Review

Deciphering the Ecology of Cystic Fibrosis Bacterial Communities: Towards Systems-Level Integration

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Despite over a decade of cystic fibrosis (CF) microbiome research, much remains to be learned about the overall composition, metabolic activities, and pathogenicity of the microbes in CF airways, limiting our understanding of the respiratory microbiome's relation to disease. Systems-level integration and modeling of host–microbiome interactions may allow us to better define the relationships between microbiological characteristics, disease status, and treatment response. In this way, modeling could pave the way for microbiome-based development of predictive models, individualized treatment plans, and novel therapeutic approaches, potentially serving as a paradigm for approaching other chronic infections. In this review, we describe the challenges facing this effort and propose research priorities for a systems biology approach to CF lung disease.

The CF Microbiota: Where Are We Now?

CF (OMIM 219700) is the most common life-shortening Mendelian disease in the white populations of Europe and North America, with an incidence of approximately one in 2500–6000 live births and a prevalence of more than 70 000 people living with CF worldwide [1,2]. Disease is caused by mutations in the *CF transmembrane conductance regulator* (*cftr*) gene, which encodes an anion channel (CFTR) found in both secretory and absorbing epithelia. This defect results in abnormal sodium, chloride, and bicarbonate transport across these epithelia, altering the composition of secretions in the lung, gastrointestinal tract, pancreas, biliary ducts, and other secretory glands. In the airways, absent or dysfunctional CFTR results in thick and tenacious mucus that compromises mucociliary clearance. This condition predisposes individuals to chronic bacterial infections and airway inflammation. During chronic infection, bacterial pathogens adapt to the microenvironment of CF airways [3,4], such as a thickened mucus layer and steep hypoxic gradients [5,6], possibly also modulating virulence mechanisms and resulting in damage to the airways and consequent chronic lung disease.

Bacterial lung infections are associated with reduced quality and length of life in CF (median predicted survival age of 43.6 years between 2013 and 2017, according to the Annual Data Report 2017 of US Patient Registry Data [7]). These infections are key drivers of a pathophysiological cascade that leads to progressive and irreversible airway damage [8]. Affected individuals consistently maintain high bacterial loads in their airways both during periods of clinical stability and episodic increases in symptoms known as **pulmonary exacerbations (PEx)**, (see Glossary). Standard clinical microbiology of CF, both during stability and PEx, usually identifies a relatively low number of bacterial species that are often thought to be important for driving PEx (Table 1), occasionally also including nontuberculous mycobacteria (NTM) and fungi, such as *Candida* spp., *Aspergillus* spp., and *Scedosporium* spp. [9,10].

Highlights

The analysis of CF respiratory microbiome data is rapidly increasing our understanding of the microbiological correlates of lung disease, fueling hope for rational design of therapeutics that target the microbiota or its behavior to improve the health of the CF lung.

Recent work using metagenomic techniques has not only identified variable taxonomic composition of microbiota, but also a conserved set of functional microbial genes in patients with similar disease severity, suggesting functional redundancy that may present opportunities for treatments. These findings have led to harnessing the sputum microbiome composition as predictive over disease progression.

Among the members of the microbiota, agonistic and antagonistic interactions have been disclosed, allowing the hypothesis of interventions based on the restoration of healthy ecological relationships inside the CF microbiome.

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Table 1. Traditional CF Pathogens (Left) and Taxa Commonly Detected by Molecular Methods (Right) in CF Respiratory Secretions

Traditional CF pathogens	CF respiratory secretion-associated genera
Pseudomonas aeruginosa[c] *Staphylococcus aureus*[b] *Stenotrophomonas maltophilia*[c] *Burkholderia cepacia* complex[c] *Haemophilus influenzae*[b] Nontuberculous mycobacteria[c] *Achromobacter xylosoxidans*[c]	*Neisseria*[c] *Rothia*[c] *Atopobium*[a] *Capnocytophaga*[a] *Leptotrichia*[a] *Porphyromonas*[a] *Prevotella*[a] *Veillonella*[a] *Actinobacillus*[a,b] *Actinomyces*[a,b] *Abiotrophia*[b] *Gemella*[b] *Granulicatella*[b] *Lactobacillus*[b] *Streptococcus*[b]
Detected with classical culture	Detected with molecular methods
Typically identified down to the species level	Typically, only identified to the genus level
Often remain stable; decrease in relative abundance prior to exacerbation	May increase in relative abundance with exacerbation
High relative abundance associated with more advanced disease	High relative abundance associated with healthier lung function
Many associated with faster lung function decline	

[a]Contains/is an anaerobic species.
[b]Contains/is a facultative anaerobic species.
[c]Contains/is an aerobic species.

