated with the both the lung and gut CF microbiome [21, 22].

12.6.1 Multilocus sequence typing and whole genome sequencing

In some cases, sequencing of a single gene or 16S rRNA sequencing may be insufficient to provide accurate species-level discrimination. For example, subspecies identification within the *M. abscessus* complex requires the sequencing of multiple genes due to the well-documented horizontal gene transfer and recombination of housekeeping genes within this complex [23]. Multilocus sequence typing (MLST) is particularly useful in such cases. The sequences of the seven housekeeping genes of an MLST scheme may be compared to curated databases (https://pubmlst.org/ and https://bigsdb.pasteur.fr/), which provide both identification and typing information.

Increasingly the method of choice for accurate bacterial identification is whole genome sequencing (WGS). WGS offers the advantage of providing accurate identification by detecting short DNA oligomers known as *k*-mers, in addition to typing information based on standard MLST. Additional tools may be used, such as core genome MLST, which uses a much greater number of genes to define isolates, and ribosomal MLST, which analyses the variation of 53 genes of the bacterial ribosome protein subunits, providing information on both taxonomy and typing [24].

Publicly available resources hosted by sites such as the Center for Genomic Epidemiology (https://www.genomicepidemiology.org/) and Pathogenwatch (https://pathogen.watch/) offer automated screening of the WGS-generated fastq files to search for virulence and resistance mechanisms, MLST and *k*-mer based identification. SNP trees may also be generated if comparison with other sequenced data is required.

One caveat for the routine clinical use of WGS would be the requirement for bioinformatics expertise, since factors such as sequence quality, appropriate reference strain use, genomic recombination events and mutation rate will have an impact on the accuracy of fine typing, such as single nucleotide polymorphisms (SNP)-based analyses. SNP genotyping is the measurement of genetic variations of SNPs between members of a species.

As WGS is highly discriminatory, becomes more cost-efficient and accurate, and long-read technologies become more readily available, it seems likely that this method will become the gold standard for both identification and typing [25, 26].

12.7 Antimicrobial susceptibility testing

Several studies have questioned the clinical relevance of performing routine susceptibility testing in CF [27, 28]. To date, neither combination antimicrobial testing nor biofilm antimicrobial susceptibility testing approaches can reliably predict response of patients to antibiotic treatment [28]. Certainly, it is challenging to predict antimicrobial resistance in CF due to the complexities of the heterogeneous CF lung environment. Nonetheless, antimicrobial susceptibility testing can distinguish methicillin-susceptible *S. aureus* (MSSA) from MRSA, identify multidrug resistance patterns, and has been crucial for epidemiologic investigations (e.g. transmission of epidemic and/or multi-resistant strains of *P. aeruginosa*). Phenotypic susceptibility testing is thus still recommended. At present, more robust data are needed to define if molecular-based technologies including whole genome sequencing, metagenomic and proteomic approaches have the potential to better predict antimicrobial resistance in the future.

12.8 Direct detection of CF pathogens in respiratory samples by PCR

Molecular assays for the direct detection of CF pathogens from sputum have been described in the literature but few studies have extensively evaluated these methods alongside conventional culture. For *P. aeruginosa*, studies have focused on the detection of targets such as *oprI, oprL, algD* and *exoA* by standard or quantitative PCR [29-31]. An advantage of these methods may be the increased sensitivity of PCR when compared with standard culture, providing early detection of infection prior to patients becoming culture-positive [32]. Some studies found dual targets, or a combination of PCR assays provided better sensitivity and specificity [31, 33].

Similar studies using 16S rRNA gene loci and *recA* have been used to identify Bcc directly from sputum, in some cases improving the detection rate for these organisms when compared with culture alone [34, 35]. Species-level identification for this genus remains challenging, however the use of multiplex real-time PCR-based assays with species-specific probes may be a promising approach provided cross-reactions between the species can be excluded [36].

Currently, there are no consensus recommendations regarding the detection of CF pathogens by PCR or non-culture-based methods, but it is likely that these technologies will be increasingly implemented in the future.

Table 1

Media and culture conditions for the processing of CF respiratory specimens.

Agar medium	Culture conditions	Target organism
Columbia blood agar bacitracin disc (30 µg)	5 days 36°C ± 1°C	*S. aureus* *P. aeruginosa* *S. pneumoniae* *M. catharralis* Non-fermenters Filamentous fungi
Chocolate agar (e.g. with bacitracin)	5 days at 36°C ± 1°C	*H. influenzae* *S. aureus* *P. aeruginosa* Non-fermenters Filamentous fungi
MacConkey agar	5 days at 36°C ± 1°C	*P. aeruginosa* Non-fermenters Enterobacterales
S. aureus selective agar (mannitol salt agar, chromo-genic *S. aureus* agar)	at least 72 h at 36°C ± 1°C	*S. aureus* *S. aureus*-SCV
B. cepacia complex selective agar 1 [6, 7]	48 h at 36°C ± 1°C + 8 days at 32°C ± 1°C	*B. cepacia* complex *Pandoraea* spp. Filamentous fungi *E. dermaditidis*

Abbreviations : SCVs=Small Colony Variants

Several guidelines recommend inoculation of at least 100 µL of liquid sample.

Note: All agar media should be examined after overnight incubation and daily until the end of the suggested incubation period.

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Chapter 13
Detecting fungi

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Introduction
The most common fungal species reported in cystic fibrosis (CF) remain by far *Aspergillus fumigatus* for filamentous fungi and *Candida albicans* for yeasts. Several other fungal species may be recovered from respiratory secretions of people with CF (pwCF), however reports of their frequencies greatly vary from one study to another. Variability in the isolated microorganisms observed in laboratories worldwide does not exclusively reflect variations in the geographical distribution of fungal species. It can also be impacted by differences in the antibiotic treatment of bacterial infections, antifungal use in medicine and agriculture, patient lifestyle, and the wide heterogeneity of laboratory mycological protocols.

Screening procedures used for fungal pathogen detection are mostly only suitable for fast-growing fungal pathogens such as *Candida* and *Aspergillus*. Slow-growing fungi like *Scedosporium* species, *Lomentospora prolificans* and *Exophiala* species are frequently concealed. The lack of standardized laboratory practices for detecting fungi in CF respiratory specimens is a major limitation to the robust understanding of the epidemiology of fungal respiratory infections in CF, the identification of risk factors and the elucidation of the clinical and functional impact of the airway colonization [1-4].

To improve the microbiological diagnosis, medical microbiologists should be aware of the spectrum of pathogens that is expected from respiratory specimens from pwCF. This is particularly important to determine the analytical procedures that should be followed. Although the clinical significance of their isolation is still debated, the most frequently isolated yeast species belong to the *Candida* genus, especially *C. albicans*, but also *C. dubliniensis*, *Nakaseomyces glabrata*, and *C. kefyr*. Other yeast species may also be encountered in CF samples, some of which can cause severe respiratory infections like *Apiotrichum mycotoxinivorans* (formerly *Trichosporon mycotoxinivorans*), *Cutaneotrichosporon cyanovorans* (formerly *Cryptococcus cyanovorans*) or, in lung transplant recipients, the well-known *Cryptococcus neoformans*. Other yeast species occasionally reported in CF are usually recovered only transiently and do not seem to be capable of chronically colonizing the airways, such as *Pichia kudriavzevii* formerly known as *Candida krusei, Geotrichum* spp., *Magnusiomyces* (formerly *Saprochaete*) spp., or *Saccharomyces* spp. Various filamentous fungi may also be recovered transiently from respiratory specimens, like some *Penicillium, Paecilomyces* or *Fusarium* species, as well as fungi of the order Mucorales. Conversely, the clinical relevance is now established of the isolation of *Aspergillus* spp., especially *A. fumigatus*, but also other species of the sections *Fumigati* like *A. lentulus* and *A. thermomutatus* (formerly *Neosartorya pseudofischeri*), or the sections *Terrei, Flavi* or *Nigri*, as well as *Scedosporium* spp., *L. prolificans, Exophiala* spp., species of the *Rasamsonia argillacea* complex or *Arthrographis kalrae* [5].

13.1 Standardizing detection

How can microbiological methods for the detection of fungi be standardized, and comparability of epidemiological studies improved? Raising awareness is always the first step, followed by proper guidelines and training of laboratory microbiologists and health care staff.

In the last decade, many protocols and research papers were published by international or local teams to standardize routine diagnostic procedure for the detection of fungal pathogens [6-9]. Here we aim to summarize key points of their proposals.

The principles of proper conventional, mycological laboratory diagnostics are described in the following sections.

13.1.1 Pre-analytical phase

Sample collection, frequency of sampling and sample transport to the laboratory can significantly impact the detection of fungal species.

Bronchoalveolar lavage (BAL) fluid is the best sample for diagnosis, but BAL is rarely performed in non-transplanted pwCF, because of its invasive nature and the risk of complications, such as acute respiratory failure. The optimal method for sampling should be refined, with consideration for the risks and benefits of such invasive techniques, and the increasing understanding of the spatial variations between upper and lower airway microbiota. Pharyngeal swabbing and nasal washings are less efficient for the detection of fungal pathogens in CF, however the preferred sample is sputum. In non-expectorating pwCF, particularly young children and those treated with CFTR modulators, sputum induced by nebulization with hypertonic saline should be collected directly into a sterile pot or via a mucus trap suction catheter, during a physiotherapy session [5-11].

Theoretically, the volume of the sample is an important factor for the yield of fungal cultures; the higher the sample volume, the better the yield of cultures. Nevertheless, this is still debated since the recovery of some filamentous fungi may be improved by dilution of the clinical samples [6-9].

The sample must be sent to the laboratory quickly, at room temperature, and processed as soon as possible, preferably within 4 hours of collection. If this is not possible, it is recommended to store the sample at a temperature ranging from +2°C to +8°C. This protects from overgrowth of culturable bacteria [6-9].

The frequency of sampling also impacts the rate and clinical relevance of fungal detection. Successive positive sputum samples indicate colonization and help exclude transient carriage of environmental molds [6-9].

13.1.2 Analytical phase

Because of the sticky nature, sputum samples and BAL fluid if highly viscous should be first homogenized to increase sensitivity of the detection procedures. The most widely used homogenization procedure involves sterile pretreatment for 20 to 30 minutes with a mucolyticum like N-acetyl-cysteine, or dithiothreitol which cleaves the disulfide bonds linking the mucin molecules, and thus decreases viscosity [12]. Nevertheless, mechanical liquefaction (for example with glass beads or intermittent sonication for 120 seconds) in the presence of a mucolytic agent should be considered for complete homogenization of the samples [13].

Direct microscopic examination may be performed on the pretreated sample, to search for fungal elements, although this has limited interest because of its low sensitivity [7, 14].

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Inoculation of the digested sample on appropriate culture media remains the gold standard. Of note, fungi are not the only microorganisms colonizing the respiratory tract of pwCF. Microbiologists should be aware of the common occurrence of mixed bacterial-fungal infections in pwCF, especially with multidrug-resistant bacteria. That is why conventional bacteriological culture media are not appropriate. For an accurate diagnosis of fungal infections, mycological culture media containing antibiotics are required to inhibit bacterial growth and unmask the presence of fungi [14]. Additionally, the use of agar plates is highly recommended. Agar slants are not recommended since their surface does not provide enough space for fungal growth, which may hinder the recovery of some slow-growing fungi, in case of association with the more rapidly and more extensively growing fungus *A. fumigatus*. Because of this, in addition to the general culture medium Sabouraud dextrose agar supplemented with chloramphenicol and gentamicin, the use of additional mycological culture media is also required to detect slow-growing fungal species [13, 14]. Several culture media may be used:

- Chromogenic agar for the detection of mixed populations of yeasts that allows direct identification of *C. albicans*/*C. dubliniensis* and presumptive identification of other yeast species,
- Sabouraud agar supplemented with chloramphenicol and gentamicine or the B+ culture medium which also contains various antibiotics and allows inhibition of bacterial growth,
- Sabouraud-chloramphenicol agar supplemented with cycloheximide or some agar-benomyl (like Dichloran-Rose Bengale-chloramphenicol agar supplemented with benomyl, Sce-Sel + or Scedo-Select III) inhibit growth of filamentous fungi such as *A. fumigatus* and facilitate the detection of *Scedosporium* species,
- Erythritol agar for the specific detection of *E. dermatitidis*,
- BHI agar or Sabouraud dextrose slants with antibiotics for long-term incubation [13, 14].

Incubation temperature and duration impact culture sensitivity. While a 48-hour incubation time may be sufficient for growth of *C. albicans* and *A. fumigatus*, most other pathogenic fungi require a longer incubation period. For example, until 2012, the UK national standards for mycological examination of respiratory secretions from pwCF recommended plating of the samples onto a single culture medium (Sabouraud with antibiotics) and incubating them for at least 48 hours at 37°C. In practice most laboratories only incubated the samples for 48 hours. To increase fungal yield, these recommendations were revised in 2012, to prolong the incubation time up to 7 days, and incubate the cultures at different temperatures, implying the use of additional culture media [8].

13.2 General logistics for fungi sampling in CF

Guidelines should be usable by all laboratories involved in the care of pwCF. In France, microbiological analysis of samples from pwCF may also be performed in non-specialized laboratories, including microbiology laboratories of general hospitals as well as private laboratories. However home-made culture media cannot be prepared in most of these non-specialized laboratories. Therefore, the culture conditions allowing detection of clinically relevant fungal species in CF should also consider the commercial availability of the culture media. For example, in the recommendations published together by the French Society for Microbiology and the French Society for Medical Mycology, the digested samples should be inoculated in parallel on a chromogenic agar plate for detection of yeasts, a gen-

eral Sabouraud agar plate with antibiotics, and a Sabouraud agar plate with chloramphenicol and cycloheximide as *Scedosporium*-selective culture medium. These three culture media should be incubated at 35°C ± 2°C for two weeks [9]. If possible, an erythritol agar slant should also be inoculated, and incubated at 25°C ± 5°C for one month [9].

The isolated fungi are finally identified on the basis of their cultural features and macroscopic and microscopic morphology, and by their biochemical characteristics revealed by MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry). Sequencing of appropriate loci, such as Sanglard sequencing, or next-generation sequencing (NGS) techniques, can identify isolated fungi, especially environmental molds, that do not sporulate or cannot be identified with routine methods [9]..

If needed, *in vitro* antifungal susceptibility testing should be performed using standardized methods according to the EUCAST or CLSI recommendations, but E-test strips may also be used [15].

13.3 Detecting *Pneumocystis jirovecii*

If *Pneumocystis jirovecii* infection is suspected, BAL fluid is the preferred sample, but induced sputum specimens may also be used for PCR detection. The diagnostic procedures, therefore, depend on the clinical sample, and on the laboratory resources. The first step is a chemical liquefaction of the sample using a mucolytic agent, or homogenization using both glass beads and a mucolyticum. In microbiological laboratories, cytocentrifugation of the BAL fluid should be performed, followed by May-Grünwald Giemsa staining, which reveals morphological stages of the fungus. In parallel, Gomori-Grocott methenamine silver staining should be performed, which reveals only the asci. Alternatively, asci can be detected using commercial immunofluorescence kits, or Calcofluor white if a fluorescence microscope with appropriate excitation/emission filters is available. Regarding molecular detection, several kits are now available for real-time quantitative PCR. All these methods are standardized and widely used in routine laboratory practice [16-19].

13.4 Specific recommendations for CF laboratories

Advances in medicine and the use of CFTR modulator therapy may lead to great difficulties in the obtaining sputum samples. In addition to culture-based methods, molecular approaches are currently being developed based on real-time PCR techniques, or on DNA-chips. One limitation of these possible new approaches will be the differentiation between infection, colonization, and transient carriage of recently inhaled fungal spores [9, 18, 19].

Reference laboratories may use NGS for secondary identification of the fungi isolated. Genotyping of fungal isolates can be applied to describe transmission patterns between patients or to identify an environmental source of contamination at home. Genotyping can also be used to differentiate between transient carriage and true chronic colonization [20]. In addition, serological tests (for detection of specific IgG antibodies) may be useful, for culture positive patients, to differentiate airway colonization from respiratory infection [20]. Likewise, detection of total serum IgE level, specific IgE antibodies and circulating antigens are complementary tests allowing the differentiation of the clinical entities caused by *A. fumigatus*, especially for the diagnosis of an allergic bronchopulmonary aspergillosis [21].

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13.5 Conclusion

Currently, the standard protocol for analyzing CF respiratory samples in medical microbiology laboratories involves conventional culture techniques. Nevertheless, in the near future the newer methods discussed above will move beyond research institutes to become routine procedures in detecting fungi [6, 7, 9, 22].

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Chapter 14
Detecting viruses

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Introduction

The thick, sticky mucus in cystic fibrosis (CF) is a major contributor to recurrent airway infections and exacerbations, which are fundamental in disease burden and reduced lifespan of patients. Modern therapies with CFTR modulators represent a significant improvement, and further therapeutic strategies are currently in progress [1]. Anti-infective treatment remains a challenge, particularly against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but also against a broad spectrum of other pathogens [2]. Beside bacteria, viruses have also been shown to frequently cause respiratory infections in people with CF (pwCF) [3].

14.1 Viral infections in patients with cystic fibrosis

CF itself does not appear to affect susceptibility to infections, as viral respiratory infections occur with similar frequency in pwCF and healthy individuals [4, 5]. Viral species found in respective studies represent the typical spectrum found in airways, i.e. rhinoviruses, adenoviruses (AdV), influenza and parainfluenza (PIV) viruses, respiratory syncytial virus (RSV), coronaviruses, metapneumovirus (MPV), cytomegalovirus (CMV), bocavirus and enteroviruses [3, 6-9]. According to several investigations, human rhinovirus was the most frequent virus found in pwCF during exacerbations [3, 9-14].

The predominant bacteria *S. aureus* and *P. aeruginosa* are well known to cause disease progression and respiratory failure [2]. In contrast, the disease-causing role of viruses was debated for a longer time. Previous studies have reported that pwCF with viral infections have increased respiratory symptoms, worsening Shwachman scores and radiological scores, longer hospital stays, persistent declines in pulmonary function, increased use of antibiotics, higher frequency of exacerbations, lower percentage of ideal weight for height, and greater frequency (r = 0.53) and duration (r = 0.84) of hospitalizations for respiratory exacerbations [4, 8]. Of respiratory viruses, infection with RSV and influenza had the most severe impact and were associated with the greatest decrease with lung functions in pwCF [8]. In another study, however, the authors were unable to demonstrate any significant adverse effect of respiratory viral infection on pulmonary function in 19 pwCF aged 5 to 21 years, compared to their siblings without CF [5]. Other studies reported a weak, not significant association between viral seroconversion and the isolation of *P. aeruginosa* from sputum [7]. In the majority of recent studies, respiratory viruses have been associated with higher symptom scores and deterioration of pulmonary function, exacerbations, facilitation of bacterial colonization, disease progression, prescription of antibiotics, and hospitalization [9-13, 15, 16]. In another study of 50 pwCF with pulmonary exacerbation, the virus-positive group presented more frequently with hypoxia than the negative group [17]. The virus-positive group did not, however, have increased rates of colonization and lung function decline over 12 months [17].

The sensitivity of detecting viruses using polymerase chain reaction (PCR) improved from 49% in sputum alone to 56% with a combination of sputum and additional mid-turbinate swab sampling [17]. A 2-year prospective study collected nasopharyngeal swabs and sputum samples for viral detection with multiplex PCR, as well as culturing and biochemical testing for bacteria detection. The study found that viruses were more commonly detected during the autumn and winter months, while bacteria were more commonly detected during the spring and summer months [14]. In a large investigation involving 12,702 children with CF and 8,320 adults with CF, surveillance tests positive for influenza or RSV were significantly associated with increased risk for pulmonary exacerbation in children. In adults this association was found for influenza, but not RSV [16].

In the recent SARS-CoV-2 (Covid-19) pandemic, it was feared that pwCF could be at risk of worse outcomes. It turned out, however, that pwCF are not more likely to be affected by this virus than the general population [18, 19]. As possible explanations, elevated mRNA for angiotensin converting enzyme 2 (ACE2) and decreased mRNA for TMPRSS2, as well as high levels of serine protease inhibitors in CF have been reported [19]. Severe Covid-19 outcomes in CF were associated with additional risk factors such as low lung function and immune suppression [18].

In general, viral infections are regarded as significantly involved in the progress of CF disease. Routine diagnostic examinations are necessary to determine causal viral or bacterial agents responsible for symptomatic disease.

Conventional viral diagnostic methods have several disadvantages: they are slow, expensive, require specialized expertise and are often not meaningful for therapeutic decisions. However, novel specific antiviral therapeutics require accurate information about the viral species, creating a clear need for precise viral diagnostics. Technological advances in modern diagnostic methods, particularly in nucleic acid chemistry, rapidly identify viruses from various clinical samples including serum, blood, cerebrospinal fluid, urine, bronchoalveolar lavage (BAL), nasopharyngeal swab, tracheal secretions, and biopsies. In the following sections, we summarize methods suitable for identifying viral infections in pwCF.

14.2 Direct detection

14.2.1 Virus isolation

Cell culture-based detection and isolation of viruses from clinical samples represents a traditional technique, utilizing primary cell types or established cell lines. Primary cells used for virus culture include Rhesus monkey kidney cells (RhMK), human foreskin fibroblasts, human embryonic kidney (HEK) cells. Ethical considerations, difficulties in availability and reproducibility and difficult culture conditions directed the cell culture-based virus isolation and detection towards using established cell lines. Examples of such cell lines include the human fibroblast MRC-5, the human lung epithelial A549, the human epithelial HEp-2, the African green monkey kidney epithelial Vero and the human kidney epithelial HEK293. Most viruses that infect pwCF can be detected by cell lines susceptible for the virus concerned [20]. Some difficulties have been reported in isolating viruses from samples from pwCF, probably due to unidentified substances in the patient samples that hinder successful virus cultivation [4]. Traditionally detection of viral infection in cell culture relies on morphological and biochemical alterations of infected cells leading to visible cellular changes, referred to as cytopathic effect (CPE) [21, 22].

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Several types of CPE can be observed, and some are sufficient to provisionally identify unknown viruses. However, fixation and staining are often necessary to clearly detect signs of infection such as inclusion bodies. CPE includes rapid total destruction of the cell monolayer (1-2 days after virus inoculation). The most severe form of CPE mostly occurs at high multiplicity of infections (MOI) or in the case of highly cytopathic viruses. Subtotal destruction, a less severe form of CPE, can be caused by some viruses by killing (detaching) of some but not all cells in the monolayer. The characteristic CPE for herpesviruses and poxviruses is focal degeneration, which appears as destruction of a localized area (foci) in the monolayer. Swelling and clumping of cells before detachment is a typical CPE, occurring for example with adenovirus. Retroviruses, paramyxoviruses, and flaviviruses can cause foamy degeneration due to vacuolization, a form of CPE which is challenging to detect without staining. Some viruses like paramyxoviruses induce cell fusion and syncytia formation, resulting in enlarged cells containing four or more nuclei. CPE usually appears 4-5 days after inoculation, but in case of certain viruses CPE can be first detected after 1-2 weeks or even later. Unfortunately, the appearance of CPE may differ between different viruses and the cell lines used for isolation. It can also depend on the MOI inoculated, and therefore requires specific expertise for detection [23]. In addition, noncytopathic viral infections do not induce CPE. To overcome these limitations, more informative methods have been developed, whereby novel genetically modified reporter cell lines turn on reporter genes in response to specific viral infections [21].

