Chapter

The Pulmonary Microbiome in Cystic Fibrosis

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Abstract

The chronic colonisation of the lower airways by bacterial pathogens is the leading cause of morbidity and mortality in patients with cystic fibrosis (CF). The use of novel culture-independent techniques such as next-generation sequencing (NGS) to analyse the lungs has allowed us to further understand the diversity, the complexity, the effects of acute exacerbations and the use of antibiotics on the bacterial communities. The understanding of the CF microbiome to airway disease remains a fascinating area of research; it presents new opportunities for disease management in CF and has the potential to explore the effects of cystic fibrosis transmembrane conductance regulator (CFTR) modulators. It also allows further appreciation regarding the roles played by anaerobic organisms within the CF airways. It is also of interest that a number of studies have demonstrated that the fluctuations of microbiome are not necessarily associated with the patient's clinical status. Despite the available evidence, there remain many challenges that must be overcome if microbiome profiling is going to influence future clinical practice. The effects of fungus and the emergence of nontuberculous mycobacteria in CF are also briefly discussed in this chapter.

Keywords: cystic fibrosis, microbiome, CFTR modulators, nontuberculous mycobacteria, *Aspergillus*

1. Introduction

Traditional culture techniques rely on growing bacteria on media in laboratory conditions often optimised for growth of specific organisms so that they can then subsequently be identified. In the last 20 years, novel techniques utilising next-generation sequencing (NGS) to identify bacteria have become available, enabling detection and description of bacterial communities without the need for conventional culture. These technologies have allowed a greater understanding of bacterial communities throughout the human body and have revealed functional roles in both health and disease.

A healthy human gut, for example, is home to a highly diverse community of bacteria, termed as microbiome, which has symbiotic functions including metabolism of otherwise indigestible compounds and defence against opportunistic pathogens [1, 2]. Furthermore, bacteria in the gut influence the stimulation and development of the innate mucosal immune system [3]. In addition to the roles in health, there has been significant interest in the relationship between microbiomes and diseases such as obesity, inflammatory bowel disease and diabetes mellitus [4–6].

Studies utilising culture-independent techniques to analyse the lungs have identified the presence of bacterial communities that are much more complex than the previously appreciated. The lungs were long considered to be an inherently sterile environment, in part due to the fact that conventional culture techniques often yielded negative results during health and it was only during disease that pathogens were detected. However, the advent of culture-independent techniques has demonstrated that multiple organisms comprise a community, termed the 'microbiome', in the lungs of patients, both healthy and diseased [7–9]. In this chapter, we discuss the techniques employed in 16S rRNA sequencing and the evidence these techniques have generated so far in relation to cystic fibrosis.

2. 16S rRNA gene sequencing

The 16S rRNA gene codes for a ribosomal subunit present in nearly all bacteria. The gene itself is approximately 1.5 kb long and consists of conserved regions, similar in nearly all microorganisms, and nine variable regions labelled, V1–V9, which are practically specific to each microorganism [10]. The identification of a specific DNA sequence that corresponds to the known variable region of 16S rRNA gene can allow discrimination of the presence and relative abundance of different microorganisms (**Figure 1**).

Once the samples have been processed, DNA is extracted, and the 16S rRNA gene is amplified using polymerase chain reaction (PCR). Next-generation sequencing allows elucidation of the precise gene sequences, and online reference databases can then be used to match each sequence to an organism and quantify its relative proportion within a multispecies population. However, it is important to note that sequencing of the 16S rRNA gene has limited resolution and often cannot distinguish species with similar gene sequences apart. Therefore, instead of distinct species, sequences are referenced against and assigned into operational taxonomic units (OTU) (see **Table 1**).

Given the large number of species identified even in healthy lungs, ecological theory and analyses are often employed to understand community dynamics [9]. According to ecological principles, the composition of the lung microbiome is determined by three factors:

1. Immigration of organisms into the lung

2. Elimination of microbes from the airways

3. Regional growth factors [11]

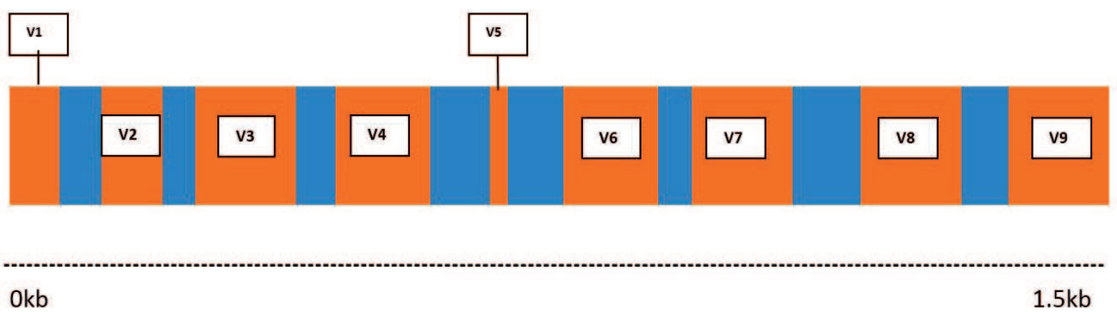

Figure 1.
Schematic illustration of structure of the 16S rRNA gene. Orange regions—variable regions; blue region—conserved region.

