State of the Art

THE AIRWAY MICROBIOTA IN EARLY CYSTIC FIBROSIS LUNG DISEASE¹

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SUMMARY

Infection plays a critical role in the pathogenesis of cystic fibrosis (CF) lung disease. Over the past two decades the application of molecular and extended culture-based techniques to microbial analysis has changed our understanding of the lungs in both health and disease. CF lung disease is a polymicrobial disorder, with obligate and facultative anaerobes

recovered alongside traditional pathogens in varying proportions, with some differences observed to correlate with disease stage. While healthy lungs are not sterile, differences between the lower airway microbiota of individuals with CF and disease-controls are already apparent in childhood. Understanding the evolution of the CF airway microbiota, and its relationship with clinical treatments and outcome at each disease stage, will improve our understanding of the pathogenesis of CF lung disease and potentially inform clinical management. This review summarizes current knowledge of the early development of the respiratory microbiota in healthy children and then discusses what is known about the airway microbiota in individuals with CF, including how it evolves over time and where future research priorities lie.

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ABBREVIATIONS IN THE TEXT

16S rRNA	16S ribosomal RNA
ARI	acute respiratory infection
BAL	bronchoalveolar lavage
BLAST	basic local alignment search tool
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CI	confidence interval
FEV ₁	forced expiratory volume in one second
HRV	human rhinovirus

IQR	interquartile range
OTU	operational taxonomic unit
qPCR	quantitative PCR
RSV	respiratory syncytial virus
SDI	Shannon Diversity Index
T-RFLP	terminal restriction fragment length polymorphism

Introduction

Infection plays a critical role in the pathogenesis and progression of cystic fibrosis (CF) lung disease. In infants with CF, pathogenic organisms in the lower airways are associated with elevated inflammatory markers¹⁻³, earlier onset of structural lung disease^{3,4}, and poorer lung function outcomes^{5,6}. In older individuals, persistent infection with multi-drug resistant bacteria is associated with clinical decline^{7,8}. Historically, CF airway microbiology was thought to follow a predictable trajectory of infection with a small range of “typical” pathogens, commencing with *Staphylococcus aureus* and *Haemophilus influenzae* in early childhood and progressing to persistent infection with gram-negative organisms, most notably *Pseudomonas aeruginosa* and, to a lesser extent *Burkholderia cepacia complex* and *Stenotrophomonas maltophilia*^{8,9}. However, neutrophilic inflammation, itself strongly associated with the early development of bronchiectasis, has also been detected in the lower airways of infants and young children in the absence of bacteria using classical culture methodologies^{3,4}. While such observations may be explained by limited sampling of multiple lung lobes and recent antibiotic treatments^{10,11}, they also raise the possibility of infection

with either non-cultivable organisms or with those requiring specialised media and/or culture conditions.

Over the past two decades, molecular techniques, including those targeting the variable regions of the bacterial 16S ribosomal RNA (16S rRNA) gene, next generation sequencing and metagenomic analyses, have changed our understanding of the human microbiome, including the respiratory microbiome, in health and disease. At each body habitat, the microbiome is believed to have complex relationships, both in the dynamics of its core and satellite organisms, and with its host, where it has potential interactions with the developing immune system and regulation of the inflammatory response^{12,13}.

CF lung disease is now understood to be polymicrobial. Numerous studies, using both extended microbiological culture and molecular analysis techniques, have reported the frequent presence of obligate and facultative anaerobes in CF airway specimens, and there are now cross-sectional descriptions of the airway microbiota at various stages of CF lung disease. What remains to be determined however, is the evolution of the lower airway microbiota in CF and its role in disease pathogenesis and progression. Such exploratory studies are needed to begin from early infancy since infants with CF may develop structural lung disease as young as 3-months of age following their diagnosis by newborn screening⁴. Unfortunately, repeated longitudinal examination of the lower airway microbiota in non-expectorating infants and young children is limited by requiring bronchoscopy under general anesthesia to obtain appropriate samples.

Understanding the composition and structure of the airway microbiota in healthy infants and children is also critical for interpreting changes that occur in disease. This review summarizes current knowledge of the evolution of the respiratory microbiota from birth through early childhood. It then discusses the perturbations in microbial community composition and structure that develop in CF lung disease, focussing on infancy and early childhood as a critical period for both disease pathogenesis and maturation of the immune response, and a potential opportunity for therapeutic intervention.

Methods

Studies were identified by an electronic search of the PubMed and Medline databases. The search strategy combined the following key words: microbiota or metagenome and cystic fibrosis, respiratory system, respiratory system abnormalities, lung or lung diseases in titles and abstracts; and was limited to articles published in English prior to 31st January 2017. The reference lists of articles identified by this search strategy were also searched and relevant articles retrieved.

The airway microbiome

In health, the lower airways are not sterile, but rather also contain a diverse microbial environment¹⁴⁻¹⁶. In disease however, microbial community structure appears to be altered, as either a primary or a secondary phenomenon, with consequent perturbations in the host-microbiome interactions. In chronic respiratory disease, it has been suggested that instability or “dysbiosis” in the airway microbiota is antecedent to pulmonary exacerbation, and that such changes in the host-microbiome relationship may further contribute to developing lung disease¹⁵⁻¹⁸.

The airway microbiota in infancy and early childhood

The initial neonatal microbiota reflects the mode of delivery, but is undifferentiated across multiple body habitats^{19,20}. Whilst the lower airway microbiota of healthy infants has not been described, numerous studies highlight the dynamic nature of the nasopharyngeal microbiota. The latter appears to progress from an environmentally or maternally transmitted profile at 24-hours of age, to *S. aureus* predominance at 1-week of age²⁰. Over the next 1-2 years, infants develop distinct nasopharyngeal microbial profiles. These are influenced by gestational age, hospitalization, breastfeeding, diet, antibiotic use, immunization status, seasonal and local environmental factors, including cigarette smoke exposure, child care attendance, family dynamics, and complex bi-directional relationships with respiratory viruses²¹⁻²⁵. Typically, the nasopharyngeal microbiota of infants are dominated by one or two members of the six most commonly encountered genera, *Staphylococcus*, *Dolosigranulum*, *Corynebacterium*, *Haemophilus*, *Streptococcus* and *Moraxella*, with the *Staphylococcus*-dominance during early infancy decreasing after the first few months of life.

Associations between the airway microbiota and respiratory health in childhood

The nasopharyngeal microbiota in infancy and early childhood may be associated with later respiratory health. Analysis of 1021 serial nasal swabs from 234 infants found that the microbial profile dominance by *Haemophilus*, *Streptococcus* and *Moraxella* were associated with increased risk of acute respiratory infection (ARI; odds ratio (95% confidence interval (CI)): 12 (4.2-35), 1.2 (1.1-2.5) and 2.2 (1.5-3.2) respectively), whilst in a subset of 160

infants sampled prior to their first respiratory infection, high (>20%) relative abundance of *Streptococcus* in the nasopharyngeal microbiota at age 7-weeks or younger was associated with increased risk of wheeze at 5-years of age (odds ratio 3.8 (1.3-12))²⁶. Similarly, a longitudinal study of 60 healthy children found that early presence and high abundance of *Moraxella* and *Corynebacterium/Dolosigranulum* were associated with microbial stability and reduced ARI, whilst *Haemophilus* and *Streptococcus* were associated with less stable microbial profiles²⁷. The 3-year follow up of 265 children enrolled in the COPSAC2000 birth cohort reported *S. pneumoniae*, *H. influenzae* and/or *M. catarrhalis* in hypopharyngeal aspirates at age 4-weeks was associated with increased risk of lower respiratory tract infection (adjusted incidence rate ratio 1.79 (95%CI 1.29-2.48))²⁸, and in a prospective study of 40 children aged 49-84 months, decreased upper airway microbial diversity was associated with increased frequency of upper respiratory tract infection²⁹. Taken together, these data suggest that *Moraxella*, *Streptococcus* and *Haemophilus* genera are more common in the upper airways of infants and children experiencing an increased frequency and severity of ARIs. Furthermore, abundance of these organisms might identify those at risk of such infections^{26,30}.

Virus-bacteria interactions

There is increasing evidence of a bi-directional relationship between respiratory viruses and the airway bacterial microbiota. In 99 infants with respiratory syncytial virus (RSV) infection (median age 22-weeks, interquartile range (IQR) 13-27 weeks), the abundance of *Streptococcus*, *Haemophilus* and *Moraxella* was increased compared with healthy controls (n=33; median age 5-weeks, IQR 2-9 weeks)³¹. Two additional studies comparing infants

with RSV and human rhinovirus (HRV) infection suggest that individual respiratory viruses may have particular effects on the host microbial profile. In the first study, infants hospitalized with RSV (n=580) and HRV bronchiolitis (n=100) demonstrated increased abundance of *Firmicutes* (*Streptococcus*) and *Proteobacteria* (*Moraxella* and *Haemophilus*) respectively³², whilst in the second study, *Staphylococcus* was increased in infants with RSV (n=83) and alpha-diversity (within samples) was higher in those with HRV infection (n=52)³³. Conversely, a longitudinal study of 559 nasopharyngeal samples from 32 infants in the first-year of life, demonstrated that symptomatic HRV infection was associated with decreased alpha-diversity and increased bacterial density (SDI 0.9 ± 0.7 vs 1.3 ± 0.9 ; mean polymerase chain reaction concentration $44 \pm 35 \text{ ng}/\mu\text{L}$ vs $29 \pm 29 \text{ ng}/\mu\text{L}$ respectively), while frequent HRV infection was associated with reduced alpha-diversity at the completion of the study (Shannon Diversity Index (SDI) coefficient -0.5 (95% CI -1.0-0.01))³⁴. Lastly, in 106 infants with RSV infection compared with 26 healthy controls, dominance of *Streptococcus* or *Haemophilus* in the nasopharyngeal microbiota was positively associated, and *Staphylococcus*-dominance inversely associated, with hospitalization³⁵. Expression of toll-like receptor genes and of pathways involved in neutrophil and macrophage activation was increased in infants with RSV infection and *Streptococcus*- or *Haemophilus*-dominance. Altogether, these studies suggest that the local bacterial microbiota may influence both the systemic immune response to and clinical manifestations of, bacterial-viral co-infections.

Sampling methods

A recent study focussing on children with chronic respiratory symptoms secondary to either protracted bacterial bronchitis or chronic suppurative lung disease, highlighted the

importance of lower airway samples in defining the respiratory microbiota and its contribution to health and disease³⁶. When comparing 16S rRNA gene sequencing results from oropharyngeal, nasopharyngeal and bronchoalveolar lavage (BAL) specimens from 68 children with chronic lung disease and 10 'disease controls' undergoing investigation for congenital stridor (median age 2.2-years across all groups), the microbial composition of the combined upper airway samples was at least 50% similar to the paired BAL samples in only 69% of subjects.

In summary, the nasopharyngeal microbiota evolves during infancy, with the development of profiles dominated by one or two of six common genera (*Staphylococcus*, *Corynebacterium*, *Dolosigranulum*, *Streptococcus*, *Haemophilus* and *Moraxella*). While dominance of the latter three genera may predispose to ARIs, the role of bacterial-viral co-infection in healthy infants remains to be clarified. Respiratory viruses likely influence the composition of the local bacterial community, and the local microbiota may modulate the clinical manifestations of viral infections. Upper airway samples are unlikely to be completely representative of the lower airway microbiota, and as such, current understanding of the healthy lower airway microbial ecosystem and of the changes that occur in disease is limited.

The airway microbiota in infants and young children with cystic fibrosis (Tables 1, 2 and 3)

Numerous cross-sectional studies of sputum samples from adults with CF, using both extended microbiological culture and culture-independent techniques, highlight both the presence of obligate and facultative anaerobes, and the extent of microbial diversity within

the CF lung. While the organization and structure of individuals' airway microbiota are unique, particular genera, including *Pseudomonas*, *Prevotella* and *Streptococcus*, are consistently present in adults with CF. Initial metagenomic analyses suggest that despite taxonomic differences, there are functional similarities between the airway microbiota of individuals with CF³⁷, and that bacteriophages in the CF lung may share similar metabolic profiles³⁸.