In summary, while cell-based methods for virus detection have certain drawbacks, they allow for the isolation and investigation of intact viral particles.

14.2.2 Antigen detection

Antigen detection for diagnosis of respiratory viruses aims to recognize viral proteins in patient samples. Generally, traditional antigen detection methods have a lower sensitivity compared to nucleic acid-based tests and are being replaced by more sensitive and less time-consuming molecular approaches [24]. Immunoassays like enzyme-linked immunosorbent assays (ELISA) and chemiluminescence immune assay (CLIA) were introduced in the early 1990s for the diagnosis of infectious diseases [25]. The direct fluorescence antibody (DFA) test involves direct staining of epithelial cells derived from nasopharyngeal swabs or aspirates by fluorescence-labeled virus-specific antibodies, followed by microscopic examination. The performance of DFA and other rapid point of care tests was compared to reverse transcriptase (RT)-PCR detection during the A/H1N1/2009 influenza pandemic. Study results suggested that, due to its lower sensitivity, negative test results by DFA should be confirmed by PCR [26]. Rapid diagnostic tests (RDT) are useful for detecting various viral infections in point of care settings [27, 28]. Usually, RDTs are based on viral antigen detection using specific antibodies by immunochromatographic (lateral flow) techniques in respiratory samples. RDT assays can be performed in 20-30 minutes, and a positive reaction results in color changes or optical signals. Commercially available RDTs have rather low sensitivity and specificity compared to nucleic acid-based detection methods. Additionally, sensitivity and specificity may vary between the viral specimen and the test manufacturer [24, 29, 30]. Efforts to improve RDTs for rapid and reliable detection of viral antigens during the Covid-19 pandemic may accelerate development of reliable diagnostics for other viral infections in the future [31].

14.2.3 Nucleic acid detection

PCR is one of the most widely adopted methods to detect nucleic acids. It can detect very low

amounts of DNA, or RNA via RT-PCR, with very high sensitivity from a variety of patient samples.

Thermal cycling of DNA comprises repeated denaturing of a double-stranded DNA template at high temperatures, followed by annealing sequence-specific oligonucleotides at lower temperatures, and synthesizing the new strand by a DNA polymerase. The discovery that *Thermus aquaticus* DNA polymerase is not inactivated by the high temperatures needed to melt DNA laid the foundation for a broad range of uses. In particular, this enzyme allows continuous cycling in a closed system, without the need to add fresh polymerase in each cycle [32, 33]. PCR was initially used to exponentially amplify small DNA fragments for diagnosing sickle cell anemia. Two years later, in 1987, researchers described a method to detect the presence of HIV, paving the way for detection of viruses using nucleic acid amplification [32, 34].

The use of intercalating fluorescent dyes such as SYBR Green I of the SYBR family, which only bind to double-stranded DNA, allows quantification of the original amount of DNA in a sample. Quantification is calculated by measuring the fluorescence intensity after each amplification cycle, and counting the number of cycles needed for the fluorescence intensity to cross a detection threshold, [35, 36]. This method, called real-time PCR or quantitative PCR (qPCR), can quantify viral load over the course of an infection, and can also determine different phases of an infection when combined with serological testing. The utility of qPCR was repeatedly demonstrated during the Covid-19 pandemic [37, 38].

Instead of intercalating dyes, pathogen-specific and different fluorescent-labeled oligonucleotides can be used to simultaneously detect many different viral pathogens, such as RSV, rhinovirus, PIV, influenza, human coronaviruses, MPV, AdV and enterovirus in only a few reactions, also allowing the detection of co-infections [39-41]. Such simultaneous detection of multiple targets, often from a single patient sample, is known as multiplex methodology. Numerous commercially available products target a broad range of viral and respiratory pathogens in parallel, such as the RespiFinder2Smart Kit (PathoFinder BV, Netherlands). Custom in-house developed assays are also common [24, 42].

Primer design must account for possible variations in the viral genome sequence, since false negative results can arise if mutations in the primer binding site prevent primers annealing [43]. To detect known genetic mutations in a specific pathogen, such as the N501Y mutation in the spike protein of SARS-CoV-2, qPCR can be followed by cooling down the reaction and performing a melting curve analysis. Here, small temperature increments and measuring fluorescence intensity to determine the melting temperature can detect relevant mutations [44].

Several sequencing technologies yield complete genomic information. These technologies can also be used to monitor mutations with potential impact on treatment or immunity, e.g. in HIV or SARS-CoV-2 [45, 46].

PCR assays to amplify and thus detect even minute amounts of DNA require the utmost care when handling samples and preparing reactions, to avoid the introduction of contaminants. In practice, this requires specialized laboratories. To avoid false positives, samples should be sequentially processed through different areas of the laboratory for 1) extraction of nucleic acids from patient material, 2) reaction set-up, 3) post-PCR activities. In addition, a space dedicated to preparing master mixes is required [47, 48].

PCR can easily detect pathogens with known genomic information. If, however, commercially available PCR kits fail to detect nucleic acids from unknown pathogens, different approaches are needed. To design targeted PCR probes, the genome of the pathogen in question must be known. As seen at the beginning of the Covid-19 pandemic, unbiased sequencing methods can generate genomic information about an emerging virus, and subsequently enable design of specific PCR primers [49].

Pathogen sequence can be determined by methodologies such as chain-termination based Sanger sequencing, next-generation sequencing (NGS) such as short-read sequencing by synthesis (SBS) by Illumina and long read sequencing technologies such as single molecule, real-time sequencing by Pacific Biosciences, or nanopore sequencing by Oxford Nanopore Technologies. Nanopore sequencing reads long strands in the range of thousands of continuous bases, by measuring the change in electric current as the bases in a strand pass through the nanopore. Metagenomic sequencing to nonspecifically determine viral infections has been applied in the past using Illumina SBS technology, and also more recently to characterize the monkeypox outbreak using nanopore sequencing. Long and short read technologies can be combined to improve viral metagenomic information even further [50].

PCR and the above-mentioned technologies need specialized laboratories and face the challenge of avoiding the introduction of contaminants. However technological advances to miniaturize, automate and improve nucleic acid detection have seen a considerable push during the Covid-19 pandemic, yielding point of care tests and field applications, in situations such as the recent outbreak of Ebola, and onboard the International Space Station [51-53]. This move towards improved and more accessible nucleic acid detection is demonstrated by miniaturized portable devices such as the VolTRAX for automated sequencing library preparation and the MinION sequencer by Oxford Nanopore Technology, and their integration into the MinION Mk1D platform. Future technologies will undoubtedly profit from the advances made during the Covid-19 pandemic.

14.2.4 Electron microscopy

In the second half of the 20th century, transmission electron microscopy (TEM) was essential for the discovery of many viruses and as a diagnostic tool for viral infections [54]. Molecular biology methods largely replaced TEM for routine diagnostics in the 1990s due to their higher sample throughput and high sensitivity and specificity. However, electron microscopy techniques have since improved, and the method is still useful in research, and in special diagnostic situations such as identification of poxviruses and particularly novel and emerging viruses [55, 56]. Cryo-electron tomography and subtomogram averaging have been recently developed to explore the structure of macromolecular complexes and viruses at sub-nanometer resolution [57, 58]. This methodology can visualize the assembly of virions in tissues, and the whole infection cycle [59, 60]. Researchers are increasingly using it to investigate the interaction of viral and human proteins, and the interaction of viruses with drugs and antibodies [57, 61]. The practical importance of cryo-electron microscopy was recently demonstrated by its pivotal role in elucidating how the SARS-CoV-2 spike protein interacts with the ACE-2 receptor, which was decisive for the development of vaccines and therapeutic antibodies [62]. Electron microscopy of organs and tissues contributed to understanding the pathogenesis of Covid-19 and to diagnostics in special cases [63, 64]. Another example of the utility of electron microscopy is the continuous clarification of the infection cycle of adenoviruses in molecular detail by the combination of cryo-electron microscopy and atomic force microscopy [65, 66].

As evident with SARS-CoV-2, it can be challenging to differentiate virus particles from subcellular structures such as coated vesicles, microvascular bodies, and rough endoplasmic reticulum [67, 68]. Moreover, tissue quality is important, and depends on the interval between sample collection and processing, and the processing methods [64]. To overcome these problems, the establishment and adherence to rigorous criteria has been suggested [67, 69]. The utility of electron microscopy for virus detection can be further optimized in the future by continuous improvement of methods and of software for evaluation as well as combination of, for instance, cryo-electron microscopy, atomic force microscopy and confocal laser scanning microscopy [58, 61, 66, 70, 71].

14.3 Indirect detection

14.3.1 Virus serology

Currently, monitoring respiratory infections relies on the detection of viral load in the respiratory specimens and the measurement of plasma levels of specific antibodies (IgM, IgG and IgA). Whereas viral load indicates viral replication in the primary infection site, qualitative and quantitative serological analysis reveals information on the functional integrity of the host immune response to infection [72]. Seroconversion refers to the significantly elevated antibody titer in response to infection. Such an increased titer – usually a 4-fold increase – between early and follow-up clinical samples is a well-accepted diagnostic tool to prove acute viral infection [73]. Serological tests measuring IgM levels can disclose an ongoing acute infection, but they provide limited sensitivity due to relatively low serum titers and low affinity of IgM to the target antigens [74]. In contrast, specific IgG levels are less useful for a diagnosis in the early stages of infection and therapeutic decisions, but provide information about the responsiveness of host immunity to infection.

Traditional methods to measure the presence of virus-specific antibodies included hemagglutination inhibition (HAI) assays and complement fixation tests [75, 76]. Nowadays various immunoassays are used to detect and quantify virus-specific antibodies in body fluids, especially serum. These immunoassays include radioimmunoassay (RIA), Western blot, ELISA, or CLIA. Studies on the etiology for community acquired pneumonia (CAP) comparing serological examinations with PCR-based molecular diagnostics support an adjunct role of serology for surveillance of respiratory virus infections [43, 77]. Of the respiratory viruses investigated in CAP patients, serology and PCR results agreed most often for RSV, and least often for AdV [43]. Generally, the highest number of positive detections related to RT-PCR tests, but serology increased the yield of positive cases for RSV, HMPV, AdV and PIV [43, 78]. Similarly, in another study, PCR detected more positive cases but the difference was significant only for AdV [77]. Importantly, cases of positive PCR but negative serological results were more common in the younger age groups (children aged <5 years) [43, 77]. Antibody serology reflects on the immune status in response to virus infection. Thus the presence and quantity of virus-specific antibodies represent important measures, but the level of protection provided by specific antibodies is often more relevant in clinical settings [79]. Therefore, determination of neutralizing antibody titers from patient sera is an important factor to predict immune protection against a virus infection. Virus-specific binding and neutralizing antibody titers usually correlate with each other. However, the potential occurrence of mutated virus variants resulting in immune escape should be considered [80]. Thus, virus neutralization assays should always include variants actually circulating in the population [46, 81]. Although it is sometimes difficult to predict the level of protection against infection, titers of neutralizing antibodies together with specific cellular immunity frequently correlate with protection from severe courses of disease and hospitalization [80, 82].

14.3.2 Virus-specific T cell responses

The presence and activation stage of pathogen-specific T cells in peripheral blood following viral infections may have an important diagnostic value that, together with serological and nucleic acid-based diagnostic methods, could help guide medical interventions providing the greatest patient benefit. Several assays for monitoring virus-specific T cell responses have been developed and some of them might be suitable for high-throughput diagnostic purposes. Generally, assays detecting pathogen-specific T cell reactivities require living cells, usually peripheral blood mononuclear cells (PBMCs) isolated from the patient. Therefore, rapid transport of patient material to the laboratory is crucial. HLA-peptide multimers can detect specific T cells directed against single epitopes, however this technology requires knowledge of the individual HLA haplotype of the patient as well as information about the immunodominance/availability of antigenic epitopes [83]. Thus, methods using overlapping peptide pools generated from pathogen-derived proteins for stimulating patient cells will be more suitable for future high-throughput diagnostic purposes [84]. When T cells specific for some of the peptides represented in the pool are present, they respond to these stimuli, which can be measured by various techniques.

Measurement of cytokines like interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and interleukin (IL)-2 from T cells after specific peptide or peptide pool stimulation reflects reactivity and functionality of T cell responses after infection or vaccination [85]. The enzyme-linked immunospot assay (ELISpot) has emerged as one of the most widely used methods to detect the magnitude and quality of T cell responses [86]. It measures the quantity and cytokine signature of responding T cells for specific stimulations at the single cell level. The ELISpot technology stands out with its high sensitivity for detecting antigen-specific T cells, even at low frequencies, and it is suitable for the diagnosis of infections like tuberculosis [87]. Nevertheless, ELISpot technology requires the isolation of PBMCs, which makes this analysis relatively laborious.

Alternatively, IFNγ release assays (IGRA) are available in which heparinized whole blood is directly stimulated by specific antigenic peptide pools. After a 24-hour incubation period, the level of IFNγ is measured in the plasma by ELISA, which provides information about the activation of the T cells. Such IGRAs are easy to perform, allowing rapid testing of the presence of antigen-specific T cell responses, and are available commercially for a number of different pathogens [88, 89]. Besides cytokine production upon stimulation, upregulation of surface molecules on T cells marks functional response to specific stimulations. The so-called activation induced marker (AIM) test is suitable to measure specific T cell activation based on the upregulation of OX40 and CD137 on CD4+ T cells or CD69 and CD137 on CD8+ T cells in response to peptide stimulation [84, 90, 91]. The major advantage of AIM tests compared to IGRA tests is that they give information on the particular T cell subsets (CD8+/CD4+) responding to antigenic stimuli [84]. Such flow-cytometry-based analyses, however, are challenging to standardize [92].

14.4 Conclusion

Viral infections play a role in the health of pwCF, and they very probably contribute to the frequency of exacerbations, deterioration of lung function, and disease progression. Viral diagnostics are key to identifying the causative pathogen and guiding therapy. Several direct and indirect methods of detecting viruses are available. The most common methods in routine practice include antigen detection including many rapid tests and nucleic acid detection, as well as detection of specific antibodies. Virus culture, electron microscopy, and specific cellular responses to viruses are used in special situations and mainly in research. The appearance of new viruses and new variants requires permanent adjustment of procedures. New developments, however, significantly contribute to the improved management of viral infections.

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Chapter 15
Eradication strategies for *Pseudomonas aeruginosa*

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Introduction
Recurrent and chronic bacterial infection are hallmarks of cystic fibrosis (CF) lung disease. A range of organisms are recognized as pathogens in the CF airway but one of the most problematic is *Pseudomonas aeruginosa*. Chronic infection with *P. aeruginosa* is common and is associated with increased mortality, progressive decline in pulmonary function and more frequent pulmonary exacerbations [1]. *P. aeruginosa* has innate resistance to many antibiotics and inducible resistance may manifest in the CF airway. Furthermore, *P. aeruginosa* may switch to a biofilm mode of growth in the CF airway, which acts as a barrier to antibiotic penetration and may allow the organism to evade the host immune system [2].

The European CF Society Patient Registry (ECFSPR) data from 2020 show that 11.7% of children and 41.0% of adults are chronically infected by *P. aeruginosa* [3]. In the UK, the Cystic Fibrosis Trust Registry Report 2020 showed that 4.7% of children under 16 years and 31.9% of people aged ≥16 years had chronic *P. aeruginosa* infection [4]. In the US, using the Leeds Criteria (where >50% of sputum cultures are positive for *P. aeruginosa* over a 12 month period), in 2019 the prevalence of chronic *P. aeruginosa* was 27.7% [5, 6]. In the 2020 CF Foundation registry report, the detection rate of people with CF (pwCF) with *P. aeruginosa* infection fell, with 43.2% having a positive culture sample in 2019 compared to 32.0% in 2020. The largest fall in prevalence was seen in the group under 18 years of age, falling from 44.5% in 2000 to 18.1% in 2020 [7].

Initial infection is usually with environmental strains which undergo progressive gene and behavioral adaptations to support persistence in the CF airways [8, 9]. Cross-infection of *P. aeruginosa* between pwCF has also been described [10]. People with minimal function mutations in the CF transmembrane conductance regulator (*CFTR*) gene may become infected with *P. aeruginosa* earlier than people with residual function *CFTR* mutations [11].

It has been established that early antibiotic treatment of initial *P. aeruginosa* infection in CF is more likely to result in eradication than no treatment or placebo [12-16]. In the initial phase of early infection, *P. aeruginosa* is not yet fully organized into mature biofilms and may be eradicated from the airways. This rationale led to one of the major advances in CF therapy; the adoption of early, aggressive eradication treatments in an effort to delay and prevent chronic *P. aeruginosa* infection. However, no single regimen has been proven to be more effective than another in early eradication of *P. aeruginosa*, and treatment regimens vary between countries [16]. In this chapter we will summarize the evidence surrounding early eradication of *P. aeruginosa*.

15.1 Guidelines and evidence

Today, antibiotic recommendations vary in different parts of the world. UK guidelines recommend an inhaled antibiotic (e.g. colistimethate sodium) in combination with an oral antibiotic (e.g. ciprofloxacin), as these antibiotics are thought to be effective against different components of the *P. aeruginosa* biofilm [17]. In the UK, a warning was recently issued about the rare musculoskeletal side effects of ciprofloxacin [18]. US guidelines favor tobramycin solution for inhalation (TIS) as first treatment [19]. The ECFS Standards of Care suggest 28 days tobramycin solution for inhalation (TIS) or up to 3 months of a combination of nebulized colistimethate sodium and oral ciprofloxacin [20]. Aztreonam has been evaluated for *P. aeruginosa* eradication, but is not currently first line in the US, UK or Europe. In an open-label study, inhaled aztreonam was effective in eradicating *P. aeruginosa* infection in pediatric patients, however this study is limited by the absence of a control group [21].

Only a limited number of randomized control trials (RCTs) of early eradication treatment have been conducted. A Cochrane systematic review concluded that eradication was more effective than placebo (or no treatment) but that no one eradication regimen has been shown to be better than another. Meta-analysis of the trials identified in the Cochrane review was hampered by the variety of outcome measures used to establish the success or failure of eradication regimens [16]. Table 1 summarizes the published RCTs and crossover trials evaluating eradication of *P. aeruginosa*, and outlines the regimens tested, duration of follow-up and outcomes.

15.2 Adjuvent treatment

Recently a multicenter, double-blind, randomized, placebo-controlled trial tested the hypothesis that the addition of azithromycin to TIS in children with CF (aged 6 months to 18 years) during their initial *P. aeruginosa* infection would reduce the risk of pulmonary exacerbation and prolong the time until *P. aeruginosa* recurrence [22]. Azithromycin was associated with a significant reduction in the risk of pulmonary exacerbation compared to placebo (hazard ratio 0.56; 95% CI 0.37-0.83; p=0.004), but no impact was observed on microbiological outcomes [22].

15.3 Importance of timely eradication treatment

The double-blind placebo-controlled EARLY study evaluated the efficacy and safety of TIS twice daily for 28 days in treating early *P. aeruginosa* infection in 51 children with CF aged 3 months to 7 years [23]. After the first double-blind period, all *P. aeruginosa* positive patients received TIS for 28 days whereas *P. aeruginosa* negative patients were given the option of crossover. After the first double-blind period, 85% of patients in the TIS arm were *P. aeruginosa* free, versus 24% in the placebo arm. At the end of the crossover period, 76% patients who had received TIS in the initial 28 days were *P. aeruginosa* free compared to 48% who had initially received a placebo. This trial suggests that eradication treatment should be started promptly and not delayed more than 28 days after *P. aeruginosa* isolation.

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15.4 Intravenous versus oral eradication treatment

Intravenous therapy against initial *P. aeruginosa* infection has long been used in clinical practice, but has not been shown to be more effective than oral treatment in RCTs. A recent large RCT compared the efficacy of 14 days of intravenous ceftazidime and tobramycin to 12 weeks of oral ciprofloxacin [24]. Both regimens were combined with 12 weeks inhaled colistimethate sodium. The primary outcome was percentage of patients with *P. aeruginosa* eradication at 3 months, remaining free of infection up to 15 months. Compared with oral therapy, intravenous therapy was not more effective, and the cost of intravenous treatment was higher.

15.5 Sustained success versus failure of eradication

Early and sustained eradication of *P. aeruginosa* is of particular clinical benefit since chronic infection is associated with progressive deterioration of pulmonary function. In addition, increasingly aggressive antibiotic treatments negatively impact the person with CF's quality of life [1].

The concept of "sustained eradication" refers to patients with negative cultures for 12 months after treatment ended. In the recent TORPEDO trial, the primary outcome was eradication after 3 months of treatment *and* remaining free of infection for a further 12 months (achieved in 44% of participants in the intravenous treatment group and 52% in the oral treatment group) [24]. People with sustained eradication over a 5 year period had a 74% reduction in the risk of developing chronic *P. aeruginosa* infection (hazard ratio 0.26; 95% CI 0.17-0.4) and reduced use of anti-pseudomonal antibiotics by comparison to those without sustained eradication [25]. In addition, those with sustained eradication had reduced risk of having mucoid *P. aeruginosa* compared to those without sustained eradication (HR 0.43%; 95% CI 0.25-0.73) [25].

Eradication treatment may fail in 28-40% of cases [26]. To date, no significant differences have been found regarding the clinical characteristics of the patients (age or lung function) or the type of treatment. However, *P. aeruginosa* characteristics found to be associated with chronicity were mucoidy, lack of twitching motility, wrinkly colony surface and colonies with irregular edges [27]. These characteristics may relate to differential resistance in *P. aeruginosa* to the actions of neutrophils [28].

15.6 Persitent or new infection, despite eradication treatment

Patients who have successfully undergone early eradication treatment can still manifest *P. aeruginosa* infection later, prompting the question "is this persistence of the initial infection or a new organism?" Molecular studies can help answer this question. The TORPEDO trial used variable number tandem repeat (VNTR) typing to determine if further *P. aeruginosa* infection, following apparently successful eradication, was with a genetically identical strain [24]. Of participants without sustained eradication, VNTR typing detected the same strain of *P. aeruginosa* at baseline and recurrence for 76% of participants in the intravenous group and 71% in the oral group. This suggests that eradication therapy may have rendered the organism undetectable but did not completely eradicate *P. aeruginosa* from the airways. A recent study used whole genome sequencing and clone typing to study recurrence of *P. aeruginosa* in pwCF from a Danish clinic [29]. Participants had a median maximum eradication period of 1.1 years (range 0.5-3.6) and 43% had the same clone of *P. aeruginosa* before

and after their maximum eradication period. The authors suggest that persisting clone types result from re-emergence of the same strain and not reinfection from the environment [29], and suggest that persistent sinus infection may serve as a reservoir of *P. aeruginosa* infection which may then reinfect the lungs [29].