Term	Definition
Microbiome	The microorganisms in a particular environment
16S rRNA gene	A gene which codes for a ribosomal subunit. Present in all prokaryotes and has variable regions, which differ slightly between bacterial species
Richness	A measure of the number of species in a community
Evenness	A measure of similarity of the relative abundance for each species in a community That is, does one species dominate, or do all species have similar relative abundance?
Diversity	A measure of variety in a community. Combination of richness and evenness
Alpha diversity	Within-sample diversity
Beta diversity	Between-sample diversity
Operational taxonomic unit (OTU)	Group of strains/species with similar 16S rRNA gene sequences

Table 1.
Glossary of terms and definitions.

The lung microbiome in healthy individuals is dictated largely by immigration and elimination and hence generally consists predominantly of those Gram-negative anaerobes also resident in the oral flora such as *Prevotella* and *Veillonella* spp. [11]. However, in disease the regional growth conditions are altered, and niches for other species to thrive are created. In CF, for example, viscous secretions, altered pH, nutrient availability and architectural disturbance may all help select for a community of altered composition to that of healthy individuals. How this community changes over time, in response to the intensive antibiotic treatment that people with CF are exposed to, has been the subject of much interest in the last decade.

3. CF respiratory microbiota in early life

Understanding the development of the CF respiratory microbiota in early life has attracted interest in order to appreciate the driving factors behind the distinct microbiota seen later in life and also to identify potential opportunities for intervention. Neonates and infants cannot expectorate sputum independently, and bronchoalveolar lavage (BAL) is only used sparingly; hence, upper respiratory tract samples are often used as surrogates. The imperfection of this approach was recently highlighted where large differences in concordance between BAL samples and upper respiratory tract (URT) samples were observed in some taxa [12]. Nevertheless, concordance was high for some important taxa such as *Moraxella* and *Staphylococcus,* and in the absence of less invasive techniques, URT sampling enables early estimation of the neonatal lower respiratory tract.

The composition of CF nasopharyngeal microbiota diverges from that of non-CF infants as early as the first few months of life [13, 14]. Newborn healthy infants appear to have nasopharyngeal microbiota dominated by *Moraxella* spp., *Corynebacterium spp.* and *Haemophilus* spp., a community structure that persists for at least the first 6 months of life. Conversely the CF nasopharynx microbiome is initially dominated by *Staphylococcus aureus* before a gradual increase in *Streptococcus* spp. and *Moraxellaceae* at 3 months of age [13]. Despite the increased *S. aureus* seen in CF, there were no decreases in measures of richness or diversity indicating that changes are due to differing microenvironment rather than interspecies competition [15].

The divergence observed in the first few months of life usually precedes antibiotic administration and demonstrates that CF itself is associated with compositional changes in the microbiota, but as CF infants grow older, exposure to antibiotics, either via acute treatment for respiratory illnesses or prophylaxis against classic CF pathogens, becomes inevitable. Mika et al. investigated the relationship between antibiotics and the nasal microbiota by prospectively following 30 newborn infants with CF with fortnightly sampling for the first 12 months of life [14]. Antibiotic administration was associated with an increase in the Shannon diversity measure (a measure of the richness and evenness of a community), but this was judged to be most likely secondary to an increase in transient colonisers. Interestingly, antibiotic therapy was staphylococcal directed, but decreases in *Staphylococcus* OTUs were not seen. Instead significant reductions in *Moraxellaceae* were observed, and when oligotyping was used to observe changes in *Staphylococcus* at the species level, mild reductions in *S. aureus* were offset by increases in *S. epidermidis*, leading the authors to suggest that *S. epidermidis* may act as a reservoir of resistance. These findings were supported by Prevaes et al. who conducted a similar study in a slightly older population (mean age 2 years old) [13]. Antibiotic treatment was observed to be associated with reductions in *Moraxellaceae* and *S. aureus* OTUs, with increases in other staphylococcal OTUs, although more specific oligotyping was not performed.

As babies grow older, sampling from the lower airways becomes more common, and comparisons between the lower and upper airways become feasible. Given the close proximity and interrelated spaces of the nose, throat and lungs, it could be expected that they all may share similar community structures; however, the reality is that the nasal community appears different from that of the throat and lung, which are much more closely aligned. Boutin et al. found differences in the community structure of the nasal cavity compared to throat and sputum samples; in that diversity, richness and evenness were significantly higher in nasal samples, and up to 21 of the 76 most abundant nasal OTUs were not present in the throat or sputum samples [16]. Interestingly, the authors also found that subjects could be broadly defined into one of two ecotypes based on the presence or absence of *Pseudomonas*, and the similarities between throat and sputum samples began to diminish once *Pseudomonas* was present. Muhlebach et al. supported these findings in a recently published study, which for the first time included routine sequential BAL sampling in young children as part of the large AREST-CF cohort in Australia and the USA. They showed that lower airway cultures mirrored that of the oral cavity until approximately age 2, when increasing predominance of known CF pathogens was observed and communities diverged [17]. This has a number of clinical implications in that, firstly, throat swabs can provide adequate representation of the lower airways in very young children and, secondly, prevention or delay of this transition point by manipulation of the microbiota could theoretically be a strategy to improve outcomes later in life.

4. Progressive loss of diversity

Once the lungs are colonised with CF pathogens, a pattern of progressively uneven community structures ensues. Cox et al. examined biobanked sputum samples from a cohort of 63 clinically stable people with CF of ages ranging from 9 months to 72 years [7]. This cross-sectional approach identified the loss of community richness, evenness and diversity as age increased. *Pseudomonas* and *Burkholderia* OTUs began to progressively dominate in older subjects, and the changes in community structure were inversely associated with pulmonary function. In a similar study design with 269 patients, Coburn et al. also found sample

diversity inversely correlated with age and disease stage. Progressive loss of diversity was particularly correlated with *Pseudomonas* and *Burkholderia* abundance, which notably increased after the age of 25 [18].

