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S Campbell, K Gerasimidis, S Milling, AJ Dicker, R Hansen, RJ Langley

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The Lower Airway Microbiome in Paediatric Health and Chronic Disease

Campbell S[1], Gerasimidis K[1], Milling S[2], Dicker AJ[3], Hansen R[3] Langley RJ[4-5*]

1. School of Medicine, Dentistry and Nursing, University of Glasgow
2. School of Infection & Immunity, University of Glasgow
3. Division of Molecular and Clinical Medicine, School of Medicine, University of Dundee
4. Department of Paediatric Respiratory & Sleep Medicine, Royal Hospital for Children, Glasgow
5. Department of Maternal and Child Health, School of Medicine, Dentistry and Nursing, University of Glasgow

*Corresponding author:

* Professor Ross Langly

Department of Respiratory and Sleep Medicine,

Royal Hospital for Children, Glasgow,

1345 Govan Road, Glasgow

Scotland, G51 4TF.

ross.langley@ggc.scot.nhs.uk

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Educational Aims

The reader will gain an improved understanding of:

- Key terminology used in microbiome research for respiratory paediatrician.

The methods used to investigate microbial communities in the lungs, including their advantages and limitations.

- Differences in the development of lung microbiota in preterm and term infants
- Associations between lung microbiota in chronic lung disease in children and the contribution role of oral taxa in disease.
- The gut-lung axis.

Abstract

The advent of next generation sequencing has rapidly challenged the paediatric respiratory physician's understanding of lung microbiology and the role of the lung microbiome in host health and disease. In particular, the role of "microbial key players" in paediatric respiratory disease is yet to be fully explained. Accurate profiling of the lung microbiome in children is challenging since the ability to obtain lower airway samples coupled with processing "low-biomass specimens" are both technically difficult. Many studies provide conflicting results.

Early microbiota-host relationships may be predictive of the development of chronic respiratory disease but attempts to correlate lower airway microbiota in premature infants and risk of developing bronchopulmonary dysplasia (BPD) have produced mixed results. There are differences in lung microbiota in asthma and cystic fibrosis (CF). The increased abundance of oral taxa in the lungs may (or may not) promote disease processes in asthma and CF. In CF, correlation between microbiota diversity and respiratory decline is commonly observed. When one considers other pathogens beyond the bacterial kingdom, the contribution and interplay of fungi and viruses within the lung microbiome further increase complexity. Similarly, the interaction between microbial communities in different body sites, such as the gut-lung axis, and the influence of environmental factors, including diet, make the co-existence of host and microbes ever more complicated. Future, multi-omics approaches may help uncover novel microbiome-based biomarkers and therapeutic targets in respiratory disease and explain how we can live in harmony with our microbial companions.

Keywords

Lung Microbiome, Children, Chronic Respiratory Disease, Gut-Lung Axis, Nasopharyngeal-lung Axis

Definitions

Term	Definition
Microbiota	The interacting bacteria, archaea and fungi (1).
Microbiome	The microbiota, their functions and viral elements (1).
Lungs/Lower Airway Microbiota	Microbiota sampled from anatomical sites below the vocal cords, outlined in Figure 1. The tracheal aspirate (TA), sputum or bronchoalveolar (BALF) microbiota refers to the microbes collected from the lower airways via the respective sampling methods.

α-diversity	Diversity within an individual/sample. Characterised by species richness (number of different taxa) and evenness (the relative abundance of different taxa), or both (2).
β-diversity	Comparison of microbial communities between individuals/samples. Generally based on distances between points on a multidimensional plot (2).

Abbreviations

16S ribosomal rRNA (16S rRNA); bronchoalveolar fluid (BALF); bronchopulmonary dysplasia (BPD); CCL4 = Chemokine (C-C motif) ligands 4; in chronic suppurative lung disease (CSLD); cystic fibrosis (CF); cystic fibrosis transmembrane conductance regulator (CFTR), GPR41 = G-protein coupled receptor 41; human microbiome project (HMP); immunoglobulin A (IgA) ; internal transcriber spacer (ITS); nasopharyngeal (NP); programmed death-ligand 1 (PD-L1); pulmonary exacerbations (PEx), regulatory T-cells (Treg cells) respiratory syncytial virus (RSV); primary ciliary dyskinesia (PCD); short chain fatty acid (SCFA); sn-glycerol

Introduction

When one considers the importance of communities in biological systems and ecology, it is important to reflect that human society thrives on diversity and dynamism – the same could be hypothesised for the lung microbiota.

The Human Microbiome Project (HMP), undertaken in 2007, marked a turning point in microbiology with a rapid shift away from the reliance on culture-based techniques towards molecular/genetic characterisation. HMP set out to characterise the microbial colonisers at various mucosal surfaces in humans answering the question "what microbes are there", whilst also providing links between microbiota in health and disease (3). Yet, the lungs were not explored, likely due to the long-held belief that the lower airways were sterile (4). Hilty and colleague's seminal work produced a paradigm shift, revealing an array of bacterial genetic material which differed between the healthy/diseased lungs of children and adults - the "lung microbiota" (5). Such findings stimulated interest in the role of lung microbiota and the application of ecology theory to gain a better understanding of chronic respiratory diseases (6, 7).

The Lung Microbiota

The respiratory tract can be anatomically classified into upper and lower compartments, with the lower airways conferring their own unique microbiota, is the least understood microbiota niche within the total "airway system" (Figure 1) and may be critical to pulmonary development in early life.