15.7 Recommendations for practice

Based on the studies described above, we provide some practical clinical recommendations.

- When *P. aeruginosa* is identified in any respiratory sample from a person with CF who has previously been free from infection, an appropriate eradication regimen should be commenced (within 28 days).
- A sample (preferably induced sputum or bronchoalveolar lavage) should be taken once the eradication regimen has been completed to confirm whether eradication has been successful. If not successful, a further course of eradication treatment should be considered.
- There is insufficient evidence, at present, to recommend one eradication regimen over another, though an agent shown to be effective in RCTs should be selected.
- Intravenous therapy offers no advantage over oral (provided inhaled therapy is also given).
- The book published by ECFS in 2020 "Optimizing pharmaceutical care in cystic fibrosis" can be helpful (https://www.ecfs.eu/content/ecfs-book) [30].

We also provide some recommendations for research:

- Clinicians, researchers and patients should agree on the most meaningful outcomes and endpoints for clinical trials of eradication regimens, to reduce research waste and maximize opportunities for demonstrating meaningful differences between regimens.
- Future trials should measure sustained eradication for ≥12 months and incorporate measures to assess whether subsequent strains are the same of different from the strain causing initial infection.
- These trials should evaluate the comparative efficacy of eradication regimens in patients receiving CFTR modulator therapy.
- In the patients on CFTR modulators, expectoration of sputum may be challenging; sputum induction with hypertonic saline may address this need. Research focused on alternative methods of detecting infection is needed.

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Table 1

Published randomized control or crossover trials of the eradication of *P. aeruginosa*.

Author	No. of patients	Age	Regimen A (RegA)	Regimen B (RegB)	Duration of follow-up	% neg. RegA	% neg. RegB	Outcome measure for successful eradication
Valerius 1991 [13]	26	2-9 years	Colistin and ciprofloxacin	No treatment	3 weeks	86	42	Time to chronic infection (monthly for 6 months)
Wiesemann 1998 [14]	22	4-18 years	TIS (80 mg twice daily)	Placebo	12 months	87	0	Time to convert from culture positive to culture negative
Gibson 2003 [31]	21	6 months to 6 years	TIS	Placebo	28 days	100	8	Bronchoalveolar lavage *P. aeruginosa* positive on day 28
Ratjen 2010 [32]	88	>6 months	TIS 28 days	TIS 56 days		93	92	Time to recurrence following end of treatment.
Treggiari 2011 [33]	304	1-12 years	Regular TIS 28 days / quarter	As required TIS	28 days	72	56	Odds ratio of being *P. aeruginosa* positive during the study
Taccetti 2012 [34]	223	1-35 years	Colistin and ciprofloxacin	TIS and ciprofloxacin	28 days	63	65	Eradication = 3 successive negative cultures in 6 months
Proesmans 2013 [35]	58	<18 years	TIS 28 days	Colistin and ciprofloxacin	3 months	79	90	Respiratory sample (any) negative at the end of treatment
Ratjen 2019 [23]	51	3 months to <7 years	Crossover TIS 28 days	Crossover Placebo 28 days	90 days	84.6	24	Eradication = negative sputum/throat swab culture on day 29 (completion of first treatment period) or 28 days post crossover treatment
Langton-Hewer 2020 [24]	286	>28 days	Colistin and intravenous ceftazidime and tobramycin	Colistin and oral ciprofloxacin	3 months	44	52	Composite: negative after 3 months of treatment plus remain negative for 1 year
Total	1079							

Abbreviations: Neg=negative, RegA=regimen A, RegB regimen B, TIS=tobramycin inhalation solution

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Chapter 16
Suppression therapy for chronic *Pseudomonas aeruginosa*

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Introduction

Pseudomonas aeruginosa is a highly adaptive gram-negative bacterium commonly isolated from the airways of people with cystic fibrosis (CF), which becomes more prevalent with increasing age and lung function severity [1]. According to the European CF Society (ECFS) Patient Registry, the prevalence of chronic infection in 2020 was 5-45% in children (<18 years) compared with 35-90% in adults (≥18 years), varying by country [2]. Thanks to effective segregation and eradication strategies, the prevalence has steadily fallen over the years in many countries. For example, in the UK prevalence dropped from 35.5% in 2010 to 19.4% in 2020 [3]. Nevertheless, chronic infection with *P. aeruginosa* remains a major challenge in CF, as it is a significant risk factor for pulmonary exacerbations, accelerated lung function decline and increased mortality [4].

The natural history of *P. aeruginosa* infection is usually of intermittent culture positivity in the early stages, with cultures usually becoming persistently positive over time [5]. This adaptive ability is thought to be due to the relatively large genome of *P. aeruginosa*, with selection for mucoid phenotypes and growth in both planktonic and biofilm states, thus enhancing its ability to withstand the relatively harsh biophysical and immunological environment of CF airways [6]. Once chronic infection is established, bacterial clearance is unlikely, so treatment strategies switch from an eradication approach to one of bacterial suppression, to reduce the risk of accelerated health decline [7]. Strategies for chronic suppression can include the inhaled, oral and intravenous (IV) routes. In practice, however, the majority of patients are treated with inhaled antibiotics, as supported by the strongest evidence base [8]. Delivering the antibiotic directly to the source of infection by inhalation achieves drug concentrations that far exceed the levels achieved by the systemic route. The inhalation route therefore allows inhibition of bacterial growth in biofilms, while minimizing systemic drug levels and the associated risk of toxicity and side effects [9].

16.1 Pharmacological considerations

Antibiotic effectiveness relates to pharmacokinetic (PK) and pharmacodynamic (PD) properties, and the target organism's susceptibility which is determined *in vitro* by the minimum inhibitory concentration (MIC) required to suppress bacterial growth [10]. A key risk factor for the selection of antibiotic-resistant strains is recurrent bacterial exposure to sub-inhibitory concentrations at the site of infection [11]. Therefore, targeted maintenance of high antibiotic concentrations at the infection locus is critical to controlling infection and minimizing antibiotic selection pressure.

It is useful to understand how lung anatomy relates to CF lung infection, when reviewing

chronic suppression strategies for *P. aeruginosa*. The smaller conducting zone (nasal cavity, pharynx, trachea, the bronchi and the terminal bronchioles), lined by mucus-secreting goblet cells and submucosal glands, is considered the primary bacterial reservoir, where bacteria are organized in mucoid biofilms within the mucus, protected against antibiotics and host defenses [12]. In addition, the unique properties of CF sputum (e.g. low oxygen content, high viscosity) can independently limit the therapeutic effect of certain antibiotics [13, 14].

Studies reporting on drug bioavailability have demonstrated very high drug concentrations in sputum following inhalation, above the conventional MICs used for systemic administration [15, 16]. By contrast, little aerosolized drug reaches the respiratory zone (respiratory bronchioles and alveoli) as evidenced by the very low drug concentrations detectable in serum (reflecting respiratory tissue concentrations) [12, 17]. The opposite is true for oral/parenteral administration, providing strong rationale for the use of inhaled antibiotics to achieve high MICs for effective suppression therapy of chronic *P. aeruginosa* in CF.

16.2 Inhaled antibiotics

Inhaled antibiotics are the mainstay of suppression therapy for chronic *P. aeruginosa* infection in CF, with evidence first emerging in the 1940s [18-20]. Published guidelines support their use based on data from large randomized trials showing benefits on bacterial load, lung function, quality of life (QoL) and pulmonary exacerbations [19, 20].

At present, two inhaled solutions are approved for use in the USA (tobramycin and aztreonam lysine) and four in the UK and Europe (colistimethate sodium, tobramycin, aztreonam lysine and levofloxacin inhaled solution; LIS). Dry powder inhalation formulations of tobramycin (TOBI® Podhaler®, Novartis) and colistimethate sodium (Colobreathe®, Forest Laboratories) have also been approved in the UK and Europe for chronic *P. aeruginosa*; the tobramycin formulation is also approved for use in the USA [21-23]. The use of these medicines is summarized in Table 1 and their mode of action is described in Figure 1. Clinical efficacy is discussed below.

Colistimethate sodium, the prodrug of colistin, is a polymyxin antibiotic. It breaks down the bacterial cell's permeability barrier by disrupting the integrity of the gram-negative outer membrane, leading to leakage of cytoplasmic contents and cell death [24]. Inhaled colistin solutions (colomycin and promixin) have been used for decades throughout the UK and Europe [25]. However, there have been limited randomized trials to support their long-term efficacy. Only a single phase III open-label study has assessed the safety and efficacy of Colobreathe® and found it to be non-inferior to tobramycin inhalation solution (TIS) based on the change in percent predicted forced expiratory volume in 1 second (FEV_1) after 24 weeks [23]. Nonetheless, inhaled colomycin products are used widely in both pediatric and adult CF centers, especially given the low rates of microbial resistance despite continuous use [26].

Tobramycin, an aminoglycoside, inhibits protein synthesis initiation by disrupting formation of the initiation complex between mRNA and the bacterial small 30S ribosomal subunit [24]. In 1998, in the form TOBI® (Novartis), tobramycin became the first antibiotic approved for inhaled use in CF in individuals chronically infected with *P. aeruginosa* [15, 27]. The efficacy and safety of TIS in CF is well established with large randomized controlled trials (RCTs) demonstrating improvements in lung function and nutritional state, reduced *P. aeruginosa* bacterial load and decreased hospitalizations [15, 27, 28]. Of note, although TIS achieved significantly high peak sputum levels of drug, there was minimal systemic absorption. In addition, two controlled clinical studies comparing the safety and efficacy of tobramycin inhalation powder to TIS demonstrated similar tolerability and efficacy with higher rates of adherence and treatment satisfaction with the inhalation powder [21, 22].

Aztreonam, a monobactam antibiotic, interrupts cell wall synthesis by interacting with penicillin-binding protein 3 of susceptible bacteria [29]. Its novel reformulation as a lysine salt for inhalation (marketed as Cayston®, Gilead) removed arginine which can cause airway inflammation [30]. It was approved in 2010 by EMA and has been shown in several clinical trials to be safe and effective in pwCF aged ≥6 years, improving respiratory symptoms and lung function, and reducing the need for additional anti-pseudomonal antibiotics [30-32]. Notably, no evidence for the development of antibiotic resistance was found in studies extending beyond 12 and 18 months [32, 33].

Levofloxacin, a fluroquinolone, blocks bacterial cell growth by inhibiting bacterial DNA gyrase and topoisomerase IV activity. Significantly, its activity in CF sputum is not reduced, unlike tobramycin and aztreonam [34]. RCTs have shown that LIS reduces *P. aeruginosa* bacterial load and improves lung function, with dysgeusia the most common adverse event [35, 36]. A systematic review and Bayesian network meta-analysis showed LIS to be as effective as other inhaled antibiotics in treating chronic *P. aeruginosa* [37].

No clear recommendations exist to guide selection of inhaled antibiotic or choice of treatment regimen (e.g. continuous versus on/off monotherapy, or continuous alternating therapy with two or more inhaled drugs). Inhaled antibiotics typically have been cycled in a 28-day on/off strategy. This is based on observations from the pivotal TIS trials where lung function benefit peaked at 4 weeks of treatment, and the hypothesis that continuous use might promote antimicrobial resistance [6]. Studies suggest that *in vitro* drug sensitivity is modestly reduced with long-term inhaled antibiotic use, but high level drug resistance is rare [30, 38, 39]. Furthermore, real-world experience has shown that lung function loss and symptom worsening tend to accompany the 4-week off periods, and this has driven the development of continuous alternating regimens in the absence of RCTs. Some support for this strategy was provided by Rojo-Molinero and colleagues who used an *in vitro* biofilm model to show that alternating treatment with inhaled aztreonam and tobramycin had increased antibiofilm activity compared to tobramycin alone [40]. However, the optimal regimen for combining and sequencing inhaled antibiotic therapy as long-term suppressive therapy remains to be determined.

16.3 Oral antibiotics

The use of oral antibiotics for the chronic suppression of *P. aeruginosa* is not standard of care as there is minimal evidence for this approach. In fact, only the fluroquinolone class of oral antibiotic has direct anti-pseudomonal properties, but their use (usually ciprofloxacin) is usually reserved for the treatment of pulmonary exacerbations [41].

The only oral antibiotic recommended for chronic use in CF is the macrolide azithromycin, although as it does not have any direct anti-pseudomonal properties, it might not be considered a suppressive therapy by strict definition [42]. It is widely used, with ~50% of patients in the UK CF Registry receiving chronic treatment in 2020 [3].

Its clinical benefit is thought to derive from its immune-modulating properties, particularly in the context of chronic *P. aeruginosa*, with evidence of downregulated neutrophil chemotaxis and reduced production of interleukin (IL)-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) by bronchial epithelial cells [43, 44]. Using data from the CF Foundation Patient Registry, a study reported the long-term benefit of chronic azithromycin, showing a rate of FEV_1 decline 40% lower in azithromycin-treated patients with chronic *P. aeruginosa* compared with matched controls over a three year period [45]. This difference was not seen in patients without *P. aeruginosa*. Additionally, these benefits were not seen in patients concomitantly prescribed inhaled tobramycin, consistent with previous post hoc and *in vitro* data, suggesting a possible antagonistic effect [46]. However, a recent RCT did not show a deleterious effect in patients on both drugs, providing some reassurance to clinical teams of this common prescribing practice [47].

The chronic use of doxycycline or co-trimoxazole as suppressant therapies has been reported anecdotally by some clinicians when other treatment options have failed, but supporting evidence is scant and registry data is not granular enough to estimate use. Neither doxycycline nor co-trimoxazole have direct anti-pseudomonal properties. Of the two, doxycycline probably has the most mechanistic data, as it is known to inhibit matrix metalloproteinase 9, a protease associated with the chronic neutrophilic infection of CF lung disease [48]. A double blind RCT of eight days of doxycycline treatment in addition to standard IV antibiotics for a pulmonary exacerbation in patients with chronic *P. aeruginosa* infection demonstrated improvements in inflammatory markers and clinical outcomes including FEV_1 [47]. While these results show promise, further studies are required, particularly in the context of chronic treatment. To our knowledge no CF studies have been conducted to support the use of co-trimoxazole in this context, although plausible explanations for benefit might include the treatment of co-existing susceptible pathogens.

16.4 Intravenous antibiotics

Apart from treating pulmonary exacerbations, IV antibiotics are not usually considered for chronic suppression of *P. aeruginosa*. The reasons for this include toxicity, side effects, inconvenience and treatment burden. However, patients with severe disease, high sputum burden and highly resistant *P. aeruginosa* isolates often require longer and more frequent IV courses, particularly in the context of end-stage disease, which might include bridging to lung transplant [49]. In this situation, it can be difficult to differentiate between genuine "acute" exacerbations and chronic pulmonary sepsis. From the UK Registry, in 2020, the median (interquartile range) annual IV antibiotic days for adults (≥18 years) was 25 [10-43, 45-50] with a maximum of 90 days [3]. Although the data do not report the actual length of each treatment course, it is likely some patients will have had treatment significantly beyond the standard 14 days in an attempt to further suppress bacteria. However, the practice of administering longer courses is largely based on clinical experience and recently has been challenged by the results of the STOP2 RCT of optimal IV antibiotic treatment [50]. This showed no added benefit of 21 days of treatment versus 14 days, although it is important to highlight that patients with a history of frequent IV antibiotic treatment were excluded from the study, which is likely to be relevant to some of the patients discussed here.

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16.5 Future therapies

Amikacin liposome inhalation suspension (ALIS; Arikace®, INSMED) was developed for the treatment of chronic *P. aeruginosa* in CF, but is not yet licensed [51, 52]. Its sustained-release liposomal formulation was designed to better penetrate viscid mucus and pseudomonal biofilms [51, 53]. As well as once-daily dosing, an added advantage is site-specific release of drug from the liposomes by bacterial rhamnolipids. A phase III open-label study assessing efficacy and safety of ALIS showed non-inferiority to TIS with respect to lung function change over 3 treatment cycles (28-days on/28-days off) [51]. In the subsequent open-label extension study, ALIS was shown to be well tolerated with a favorable safety profile and sustained antibacterial activity over 96 weeks [52], however the ALIS development program appears to be on hold.

Fosfomycin, a phosphonic acid antibiotic, with a broad spectrum of activity, has been combined with tobramycin, to take advantage of synergistic effects [41]. Despite promising results of a phase II study, the program is currently on hold [54, 55].

Ciprofloxacin dry powder for inhalation was trialed in a randomized, placebo-controlled, phase IIb study, but unfortunately failed to meet its primary endpoint of change in FEV_1, and the CF program was terminated [56].

Several novel non-antibiotic approaches are also being explored to treat chronic pseudomonal infection in CF, with gallium and bacteriophage therapy currently in clinical trials [55]. Gallium, a group 13 metal, disrupts key iron-dependent processes in bacteria [57]. A phase II study (NCT02354859) evaluating the safety and efficacy of a five-day IV infusion in adults with chronic *P. aeruginosa* has been completed. A phase I/IIa study of inhaled gallium (NCT03669614) is underway, to evaluate the efficacy and PK profile in healthy adults and the safety, PK and efficacy of repeat administrations in *P. aeruginosa* infected pwCF.

Lytic bacteriophages are strain-specific viruses that infect and replicate within bacterial cells, lysing the host cell when releasing viral progeny [58]. In chronic and acute murine models of *P. aeruginosa* infection, lytic phage preparations have shown efficacy, reducing bacterial load and inflammation with no evidence of toxicity [59, 60]. Armata Pharmaceuticals have formulated an inhaled multi-phage cocktail (AP-PA02). It is under investigation in a phase Ib/IIa study (NCT04596319) to determine safety and tolerability in CF.

16.6 Old and new challenges

With the growing adoption of highly effective CFTR modulator therapies it remains to be seen how chronic pseudomonal infection will continue to impact CF lung pathophysiology and if inhaled antimicrobial therapies will continue to play as critical a role in CF pulmonary management. However, some pwCF do not tolerate or do not respond to commercially available inhaled therapies or have resistant bacteria other than *P. aeruginosa*. Therefore, antibiotic drug development is still needed, to provide options to these patients, and to patients not receiving CFTR modulators, either because of ineligibility or intolerance.

Strategies to simplify dosing regimens, encourage adherence and promote patient satisfaction remain important priorities. Old challenges persist, including determining the impact of long-term use of suppression therapy on quality of life and survival. The evolution of culture-inde-

pendent techniques for microbial analysis may provide better insights into the relationship between the CF lung microbiome and inhaled antibiotic efficacy and enable better identification of new therapies. However, new challenges in the post modulator era are emerging, particularly around airway sampling to guide decisions on safe withdrawal of suppression therapy, as patients are producing less sputum and are increasingly keen to reduce treatment burden as their clinical outcomes improve.

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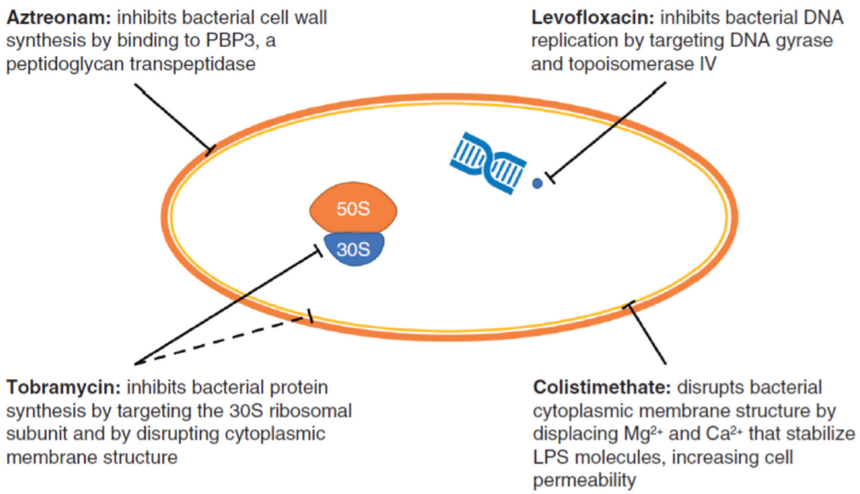
Figure 1

Modes of action of inhaled antibiotics approved for chronic suppression of *P. aeruginosa* infection in CF.



Abbreviations: BP3=Penicillin-binding protein 3; LPS=Lipopolysaccharide. Reproduced with permission from [29]

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Table 1

Summary characteristics of commercially approved inhaled antibiotics for chronic suppression of *P. aeruginosa* infection in CF

Drug preparation	Brand name(s)	Inhalation device(s)	Dose		Inhalation time (min)	Frequency of device sterilisation	Preparation	Need for refrigeration	Selected common or undesirable side effects
Tobramycin inhalation solution	TOBI Bramitob	Pari-LC Plus E-flow rapid (Off-label) I-neb (Off-label)	300 mg BD		~4-20	Each use	Ampoule, ready to use	Yes	Cough, tinnitus, hearing loss, dysphonia*
Tobramycin inhalation powder	TOBI Podhaler	Podhaler DPI	112 mg BD		~5	Not required. Device discarded after 1 week	Four capsules	No	Cough, tinnitus, hearing loss, dysphonia*
Colomycin inhalation suspension	Colistin Promixin	Pari-LC Plus E-flow rapid I-neb	1-2 million IU BD		~3-15 depending on inhalation device used	Each use	Ampoule, dilution required	No	Cough, bronchospasm, throat irritation
Colomycin inhalation powder	Colobreath	Turbospin DPI	1 662 500 IU BD		~1	Not required. Clean with a dry wipe	One capsule	No	Dyspnoea, dysphonia, cough, throat irritation
Aztreonam lysine for inhalation	Cayston	Altera nebuliser with E-flow rapid	75 mg TDS (at least 4 h between doses)		~2-3	Each use	Ampoule, dilution required	Yes	Cough, nasal congestion, wheeze, dyspnoea
Levofloxacin inhalation solution	Quinsair	Zirela nebulizer with E-flow rapid	240 mg BD		~5	Each use	Ampoule, ready to use	No	Dysgeusia

Abbreviations: BD=twice a day; DPI=day power inhaler; TDS=three times daily

Adapted with permission from [29]. Refer to the respective summary of product characteristics for specific drugs for more detailed information.
*Tinnitus, hearing loss and dysphonia were reported in controlled trials of nebulized tobramycin events. The episodes were transient and resolved without discontinuation of tobramycin and were not associated with permanent deficit on audiology testing.

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Chapter 17

Staphylococcus aureus in the airways of people with cystic fibrosis

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Introduction

The relationship between *Staphylococcus aureus* and humans is complex and that complexity is heightened for people with CF (pwCF). This chapter will consider and discuss the role of *S. aureus* in CF lung disease, including its methicillin resistance form (MRSA). Information will be provided on epidemiology, virulence factors, diagnosis and treatment approaches.