Subsequent studies utilizing culture-independent analysis demonstrated that a complex mix of bacteria, fungi, and viruses form the airway microbiota (i.e., the communities of microorganisms that inhabit a given environment) of chronically infected individuals with CF [11]. Pioneering studies by Rogers and colleagues [12,13] identified bacterial species not previously associated with CF airways in many CF sputum samples, expanding the list of potential CF pathogens and chronic airway colonizers, and establishing the polymicrobial nature of most CF respiratory infections (Table 1). Research during the past decade using next-generation sequencing confirmed and broadened this list of CF respiratory microbiota [14] and added to the list of potential 'atypical CF pathogens' both obligate and facultative anaerobes, such as *Gemella*, *Rothia*, and members of the *Streptococcus anginosus* group [15,16]. Recent metagenomic and **metatranscriptomic** studies on sputum samples suggested a role for these taxa in the CF airway microbiome (i.e., all microorganisms colonizing a defined environment as well as their genomic content and the surrounding environmental conditions) [17].

Finally, next-generation sequencing has also identified diverse fungi (including novel taxa from *Candida* spp. and *Aspergillus* spp.) [18] in the airways of people with CF, although their involvement in respiratory disease remains controversial and largely understudied [10,19,20]. Metagenomic studies have also revealed an impact of respiratory viral infections in CF airway damage [21–23], as well as the presence of bacterial viruses (phages) in CF airways [24,25]. Phages may play roles in infection dynamics, perhaps by limiting specific bacterial populations and facilitating the adaptation of bacteria to CF airway conditions and/or the spread of antimicrobial resistance (e.g., through transduction) [26], as well as providing a potential therapeutic avenue. However, due to the lack of universal gene markers for viruses (as opposed to bacterial 16S ribosomal RNA gene), the overwhelming majority of investigations of CF microbiota have

focused on bacteria [22]. Consequently, we will mainly review studies on the bacterial fraction of the CF airway microbiome, beginning with an overview of the CF microbiota and the factors that potentially affect its community structure, composition, and diversity, then moving on to the important role of the functional interaction patterns in development of precision medicine tailored to the microbiome of individual people with CF.

Factors Influencing the Evaluation of CF Microbiota

Study Design

Both **longitudinal** and **cross-sectional study** designs have been applied to investigate CF respiratory microbiota. Each of these approaches offers distinct advantages and disadvantages, providing complementary insights into CF respiratory microbiology (Figure 1). In longitudinal studies, individuals act as their own controls, allowing for a more fine-tuned analysis of how individual taxa correlate with different clinical and patient factors. Longitudinal studies have demonstrated, for instance, interpatient heterogeneity in CF respiratory microbiota to be higher than intrapatient heterogeneity; these studies have also identified great variation in the rate of microbiota change within patients [27,28]. The complexity of performing longitudinal studies, however, often limits sample size and, therefore, generalizability [29].

Conversely, cross-sectional studies tend to be larger, helping to mitigate the confounding effects of interpatient heterogeneity. Cross-sectional studies have also identified substantial interpatient
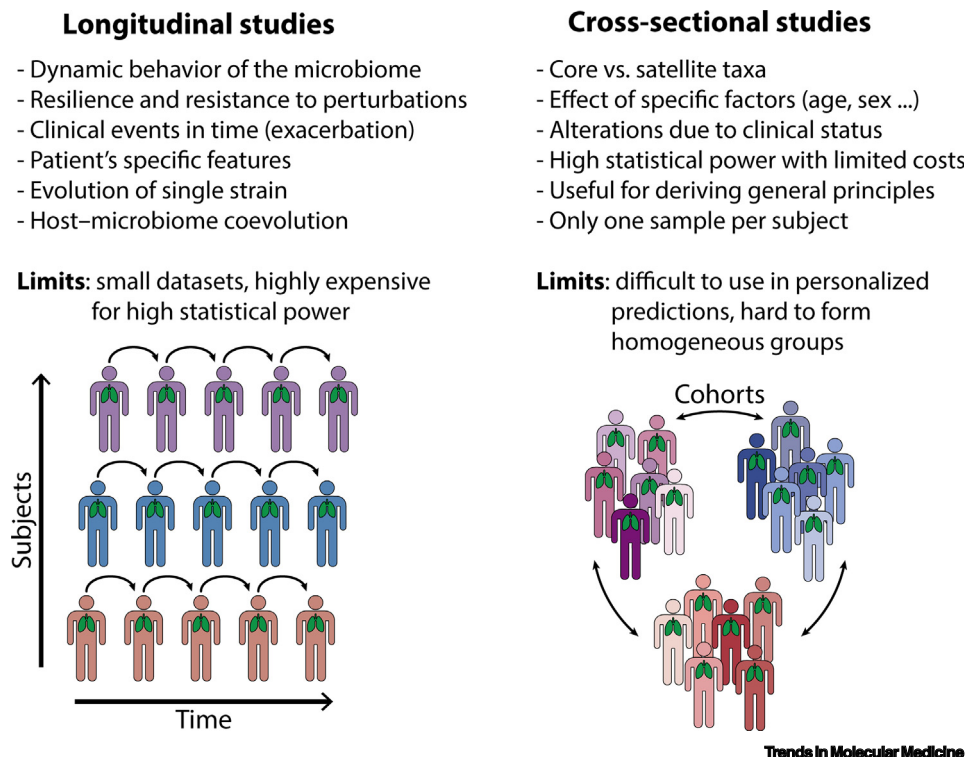