Zhao et al. were the first to confirm these findings longitudinally when they followed up six patients over a 9-year period with serial sputum collections. It was observed that the three patients with what they termed as more 'progressive' disease had significant decreases in community diversity over the course of several years. Decreasing lung function and increasing age were also associated with decreasing community diversity [19]. This study was soon followed by Fodor et al. who focussed more on changes in the microbiota associated with acute changes in clinical status but did observe a strong correlation between low species richness and poor lung function [20]. Stokell et al. followed up a single patient up to over 3 years and observed increasing total bacterial load as well as diminishing community richness and diversity [21].

Contrastingly, Whelan et al. recently published a study of six patients who submitted thrice-weekly sputum samples for a year [22]. No overall changes in community structure were observed over the course of the year, and the authors concluded that the respiratory microbiome is unique to each patient and the previously reported associations between community structure and clinical parameters may be true on a cohort/population level but not at an individual level. There is some merit in this argument, but it is also worth noting that the six patients in the study appeared relatively stable with a median of only one exacerbation in the 12-month study period. It is also therefore a possibility that the follow-up period was not long enough to capture the more indolent changes likely to be present in those patients [23]. A much longer study period was adopted by Acosta et al. [24] who analysed samples from matched patients with biobanked sputum samples in three historic cohorts spanning nearly 20 years at a single centre. Across all the three cohorts, the core microbiome constituents were preserved, but the proportion of *Pseudomonas*-dominated communities was reduced, and overall diversity increased in the more recent cohorts. Community structure improved gradually from the most historic cohort to the most recent, and these changes appeared to correlate with the generally improving clinical status of people with CF, confirming the previously described observed association between community structure and clinical outcomes [24].

The association reported in most studies between community structure and clinical outcomes has inevitably led to the question of whether a less diverse or even rich microbiome is simply a marker of increased pulmonary disease or is itself a driver in disease pathogenesis [25]. If the latter were true, efforts to promote a more diverse community could have the potential to slow pulmonary disease progression. An Italian group has led efforts to find patterns or signatures in the microbiome that may predispose patients to accelerated lung function decline; however, no causal association has been elucidated [26–28]. Instead, Zhao et al. found that the relationship between age, lung function and community diversity disappeared once controlled for antibiotic use, thus suggesting antibiotic therapy is the predominant driver of reducing community diversity [19]. The same group later developed a statistical approach to more precisely correct the antibiotic exposure when examining relationships between microbiota and clinical outcomes. The approach was applied to 478 sputum samples and confirmed that antibiotic use was an independent predictor for decreased diversity [29].

Accurately recording antibiotic use is troublesome in longitudinal studies due to the frequent episodic use of antibiotics in CF which is often self-directed by patients themselves, due to the widespread use of long-term antibiotics for which compliance may be heterogeneous and also due to the retrospective nature of a number

of CF microbiome studies [30]. However, Pittman et al. were able to prospectively perform bronchoscopy and record antibiotic exposure of 32 subjects as part of the AREST-CF study. In that study, community diversity was much lower in the BAL of those patients receiving antibiotics [31].

Thus it appears likely that the strong association between community structure and degree of lung disease is related to the inevitable prolonged and aggressive use of antibiotics in CF rather than direct pathogenesis from a less diverse microbiome.

5. Community changes with acute pulmonary exacerbations

Despite the importance of exacerbations on long-term outcomes of people with CF, the pathophysiology of these events remains undefined [32, 33]. Clinically, exacerbations are frequent and are characterised by rapid changes in symptoms such as an increase in sputum volume or purulence, shortness of breath and fatigue. The precise mechanisms underlying these important events remain elusive, and studies looking for answers using culture-independent techniques have not found consistent answers. For example, one may expect to find evidence of increases in known pathogens at the time of exacerbations, yet there is no consistent evidence of this [23]. In fact, a number of studies have found the CF microbiota to be extremely stable over time and resilient to change at exacerbation and following subsequent treatment [19, 20, 34, 35].

However, when the community structure as a whole is considered, a number of larger studies have found reduced diversity or richness at the times of exacerbation compared to clinical stability. Coburn et al. found small decreases in Shannon diversity in exacerbation samples compared to their baseline study of 269 people with CF [18]. Similarly, Filkins et al. found that samples taken during exacerbations had significantly lower diversity than samples taken when patients were stable [36]. Perhaps most convincingly, Li et al. collated data from 18 previous studies to analyse over 700 sputum samples and found that there were significant reductions in community richness at exacerbation [37].

Whilst increases in *P. aeruginosa* at the time of exacerbation have not been seen consistently, they have been observed in some cases. Carmody et al. followed up four patients for 3 months with daily sputum sampling and observed daily stability between exacerbations but increased *P. aeruginosa* abundance at the time of exacerbation in some patients and increases in *Prevotella* in others [38]. These findings help introduce two new concepts: firstly, the potential for exacerbations to appear similar phenotypically but have different underlying aetiology with only some being due to changes that can be observed in the microbiota and, secondly, that previously overlooked anaerobes may play a pathogenic role.