17.1 Biology of *Staphylococcus aureus* infection

In contrast to most CF pathogens, *S. aureus* is not an environmental bacterium but rather a natural colonizer of humans and other domesticated and wild animals and birds [1, 2]. Colonization typically occurs on skin and mucus membranes, with about 30% of humans having chronic nasal colonization. Nasal colonization is lower for MRSA with prevalence ranging from <0.5% in Scandinavian countries, to 1-4% in the USA or India among healthy people not working in healthcare settings [3]. Chronic MRSA colonization may be associated with an increased risk of post-operative infections in adults, however confounders are underlying disease and length of surgery [4]. Colonization rates vary by region of residence but are often higher in children who attend daycare or school [5]. Several countries also report livestock associated MRSA, predominantly the ST398 strain, mostly in people with close contact with the animals [6].

The identification of numerous strains of *S. aureus* using a variety of genetic testing approaches has provided key epidemiological information. The most established method is pulse-field gel electrophoresis (PFGE), which compares gel electrophoresis patterns of each strain. This involved method has now been replaced by more efficient sequencing methods such as multilocus sequence typing (MLST) and typing of the gene encoding the variable tandem repeat region of staphylococcal protein A (*spa* typing). For more granular, mostly research-related, tracking, whole genome sequencing has become cheaper and faster to perform. One particular gene sequence (the mobile genetic element, SCC*mec*) is particularly relevant to MRSA, as certain strains are associated with resistance to the penicillin-binding protein [7]. Of the 12 currently recognized SCC*mec* variants, the *mec* II type is most frequent in the healthcare setting and *mec* IV type is most frequent in the community. However, the smaller size of the *mec* IV confers a higher fitness. Therefore, the SCC*mec* IV strains have become highly prevalent in healthcare settings, contributing to nosocomial outbreaks [8].

S. aureus causes a wide range of infections in humans ranging from minor skin infections to overwhelming sepsis. The steps leading from colonization to infection and the associated changes in bacterial virulence and metabolism during this transition have been intensively studied, but many aspects remain incomplete-

ly understood. Key processes include adhesion, changes in multiplication rate, and production and regulation of virulence factors that either cause toxicity or inhibit host immune functions. Triggers for infection include changes in the host, for example viral infections in the context of *S. aureus* lung infections. No biomarkers have been found to reliably predict transition from colonization to infection. Such biomarkers would be beneficial, and possibly very relevant for pwCF, to distinguish upper airway colonization from airway infection.

17.2 *Staphylococcus aureus* epidemiology

Data from the US patient registry suggest that *S. aureus* is a regular isolate from the respiratory cultures of pwCF, in contrast to registry data from the UK [9, 10]. Of note, UK data reports chronic infection whereas US data reports any *S. aureus* identification. Anti-staphylococcal antibiotic prophylaxis is recommended in the first three years of life in the UK [11]. A detailed longitudinal observational study of children with CF comparing US and UK registry data and adjusting for significant confounding variables, suggested that US children had an increased risk for *S. aureus* detection (hazard ratio 5.79; 95% confidence interval [CI], 4.85-6.90) and an increased risk of *P. aeruginosa* detection (1.92, 95% CI, 1.65-2.24) [12]. Children in the UK on flucloxacillin prophylaxis were at increased risk of *P. aeruginosa* detection. The observation of higher *P. aeruginosa* detection despite higher rates of *S. aureus* [12], and studies on sputum cultures and the US patient registry, do not support the notion that *S. aureus* colonization protects against *P. aeruginosa* airway infection [13, 14].

The relationship between *P. aeruginosa* and *S. aureus* in CF has been investigated extensively and data from *in vivo* and laboratory work are sometimes contradictory. Environmental *P. aeruginosa* and *S. aureus* co-exist. However, *P. aeruginosa* that has adapted to the CF airway environment has a more commensal-like approach, and may even encourage *S. aureus* resistance to certain antibiotics. In addition there is good evidence that in this environment the *S. aureus* may itself adapt to small colony forming variants (SCVs) and with this phenotype co-exist with *P. aeruginosa* [15] as well as other pathogen-pathogen interactions [16]. SCV *S. aureus* has been associated with worse clinical outcomes for CF respiratory health, yet can be easily missed by standard microbiological culture techniques.

The epidemiology of MRSA also differs significantly between different countries and typically mirrors rates of MRSA in non-CF populations [17]. The prevalence of MRSA in pwCF is around 25% in the US, with higher rates in the south and southwest regions of the US compared to the west. In contrast MRSA is almost never reported in pwCF from the Netherlands or Denmark. Across the countries contributing data to the ECFS Patient Registry (ECFSPR), the average rate of MRSA in 2021 was 4.56% ranging from 0.5 to 22% [18].

17.3 Resistance, virulence and persistence

17.3.1 Antibiotic resistance

S. aureus has developed resistance to many antibiotics; initially to oxacillin, then to methicillin shortly after its introduction and for almost all newly developed antibiotics within a few years of their clinical use. In pwCF in the US, there is a high rate of MRSA resistance to clindamycin (70-90%), likely related to frequent use of macrolides [19]. Resistance to fluoroquinolones is equally frequent and fluoroquinolones can induce high level oxacillin resistance and cross-resistance to other antibiotic classes [20]. Resistance to linezolid and vancomycin intermediate and vancomycin heteroresistance (i.e., a sub-population of resistant isolates) is also recognized albeit less frequently [21]. As routine clinical susceptibility testing only evaluates a few isolates per sample, heteroresistance can be missed and more extensive testing should be considered in patients with MRSA airway infection failing to respond to therapy.

17.3.2 Virulence factors, resistance and tolerance

In CF respiratory infections, the persistence of the organism remains the challenge, rather than fulminant infections as seen in non-CF *S. aureus* pneumonia. This persistence is enhanced due to the versatility and adaptation of *S. aureus* to the CF lung environment leading to immune evasion and failure of antibiotics. Factors that contribute to persistent infection include adherence processes, toxin production, adaptation to the CF airway environment and evasion of the immune system.

17.3.3 Adherence processes

Adherence to the cell surface is mediated by surface proteins anchored in the bacterial cell wall with currently about 24 different *S. aureus* known surface proteins. These surface proteins possess specificity to different host structures (e.g., binding to fibronectin, complement factors or hemoglobin), yet show variable expression depending on growth conditions and growth phase of the organism [22].

17.3.4 Toxin production

S. aureus produces a variety of toxins including α-hemolysin, toxic shock syndrome toxin (TSST-1), and SEA (Staphylococcal enterotoxin A). The interaction of toxins, severity of disease and potential for chronic infection is complex. High toxin-producing strains are more transmissible and more likely to transition from colonization to mild infection. In contrast, low toxin-producing strains may be more successful in establishing chronic infection [23]. Severe fulminant infections are unusual for CF respiratory infections, where even exacerbations are not typically associated with signs of systemic inflammation.

The accessory gene regulator (*agr*) is a quorum sensing system regulating toxin production and growth mode. This system senses environmental conditions (e.g., nutrient limitation and *S. aureus* cell density) for regulation of toxin expression, cell wall surface attachment and biofilm formation. Mutations in the *agr* locus have been shown to be present in *S. aureus* in CF chronic lung disease [24]. Interestingly certain *agr*-types may be associated with clinical outcomes in CF, yet virulence factors were not, and over time a decrease in virulence genes was noted [25]. This down-regulation of toxins has been described as part of adaptation to chronic infection in CF *S. aureus* isolates [26, 27].

As the virulence and toxin profiles overlap between methicillin sensitive *S. aureus* (MSSA) and MRSA, it is unlikely that MRSA-specific virulence factors cause the worse outcomes seen with chronic MRSA compared to chronic MSSA infections in pwCF [28, 29]. However, the Panton Valentine Leukocidin (PVL) toxin is expressed about twice as often by MRSA compared to MSSA strains. The MRSA strain USA300 was associated with a surge in community associated MRSA infections in the early 2000s and encoded the *pvl* gene in 80-100% of isolates [30]. PVL attaches to specific leukocyte membrane receptors causing perforation and necrosis, and was initially considered to be the key toxin responsible for necrotic skin and lung infections. Despite initial epidemiologic reports showing a higher mortality with PVL positive strains, the contribution of PVL to poor outcomes has not been confirmed [31, 32]. Similar to the high rate of USA300 in the general population, there was also a spike in in-

cident MRSA among the US CF population and a shift from SCC*mec* II to SCC*mec* IV, many of which were PVL positive [33, 34]. Yet, the duration of infection rather than *mec* type was a more important determinant of outcome [34].

17.3.5 Adaptation

As with other bacteria, *S. aureus* phenotypic and genotypic adaptability is associated with slower growth, decreased metabolic activity and escape from the immune system. *S. aureus* isolates from respiratory cultures from pwCF often show slow growth, likely due to decreased anabolic activity, with heterogeneity of growth within a given sputum [35]. Biofilm formation, mucoidity and growth under micro-aerophilic or hypoxic conditions have all been described in CF lung disease [36, 37]. Bacterial biofilms represent a community of bacteria (single- or mixed-species) with different functional and structural properties embedded in an extracellular matrix. Relevant to therapy, antimicrobial susceptibility testing under biofilm conditions does not improve predictive value of MIC related to clinical outcomes [38].

SCV of *S. aureus* have characteristically slow growth, are metabolically less active and can persist intracellularly, all of which renders them refractory to antibiotic therapy. These SCV are also called auxotrophs as they require specific substrates for growth that are not required by other isolates. The most frequent substrates that support or repress growth are menadione, haemin, thymine, and CO_2. In pwCF, SCV are most often thymidine auxotrophs associated with extensive prior use of trimethoprim-sulfamethoxazole (TMP-SMX). Other antibiotics associated with auxotrophs are aminoglycosides with auxotrophism to the substrate haemin and/or meandione. Reports from European and North American CF centers have shown that SCV *S. aureus* (either as MSSA or MRSA) is associated with worse clinical outcomes [39-42]. Despite the clinical relevance, SCV may be underreported by clinical laboratories given their slow growth, being overgrown by regular phenotypic *S. aureus*, and their requirements for special growth substrates. Reports on their prevalence range between 8% and 24% in *S. aureus* positive cultures [40, 42].

Another phenotype of decreased metabolic activity are "persister cells" defined as isolates within a bacterial population that can withstand bactericidal doses of antibiotics and then regrow from their dormant state. Growth under reduced oxygen or anaerobic conditions is also associated with decreased metabolic activity.

17.3.6 Evasion of the immune system

S. aureus can evade the immune system at a number of levels: inhibition of the complement system, decreased neutrophil recruitment, reduced neutrophil extracellular traps (NETs), and diminished phagocytosis provided by staphylxanthin. *S. aureus* possesses many mechanisms that interfere with phagocytosis and intracellular killing by macrophages and neutrophils [43]. In addition, *S. aureus* can affect T cell memory function at multiple steps including differentiation and proliferation of memory and effector cells [44].

Such observations are relevant to the as yet unsuccessful vaccine development for both non-CF and CF populations [45]. Antibody levels are lower in infants and increase with age, indicating early exposure and likely sub-clinical infections contributing to immune response. Yet humans can have recurrent infections indicating lack of competent immunity. Antibody detection for diagnosis of invasive *S. aureus* infections is of interest in CF. Antibody levels against Hla, LukA, LuKS-PVL showed initially promising results but were not sufficiently sensitive [46]. A recent study showed that auto-antibodies to the enzyme peptidylarginine deiminase 4 (PAD4), which is required for NET formation, were elevated in CF compared to healthy controls, increase with age and correlated negatively with lung function [47]. Such serum diagnostic markers may be of increasing relevance to distinguish infection from upper airway colonization as fewer patients produce sputum. However more work is required to determine the validity of this approach.

17.3.7 *In vivo* pathogenicity of *Staphylococcus aureus* in CF

The early occurrence of *S. aureus* positive cultures and its presence as an indolent colonizer contribute to the ongoing debate about treatment. Arguments for treatment are the better survival of children on anti-staphylococcal therapy in the 1960s, the beneficial effect of antibiotics in patients with *S. aureus* as sole organism and pediatric studies showing increased inflammation in bronchoalveolar lavage with *S. aureus* isolation [48, 49]. The argument against treatment is our poor ability to distinguish colonization from infection especially in people who do not expectorate sputum and with unchanged clinical status. The complex interaction between *S. aureus* and the airways of pwCF underlies the varied approaches to treatment that are covered in the next section.

17.4 Therapeutic approaches for *Staphylococcus aureus*

17.4.1 Methicillin Sensitive *Staphylococcus aureus* (MSSA)

There is significant global disparity in the approach to acute and prophylactic treatment of MSSA in the airways of pwCF. The US antibiotic guidelines recommend against the routine use of anti-staphylococcal antibiotic prophylaxis (ASAP). The rationale for this recommendation is that ASAP may predispose pwCF to earlier airway infection with *P. aeruginosa*, a gram-negative bacterium that is challenging to treat in CF (see Chapters 15 and 16). This hypothesis relates to the results of a large double-blind study undertaken in the US, where children diagnosed with CF were randomly allocated to receive cefalexin as ASAP or a placebo [50]. The study duration was for up to seven years and the primary outcome was spirometry when the children were old enough to perform this measure. The children on cefalexin had significantly less isolation of *S. aureus* from respiratory cultures but an increased tendency to grow *P. aeruginosa*, although not at the expense of decreased lung function. These results underpin the recommendation against ASAP in the US. In addition, most US centers disregard the identification of *S. aureus* on a routine respiratory sample in an asymptomatic patient [51]. The high rates of *S. aureus* identification reported on the US patient registry is a consequence of this approach, yet supports the rationale that *S. aureus* may not be a significant pathogen in well children and adults with CF.

In contrast, most centers in Europe take a more active approach to treating *S. aureus* isolated from a routine respiratory sample, even in a well child with CF. Some countries, most notably the UK, have had a culture of using ASAP, generally with the narrow spectrum antibiotic flucloxacillin. The UK guidelines suggest a twice daily reduced dose of this antibiotic in pre-school children. This is based on a small study undertaken in the 1980s, which demonstrated reduced *S. aureus* isolation and hospitalization in infants on flucloxacillin ASAP compared to those given antibiotics in a more targeted manner [52]. A larger study (CF START) is now revisiting this question and has enrolled over 400 infants in the UK to assess the impact of ASAP on age of growth of *P. aeruginosa*, compared to infants treated in a more targeted manner (https://www.isrctn.com/ISRCTN18130649). The study will also assess the need for antibiotic treatment and a measure of respiratory function (lung clearance

index) when the child is 40 months of age. Although the use of ASAP is less embedded in UK practice than previously, partly as a result of CF START, the study will provide a clearer picture of the impact of ASAP and provide evidence to guide early antibiotic treatment in infants with CF.

17.4.2 MRSA

European and US CF care guidelines do not recommend prophylactic or maintenance antibiotics for MRSA [51]. Similar to MSSA, ECFS and UK guidelines recommend treatment of MRSA for incident infections or when patients are symptomatic without guidance on antibiotic choices. The US guidelines do not specify indications or antibiotic choices for MRSA, which may explain the wide variation in treatment choices found in a survey of US CF care centers [53]. At that time, TMP-SMX was the most frequently prescribed therapy for outpatient therapy (~35% of respondents) and linezolid for inpatient therapy (~18% of respondents).

Incident MRSA can be treated and eradicated, as shown by several case series and small retrospective analyses without control groups. Various medications and regimens were used in the retrospective studies, most frequently oral fusidic acid in combination with rifampin. Other medications included tetracyclines, TMP-SMX and oral linezolid. Success rates were 66% (19/29) at end of dual therapy and 52% (16/31) at 6 months [54], and 73% (27/37 participants) at 6 months in two of these studies [55]. A more aggressive approach used dual IV therapy (with various antibiotics used) followed by 6 weeks of inhaled vancomycin and reported an 84% (31/37) eradication success rate at 3 years [56].

Two randomized controlled trials, one in the US and one in Italy, assessed eradication of incident MRSA. Both trials used TMP-SMX and rifampin (substituting for minocycline in case of TMP-SMX intolerance) compared to observation only. The US trial showed significantly lower MRSA positivity in the treatment group compared to the observational group at day 28 (26% versus 82%, $p<0.001$) but this difference was no longer significant at 6 months [57]. Many patients in the Italian trial were excluded from analyses due to the use of additional antibiotics, and the per protocol analysis did not show significance with 63% (12/19) of patients achieving eradication at 6 months versus 39% (5/13) in the treatment and observational arms, respectively [58]. Both studies showed trends for better lung function and fewer antibiotic courses after intervention in the treated compared to the observational group. One consistent approach across all studies has been environmental cleaning (including equipment, e.g. nebulizer) and, in most trials, topical nasal therapy with mupirocin [53-56].

Treatment and eradication of chronic MRSA may be more difficult to achieve due to adaptation of MRSA. Two prospective studies in pwCF evaluated a 6 months of treatment with fusidic acid and rifampin combination therapy, resulting in clearance in 5/7 and 10/11 participants [59, 60]. A more recent prospective controlled trial evaluated one month of treatment with oral antibiotics (TMP-SMX or doxycycline), with or without addition of inhaled vancomycin. Clearance of MRSA was seen in 20% of participants in either arm a month after completion, however 4/15 participants in the vancomycin arm experienced bronchospasm that led to drug discontinuation [61]. A dry powder inhaled vancomycin formulation had shown promising results in phase I, but development of this medication subsequently stopped for unknown reasons [62].

Currently available antibiotics, dosing and drug interactions that are especially relevant to CF are listed in Table 1. Novel antibiotics licensed for skin and soft-tissue infections or pneumonia are omadacycline and lefamulin, and both are available as intravenous and oral formulations. Lefamulin is a pleuromutilin antibiotic, which inhibits protein synthesis via unique binding sites to the 50S bacterial ribosome. Two randomized, double-blind trials in community acquired pneumonia totaling approximately 1200 adults showed non-inferiority to moxifloxacin (or moxifloxacin-linezolid combination if MRSA was detected) [63-65]. Studies in children are ongoing but so far, no data in CF is available. Omadacycline is a tetracycline-class antibiotic that showed non-inferiority to moxifloxacin in adults with community acquired pneumonia (without MRSA positive sputum).

17.5 Conclusion

S. aureus, both MSSA and MRSA, is a prevalent isolate from the respiratory cultures of pwCF, both children and adults. Despite a considerable amount of research on genetic and phenotypic factors, there are no key bacterial biomarkers to highlight the potential for significant airway infection in pwCF. It is clear that chronic airway infection with MSSA and MRSA is relevant to some pwCF, who will respond to acute anti-staphylococcal treatment regimens, but there is no clear consensus on approaches to prophylaxis and eradication for early identification. Large ongoing studies will improve the evidence base to guide treatment approaches. For the moment, treatment should be tailored to the individual patient, monitoring clinical impact and applying good antibiotic husbandry (for example, avoiding broader spectrum antibiotics for MSSA). It is too early to determine the impact of emerging therapies (such as CFTR modulator treatments of the underlying molecular defect) on airway microbiology. However, given the nature of *S. aureus* it is likely that this will remain a relevant pathogen in the lives of pwCF.

Table 1

Currently available anti-staphylococcal treatments in CF.

Name	Class/Mechanism	Dosing in CF for pediatrics (P)[1] and adults (A)	Interaction / side effects[2]
Fusidic Acid	Fucidane Inhibits protein synthesis	P: 20 mg/kg/day as TID dose max 500 mg A: 500 mg TID	Metabolism altered when also taking rifampin or azoles
Trimethoprim-sulfamethoxazole (TMP-SMX)	Sulfa drug Folic Acid Inhibitor	P: 8-10 mg TMP/kg BID A: 320 mg TMP BID	Risk of SCV *S. aureus* with prolonged use
Doxycycline, minocycline	Tetracycline derivatives Inhibit protein synthesis by binding to the 30S ribosomal subunit	Doxy 1-2 mg/kg BID max 100 mg Mino: 4 mg/kg once, then 2 mg/kg Q12. Max 100 mg BID.	Teeth discoloration in children <8 years. Pseudotumor cerebri
Linezolid	Oxazolidinone Inhibits protein synthesis	<12 years: 10 mg/kg TID >12 years: 10 mg/kg BID Same dosing IV and po. Clearance >CF children > CF adults [66]. If inadequate response increase dosing frequency [67]	Good sputum penetration [68]. Myelosuppression with prolonged therapy. Interactions with serotoninergic and adrenergic agents.
Tedizolid	Oxazolidinone Inhibits protein synthesis	≥12 years: 200 mg daily (IV or po)	Better dosing and safety profile compared to linezolid
Ceftaroline	5th generation cephalosporin Inhibits cell wall synthesis	<6 months: 8 mg/kg TID >6 months & <33 kg: 12 mg/kg TID >33 kg: 400 mg TID Max: 600 mg TID	In CF, higher clearance in CF children and adults compared to non-CF [69, 70]. Higher doses or shorter intervals recommended
Ceftobiprole (not approved in US- as of June 2022)	5th generation, extended-spectrum cephalosporin	15 mg/kg q 8 hours (2 hour infusion)	Higher clearance in younger patients. No CF-specific PK data available. ELF penetration ~25%.
Vancomycin	Glycopeptide Inhibits cell wall synthesis by blocking glycopeptide polymerization	15-20 mg/kg q 6-8 hour (adjust per trough level). Also used as continuous infusion to reduce red-man.	Renal toxicity and flushing. Interaction with aminoglycosides reportedly less concerning with newer vancomycin formulation
Teicoplanin	Glycopeptide Inhibits cell wall synthesis by blocking glycopeptide polymerization	Loading dose 3 times every 12 hours then once-daily per levels. Loading dose: 6-10 mg/kg ages 2 months to 12 years. >12 years 6 mg/kg	Variable PK in pediatric patients – check levels. Goal: ≥20 - 30 mg/L for invasive infections (<60 mg/L to minimize risk for nephrotoxicity).
Televancin	Glycolipopeptide	10 mg/kg q 24 hours for CF adults [71]	Renal toxicity and flushing as for vancomycin. Metallic taste as side-effect.
Rifampin	Antimycobacterial Inhibits RNA synthesis through blocking RNA polymerase and RNA transcription	10 mg/kg/dose po q 12 hours. Max. 600 mg/day as q day or BID per GI tolerance	Only as antibiotic combination therapy. Interactions with ivacaftor, antifungals, linezolid. CYP 3A induction.

Abbreviations: BID=two times a day; ELF=epithelial lining fluid; GI=gastrointestinal; IV=intravenous; PK=pharmacokinetics; PO=per oral; Q=every; TID=three times a day.

1 Maximum dose in pediatrics is the adult dose.

2 As almost all antibiotics listed can cause GI side effects, allergic responses, and rash only side effects specific to this class is listed.