Longitudinal studies

- Dynamic behavior of the microbiome
- Resilience and resistance to perturbations
- Clinical events in time (exacerbation)
- Patient's specific features
- Evolution of single strain
- Host–microbiome coevolution

Limits: small datasets, highly expensive for high statistical power

Cross-sectional studies

- Core vs. satellite taxa
- Effect of specific factors (age, sex ...)
- Alterations due to clinical status
- High statistical power with limited costs
- Useful for deriving general principles
- Only one sample per subject

Limits: difficult to use in personalized predictions, hard to form homogeneous groups

Subjects

Time

Cohorts

Trends in Molecular Medicine

Figure 1. Cystic Fibrosis Microbiome Study Designs: Features and Insights from Longitudinal and Cross-Sectional Studies. The main advantages and limits are reported for each strategy. The left panel refers to longitudinal studies where subjects are sampled over time. Each patient is represented by a different color, sampling points are reported using arrows. On the right panel, a general scheme of cross-sectional studies shows cohorts of patients colored using the same colors in different shades. Shades are used to represent differences between patients belonging to the same cohort. Arrows indicate possible comparisons between patient cohorts.

Glossary

CFTR modulator therapy: recently-developed drugs targeting the causative defect in the CFTR protein, enhancing function of the ion channel. Such drugs are partially specific to CF mutation.

Cross-sectional studies: refers to a snapshot of a particular group of people who differ on one key characteristic at one specific point in time. They are used to describe what is happening at the present moment.

Culturomics: refers to the collection and analysis of microbial composition by the application of high-throughput culture conditions.

α-Diversity: taxonomic diversity within a given habitat or environment. Commonly described using richness (the number of taxa), Shannon, or Simpson diversity indices in microbiota studies. An additional diversity measure is evenness, which quantifies how numerically equal the taxonomic composition of the microbiota is.

Holobiont: an assembly of different species that, together, form an ecological unit. In the CF airway, this includes all of the microbes as well as the host.

Longitudinal studies: look at a group of people over an extended period. They involve taking multiple measures over a period of weeks, months, or even years.

Metabolomics: the global metabolite profiles in a system (cell, tissue, or organism) under a given set of conditions.

Metagenome: the collection of genomes and genes from the members of a microbiota.

Metaproteomics: the large-scale characterization of the entire protein complement of a defined sample at a given point in time.

Metatranscriptomics: the analysis of the suite of expressed RNAs of the corresponding meta-cDNAs by high-throughput sequencing.

Pulmonary exacerbations (PEx): refers to the acute worsening of symptoms, entailing an increase in respiratory symptoms (e.g., increased cough, sputum production, shortness of breath) accompanied by an acute decrease in lung function. These episodes are usually treated with antibiotics.

16S rRNA amplicon sequencing: relies on sequencing of the 16S ribosomal RNA (rRNA) gene as the

variation in microbiota composition, albeit generally at single time points and without providing a view of intrapatient variation. However, the relatively large sizes of many cross-sectional studies have facilitated the distinction of common CF respiratory microbes from uncommon ones (i.e., core and satellite taxa) [30]. These large sample sizes have allowed several investigators to identify important differences in average microbiota with varying age and disease state [31].

Antimicrobial Treatment and Exacerbation Events

Collectively, both longitudinal and cross-sectional CF respiratory microbiota studies have identified important correlations with patient characteristics, treatments, and outcomes (Table 2). While it is difficult to either predict or demonstrate causality (i.e., whether microbiology drives disease, or whether the diseased airway environment or its treatments drive microbiology), the relative dynamics of both microbiota and clinical characteristics from observational trials over time can help to suggest clinically relevant relationships. Many longitudinal studies have identified remarkable stability among CF respiratory microbiota even during clinical changes [27,32,33]. In some studies, PEx onset was associated with transient changes in abundance of a number of taxa, such as anaerobes [34] and *Gemella* [35]. However, studies have also shown that sputum microbiota within patients exhibited remarkable resilience, returning to their baseline states after treatment ceased [27,28,32,33,36,37]. Interestingly, there is little evidence that the density of canonical CF pathogen *Pseudomonas aeruginosa* changes appreciably during exacerbation [38], and the degree to which antibiotics reduce *P. aeruginosa* levels following treatment has not been correlated with therapeutic success [39]. Transient microbiological changes with antibiotic therapy are often most pronounced among aerobic taxa, most notably in *P. aeruginosa* [32,33], a common target of antibiotics in CF. However, these changes in sputum microbiota do not necessarily signify the mechanism of the clinical effects of these antibiotics

genetic marker to study bacterial phylogeny and taxonomy (who is there?).
Shotgun metagenomic sequencing: refers to massive parallel sequencing of DNA samples. It involves random fragmentation of DNA, sequencing of these fragments, followed by reconstruction and assembly of overlapping sequences into a continuous sequence. It provides insight into community biodiversity (who is there?) and function (what is microbiota capable of doing?).
Systems biology: refers to the systematic study of complex interactions in biological systems (i.e., between community microbes, and between the microbial community and the host) using integration models.