The first concept is supported by Whelan et al. who found in longitudinal sampling of six patients that some but not all exacerbations were associated with changes in the microbiota [22]. Attempts to identify different types of exacerbations in COPD have identified four distinct aetiological clusters, bacterial, viral, eosinophilic predominant and 'paucinflammatory', and even though these clusters may not be mirrored in CF, it is plausible that not all exacerbation clusters would be associated with changes apparent in either individual taxa or overall bacterial community structure [39].

Changes in the metabolic activity of specific taxa or the community as a whole triggering an exacerbation could be another explanation for an apparent lack of change in the community structure seen in some studies. The metabolites lactate and putrescine were found by Twomey et al. to increase during exacerbation in the absence of clear changes in the community structure [40]. Quinn et al. used

the ecological functional networking to identify the non-mevalonate pathway of isoprenoid synthesis as a 'keystone' pathway in CF infections. Intriguingly fosmidomycin, an antimalarial agent, is known to be effective at targeting this pathway [41].

The second concept to emerge from the study of Carmody et al. relates to the changes in *Prevotella* abundance at the time of exacerbation and raises the prospect that species not considered conventional CF pathogens may play a role in exacerbations [42]. Anaerobic species are easily overlooked in conventional selective culturing due to the requirement for anoxic culture yet are identified frequently in culture-independent analyses of the CF lower airways. In addition to *Gemella*, both *Prevotella* and *Streptococcus anginosus (milleri)* have been found to have associations with clinical stability [36, 38, 42]. Anaerobes have been shown to have the potential to modulate *P. aeruginosa* gene expression in the polymicrobial setting; hence, even if they are not directly pathogenic, they may still play a contributory role to the pathogenesis of some exacerbations [43, 44].

To summarise, the aetiologies underpinning the transition from a stable state to an acute exacerbation are not well understood. It is likely that there multiple aetiological clusters but only some of which may be associated with changes in community structure.

6. Community changes associated with treatment for acute pulmonary exacerbations

Traditional dogma would dictate that intensive, targeted antimicrobial therapy with dual anti-pseudomonal agents will result in significant reductions in abundance of *P. aeruginosa*; however, in a similar vein to the findings from studies of the onset of exacerbations, microbiota responses to treatment for acute pulmonary exacerbations in CF have not aligned with this conventional understanding of infections.

One of the predominant themes that has emerged from studies of the respiratory microbiota response to acute antibiotics is that *P. aeruginosa* is not impacted to the same degree as other members of the community. For example, Daniels et al. studied 12 adult CF subjects across the cycle of an exacerbation and treatment and found that following initiation of anti-pseudomonal antimicrobials, the relative abundance of *P. aeruginosa* actually increased, alongside a reduction in the total number of species detected [45]. Cuthbertson et al. also found no evidence of reduced *P. aeruginosa* abundance in a study of 12 CF patients receiving treatment for pulmonary exacerbations [45]. Instead, reductions in *Streptococcus sanguinis*, *Prevotella* and *Porphyromonas* OTUs were observed [35]. Similarly, Li et al., in their analysis of over 700 sputum samples, found that antibiotic treatment had no effect on *Pseudomonas* abundance but did have significant effects on *Gemella*, *Staphylococcus*, *Actinomyces, Moraxellaceae* and *Fusobacterium* [37]. Further, Fodor et al. again found that dominant taxa such as *Pseudomonas* and *Burkholderia* were unchanged when compared at the beginning and end of an exacerbation but the relative abundances of *Gemella, Streptococcus* and a small number of other less abundant OTUs were all reduced [20].

In contrast, two studies have found reductions in *P. aeruginosa* following treatment. Firstly, Zemanick et al. investigated the association between inflammation and changes to the airway microbiota during treatment for exacerbations and found that although bacterial load did not change, the relative abundance of *P. aeruginosa* was observed to decrease and that these changes correlated with improved lung function [46]. A reduction in *P. aeruginosa* abundance following treatment was also reported by Smith et al., who noted rapid decreases in *P. aeruginosa* abundance and

an associated increase in diversity following the initiation of intravenous antibiotic treatment for pulmonary exacerbations in CF, although these changes were transient and returned to baseline following the completion of treatment [47].

There are a number of factors that may explain the differences between the studies mentioned in this section, and many of them apply to studies of the CF microbiome in general. The most obvious is the heterogeneous study designs, which are mostly retrospective and observational in nature and include a wide range of antibiotic regimens. For example, some authors such as Cuthbertson and Daniels included exacerbations treated with oral antibiotics as well as those requiring intravenous therapy [35, 45]. Milder exacerbations are often treated with oral antibiotics, and hence associated changes in the microbiota may also be expected to be more subtle. Even in those studies where only intravenous regimens were used, the antibiotic regimens or doses given are often not listed. The lack of a control or comparator group further makes interpreting results difficult [35, 45].

A further consideration is the sampling timeframes in each study, where again there exists a considerable variation that may have implications for interpreting results, particularly given that Smith et al. reported significant but transient reductions in *P. aeruginosa* abundance in the first few days of treatment [48].

Sample collection, storage, handling and DNA extraction techniques all also have the potential to impact on subsequent sequencing results. For example, multiple freeze–thaw cycles have been demonstrated to affect the results of microbiota analysis in respiratory samples [49]. Furthermore, different sequencing platforms can also produce different profiles [50].