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Chapter 17 - *Staphylococcus aureus* in the airways of people with cystic fibrosis

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Chapter 18
Nontuberculous mycobacteria in cystic fibrosis

Authors
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Introduction

The question *"What is the best treatment for nontuberculous mycobacteria"* came third in a 2018 survey of the top ten research priorities in cystic fibrosis (CF) [1]. The survey was answered by a partnership of people living with CF (pwCF) and their healthcare providers and reflects the growing concern for this pathogen in the CF community. Pulmonary infection with nontuberculous mycobacteria (NTM) is now estimated to affect up to one in five pwCF, causing considerable burden and cost [2]. The most worrisome species of NTM in CF is *Mycobacterium abscessus* complex (MABSC), a rapidly growing, multidrug-resistant NTM, which is widespread in the environment, but is now also understood to be transmitted between patients [3]. Progress has been made on how to manage NTM clinically, but the lack of effective and straightforward treatment poses a continuous problem. This chapter outlines current knowledge on epidemiology and clinical aspects of NTM, and highlights challenges facing CF centers such as transmission and the role played by specific niches for NTM in the environment.

18.1 Pathogenic NTM species in CF and their distribution

MABSC is the most common NTM in Europe (Table 1) and comprises the subspecies *M. a. subsp. bolletii*, *M. a. subsp. massiliense* and *M. a. subsp. abscessus*. All three subspecies can cause persistent and severe pulmonary disease in children and adults with CF, although the clinical spectrum is wide and transient infections are also common [4]. The slow-growing *Mycobacterium avium complex* (MAC) is the second most common NTM in Europe and comprises *M. avium, M. intracellulare* and *M. chimaera*. The species *M. simiae, M. kansasii, M. fortuitum* and rarer NTM species are less common, often with poorly understood geographical distribution. Overall, there has been a trend towards increasing prevalence since the 1980s.

18.2 Sources of infection and transmissibility

NTM are environmental and biofilm-forming bacteria found in water, soil, dust, and inside amoeba [5-7]. The natural environment is the main source of infection for pwCF, but recent evidence has demonstrated transmission of MABSC between patients [8]. Genome analysis has revealed the presence of seven dominant circulating clones (DCCs), supporting the existence of global human transmission networks [9]. Following initial reports of possible transmission, the CF Foundation and European CF

Society (ECFS) quickly highlighted the issue, stressing the importance of prevention through infection control [10]. The sources of these transmission events were not clear at first, but indirect contact through fomites and long-lived aerosols was suggested. Subsequent studies performed at various CF centers also showed DCC-strains, but without support for transmission by epidemiological observations or single nucleotide polymorphism (SNP) analysis [11, 12]. Patient-to-patient transmission is now thought to involve intermediates from the environment or persons without CF, who may have acted as reservoirs (albeit inefficiently) and conceivably contributed to long distance transmission [9]. A role for non-CF smokers in driving transmission was also suggested, although it is not know to what extent intermediaries are still important in driving outbreaks [9]. DCC establishment may have been supported by long-term antibiotic pressure, causing changes in the CF lung microbiomes over time. The extent and mechanism of human transmission of NTM needs further investigation. Worryingly, NTM can survive some disinfection routines. For example, nosocomial outbreaks are well-known from cosmetic surgery and in patients undergoing heart surgery in the presence of contaminated heater-cooler-instruments, where *M. chimaera* has caused serious infections [13-15]. Zoonotic transmission has been considered, but not proven. *M. marinum* is known to be transmitted by fish, but the zoonotic potential of other NTM remains unknown.

18.3 Diagnosis of NTM pulmonary disease

Current diagnostic guidelines comprise a series of pragmatic, evidence-based recommendations from the US CF Foundation and the ECFS for the screening, investigation, and diagnosis of NTM infection in pwCF [10]. The diagnostic criteria stem from the original American Thoracic Society (ATS) / Infectious Diseases Society of America (IDSA) criteria for diagnosing NTM pulmonary disease (NTM-PD) [37]. The criteria state that the same NTM species needs to be cultured more than once (or just once if from a bronchoscopy sample), there must be clinical and radiological signs of disease, and other causes must be ruled out.

18.4 Effect on lung function

In 2003, Olivier *et al.* showed that 18 pwCF with ATS/IDSA defined NTM disease did not have a significantly accelerated lung function decline over a 15-month period [38]. They suggested that a longer observation time might reveal a difference. In 2010, Esther *et al.* examined longitudinal data from 23 patients with MABSC and found an excess lung function decline of -0.78% per year [25]. Martiniano *et al.* followed 70 MAC and 24 MABSC cases for three years after NTM infection and found excess annual rates of decline of -4.1% for those who fulfilled ATS/IDSA criteria, and -1.6% per year for those with only one positive culture [4]. The authors did not distinguish between the effects of MABSC and MAC. In 2016, Qvist *et al.* demonstrated that pwCF with MABSC had an -2.2% of excess lung function decline per year, while MAC infection had no significant effect [39]. A negative effect of 2.2% translates to the development of end-stage lung disease within approximately 14 years from acquisition, ranking MABSC among the most serious CF pathogens [39].

18.5 Treatment

Management of NTM infection is a clinical challenge as disease courses can be highly variable, ranging from transient colonization to invasive pulmonary disease. Treatment regimens for NTM are not evidence-based and involve complex and often poorly tolerated drug combinations for prolonged periods (>12 months) [40]. Therefore, it is difficult to decide when NTM infection is serious enough that the possible benefit of treatment outweighs the burden and side effects of treatment. Therapy for MABSC should start with an induction phase with intravenous (IV) antibiotics, followed by a longer oral maintenance phase. Treatment is burdensome for patients with considerable toxicity, high costs and disappointingly poor eradication results. Eradication of MABSC has been reported to occur in 48 % to 65 % of children and less often in adults [41, 42]. A proposed eradication regimen comprises an intensive phase of 4-12 weeks of IV antibiotics (usually amikacin, cefoxitin or imipenem + tigecycline) plus an oral macrolide. Maintenance therapy, also called consolidation therapy, includes oral drugs (usually a macrolide, plus others based on antibiograms, tolerability and experience) and an inhaled IV formulation of amikacin for 3 to >12 months. Before approval of inhaled amikacin, the IV form (delivered via a nebulizer) was used widely off-label.

18.6 Evolution of *M. abscessus* complex

MABSC causes chronic infections which can last decades giving the bacteria sufficient time for within-patient evolution. This may help the bacterial population adapt to changing conditions within the lung such as host immune responses, microbiome composition, and antibiotic pressure which is often considerable in CF. The evolution of MABSC from a saprophytic to a pathogenic bacterium was analyzed in depth by Bryant *et al.* [43]. They postulated a stepwise evolution starting with gain of methylation and transcription factors by horizontal gene transfer, resulting in a saltatory jump in evolution towards increased virulence in human infection. Within-patient evolution generated genetic diversification, especially towards resistance to macrophage killing. In this model, the need for environmental intermediates for transmission constrained further evolution. However, an increased frequency of direct person-to-person transmission might now result in unconstrained development towards increased pathogenicity.

18.7 Future perspectives

Consensus is building on how to manage NTM in CF, but the lack of effective and straightforward treatment remains a central challenge. Recommended NTM treatment consists of at least 12 months of three or more antibiotics, resulting in considerable toxicities and a high burden of care. Treatment outcomes are not satisfactory and chronic infection with particularly MABSC leads to high morbidity and mortality. Randomized clinical trials are urgently needed and are now finally under way (see FORMaT, NCT04310930). The central question of how transmission takes place is not fully resolved. Prevention through infection control is paramount, regardless of transmission route. More effort should be invested in investigating the potential environmental and zoonotic reservoirs of NTM. Advances in the understanding of MABSC genomics have been considerable, but efforts need to be broadened to include the other pathogenic NTM species, which remain understudied. A surveillance system for all pathogenic NTM should be established to trace NTM evolution and monitor changes in population structure.

Inflammation and infection in cystic fibrosis
Chapter 18 - Nontuberculous mycobacteria in cystic fibrosis

Table 1

Studies reporting the prevalence of nontuberculous mycobacteria in cystic fibrosis 1980-2020.

Study	Year(s) of prevalence	Location	Design	People with CF	NTM prevalence	% MABSC	% MAC
Boxerbaum B. [16]	1980	OH, USA	Prospective	430	1.8	75[1]	0
Smith MJ *et al.* [17]	1984	UK	Prospective	223	1.8	25[1]	0
Hjelte L *et al.* [18]	1990	Sweden	Prospective	54	9.2	0	60
Kilby JM *et al.* [19]	1992	NC, USA	Prospective	87	19.5	29[1]	76
Aitken ML *et al.* [20]	1993	WA, USA	Prospective	64	12.5	0	88
Hjelt K *et al.* [21]	1994	Denmark	Prospective	185	3.8	71[1]	29
Sermet-Gaudelus I *et al.* [22]	1996-99	France	Prospective	296	9.8	5[2]	21
Fauroux B *et al.* [23]	1997	France	Prospective	106	6.6	43[1]	0
Mussaffi H *et al.* [24]	1997-02	Israel	Retrospective	139	8.6	67[2]	252
Esther CR Jr *et al.* [25]	2000-07	NC, USA	Registry	1,216	13.7	41	59
Pierre-Audigier C *et al.* [26]	2000	France	Prospective	385	8.1	42	23
Oliver A *et al.* [27]	2000	Spain	Prospective	37	16.2	50[1]	33
Radhakrishnan *et al.* [28]	2004	Canada	Prospective	98	6.1	33	67
Levy I *et al.* [29]	2001-03	Israel	Cross-sectional	186	22.6	31	14
Olivier KN *et al.* [30]	2002	USA	Prospective	986	13.0	20	72
Roux AL *et al.* [31]	2004	France	Prospective	1,582	6.6	48	22
Valenza *et al.* [32]	2006	Germany	Prospective	60	13.3	50	50
Chalermskulrat W *et al.* [33]	2006	OH, USA	Retrospective	132	19.7	46	50
Binder AM *et al.* [34]	2011	USA	Registry	5,403	3.5	36	64
Qvist T *et al.* [35]	2000-12	Scandinavia	Retrospective	1,411	11.1	45	32
Adjemian J *et al.* [36]	2010-14	USA	Registry	16,153	20	39	61
ECFS Patient Registry Report	2019	Europe	Registry	22,756	5.5	-	-
US Patient Registry Report	2020	USA	Registry	10,220	10	40	54

Abbreviations: NTM=nontuberculous mycobacteria, MABSC=*M. abscessus* complex, MAC=*M. avium* complex

1 percentage based on six chronic NTM cases

2 *M. chelonae* reported, but is here included as MABSC due to historical changes in taxonomy

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Chapter 19
Other bacteria

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Introduction

People with CF (pwCF) host polymicrobial communities in their lungs [1]. Less typical gram-negative bacterial infections are increasingly prevalent members of this polymicrobial ecosystem.

The changes in the CF polymicrobial communities have been driven by changes in the care and treatment provided to pwCF. Most importantly, the key change has been the increase in life expectancy of pwCF. Furthermore, the greater availability and more aggressive use of antibiotics, such as quinolone and inhaled antibiotics, have become a standard part of CF care. Antimicrobial treatment has also moved from the episodic treatment of respiratory exacerbations toward chronic suppressive antibiotic therapy, as well as early aggressive treatment to eradicate initial respiratory tract colonization/infection. These strategies have placed greater selective pressures on the microbes causing infection in CF, possibly facilitating the emergence of species with multidrug resistance phenotypes.

There is a sense that the spectrum of species involved in CF infection is expanding, thanks to broader detection of microbial species in clinical specimens and more accurate methods of identifying existing species, sometimes leading to reclassification [2]. However, identification of the presence of new/unusual bacteria using methods such as MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry), is not always accompanied by additional symptoms or worse clinical presentation such as decrease in forced expiratory volume (FEV_1) or body mass index (BMI).

Bacteria that infrequently cause disease in healthy hosts, such as *Burkholderia* species, *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*, can cause both acute and chronic infection in CF [3]. Although some of these species have been associated with poor outcomes in pwCF, the role that other species such as *Ralstonia, Pandoraea* and *Bordetella* may play in contributing to disease progression as yet remains uncertain.

Longitudinal CF registry data show that the infections most significantly impacting decline in percent predicted FEV_1 were *Mycobacterium abscessus* complex (2.22% points per year), followed by *Burkholderia cepacia* complex (Bcc) (1.95%), *A. xylosoxidans* (1.55%) and *Pseudomonas aeruginosa* (0.95%) (Figure 1) [4].

19.1 *Burkholderia cepacia* complex

The reported prevalence of chronic Bcc infection varies from 1% in children to 4% in adults with CF in Europe [5], with approximately similar prevalence in the USA. Bcc infections occur less often than other pathogens such as *P. aeruginosa* and are restricted to older pwCF, typically teenagers and adults [6]. The 2021 CFF Annual Data Report

identifies numerous distinct subspecies [7], with 24 related genetically distinct species identified to date [6]. Bcc bacteria are highly problematic pathogens for multiple reasons, including

- their high antibiotic resistance [8]
- the potential for patient-to-patient transmission [9]
- the subspecies B. *cenocepacia* and B. multivorans are associated with higher morbidity and mortality. Patients infected with Bcc can rapidly progress to "cepacia syndrome", a fatal necrotizing pneumonia and bacteremia [10]
- the recognition of epidemic spread, and the increased mortality rates associated with the epidemic strains, has led to the policy of strict segregation in clinical units since the 1990s [11]

Bcc is typically identified by isolation on selective culture media following culture of sputum or other respiratory samples [12]. However, methodological advances using MALDI-TOF allow more accurate identification [13].

Susceptibility is multifactorial, with recent research suggesting the role of T6SS-expressing *P. aeruginosa* and its possible role in preventing Bcc superinfection and its sequelae [6, 14].

Inconsistency exists in guidance for antimicrobial susceptibility testing (AST). For example, the Clinical and Laboratory Standards Institute recommends performing antimicrobial susceptibility testing (AST) for Bcc. In contrast, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) does not recommend AST since performance of this method is poor, with little correlation with clinical outcomes [15].

Therapeutic strategies vary [16] (Table 1), and can include antioxidant/antibiotic combination therapy [17]. Therapies using an N-acetylcysteine (NAC) effective disruptor such as OligoG may allow lower concentrations of antibiotic and therapies for Bcc/biofilm disruption [18, 19].

19.2 *Stenotrophomonas maltophilia*

S. maltophilia is an increasingly prevalent CF pathogen [17, 20, 21], with a reported incidence of 7% in children and 9% in adults with CF in the ECFS Patient Registry (ECFSPR) 2020 report [5]. Colonization with *S. maltophilia* is associated with increased risk of pulmonary exacerbations, lung function decline and worse outcomes in adults [20, 22-24] and children with CF [25, 26]. Extensive drug resistance has been reported [27-30] and evidence of effective antibiotic treatment is variable [22]. Bacterial recolonization occurs early after lung transplantation, but survival is not influenced by recolonization or species [31]. *S. maltophilia* has complex colonization interactions with other pathogens [32, 33]. Additionally fungal infection in CF is associated with *P. aeruginosa* and *S. maltophilia* co-infection [34].

Novel therapeutic strategies are actively being sought, including siderophore-based agents, tetracyclines, ß-lactamase inhibitors, antimicrobial peptides, cell envelope targets, silver, NAC, and plant compounds [20].

19.3 *Achromobacter* species

This section focuses on *A. xylosoxidans*, previously referred to as *Alcaligenes* spp.
A. xylosoxidans is the most prevalent *Achromobacter* species in pwCF, with a reported incidence of 3% in children and 7% in adults with CF [5]. Prevalence has increased [35]. Prolonged treatment with IV antibiotics may predispose pwCF to increased risk of *A. xylosoxidans* infection [25]. Further studies are required to explore *A. xylosoxidans* inhibitory mechanism upon *P. aeruginosa* [33].

Whole genome sequencing is needed to identify and type *A. xylosoxidans*. The new duplex assay has been demonstrated to be accurate and inexpensive [36, 37].

Chronic colonization of *A. xylosoxidans* in CF is associated with decline in lung function, increased risk of pulmonary exacerbations [38] and more severe disease outcomes [35, 37, 39-43] and increased risk of transplant and death [41]. However, survival is not influenced by recolonization [31] and its presence should not preclude transplant [44].

A. xylosoxidans has poor susceptibility to most conventional IV antibiotics [45], with inconsistent outcomes reported for combination therapies [46]. It is susceptible to broad spectrum gallium-based therapy $Ga(NO_3)_3$ and Ga(III)-maltolate [47]. The synergistic activity of gallium-based therapies with IV antibiotics requires further study. Bacteriophages [48, 49] and approaches targeting biofilm or quorum sensing communication signals offer possible new treatment pathways [33].

19.4 Treatment strategies

There is widespread variability in treatment regimens for less typical pathogens such as Bcc, *S. maltophilia* and *A. xylosoxidans*, with a lack of consensus regarding antibiotic cycling [16]. Exploratory treatments exist, such as OligoG as therapy for Bcc/biofilm disruption [18], while antioxidant/antibiotic combination therapy and phage therapy offer alternative strategies [50, 51]. Newly approved broad spectrum options include cefiderocol [52] and gallium compounds [47]. Immunotherapy and immunization strategies for pathogens such as Bcc are still in the exploratory stages [53-56].

19.5 Rare pathogens

The following is a non-exhaustive selection of key less frequent pathogens.

Pandorea species infection in pwCF has been reported [67-69], with severe lung disease, bacteremia and multidrug resistance.

Bordetella species (*petrii or bronchispectica*) infection has been correlated with pulmonary exacerbation [70-72]. The *Bordetella* spp. are considered multidrug resistant, with case reports demonstrating sensitivity to piperacillin/tazobactam and minocycline but resistance to erythromycin and aztreonam [73].

Ralstonia has a low but possibly increasing prevalence (0.8% to 2.1% in pwCF in a single center), and is correlated with moderate to severely impaired lung function. *Ralstonia* is considered multidrug resistant, with evidence of possible cross-infection [38, 74].

19.6 Challenges

The polymicrobial community of the CF airways presents a complex challenge in terms of detection and treatment [1], compounded by adaptation and diversification of bacteria. Longitudinal analysis is needed to understand evolutionary patterns [75]. Diagnostic challenges also exist in terms of the selection of culture media.

Multi-resistant bacteria pose a threat to

post-transplant pwCF [76, 77] for example, in relation to Bcc. However, not all species of Bcc should be considered contraindications to lung transplant, as post-transplant survival may not be influenced by recolonization [31]. Nasal and oral sites may also act as reservoirs of bacterial infection, facilitating transmission to lower airways [78].

19.7 Conclusion

Bacterial competitiveness in the polymicrobial community of the CF lung presents ongoing research challenges, requiring new antimicrobial strategies beyond conventional treatments. Where microbiology might play a role, trials should include the identification of the microbiota profiles of participating pwCF in advance, assisting in the stratification of patients [19].

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Figure 1

Effect on lung function of chronic infection from onset to end-stage lung disease in Danish cystic fibrosis patients. (Kindly reproduced from Qvist et al, 2016) [4].

The figure visualizes the impact of onset of chronic infections by plotting population averaged %FEV_1 trajectories before and after onset of infection with other model parameters held constant. Thus, for a patient born in 1994 and infected at age 20, all other things being equal, end-stage lung disease will occur after 13.6, 14.7, 16.8, 21.3, 24.4 and 35.6 years for MABSC, *B. cepacia* complex, *A. xylosoxidans, P. aeruginosa, S. maltophilia* and MAC respectively. Trajectories plotted and held constant for a person born in the 1994–2004 birth cohort, mutually adjusted for other co-variates in the model, with onset of infection occurring at age 20.

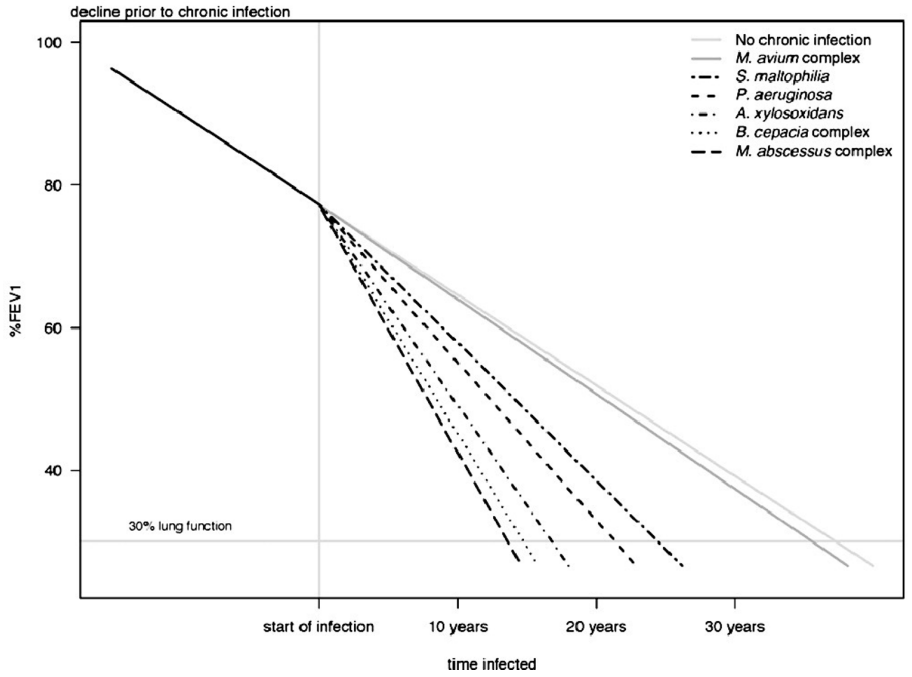

Table 1

Expert opinion on suggested treatments by pathogen. Adapted from Spencer et al, 2020 [57].

Pathogen	First-line-agents	Alternative agents	Other/Emerging therapies
Burkholderia cepacia complex	Trimethoprim-sulfamethoxazole Meropenem Ceftazidime Chloramphenicol	Minocycline, ceftazidime-avibactam [58], Ceftazidime-avibactam + pipercillin-tazobactam Other recommended: e.g. imipenem-relebactam as possible alternative where isolates may be resistant to ceftazidime-avibactam [59]	Other strategies include antibiotic cycling in regard to *Burkholderia mulitvorans*: reversion of resistance [60] Exploratory approaches to vaccine development/progress [61]
Stenotrophomonas maltophilia	Trimethoprim-sulfamethoxazole (Co-trimoxazole)	Levofloxacin, tetracyclines (minocycline, tigecycline & eravacycline), colistin, ceftazidime-avibactam + aztreonam, amoxicillin-clavulanate + aztreonam, ticarcillin/clavulanate, ciprofloxacin, moxifloxacin, tobramycin, minocycline, chloramphenicol	Combination therapies: *in vitro* combination testing for tigecycline-cefoperazone-sulbactam and tigecycline-levofloxacin [62] Cefiderocol [20, 52] Ga(III)-based therapy as a broad-spectrum strategy [47] Gepotidacin (*in vitro* and animal studies) [63] Antibiofilm compounds [28] PYED-1 in combination with aminoglycosides [64] Celastrol [65]
Achromobacter xylosoxidans	Trimethoprim-sulfamethoxazole (Co-trimoxazole) Piperacillin-tazobactam Meropenem Imipenem Ceftazidime	Minocycline Chloramphenicol	Cefiderocol; *in vitro* studies [52] Ga(III)-based therapy as a broad-spectrum strategy [47] Alternative therapies: essential oils [66]

Abbreviations: Ga(III)=Gallium III, PYED-1=pregnadiene-11-hydroxy-16α,17α-epoxy-3,20-dione-1

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Chapter 20
Treating bronchopulmonary exacerbations due to bacteria

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Introduction

Pulmonary exacerbations (PEx) are common events in the lives of people with cystic fibrosis (pwCF). Although there is no specific accepted definition, a PEx is generally described as a worsening of respiratory health, typically manifested by an increase in cough and sputum production, often accompanied by reduced pulmonary function as measured by spirometry [1], and a clinician based assessment of the patient leading to the decision to treat the event. The patient often has reduced appetite, weight loss and greater fatigue. Changes in respiratory exam and respiratory rate may also be evident.