The lower airway microbiota in children with CF

Differences between the microbiota in the lower airways of healthy children and those with CF are evident from early childhood. Examination of BAL fluid from 13 clinically stable children with CF (mean age 3.95-years, range 1.1-8.1 years) demonstrated a less rich and less even microbial community than observed in the BAL fluid from nine disease-controls (mean age 3.78-years, range 0.9-9.75 years)³⁹. A *Corynebacterium* species, a *Streptococcus* species and an unclassified bacterium were detected in CF samples only, while *Prevotella veroralis*, and unclassified *Pseudomonas*, *Acintomyces*, *Selenomonas*, *Leptotrichia* and TM7 species were recovered from control airways only. In the CF airway, the presence of *P. aeruginosa* and *S. pneumoniae* in microbiological culture appeared to influence microbial community structure whereby each was associated with reduced species diversity. These observations support an earlier study where distinct differences in BAL fluid microbial communities were reported in 28 children with CF (female median age 9.1-years, male median age 8.5-years, range 0.9-20.9 years) and 14 disease-controls (female median age 3.5-years, male median age 2.7-years, range 0.7-17.7 years)⁴⁰. Almost two-thirds (61%) of the 16S rRNA gene libraries from CF samples were dominated (>85% of total sequences) by

established CF pathogens, including 50% of samples dominated by a single pathogen. In contrast, the control samples were generally more complex, and only four contained five or fewer species. Cross-sectional analysis of respiratory specimens from 56 children aged 6-months to 18-years (BAL or sputum) and 68 adults (sputum only) with CF, bronchiectasis and persistent bacterial bronchitis, suggest that children with these chronic suppurative lung disorders may share a common core microbiota early in their disease course, with divergence according to disease and possibly its treatment occurring before adulthood⁴¹.

Further cross-sectional studies suggest associations between microbial diversity and age, cystic fibrosis transmembrane conductance regulator (CFTR) genotype and bile aspiration⁴²⁻⁴⁶. A comparison of 'deep' throat swabs from 26 children with sputum specimens from 37 adults found that the microbiota of older patients possessed less richness, evenness and diversity than that of younger patients, and that increased age was positively associated with the presence of *S. maltophilia* and *P. aeruginosa* and negatively associated with *H. influenzae*⁴². These results though need to be interpreted with caution as upper airway specimens were being compared with sputum from the lower airways^{36,47}.

Sputum samples from 76 children (median age 12-years, range 4-17 years) and 193 adults (median age 29-years, range 18-64 years) with CF revealed five core genera, *Streptococcus*, *Prevotella*, *Rothia*, *Veillonella* and *Acintomyces*⁴⁸. These genera are not considered traditional CF pathogens and were seldom or never the dominant genus. *Neisseria*, *Haemophilus* and *Gemella* were unique core genera in pediatric samples only, while *Pseudomonas* was unique to the adult core microbiota. In older subjects, decreased

diversity and lung function coincided with an increased proportion of sputum microbial communities dominated by *Pseudomonas* or *Burkholderia* and a decreased proportion of sputum samples either dominated by *Streptococcus* or lacking a dominant organism.

Differences between the upper and lower airways in relation to clinical status in the CF pediatric population have been explored in a single study involving 61 samples of saliva, oropharyngeal swabs, and induced and spontaneously expectorated sputum from 16 children and young adults, aged 8-21 years, with mild CF lung disease⁴⁹. Greater variation in microbial community composition was documented between, rather than within, subjects. In addition, *Pseudomonas* and *Staphylococcus* were detected in higher relative abundance in lower airway samples compared with those from the upper airway, although analyses did not adjust for subject age. Divergence between upper and lower airway microbial community structures was observed and associated with increased sputum neutrophil elastase, but not pulmonary function⁴⁹. However, the latter observation may have been influenced by the small sample size and that participants had mild lung disease. Interestingly, in this study the previously identified core pediatric microbiota genera⁴⁸ were either not associated or had a negative association with sputum inflammatory markers.

The airway microbiota at different disease stages

Culture-independent analyses of lung tissue specimens obtained at the time of transplant demonstrate a relatively homogenous population of typical CF pathogens, with the dominant organisms reflecting those found in contemporaneous sputum cultures^{47,50}. In a comparison of matched lung, sputum and upper airway samples, the same dominant

organism was recovered from lung and sputum, albeit with increased microbial diversity in the latter, whilst the microbiota of the upper airways was discordant⁴⁷. In contrast, molecular analysis of lung tissue specimens from a 3-year-old child with CF, obtained at lobectomy for severe localized disease, demonstrated a diverse, heterogeneous bacterial population, which correlated poorly with the species identified on conventional culture of both BAL fluid and oropharyngeal swabs⁵¹, suggesting that microbial diversity persists even in severely affected children and raising more questions regarding the “optimal” sample type for identifying airway microbiota in early CF lung disease.

Further studies of adults with CF suggest an association between microbial diversity in the lower airway, clinical stability and higher pulmonary function^{48,52}. A comparison in adults of 22 stable outpatients and 13 inpatients experiencing an acute exacerbation (median age in both groups: 27.5-years; median forced expiratory volume in one second (FEV₁): 56% and 27% predicted in outpatient and inpatient groups respectively), found that bacterial diversity was significantly higher, and the relative abundance of *Streptococcus*, *Gemella* and *Haemophilus* genera was increased in the outpatient group, although adjustment was not made for current or recent antibiotic exposure⁵³. In a terminal restriction fragment length polymorphism (T-RFLP) analysis of monthly sputum samples, collected over a 12-month period from 14 adults (mean FEV₁: 49.1±17% predicted), pulmonary function was related positively to the number of species per sample, and negatively to the rank abundance slope and the relative abundance of *P. aeruginosa*. The relative abundance of *P. aeruginosa* was negatively associated with species number, rank abundance slope and community stability over time⁵⁴. The contribution of long-term therapies, including antibiotics and

corticosteroids, to microbial diversity is still to be determined, although lifetime exposure to these agents is potentially higher in individuals with more severe pulmonary disease.

The airway microbiota during pulmonary exacerbations

A microbial signature marking the onset or resolution of pulmonary exacerbations in CF has not been identified. Multiple analyses of sputum samples obtained at each stage of the “exacerbation cycle” (baseline, onset, treatment and recovery) found no consistent changes in microbial community structure, and indeed, highlight the resilience of the CF airway microbiota⁵⁵⁻⁶² (Supplement Table). Microbial community composition varied between individuals and across sample cohorts, and changes were often observed following the introduction of antibiotics, however community structure consistently returned to baseline during the recovery period.

The development of airway microbiota in CF

The evolution of the CF airway microbiota over time is poorly understood. While longitudinal studies of lower airway specimens from the same individuals help minimize the confounding impact of genetic and local environmental factors and maximize opportunities for causal inferences, these studies have focused on adults with established lung disease^{42,54,63-66} (Supplement Table). In 126 sputum samples from six age-matched adults collected over a period of 8-9 years, those with progressive lung disease had decreased microbial diversity in association with increasing age and decreasing pulmonary function⁶⁵. However, after adjustment for antibiotic load, these changes were not statistically significant, suggesting that cumulative antibiotic exposure may be associated with changes

in microbial community composition. In contrast, patients with stable lung disease maintained their microbial diversity. In both groups, community structure was dynamic, with changes in the occurrence and relative abundance of operational taxonomic units (OTUs), but total bacterial density remained unchanged over time. There were also differences in community composition between the two groups, and in particular, *Staphylococcus* was found in 67% and 6% of samples from those with progressive and stable lung disease respectively⁶⁵. In adults, progression of the airway microbiota over time appears to vary substantially between individuals. In monthly T-RFLP analysis of sputum samples from 14 adults, the group mean change in bacterial composition was 35.5%±18.2% over the 12-month study period, with individuals' changes ranging from 4.3% to 70.1%. There was no relationship between community variability and richness⁵⁴.

Longitudinal studies of infants and young children with CF have predominantly explored the upper airway microbiota (Table 2). This is understandable given the difficulties in collecting lower airway samples from CF patients who are too young to expectorate, the inaccuracy and inconvenience of induced sputum specimens in this age group⁶⁷ and infeasibility of performing frequent BAL procedures¹⁻⁴. Differences are apparent in the nasopharyngeal microbiota of subjects with CF and healthy controls from early infancy, prior to exposure to antibiotics. Two recent prospective cohort studies have described an increased relative abundance of *Staphylococcus* early in life^{68,69}. The first was a longitudinal study of 324 nasopharyngeal samples collected monthly for the first 6-months of life from 20 Dutch CF infants identified by a newborn screening program⁶⁸. Quantitative polymerase chain reaction (qPCR) assays amplified the 16S rRNA gene, followed by sequencing the V5-V7 hypervariable region. Different OTUs were confirmed at species level using the Basic Local

Alignment Search Tool (BLAST) algorithm and for *Streptococcus* OTUs by culture, *lytA* qPCR and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *S. aureus*, *S. mitis*, *Corynebacterium accolens* and gram-negative bacilli were more abundant than found in nasopharyngeal samples from age-matched healthy controls where *Moraxella* species, other *Corynebacteria* species and *H. influenzae* were more abundant⁶⁸. Antibiotic use in CF infants was associated with decreased total bacterial density, increased detection of gram-negative bacilli, including *Burkholderia*, and a reduction in *Moraxella*, *Corynebacteria* and *Dolosigranulum* genera. These latter genera in healthy infants are associated with a more stable nasopharyngeal microbiota and fewer respiratory illnesses^{26,27}.

The second study reported on 461 nasal swab samples collected fortnightly from ages 2 to 12-months in 30 infants diagnosed following newborn screening in Switzerland and compared these with 872 samples from healthy controls⁶⁹. Significant differences in nasal microbiota, independent of exposure to antibiotics, were observed between CF and healthy infants, with the increased relative abundance of *Staphylococcae* and reduced relative abundance of *Corynebacteriaceae* and *Pasterellaceae* as the major contributors. Surprisingly, while antibiotic treatment was associated with a decrease in *S. aureus* in CF infants, it was also accompanied by increased microbial diversity and richness, including increased relative abundance of coagulase-negative *Staphylococci*. The authors speculated that this may have resulted from transient colonization by organisms occupying an ecological niche made vacant by antibiotic treatment, or alternatively, for technical reasons

where the compositional and relative nature of 16S rRNA sequencing means loss of dominant organisms leads to increased relative abundance in the remaining microbiota⁷⁰.

Finally, 3-monthly analysis of the oropharyngeal and gastrointestinal microbiota of seven infants with CF, aged 1 to 9-21 months, revealed a distinct, but overlapping core microbiota dominated by *Streptococcus* and *Veillonella*⁷¹. Bacterial diversity increased in both sites over time, occurring more rapidly in the upper airway than in the gastrointestinal tract. The upper airway microbiota was dominated by *Streptococcus*, *Veillonella* and *Prevotella*, and while the microbiota of each habitat was different, there were 16 shared genera, 14 of which increased in parallel. Changes in diet were also associated with altered respiratory microbiota, suggesting that nutritional factors and gastrointestinal microbiota may influence respiratory microbial development in CF infants.

Three longitudinal studies have explored the development of the lower airway microbiota of infants with CF. Analysis of 11 BAL samples from eight infants, including repeated samples from three infants, obtained at median ages of 6.8 and 12.4-months (range 5.7-11.8 months and 11.9-13.2 months) respectively, found that *Streptococcus* was the most abundant genus (relative abundance 31.7%), and along with *Burkholderia*, *Prevotella*, *Haemophilus*, *Veillonella*, *Gemella*, *Neisseria* and *Methylobacterium*, was common to all eight initial samples⁷². Two infants with repeated samples demonstrated substantial interval change in their microbiota (Morisita-Horn beta-diversity index ≤ 0.38), and while there was a positive association between *Streptococcus* relative abundance and FEV_{0.5}, there was no association between alpha-diversity or bacterial load and BAL neutrophilia. Analysis of 25

nasopharyngeal, oropharyngeal and BAL sample sets from 17 infants, obtained in early and late infancy (median age (range): 4.6 (3-8) and 12.4 (9-13) months respectively) demonstrated variable intra-individual concordance between both nasopharyngeal and BAL and oropharyngeal and BAL microbiota, and in serial BAL samples⁷³. A larger study of 95 BAL samples from 47 infants aged 1.2-78.3 months, including longitudinal samples from 27 subjects (13 samples obtained before age 6-months), demonstrated increased lower airway inflammation in the setting of decreased alpha-diversity and dominant pro-inflammatory genera⁷⁴. Typical CF pathogens, when present, were often dominant. In this cohort, the beta-diversity (between samples) of serial samples from individual subjects was comparable to that of samples from different subjects, indicating that there are substantial changes in individuals' lower airway microbiota during infancy and early childhood. There was no relationship between the early BAL microbiota and FEV₁ at 6-years, although more sensitive measures of lung disease and further longitudinal follow up would further define the relationship between the lower airway microbiota and the earliest pathogenesis of CF lung disease.

Limitations

Comparing studies of the airway microbiota is limited, not only by differences in specimen type (i.e. oropharyngeal, nasopharyngeal, sputum, BAL, lung tissue), but also by differences in laboratory methods. The latter include using various DNA extraction protocols for handling samples with low bacterial biomass^{75,76} and adopting different statistical analysis tools. Failure to estimate total bacterial density is commonplace and compromises measures of relative abundance and inferences on airway microbiota composition, while

inconsistent definitions of common terms, such as “dominance” add to these difficulties⁷⁷. Clinical interpretation may be further confounded by differences in patient age, clinical and infection status (for example, intercurrent viral illness) and medication exposure, including long-term treatment or prophylactic antibiotics and potentially CFTR modulators. Nonetheless, these studies suggest substantial differences in the structure and composition of the airway microbiota of young children with CF, compared to both healthy children and to adults with CF.