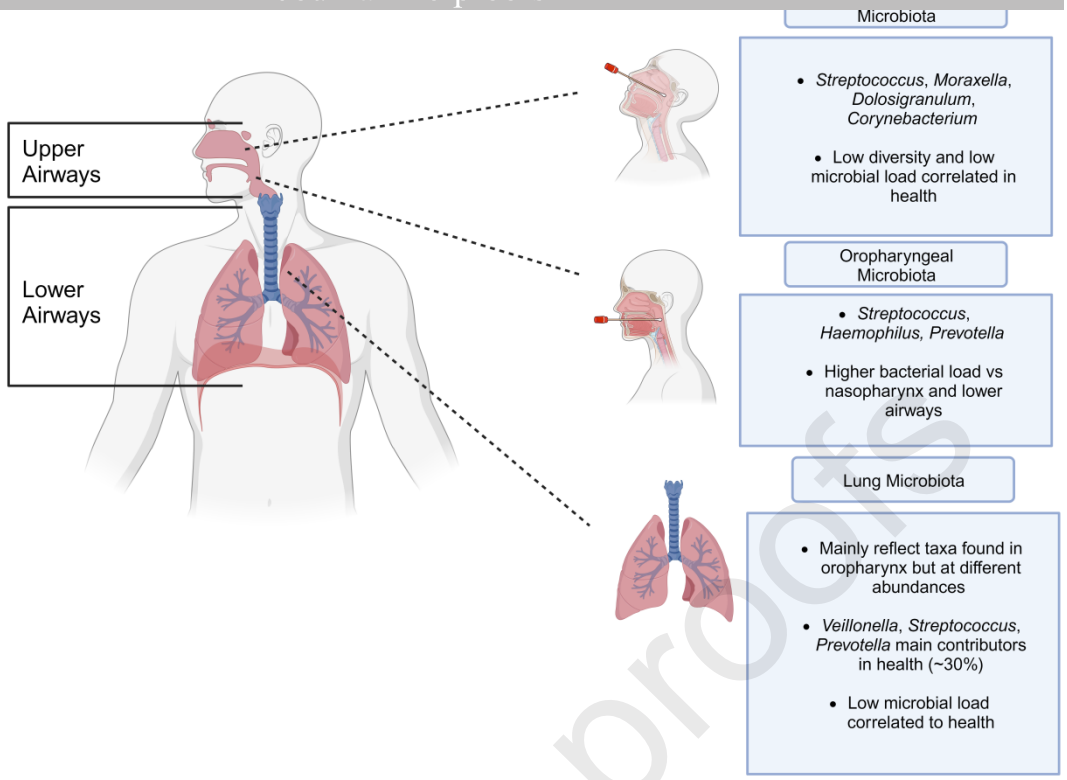

Figure 1: The upper and lower airway microbiota. The airways are broadly separated into upper and lower compartments. Microbiota sampling of the nasopharynx, oropharynx and lung, has demonstrated each site possesses a unique microbiota.

"The Hygiene Hypothesis" suggests that more diverse microbiota-host interactions are beneficial to host health (8). Furthermore, *In vivo* evidence supports causal relationships between lung microbiota and lung physiology (8). In murine neonates, the absence of airway commensals disrupts pulmonary tolerance to allergen exposure, by inhibiting the formation of regulatory immune cells, and is linked to development of experimental asthma. This process can be abrogated by introducing lung commensals in the first 2 weeks of life, but not afterward, suggesting a 'window of opportunity' exists shortly after birth where microbiota-host interactions shape pulmonary development (9). Lung microbiota differences have been demonstrated between term and preterm infant in the first weeks of life. Clinically, the preterm lung microbiota is less diverse than full-term counterparts, suggesting lung microbiota could in part explain the observation that premature infants are at higher risk for the development of chronic respiratory issues (10),

Furthermore, unlike the gut and oral microbiota which are thought to be relatively stable, the lungs likely possess a 'dynamic' polymicrobial environment which exhibit bidirectional movement (upward and downward) within the airways (11). Lung microbiota are predominantly derived from the oral cavity/ oropharynx although the relative abundances of microbiota species differ along the respiratory tract (12-17). Microbiota translocate into the lungs mainly via micro-aspiration of bacteria and inhalation of fungal spores (18, 19). Microbial load declines distally in the lower airways with numbers 100-fold lower at alveolar regions than at the oropharynx (20, 21) [Figure 2]. Immigration events are finely balanced by clearance mechanisms in the lungs. Immune factors

particles upwards and out of the lower airways. Additionally, the lungs naturally possess an array of "local factors", creating a harsh environment that inhibit microbial growth (22, 23). This microbial immigration-to-clearance equilibrium maintains a dynamic, diverse, but low abundant, microbiota – potentially a marker of lung health (17).

In chronic lung disease, disruption to local factors may create an imbalance between microbial immigration and clearance mechanisms, leading to disrupted microbial communities in the lungs which may contribute to disease processes (12). This transition from dynamism to stability may be a key metric of severe respiratory decline (24). Moreover, the increased presence of oral taxa in the lower airway mays contribute to inflammatory processes and worse outcomes (18, 19). For example, in CF, impaired lower airway clearance mechanisms may create niche opportunities that permit an increase in the abundance of oral taxa, in turn, providing nutritional support for pathogenic growth in the lungs (25). Furthermore, increased presence of orally derived microbiota within the lower airways of children with asthma have been linked to exacerbations (26).

Herein we examine clinical evidence comparing lung microbiota composition between healthy term and preterm infants and review microbiota involvement in three chronic lung conditions: BPD, asthma and CF. Additionally, we consider whether oral-associated taxa may promote disease processes in asthma and CF.

Although general microbiological trends emerge, high variability is commonly reported between patients, indicating highly personalised lung microbiota, like data from gut studies (22, 23). Relationships between lung microbiota diversity and paediatric lung disease is unclear with the exception of more advanced stages in CF, suggesting taxonomic descriptions alone may be insufficient, underlining the need to combine microbiota presence with functions such as the metabolites generated and genes expressed in samples which is currently lacking (27, 28). The issue of obtaining appropriate lower airway samples in paediatrics and handling specimens that have low microbial biomass have complicated research. Further challenges reflect technical issues with experimental reproducibility, and availability of appropriate controls (29).

Despite these challenges and complexities, it is essential that respiratory paediatricians understand the role of microbiota in lung disease since future treatment strategies may involve manipulating lung microbiota to improve disease outcomes (30).

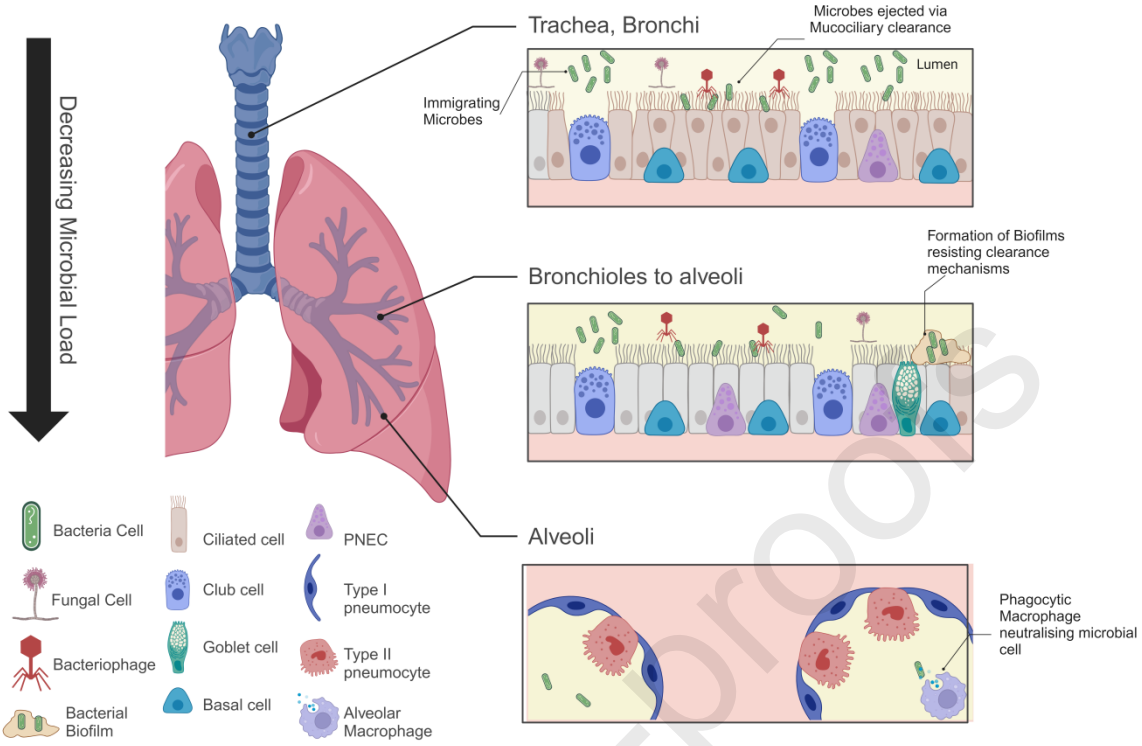

Figure 2: Microbial load declines distally within the lower airways. Microbial immigration from the upper airways is juxtaposed by microbial clearance mechanisms in the lower airways, maintaining a dynamic, low microbial biomass, environment in respiratory health. Microbial numbers progressively decline in more distal lung regions. Bacteria are thought to outnumber fungi and viruses in the lungs, although more work is required to validate the contribution of viruses and fungi to the lung microbiome (19). The mucus lining the epithelium creates a possible niche for bacteriophages, which may act as a non-host derived form of bacterial immunity. The formation of bacterial biofilms could insulate bacteria from phage and host defences including mucociliary clearance. The presence of bacteriostatic substances and phagocytic macrophages likely explain the low microbial load at the alveolar regions. PNEC = pulmonary neuroendocrine cells.