Table 2. Effects of Patient Characteristics on Airway Secretion Microbiota

Effector	Effect on microbiota
Age	Relatively sterile airways in infancy Gradual rise in total microbial abundance throughout childhood and early adulthood Transition from dominance by oral anaerobes to 'traditional' pathogens (*S. aureus*, *P. aeruginosa*), generally during childhood Decreased community diversity and dominance of traditional CF pathogens in adulthood
Pulmonary exacerbations (PEx)	Stability and resilience: few microbiota changes, often involving shifts to higher anaerobe abundances, preceding or during exacerbations. Where changes occur, community often rebounds afterwards to pre-exacerbation state Persistent communities (climax microbiomes) during periods of clinical stability Transient communities (attack microbiomes) associated with PEx
Antibiotic exposure	Stability and resilience: few changes with antibiotic therapy for PEx. Where changes occur, community often rebounds afterwards to pretreatment state Gradual decrease in microbial diversity over individuals' lifetimes correlates with cumulative antibiotic exposure load
Host immunity	Limited and contradictory data Decreased inflammation associated with increased α-diversity Increased inflammation associated with specific anaerobes, traditional pathogens, and increased absolute bacterial abundance
Genetic background	Limited data, more investigation needed Trends towards higher *P. aeruginosa* prevalence with F508del CFTR mutation F508del CFTR mutation associated with loss of airway bacterial diversity and conserved community composition
Disease state/Lung function decline	Stability over months and decades, less stability during periods of rapid clinical decline Decrease in α-diversity, increase in absolute and relative abundance of traditional pathogens with advancing disease Decreased diversity early in life associated with faster rate of decline

[36,40]. Thus, it is important to be conservative when assigning any change in microbial community constituency as causally related to therapeutic success or lung function decline.

Host and Therapeutic Factors

While stability and resilience are core features of the CF respiratory microbiome, both cross-sectional and long-term longitudinal studies have identified a number of host and therapeutic factors that are associated with differences (cross-sectional) and changes (longitudinal) in microbiota, many of which vary over time, not only within individuals, but also in CF populations, as care evolves (Table 2) [41]. Studies have demonstrated that, on average, CF respiratory communities are usually dominated by oral anaerobes early in life, after which **α-diversity** (diversity within sample; i.e., the number and/or evenness, and types of taxa within a local community) usually decreases due to increasing abundances (relative and absolute) of 'traditional' CF pathogens [42–45]. These changes are associated both with decreasing lung function and also increasing antibiotic exposure [30,46]. Treatment with CFTR modulators, which address the underlying defect in CF, was recently shown to be followed by an initial decrease in absolute and relative abundances of *P. aeruginosa* and with an accompanying increase in relative abundances of *Streptococcus* and *Prevotella* spp. These changes resulted in increased overall α-diversity, all of which accompanied an increase in lung function [47]. Interestingly, pre-exacerbation α-diversity and disease severity also appear to predict the degree of community perturbation at exacerbation [34,35], and decreased microbial diversity early in life has also been associated with a faster rate of lung function decline [48]. Such findings underscore the importance of systems-level approaches that more fully reflect the complexity of these microbiota. Additionally, agonistic and antagonistic interactions among members of the CF airway microbiota can occur, influencing community composition dynamics, as suggested by various reports and confirmed on some microbial species under *in vitro* conditions [49–53]. While host factors, including genetic background and immune responses, also play important roles in CF pathogenesis, their contribution in shaping respiratory microbiota is not well studied. CF genotype has not been associated with microbiota composition [54], although small sample sizes and diversity of CF genotypes may have limited this analysis. Similarly, while few studies have investigated relationships between host immune responses and the respiratory microbiota, inflammation has been found to be inversely associated with α-diversity in some studies [45].