Lessons learned and directions for future study

In infancy and early childhood, the nasopharyngeal microbiota is typically dynamic, with environmental factors, including viral infection, likely contributing to change over time. Particular community structures, including dominance of *Streptococcus*, *Haemophilus* and *Moraxella*, may predispose to ARIs. The lower airway microbiota of healthy infants has not been described, and whilst tempting, it is difficult to infer its composition and structure from analyses of the upper airways.

Existing studies of very early CF lung disease focus predominantly on the upper airways, where microbial community composition and structure differs between infants with CF and healthy age-matched controls, even prior to the introduction of antibiotics. Substantial changes in individuals’ lower airway microbiota over time have been described, but the association with clinical outcomes remains uncertain⁷⁴. However, by adulthood the sputum microbiota of those with advanced CF lung disease is less diverse than that of those with

milder disease, and indeed, in end-stage CF, the lower airway microbiota is composed almost entirely of 1-2 well-recognized pathogens.

Further longitudinal studies, where individuals serve as their own controls, combined with improved descriptions and understanding of the ontogeny of the healthy lung microbiome during infancy and early childhood are required to improve our understanding how airway microbiota contribute to the pathogenesis of CF lung disease. Future research will need to focus upon correlating lower airway microbial profiles that also include viruses and fungi with markers of disease severity in early childhood. This includes infant and pre-school lung function, airway inflammatory markers, computed-tomography evidence of structural lung disease, frequency of exacerbations, and nutritional and growth parameters. The relationship between airway microbiota and established CF therapies, such as prophylactic and treatment antibiotics, inhaled mucolytics and corticosteroids, nutritional support and CFTR modifiers also needs exploring. In the longer term, intervention trials, with, for example, targeted antibiotics, and pre- or pro-biotics, are required to identify if the lower airway microbiome can be intentionally manipulated to provide a therapeutic benefit.

This review summarizes current knowledge of the developing airway microbiota during infancy and childhood. In CF, the microbiota changes with disease status, however the interplay between this dynamic community and the early development of structural and functional lung disease remains uncertain. Future research is required to identify pathogenic and protective factors to help direct therapy.

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TABLE 1: CROSS-SECTIONAL STUDIES OF THE RESPIRATORY MICROBIOTA OF CHILDREN WITH CYSTIC FIBROSIS

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
Harris <i>et al</i> 2007, United States ⁴⁰	Subjects: 28 Age (males), median (range): 8.5 (0.9-20.9) years Age (females), median (range): 9.1 (1.0-20.5) years Male 47%. P.Phe508del homozygous: 50%. Disease controls ⁱ : 14 Age (males), median (range): 2.7 (0.7-17.7) years Age (females), median (range): 3.5 (0.5-9.1) years	BAL fluid	T-RFLP	To test the hypothesis that organisms not routinely identified by culture occur in the CF airway and may contribute to disease.	121 bacterial species identified (65 in CF, 33 in both groups). CF libraries: 61% dominated ⁱⁱ by CF pathogens (<i>S.aureus</i> , <i>S. maltophilia</i> , <i>P. aeruginosa</i> , <i>S. mitis</i> group, <i>H. influenzae</i> , <i>A. xylosoxidans</i> , <i>M. abscessus</i>). ~50% contained 1 dominant pathogen. 25% did not contain typical CF pathogens and had higher relative abundance of anaerobes. 39% contained bacteria not routinely identified by culture. Potential novel pathogens identified in 4 specimens (<i>Prevotella denticola</i> , a <i>Lysobacter</i> sp., members of the <i>Rickettsiales</i>). Limitations to interpretation: CF and disease control subjects not age-matched; wide age range of included subjects, spanning infancy to

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					adulthood.
Klepac-Ceraj <i>et al</i> 2010, United States ⁴⁵	Subjects: 45 Age, mean (range): 8.6 (2-16) years. FEV ₁ % predicted (mean): 97.6	Oropharyngeal swabs	High-density 16S rRNA gene microarray (PhyloChip)	Primary: to assess the influence of age, CFTR genotype, antibiotic therapy and <i>P. aeruginosa</i> on respiratory tract bacterial community composition. Secondary: to explore potential interactions among these different parameters on community composition and clinical disease markers.	2051 taxa from 43 phyla. Three dominant phyla: <i>Firmicutes</i> , <i>Proteobacteria</i> , <i>Bacteroidetes</i> . Range in bacterial diversity: 206-1329 species per person. Inverse relationship between age and richness, evenness and diversity. Decreased richness and increased beta-diversity in samples with <i>P. aeruginosa</i> . Lower beta-diversity in subjects with at least one P.Phe508del allele. Limitations to interpretation: wide age range; cross-sectional design with small number difficult to discern influence of antibiotic exposure and <i>P. aeruginosa</i> .
Guss <i>et al</i> 2011, United States ⁷⁸	Subjects: 4 Samples: 4 (pyrosequencing, n=3)	Sputum	16S rRNA clone libraries and pyrosequencing (V1- V2 variable regions) Microbiological culture (directed by 16S rRNA sequencing results)	To examine the microbial communities present in CF sputum samples using a combination of techniques.	Diverse microbial communities detected by all modalities. 16S rRNA libraries: 38 OTUs, representing 22 genera from 6 phyla. Most diversity within the <i>Bacteroidetes</i> ,

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					<p><i>Fusobacteria</i> and <i>Firmicutes</i> phyla. Sequences from typical CF pathogens, organisms not routinely cultured (including facultative and obligate anaerobes i.e. <i>Streptococcus</i>, <i>Rothia</i>, <i>Bacteroides</i>, <i>Prevotella</i>, <i>Prophyromonas</i> and <i>Veillonella</i>) and phylotypes and clones not previously cultured with no known corresponding species.</p> <p>Pyrosequencing: 200-300 sequence clusters per sample with greater than 6 genera from 8 phyla. <i>Tenericutes</i> and <i>Spirochetes</i> were not identified by other methods. 19 genera were shared between all analysed samples, also found using 16S rRNA clone libraries.</p> <p>Most common: <i>Pseudomonas</i> (<i>P. aeruginosa</i>-like) 67%, <i>Streptococcus</i> 21%, <i>Rothia</i> 3%, <i>Prevotella</i> 3%.</p> <p>Limitations to interpretation:: Sm</p>

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					number of sample from anonymous subjects, lacks accompanying clinical/demographic information.
Brown <i>et al</i> 2014, United States ⁵¹	Subject: 1 Age: 3-years Male P.Phe508del homozygous	Lung tissue (right lower lobe). Oropharyngeal swab	16S rRNA gene pyrosequencing (V1-V3 variable region). Computational phylogenetic analysis. Microbiological culture.	To define the microbiota in the diseased lung of a child who underwent lobectomy for severe localised CF lung disease.	Diverse and anatomically heterogenous bacterial populations in the lung tissue. Culturable and non-culturable species, including abundant <i>Haemophilus</i> , <i>Ralstonia</i> and <i>Propionibacterium</i> species. Oropharyngeal swab: predominantly <i>Streptococcus</i> species. Limitations to interpretation: Samples from a single subject with an unusual clinical presentation.
Hampton <i>et al</i> 2014, United States ⁴⁴	Subjects: 23 Samples: 43 Pediatric siblings: 13, from 6 families (monozygotic twins: 8; non-twin siblings: 5). Age, median: 14-years Clinically stable Adults: 10 Age, median: 30-	Induced sputum (pediatric samples). Spontaneously expectorated sputum (adult samples)	16S rRNA gene sequencing	To determine the influence of environmental factors on the development of the CF pediatric microbiome.	Pediatric subjects: 19 genera accounted for 85% of total reads. Dominated by <i>Streptococcus</i> sp.. Microbial diversity increased compared with adults. Microbiota of subjects living together (monozygotic twin

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
	years Clinically stable.				and non-twin siblings) more similar than of subjects living apart. Adult subjects: 15 genera accounted for 85% of total reads. Often dominated by <i>Pseudomonas</i> . Limitations to interpretation: small sample size.
Reen <i>et al</i> 2014, Ireland ⁴³	Subjects: 10 Bile-aspirating ⁱⁱⁱ : 5 Non-bile aspirating: 5 Age, range: 6-19 years FEV ₁ % predicted, range: 30-118	Sputum	Denaturing gradient gel electrophoresis (DGGE) 16S rRNA 454 pyrosequencing (V1-V3 variable regions)	To investigate the impact of bile aspiration on the lung microbiome of respiratory patients.	Bile-aspirating subjects: reduced biodiversity and richness; genera associated with the CF microbiome, including dominant proteobacterial genera. Lower relative abundance of <i>Bacteroidetes</i> and <i>Fusobacteriaceae</i> phyla and of <i>Veillonellaceae</i> , <i>Fusobacteriaceae</i> , <i>Prevotellaceae</i> , <i>Pasteurellaceae</i> families. <i>Haemophilus</i> almost completely absent (relative abundance 0.1% in 1 subject). Higher ratio of Firmicutes to <i>Bacteroidetes</i> ratio. Non-bile aspirating subjects: bacteria associated with the healthy lung were

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					most abundant (<i>Prevotellaceae</i> , <i>Veillonaceae</i> , <i>Pasteurellaceae</i>). Limitations to interpretation: small sample size, impact of antibiotics and CFTR modulators not explored.
Renwick <i>et al</i> 2014, Ireland ³⁹	Subjects: 13 Age, mean (range): 3.95 (1.1-8.1) years. Male: 69% P.Phe508del homozygous: 92% Clinically stable Disease controls ^{iv} : 9 Age, mean (range): 3.78 (0.9-9.75) years	BAL fluid. Oropharyngeal swabs.	Microbiological culture 16S rRNA microarray (G3 Phylochip array)	To describe and compare the microbial communities of the lower airway of clinically stable children with CF and children without CF.	Diverse bacterial communities in both groups. Average number (range) of species, control lower airway: 167 (45-202); CF lower airway: 133 (67-163); CF upper airway: 130 (71-167). Most prevalent phyla: <i>Proteobacteria</i> , <i>Firmicutes</i> , <i>Bacteroidetes</i> , unclassified, <i>Tenericutes</i> . Increased lower airway diversity in control group. Significant differences in taxa present in CF upper and lower airway samples compared with controls. <i>Prevotella veroralis</i> and CW040 less abundant in CF airways; a <i>Corynebacterium</i>

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					<p>was more abundant. Also differences in <i>Prevotella</i>, <i>Bacteroides</i>, <i>Pseudomonas</i>, <i>Streptobacillus</i>, <i>Actinomyces</i>, <i>Selenomonas</i>, <i>Blautia</i>, <i>Leptotrichia</i>. 58 eOTUs present >30% of one group and completely absent from the other. <i>Prevotella veroralis</i>: 78% control samples, 0 CF samples. <i>P. aeruginosa</i> and <i>pneumoniae</i> in microbiological culture abundance were associated with microbial community structure. Limitations to interpretation: many bacterial taxa not classified to genus level, control subjects with respiratory symptoms.</p>
Van der Gast <i>et al</i> , 2014, United States and Australia ⁴¹	Protracted bacterial bronchitis (PBB): 12 Samples (BAL): 12 Age, mean (range): 2.3±1.7 (0.6-5.5) years. Male: 75%	Sputum. BAL fluid.	Quantitative PCR 16S rRNA gene pyrosequencing (V1-V3 variable regions) Phylogenetic analysis Ecological statistical tools	Primary: to compare the core and satellite microbiota in children with different chronic lung infections (PBB, bronchiectasis, CF).	The 3 pediatric disease groups had similar core respiratory microbiota, differed from adult disease groups. Similar total bacterial abundance.