There is no universally standardised protocol for the extraction of DNA from respiratory samples, and hence methods are often inconsistent between study groups. One obvious inconsistency is the use of propidium monoazide (PMA), a chemical compound that binds DNA in cells with damaged membranes and hence allows exclusion of non-viable DNA from sequencing. Excluding non-viable DNA has been suggested to be important for accurately identifying which members of the community are active at times of exacerbation and helps to avoid overestimation of viable microorganisms following treatment with antibiotics, but it is not utilised by all groups [51, 52]. There are concerns that PMA may incompletely penetrate sputum, hence only identifying a portion of non-viable cells. PMA is also known to stain viable cells of some species and stain dead cells in others [53]. In CF exacerbations, PMA treatment was not found to significantly alter the community as a whole, and only changes in low abundance 'satellite' taxa were apparent [51].

Overall, there is certainly evidence that acute antibiotic administration alters the respiratory microbiota; however, in the absence of prospective controlled trials, it is difficult to interpret these results given the confounders mentioned above. Indeed there have been calls for future clinical trials in CF to include biobanking of samples to allow a more rigorous scrutiny of the effect of antibiotic agents on the microbiome [54].

7. Community changes associated with chronic suppressive antibiotics

Inhaled antibiotics such as colistimethate (COL), tobramycin (TOB), aztreonam (AZLI) and levofloxacin (LIS) preparations are all licenced in the UK for the treatment of chronic *P. aeruginosa* infections and have, to varying degrees, demonstrated improvements in lung function and exacerbation rates as well as sputum density of *P. aeruginosa* [55–58]. However, despite the near ubiquitous use of these inhaled anti-pseudomonals in the chronic *P. aeruginosa* treatment in CF, the effect of these treatments on the microbiome remains poorly defined. Furthermore,

many patients receive chronic macrolide therapy over many years, at least in part for its immunomodulatory effects, yet similarly little is known about the effects of this persistent selective pressure on the microbiota. When considering inhaled antibiotics, there is contrasting evidence as to their influence on the CF microbiome. For example, Kramer et al. [59] did not find any correlation between bacterial community structure and inhaled antibiotic treatments, although it is unclear which agents patients were using in that study. More recently Acosta et al. [24] utilised a prospectively collected Canadian sputum biobank primarily to investigate changes in CF cohort microbiota over time but also assessed whether different long-term antibiotics were associated with distinct microbiota. Eighty-two samples from 42 patients were sequenced, and those receiving long-term tobramycin and colistimethate harboured significantly different respiratory communities compared to those who were not, but interestingly no differences were seen in people receiving AZLI. The authors also assessed the impact of long-term oral azithromycin and nebulised dornase, but these agents were not associated with any differences. Perhaps intrigued by the failure of AZLI to be associated with microbiomic differences given its proven clinical benefits, the same group investigated its effects in more detail. In the only published study focussing on the microbiomic outcomes of a specific inhaled antibiotic, Heirali et al. [54] utilised the same Canadian biobank and sequenced 80 samples from 24 patients naive to AZLI and 82 samples from the same patients following initiation of AZLI. Overall no differences were observed in alpha or beta diversity measures, but at the OTU level, significantly lower relative abundances of *Prevotella* were seen following AZLI initiation. The authors then subclassified patients into AZLI 'responders' and 'non-responders' based on clinical outcomes and found 'non-responders' to have lower abundance of *Pseudomonas* and higher abundance of *Staphylococcus*. This novel approach raises the prospect of signatures in an individual's microbiota acting as a biomarker for response to antibiotics and may represent an important step in the march towards personalised precision medicine in CF. Further studies are required to explore the potential for an individual's microbiota to guide treatment.

8. Community changes associated with CFTR modulators

In the last 5 years, treatments targeted towards correcting the underlying defect in CF have become available. Ivacaftor, a cystic fibrosis transmembrane conductance regulator (CFTR) potentiator, is licenced specifically for the treatment of people with a G551D mutation and a number of other rare gating mutations, which together account for approximately 5–10% of the CF population in the UK [60]. In this subset of the CF population, ivacaftor use has been associated with improvements in lung function, reductions in exacerbations, reductions in sweat chloride, improved weight gain and improved quality of life [61, 62]. The restoration of CFTR activity by ivacaftor and the associated clinical benefits, in particular improved lung function and reductions in exacerbation, has inevitably raised questions as to whether ivacaftor has an antimicrobial effect.

Theoretically, ivacaftor could have an antimicrobial effect in a number of ways. Firstly, the restoration of CFTR activity should result in a rehydrated airway surface layer, and this in turn will allow the mucociliary escalator to function physiologically. The improved clearance of airway secretions would then result in the elimination of bacteria. Secondly, the restoration of CFTR activity could result in a dramatic change in the local pulmonary microenvironment, turning a previously favourable environmental niche into an inhospitable one for resident

microbiota. There is evidence to support a similar effect in the GI tract, where CFTR modulation with ivacaftor was associated with improved proximal small intestinal pH, likely secondary to improved bicarbonate secretion [63]. Thirdly, a direct bactericidal effect of ivacaftor itself has been postulated given that its chemical structure contains a quinolone ring and many quinolone derivatives have antimicrobial properties [64]. This theory is supported by evidence that ivacaftor exerted in vitro antibacterial effects on clinical respiratory isolates of *S. aureus* (MSSA and MRSA) and *Streptococcus pneumoniae* [65, 66]. However, these studies found very little activity against Gram-negative organisms, which is uncharacteristic of a quinolone-based antibiotic [65]. Interestingly, the authors of the same study noted synergism of ivacaftor with antibiotics commonly used in CF, and this was recently supported by Payne et al., who again found that ivacaftor had activity against *Streptococcus* spp. and *S. aureus* and that the effect was potentiated in the presence of tobramycin. Again there was no evidence of direct antibacterial activity against *P. aeruginosa* [67].