Methods in lung microbiome research

Historically, our understanding of pulmonary microbiology has been driven by a reliance on culture-based microbiology and the need to "grow" bacteria on growth media in particular environmental conditions. The arrival of amplicon sequencing (Figure 3), mediated by next-generation sequencing (NGS) technologies, and metagenomics, overcomes these culture-dependent biases. DNA from hard-to-culture anaerobic organisms has been consistently identified in the lungs. These approaches are rapidly challenging our understanding of lung microbiology, however, NGS also confer limitations.

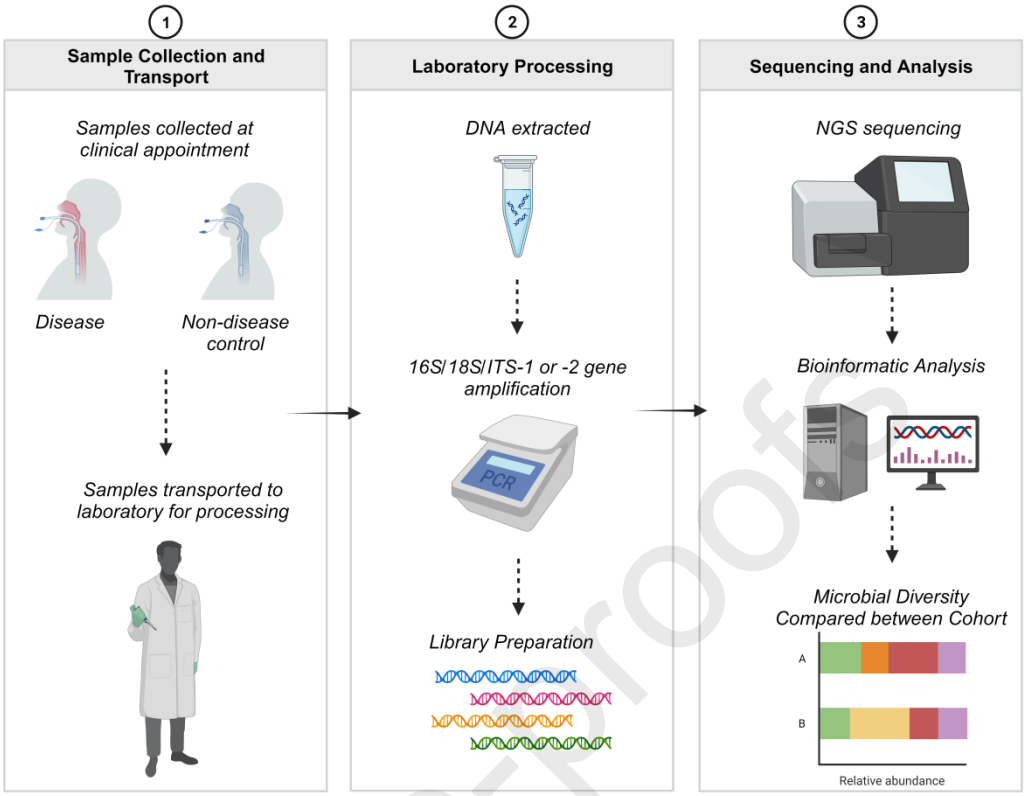

Figure 3: Example of amplicon sequencing workflow (e.g. 16S rRNA gene sequencing) in lower airway microbiome studies informing 'what is there' in the samples. 1: Lower airway samples collected and transported to the laboratory. 2: DNA is isolated from samples (host and microbial DNA). Most studies to date have employed amplicon sequencing PCR approaches by targeting variable regions of the highly conserved genes in bacteria (16S gene) or fungi (18S gene, ITS-1, ITS-2 gene), allowing selective targeting of microbial DNA (and not host DNA). Library preparation enables 'barcoding; of DNA fragments permitting sample multiplexing and identification during sequencing and bioinformatics analysis. 3: Libraries are then typically outsourced for NGS. Bioinformatics analyses are performed where sequencing 'reads' are mapped to a reference database that can accurately identify microbes with the taxonomic level depending on the length of sequence and adequacy of match, but as low as genus level. Expression of the relative abundance of phyla and genera of microbes present in samples between groups e.g. healthy vs disease that are compared in terms of diversity (31).

Amplicon sequencing

Bacteria are the main contributors to the lung microbiome (32, 33). Molecular probes target hypervariable portions (V1-V9) of the bacterial/archaeal 16S rRNA gene [Figure 3]. Selectively targeting a particular hypervariable region can identify bacteria/archaea down to the genus level, however, the choice of hypervariable region amplified can significantly impact downstream results, since no single region can discriminate between all taxa (34). Full-length 16S sequencing may provide species-level resolution but has rarely been applied to date (35, 36). *Prevotella*, *Veillonella* and *Streptococcus* may account for approximately one-third of species in healthy lungs (37). Others have found that, in some individuals, the heathy lung microbiota signatures are indistinguishable from microbial DNA found in negative control samples, whilst the identification of orally-derived taxa, such as *Prevotella* and *Veillonella,* are associated with

subjects (18, 19).

Fungi and viruses are less well characterised in the airways. Fungi can be examined by targeting the 18S rRNA gene or internal transcriber spacer (ITS)-1 or ITS-2 regions and are found at low levels in health with prevalent taxa including *Aspergillus* and *Cladosporium* (38, 39). The durable fungal cell wall requires a more vigorous cell-lysis approach to release DNA prior to nucleic acid extraction, though this can also be relevant to a lesser extent for Gram-positive bacteria (40, 41). Viruses do not contain highly conserved genetic elements, however, can be investigated via metagenomic sequencing (42).

Shotgun Metagenomics

Shotgun metagenomics sequences all DNA within a sample, providing the opportunity to analyse the entire microbiome including DNA-based viruses and the functional capacity of microbes, for example the presence of antimicrobial resistant genes in bacteria (43).

Bacteriophages may be the predominant viral entity in the lung; however, further work is required to fully elucidate the role of all "commensal" viruses, including RNA-based viruses, within the lungs (33, 42, 44).