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
	<p>FEV₁ % predicted, mean (range): 117±1.4 (116-118)</p> <p>Antibiotics at sampling: 8%</p> <p><i>P. aeruginosa</i>: 0</p> <p>Bronchiectasis: 19</p> <p>Samples – sputum: 10, BAL: 9</p> <p>Age, mean (range): 8.9±4.7 (1.8-16.3) years</p> <p>Male: 64%</p> <p>FEV₁ % predicted, mean (range): 85.7±21 (41-120)</p> <p>Antibiotics at sampling: 42%</p> <p><i>P. aeruginosa</i>: 0</p> <p>CF: 25</p> <p>Samples (sputum): 25</p> <p>Age, mean (range): 12.5±3.5 (2.3-17.7) years</p> <p>Male: 32%</p> <p>FEV₁ % predicted, mean (range): 77.9±24 (22.2-116.2)</p> <p>Antibiotics at sampling: 28%</p> <p><i>P. aeruginosa</i>: 34%</p> <p>Controls: 4</p> <p>Adult CF: 30, age 18-55 years.</p> <p>Adults with bronchiectasis:</p>			<p>Secondary: to compare pediatric and adult CF and bronchiectasis respiratory metacommunities (using data from other recent studies).</p>	<p>Common species between pediatric controls and subjects. Adult bronchiectasis and CF samples differed from each other.</p> <p>Limitations to interpretation: Subject groups not matched for age, disease severity, treatment or demographics (including continent of origin).</p>

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
	38, age 37-74 years.				
Boutin <i>et al</i> 2015, Germany ⁷⁹	Subjects: 20 Samples: 98 Age, mean (range): 16.1 ± 3.8 (7-22) years Male 75% P.Phe508del homozygous: 45% FEV ₁ % predicted, mean (range): 70.5 ± 24.5 (33-111.2)	Sputum: 32 Nasal swabs: 36 Throat swabs: 30	Quantitative PCR Next generation sequencing (V6 variable region)	Primary: to compare the microbial ecosystems of the nasal cavity, throat and sputum microbiota in young CF patients with mild-moderate lung disease. Secondary: to determine the onset and dynamics of abnormal microbiome composition in CF.	Throat and sputum microbiota closely related, significantly different from nasal microbiota. Nasal: greater diversity, richness and evenness. Most common OTUs: <i>Ralstonia</i> 17.5%, <i>Microbacterium sp.</i> 14.1%, <i>Phyllobacterium sp.</i> 13.4%, <i>Streptococcus sp.</i> 10.1%, Enteric bacteria cluster 6.3%. Throat: Most common OTUs: <i>Streptococcus sp.</i> 24.1%, <i>Prevotella sp.</i> 18%. Sputum: Most common OTUs: <i>P. aeruginosa</i> 15.1%, <i>Prevotella sp.</i> 9.6%. High inter-individual variability, primarily due to decrease in evenness with increased abundance of pathogens. Decreased diversity associated with increased bacterial load. High concordance between pediatric

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					<p>and adults sputum microbiota, except <i>Burkholderia</i> observed in adults only.</p> <p>Limitations to interpretation: we correlation between microbiological culture and next generation sequencing results possibly due to laboratory methodology.</p>

ⁱ Disease controls: with other respiratory conditions

ⁱⁱ >85% of total sequences.

ⁱⁱⁱ Bile-aspiration status determined by liquid chromatography-mass spectrometry.

^{iv} Children without CF undergoing bronchoscopy for clinical reasons.

Coburn <i>et al</i> 2015, Canada ⁴⁸	<p>Subjects: 269</p> <p>Pediatric: 76</p> <p>Age, median (range): 12 (4-17) years</p> <p>Male 33%</p> <p>P.Phe508del homozygous: 52.6%</p> <p>FEV₁ % predicted, median (range): 73 (26.6-117)</p> <p>Adult: 193</p> <p>Age, median (range): 29 (18-64) years</p> <p>Male: 59.2%</p> <p>P.Phe508del homozygous: 45.8%</p> <p>FEV₁ % predicted, median (range): 60.5 (15-111)</p>	Sputum	16S rRNA sequencing	To report a cross-sectional survey of the bacterial community in sputum samples spanning a 60-year age range and a wide range of lung function.	<p>Core genera – all samples:</p> <p><i>Streptococcus</i>, <i>Prevotella</i>, <i>Rothia</i>, <i>Veillonella</i>, <i>Acintomyces</i>; pediatrics only: <i>Neisseria</i>, <i>Haemophilus</i>, <i>Gemella</i>; adults only: <i>Pseudomonas</i>.</p> <p>Dominant genus^v in 45% pediatric and 57% adult samples.</p> <p>CF pathogens were less prevalent than core genera, but when present, tended to be dominant. Only <i>Pseudomonas</i>, <i>Streptococcus</i>, <i>Burkholderia</i>, <i>Haemophilus</i> and <i>Bordetella</i> accounted for >90% of of any sample, and only of adults' samples.</p> <p>Lower microbial diversity was</p>
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					<p>associated with more advanced disease and treatment in adults and with increased antibiotics in both adults and pediatrics. Bacterial density highest at 20-24 years. Microbial diversity lower in older patients (nadir 30-34 years); differences in diversity paralleled differences in lung function across age groups. In older age groups, lower diversity and lung function coincided with increased dominance of <i>Pseudomonas</i> and <i>Burkholderia</i> and decreases in <i>Streptococcus</i> dominance and in samples without a dominant genus. Limitations to analysis:</p>
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					<p>despite comparisons of between patient differences at varying disease stages, difficult to infer within-patient trends over time.</p> <p>Analysis not designed to assess impact of low prevalence taxa.</p> <p>Specimen processing and subsequent 16S rRNA gene sequencing not sensitive to <i>S. aureus</i>.</p>
<p>Zemanick <i>et al</i> 2015, United States⁴⁹</p>	<p>Subjects: 16 Samples: 61 Age, mean (range): 13.8±3.8 (8-21) years. Male: 62.5% P.Phe508del homozygous: 44% FEV₁ % predicted, mean: 95.8±17.2</p>	<p>Saliva Oropharyngeal swabs Induced sputum Expectorated sputum</p>	<p>Bacterial 454 pyrosequencing. Quantitative PCR Microbiological culture Sputum inflammatory markers (neutrophil elastase, IL-1β, IL-1ra, IL-6, IL-8, TNF-α, VEGF)</p>	<p>To compare bacteria detected by sequencing and quantitative PCR from concurrently collected saliva, oropharyngeal swabs, expectorated and induced sputum and to correlate these</p>	<p>Total bacterial load similar in expectorated and induced sputum and saliva, lower in oropharyngeal swabs. No significant differences in diversity, richness or evenness between sample types. Samples from individual subjects were similar, with greater variation between than within</p>

	Microbiology: <i>P. aeruginosa</i> 63%; <i>S. aureus</i> 69%			findings with indices of disease.	subjects. Divergence within subjects was mostly due to differences between upper and lower airway samples; those with increased divergence had higher relative abundance of <i>Pseudomonas</i> , <i>Staphylococcus</i> , <i>Enterobacteriaceae</i> and <i>Haemophilus</i> in lower airway samples. Higher relative abundance of <i>Pseudomonas</i> and <i>Staphylococcus</i> in lower airway samples and of <i>Veillonella</i> in upper airway samples. <i>Pseudomonas</i> , <i>Staphylococcus</i> and <i>Enterobacteriaceae</i> were associated with increased inflammation and divergence between
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					upper and lower airway microbiota was associated with increased sputum neutrophil elastase. Limitations to interpretation: small sample size. 16S rRNA gene sequencing processes less sensitive in detecting <i>S. aureus</i> .
Reen <i>et al</i> 2016, Ireland ⁴⁶	Subjects: 7 (bile aspirating, n=4), including longitudinal samples from 2 subjects (1 bile aspirating). Age, mean (range): 14.7 (8-19) years. Male: 5 (71.4%). P.Phe508del homozygous: 100%.	Sputum	16S rRNA sequencing (V1-V3 variable regions)	To investigate the longitudinal impact of bile acid aspiration in a clinical setting ^{vi} .	Bile acids were associated with reduced diversity and richness, increased Proteobacteria, with relative abundance 50-90%. Only one non-bile aspirating subject had an organism (<i>Rothia</i>) with relative abundance >50%. Longitudinal analysis: increase in <i>Streptococcus</i> over 4-month period in the bile-aspirating subject. Limitations to

	FEV ₁ % predicted, range: 52-9.				interpretation: small sample size.
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TABLE 1: CROSS-SECTIONAL STUDIES OF THE RESPIRATORY MICROBIOTA OF CHILDREN WITH CYSTIC FIBROSIS

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
Harris <i>et al</i> 2007, United States ⁴⁰	Subjects: 28 Age (males), median (range): 8.5 (0.9-20.9) years Age (females), median (range): 9.1 (1.0-20.5) years Male 47%. P.Phe508del homozygous: 50%. Disease controls ^{vii} : 14 Age (males), median	BAL fluid	T-RFLP	To test the hypothesis that organisms not routinely identified by culture occur in the CF airway and may contribute to disease.	121 bacterial species identified (65 in CF, 33 in both groups). CF libraries: 61% dominated ^{viii} by CF pathogens (<i>S.aureus</i> , <i>S. maltophilia</i> , <i>P. aeruginosa</i> , <i>S. mitis</i> group, <i>H. influenzae</i> , <i>A. xylosoxidans</i> , <i>M. abscessus</i>). ~50% contained 1 dominant pathogen. 25% did not contain typical CF pathogens and had higher relative abundance of anaerobes.

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
	(range): 2.7 (0.7-17.7) years Age (females), median (range): 3.5 (0.5-9.1) years				39% contained bacteria not routinely identified by culture. Potential novel pathogens identified in 4 specimens (<i>Prevotella denticola</i> , a <i>Lysobacter</i> sp., members of the <i>Rickettsiales</i>). Limitations to interpretation: CF and disease control subjects not age-matched; wide age range of included subjects, spanning infancy to adulthood.
Klepac-Ceraj <i>et al</i> 2010, United States ⁴⁵	Subjects: 45 Age, mean (range): 8.6 (2-16) years. FEV ₁ % predicted (mean): 97.6	Oropharyngeal swabs	High-density 16S rRNA gene microarray (PhyloChip)	Primary: to assess the influence of age, CFTR genotype, antibiotic therapy and <i>P. aeruginosa</i> on respiratory tract bacterial community composition. Secondary: to explore potential interactions among these different parameters on community composition and clinical disease markers.	2051 taxa from 43 phyla. Three dominant phyla: <i>Firmicutes</i> , <i>Proteobacteria</i> , <i>Bacteroidetes</i> . Range in bacterial diversity: 206-1329 species per person. Inverse relationship between age and richness, evenness and diversity. Decreased richness and increased beta-diversity in samples with <i>P. aeruginosa</i> . Lower beta-diversity in subjects with at least one P.Phe508del allele. Limitations to interpretation: wide age range; cross-sectional

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					design with small numbers, difficult to discern influence of antibiotic exposure and <i>P. aeruginosa</i> .
Guss <i>et al</i> 2011, United States ⁷⁸	Subjects: 4 Samples: 4 (pyrosequencing, n=3)	Sputum	16S rRNA clone libraries and pyrosequencing (V1- V2 variable regions) Microbiological culture (directed by 16S rRNA sequencing results)	To examine the microbial communities present in CF sputum samples using a combination of techniques.	Diverse microbial communities detected by all modalities. 16S rRNA libraries: 38 OTUs, representing 22 genera from 6 phyla. Most diversity within the <i>Bacteroidetes</i> , <i>Fusobacteria</i> and <i>Firmicutes</i> phyla. Sequences from typical CF pathogens, organisms not routinely cultured (including facultative and obligate anaerobes i.e. <i>Streptococcus</i> , <i>Rothia</i> , <i>Bacteroides</i> , <i>Prevotella</i> , <i>Prophyromonas</i> and <i>Veillonella</i>) and phylotypes and clones not previously cultured, with no known corresponding species. Pyrosequencing: 200-300 sequence clusters per sample, with greater than 60 genera from 8 phyla. <i>Tenericutes</i> and <i>Spirochetes</i> were not identified by

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					<p>other methods.</p> <p>19 genera were shared between all 3 analysed samples, 8 also found using 16S rRNA clone libraries. Most common: <i>Pseudomonas</i> (<i>P. aeruginosa</i>-like) 67%, <i>Streptococcus</i> 21%, <i>Rothia</i> 3%, <i>Prevotella</i> 3%.</p> <p>Limitations to interpretation:: Small number of samples from anonymous subjects, lacks accompanying clinical/demographic information.</p>
Brown <i>et al</i> 2014, United States ⁵¹	Subject: 1 Age: 3-years Male P.Phe508del homozygous	Lung tissue (right lower lobe). Oropharyngeal swab	16S rRNA gene pyrosequencing (V1-V3 variable region). Computational phylogenetic analysis. Microbiological culture.	To define the microbiota in the diseased lung of a child who underwent lobectomy for severe localised CF lung disease.	<p>Diverse and anatomically heterogenous bacterial populations in the lung tissue. Culturable and non-culturable species, including abundant <i>Haemophilus</i>, <i>Ralstonia</i> and <i>Propionibacterium</i> species.</p> <p>Oropharyngeal swab: predominantly <i>Streptococcus</i> species.</p> <p>Limitations to interpretation: Samples from a single subject with an unusual clinical presentation.</p>
Hampton <i>et</i>	Subjects: 23	Induced sputum	16S rRNA gene	To determine the	Pediatric subjects: 19 genera

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
<i>al</i> 2014, United States ⁴⁴	Samples: 43 Pediatric siblings: 13, from 6 families (monozygotic twins: 8; non-twin siblings: 5). Age, median: 14-years Clinically stable Adults: 10 Age, median: 30-years Clinically stable.	(pediatric samples). Spontaneously expectorated sputum (adult samples)	sequencing	influence of environmental factors on the development of the CF pediatric microbiome.	accounted for 85% of total reads. Dominated by <i>Streptococcus</i> sp.. Microbial diversity increased compared with adults. Microbiota of subjects living together (monozygotic twins and non-twin siblings) more similar than of subjects living apart. Adult subjects: 15 genera accounted for 85% of total reads. Often dominated by <i>Pseudomonas</i> . Limitations to interpretation: small sample size.
Reen <i>et al</i> 2014, Ireland ⁴³	Subjects: 10 Bile-aspirating ^{ix} : 5 Non-bile aspirating: 5 Age, range: 6-19 years FEV ₁ % predicted, range: 30-118	Sputum	Denaturing gradient gel electrophoresis (DGGE) 16S rRNA 454 pyrosequencing (V1-V3 variable regions)	To investigate the impact of bile aspiration on the lung microbiome of respiratory patients.	Bile-aspirating subjects: reduced biodiversity and richness; genera associated with the CF microbiome, including dominant proteobacterial genera. Lower relative abundance of <i>Bacteroidetes</i> and Fusobacteriaceae phyla and of Veillonellaceae, Fusobacteriaceae, Prevotellaceae, Pasteurellaceae families. <i>Haemophilus</i> almost completely absent (relative abundance 0.1% in 1 subject).