Interest has since focussed on clinical microbiological outcomes of patients commenced on ivacaftor therapy in an effort to investigate differences post-treatment initiation. Interestingly, despite ivacaftor appearing to have no innate activity against *P. aeruginosa,* a number of studies have reported reductions in counts and density post initiation, perhaps suggesting that quinolone activity is not the only mechanism at play [68, 69].

In contrast to the relative lack of focus on inhaled antibiotics, a number of studies have investigated the effects of ivacaftor on the respiratory microbiome. Peleg et al. [70] conducted the only placebo-controlled trial in this field when they performed a double-blind, placebo-controlled, cross-over study of 28-day ivacaftor treatment. Sputum was collected at the start and end of each 28-day treatment period, and 16S rRNA sequencing with qPCR correlation was subsequently performed. No significant differences were observed for either total bacterial load or *P. aeruginosa* load following ivacaftor therapy, and no significant difference in the microbiota composition (based on 16S rRNA microbiome analysis) was observed between the placebo and treatment samples. However, the authors noted that when they adjusted for consistent or changing antibiotic exposure in the 28-day study period, ivacaftor was associated with a significant reduction in *P. aeruginosa* load. That is to say, changes in the microbiota induced by acute changes in antibiotic administration during the 28-day treatment periods may have masked the effect induced by ivacaftor.

In longer-term observational studies, a number of changes have been observed. Bernarde et al. [71] noted no significant changes in bacteria load and also no significant changes in overall community composition at 1 year following ivacaftor initiation. However, individual taxa were observed to change in that the relative abundance of *Streptococcus mitis* group was significantly diminished and a *Porphyromonas* OTU was significantly increased. Elsewhere, in perhaps the most comprehensive study of microbiological outcomes following ivacaftor initiation, Hisert et al. [72] found marked reductions in *P. aeruginosa* sputum densities using conventional culture and also reduced sputum inflammatory markers in regular follow-up throughout the first 2 years of ivacaftor use. These findings were mirrored in the 16S rRNA-based analysis, where decreases in mean *P. aeruginosa* relative abundance and subsequent increases in diversity measures were observed. However, no patient eradicated *P. aeruginosa*, and after 12 months of treatment, relative abundance, sputum counts and inflammatory markers began to increase again. The authors interpreted these findings to suggest that *P. aeruginosa* may adapt to a CFTR-restored environment and this clearly has implications for the need for ongoing anti-infective chemotherapy in CF.

9. Nontuberculous mycobacteria

Nontuberculous mycobacteria (NTM) are identified in approximately 10% of CF patients, but only a small proportion will go on to develop NTM pulmonary disease (NTM-PD) warranting treatment. First and foremost, the management of CF pulmonary disease should be optimised, including antibiotic therapy targeted to the individual's usual airway bacteria, prior to considering treatment for NTM-PD. Those who fulfil criteria for NTM lung disease may not necessarily require treatment and could be monitored expectantly if symptoms and radiographic findings are minimal or stable over a period of surveillance. However, the presence of *Mycobacterium abscessus* complex (MABSC), deteriorating lung function, worsening radiology and/or anticipated lung transplant should prompt NTM therapy initiation (**Table 2**). For CF patients with *Mycobacterium avium* complex (MAC), recommended treatment includes triple antibiotic therapy with a macrolide, rifampin and ethambutol. Azithromycin is generally the preferred macrolide of choice in CF as it is better tolerated and has fewer drug–drug interactions. An initial course of injectable amikacin or streptomycin should be considered in the presence of (i) acid-fast bacillus smear-positive respiratory tract samples, (ii) radiological evidence of lung cavitation or severe infection and (iii) systemic signs of illness. MABSC treatment is more complicated and requires an induction phase (oral macrolide and two IV agents including amikacin with one or more additional intravenous antibiotics including tigecycline, imipenem, cefoxitin) for 3 to 12 weeks as well as a maintenance phase (nebulised amikacin and a macrolide with two to three oral antibiotics including minocycline, clofazimine, moxifloxacin, linezolid). Baseline and interval testing for drug toxicity is essential. The treatment duration for both MAC and MABSC is extended 1-year post-culture conversion. However, in patients who do not achieve culture negative status but tolerate therapy, ongoing treatment for mycobacterial suppression and prevention of disease progression can be considered. There are no randomised controlled trials of MABSC therapy in the general population or in CF; however, there is MABSC treatment outcome data in non-CF populations from several clinical studies [74]. In a study of 57 non-CF

Clinical (both required)
1. Pulmonary symptoms with nodular or cavitary opacities on chest radiograph or a high-resolution CT scan that shows multifocal bronchiectasis with multiple small nodules
2. Appropriate exclusion of other diagnoses.
Microbiologic (one of the following required)
• Positive culture results from at least two expectorated sputum samples. If the results from samples are non-diagnostic, consider repeat sputum acid-fast bacillus (AFB) smears and cultures.
• Positive culture results from at least one bronchial wash or lavage.
• Transbronchial or other lung biopsy with mycobacterial histopathological features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathological features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.
• Expert consultation should be obtained when either infrequently encountered NTM or those usually representing environmental contamination are recovered.
• Patients who are suspected of having NTM-PD but who do not meet the diagnostic criteria should be followed up until the diagnosis is firmly established or excluded.
• Making the diagnosis of NTM-PD does not, per se, necessitate the institution of therapy, which is a decision based on potential risks and benefits of therapy for individual patients.