Technical limitations have impacted the use of metagenomics in lung microbiome studies (45). The high host-to-microbe genetic content in samples means microbial enrichment (especially viral enrichment) and/or host depletion methods are necessary. Low viral-host genetic material ratio in the lungs means accurate viral analysis requires different sample processing compared to samples interrogated for bacterial/fungal DNA (42, 43).

Similarly, because microbial sequences identified need to be matched against databases of known organisms, fungal and viral sequences are poorly represented compared to bacteria (50, 51).

Limitations of DNA-based sequencing approaches

Since DNA-based sequencing approaches are unable to differentiate between live and dead microbes, methods have been developed that can deplete so-called relic DNA (53). Yet, relic DNA may still induce physiological effects via interactions with host immune factors and therefore contribute to the disease process (54, 55).

Thus, studies are needed that complement DNA-sequence approaches with functional investigations, providing a more comprehensive understanding of microbiota-host relationships. Metatranscriptomics can provide information on gene expression of host and microorganisms and elucidate RNA-based viruses (28, 42). Metabolites and proteins present in specimens can be investigated via metabolomics and proteomics, respectively. These approaches can be used to correlate microbiota changes to functional profiles within lower airways to determine possible "cause and effect" between the lung microbiome and pulmonary health/disease, particularly in longitudinal studies (28). Such approaches may establish novel "endotypes" – subclassifications of disease based on microbiome markers, as has been attempted in paediatric asthma (46). Multi-

between individuals in lung microbiota studies (18, 19, 28, 47, 48).

Sampling the Paediatric lung

Lung microbiota specimens in children are mainly collected during flexible bronchoscopy, sputum (spontaneously produced or induced) or tracheal aspiration (TA). All sampling modalities confer strengths and limitations and reflect distinct microbiota from the lower airways microbial entities, making cross-comparisons challenging (12-17).

Bronchoscopy

Bronchoscopy sampling via bronchoalveolar lavage fluid (BALF) can collect from 1/40 of total lung surface area including alveolar regions, whilst the use of a specimen brush may manage to dislodge mucosal-adherent taxa in the conducting airways. However, paediatric bronchoscopy is performed under general anaesthesia and are reserved for children with respiratory complications, making serial sampling and the recruitment of healthy children difficult (40).

Sputum

Sputum collection is arguably the most readily available and feasible option for serial sampling of the lungs, although younger children cannot expectorate and those who can typically present with more severe disease (40). Induced sputum (via inhalation of nebulised saline) may permit inclusion of healthy children in studies, although may be deemed unethical in infants, restricting the recruitment of younger cohorts (49). Furthermore, sputum must traverse the upper airways increasing risk of contamination from the more abundant upper airway microbiota (16, 40).

Tracheal Aspirate

Children that are intubated during a procedure or receiving mechanical ventilation for other reasons can permit TA sampling from the endotracheal tube, with the latter allowing serial sample collections. TA microbiota is likely distinct from samples collected more distally and may represent a mixture of microbiota entering from the upper airways and microbiota being removed from the lungs (40). Samples collected two-hours post intubation have also been shown to underrepresent obligate anaerobes and/or overrepresent aerobes compared to TA samples taken at time of intubation (50). Therefore, it may be to good practice to report sampling times.

Although there is no established gold-standard method of sampling, protected bronchoscopy may be the most effective to minimise pharyngeal contamination at time of sampling, since contamination is a major consideration in lower airway microbiota studies (51).

Low-biomass contamination

Lower airway specimens contain a low microbial load or "low-biomass". Low-biomass samples follow a "power-law dynamic", meaning the lower the biomass the greater the impact of sample contamination has (52). Contamination is commonly introduced from laboratory reagents, the adjacent environment and DNA extraction kits, called 'kitome' contamination; all can significantly drown the "true signal" from the test sample (53, 54). This is a difficult problem to resolve since many contaminants are microorganisms also present in the respiratory tract, therefore, complete bioinformatic removal of contaminants may result in loss of biologically relevant taxa (55). The inclusion of sampling and processing controls is highly recommended to improve contaminant detection, however many paediatric studies have not reported such strategies; this may impact accuracy of findings (55-57).

Laboratories should assess their "limit of detection" - the threshold concentration at which sample DNA is overcome by contaminant DNA. This can be achieved using a "mock community" positive control- a diverse range of *in vitro* microorganisms found in the airways of known composition and concentration, although in lung microbiota research has rarely been employed (55).

Previous reports performing such approaches indicate this limit of detection may lie between 10^4-10^6 copies of 16S rRNA, which may be the biomass of paediatric BALF samples (37, 54, 56, 58). In addition, metagenomics may require 10^8 microbial DNA to reliably classify microbiota present within samples (59). Therefore, reporting of the microbial burden in samples should be a necessary component in low-biomass studies (57). Samples that fall below an established threshold may not be used for microbiota sequencing, improving the quality of data.

Despite the technical challenges and limitations, many studies have begun to explore the role of the lung microbiota from birth and beyond.

Lung microbiota in the first weeks of life - predictors of lung disease?