Methodological Factors

Finally, there are a number of methodological factors to consider when assessing influences on any microbiota evaluation. For example, different methods for extraction [55,56] and sequencing of DNA [57] can yield different microbiota profiles, with effect sizes often similar to those of the biological factors being studied [58]. Respiratory sample type (sputa, swabs, bronchoalveolar lavage fluid, protected brush sampling, lung explant) can also influence calculated community structure, not only because they may reflect different airway locations, but also because different sampling methods tend to be used in the various life and disease stages of patients with CF (e.g., swabs are used most often in children, sputum tends to be produced at later stages, and lung explants usually reflect end-stage disease) [43,59]. In addition, some studies have reported microbial heterogeneity in different sites within the same lung [59,60], highlighting how difficult it can be to accurately sample the entire 'respiratory microbiota' at once. The heterogeneity found between studies examining microbiota dynamics during exacerbations and antibiotic treatments may be attributable in part to such variation in sampling approaches [27,54]. All of these methodological differences make it difficult to compare results across studies.

Therefore, while there are many host and therapeutic factors that influence the CF respiratory microbiome, methodological variation is equally important. In addition, the observational nature

of many studies, and the correlation between antibiotic burden, disease severity, and age in CF, make it difficult to definitively establish the causal relationships between clinical and microbiological characteristics. These concerns underscore the importance of using consistent approaches for sampling, processing, sequencing, and data analysis to allow for more rigorous evaluation of factors contributing to CF respiratory microbiota assembly and dynamics and to allow for investigation of causality. Harmonized approaches to addressing contamination, sample processing, and sequencing methodology will also help facilitate comparison across studies. Furthermore, the ecology of CF respiratory microbiology is changing as therapies evolve, which complicates the comparison of recent and past results even while providing an opportunity to better understand the microbial determinants of this disease [61], highlighting the importance of repeating these studies as treatments progress.

Many Singers, but Which Song(s)?

Most of the studies assessing CF respiratory microbiota utilize **16S rRNA amplicon sequencing** [33,34,37,62–65], limiting their ability to infer strain-level and genetically-conferred functional community characteristics [66,67]. Indeed, as different bacterial lineages may harbor largely different gene sets (and hence different functional capacities) [68], strain-level profiling may provide a more complete picture of microbiota dynamics in CF. Relevant bacterial species in CF, such as *P. aeruginosa* [69] and *Staphylococcus aureus* [70,71], possess large, flexible gene pools, including genes encoding antibiotic resistance, pathogenicity, environmental response, and metabolic flexibility, which may explain the adaptive nature of chronic infections [72]. Although the **metagenome** contributes greatly to interactions with the host [25,73], very few studies on CF metagenomes have been performed, involving a limited number of both patients [29,63,74,75] and specific metabolic functions [76]. By analyzing the abundance of specific genes, such studies found a homogeneous distribution of predicted bacterial community activities (e.g., specific metabolic pathways) across patients with similar pulmonary function, as indicated by forced expiratory volume in 1 second (FEV_1) [73], suggesting a relationship between microbiome function and clinical status. This finding is in line with other metagenomic studies in humans and other systems, suggesting that changing the taxonomic composition does not drastically alter the functional capacity of the microbiome (see [77] for a comprehensive list of studies). This interpretation is also in line with the finding that the functional capacities of human microbiomes tend to be more conserved than their taxonomic composition [77]. This concept has earned a catchphrase: 'the song not the singer', meaning that functional interaction patterns, rather than the taxa responsible for them, are the relevant factors in establishing a microbiome [77]. From this point of view, the airway microbiome in CF can be considered, as a whole, only partially dependent on the taxonomy of its members when performing its 'ecosystem services', as described for other microbiomes [77–79].

Predicting CF Patient Respiratory Microbiome Interactions

The studies that have investigated which factors influence, and are influenced by, CF respiratory microbiota have mainly been observational, as detailed earlier. To move beyond mere associations, and to investigate mechanisms by which the microbiota intersect with disease, requires a different approach. One promising framework for mechanistic studies is provided by **systems biology**, which involves the integration and interpretation of multiomics data, using concepts and methods from a wide array of disciplines, including microbiology, cell biology, biochemistry, chemistry, physics, mathematics, bioinformatics, and biostatistics [80,81].

Systems (Micro)Biology in CF

The goal of systems biology studies is to develop and validate models that accurately reflect, and predict, the behaviors of complex biological systems (i.e., a cell, an organism, or a community),

which may then be used to predict microbiome behavior in similar systems. For host–microbe interactions, this goal – the precise interpretation and prediction of phenotypic (e.g., health status or the composition of the microbiome) outcomes from currently available measures – is one of the greatest challenges in human microbiome analysis [82–85]. Nevertheless, systems biology approaches offer a promising direction in understanding the dynamics and pathophysiology of airway microbiomes in CF [25].