There are many proposed etiologies for a PEx, including viral infection [2] or change in bacteria [3], but it may also be the result of progressive accumulation of airway phlegm over time. Since bacteria are commonly present in the airways, antibiotics are commonly recommended as a component of treatment of a PEx [4]. It is important to note that antibiotics are not the sole treatment of a PEx, and clinical practice guidelines recommend airway clearance therapies and inhaled medications (e.g. hypertonic saline, dornase alfa, bronchodilators) [4]. For the purposes of this chapter, we will assume that these other aspects of care are optimized and focus our discussion on the important aspects related to antibiotic treatment.

20.1 Clinical outcomes

PEx treatment aims to relieve the symptoms that led to the diagnosis, allowing the pwCF to return to their previous baseline health status. However, when should these clinical outcomes be measured and to what should they be compared? Surveys have shown that patients are most interested in relief of symptoms while clinicians are focused on recovery of lung function [5]. Clinicians often report treating the PEx until the patient recovers to baseline status, but most clinical outcomes may lack a proximate assessment (e.g. there may not be a graded assessment of symptoms during a time of wellness) or the previous measure of lung function (percent predicted forced expiratory volume at 1 second; $ppFEV_1$) may not be a reliable measure [6]. Indeed, in an observational study of PEx, nearly 25% of patients admitted to the hospital had a $ppFEV_1$ greater than their "baseline", or best measure in the previous six months [7]. Thus, for these measures it is most practical to measure a clinical meaningful improvement compared to the status at the time of initiation of treatment. An additional lesson from observational studies is that some measures (e.g. $ppFEV_1$) begin to decline shortly after stopping PEx treatment [6]. This informed the Standardized Treatment of Pulmonary Exacerbations (STOP) program that treatment response (at least for comparison of interventions) is best measured at the end of PEx treatment (e.g. 2 weeks) [5].

Although respiratory symptoms tend to improve in the short term (i.e. during the antibiotic treatment) [8], other health related quality of life domains, especially physical functioning, can take weeks to return to baseline [9].

20.2 Selection of antibiotics

Greater variation in care is associated with worse health outcomes. Therefore, standardization of best practices can improve effective care. A standardized PEx antibiotic strategy ensures that patients are treated generally the same, while personalizing treatment based on relevant factors. Historically, clinicians have regarded the results of cultures of respiratory specimens as the likely targets for antibiotic treatment. In the absence of specific culture data for a patient, registry data describe the most common pathogens that vary with increasing age (Figure 1) [10]. Oropharyngeal swabs provide surrogate samples for those unable to expectorate but may not be truly representative of lower airway infection [11]. We also know that the CF airway microbiome is typically polymicrobial, complicating the interpretation of standard culture results. Organisms have different growth requirements, permitting one pathogen to predominate over others in culture media. For example. *Pseudomonas aeruginosa* often overgrows other bacteria (e.g. *Staphylococcus aureus*). Selective culture media are used to address this problem [12]. The other challenge of a polymicrobial infection is determining which pathogen is the culprit to target; to treat all pathogens identified in culture might require the use of many antibiotics.

Antibiotic susceptibility testing has long helped guide antibiotic decisions, but antibiotic resistance patterns may also complicate antibiotic choices. Novel laboratory methods, such as synergy testing and multiple combination bactericidal testing (MCBT) of resistant *Pseudomonas* isolates, have not been shown to improve clinical or microbiology outcomes compared to standard susceptibility testing [13]. More recently, CFMATTERS (CF Microbiome-determined Antibiotic Therapy Trial in Exacerbations Study) attempted to exploit microbiome results to see if antibacterial therapy prescribed on the basis of community composition would be beneficial. While not fully reported, the empiric addition of a third "microbiota-targeting agent" (predicted to have activity against the top four most abundant organisms) to standard of care did not improve clinical outcomes [14].

Paradoxically, antibiotic susceptibility data have not proven useful in predicting clinical outcomes [15]. A systematic review of the literature found that standard susceptibility testing was not associated with improved clinical outcomes [15]. To address this challenge, a working group on antimicrobial resistance offered several recommendations, including that antibiotic choice should be guided by respiratory culture results (i.e. genus and species) and previous treatment response [16]. These are pragmatic recommendations given the absence of better outcomes with more advanced testing.

CF treatment guidelines have long recommended two antibiotics for *P. aeruginosa*, despite lack of objective evidence of greater clinical benefit compared to a single antibiotic. *P. aeruginosa* in early infection is commonly susceptible to anti-pseudomonal β-lactam antibiotics (e.g. cephalosporins, carbapenems), aminoglycosides and fluoroquinolones. Combination antibiotic therapy for *P. aeruginosa* has been justified by either synergist effects (discounted in the studies noted above) or reduced selection for resistance. However, some studies reported that combination therapy had either no impact or increased antimicrobial resistance [17-20]. A recent *post hoc* analysis of a large study of exacerbation treatment has questioned the value added with a second anti-pseudomonal antibiotic [21].

Dosing of antibiotics is also somewhat controversial. For most drugs, pharmacokinetic parameters are similar for pwCF compared to people without CF, but some drugs require higher doses for treating infections in pwCF [22]. Pharmacodynamic principles evaluating time and concentration factors that predict microbiologic effect have been applied, through prolonged infusion of β-lactam antibiotics and by restricting aminoglycoside dosing to once-daily [23]. Although no studies have proven any dose or dosing regimen to be ideal, studies have offered guidance for clinicians to assure consistency. Table 1, while not exhaustive, is modified from protocols of exacerbation treatment studies and is a useful reference point for clinical teams identifying both oral and intravenous (IV) options [24].

Finally, the method of antibiotic delivery is of interest. Typically, antibiotics are given systemically, either oral or intravenously. However, inhaled antibiotics offer another approach to treatment. Inhaled antibiotics have long been used in the management of CF airways for either eradication of new infection or as suppressive treatment of persistent infection [25]. To date there is little information on the utility of topical antibiotic delivery (i.e. inhaled antibiotics) in acute PEx. Although there is low systemic absorption of inhaled antibiotics, one study assessing concomitant inhaled tobramycin along with IV tobramycin in acute PEx demonstrating that the timing of the inhaled dose relative to the IV dose may impact the interpretation of tobramycin pharmacokinetic parameters [26]. In addition, some patients may find tolerability of inhaled antibiotics difficult during an acute PEx.

20.3 Place of treatment

The optimal place of treatment (i.e. home versus hospital) is an unresolved question in PEx treatment, but there is growing evidence to suggest better outcomes are associated with hospital care. Observational studies report better outcomes for those treated in hospital versus at home [27]. A recent analysis of a national data registry found that inpatient treatment was associated with a greater probability of a successful treatment response than outpatient treatment [28]. A large study comparing treatment durations but controlled for place of treatment found that treatment in the hospital was associated with greater improvements in lung function, respiratory symptoms and weight as compared to treatment at home [29]. Only one comparative trial exists that demonstrated similar outcomes for home and hospital settings [30]. However, it was a small cohort (31 exacerbations in 17 patients), and the patients undergoing home therapy had spent a median of three days in hospital before home discharge (range 1–5 days).

Relevant comorbidities may influence a final decision on the place of treatment. Examples include fatigue and ability to perform therapies properly, poor nutritional status at baseline [31] (which will require even greater support during an exacerbation), CF-related diabetes (CFRD) (and typically the need for additional insulin requirement) [32], or renal disease/dysfunction and drug level monitoring. Awareness of the home environment should be an important consideration as socio-economic factors (e.g. appropriate utilities, family support) may impact outcomes [4]. Geography and the distance/ease of access from the CF center may also be a consideration for the clinical team [30]. In general, home therapy may be appropriate for many pwCF; however, if there is any doubt, hospital admission is the current preferred option.

20.4 Duration of antibiotic treatment

CF guidelines identified a lack of evidence to guide antibiotic duration [4]. A pragmatic study assessing IV antimicrobial treatment duration for PEx found that 10 days was not inferior to 14 days in pwCF who demonstrated an early response to treatment. This early response was measured between day 7 and 10 and defined as both improvement in ppFEV$_1$ of at least 8% and a decrease in chronic respiratory infection symptom score (CRISS) of at least 11 points. In addition, a treatment duration of 21 days was not superior to 14 days in pwCF who had a less robust response to treatment [24]. These data provide strong evidence for a standardized treatment duration, both in clinical practice and research trials. Some biomarkers may associate with clinical outcomes. C-reactive protein (CRP) has been proposed as a biomarker of interest. Recent data found that although it associated with an increased odds of increased future PEx there were only weak correlations between CRP changes and clinical response to treatment [33].

20.5 Conclusion

PEx significantly impact the lives of pwCF. Bacterial pathogens are persistently present in the CF airways and are often blamed for causing or playing a role in the pathology of a PEx. Therefore, antibiotics have long been considered essential in the treatment of PEx, with consensus recommendations for treatment decisions. These have been based on extensive clinical experience but without the support of high quality evidence obtained through controlled clinical trials. In addition, there has been broad heterogeneity across CF centers in their treatment decisions, suggesting a poor standardization of care.

Defining best practices, evolving treatment guidelines and standardizing approaches is critical to improve patient outcomes. Recent studies have challenged long-held beliefs about best practices, and studies over the next decade future may change our approach significantly, particularly regarding duration of treatment and selection of antibiotics. The advent of highly effective CFTR modulators (in particular ivacaftor [34] and ivacaftor/tezacaftor/elexacaftor [35]), have been demonstrated to significantly reduce exacerbation rates but have not been shown to improve clinical outcomes following treatment of a PEx [36]. In addition, pwCF on CFTR modulator therapy expectorated reduced or no sputum, posing new challenges for the surveillance of bacterial pathogens. We will have to develop new methods to understand the pathophysiology of CF airways disease and the impact of CFTR modulators on disease progression as well as PEx presentation and response to treatments.

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Figure 1
Prevalence of respiratory microorganisms by age cohort.

Data from the CF Foundation Patient Registry including cystic fibrosis patients under care at CF Foundation-accredited care centers in the United States who consented to have their data entered [37].

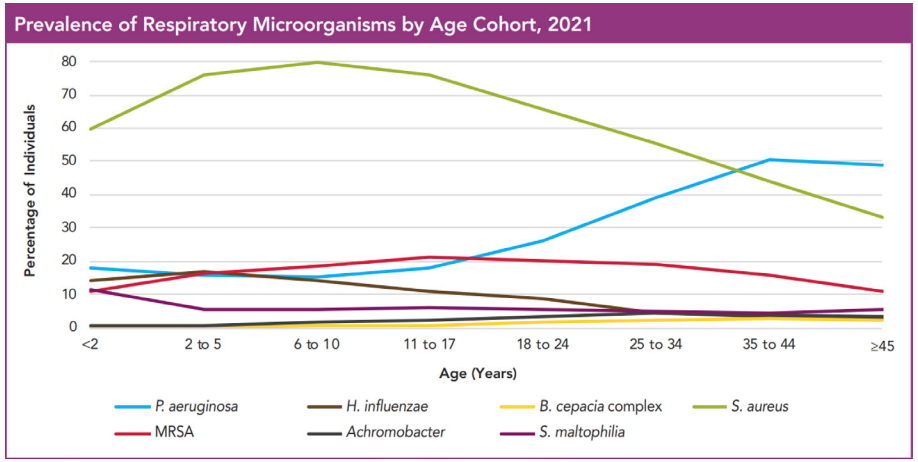

Table 1

Antibiotic dosing recommendations for the treatment of pulmonary exacerbations [24].

Antibiotics are commonly used to treat pathogens found in respiratory cultures of CF patients. These dosing recommendations are modified from a recent clinical trial of CF exacerbations [24].

Antibiotic	Pathogen	Formulation	Dose
Ceftazidime	PA, BURK	IV	Adult: 2 g q 8 h Pediatric: 100 mg/kg q 8 h
Imipenem-cilastatin	PA	IV	Adult: 1 g q 6-8 h Pediatric: 25 mg/kg q 6 h
Meropenem	PA, ACH, BURK	IV	Adult: 2 g q 8 h Pediatric: 40mg/kg q 8 h
Aztreonam	PA	IV	Adult: 2 g q 8 h Pediatric: 30 mg/kg q 6-8 h
Piperacillin-tazobactam	PA, BURK	IV	Adult: 4.5 gm IV q 6 h Pediatric: 350-600 mg/kg/day divided q 4-6 h [max 4 g/dose]
Ceftazidime-avibactam	PA, BURK	IV	Adult: 2.5 g q 8 h Pediatric: 62.5 mg/kg q 8 h
Ceftolazone-tazobactam	PA	IV	Adult: 1.5 - 3 g q 8 h Pediatric: 20/10 mg/kg q 8 h[1]
Imipenem-cilastatin-relebactam	PA	IV	Adult: 1.25 g q 6 h Pediatric: Dosing under study[1]
Meropenem-vaborbactam	PA, ACH, BURK	IV	Adult: 4 g q 8h Pediatric: Dosing under study[1]
Tobramycin	PA	IV	Adult: 10 mg/kg q 24 h Pediatric: 10 mg/kg q 24 h
Colistin	PA	IV	Adult: 2.5 mg/kg q 8 h Pediatric: 5-8 mg/kg/d divide q 8 h
Colistimethate Sodium	PA	IV	Adult: 1-2 million units q 8 h Pediatric: < 60 kg 25,000 units/kg q 8 h >60 kg 2 million units q 8h
Ciprofloxacin	PA	IV PO IV PO	Adult: 400 mg q 8-12 h Adult: 750 mg q 12 h Pediatric: 15 mg/kg q 12 h Pediatric: 250 mg q 12 h
Levofloxacin	PA	IV PO	Adult: 750 mg q d or 500 mg q 12 h Adult: 750 q d or 500 mg q 12 h
Vancomycin	MRSA	IV	Adult: 15-20 mg/kg q 12 h then adjust according to drug levels Pediatric: 15 mg/kg q 6 h
Trimethoprim-sulfameth-oxazole[2]	MRSA, SM, ACH, BURK	IV/PO	Adult: 5 mg/kg q 6-8 h Pediatric: 5 mg/kg q 6-8 h
Linezolid	MRSA	IV/PO	Adult: 600 mg q 12 h Pediatric: (<12) – 10 mg/kg q 8 h (>12) – 10 mg/kg q 12 h
Doxycycline[3]	MRSA	IV/PO	Adult: 100 mg q 12 h Pediatric: 2 mg/kg q 12 h
Tigecycline	MRSA	IV	Adult: 100 mg loading, then 50 mg q 12 h Pediatric: (> 12 years) 50 mg q 12 h (8-11 years): 1.2-2 mg/kg q 12 h
Minocycline	ACH	IV/PO	Adult: 200 mg load, then 100 mg q 12 h Pediatric: 4 mg/kg load, then 2 mg/kg q 12 h

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Abbreviations: PA=*Pseudomonas aeruginosa*; BURK=*Burkholderia cepacia*; ACH=*Achromobacter xylosoxidans*; MRSA=methicillin resistant *Staphylococcus aureus*; SM=*Stenotrophomonas maltophilia*.

Pediatric dosing applies to persons 6 years and older and generally up to weights of 40 kg and to a maximal dose equivalent to adult dosing.

1 Safety and efficacy not established in children younger than 18 years of age

Therapeutic drug monitoring (TDM) for tobramycin (adults and children): Extended interval – Peak: 20-40 mg/L; Trough: < 1mg/L, (If traditional dosing of 4 mg/kg q 8 h is used – targets are as follows: Traditional – Peak: 10-12 mg/L; Trough: < 2 mg/L)

2 Dosing of TMP-SMX is based on TMP component

3 Tetracycline antibiotics not recommended for use in persons younger than 8 years

Vancomycin TDM: Adult – Trough 15-20 mg/L; AUC/MIC ratio 400-600; If continuous infusion used, steady state concentration target of 20-25 mg/L; Pediatric – Trough 10-20 mg/L; AUC/MIC ratio of 400-600

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Inflammation and infection in cystic fibrosis
Chapter 20 - Treating bronchopulmonary excerbations due to bacteria

Chapter 21

Measuring response to treatment of bronchopulmonary exacerbations due to bacteria

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Introduction

Inflammation is the body's defense mechanism to remove harmful stimuli and initiate the healing process. While acute inflammation is beneficial, persistent inflammation due to lack of resolution of inflammation and/or persisting insult can be harmful [1]. In cystic fibrosis (CF), this "normal" process is misdirected and mainly driven by an excessive and sustained response of neutrophils relative to the burden of infection [2]. Neutrophils undergo necrosis rather than normal apoptosis and clearing by macrophages. This results in the release of damaging intracellular contents, including massive quantities of myeloperoxidase (MPO) and proteases such as neutrophil elastase (NE) and chemoattractants which further fuel neutrophil influx [3, 4]. This mechanism is reinforced by an imbalance between inflammatory and resolution mediators and finally results in a vicious cycle of neutrophilic inflammation, protease release and oxidative stress leading to tissue injury [5].

This chapter outlines biomarkers of the response to anti-inflammatory treatment – including antibiotic, anti-inflammatory, and cystic fibrosis transmembrane conductance regulator (CFTR) modulator treatments. Inflammatory biomarkers may be considered independently from clinical response as "pharmacological" indicators of biological efficacy, go/no-go indexes in proof-of-concept trials, or as responsive or prognostic biomarkers reflecting clinical response or disease progression at short or long-term. This will be considered at the systemic and the respiratory level.

21.1 Lung Biomarkers

Assessing inflammation in the airways relies on access to a biofluid reliably quantifying lower airway inflammation, and easily repeatable for longitudinal measurement. The use of bronchoalveolar lavage (BAL), the gold standard of sampling, is hampered by its invasiveness and the risk of adverse events in patients with advanced disease. Sputum (both induced and spontaneous) is mainly used although there may be some difference between induced sputum and BAL [6]. Nasal lavage content may also provide some information, but it is unclear whether this truly reflects broader pulmonary status [7]. The CF airway contains a broad spectrum of cytokines, neutrophil-related proteases and anti-proteases, growth factors, bioactive metals and co-factors of superoxide dismutase involved in oxidative stress [8]. Biomarkers present in high concentrations in CF airways, which effectively discriminate between people with CF (pwCF) and people without CF include NE, interleukin (IL)-8, tumor necrosis factor alpha (TNF-α), IL-6, IL-10, IL-1β, high mobility group box protein (HMGB-1), chemokine ligand 18 (CCL-18), and chitinase-3-like protein 1 (YKL-40) [8, 9].

Studies have highlighted that lung anatomical lesions and their progression correlate with various bronchial inflammatory markers, in particular NE and IL-8. This is clear in the pediatric population, as shown by the negative association between sputum NE increase and percent predicted forced expiratory volume in one second ($ppFEV_1$) decline in children [10] while the level of NE and IL-8 in BAL at 3 months of life is predictive for bronchiectasis at 3 years of age [11]. Furthermore, the level of IL-8 at 5-6 years of age is significantly correlated to the extent of bronchiectasis and air trapping in late childhood or adolescence (e.g. doubling of IL-8 levels in BAL at 5 years of age increases the odds of % bronchiectasis by 20% between 10 and 15 years of age) [12]. A small number of studies in pwCF with more advanced disease highlighted that lung function is negatively correlated with neutrophil count, NE and IL-8, both at definitive timepoints and longitudinally. Thus these biomarkers can be used to predict lung function decline [10, 13, 14]. Interestingly, NE level was significantly associated with microbiota diversity and evenness, and *P. aeruginosa* load while IL-1β was correlated with *S. aureus* load [15]. IL-6 and HMGB-1 demonstrated a significant correlation with survival and pulmonary exacerbation (PEx) rate at baseline (study start) [16].

The majority of studies investigating response to therapeutic interventions focused on IL-8, NE, TNF-α and IL-1β. Evidence of the responsiveness to antibiotic therapy is conflicting [10, 16-22]. One of the largest studies was carried out in 55 patients treated with IV antibiotics, demonstrating a decline in NE and IL-8 by 0.4 and 0.5 log respectively, in parallel to a 2 to 4 log reduction in lower airway bacterial density [21]. Similar results were observed, although less documented, for IL-1β, IL-6, IL-4, calprotectin and MMP-9 [10, 14, 23]. Studies on sputum biomarker response to anti-inflammatory therapies have been limited until now. Ibuprofen induced a reduction of IL-6, (but not NE), over a 28-day trial period [24]. Recombinant alpha-1-antitrypsin resulted in a significant reduction of NE, IL-8, IL-1β and TNF-α [25, 26]. Lenabasum demonstrated a significant reduction in IL-8 only [27]. Ivacaftor administration in adults resulted in a short-term reduction in arginase-1, MPO, calprotectin, with long-term reductions in NE, IL-8 and IL-1β [28].

21.2 Systemic biomarkers

The correlation of blood biomarkers to lung clinical outcomes has been less studied than sputum [29]. C-reactive protein (CRP) is the most investigated protein with a consistent correlation with exacerbations and their treatment. Serum calprotectin, a neutrophilic protein, is also predictive of PEx risk in CF and its level decreases in response to PEx treatment [30]. Interestingly, an untargeted proteomic study showed increased levels of 12 proteins during exacerbation: CRP, peroxiredoxin-2, hemoglobin subunit alpha, carbonic anhydrase, CD5, apolipoprotein C-II, C-protease C1 inhibitor, insulin-like growth factor binding protein, transthyretin, fibrinogen, serine protease inhibitor and albumin. However, only the six first met the threshold of significance [31]. One of the most important studies has been led by Ratjen and colleagues [32], who studied the effect of azithromycin in children above 6 years of age with CF uninfected with *P. aeruginosa*. Neutrophil counts, CRP, serum amyloid A (SAA), MPO and calprotectin all significantly reduced after 28 days of treatment and, apart from MPO, correlated with improvements in lung function and weight gain, providing evidence of their clinical relevance. The decrease was maintained at 6 months of treatment for neutrophil count, calprotectin and SAA. Neutrophil count remained significantly correlated with lung function change. Moreover, calprotectin was significantly correlated to PEx. 40% of participants with a calprotectin reduction less than the cutoff experienced at least one PEx compared with only 8% of participants with calprotectin reduction greater than the cutoff [33]. A secondary analysis based on untargeted proteomics showed change in various other proteins such as chemokine ligand 20 (CCL20), C-X3-C motif chemokine ligand 1, Flt3L, Fms-related tyrosine kinase 3 ligand, IL-6 IL-17A, IL-17C, IL-10 receptor subunit beta and programmed cell death ligand 1. However, only CCL20 and C-X3-C motif chemokine ligand 1 changes were significant at 28 days. These changes were not maintained after 6 months of treatment.