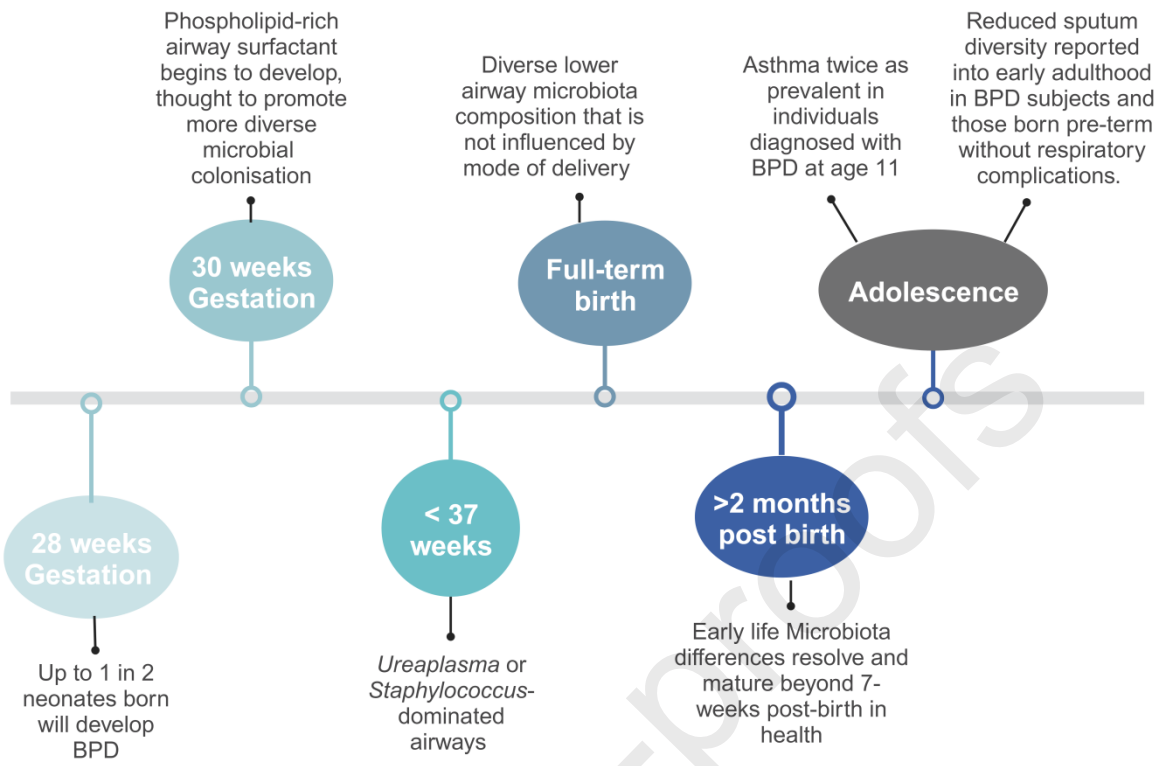

Figure 3: Development of the Lung Microbiota. Infants born before 28 weeks gestation are at high risk of developing bronchopulmonary dysplasia. Beyond 30-weeks' gestation, the production of phospholipid-rich airway surfactant begins to reach physiological concentrations and is thought to be more hospitable for diverse microbial colonisers. This may explain the reduced diversity noted in premature infants, who are more likely to present a lower airway microbiota dominated by *Staphylococcus* (C-section delivery) or *Ureaplasma* (vaginal delivery). In full-term infants, greater microbial diversity is thought to exist from birth and cannot be distinguished based on route of delivery, differing from the preterm airways. Despite these early-life differences, lung microbiota differences are thought to resolve and stabilise beyond 2 months post-birth **(10)**. How these altered colonisation patterns in the first weeks of life impact chronic airway development is unknown. At age 11, asthma was twice as common in children BPD who were born extremely prematurely compared to children born at term, but no difference in asthma incidence was found in the same cohort at age 19 **(60, 61).** Moreover, by adolescence, preterm infants (both BPD and non-BPD diagnosed) sputa displayed significantly lower α-diversity with reductions in Bacteroidota abundance, mainly *Prevotella melaninogenica*, compared to full-term subjects, hinting that early life events may chronically impact lung microbiota composition and development **(61).**

Infant host-microbiota interactions in health

The early predictors of lung health or disease in children involve ante-, peri- and post-natal factors [Figure 4]. Preterm delivery impacts normal staged lung development and early exposure to oxygen as well as other environmental insults [eg positive pressure ventilation] likely play a detrimental role. The role of infective agents is also an important consideration since preterm delivery may be triggered by *in utero* inflammatory or infectious events (62). Thus, infant host-microbe interactions likely influence longitudinal lung health (9).

Pattaroni *et al*. investigated the tracheal microbiota in ventilated preterm (n=26) and term (n=19) mechanically ventilated "healthy" infants in the first

microbiota (10).

In preterm infants, the microbiota clustered into *Staphylococcus* or *Ureaplasma*-dominated environments in caesarean-section and vaginally delivered neonates, respectively. In term infants, a more diverse microbiota colonised the trachea irrespective of mode of delivery. Beyond 30-weeks' gestation, airway surfactant increases in phospholipid content which may create a more hospitable environment, permitting more diverse microbial interactions in the lower airways in gestationally older neonates. Interestingly, in all infants, the TA microbiota stabilised, and differences resolved, beyond the second month post-birth, with communities reflective of the lower airways in adults. Abundant genera include *Veillonella*, *Porphyromonas*, *Prevotella*, *Streptococcus* and *Neisseria*, with the latter two proposed as keystone genera shaping the lower airway microbiota structure in healthy infants (10).

Transcriptomics was also performed on TA samples from a subset of participants and appeared to indicate microbial influence on the host. Immunoglobulin A (IgA) and anti-IgA pathways were upregulated, suggesting microbial 'priming' of mucosal immunity in early life, or, conversely, airway mucosal immunity influences microbiota composition/colonisation (10). Importantly, the study did not control for BPD within the study population (40% of preterm infants), questioning our understanding of the development "healthy", preterm, lung microbiota in the first weeks of life.

Bronchopulmonary Dysplasia

Up to half of infants born before 28 weeks gestation are at risk of developing BPD, since the canalicular, saccular and early alveolar stages of lung development can be impaired. BPD is characterised by altered alveolar development, impaired pulmonary vascularisation and chronic inflammation leading to prolonged requirement for ventilatory and/or supplemental oxygen support (62, 63). Numerous risk factors have been identified but the role of lung microbiota is, as yet, unclear (64). Early sampling in "at risk babies" for BPD can provide insight to the initial colonisers and/or changes to lower airway microbiotas prior to established disease.

Ureaplasma and BPD

The role of *Ureaplasma* in the lower airways and BPD development is controversial (65-68).

Staphylococcus (68%) and the vaginal commensal *Ureaplasma* (18%), were identified as most dominant microbes in the trachea over the first 3 weeks of life. Infants who developed BPD had lower abundance of *Staphylococcus* and higher abundance of *Ureaplasma* after birth plus greater microbial community instability (68). In preterm infants with increased abundance of *Ureaplasma*, reductions in total bacterial was noted, although this may have been influenced by antibiotic therapy (10). Gallacher *et al*. collected longitudinal samples in 55 preterm infants over the first month of life (50 developed BPD), linking antibiotic use to increased abundance of members of the Mycoplasmatota (previously Tenericutes) phylum, particularly *Ureaplasma* and *Mycoplasma*, microbes not susceptible to routinely prescribed antibiotics. Furthermore, Il-6

treatment, suggesting a pathogenic role for these taxa with cytokine levels peaking one-week post-birth (69). Other studies have acknowledged the presence of *Ureaplasma* but failed to find associations with BPD risk (66, 67). Therefore, the premature airways are likely more susceptible to *Ureaplamsa* colonisation in vaginally-delivered infants, and commonly prescribed antibiotic therapy may reduce competition causing an increase in the relative abundance of these intracellular bacteria such as *Ureaplasma* and *Mycoplasma* in TA samples (10, 69). However, a causal link between *Ureaplasma* abundance in early life with BPD development requires further study.

Pseudomonadota, *Lactobacillus* and BPD

Reduced microbiota diversity caused by increased abundance of the Pseudomonadota (previously Proteobacteria) phylum linked has been correlated to the development of BPD (70-72).

Stenotrophomonas (on day 1 of life) has been associated with the development of severe BPD (but not mild-moderate disease) and appears to correlate with increasing concentrations of sn-glycerol 3-phosphoethanolamine (sn-G3PE), a cell membrane constituent, involved in glycerophospholipid metabolism and potential biomarker in BPD (72).

Others found levels of Enterobacteriaceae and *Lactobacillus* were positively and negatively linked to BPD, respectively (71). Individuals with lower levels of *Lactobacillus* in TA samples collected within 6-hours of birth went on to develop BPD, suggesting a protective role in the developing airways (71). In germ-free mice, introduction of *Lactobacillus* at the nose positively influences alveolar development and lung immunity (73, 74). Further possible links between altered microbiota at birth and metabolic changes in the lower airways have been described. The airway metabolome of BPD-infants is augmented in pathways related to fatty acid-metabolism including both androgen and oestrogen generation; such findings may underlie the sex-related differences in BPD development (75).