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					<p>Higher ratio of Firmicutes to Bacteroidetes ratio.</p> <p>Non-bile aspirating subjects: bacteria associated with the healthy lung were most abundant (<i>Prevotellaceae</i>, <i>Veillonaceae</i>, <i>Pasteurellaceae</i>).</p> <p>Limitations to interpretation: small sample size, impact of antibiotics and CFTR modulators not explored.</p>
Renwick <i>et al</i> 2014, Ireland ³⁹	<p>Subjects: 13</p> <p>Age, mean (range): 3.95 (1.1-8.1) years.</p> <p>Male: 69%</p> <p>P.Phe508del homozygous: 92%</p> <p>Clinically stable</p> <p>Disease controls^x: 9</p> <p>Age, mean (range): 3.78 (0.9-9.75) years</p>	BAL fluid. Oropharyngeal swabs.	Microbiological culture 16S rRNA microarray (G3 Phylochip array)	To describe and compare the microbial communities of the lower airway of clinically stable children with CF and children without CF.	<p>Diverse bacterial communities in both groups.</p> <p>Average number (range) of species, control lower airway: 167 (45-202); CF lower airway: 133 (67-163); CF upper airway: 130 (71-167).</p> <p>Most prevalent phyla: <i>Proteobacteria</i>, <i>Firmicutes</i>, <i>Bacteroidetes</i>, unclassified, <i>Tenericutes</i>.</p> <p>Increased lower airway diversity in control group.</p> <p>Significant differences in taxa present in CF upper and lower airway samples compared with</p>

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					<p>controls. <i>Prevotella veroralis</i> and CW040 less abundant in CF airways; a <i>Corynebacterium</i> was more abundant. Also differences in <i>Prevotella</i>, <i>Bacteroides</i>, <i>Pseudomonas</i>, <i>Streptobacillus</i>, <i>Actinomyces</i>, <i>Selenomonas</i>, <i>Blautia</i>, <i>Leptotrichia</i>. 58 eOTUs present in >30% of one group and completely absent from the other. <i>Prevotella veroralis</i>: 78% control samples, 0 CF samples.</p> <p><i>P. aeruginosa</i> and <i>S. pneumoniae</i> in microbiological culture abundance were associated with microbial community structure. Limitations to interpretation: many bacterial taxa not classified to genus level, control subjects with respiratory symptoms.</p>
Van der Gast <i>et al</i> , 2014, United States and Australia ⁴¹	Protracted bacterial bronchitis (PBB): 12 Samples (BAL): 12 Age, mean (range): 2.3±1.7 (0.6-5.5) years.	Sputum. BAL fluid.	Quantitative PCR 16S rRNA gene pyrosequencing (V1-V3 variable regions) Phylogenetic	Primary: to compare the core and satellite microbiota in children with different chronic lung infections (PBB, bronchiectasis, CF).	The 3 pediatric disease groups had similar core respiratory microbiota, different from adult disease groups. Similar total bacterial abundance. Common species between

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
	<p>Male: 75% FEV₁ % predicted, mean (range): 117±1.4 (116-118) Antibiotics at sampling: 8% <i>P. aeruginosa</i>: 0 Bronchiectasis: 19 Samples – sputum: 10, BAL: 9 Age, mean (range): 8.9±4.7 (1.8-16.3) years Male: 64% FEV₁ % predicted, mean (range): 85.7±21 (41-120) Antibiotics at sampling: 42% <i>P. aeruginosa</i>: 0 CF: 25 Samples (sputum): 25 Age, mean (range): 12.5±3.5 (2.3-17.7) years Male: 32%</p>		<p>analysis Ecological statistical tools</p>	<p>Secondary: to compare pediatric and adult CF and bronchiectasis respiratory metacommunities (using data from other recent studies).</p>	<p>pediatric controls and subjects. Adult bronchiectasis and CF samples differed from each other. Limitations to interpretation: Subject groups not matched for age, disease severity, treatment or demographics (including continent of origin).</p>

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
	FEV ₁ % predicted, mean (range): 77.9±24 (22.2-116.2) Antibiotics at sampling: 28% <i>P. aeruginosa</i> : 34% Controls: 4 Adult CF: 30, age 18-55 years. Adults with bronchiectasis: 38, age 37-74 years.				
Boutin <i>et al</i> 2015, Germany ⁷⁹	Subjects: 20 Samples: 98 Age, mean (range): 16.1 ± 3.8 (7-22) years Male 75% P.Phe508del homozygous: 45% FEV ₁ % predicted, mean (range): 70.5 ± 24.5 (33-111.2)	Sputum: 32 Nasal swabs: 36 Throat swabs: 30	Quantitative PCR Next generation sequencing (V6 variable region)	Primary: to compare the microbial ecosystems of the nasal cavity, throat and sputum microbiota in young CF patients with mild-moderate lung disease. Secondary: to determine the onset and dynamics of abnormal microbiome composition in CF.	Throat and sputum microbiota closely related, significantly different from nasal microbiota. Nasal: greater diversity, richness and evenness. Most common OTUs: <i>Ralstonia</i> 17.5%, <i>Microbacterium sp.</i> 14.1%, <i>Phyllobacterium sp.</i> 13.4%, <i>Streptococcus sp.</i> 10.1%, Enteric bacteria cluster 6.3%. Throat: Most common OTUs: <i>Streptococcus sp.</i> 24.1%, <i>Prevotella sp.</i> 18%. Sputum: Most common OTUs: <i>P. aeruginosa</i> 15.1%, <i>Prevotella sp.</i>

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					<p>9.6%.</p> <p>High inter-individual variability, primarily due to decrease in evenness with increased abundance of pathogens.</p> <p>Decreased diversity associated with increased bacterial load.</p> <p>High concordance between pediatric and adults sputum microbiota, except <i>Burkholderia</i> observed in adults only.</p> <p>Limitations to interpretation: weak correlation between microbiological culture and next generation sequencing results, possibly due to laboratory methodology.</p>
Coburn <i>et al</i> 2015, Canada ⁴⁸	<p>Subjects: 269</p> <p>Pediatric: 76</p> <p>Age, median (range): 12 (4-17) years</p> <p>Male 33%</p> <p>P.Phe508del homozygous: 52.6%</p> <p>FEV₁ % predicted, median (range): 73 (26.6-117)</p>	Sputum	16S rRNA sequencing	To report a cross-sectional survey of the bacterial community in sputum samples spanning a 60-year age range and a wide range of lung function.	<p>Core genera – all samples: <i>Streptococcus</i>, <i>Prevotella</i>, <i>Rothia</i>, <i>Veillonella</i>, <i>Acintomyces</i>;</p> <p>pediatrics only: <i>Neisseria</i>, <i>Haemophilus</i>, <i>Gemella</i>; adults only: <i>Pseudomonas</i>.</p> <p>Dominant genus^{xi} in 45% pediatric and 57% adult samples.</p> <p>CF pathogens were less prevalent than core genera, but when</p>

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
	Adult: 193 Age, median (range): 29 (18-64) years Male: 59.2% P.Phe508del homozygous: 45.8% FEV ₁ % predicted, median (range): 60.5 (15-111)				<p>present, tended to be dominant. Only <i>Pseudomonas</i>, <i>Streptococcus</i>, <i>Burkholderia</i>, <i>Haemophilus</i> and <i>Bordetella</i> accounted for >90% of of any sample, and only of adults' samples.</p> <p>Lower microbial diversity was associated with more advanced disease and treatment in adults and with increased antibiotics in both adults and pediatrics. Bacterial density highest at 20-24 years. Microbial diversity lower in older patients (nadir 30-34 years); differences in diversity paralleled differences in lung function across age groups.</p> <p>In older age groups, lower diversity and lung function coincided with increased dominance of <i>Pseudomonas</i> and <i>Burkholderia</i> and decreases in <i>Streptococcus</i> dominance and in samples without a dominant genus.</p> <p>Limitations to analysis: despite</p>

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					comparisons of between patient differences at varying disease stages, difficult to infer within-patient trends over time. Analysis not designed to assess impact of low prevalence taxa. Specimen processing and subsequent 16S rRNA gene sequencing not sensitive to <i>S. aureus</i> .
Zemanick <i>et al</i> 2015, United States ⁴⁹	Subjects: 16 Samples: 61 Age, mean (range): 13.8±3.8 (8-21) years. Male: 62.5% P.Phe508del homozygous: 44% FEV ₁ % predicted, mean: 95.8±17.2 Microbiology: <i>P. aeruginosa</i> 63%; <i>S. aureus</i> 69%	Saliva Oropharyngeal swabs Induced sputum Expectorated sputum	Bacterial 454 pyrosequencing. Quantitative PCR Microbiological culture Sputum inflammatory markers (neutrophil elastase, IL-1β, IL-1ra, IL-6, IL-8, TNF-α, VEGF)	To compare bacteria detected by sequencing and quantitative PCR from concurrently collected saliva, oropharyngeal swabs, expectorated and induced sputum and to correlate these findings with indices of disease.	Total bacterial load similar in expectorated and induced sputum and saliva, lower in oropharyngeal swabs. No significant differences in diversity, richness or evenness between sample types. Samples from individual subjects were similar, with greater variation between than within subjects. Divergence within subjects was mostly due to differences between upper and lower airway samples; those with increased divergence had higher relative abundance of <i>Pseudomonas</i> , <i>Staphylococcus</i> , <i>Enterobacteriaceae</i> and

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
					<p><i>Haemophilus</i> in lower airway samples.</p> <p>Higher relative abundance of <i>Pseudomonas</i> and <i>Staphylococcus</i> in lower airway samples and of <i>Veillonella</i> in upper airway samples.</p> <p><i>Pseudomonas</i>, <i>Staphylococcus</i> and <i>Enterobacteriaceae</i> were associated with increased inflammation and divergence between upper and lower airway microbiota was associated with increased sputum neutrophil elastase.</p> <p>Limitations to interpretation: small sample size. 16S rRNA gene sequencing processes less sensitive in detecting <i>S. aureus</i>.</p>
Reen <i>et al</i> 2016, Ireland ⁴⁶	Subjects: 7 (bile aspirating, n=4), including longitudinal samples from 2 subjects (1 bile aspirating). Age, mean (range): 14.7 (8-19) years.	Sputum	16S rRNA sequencing (V1-V3 variable regions)	To investigate the longitudinal impact of bile acid aspiration in a clinical setting ^{xii} .	<p>Bile acids were associated with reduced diversity and richness, increased Proteobacteria, with relative abundance 50-90%. Only one non-bile aspirating subject had an organism (<i>Rothia</i>) with relative abundance >50%.</p> <p>Longitudinal analysis: increase in</p>

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
	Male: 5 (71.4%). P.Phe508del homozygous: 100%. FEV ₁ % predicted, range: 52-9.				<i>Streptococcus</i> over 4-month period in the bile-aspirating subject. Limitations to interpretation: small sample size.