Understanding how microorganisms interact with their hosts is not a trivial task. Indeed, host-associated microbes, as in the CF lung airways, run the gamut from innocuous environmental
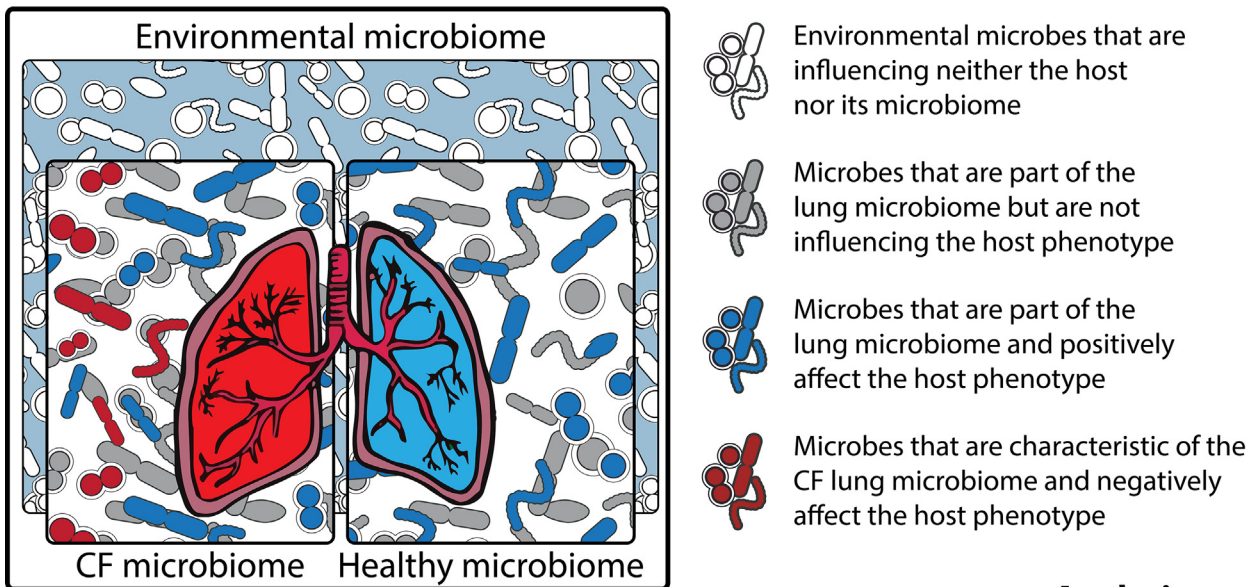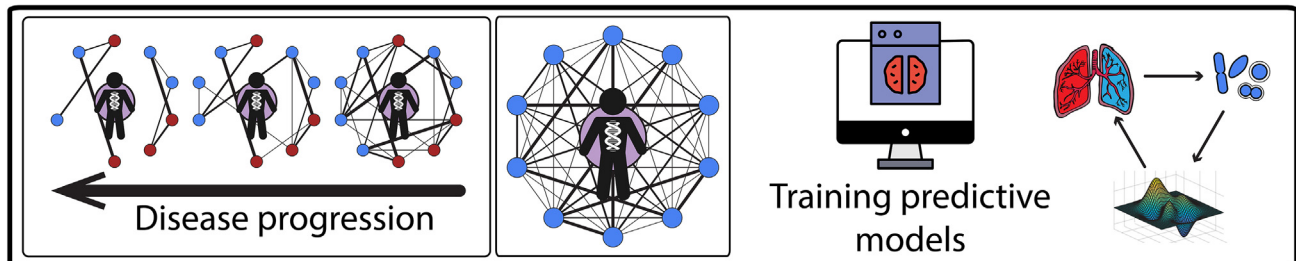
Figure 2. Towards a Prediction of the Cystic Fibrosis Lung Microbiome Dynamics. The host, and all of its microbial symbionts can be collectively defined as a single biological entity called 'the holobiont'. Upper panel: Microbes can coevolve with the host and positively affect its phenotype (blue) by forming complex community structures that prevent microbial dysbiosis. The airways of cystic fibrosis (CF) patients are also colonized by organisms that may still coevolve with the patient but negatively affect its phenotype (red), reducing the network of overall interactions. The presence of species that may not contribute directly to host fitness (gray) or that may be inconsistently acquired from the environment (white) further complicate this scenario. Such microbes are often sampled during metagenomic studies but do not provide information about the clinical condition of the host, as they do not directly alter its phenotype. Developing an accurate predictive model based on microbial assemblages implies the correct identification of both the 'blue' and the 'red' components of the microbiome. Lower panel: Reconstructing the network of interaction between microbes and the host is an essential step for discerning relevant and nonrelevant species in the context of CF disease; center: a healthy subject, left: a possible scenario of how the lung microbiome reacts to a worsening of CF disease, and right: after network reconstruction we can train predictive models in recursive ways (i.e., from microbiome data to the model and then back to the microbiome). Large-scale longitudinal studies or well-defined animal models will be needed for optimizing models useful for rapid diagnosis or to fine-tune clinical cares.