However, there are important caveats to consider. There is often a lack of adequate reporting on sampling and/or processing controls (57). Microbial DNA positively, (*Acinetobacter*, Enterobacteriaceae, *Stenotrophomonas*), or inversely (*Lactobacillus*) linked to BPD can be found in laboratory reagents (54). Moreover, BPD is characterised by disrupted alveolarisation, therefore, tracheal microbiota may not accurately reflect the alveolar ecosystem (40, 62). However, there are practical and ethical issues with repeated sampling alveolar regions in neonates (40, 69).

Whilst premature birth appears to reduce lower airway microbial diversity, further studies are required to separate specific microbiota associations with health and disease in young infants. Early-life exposure to a diverse range of microorganisms has been shown to be inversely proportional to the risk of asthma development suggesting early life exposure to diverse microbes and establishment of a varied airway microbiota may have an important role in the development of lung disease (8, 76).

Asthma

Asthma is very common in children, characterised by increased mucus production, reversible airway obstruction, airway inflammation and remodelling, resulting in impaired lung function. Airway inflammation in asthma is characterised by an exaggerated Th2, eosinophilic response, whilst increased Th1 and Th17 responses have been described in corticosteroid -resistant and difficult asthma (77). Despite asthma affecting the upper *and* lower airways, there is currently a lack of data relating to lower airway microbiota research in paediatric asthma. Moreover, studies have mainly examined small sample sizes (5, 78, 79).

Increased abundance of *Haemophilus* and *Staphylococcus*, and reductions in *Prevotella*, have been observed paediatric asthma (5). Others found no significant differences between children with severe asthma and disease controls, although evidence of bronchial inflammation was reported in controls (78). A study that incoroprated healthy controls into their study design found differences in α-and-ß-diversity between asthmatics and healthy controls (79). Asthmatic children demonstrated increased abundance of fungal *Malasezzia* and lactic acid-generating bacteria *Streptococcus* and *Enterococcus* coincided with reductions of an unknown fungus and *Lactobacillus*. Moroever, reductions in *Penicillium aethiopicum* and *Alternaria* species were viewed in children with difficult asthma. Differences in bacterial species were observed between paeditaric and adult asthmatic sputum, suggesting age-dependent changes of the lung microbiota, although longitdunal studies are needed to stenghten these age-depedent of lower airway microbiota in asthma (79).

Another important question is how therapies impact lung microbiota in asthma (or vice-versa). Drug-dependent changes in paeditrac asthmatic sputum microbiota have been shown between steroid therapy to subjects on dual steroid and leuktirene receptor anatagonsts although the clinical importance of this observation is unknown (79).

Importantly, most paediatric asthma exacerbations are triggered by viral pathogens, in particular rhinovirus, however, there are no microbiome studies which have incorporated the role of lower airway viruses in paediatric asthma (80). In asthmatic adult sputum, the integration of DNA-based viruses demonstrated greater predictability of disease severity than 16S rRNA gene analyses, therefore, should be a future research aim in paediatrics (81).

Overall, there is a lack of data investigating relationships between lower airway microbiota and asthma. Longitudinally studies sampling the lower airways shortly after birth and follow infants over childhood that assess incidence of asthma development would strengthen mechanistic animal work that implicates early-life microbiota to asthma development (9). Furthermore, our understanding of how lung microbiota develops over time in children is largely derived from studies in CF.

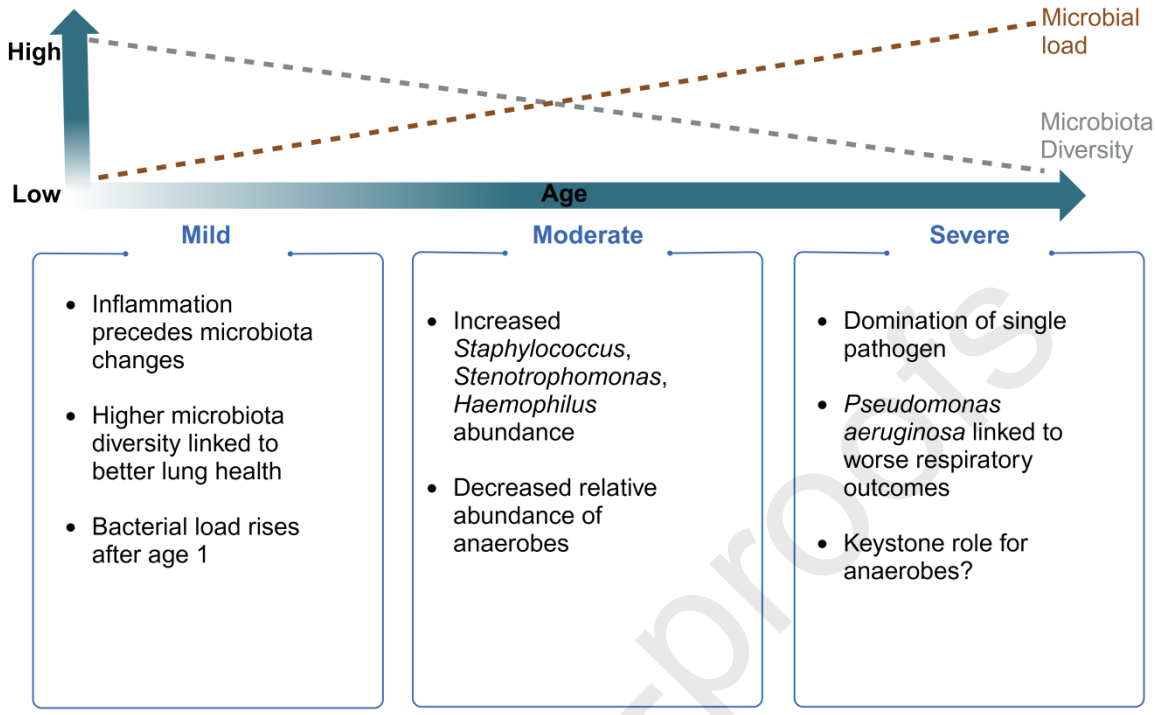

Figure 4: Polymicrobial changes in the CF lung are tightly linked with lung decline across childhood. A reduction in lower airway diversity and concomtiant rise in microbial load is seen as chuldren age with microbiota alteratons corrleated to mild, moderate and severe disease states. The advent of CF modulators may delay this process and studies investigating the effects of modulators on lung microbial diversity and mainteneance of lung health are of interest and underway.

CF lung disease is typified by a constant cycle of neutrophilic inflammation and infection, which, coupled with cystic fibrosis transmembrane conductance regulator (CFTR*) dysfunction* results in viscous secretions, impaired airway clearance and chronic infection by "traditional" CF-associated respiratory pathogens such as *Pseudomonas aeruginosa* (82). Studies utilising NGS have observed polymicrobial activity in the lungs, including *Streptococcus*, *Veillonella* and *Prevotella*, referred to as "non-traditional" taxa (83-87). Some suggest depleted lung microbiota diversity is tightly linked to disease progression in childhood (Figure 5) (88-96).