Table 2.
ATS/IDSA clinical and microbiologic criteria for diagnosing nontuberculous mycobacterial pulmonary disease (NTM-PD) [73].

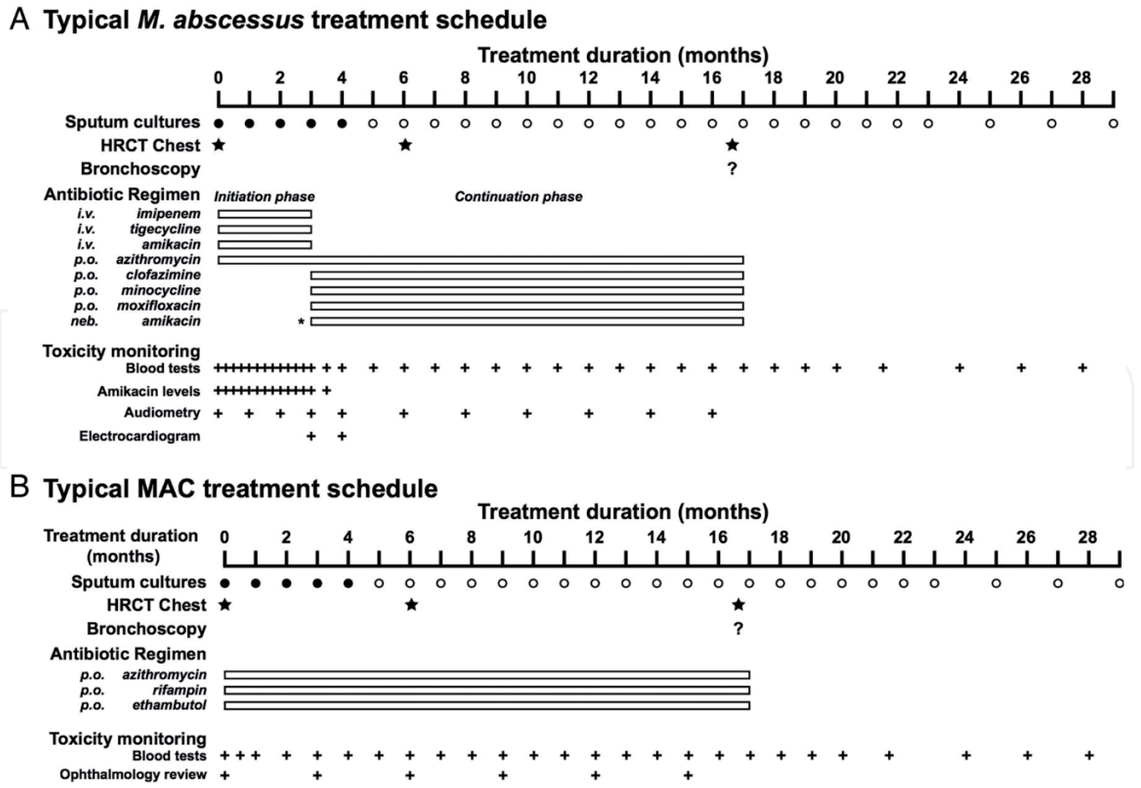

Figure 2.
Typical treatment schedules for individuals with CF with Mycobacterium abscessus *or MAC pulmonary disease [74].*

subjects that compared *M. abscessus* ssp. *massiliense* and ssp. *abscessus* infections and treatment outcomes, all individuals were treated with clarithromycin-containing regimen in combination with an initial 4-week course of cefoxitin and amikacin, was given to 57 patients (24 with *M. abscessus* and 33 with *M. massiliense*) for more than 12 months. The proportion of patients with sputum conversion and maintenance of negative sputum cultures was higher in patients with *M. massiliense* infection (88%) than in those with *M. abscessus* infection (25%; $p < 0.001$). Inducible resistance to clarithromycin (minimal inhibitory concentrations ≥ 32 μg/ml) was found in all tested *M. abscessus* isolates (n = 19), but in none of the *M. massiliense* isolates (n = 28) [75].

Inhaled liposomal amikacin for maintenance treatment has also drawn interest. In a randomised placebo-controlled trial, CF subjects with NTM lung disease refractory to standard therapy were assigned to 590 mg OD inhaled liposomal amikacin or placebo, in addition to their standard CF treatments and ongoing NTM therapy [76]. The group of 90 patients was stratified based on MAC (64%) and MABSC (36%) [77]. At the end of the 6-month treatment period, there was a statistically significant increase in culture negativity overall and for the MAC group.

Reported NTM prevalence in CF ranges from 3 [78] to 23% [79]. The majority (95%) of NTM isolated from CF patients are *Mycobacterium avium* complex (MAC) (*M. avium-intracellulare* and four *M. avium* subspecies) and *M. abscessus* complex (MABSC) (subspecies abscessus, massiliense, and bolletii) [80, 81]. Bryant et al. [82] reported the largest outbreak occurred in Cambridge, England, where 11 of 31 patients with MABSC had a shared strain of *M. abscessus* subsp. *massiliense* with similar antibiotic resistance patterns by whole genome sequencing despite a lack of exposure to the same antibiotics, raising the possibility of cross infection amongst the CF cohort albeit conventional cross infection measures (**Figure 2**).