microbes, to microbes affecting their host (positively or negatively), to microbes intimately coevolved with their hosts [86] (Figure 2). From an ecological perspective, a host and all of its associated organisms (i.e., microbes affecting and/or coevolving with the host) can be considered as an integrated unit, a **holobiont**, all of which is investigated concurrently using a systems biology approach [86]. Furthermore, the environment also affects the composition of the holobiont in most cases, including in CF lungs. The genes within microbial communities can be transmitted vertically (from mother cells to offspring) or horizontally (between cells belonging to different genealogies) and are likely acquired from environments proximal to the CF airway. In support of this idea, the microbiota of the oral and nasal cavities both share taxonomic features with the lower airway microbiota, both in healthy children and those with CF [87,88]. It is important to note that environmental microbes external to the host are not interacting members of the holobiont, although they may act as reservoirs for microorganisms introduced into the system. In this context, analyzing the relationships between the respiratory microbiome and the host in CF can be difficult, underlining the need for reliable methods to discern between 'casual' interactions and physiologically-relevant host–microbiome interactions (Figure 2).

Applying (Meta-)Omics to the CF Microbiome

Classically, meta-omics data can be useful both as markers of disease processes and to give insight into which biological pathways, processes, or taxa differ between CF and control groups. [89]. Even if resultant findings cannot be used directly in clinical settings, such association is essential for developing mechanistic models that can be used for biomarker development or applied directly in clinical settings [90]. Machine learning approaches based on microbiome composition have been used in many diseases, including colorectal cancer [91,92], Crohn's disease [93], and nonalcoholic fatty liver disease [94]. However, the complexity of performing such studies has thus far limited the utility and generalizability of their predictive outcomes. For example, in one of the largest studies, He *et al.* [92] assessed the generalizability of diagnostic models based on gut microbiota data of 7009 individuals. Their results showed that predictive diagnostic models are reliable, but only at regional scale, and that geographical variation plays a significant role in shaping host-associated microbiota. Indeed, the high complexity (elevated number of taxa) and the strong influence of the environment (e.g., diet) involved in these microbiota strongly limit the applicability of the resulting models. By comparison, CF, which is characterized by relatively low taxonomic complexity, could be ideal for testing the reliability of machine learning-based diagnostic models. Thus, further studies are needed to approach CF disease from a 'multiomics' perspective, not only focusing on individual, isolated clinical aspects but integrating taxonomical, metabolic, and functional features of the airway microbiome with diverse clinical characteristics [90,95].

Ecological Modeling in CF Microbiome Predictions

Nevertheless, to properly address predictive mathematical models of CF–microbiome interactions, both large studies (cross-sectional and longitudinal) and proper *in vitro* and *in vivo* animal models are needed (Box 1). Indeed, while *in vitro* models have been used to investigate pathogen physiology and identify molecular-level interactions between cells [96–100], animal models allow for more in depth and nuanced investigation of ecological dynamics of CF microbiota and could provide relevant opportunities to experimentally validate predictions [101–103]. These could allow researchers to investigate functional interactions among members of the microbiome *in vivo* and in simplified simulated conditions (e.g., lung organoids) as well as the role of specific taxa in microbiome dynamics in health and in response to perturbation [104]. By taking into account the spatial heterogeneity and the temporal variability of a given district of the human body [105], recent modeling of microbial interactions has also increased our knowledge of CF disease pathogenesis [105,106]. However, as noted earlier, we do not yet understand the

Box 1. Models of CF Lung Disease

Type of model	Expectations
In vitro	Glass capillaries, growth substrates (e.g., mucin), biofilm conditions, including various microbial species and viruses [94–96] are used to define the molecular aspects and physiology of microbial species.
	Cellular models can be used to evaluate the molecular determinants of microbial (and viral) infectivity [97,98].
	Organoids offer the possibility to identify interactions between the microbiome, the host, and the immune system signaling [107].
In vivo	Animal models (e.g., mouse, ferret, pig, sheep) provide opportunities to experimentally manipulate the microbiome in the host and validate predictions [99–101].

microbial determinants of exacerbations or responses to antibiotic therapies; a microbial ecology-oriented perspective may allow therapies to shift the community to an alternative, and perhaps healthier, stable state, following treatment [107,108]. Future studies using patient-specific organoids may permit the development of personalized medicines and targeted therapies for opportunistic pulmonary infections [109].

The Microbiome as a Therapeutic Target

As stated previously, lung infection is a major contributor to morbidity and mortality among people with CF. For some traditional CF pathogens, timely and aggressive antibiotic treatment can eradicate infection in early stages, and continuous suppressive therapy is indicated if eradication is not successful [110]. In addition, antibiotic treatment is given at the time of PEx, and the individual role of antibiotics in recovery from exacerbation has been shown [111], highlighting the importance of infection in PEx pathogenesis. Currently, the standard of CF exacerbation care [112] is to select antibiotics targeting traditional CF pathogens, including *P. aeruginosa*, *S. aureus*, and others. For *P. aeruginosa*, antibiotic treatment for eradication can be successful [113], although treatment failures are common and are associated with worse prognosis [114].