Inflammation in the airways may precede microbiota alterations (89). As the child ages, bacterial load rises and may be associated with structural changes in the lung (97). Reductions in Bacteroidota, Bacillota (Previously Bacteroidetes and Firmicutes, respectively) and Fusobacteria occur, with increases in Pseudomonadota (93). *Veillonella* and *Prevotella* are also abundant in youth with *Granulicatella* spp. and *Streptococcus mitis* indicative of greater microbial diversity and better pulmonary health (98). Increased abundance of *Staphylococcus*, *Stenotrophomonas* and *Haemophilus* may represent moderate stage of disease, with a single respiratory pathogen, commonly *Pseudomonas aeruginosa*, dominating the microbial environment in severe states (24, 89, 90, 93, 95, 99-101). *Staphylococcus* presence has been correlated with the amino

increased bacterial load, and may permit progression to chronic pathogen colonisation (102). Increased abundance of *H. influenzae*, may protect from *P. aeruginosa* domination, possibly via priming the innate immune system (103, 104).

Comparing the lower airways of CF and primary ciliary dyskinesia (PCD), *Haemophilus* is more prevalent in children with PCD, with *P. aeruginosa* typically emerging later, supporting a potential protective role for *Haemophilus* against *Pseudomonas* colonisation and lung function decline (94). Diverse lower airway microbiota has also been reported in children newly diagnosed with non-CF bronchiectasis. Interestingly, diversity nor microbial load differed from healthy controls (105). Furthermore, no significant differences in microbiota composition were found in children with CF, non-CF bronchiectasis and protracted bacterial bronchitis (PBB), suggesting a 'core' microbiota that is lost as disease progresses into adulthood (106). For example, in older non-CF bronchiectasis patients with more severe disease , the increased abundance of *P. aeruginosa* and *Haemophilus* results in depleted microbiota diversity, suggesting that attempts to maintain lung microbiota diversity may inhibit disease progression in chronic suppurative lung disease (CSLD) (105, 106).

The contribution of fungi and viruses to the CF microbiome is less well understood. *Candida* has been recognised as the dominant fungi in CF with *Aspergillus* contributing around 5% to the fungal community (100, 101). However, no significant differences in fungal BALF abundance have been found between CF and non-CF children (107). Lower airway viral analysis in children with CF show high levels of *Pseudomonas*, *Burkholderia* and *Streptococcus* phage, reflecting the increased abundance of these bacteria in the CF lungs (33).

Effects of exacerbations and therapeutics on CF lung microbiota

Antibiotics are commonly prescribed to improve both acute symptoms and decline in lung function during a pulmonary exacerbation (PEx); over time, the number of exacerbations is associated increased respiratory morbidity and mortality (18, 19, 21). Furthermore, the use of prophylactic antibiotics in CF differs between nations and may impact microbiota development and explain interstudy variability. Prophylactic flucloxacillin is not routinely used in the USA but is currently recommended in children until age 3 in the U.K. (108).

Interestingly, acute impact of antibiotics on airway diversity in CF are unclear. Pittman *et al*. showed that anti-Staphylococcal antibiotics were linked to reduced airway diversity and lower inflammation (109). However, results may have been confounded by patient age with better inflammatory profiles viewed in younger CF patients (89). Felton *et al* found differences in species richness with an increase in opportunistic pathogens following antibiotic treatment, with upregulation of sulphate assimilation correlated to *Escherichia* persistence. Moreover, long chain fatty acid metabolic pathways were enriched which the authors speculate are synthesised by respiratory pathogens that persist following antibiotic treatment, promoting the inflammatory responses (110). Others report microbiota resilience during antibiotic treatment (111, 112). Therapeutic intravenous beta-lactam administration reduced microbial diversity at least 1-month post treatment, compared to those who received subtherapeutic doses

However, a follow-up study incorporating a larger cohort showed the opposite effects; sub-optimal doses showed greater acute depletion in microbiota diversity (114). Long-term, airway diversity may recover following acute depletion, although lung function remains compromised compared to functioning prior to PEx (115).

CF modulators have drastically improved outcomes and PEx rates in CF patients likely by improving CFTR function and mucociliary clearance (116, 117). Such therapies are predicted to improve life expectancy (118). Modulator therapies appear to increase lung diversity and reduce pathogen colonisation, potentially by achieving a greater balance between the ratio of "immigration to clearance" in the lungs (119). One study in 3 children showed that the increased abundance of *S. mitis* corresponded to poor treatment response whilst abundance of *Prevotella* and *Porphyromonas* was linked to better response (120). Promising changes in the gut microbiome have been noted in response to treatment, thus modulators may promote better lung outcomes via the gut-lung axis (121).

Other considerations - Oral microbiota in paediatric lungs – signatures of lung disease?

Aspiration events are common in healthy subjects(122). In mice, aspiration of the human oral commensals *Prevotella melangenica, Veillonella parvula* and *S. mitis* (despite being rapidly removed from the lungs) 'primed' pulmonary innate immunity, decreasing susceptibility to *S. pneumoniae* infection long after the aspirated oral commensals had been cleared, suggesting low levels of aspiration events contribute to lung homeostasis (123). Yet, the increased rates of aspiration of these orally-derived taxa have been linked to Th17-immune response in the lungs of healthy adults, supporting the hypothesis that the imbalance between microbial immigration and clearance mechanisms is disruptive to pulmonary physiology (18, 19).

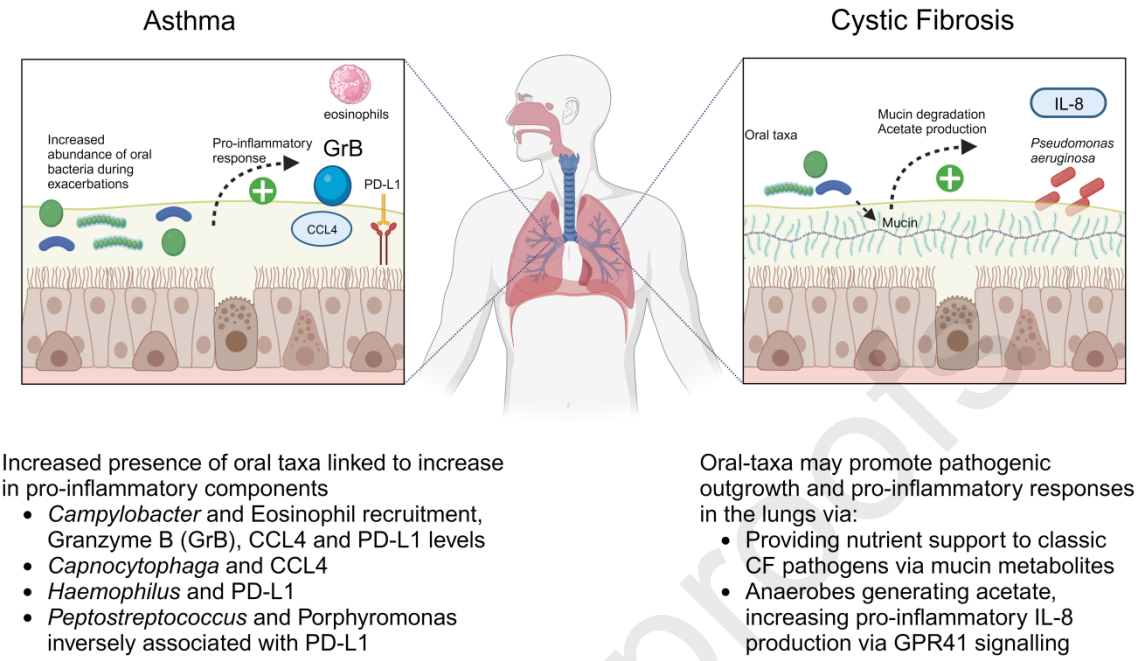

Figure 5: The posited detrimental role of oral taxa in paediatric asthma and CF. Increased oral taxa have been noted during exacerbations in sputum of paediatric asthmatic patients which may promote pro-inflammatory responses in the airways. Debate on the role of anaerobic orallyassociated taxa exists in CF. Some suggest oral taxa facilitate the domination of classical CF pathogens such as *Pseudomonas aeruginosa* by providing nutrient support via mucin degradation in the airways. CCL4 = Chemokine (C-C motif) ligands 4; PD-L1 = Programmed death-ligand 1; GPR41 = G-protein coupled receptor 41; IL-8 = interleukin-8