MABSC has been demonstrated to accelerate lung function decline in CF patients compared to uninfected CF controls [83, 84]. In CF, a common measure

of lung disease severity is percent-predicted forced expiratory volume in 1 second (FEV1), with lower values indicating more severe lung disease. Qvist et al. showed that MABSC had a greater rate of FEV1% predicted decline than other organisms, including *Pseudomonas aeruginosa* and *Burkholderia cepacia* [84]. However, no lung function decline was noted in patients growing MAC [83]. Likewise, a large study found no significant effect of NTM infection on lung function decline, although analyses were not based on different NTM subtypes [80]. A diagnosis of NTM pulmonary disease must meet both clinical and microbiologic criteria as outlined by ATS guidelines, with exclusion of other aetiologies [73].

Individuals who are NTM culture positive but who do not meet ATS criteria for disease should be monitored closely [74]. Patients with CF meeting criteria for NTM-PD should be considered for therapy; however, treatment decisions should be individualised [74, 85]. It may be reasonable to monitor individuals with mild CF lung disease, MAC lung disease with mild symptoms and radiographic changes or a high possibility of drug intolerance or drug interactions [85]. However, CF patients with MABSC and/or severe CF lung disease should generally be treated in the absence of contraindications [85].

10. Fungal lung disease in CF

Clinical manifestations of respiratory fungal diseases in adult CF patients are very heterogeneous, ranging from asymptomatic colonisation to chronic infections, allergic disorders or invasive diseases in immunosuppressed CF patients following lung transplantation.

Aspergillus spp. are amongst the most widespread filamentous fungi in the environment, especially in areas with high humidity [86]. In CF patients, the most frequently isolated species is *Aspergillus fumigatus*, accounting for 67–73% of *Aspergillus*-positive sputum cultures [87]. Isolation of other species such as *A. flavus*, *A. niger* and *A. terreus* is less frequent but not rare (4, 4 and 2% of *Aspergillus*-positive sputum cultures, respectively) [87]. Notably, the prevalence of isolation of *Aspergillus* spp. from sputum cultures in CF patients increases with age, possibly reaching 46–78% in adult CF patients, although with important inter-region and inter-centre variability [88–90]. However, knowledge of the prevalence of *Aspergillus* spp. isolation from sputum does not automatically allow to infer the prevalence of the various *Aspergillus*-related manifestations in CF patients, which range from asymptomatic colonisation to invasive diseases, especially in patients post-lung transplantation.

Allergic bronchopulmonary aspergillosis (ABPA) refers to a complex hypersensitivity reaction which often occurs in patients affected by CF or asthma. ABPA is beyond the scope of discussion in this chapter.

In CF patients, the disease-related progressive damage of the lungs may favour the development of chronic *Aspergillus* infection, commonly defined as '*Aspergillus* bronchitis', although aspergilloma(s) might also develop in some cases, especially in pre-existing cavities or bronchiectasis [91–93]. *Aspergillus* bronchitis has an estimated prevalence of ~2–8% in CF patients and may be suspected in the case of pulmonary exacerbation unresponsive to antibacterial treatment [94, 95]. As per Baxter et al.'s [96] classification, diagnosis of *Aspergillus* bronchitis can be made in the presence of a positive sputum galactomannan, high levels of *Aspergillus*-specific IgG and negative total and *Aspergillus*-specific IgE. Since *Aspergillus* bronchitis does reflect infection and not an immune-mediated response as in ABPA, corticosteroids are not the cornerstone of treatment. Treatment with azole derivatives is the current standard of care, although the overall duration of treatment is still not clearly

defined [97]. Invasive aspergillosis is described as the infection progresses across tissues and invades the vessels, with subsequent necrosis [96]. Usually, invasive aspergillosis is observed in severely immunocompromised non-CF populations, such as haematology patients with prolonged neutropenia and patients receiving high dosages of corticosteroids or other immunosuppressive agents. Clinical symptoms usually include fever, chest pain, shortness of breath and/or cough [96]. Haemoptysis and pneumothorax might also develop in some cases [98, 99]. Invasive aspergillosis is rarely seen in patients affected by CF. However, it could develop in end-stage CF patient and in immunosuppressed CF patients following lung transplantation, frequently in the form of *Aspergillus* tracheobronchitis, occurring mainly in the first 3 months after transplants and associated with increased mortality (39%) [100, 101]. Frequent symptoms of *Aspergillus* tracheobronchitis are severe dyspnoea, cough and wheezing [97]. The histopathological examination may show different features: (1) obstructive bronchial aspergillosis; (2) ulcerative tracheobronchitis (characterised by the invasion of the tracheobronchial mucosa and cartilage); and (3) pseudomembranous tracheobronchitis (characterised by inflammation and invasion of the tracheobronchial tree). Invasive aspergillosis is usually treated with systemic azole therapy (possibly associated with nebulised amphotericin B in some cases of tracheobronchitis) [97]. The US guidelines recommend a minimum of 6 to 12 weeks of therapy for patients with invasive pulmonary aspergillosis and at least 3 months in the case of *Aspergillus* tracheobronchitis [97].

11. Conclusion

The pulmonary microbiome of people with CF diverging significantly from that of the healthy individuals has been the focus of much research in the last 5 years often producing more questions than answers. As the disease progresses, community structure becomes progressively less diverse, most likely as a consequence of long-term aggressive antibiotic therapy. The impact of acute antibiotic therapy, antifungal treatments and CFTR modulators are less well defined, and prospective clinical trials with sputum biobanking are needed to answer these questions.

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