TABLE 2: LONGITUDINAL STUDIES OF THE RESPIRATORY MICROBIOTA OF CHILDREN WITH CYSTIC FIBROSIS

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
Worlitzsch <i>et al</i> 2009, Germany ⁶⁴	Subjects: 45 Samples: sputum 114, throat swabs 7 Longitudinal samples (≥2 timepoints): 58% Age, mean (range): 24.6±9.7 (6-64) years. Male: 40% Pediatric subjects: 9 (20%), age range 6-18 years Acute exacerbation/s	Sputum: 114 Throat swabs: 7 (BAL fluid: 6 disease controls)	Microbiological culture	Primary: to identify obligate anaerobes and to determine cell numbers and antibiotic susceptibilities of facultative and obligate anaerobes in sputum and throat swab samples from children and adults with CF. Secondary: to assess whether intravenous antibiotic therapy for acute exacerbations would affect the numbers of facultative and obligate	≥1 obligate anaerobe identified in 91% of CF subjects, at comparable rates in children and adults. Up to 4 different species per sputum sample. Total 168 isolates from 16 genera. Most prevalent: <i>Staphylococcus</i> , <i>Peptostreptococcus</i> , <i>Actinomyces</i> , <i>Veillonella</i> , <i>Clostridium</i> . Species persisted in 42% of longitudinal samples. <i>P. aeruginosa</i> infection did not increase the likelihood of anaerobic bacteria. Presence of obligate anaerobes not associated with pulmonary

	during study period: 27% Disease controls ^{xiii} : 6. Age, mean (range): 7.2±5.3 (2-16) years			anaerobes and lung function.	function. No significant change in numbers of facultative or obligate anaerobes with anti-pseudomonal antibiotic therapy, although FEV ₁ improved. Limitations to interpretation: controls with pulmonary disease.
Cox <i>et al</i> 2010, United States ⁴²	Subjects: 51 <i>Pediatric</i> : 26 <i>Adult</i> : 37 Longitudinal (>1 sample): 13 <i>Pediatric</i> : 13, age 9-months to 11-years <i>Adult</i> : 3	Deep throat swabs (pediatric). Sputum (adult).	16S rRNA PhyloChip; Quantitative PCR (14 adult samples)	To perform an in-depth characterisation of age-based bacterial composition of CF airways (analysed according to 5-year age brackets)	33% of community variance explained by age group. Airway microbiota was less rich, less even, less diverse and more related to each other in older patients. Abundance of particular CF pathogens correlated with age: <i>H. influenzae</i> (r=-0.31); <i>S. maltophilia</i> (r=0.31), <i>P. aeruginosa</i> (r=0.42). Changes in community diversity over time: net increase in pediatric samples, net decrease in adult samples. Limitations to interpretation: small numbers with limited information regarding subject age and time interval between serial samples.

Madan <i>et al</i> 2012, United States ⁷¹	Subjects: 7 Samples: 26. Initial sample: <1-month; Sampling interval: 3-monthly Sampling duration: 9-21 months	Oropharyngeal swabs (n=26); Stool samples (n=33)	16S rRNA gene pyrosequencing	To investigate the developing respiratory and intestinal microbiome in infants with CF.	79 unique genera, including 8 that represented 74% and 72% of the intestinal and respiratory microbiome sequences respectively. Most abundant genera – respiratory: <i>Streptococcus</i> , <i>Veillonella</i> and <i>Prevotella</i> ; Intestinal: <i>Bacteriodes</i> , <i>Bifidobacterium</i> and <i>Veillonella</i> . Common core of bacteria (<i>Streptococcus</i> and <i>Veillonella</i>) in respiratory and intestinal samples at all timepoints. Two large clusters of bacteria represented in respiratory tract only. 14 of 16 genera increased over time in both sites; subset detected in gut prior to respiratory tract. Significant increase in diversity over time, more acute increase in respiratory tract. Limitations to interpretation: small number of subjects, including three requiring prolonged hospitalisation (due to prematurity (n=1) and surgically-
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					managed meconium ileus (n=2).
Bernarde <i>et al</i> 2015, France ⁸⁰	Subjects: 3 Samples: 6-7 per subject Female: 100% Ages: 10, 11 and 16-years. Sampling interval: 3-months (mean) prior to commencement of ivacaftor, and then 2-monthly. Sampling duration: 10-months, during ivacaftor treatment.	Sputum	Quantitative PCR 16S rRNA gene pyrosequencing	To analyse the evolution of the airway microbiota in patients treated with ivacaftor.	No significant differences in total bacterial load or global community composition before and after ivacaftor treatment. Relative abundance of <i>Porphyromonas</i> increased and of <i>Streptococcus</i> (<i>S. mitis</i> group) decreased. Limitations to interpretation: small number of participants with differences in age, antibiotic treatment and lung function.
Hoen <i>et al</i> 2015, United States ⁸¹	Subjects: 13 Samples: 54 Enrolment: <1-month; Sampling interval: 3-months Sampling duration: 6-34 months	Oropharyngeal swabs (n=54); Stool samples (n=66)	16S rRNA gene pyrosequencing (V6 or V4-V6 variable regions)	To examine patterns of microbial colonization in the respiratory and intestinal tracts in early life and their associations with breastfeeding and clinical outcomes (pulmonary exacerbation, growth failure,	Group of bacteria with high relative abundance in both intestinal and upper respiratory tracts: <i>Veillonella</i> , <i>Streptococcus</i> , <i>Bifidobacterium</i> , <i>Bacteroides</i> . Second cluster of bacteria dominated in respiratory samples, tended to be derived from later time points

				<p>hospitalisation, <i>P. aeruginosa</i> colonisation). To investigate the hypothesis that microbial acquisition patterns relate to risk of CF-related complications and clinical markers of CF disease progression.</p>	<p>(<i>Acintomyces</i>, <i>Granulicatella</i>, <i>Haemophilus</i>, <i>Prevotella</i>, <i>Staphylococcus</i>, <i>Ralstonia</i>, <i>Rothia</i>, <i>Gemella</i>). Prior to <i>P. aeruginosa</i> colonization, relative abundance of <i>Panbacteroides</i> in the intestinal tract decreased and of <i>Salmonella</i> in the respiratory tract increased. Significant association between pulmonary exacerbations and gut but not respiratory microbiome composition. Trend towards longer time to first exacerbation with breast milk exposure. Trend towards longer time of exacerbation and <i>P. aeruginosa</i> colonization with increased intestinal microbial diversity. Limitations to interpretation: intestinal microbiota more likely similar to oropharynx than to other airway sites.</p>
Keravec <i>et al</i> 2015, France ⁸²	Subjects: 5 Samples: 20 (4 per subject).	Sputum	Quantitative PCR to determine presence of <i>P. aeruginosa</i>	To investigate whether early <i>P. aeruginosa</i> colonization was	High bacterial diversity. Core microbiota ^{xiv} : 57 OTUs from 5 phyla (<i>Firmicutes</i> ,

	<p>Age range: 5-19 years.</p> <p>Sampling duration: median 22 months (range 16-39 months).</p> <p>Microbiology: <i>P. aeruginosa</i> negative, n=3; never detected, n=2. All subjects became <i>P. aeruginosa</i> positive during follow-up</p>		<p>16S rRNA gene sequencing (V3-V4 variable regions)</p> <p>Respiratory viruses screening</p> <p>Cloning and sequencing analysis</p>	<p>accompanied by significant changes in the respiratory microbiota (community structure, relative abundance).</p> <p>To identify potential biomarkers or predictive factors of <i>P. aeruginosa</i> implantation.</p>	<p><i>Proteobacteria</i>, <i>Actinobacteria</i>, <i>Bacteroidetes</i>, <i>Fusobacteria</i>). 13 predominant genera (relative abundance >1%), accounting for 94% of “core” reads.</p> <p>High prevalence of viruses: at least 1 detected in 52.6% of samples, ≥2 in 15.8%.</p> <p>9 OTUs from 6 genera (<i>Veillonella</i>, <i>Actinomyces</i>, <i>Prevotella</i>, <i>Haemophilus</i>, <i>Granulicatella</i>, <i>Streptococcus</i>) were persistent through early <i>P. aeruginosa</i> colonization.</p> <p>Limitations to interpretation: small numbers of participants with wide age range, no information regarding antibiotic exposures.</p>
Prevaes <i>et al</i> 2016, Netherlands ⁶⁸	<p>Subjects: 20</p> <p>Samples: 324</p> <p>Males: 50%</p> <p>Age, mean (range): 2 (0.6-6.1 months).</p> <p>P.Phe508del homozygous: 80%</p> <p>Asymptomatic at</p>	Nasopharyngeal swab	<p>Quantitative PCR</p> <p>16S rRNA sequencing (V5-V7 variable regions).</p> <p>Microbiological culture</p> <p>Viral PCR</p>	<p>To investigate the development of the nasopharyngeal microbiota profiles in infants with CF compared with healthy controls during the first 6 months of life.</p>	<p>Differences in microbiota of infants with CF compared with controls.</p> <p>CF: early <i>S. aureus</i> and to a lesser extent, <i>Corynebacterium</i> spp. and <i>Moraxella</i> spp. dominance, followed by a switch to <i>S. mitis</i> after 3-months. <i>S. aureus</i>, <i>S. mitis</i>, <i>Corynebacterium accolens</i></p>

	<p>diagnosis: 70%</p> <p>Sampling interval: monthly and with acute respiratory tract infection</p> <p>Controls: 45</p>				<p>and bacilli more abundant. Lower bacterial density, particularly >4-months, related to antibiotic use.</p> <p>No significant differences in alpha-diversity between the 2 groups.</p> <p>Antibiotic use associated with increased colonisation of gram-negative and reduced colonisation of potential beneficial commensals.</p> <p>Controls: <i>Moraxella</i> spp. enrichment throughout. <i>Moraxella</i> spp., <i>Corynebacterium pseudodiphthericum</i>, <i>Corynebacterium propinquum</i> and <i>H. influenzae</i> more abundant.</p> <p>Limitations to interpretation: single center, short follow-up, small numbers and different environmental exposures between cases and controls for antibiotics and day care attendance.</p>
Mika <i>et al</i>	Subjects: 30	Nasal swabs	16S rRNA gene	Primary: to compare the	Different composition of nasal

2016, Switzerland ⁶⁹	<p>Samples: 461, mean 15.4 per subject (range 4-24)</p> <p>Male: 43%</p> <p>P.Phe508del homozygous: 43%</p> <p>Sampling interval: fortnightly.</p> <p>Healthy controls: 47.</p>		<p>pyrosequencing</p> <p>Oligotyping analysis for differentiation between <i>Staphylococcus</i> species</p>	<p>nasal microbial composition of infants with CF with healthy controls during the first-year of life.</p> <p>Secondary: to examine the effect of antibiotic treatment on the nasal microbiota of infants with CF.</p>	<p>microbiota of infants with CF compared with healthy controls, even in the absence of antibiotic exposure. Increased relative abundance of Staphylococcae and decreased relative abundance of Corynebacteriaceae and Pasteurellaceae in infants with CF.</p> <p>At and after antibiotic treatment: decrease in <i>S. aureus</i> and increase in coagulase-negative Staphylococci.</p> <p>Limitations to interpretation: varying numbers of participants exposed/not exposed to antibiotics.</p>
Laguna <i>et al</i> 2016, United States ⁷²	<p>Subjects: 8</p> <p>Samples: 12</p> <p>Serial samples: 3 subjects</p> <p>Initial BAL (n=8):</p> <p>Male: 25%</p> <p>Age, median (range): 6.8 months (5.7-11.8)</p> <p>FEV_{0.5} (% predicted),</p>	<p>BAL fluid</p> <p>Nasopharyngeal swab</p>	<p>16S rRNA sequencing (V1-V2 variable regions)</p>	<p>Primary: to describe the BAL fluid microbiota of asymptomatic infants with CF during the first year of life.</p> <p>Secondary: to describe the relationship between BAL fluid microbiota, standard BAL fluid and NP culture and clinical characteristics at up to 5-years of follow-</p>	<p>Cross-sectional samples (n=8):</p> <p>Total bacterial load, median (range): 7.8-log₁₀ rDNA copies/mL (7.1-9.2).</p> <p>SDI, median (range): 2.2 (1.1-3.2).</p> <p>Richness, median (range): 70.9 (17.1-92.6).</p> <p>Evenness, median (range): 0.4 (0.2-0.6).</p> <p>232 taxa detected across all samples, 10 most abundant:</p>

	<p>median (range): 94 (69-116) P.Phe508del homozygous: 88% Second BAL (n=3): Male: 0 Age, median (range): 12.4 (11.9-13.2) months. FEV_{0.5} (% predicted), median (range): 124 (97-145) P.Phe508del homozygous: 100%</p>			up.	<p><i>Streptococcus</i> (31.7% median relative abundance), <i>Burkholderia</i>, <i>Prevotella</i>, <i>Haemophilus</i>, <i>Porphyromonas</i>, <i>Veillonella</i>, <i>Gemella</i>, <i>Neisseria</i>, <i>Lactobacillales</i>, <i>Methylobacterium</i>. <i>Streptococcus</i>, <i>Burkholderia</i>, <i>Prevotella</i>, <i>Haemophilus</i>, <i>Veillonella</i>, <i>Gemella</i>, <i>Neisseria</i> and <i>Methylobacterium</i> detected in all 8 initial samples; <i>Burkholderia</i> and <i>Prevotella</i> dominant in 3 and 1 sample respectively. Longitudinal samples: Beta-diversity (Morisita-Horn) ≤0.38 in 2 samples, ~1 in the 3rd sample. No association between BAL fluid neutrophil count and total bacterial load or SDI. Positive correlation between relative abundance of <i>Streptococcus</i> and FEV_{0.5}. Limitations to interpretation: unexpectedly high relative abundance of <i>Burkholderia</i>,</p>
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					particularly in samples with low total bacterial loads raises possibility of sample contamination; small number of participants with serial samples.
Prevaes <i>et al</i> 2017, Netherlands ⁷³	Subjects: 17 Samples: 25 Serial samples: 8 subjects Male: 47% P.Phe508del homozygous: 88% Early BAL (n=13): Age, median (range): 4.6 (3-8) months Late BAL (n=12): Age, median (range): 12.4 (9-13) months	Nasopharyngeal swab Oropharyngeal swab BAL fluid.	16S rRNA sequencing (V5-V7 variable regions)	To assess inter- and intra-individual concordance of the microbiota at different “respiratory niches”. To explore the benefits of 16S rRNA gene sequencing compared with microbiological culture in clinical management of patients with CF. To evaluate temporo-spatial variation in lung microbiota in infants with CF.	There were significant differences in microbial diversity between the sample sites. The SDI was lowest in the nasopharyngeal samples and highest in the oropharyngeal samples. Intra-individual concordance between nasopharyngeal and BAL and oropharyngeal and BAL samples was variable, and both upper airway sites appear to contribute, but are not identical, to the lower airway microbiota. In those who contributed both “early” and “late” BAL samples, diversity increased over time, but microbial community composition varied. Limitations to interpretation: Unable to assess impact of antibiotic exposure on microbiota composition.
Frayman <i>et al</i>	Subjects: 48	BAL fluid.	16S rRNA	To describe the lower	Most prevalent genera:

2017, Australia ⁷⁴	<p>Samples: 95</p> <p>Serial samples: 27 subjects</p> <p>Male: 52%</p> <p>Age, median (range): 20.4 (1.2-78.3) months.</p> <p>P.Phe508del homozygous: 68%</p>		sequencing (V1-V3 variable regions)	<p>airway microbiota in infants and young children with CF.</p> <p>To explore the relationship of the lower airway microbiota to lower airway inflammation and pulmonary function at 6-years.</p>	<p><i>Staphylococcus</i> (36.6%), <i>Streptococcus</i> (13.7%), <i>Pseudomonas</i> (6.1%), <i>Neisseria</i>, <i>Haemophilus</i>, <i>Gemella</i>, <i>Granulicatella</i>, <i>Prevotella</i>, <i>Veillonella</i>, <i>Streptomyces</i>.</p> <p>Microbial diversity decreased with increasing subject age (beta=-0.01, p=0.03).</p> <p>Detection of typical CF bacterial pathogens was associated with lower microbial diversity and with higher lower airway inflammatory indices.</p> <p>The composition of individuals' lower airway microbial communities varied considerably over time.</p> <p>There was no relationship between lower airway microbial diversity in early childhood and FEV₁ at age 6-years.</p> <p>Limitations to interpretation: Secondary analysis of BAL samples collected two decades prior.</p>
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TABLE 3: THE AIRWAY MICROBIOTA IN EARLY CYSTIC FIBROSIS LUNG DISEASE*

UPPER AIRWAY	LOWER AIRWAY
Oropharyngeal specimens: Studies: 4 ^{51,71,73,81} Participants: 38 Samples: 106 Common genera: <i>Bacterioides</i> <i>Gemella</i> <i>Granulicatella</i> <i>Haemophilus</i> <i>Neisseria</i> <i>Prevotella</i> <i>Rothia</i> <i>Staphylococcus</i> <i>Streptococcus</i> <i>Veillonella</i>	Bronchoalveolar lavage specimens: Studies: 3 ⁷²⁻⁷⁴ Participants: 73 Samples: 132 Common genera: <i>Gemella</i> <i>Haemophilus</i> <i>Neisseria</i> <i>Prevotella</i> <i>Staphylococcus</i> <i>Streptococcus</i> <i>Veillonella</i>
Nasopharyngeal specimens: Studies: 3 ^{68,69,73} Participants: 67 Samples: 810 Common genera: <i>Corynebacterium</i> <i>Moraxella</i> <i>Staphylococcus</i> <i>Streptococcus</i>	Lung tissue specimens: Studies: 1 ⁵¹ Participants: 1 Samples: 6 Common genera: <i>Anaerococcus</i> <i>Haemophilus influenzae</i> <i>Propionibacterium spp.</i> <i>Propionibacterium acnes</i> <i>Ralstonia pickettii</i> <i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i>

*Studies of the airway microbiota in children with cystic fibrosis of average age 6-years or younger and their most prevalent genera are summarized. Genera with an overall prevalence and/or average relative abundance $\geq 2\%$ in two or more studies of individual specimen types are included. As there is only one study of lung tissue, involving six specimens from a single subject, all genera with a relative abundance of $\geq 2\%$ listed. An early study of the BAL and upper airway microbiota is it combined data regarding the most prevalent genera in the upper and lower airway samples³⁹.

^v Dominant genus defined as the most abundant genus, with at least twice the abundance of the second most abundant genus.

^{vi} Sub-component of larger study aiming to explore the molecular mechanisms involved in the impact of aspirated bile acids on the respiratory microbiome.

ABBREVIATIONS:

16S rRNA 16S ribosomal RNA

OTU

operational taxonomic unit

BAL	bronchoalveolar lavage	PCR	polymerase chain reaction
CF	cystic fibrosis	PBB	protracted bacterial bronchitis
CFTR	cystic fibrosis transmembrane regulator	TNF- α	tumour necrosis factor alpha
DGGE	denaturing gradient gel electrophoresis	T-RFLP	terminal restriction fragment length polymorphism
FEV ₁	forced expiratory volume in one second	VEGF	vascular endothelial growth factor
IL	interleukin		