21.3 Other lung biomarkers

21.3.1 Pulmonary exacerbations

As outlined above, PEx are correlated to lung inflammation and, as such, represent a meaningful clinical biomarker. Indeed, dornase alfa, tobramycin, hypertonic saline and CFTR modulators have shown PEx reduction compared with placebo although PEx reduction is not always discriminative in children [34-37]. The paradox however is that we still require international consensus for the definition of an exacerbation. Historically, a PEx was defined as a deterioration of patient status leading to therapeutic changes, either at the clinical level, such as sputum volume and color or at the paraclinical level such as change in FEV_1. This definition is obviously dependent upon variations in practice [38]. Nevertheless, the definition by Fuchs *et al.* remains the most widely used [34]. The improved health of pwCF now requires development of more sensitive clinical markers such as new patient reported outcome measures (PROMs) [39-41].

21.3.2 Respiratory function

Improving inflammation in the lungs should be reflected by improved respiratory function. Nevertheless, anti-inflammatory clinical trials did not show improvement of FEV_1, including the LTB_4 receptor antagonist acebilustat, [42], lenabasum [27] and high dose ibuprofen [43]. For ibuprofen nevertheless, longitudinal follow-up showed slower decline of FEV_1, with a more potent effect in children under 13 years of age [44]. A more sensitive lung function marker, and surrogate of inflammation is the lung clearance index (LCI), derived from the multiple breath washout (MBW) test. It indicates pulmonary disease even when FEV_1 is normal, and correlates with markers of systemic inflammation including CRP and calprotectin and the extent of airway inflammation [14, 45, 46].

21.3.3 Lung imaging

Observational data from pediatric studies as well as animal models show that airway obstruction is present from the first days of life, which favors early infection and triggers inflammation. As a result, mucus plugging and airway wall thickening are the earliest structural changes evidenced by lung imaging in the first months of life [47]. This is associated with low attenuation regions with a mosaic pattern as a consequence of small airway pathology, corresponding to a mix of trapped air and parenchymal hypoperfusion. These abnormalities progress over time and lead to a diffuse widening of the airways defined as bronchiectasis, indicating irreversible injury and the inevitable progression of CF lung disease [48].

Over the last decade, the Perth-Rotterdam Annotated Grid Morphometric Analysis for Cystic Fibrosis (PRAGMA-CF) has been developed to quantify early and subtle structural lung changes in children with CF, because other scoring methods were not sensitive or reproducible enough [49]. Recent studies including low dose

chest CT have shown significant improvement in the short term with CFTR modulator therapy [50, 51]. Based on previous studies showing correlation between structural lung damage and inflammation, there is strong argument that these CT modifications reflect the beneficial impact on the infection and inflammatory components of lung disease.

The main advantage of pulmonary MRI is that it does not use ionizing radiation, and provides high-resolution images within a single breath-hold, sensitive to inflammation [52].

Other techniques, such as positron emission tomography (PET), directly quantify inflammation, as fluorodeoxyglucose is taken up by activated neutrophils, macrophages, and lymphocytes. Unfortunately, its use is limited by massive radiation [53].

21.4 Lessons and future challenges for inflammation biomarkers

Overall, data show that the biomarkers with the most reliable validity and responsiveness are NE, IL-8, TNF-α and IL-1β in sputum, and CRP and calprotectin in serum. Other biomarkers may be very relevant in sputum such as IL-6, calprotectin, HMGB-1, YKL-40 and IL-17. Difficulties in developing new anti-inflammatory therapies can, in part, be attributed to a lack of accurate, reproducible, non-invasive biomarkers.

First, for validated biomarkers, we lack knowledge regarding changes that can be considered clinically meaningful. Large studies are necessary to establish inter and intra-patient variability, temporal variation, reproducibility and reliability. Sputum analysis depends on the quality of the sample. Only samples expectorated from the airways are suitable for longitudinal follow-up of pwCF [8]. Collection, storage, and processing of sputum is highly inconsistent across studies, impacting biomarker levels and generating wide variability in measured values. Harmonization and standardization of these steps is mandatory. We still lack a valid definition of PEx, and identification of which measure we should use to compare the outcome of studies: frequency, time to the next pulmonary exacerbation event, or risk/odds ratio of PEx.

Second, the complexity of the inflammatory process in CF provides a lot of potential biomarkers. Therefore, a panel of multiple biomarkers should be studied rather than just a handful. Better knowledge is needed about how these biomarkers vary over time. For example, our understanding of how sputum biomarkers respond to anti-inflammatory therapies is hampered by short study durations. Long-term studies are needed to establish their trajectory over time, as well as their absolute variation at specific time points.

Third, one size does not fill all. It may be that some biomarkers are more correlated to short-term inflammatory processes, reflecting muco-obstructive hypoxia, whereas others may be more related to processes involved in resolution of inflammation and lung remodeling. Most importantly, it is likely that different endpoints should be used in “pre-symptomatic” children and in adults with evolving disease. New methodology investigating heterogeneity of the response in different subgroups may also help our understanding of the mechanisms of CF lung inflammation [54]. Future clinical trial endpoints of inflammation should be specifically designed based on the study population and the mechanism of action of the intervention.

Finally, a major issue in anti-inflammatory therapies is that suppression of the inflammatory response in a chronically infected airway may impair host defense, exacerbate infections and harm the patient. The LTB_4 receptor antagonist clinical trial was interrupted because of increased pulmonary exacerbations, decreased pulmonary function, and increased circulating neutrophils [55]. Studies in animal models showed that the high dose used in the trial downregulated inflammation and increased bacterial colony counts. Therefore, immune cell function should also be investigated in depth, in addition to biochemical biomarkers.

21.5 Conclusion

Further research is needed to extend our knowledge of the most critical biomarkers to monitor change in inflammation [56]. This includes new molecular and cellular biomarkers in the airway biofluids, serum and other target organs, but also PROMs as well as omics-derived biomarkers still at the preclinical stage using exhaled breath, and breath condensate. Longitudinal follow-up with large samples of patients is needed to assess the trajectory of pulmonary status overall, and in different patient subgroups.

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Chapter 22
Allergic bronchopulmonary aspergillosis (ABPA) in cystic fibrosis

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Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a lung hypersensitivity disease mediated by an allergic late-phase inflammatory response to specific antigens of *Aspergillus fumigatus* [1, 2]. *A. fumigatus* is a ubiquitous, spore-forming fungus, that has been linked to several pulmonary disorders including *Aspergillus* bronchitis, allergic asthma, ABPA, aspergilloma, invasive aspergillosis, and hypersensitivity pneumonitis [3, 4].

22.1 Pathophysiology and epidemiology

The development of ABPA is caused by the repeated inhalation of *A. fumigatus* spores by susceptible people (hosts). Colonization of the airway is mediated by allergens from the fungus resulting in epithelial damage, leading to higher adherence and bronchial penetration of *A. fumigatus* [5]. In people with cystic fibrosis (pwCF), the impaired mucociliary clearance resulting from CF transmembrane conductance regulator (CFTR) dysfunction predisposes the airways to colonization, leading to abnormal antimicrobial activity and increased mucus viscosity [6]. A consequence of impaired mucociliary clearance in pwCF is the accumulation and persistence of fungal spores within the smaller airways [7]. These spores eventually germinate, leading to the formation of fungal hyphae and the accumulation of proteases, phospholipases, and other virulence factors that damage the airways [8]. In addition, studies of *CFTR* mutant mice have shown that the immune response to ABPA in pwCF is mediated by T helper cell (Th) type 2 and is predominantly interleukin (IL)-4–mediated [1, 9]. Additionally, both *CFTR* gene mutations [10] and environmental factors [11] were shown to be associated with the development of ABPA.

The prevalence of *A. fumigatus* in pwCF differs greatly between regions, but continues to increase, and has been reported to be more than 25% [12]. However, molecular methods suggest that *A. fumigatus* colonization rates are even higher [13]. Many colonized pwCF become sensitized to *A. fumigatus*, however, only a small proportion develops ABPA [14, 15]. The prevalence of ABPA in pwCF was reported to range from 1 to 15% [16-19]. Prevalence varies between different regions, and increases with age [20].

22.2 Diagnosis and clinical manifestations

The clinical manifestations of ABPA include respiratory deterioration associated with elevated serum immunoglobulin E (IgE) and precipitin levels, and evidence of immediate cutaneous reactivity to *A. fumigatus*. In 30% of cases, pwCF are asymptomatic and are diagnosed in routine follow-up [21]. PwCF often experience wheezing, pulmonary infiltrates, and a central pattern of bronchiectasis [22]. The non-specific nature of ABPA symptoms, which overlap with many com-

mon manifestations of CF make it challenging to diagnose ABPA. This can result in delayed or even under-diagnosis. ABPA should be suspected when there is clinical deterioration that does not respond to appropriate antibiotic therapy, and cannot be attributed to another cause. Other signs that suggest ABPA include the presence of new radiological signs, a markedly elevated total serum IgE level, and evidence of *A. fumigatus* sensitization [23]. While the impact of fungal infections remains unclear [24], an early diagnosis of ABPA seems to be crucial to avoid deterioration of lung function [25]. Some studies found that pwCF and ABPA had no significant deterioration in FEV_1 [26], while others describe worse pulmonary function in these patients [27].

Several diagnostic criteria have been published over the last decades [15, 23, 28]. All pwCF should be considered at high risk for ABPA [29]. More than four decades ago, Rosenberg and Patterson proposed both primary and secondary diagnostic criteria for ABPA [30]. In 2013, the International Society for Human and Animal Mycology (ISHAM) recommended similar diagnostic criteria for ABPA, both for patients with asthma and pwCF. Some of these criteria are mandatory for diagnosing ABPA, such as total serum IgE levels >1000 UI/mL [31]. Still, there is no consensus on how ABPA can better be diagnosed in pwCF [32], and unfortunately diagnosis can still be delayed or missed.

Different biomarkers associated with ABPA have been investigated, in order to differentiate between *Aspergillus* sensitization and ABPA [33]. Keoun *et al.* found that in children with CF, fractional exhaled nitric oxide (FeNO) and peripheral eosinophil counts are elevated in ABPA compared to those who are just sensitized to *Aspergillus*. These biomarkers may serve as useful diagnostic tests [34].

Besides clinical manifestations and laboratory findings, certain radiological features also suggest the presence of ABPA in pwCF. High attenuation mucous (HAM) was described as the most accurate computed tomography (CT) sign of ABPA in CF, with high sensitivity and 100% specificity [35]. The presence of HAM can be considered as a firm diagnostic criterion for ABPA (a pathognomonic feature) [32]. Lu *et al.* found that there is a correlation between disease severity and mucus plugs in the chest CT scans [36]. Airway mucus contrast in magnetic resonance (MR) imaging was also shown to be a good tool for ABPA diagnosis [37].

22.3 Treatment

The main objective of ABPA treatment in pwCF is to control the acute episodes by reducing the inflammatory and immunological mediated damage of tissue, to further avoid the development of chronic lung disease.

Treatment is mainly based on systemic corticosteroids, antifungal and biological therapy [38]. Environmental factors have been found to be associated with the development of ABPA [11], so it is important to take steps to avoid them.

22.3.1 Oral corticosteroids

The mainstay of treatment of acute episodes of ABPA is corticosteroids in combination with oral antifungal agents. There is no uniform dosing protocol regarding the recommended dose. The CF Foundation Consensus Conference report recommended an initial dose of prednisolone as 0.5–2.0 mg/kg/day (maximum 60 mg) for 1–2 weeks, followed by an attempt to taper within two to three months to 0.5–2.0 mg/kg/day every other day for 1–2 weeks, followed by another taper 2–3 months later [23]. Antifungal agents are recommended as a second line, as discussed later. Recently, Gothe *et al.* [39] evaluated the combination of a short-term of prednisone with long-term itraconazole treatment. Prednisone was used at a starting dose of 2 mg/kg body weight for 3 days before being tapered every 5 days to 1, 0.5, and 0.25 mg/kg body weight and discontinued after 18 days in total. Itraconazole was commenced at a daily dose of 10 mg/kg for capsules and 5 mg/kg for suspension (in 18% of cases) from day 1 of treatment and continued for at least 12 months. Studies have shown that steroid treatment of ABPA in pwCF does not have adverse effects on their long-term pulmonary outcomes. If steroids are used for a long time or repeatedly, side effects including weight gain, hyperglycemia, cataracts, growth retardation, cushingoid facies, hypertension, and osteoporosis should be monitored.

22.3.2 Intravenous pulse doses of methylprednisolone

Pulse doses of intravenous (IV) steroids have shown efficacy [40, 41]. Cohen-Cymberknoh *et al.* [41] reported their experience in nine pwCF and ABPA (five females, aged 7-36 years) who were treated by high doses of IV pulses of methylprednisolone (10-15 mg/kg/d) for three consecutive days, once per month, together with itraconazole, until clinical and laboratory resolution of ABPA (6-10 pulses). All participants had improved clinical (increase in lung function) and laboratory (decrease in total serum IgE levels and eosinophil counts) outcomes. Adverse effects were minor and disappeared shortly after each IV pulse therapy. They concluded that high dose IV pulse methylprednisolone is an effective treatment for pwCF and ABPA, and it may be useful as an alternative treatment with fewer and reversible side effects.

22.3.3 Inhaled corticosteroids

Clinical evidence of efficacy with inhaled glucocorticoids (ICS) is lacking. ICS can be used as an add-on therapy for controlling asthmatic symptoms but not alone, and not as a first line of treatment [42].

22.3.4 Antifungal agents

Antifungal agents have become an important component of ABPA treatment as they are thought to decrease the fungal burden in the airways, reduce inflammation, hence improving symptoms, and thus acting as steroid-sparing agents. The antifungal agents commonly used are azoles, including itraconazole, voriconazole and posaconazole. A recent Cochrane analysis concluded that at present "there are no randomized controlled trials that evaluate the use of antifungal therapies for the treatment of ABPA in pwCF, although one trial is currently ongoing" [43]. The CF Foundation Consensus Conference report recommended treatment with oral itraconazole 5 mg/ kg/day once or twice daily (maximum 400 mg per day) for 3-6 months unless low serum itraconazole levels are obtained [23]. Oral itraconazole is used with steroids to provide a steroid-sparing effect, for the prevention of exacerbations [44]. Itraconazole has some limitations, including poor gastrointestinal absorption (should be taken with acidic drink), the need to closely monitor therapeutic levels, and some drug interactions (antiacid agents or calcium supplements should be avoided). The duration of treatment is 3-6 months or longer, depending on the patient´s response, although long treatments may predispose to azole-resistant *Aspergillus* species. Itraconazole induces liver enzymes and interacts with several medications, therefore liver function tests should be performed periodically (at baseline, 1 month later and then after 2- 3 months). When there is a lack of response, intolerance or side effects, voriconazole is an alternative to itraconazole [45], even as monotherapy, when oral corticosteroids may not be suitable. In a retrospective review, Glackin *et al.* found a significant drop in IgE levels post-treatment as well as a decrease in steroid dosing with voriconazole therapy [46].

Oral absorption of voriconazole is acceptable, however, serum levels should be monitored due to side effects such as liver toxicity, transient vision changes, visual hallucinations, hair loss, photosensitivity or skin rashes that may limit its use. Few studies have been published regarding posaconazol use in pwCF. In a retrospective study, there was a significant reduction in specific IgE to *Aspergillus* with posaconazole, compared with itraconazole and voriconazole [47]. Patel *et al.* reported an improvement in FEV_1 and serologic parameters in a prospective single-center, non-randomized, open-label observational study, and posaconazol in children was shown to be effective, safe and with good tolerability [48]. Both studies demonstrated a better oral bioavailability, good tolerability and better effectiveness than both voriconazole and itraconazole. There has been one report of isavuconazole treatment of ABPA (in a person without CF) without adverse effects [49], with no reports of isavuconazole use in CF. The higher cost of newer azoles is a limitation for prescription and therefore these medicines are frequently reserved for cases of itraconazole or voriconazole resistance [50] and failure.

A challenging situation that should be addressed is the drug-drug interaction between antifungal agents and CFTR modulators [51]. Ivacaftor, the first CFTR modulator approved, is a CYP3A4 substrate and its concentration in blood is affected when administered with CYP3A4 inducers. Therefore, the dosing should be reduced to twice weekly when given concomitantly with moderate CYP3A4 inhibitors such as azole antifungals (itraconazole, voriconazole, posaconazole). Lumacaftor-ivacaftor decreases azole exposure and therefore, a daily dose is recommended when lumacaftor/ivacaftor and azoles are used concomitantly. On the other hand, azole antifungals increased tezacaftor-ivacaftor exposure, and when used in combination, the dose should be reduced to twice weekly [51, 52]. Finally, the dosage of elexacaftor-tezacaftor-ivacaftor should be lowered when it is co-administered with strong CYP3A inhibitors such as azole antifungals.

22.3.5 Inhaled amphotericin B

Inhaled amphotericin B was suggested as an alternative option to adjunctive antifungal therapy for the treatment of ABPA in pwCF [53]. As nebulized amphotericin is poorly tolerated, the use of a short-acting bronchodilator 15 minutes before dosing may prevent bronchospasm and improve tolerability. Trials of dry powder formulations for inhalation of both itraconazole (NCT03479411) and voriconazole (NCT04872231) are also underway.

22.3.6 Monoclonal antibodies

Omalizumab, a humanized anti-IgE monoclonal antibody has been suggested as an effective alternative in pwCF with ABPA who are refractory to other treatments or with side effects to steroids [54-56]. The benefits reported in case reports or series include improvements in lung function, decreased exacerbation rates and discontinuation of oral steroids. There is currently no data from large prospective randomized controlled studies [57]. Omalizumab is not yet recommended for routine use in pwCF with ABPA but should be considered on a case-by-case basis. In ABPA, the IgE levels are very high, therefore, doses of 750 mg monthly have been used, resulting in a significant reduction in exacerbations.

22.3.7 Anti-Th2 therapies: newer treatment

Anti-Th2 therapies certainly have a role in the management of ABPA as the immune response is predominantly Th2 mediated (IL-4, IL-5 and IL-13). Recently, the benefits of anti-IL-5 antibodies (mepolizumab and benralizumab) in ABPA have been described in several case series. Mepolizumab, a humanized monoclonal antibody for IL-5, has demonstrated good tolerability and a beneficial effect in discontinuation of oral steroids in three pwCF with ABPA [58]. Benralizumab, an anti-IL-5 receptor alpha (IL-5Rα) antibody targeting the IL-5 receptor on eosinophils [59], was also suggested for the treatment of ABPA. However, more evidence and more randomized trials are needed before anti-Th2 therapies should be recommended as an alternative to glucocorticoids or triazoles in the management of ABPA.

22.3.8 Vitamin D

Some studies have investigated the role of vitamin D in the treatment of ABPA [60]. A phase I clinical trial investigated the safety and effectiveness of daily vitamin D3 supplementation in pwCF with ABPA. Treatment was associated with reduced *Aspergillus* induced IL-13 responses, and the study supports further investigation of the use of vitamin D supplementation in Th2 mediated diseases.

22.4 Conclusion

ABPA prevalence ranges from 1-15% in pwCF, and prolonged and recurrent episodes of ABPA exacerbations are common. As a result, maintaining a high level of suspicion of ABPA is crucial for prompt diagnosis to prevent clinical deterioration and irreversible lung damage. Diagnosing ABPA can be challenging and is based on clinical manifestations, laboratory tests, and radiological imaging that are compatible with ABPA.

The optimal clinical management of ABPA in pwCF is still under investigation. The main treatment for acute exacerbations of ABPA is corticosteroids. Pulse doses of steroids have shown efficacy with fewer side effects and may be useful as an alternative treatment. Antifungal agents can be added, especially if there is a corticosteroid toxicity, a poor response to corticosteroids, relapse of ABPA, or corticosteroid dependent ABPA. Triazoles are used to treat ABPA, but vigilance should be exercised regarding potential drug-drug interactions with CFTR modulators, as well as possible side effects and difficulty achieving optimal serum levels despite maximum doses. Omalizumab is not yet recommended for routine use in pwCF and ABPA but can be useful in individual case. Clinical trials for commonly used therapeutic strategies for ABPA in pwCF are in progress. Several newer treatment modalities such as anti-Th2 therapies are likely to be available in the next years.

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Chapter 23
Fungal bronchitis in cystic fibrosis

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Introduction
Lung disease in CF is characterized by a vicious cycle of inflammation and chronic endobronchial infection (bronchitis), punctuated and propagated by acute exacerbations. Lung infection is dominated by a number of typical bacterial species, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and other gram-negative organisms such as *Burkholderia* and *Achromobacter* species, which often result in chronic infection. However, fungal species are also frequently isolated from the lower respiratory tract of people with CF (pwCF). While some fungi can cause potential serious complications post-lung transplantation, the clinical significance of their isolation in immunocompetent pwCF is less clear. The most common filamentous fungi encountered in CF are the yeast *Candida albicans*, *Aspergillus* species, *Scedosporium* species, *Lomentospora prolificans*, and *Exophiala dermatitidis*.

23.1 Fungal bronchitis

The ability of fungal species to lead to disease is not disputed in CF. Allergic bronchopulmonary aspergillosis (ABPA) and pneumonia are well described entities requiring specific treatment and are covered comprehensively elsewhere (see Chapters 22 and 24). Conversely the concept of fungal bronchitis in CF is proposed but is somewhat controversial and lacking in defined consensus diagnostic criteria. In many cases there can be repeated isolation of a fungal species. In addition, immune responses to fungal species can be demonstrated in some pwCF, both as sensitization or immune hypersensitivity, and as host IgG antibody responses. Clinicians may assess such results as being suggestive of fungal bronchitis. Pragmatically, clinical approaches have mostly relied on ruling out other reasons for increased pulmonary symptoms and instituting empirical trials of treatment targeting colonizing bacterial or mycobacterial species, while investigating other systemic issues such as glycemic control before embarking on antifungal therapy. This review will highlight distinct fungal entities and current evidence of their pathogenicity in causing fungal bronchitis.

23.2 *Aspergillus* species

Aspergillus fumigatus is frequently isolated from sputum samples of pwCF [1]. The significance in CF of the presence of *Aspergillus* in a sputum sample, or of a host immunological response to *Aspergillus*, without evidence of invasive infection, aspergilloma formation or a heightened sensitization (ABPA) is clinically uncertain, particularly whether this represents just simple colonization or an infection [1].

In vitro models suggest that *Aspergillus* can cause inflammation in CF [2, 3]. The presence of

Aspergillus in lower respiratory tract samples has been associated with increased risk of radiological abnormalities [4, 5], respiratory symptom scores [6], respiratory exacerbations [5, 7] and lung function decline [8] in pwCF. However, these finding are not universal and other CF studies have found no difference in pulmonary exacerbation rates or decline in lung function [9, 10] with isolation of *Aspergillus*.