From a microbiome perspective, current antimicrobial strategies can be perceived as focusing on only one side of a coin. Focus on *P. aeruginosa* or *S. aureus* may ignore either the pathological or healthy effects of 'nontraditional' CF organisms, whether direct or indirect. Specifically, our current strategies may either not be effective against undetected pathogens, or they may be adversely impacting beneficial organisms through off-target effects (see Clinician's Corner). The first notion – of treating PEx with additional antibiotics, selected according to patient's personal microbiome – is being evaluated in the first microbiome-based, interventional clinical trial: CFMATTERS (clinicaltrials.gov: NCT02526004; www.cfmatters.eu). This study aims to determine whether targeting nonclassical (e.g., anaerobic) species with antibiotics will improve CF outcomes.

Perhaps more likely is the second notion: that current strategies targeting classical pathogens inadvertently kill beneficial members of the CF microbiome, to the detriment of the holobiont (including the lung microbial ecosystem). It may be possible to tailor therapeutic interventions considering the entire microbiome, including the microbial taxa present, their abundances, and the nature of their interactions with each other and with the host. Deciphering the intimate interactions within the microbiome and the 'ecosystem service' it provides to the holobiont (the 'song' referred to previously [77]) could lead to identification of keystone functions and taxa and allow for the maintenance of a healthy respiratory microbiome [75]. Moreover, in a holobiont framework, considering the data already collected on the gut–lung axis [115], it may be reasonable to hypothesize that manipulation of the airway and gut microbiomes through dietary

episodic regimens, with the intention of targeting traditional CF-associated pathogens (*P. aeruginosa*, *S. aureus*, *B. cepacia* complex, etc.). However, these treatments also impact other members of the microbiota, often in patient-specific fashions, including anaerobic taxa. These 'off-target effects' may, in part, explain why a majority of patients benefit from antibiotic treatment despite failure to eradicate the classical 'pathogens', and why clinical and traditional microbiological outcomes do not always correlate. Nevertheless, the complexity of microbiome data suggests that our current therapeutic approach is simplistic and relatively uninformed. To develop rational antimicrobial interventions, we must understand not only which species are truly pathogenic or 'healthy', whether generally or only in specific contexts or patients, but also both the beneficial and negative effects of antibiotics on respiratory microbiomes.

intervention, probiotic cocktails, CFTR modulators, or others therapeutic interventions (like vitamin D supplementation, sodium chloride supplementation, or antioxidant vitamin intake), could synergize with antimicrobial treatment and may be used for designing personalized precision approaches for prevention as well as treatment of CF lung disease.

Concluding Remarks

Although a substantial number of CF respiratory microbiome studies have described changes related to PEx and to the use of antibiotics [32,33,37,40,64], the lack of robust predictive models has limited discovery of novel, ecology-minded interventions that could be more beneficial to patients. We are therefore not ready to rationally modify antibiotic regimens in a way that we can be certain will promote healthier individual microbiomes (see Clinician's Corner). Yet we can foresee microbiome-directed treatment as part of a new CF precision medicine era that will tailor therapies to individual patient characteristics, including high-resolution microbiological data, similar to tailoring **CFTR modulator therapy** according to CFTR genotype/theratype [116].

Optimizing study design and focus is integral in this effort and there are a number of considerations that, we believe, will facilitate development of such personalized treatments. The field of microbiome research has begun deemphasizing 16S rRNA amplicon sequencing to focus on **shotgun metagenomic sequencing**, as well as other multiomic approaches (like metatranscriptomics, **metabolomics**, and **metaproteomics**), that afford more complete and nuanced pictures of microbial communities [117]. These newer descriptions may allow for selection of antibiotic therapies based on the composition and relative abundance of antibiotic resistance genes within the respiratory microbiome. As mentioned previously, CF respiratory microbiota vary considerably between individuals, so designing longitudinal studies with frequent sampling together with complementary cross-sectional studies with larger sample sizes to fully account for this interpatient variability will be necessary to formulate such generalizable models of disease progression and response to therapy. Finally, the field of CF clinical care is rapidly evolving, and new drugs (e.g., CFTR modulators) are predicted to improve lung function and clinical outcomes. Proactive study designs that collect samples before and after starting such novel therapies provide a unique opportunity to examine causality and should be leveraged if possible. Finally, more complete knowledge of the CF microbiome will permit identification and possibly culture (by **culturomics** approach) of still unknown bacteria [118], widening the diagnostic capabilities of CF clinical microbiology.

A substantial number of CF respiratory microbiome studies have described changes related to PEx and to the use of antibiotics. However, we still lack strong functional and cause–effect data to build predictive models to foresee ecology-minded interventions that could be beneficial to patients. The more relevant questions to address this main goal are listed (see Outstanding Questions).

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