TABLE 2: LONGITUDINAL STUDIES OF THE RESPIRATORY MICROBIOTA OF CHILDREN WITH CYSTIC FIBROSIS

STUDY	POPULATION	SAMPLE TYPE	METHODOLOGY	OBJECTIVES	OUTCOMES AND COMMENTS
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Worlitzsch <i>et al</i> 2009, Germany ⁶⁴	<p>Subjects: 45</p> <p>Samples: sputum 114, throat swabs 7</p> <p>Longitudinal samples (≥ 2 timepoints): 58%</p> <p>Age, mean (range): 24.6$\pm$9.7 (6-64) years.</p> <p>Male: 40%</p> <p>Pediatric subjects: 9 (20%), age range 6-18 years</p> <p>Acute exacerbation/s during study period: 27%</p> <p>Disease controls^{vi}: 6.</p> <p>Age, mean (range): 7.2$\pm$5.3 (2-16) years</p>	<p>Sputum: 114</p> <p>Throat swabs: 7 (BAL fluid: 6 disease controls)</p>	Microbiological culture	<p>Primary: to identify obligate anaerobes and to determine cell numbers and antibiotic susceptibilities of facultative and obligate anaerobes in sputum and throat swab samples from children and adults with CF.</p> <p>Secondary: to assess whether intravenous antibiotic therapy for acute exacerbations would affect the numbers of facultative and obligate anaerobes and lung function.</p>	<p>≥ 1 obligate anaerobe identified in 91% of CF subjects, at comparable rates in children and adults. Up to 4 different species per sputum sample.</p> <p>Total 168 isolates from 16 genera. Most prevalent: <i>Staphylococcus</i>, <i>Peptostreptococcus</i>, <i>Actinomyces</i>, <i>Veillonella</i>, <i>Clostridium</i>. Species persisted in 42% of longitudinal samples.</p> <p><i>P. aeruginosa</i> infection did not increase the likelihood of anaerobic bacteria.</p> <p>Presence of obligate anaerobes not associated with pulmonary function.</p> <p>No significant change in numbers of facultative or obligate anaerobes with anti-pseudomonal antibiotic therapy, although FEV₁ improved.</p> <p>Limitations to interpretation: controls with pulmonary disease.</p>
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Cox <i>et al</i> 2010, United States ⁴²	Subjects: 51 <i>Pediatric</i> : 26 <i>Adult</i> : 37 Longitudinal (>1 sample): 13 <i>Pediatric</i> : 13, age 9-months to 11-years <i>Adult</i> : 3	Deep throat swabs (pediatric). Sputum (adult).	16S rRNA PhyloChip; Quantitative PCR (14 adult samples)	To perform an in-depth characterisation of age-based bacterial composition of CF airways (analysed according to 5-year age brackets)	33% of community variance explained by age group. Airway microbiota was less rich, less even, less diverse and more related to each other in older patients. Abundance of particular CF pathogens correlated with age: <i>H. influenzae</i> (r=-0.31); <i>S. maltophilia</i> (r=0.31), <i>P. aeruginosa</i> (r=0.42). Changes in community diversity over time: net increase in pediatric samples, net decrease in adult samples. Limitations to interpretation: small numbers with limited information regarding subject age and time interval between serial samples.
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Madan <i>et al</i> 2012, United States ⁷¹	Subjects: 7 Samples: 26. Initial sample: <1-month; Sampling interval: 3-monthly Sampling duration: 9-21 months	Oropharyngeal swabs (n=26); Stool samples (n=33)	16S rRNA gene pyrosequencing	To investigate the developing respiratory and intestinal microbiome in infants with CF.	79 unique genera, including 8 that represented 74% and 72% of the intestinal and respiratory microbiome sequences respectively. Most abundant genera – respiratory: <i>Streptococcus</i> , <i>Veillonella</i> and <i>Prevotella</i> ; Intestinal: <i>Bacteriodes</i> , <i>Bifidobacterium</i> and <i>Veillonella</i> . Common core of bacteria (<i>Streptococcus</i> and <i>Veillonella</i>) in respiratory and intestinal samples at all timepoints. Two large clusters of bacteria represented in respiratory tract only. 14 of 16 genera increased over time in both sites; subset detected in gut prior to respiratory tract. Significant increase in diversity over time, more acute increase in respiratory tract. Limitations to interpretation: small number of subjects, including three requiring prolonged hospitalisation (due to prematurity (n=1) and surgically-managed meconium ileus (n=2)).
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Bernarde <i>et al</i> 2015, France ⁸⁰	<p>Subjects: 3</p> <p>Samples: 6-7 per subject</p> <p>Female: 100%</p> <p>Ages: 10, 11 and 16-years.</p> <p>Sampling interval: 3-months (mean) prior to commencement of ivacaftor, and then 2-monthly.</p> <p>Sampling duration: 10-months, during ivacaftor treatment.</p>	Sputum	Quantitative PCR 16S rRNA gene pyrosequencing	To analyse the evolution of the airway microbiota in patients treated with ivacaftor.	<p>No significant differences in total bacterial load or global community composition before and after ivacaftor treatment.</p> <p>Relative abundance of <i>Porphyromonas</i> increased and of <i>Streptococcus</i> (<i>S. mitis</i> group) decreased.</p> <p>Limitations to interpretation: small number of participants with differences in age, antibiotic treatment and lung function.</p>
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<p>Hoen <i>et al</i> 2015, United States⁸¹</p>	<p>Subjects: 13 Samples: 54 Enrolment: <1-month; Sampling interval: 3-months Sampling duration: 6-34 months</p>	<p>Oropharyngeal swabs (n=54); Stool samples (n=66)</p>	<p>16S rRNA gene pyrosequencing (V6 or V4-V6 variable regions)</p>	<p>To examine patterns of microbial colonization in the respiratory and intestinal tracts in early life and their associations with breastfeeding and clinical outcomes (pulmonary exacerbation, growth failure, hospitalisation, <i>P. aeruginosa</i> colonisation). To investigate the hypothesis that microbial acquisition patterns relate to risk of CF-related complications and clinical markers of CF disease progression.</p>	<p>Group of bacteria with high relative abundance in both intestinal and upper respiratory tracts: <i>Veillonella</i>, <i>Streptococcus</i>, <i>Bifidobacterium</i>, <i>Bacteroides</i>. Second cluster of bacteria dominated in respiratory samples, tended to be derived from later time points (<i>Acintomyces</i>, <i>Granulicatella</i>, <i>Haemophilus</i>, <i>Prevotella</i>, <i>Staphylococcus</i>, <i>Ralstonia</i>, <i>Rothia</i>, <i>Gemella</i>). Prior to <i>P. aeruginosa</i> colonization, relative abundance of <i>Panbacteroides</i> in the intestinal tract decreased and of <i>Salmonella</i> in the respiratory tract increased. Significant association between pulmonary exacerbations and gut but not respiratory microbiome composition. Trend towards longer time to first exacerbation with breast milk exposure. Trend towards longer time of exacerbation and <i>P. aeruginosa</i> colonization with increased intestinal microbial diversity. Limitations to interpretation: intestinal microbiota more likely similar to oropharynx than to other airway sites.</p>
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Keravec <i>et al</i> 2015, France ⁸²	<p>Subjects: 5</p> <p>Samples: 20 (4 per subject).</p> <p>Age range: 5-19 years.</p> <p>Sampling duration: median 22 months (range 16-39 months).</p> <p>Microbiology: <i>P. aeruginosa</i> negative, n=3; never detected, n=2. All subjects became <i>P. aeruginosa</i> positive during follow-up</p>	Sputum	<p>Quantitative PCR to determine presence of <i>P. aeruginosa</i> 16S rRNA gene sequencing (V3-V4 variable regions)</p> <p>Respiratory viruses screening</p> <p>Cloning and sequencing analysis</p>	<p>To investigate whether early <i>P. aeruginosa</i> colonization was accompanied by significant changes in the respiratory microbiota (community structure, relative abundance).</p> <p>To identify potential biomarkers or predictive factors of <i>P. aeruginosa</i> implantation.</p>	<p>High bacterial diversity. Core microbiota^{vi}: 57 OTUs from 5 phyla (<i>Firmicutes</i>, <i>Proteobacteria</i>, <i>Actinobacteria</i>, <i>Bacteroidetes</i>, <i>Fusobacteria</i>). 13 predominant genera (relative abundance >1%), accounting for 94% of “core” reads.</p> <p>High prevalence of viruses: at least 1 detected in 52.6% of samples, ≥2 in 15.8%.</p> <p>9 OTUs from 6 genera (<i>Veillonella</i>, <i>Actinomyces</i>, <i>Prevotella</i>, <i>Haemophilus</i>, <i>Granulicatella</i>, <i>Streptococcus</i>) were persistent through early <i>P. aeruginosa</i> colonization.</p> <p>Limitations to interpretation: small numbers of participants with wide age range, no information regarding antibiotic exposures.</p>
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Prevaes <i>et al</i> 2016, Netherlands ⁶⁸	<p>Subjects: 20</p> <p>Samples: 324</p> <p>Males: 50%</p> <p>Age, mean (range): 2 (0.6-6.1 months.</p> <p>P.Phe508del homozygous: 80%</p> <p>Asymptomatic at diagnosis: 70%</p> <p>Sampling interval: monthly and with acute respiratory tract infection</p> <p>Controls: 45</p>	Nasopharyngeal swab	<p>Quantitative PCR</p> <p>16S rRNA sequencing (V5-V7 variable regions).</p> <p>Microbiological culture</p> <p>Viral PCR</p>	To investigate the development of the nasopharyngeal microbiota profiles in infants with CF compared with healthy controls during the first 6 months of life.	<p>Differences in microbiota of infants with CF compared with controls.</p> <p>CF: early <i>S. aureus</i> and to a lesser extent, <i>Corynebacterium</i> spp. and <i>Moraxella</i> spp. dominance, followed by a switch to <i>S. mitis</i> after 3-months. <i>S. aureus</i>, <i>S. mitis</i>, <i>Corynebacterium accolens</i> and bacilli more abundant.</p> <p>Lower bacterial density, particularly >4-months, related to antibiotic use.</p> <p>No significant differences in alpha-diversity between the 2 groups.</p> <p>Antibiotic use associated with increased colonisation of gram-negative and reduced colonisation of potential beneficial commensals.</p> <p>Controls: <i>Moraxella</i> spp. enrichment throughout. <i>Moraxella</i> spp., <i>Corynebacterium pseudodiphthericum</i>, <i>Corynebacterium propinquum</i> and <i>H. influenzae</i> more abundant.</p> <p>Limitations to interpretation: single center, short follow-up, small numbers and different environmental exposures between cases and controls for antibiotics and day care</p>
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Mika <i>et al</i> 2016, Switzerland ⁶⁹	Subjects: 30 Samples: 461, mean 15.4 per subject (range 4-24) Male: 43% P.Phe508del homozygous: 43% Sampling interval: fortnightly. Healthy controls: 47.	Nasal swabs	16S rRNA gene pyrosequencing Oligotyping analysis for differentiation between <i>Staphylococcus</i> species	Primary: to compare the nasal microbial composition of infants with CF with healthy controls during the first-year of life. Secondary: to examine the effect of antibiotic treatment on the nasal microbiota of infants with CF.	Different composition of nasal microbiota of infants with CF compared with healthy controls, even in the absence of antibiotic exposure. Increased relative abundance of Staphylococcae and decreased relative abundance of Corynebacteriaceae and Pasteurellaceae in infants with CF. At and after antibiotic treatment: decrease in <i>S. aureus</i> and increase in coagulase-negative Staphylococci. Limitations to interpretation: varying numbers of participants exposed/not exposed to antibiotics.
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Laguna <i>et al</i> 2016, United States ⁷²	<p>Subjects: 8 Samples: 12 Serial samples: 3 subjects Initial BAL (n=8): Male: 25% Age, median (range): 6.8 months (5.7-11.8) FEV_{0.5} (% predicted), median (range): 94 (69-116) P.Phe508del homozygous: 88% Second BAL (n=3): Male: 0 Age, median (range): 12.4 (11.9-13.2) months. FEV_{0.5} (% predicted), median (range): 124 (97-145) P.Phe508del homozygous: 100%</p>	BAL fluid Nasopharyngeal swab	16S rRNA sequencing (V1-V2 variable regions)	<p>Primary: to describe the BAL fluid microbiota of asymptomatic infants with CF during the first year of life. Secondary: to describe the relationship between BAL fluid microbiota, standard BAL fluid and NP culture and clinical characteristics at up to 5-years of follow-up.</p>	<p>Cross-sectional samples (n=8): Total bacterial load, median (range): 7.8-log₁₀ rDNA copies/mL (7.1-9.2). SDI, median (range): 2.2 (1.1-3.2). Richness, median (range): 70.9 (17.1-92.6). Evenness, median (range): 0.4 (0.2-0.6). 232 taxa detected across all samples, 10 most abundant: <i>Streptococcus</i> (31.7% median relative abundance), <i>Burkholderia</i>, <i>Prevotella</i>, <i>Haemophilus</i>, <i>Porphyromonas</i>, <i>Veillonella</i>, <i>Gemella</i>, <i>Neisseria</i>, <i>Lactobacillales</i>, <i>Methylobacterium</i>. <i>Streptococcus</i>, <i>Burkholderia</i>, <i>Prevotella</i>, <i>Haemophilus</i>, <i>Veillonella</i>, <i>Gemella</i>, <i>Neisseria</i> and <i>Methylobacterium</i> detected in all 8 initial samples; <i>Burkholderia</i> and <i>Prevotella</i> dominant in 3 and 1 sample respectively. Longitudinal samples: Beta-diversity (Morisita-Horn) ≤0.38 in 2 samples, ~1 in the 3rd sample. No association between BAL fluid neutrophil count and total bacterial load or SDI. Positive correlation between relative abundance of</p>
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Prevaes <i>et al</i> 2017, Netherlands ⁷³	Subjects: 17 Samples: 25 Serial samples: 8 subjects Male: 47% P.Phe508del homozygous: 88% Early BAL (n=13): Age, median (range): 4.6 (3-8) months Late BAL (n=12): Age, median (range): 12.4 (9-13) months	Nasopharyngeal swab Oropharyngeal swab BAL fluid.	16S rRNA sequencing (V5-V7 variable regions)	To assess inter- and intra-individual concordance of the microbiota at different “respiratory niches”. To explore the benefits of 16S rRNA gene sequencing compared with microbiological culture in clinical management of patients with CF. To evaluate temporo-spatial variation in lung microbiota in infants with CF.	There were significant differences in microbial diversity between the sample sites. The SDI was lowest in the nasopharyngeal samples and highest in the oropharyngeal samples. Intra-individual concordance between nasopharyngeal and BAL and oropharyngeal and BAL samples was variable, and both upper airway sites appear to contribute, but are not identical, to the lower airway microbiota. In those who contributed both “early” and “late” BAL samples, diversity increased over time, but microbial community composition varied. Limitations to interpretation: Unable to assess impact of antibiotic exposure on microbiota composition.
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<p>Frayman <i>et al</i> 2017, Australia⁷⁴</p>	<p>Subjects: 48 Samples: 95 Serial samples: 27 subjects Male: 52% Age, median (range): 20.4 (1.2-78.3) months. P.Phe508del homozygous: 68%</p>	<p>BAL fluid.</p>	<p>16S rRNA sequencing (V1-V3 variable regions)</p>	<p>To describe the lower airway microbiota in infants and young children with CF. To explore the relationship of the lower airway microbiota to lower airway inflammation and pulmonary function at 6-years.</p>	<p>Most prevalent genera: <i>Staphylococcus</i> (36.6%), <i>Streptococcus</i> (13.7%), <i>Pseudomonas</i> (6.1%), <i>Neisseria</i>, <i>Haemophilus</i>, <i>Gemella</i>, <i>Granulicatella</i>, <i>Prevotella</i>, <i>Veillonella</i>, <i>Streptomyces</i>. Microbial diversity decreased with increasing subject age (beta=-0.01, p=0.03). Detection of typical CF bacterial pathogens was associated with lower microbial diversity and with higher lower airway inflammatory indices. The composition of individuals' lower airway microbial communities varied considerably over time. There was no relationship between lower airway microbial diversity in early childhood and FEV₁ at age 6-years. Limitations to interpretation: Secondary analysis of BAL samples collected two decades prior.</p>
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^{vi} Disease controls: with other pulmonary diseases.

^{vi} “Core” microbiota: OTUs present in $\geq 50\%$ of samples at $\geq 0.1\%$ relative abundance.

ABBREVIATIONS:

16S rRNA	16S ribosomal RNA	NP	nasopharyngeal
BAL	bronchoalveolar lavage	OTU	operational taxonomic unit
CF	cystic fibrosis	PCR	polymerase chain reaction
FEV ₁	forced expiratory volume in one second	SDI	shannon diversity index

TABLE 3: THE AIRWAY MICROBIOTA IN EARLY CYSTIC FIBROSIS LUNG DISEASE*

UPPER AIRWAY	LOWER AIRWAY
Oropharyngeal specimens: Studies: 4 ^{51,71,73,81} Participants: 38 Samples: 106	Bronchoalveolar lavage specimens: Studies: 3 ⁷²⁻⁷⁴ Participants: 73 Samples: 132

<p>Common genera:</p> <p><i>Bacterioides</i></p> <p><i>Gemella</i></p> <p><i>Granulicatella</i></p> <p><i>Haemophilus</i></p> <p><i>Neisseria</i></p> <p><i>Prevotella</i></p> <p><i>Rothia</i></p> <p><i>Staphylococcus</i></p> <p><i>Streptococcus</i></p> <p><i>Veillonella</i></p>	<p>Common genera:</p> <p><i>Gemella</i></p> <p><i>Haemophilus</i></p> <p><i>Neisseria</i></p> <p><i>Prevotella</i></p> <p><i>Staphylococcus</i></p> <p><i>Streptococcus</i></p> <p><i>Veillonella</i></p>
<p>Nasopharyngeal specimens:</p> <p>Studies: 3^{68,69,73}</p> <p>Participants: 67</p> <p>Samples: 810</p> <p>Common genera:</p> <p><i>Corynebacterium</i></p> <p><i>Moraxella</i></p> <p><i>Staphylococcus</i></p> <p><i>Streptococcus</i></p>	<p>Lung tissue specimens:</p> <p>Studies: 1⁵¹</p> <p>Participants: 1</p> <p>Samples: 6</p> <p>Common genera:</p> <p><i>Anaerococcus</i></p> <p><i>Haemophilus influenzae</i></p> <p><i>Propionibacterium spp.</i></p> <p><i>Propionibacterium acnes</i></p> <p><i>Ralstonia pickettii</i></p> <p><i>Staphylococcus aureus</i></p> <p><i>Staphylococcus epidermidis</i></p>

*Studies of the airway microbiota in children with cystic fibrosis of average age 6-years or younger and their most prevalent genera are summarized. Genera with an overall prevalence and/or average relative abundance $\geq 2\%$ in two or more studies of individual specimen types are included. As there is

only one study of lung tissue, involving six specimens from a single subject, all genera with a relative abundance of $\geq 2\%$ listed. An early study of the BAL and upper airway microbiota is it combined data regarding the most prevalent genera in the upper and lower airway samples³⁹.

^{vii} Disease controls: with other respiratory conditions

^{viii} $>85\%$ of total sequences.

^{ix} Bile-aspiration status determined by liquid chromatography-mass spectrometry.

^x Children without CF undergoing bronchoscopy for clinical reasons.

^{xi} Dominant genus defined as the most abundant genus, with at least twice the abundance of the second most abundant genus.

^{xii} Sub-component of larger study aiming to explore the molecular mechanisms involved in the impact of aspirated bile acids on the respiratory microbiome.

ABBREVIATIONS:

16S rRNA	16S ribosomal RNA	OTU	operational taxonomic unit
BAL	bronchoalveolar lavage	PCR	polymerase chain reaction
CF	cystic fibrosis	PBB	protracted bacterial bronchitis
CFTR	cystic fibrosis transmembrane regulator	TNF- α	tumour necrosis factor alpha
DGGE	denaturing gradient gel electrophoresis	T-RFLP	terminal restriction fragment length polymorphism
FEV ₁	forced expiratory volume in one second	VEGF	vascular endothelial growth factor
IL	interleukin		

^{xiii} Disease controls: with other pulmonary diseases.

^{xiv} “Core” microbiota: OTUs present in $\geq 50\%$ of samples at $\geq 0.1\%$ relative abundance.

ABBREVIATIONS:

16S rRNA	16S ribosomal RNA	NP	nasopharyngeal
BAL	bronchoalveolar lavage	OTU	operational taxonomic unit
CF	cystic fibrosis	PCR	polymerase chain reaction
FEV ₁	forced expiratory volume in one second	SDI	shannon diversity index

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