The possibility of a clinical entity of "*Aspergillus* bronchitis" was highlighted in a case series detailing six pwCF who were failing to respond to conventional antibiotic therapy but who had persistent growth of *A. fumigatus* in their sputum samples. These patients had either no or minimally elevated IgE serological markers of sensitization to *Aspergillus* and hence a separate clinical entity of *Aspergillus* bronchitis was proposed [11]. In an observational study, 13 pwCF with chronic *Aspergillus* in lower airway samples and without significant evidence of sensitization were treated for 6 weeks with itraconazole. They had reduced levels of inflammation and exacerbation rates, and improvements in symptom scores, but did not have improvements in lung function [2]. In a retrospective German registry study, Brandt and colleagues defined *Aspergillus* bronchitis as repeated positive sputum culture findings for *A. fumigatus*, no antibiotic treatment response, total serum IgE levels <200 kU/l, no observation of new pulmonary infiltrates and appropriate antifungal treatment response [12]. They identified ten cases with bronchitis, with improvements in lung function and clinical symptoms following 2-6 weeks of antifungal treatment.

PwCF can show discretely different responses to *Aspergillus* and without consistent diagnostic criteria it is difficult to evaluate clinical outcomes in published literature for different phenotypes of *Aspergillus* disease. Baxter and colleagues established a novel classification for *Aspergillus* in CF, using a combination of two methods of *Aspergillus* detection, sputum galactomannan and real-time *Aspergillus* PCR, alongside serologic markers. In 130 triazole-naive pwCF, the team identified a non-*Aspergillus* disease group (n=49) and three distinct classes of aspergillosis: ABPA (n=23), *Aspergillus* sensitization (n=19) and *Aspergillus* bronchitis (n=39) [9]. After 10 years of follow-up, the participants classified as having *Aspergillus* bronchitis did not have increased adverse clinical outcomes compared to the other pwCF studied (authors own unpublished data).

In a prospective randomized study of pwCF with positive sputum cultures for *Aspergillus,* participants treated with 24 weeks of itraconazole (n=18) or placebo (n=17) had no differences in exacerbation frequency, decline in forced expiratory volume in one second (FEV_1) or quality of life. However, the study failed to recruit to its target number of participants and, in a *post hoc* analysis, it was found that 43% of participants taking itraconazole had failed to achieve therapeutic drug levels [13].

The concept of *Aspergillus* bronchitis in CF is still controversial. An international survey of CF clinicians identified differences both in the diagnostic criteria for *Aspergillus* bronchitis, and opinions as to whether *Aspergillus* bronchitis as a clinical disease warranted therapy. A significantly greater proportion of North American clinicians were unable to define *Aspergillus* bronchitis compared to those from Europe and other regions [14]. In another recent survey 73% of United Kingdom clinicians reported that they had experience of treating *Aspergillus* bronchitis [15].

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23.3 *Candida* species

Candida is a genus of yeasts and is a frequently isolated organism in lower respiratory tract secretions of pwCF. Although it is the leading cause of fungal infections worldwide, its role in CF lung disease and specifically as a cause of pulmonary exacerbations is less well defined. A retrospective 2-year study in 55 adult pwCF in the UK revealed that 69% had chronic colonization [9]. *Candida* colonization or sensitization as measured by specific ImmunoCAP IgE levels were not associated with worse pulmonary outcomes in terms of FEV_1 decline or pulmonary exacerbations. In contrast, a French study that followed 237 pwCF for 2 years found that the presence of *C. albicans* was associated with a higher rate of lung function decline in a multi-variate model but not with an increased risk of lung transplant or death [16]. The authors concluded that the presence of *Candida* was associated with worse lung function and appeared to precede lung function decline. An 11-year prospective cohort study in Ireland found that *Candida* colonization is associated with an accelerated rate of decline in lung function and an increased risk of pulmonary exacerbations, but again conclusive evidence of the role of yeast colonization could not be robustly derived from this study [17]. Small studies found the presence of *C. dubliniensis* to be associated with lung function decline [18, 19].

The presence of *Candida* in the lower airway of pwCF therefore is not necessarily benign. Researchers have suggested a complex interplay between *Candida* and other more notable pathogens including *P. aeruginosa* with impacts on virulence [20]. There is some evidence that *Candida* may increase *Pseudomonas* biofilm tolerance to meropenem [21]. There is some evidence to suggest that the presence of *C. albicans* in the lower respiratory tract is associated with the co-administration of nebulized antibiotics [22].

It is unclear if *Candida* can cause pulmonary exacerbations. Treatment guided only by the isolation of this organism has not been extensively evaluated and cannot be routinely recommended.

23.4 *Scedosporium* species and *Lomentospora prolificians*

After *Aspergillus, Scedosporium* species and *Lomentospora prolificians* rank as the second most common isolated filamentous fungus in the lower respiratory tract secretions of pwCF. In a study from the Netherlands, 94 of 1312 patients from five CF centers had at least one positive sputum sample of *Scedosporium* spp. [23]. A positive isolation was associated with a severe CF genotype, CF-related diabetes mellitus, the presence of other microorganisms, and the use of inhaled antibiotics. Participants with and without *Scedosporium* spp. were comparable with regards to body mass index (BMI) and lung function. In a multicenter German study, 29 of 161 pwCF were found to be colonized with *Scedosporium* or *Lomentospora* species, giving rise to an estimated prevalence of 4%. Colonization was associated with younger age, less colonization with *H. influenzae*, higher frequency of ABPA, and co-colonization with mucoid *P. aeruginosa* [24]. This contrasts with an Australian case series which found that *Scedosporium* colonization was associated with absence of mucoid *P. aeruginosa* [25]. A French study examining seroprevalence to *Scedosporium apiospermum* found that antibodies were present in 9.7% of children, but this was not associated with clinical outcomes [26].

Scedosporium spp. has however been associated with symptomatic infection in pwCF. In a Greek study of ten pwCF, two had symptoms of an infection when colonized with *Scedosporium* spp. and were unresponsive to conventional

antibiotic treatment targeted at their colonizing bacterial pathogens [27]. Both experienced a resolution of symptoms with antifungal therapy according to sensitivity testing. In a case series of five children treated with a combination of posaconazole and terbinafine, *Scedosporium* spp. was not eradicated but lung function had significantly improved in two children and stabilized in another two children [28]. This retrospective report did not describe the health status of symptomatic patients at commencement of therapy and whether respiratory symptoms were attributed to the fungal pathogens.

In certain clinical scenarios, treatment for *Scedosporium* spp. and *L. prolificans* may be indicated. In a multinational survey of 45 lung transplant centers, 10% of respondents deemed *Scedosporium* spp. to be a contraindication to lung transplantation, rising to 31% for *L. prolificans* [29]. Treatment decisions can be difficult due to inherent resistance, with the best evidence favoring a combination antifungal approach [30]. Treatment is likely best guided by sensitivity testing.

23.5 *Exophiala dermatitidis*

E. dermatitidis is another fungal species that may be isolated from lower respiratory tract samples from pwCF. Prevalence of this organism varies in clinical studies and may relate to culture techniques, particularly the duration of the culture, with longer incubation periods tending to generate greater yields. One study identified *E. dermatitidis* in the sputum of 17 out of 98 pwCF. Of the 17 patients identified with *E. dermatitidis*, four were diagnosed as having symptomatic infection with *E. dermatitidis* and received treatment with broad spectrum antifungals. There was apparent clearance of the organism from subsequent sputum samples in only one patient, but partial reduction in isolation of *E. dermatitidis* in the other three cases [31]. In two of the cases, treatment was associated with an improvement in clinical symptoms. In one of these cases, there was also a radiological improvement, but this patient had a concomitant history of ABPA. Patients had evidence of IgG antibodies to *E. dermatitidis* and increased levels were associated with a greater number of IV antibiotic courses received during the preceding year, worse percentage predicted FEV_1 and increased levels of inflammation. However, comorbidities and co-infections were potential confounders, as higher levels of antibodies were seen in patients with exocrine pancreatic insufficiency and higher rates of colonization with other pathogenic microorganisms [32]. In a recent UK monocenter retrospective study, 31 pwCF with a history of *E. dermatitidis* were matched with a control population of 62 pwCF who had never had *E. dermatitidis* cultured from the respiratory tract [33]. The rate of lung function decline was higher following isolation of *E. dermatitidis* (-1.824 $\%FEV_1$/year, $p<0.01$). However, although 14 patients received antifungals, their rates of lung function decline were not significantly different from the other 17 patients who were not treated. Several more recent studies have found that the presence of *E. dermatitidis* in sputum from adults with CF is not associated with adverse health status [23, 34, 35]. *E. dermatitidis* was isolated at least once from a sputum sample in 31 of 1312 Dutch pwCF, with no difference in lung function or BMI between those with and without *E. dermatitidis* [23]. In another study at a UK pediatric CF center, *E. dermatitidis* was isolated from 27 of 244 patients (11%) on more than one occasion [34]. There were no significant differences in spirometry, number of intravenous antibiotic days and BMI between cases and controls. In addition, there was also no clinically significant difference in spirometry, mean number of IV antibiotic days and BMI in cases pre and post *E. dermatitidis* isolation. Another recent UK study identified 21 pwCF with E. *dermatitidis* and 105 pwCF without *E. dermatitidis* [35]. There were no differences in baseline lung function or BMI, or rate of change in lung function or number of exacerbations over 12 months between the two groups. The study also evaluated treatment outcomes for participants with and without *E. dermatitidis* who had received treatment with intravenous antibiotics for an infective exacerbation. There were no difference in clinical outcome measures between pwCF with and without *E. dermatitidis*, despite the omission of specific antifungal therapy [35]. At present, most published studies do not support routine targeted antifungal therapy for the majority of pwCF who harbor *E. dermatitidis*.

23.5 Other fungal species

Other fungal species including *Alternaria, Cladosporium, Penicillium, Trichosporon* spp. are also occasionally encountered in CF respiratory samples. In many cases carriage may be transient, and the clinical significance is unclear. Apart from a few case reports, there is little to inform about potential fungal bronchitis.

23.7 Impact of CFTR modulator therapy

CFTR modulator therapy has been shown to alter the CF airway microbiome and circulating inflammatory markers [36]. A reduced rate of isolation of *Aspergillus* in pwCF receiving ivacaftor has been reported in one study [37] and CFTR modulators have been shown to have the potential to dampen *Aspergillus*-induced host inflammation which may be more relevant for bronchitis than for ABPA [38, 39]. At present, it is too early to speculate whether CFTR modulation will change the natural history of fungal bronchitis in CF.

23.8 Lung transplant recipients

There are higher rates of invasive fungal disease in CF lung transplant recipients, with *Aspergillus* species were most commonly responsible for colonization. Many transplant centers provide antifungal prophylaxis to lung transplant recipients, although there is no consensus on the choice of antifungal agent. The most common antifungal used is voriconazole, followed by itraconazole and inhaled amphotericin B [40]. Evidence is currently lacking to support the use of inhaled amphotericin B alone for the primary treatment of *Aspergillus* tracheobronchitis.

23.9 Conclusion

In conclusion, fungal species can be frequently isolated from the lower airways of pwCF. The clinical relevance is often uncertain, and in many cases the fungi are often regarded as bystanders rather than as significant respiratory pathogens. Comparison between studies is often hampered by a lack of standardization of methodologies for detecting fungi and/or host responses, a lack of a uniform definition of fungal bronchitis, and potential confounding factors such as patient comorbidities and co-pathogens. Individual patient factors are also likely to play a role. Antifungal medications can have significant adverse effects including hepatotoxicity, gastrointestinal disturbance and QTc prolongation, and can interact with other CF medications including CFTR modulators [41, 42]. At present, there is insufficient evidence to support routine targeting of fungi with antifungals either during acute exacerbations or for longer term colonization, without first optimizing all other standard CF therapies. Future prospective studies are required to further elucidate the clinical relevance of fungal bronchitis in CF, and in particular if treatment leads to improved clinical outcomes.

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Chapter 24
Pulmonary fungal infections in cystic fibrosis

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Introduction

In recent decades, several studies have been conducted regarding fungal colonization and infection of the airways in people with cystic fibrosis (pwCF). The most common fungal species in pwCF are, by far, *Aspergillus fumigatus* for filamentous fungi and *Candida albicans* for yeasts. However, the pattern of fungal species associated with CF has considerably diversified recently [1-10]. Thus, some *Scedosporium* species (*Scedosporium boyidii, Scedosporium apiospermum, Scedosporium aurantiacum, Scedosporium minutisporium*) and the closely related species *Lomentospora prolificans* have been globally recognized as significant fungal pathogens in CF, potentially causing severe fungal infections that are frequently difficult to treat. In addition, very rare fungal species such as *Exophiala dermatitidis, Arxula adeninivorans* and *Trichosporon mycotoxinivorans* have also been described to cause severe fungal pneumonia in pwCF [11-16]. Frequently, invasive fungal infections have delays in diagnosis and therefore inadequate treatment.

In this chapter, we will define pulmonary fungal infections, and describe the cornerstones of the diagnostic and therapeutic approach.

24.1 Definition of pulmonary fungal infections

In CF, different entities or categories in terms of pulmonary fungal diseases have been discovered. Beside fungal airway colonization, host responses can be divided into the allergic and the infectious groups. The allergic group present as sensitization or allergic bronchopulmonary aspergillosis or mycosis. The infectious group includes pulmonary fungal infection (invasive pulmonary infection, IPI), fungal bronchitis and aspergilloma [4, 5, 17-24]. In this chapter, we focus on IPI. As bronchopulmonary infections or exacerbations in CF are usually caused by bacteria, no commonly agreed upon definition for pulmonary fungal infections existed in the past. However, in recent years, a definition has been developed and published by international experts in the field [8, 21, 25, 26]. This internationally accepted definition of pulmonary fungal infection in CF is:

- Increased sputum production
- Multiple isolation of the same fungal species from sputum or bronchoalveolar lavage (more than twice over a 6-month period)
- Pulmonary infiltrate(s) on chest CT, MRI imaging or chest X-ray
- Antibiotic treatment failure (more than two antibiotic treatments, duration ≥ 2 weeks)
- Unclear lung function decline (exclusion of newly diagnosed CF-related diseases, e.g. CF-related diabetes mellitus)
- Exclusion of new/other bacteria (e.g. non-tuberculous mycobacteria or *Pseudomonas aeruginosa*)

24.1.1 Exclusion of ABPA

Although this definition of pulmonary fungal infection in CF probably helps to identify patients with fungal pneumonia, it can still be challenging to confirm the diagnosis. CT or MRI imaging can help diagnose fungal pneumonia, based on the typical findings previously described [11, 22] of ground glass density surrounding a nodule, also known as "halo sign", or consolidations with internal ground glass density. But other specific hints for severe non-specific pulmonary infections in CF include findings like semi-solid and ground glass nodules, and particularly, peripheral well-circumscribed nodules [27, 28]. The definitive diagnosis of IFI is by microbiology or histopathology. However, many pwCF receiving highly effective CF transmembrane conductance regulatory (CFTR) modulator therapy produce less sputum, which complicates collection of sputum samples for analysis. Meanwhile, bronchoalveolar lavage (BAL) or induced sputum is not feasible in a routine setting. For this reason, new methods are needed to detect microbiological colonization or infections with bacteria or fungi.

A novel diagnostic tool has been proposed in recent years relying on the measurement of fungus-specific host response. This antigen-reactive T cell enrichment assay (ARTE) might be useful to measure fungus-specific T cell responses during the onset of fungal infections [23, 24, 29, 30]. This method reflects the direct host-pathogen reaction, therefore identifying the relevant pathogen in usually co-colonized pwCF could be possible, despite negative culture-based detection methods or PCR-based methods from low quality samples. In addition, an easier and less time-consuming diagnostic tool is the above-mentioned fungus-specific IgG. In a recent publication, it could be shown that *A. fumigatus* specific IgG might be helpful in the diagnosis and treatment follow-up of *A. fumigatus* infection, as *A. fumigatus*-specific IgG decreases after therapy [31].

Serological techniques such as galactomannan in blood or BAL, or other *Aspergillus* antigens measured by lateral flow devices have also helped to diagnose aspergillosis within hours with a specificity and sensitivity of more than 80%, particularly in severe, life-threatening cases [32-34]. Although not commercially available, some serological methods have recently also been proposed for *Scedosporium*/*Lomentospora*. Two of them are based on ELISA systems, using a total extract or recombinant proteins as antigen [35, 36]. An additional, newly developed assay based on a rapid dot immunobinding assay (DIA), which detects serum IgG against a total extract of these fungi in less than 15 minutes with 90.48% sensitivity and 79.30% specificity [37].

A. fumigatus or non-*Aspergillus* species pneumonia is a new disease entity in pwCF which is not easy to diagnose as bacteria are the main reason for infections with similar symptoms [38, 39]. Therefore this pathology needs the attention of radiologists and clinicians. The usual gold-standard diagnostic approach would be transbronchial biopsy of the region with infiltrate to directly detect fungal growth [40]. In CF, this intervention is usually too dangerous as pneumothorax can cause severe clinical deterioration [41].

24.2 Treatment of pulmonary fungal infections

The treatment of fungal infections can be challenging in pwCF. *A. fumigatus* infection is usually treated with monotherapy using voriconazole, posaconazole, isavuconazole, caspofungin, micafungin or anidulafungine or lipidic amphotericin B [22, 42]. Susceptibility testing is considered important if clinical response lacks, particularly since azole resistance has recently been described [43, 44].

Candida spp. are the most common yeast isolated in respiratory samples in pwCF. A 6-year retrospective study in 9 CF centers in Europe found that *Candida* spp. were present with a mean frequency of detection ranging from 33.8% up to 77.9% [3]. However, infections caused by this fungus are only rarely described [3, 22]. In fact, the pathogenicity of *Candida* spp. in CF and their influence on disease progression is less clearly understood than with filamentous fungi and continues to be debated. In the late 1990s, registry data from 7010 pwCF showed the association of *Candida* spp. and lower FEV_1 [41], although it is unknown whether this was due to a direct effect of *Candida* spp., an observation for its predilection for damaged pulmonary parenchyma or a finding associated with antibiotic treatment used more often in those with greater disease severity. More recently, the potential of *Candida* spp. to cause lung function decline was again demonstrated, as well as the yeast's ability to cause both localized and systemic infections, and induce oral and genital thrush, vascular access device-related infections and post-transplantation complications [7, 39, 45]. However, whether treatment against *Candida* spp. influences the course of disease or the drop in lung function remains unknown and needs further investigation. In the very rare cases of pulmonary infection highly probably due to *Candida* spp., the accurate identification of the infecting *Candida* species is crucial in determining which antifungal agent to use, because of the occurrence of fluconazole-resistant *Candida* species [42]. In *C. albicans* infections, it is recommended to start with an azole, preferably fluconazole, and to modify treatment if needed, according to susceptibility tests. Echinocandins (e.g. caspofungin, anidulafungin, micafungin) are effective drugs for *C. glabrata* and *C. tropicalis* infections. Amphotericin B is also useful for *Candida* spp. infections but has the disadvantage of nephrotoxicity, hypokalemia and acute infusion-related side effects [10]. However, *Candida* spp. are still rarely identified as causing acute pulmonary infection in CF requiring treatment. Further research is needed to determine their true position as pathogenic organisms in CF.

Treatment of pulmonary fungal infections in CF remains challenging when they are caused by multi-resistant fungi such as *Scedosporium* species and *Lomentospora prolificans*, or the emerging *Rasamsonia* species. These fungi exhibit a primary resistance or low susceptibility to most available antifungal drugs [6, 46]. In recent years, a lot of effort has been invested performing studies to address this issue. Firstly, susceptibility testing is mandatory for all antifungal treatments, as resistance can occur [6, 8, 47]. Single studies on *Scedosporium*/*Lomentospora* infections recommend the option of combined antifungal therapy [8, 25, 48]. In those clinical settings, a combination of two or even three different antifungals might be necessary and is therefore recommended [8, 22, 25, 26, 49]. Ideally, the treatment combination should combine an oral azole (voriconazole, posaconazole, and isavuconazole), an intravenous echinocandin, and inhaled lipidic amphotericin B. In *Rasamsonia* infection, micafungin would be preferred in combination, and in infections with *L. prolificans*, terbinafine might be suitable as well as miltefosine, in combination with an echinocandin and azole [8, 22, 25, 50]. *E. dermatitidis* can also cause pulmonary fungal infections in pwCF, and it was first described in the 1990s [13, 14]. Antifungal therapy recommendations are mainly focused on therapy with amphotericin B, flucytosine, or azoles [13, 14, 21], but susceptibility testing should also be performed initially. In rare, single cases, *T. mycotoxinivorans* could also cause severe infections in pwCF, as described in single publications. The recommended treatment regimens in such cases are a combination of amphotericin B (intravenous or inhaled) and an azole as with monotherapy; however, breakthrough infections might occur [15, 51-54]. As recommended for other fungi, it is also crucial to perform susceptibility testing in

pwCF with lung infections with *Trichosporon* spp.

All detected fungal species in respiratory samples of pwCF should be considered as potentially pathologic. This is illustrated by first in-human detection of *A. adeninivorans* in a pwCF causing a life-threatening invasive pulmonary infection [11].

An additional important question for clinicians is whether colonization without clinical implications should be treated to prevent clinical deterioration and chronic lung damage, in particular, if multi-resistant fungi such as *L. prolificans* are detected, which potentially contraindicate lung transplantation [4, 55].

24.3 Specific aspects in CF

Some CF-specific aspects differ from other diseases, requiring adjustment of azole dose and drug level monitoring for drugs with a low volume of distribution. The following factors can potentially require higher dosing and therapeutic drug monitoring: delayed absorption of oral antifungal drugs due to pancreatic insufficiency and fat malabsorption, increased total body volume leading to higher volume distribution with low drug concentrations, enhanced hepatic and renal drug clearance due to hypermetabolic state [56, 57]. In addition, pwCF usually have many co-medications, particularly drugs with severe interactions [58]. Drug-drug interactions with CFTR modulators are typical and in patients with elexacaftor, tezacaftor and ivacaftor, the modulator therapy needs to be adjusted to a lower dosage when given with concomitant drugs [59-61].

24.4 Future treatment direction

As pwCF live longer, they have greater opportunity to become infected or colonized with multidrug-resistant bacteria and fungi. Therefore, new therapies are needed. The antifungal pipeline currently includes different, very potent drugs such as fosmanogepix, ibrexafungerp, olorofim, opelconazole, and rezafungin [62]. The drug olorofim is particularly relevant for CF. This orotamide will have a central role in the treatment of multi-resistant mold infections, including azole-resistant aspergillosis, *L. prolificans*, and endemic mycoses. In addition, opelconazole is the first broad spectrum triazoles antifungal drug formulated for inhalation. A phase II study of opelconazole in lung transplant recipients is starting and is will likely have a superior safety profile than systemic azole treatment [62].

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