Oral taxa and asthma

Increased abundance of orally-derived (putative) commensals *Prevotella* and *Veillonella* at the hypopharynx in young infants were linked with asthma risk at the age of 6 (124). Moreover, increases in orally derived bacteria during asthma exacerbations were noted in the largest study to date. Kim *et al* found significant differences in ß-diversity between stable asthma and those with asthma exacerbations, indicating communities abundant in oral taxa were linked with pulmonary inflammation during PEx (26).

Numerous bacterial genera were associated with exacerbations including *Campylobacter*, *Haemophilus*, *Neisseria*, *Granulicatella*, *Peptostreptococcus*, *Fusobacterium*, and *Streptococcus (26)*. The authors demonstrated changes in inflammatory proteins between stable and asthma exacerbations outlined (Figure 6). Interestingly, *Camplyobacter* showed significant correlation with eosinophil recruitment, implying increased aspiration of this orally derived microbe could worsen asthma symptoms (26). Similar observations have been noted in adults with eosinophilic-driven chronic obstructive pulmonary disease (COPD), supporting microbiota associations with an eosinophilic response (125). In 83 asthmatic children, predominantly with Th2-driven asthma, sputum

Haemophilus and *Neisseria* correlated to a mixed neutrophillic and eosinophillic inflammation, worse lung function and PD-L1 levels. Clusters with higher relative abundances of *Prevotella*, *Veillonella* and *Actinomyces* correlated to better outcomes (46). Whether microbial presence is a cause or consequence of inflammatory endotypes needs to be established.

Oral taxa and CF

Rothia and *Fusobacterium* in sputum have been suggested as indicators of better lung function, with correlation between higher abundance of oral taxa to lower inflammatory markers, in CF (126). Others have linked a single pathway in the oral microbe *Veillonella atypica* with better lung function. Moreover, the authors associated *Staphylococcus aureus* abundance with PEx suggesting its metabolite and nucleotide generation may permit long-term infection and inflammation in the lungs in a nutrient-poor environment, a potential trigger for PEx, supporting positive associations with oral taxa and detrimental effects of "classical pathogens" in CF (127).

Alternatively, orally derived taxa, persisting at lower levels, could mediate the transition toward a pathogen-dominated lung. The increased generation of mucus flakes and lactate can provide niche's for oral anaerobes, which in turn, may provide nutritional support via mucin degradation for pathogens such as *P. aeruginosa,* which cannot metabolise mucin in isolation (25, 96, 128). Additionally, oral anaerobes may directly exacerbate inflammation via the fermentation of short chain fatty acids (SCFA's), with acetate generation increased in more advanced disease. Acetate can mediate its pathogenicity through G-protein-coupled receptor 41 (GPR41), which is upregulated in the CF bronchi, increasing IL-8 production, potentially exacerbating neutrophil activity in the CF airways (129).

However, one study has challenged the presence of oral taxa in CF. The study collected protected BALF specimens and retrieved paired samples from oral and pharyngeal sites in addition to collecting negative controls during sampling by rinsing sterile saline through the bronchoscope. The authors delineated that oral taxa DNA was present at similar levels in the sampling instrument compared to CF BALF samples, which were characterised by the domination of a single pathogen. *Streptococcus*, *Veillonella* and *Prevotella* were more abundant than controls, albeit at very low levels, therefore their role as 'keystone species' for pathogen takeover in the CF lung cannot be excluded (130). Children under the age of 5 were not included in this study; it is difficult to extrapolate this data in younger patients with greater microbial diversity, although these findings highlight the importance of collecting control samples at time of sample collection (96, 109, 131).

The unified airway hypothesis

The NP microbiota has been hypothesised as gatekeepers of respiratory health (133). The 'unified airway hypothesis', considers the upper and lower airways as a single-interconnected organ, where pathology in one site can induce changes at another (133). Moreover, bacterial and viral pathogens can multiply at the NP before migrating down the respiratory tract, exacerbating symptoms in lung disease (134-136). Two-thirds of participants suffering asthma PEx contained

disturbances may impact lower airway microbiota composition and play a role in asthma severity (26).

Chun *et al* investigated the relationship between the NP and lower airways in paediatric asthma. Despite failing to show differences in diversity between the NP of healthy and asthmatic children, an increase in *Streptococcus* and reduction in *Corynebacterium* relative abundances were noted in the NP of asthmatics. The microbial-transcriptome relationships between nasal and bronchial brushings in asthmatics suggested *Actinomyces* presence in the lungs was inversely linked with bronchial inflammation (137). Moreover, reductions of NP *Corynebacterium* correlated with airway inflammation, supporting findings in adult subjects that found reductions in *Cornynebacterium* was inversely associated with proinflammatory molecules in the lungs (137, 138).

The gut-lung axis

The gut-lung axis is a postulated bi-directional link between the gut and lung microbiomes, mediated via microbiota interactions with immune cells and metabolic-derived molecules (139). Disruptions to gut microbiota have been illustrated in early life in respiratory disease (139). Furthermore, a causal association has been found between inflammatory bowel disease (IBD) and future development of interstitial lung disease (but not vice-versa), supporting a causal link between gut and lung disorders (140). Gut microbiota may influence lung immunity indirectly via interactions with intestinal immune cells that can subsequently migrate via the lymphatic system or bloodstream, mediating microbiota-host interactions at the lungs. Moreover, a fibre-rich diet has been linked to improved pulmonary function, potentially via the actions of SCFA's, predominantly synthesised by gut microbiota, from dietary fibre (141). Mckay *et al* examined dietary effects on the gut and lung microbiota of children with CF, demonstrating that a high-fat diet, characteristic in CF patients, could impact gut microbiota composition (142). The study postulates that diet could indirectly modulate lung physiology via changes to gut microbiota impairing metabolites generation such as SCFA's. Anti-inflammatory gut microbiota including A*kkermensia* abundance in stool correlated with better lung function, although the mechanistic relationships were not investigated(142). Direct transit of gastric materials into the lungs can also occur. Gastric-derived bile acids have been noted in the lower airways of CF patient, directly correlating to levels of pulmonary inflammation, suggesting reflux and subsequent aspiration of contents may also be a mechanism driving interactions between the gut and lungs (143-145).

Gut microbiome alterations have long been appreciated in asthma. Reductions in anti-inflammatory-associated microbiota such as *Faecalibacterium* and increased abundance of potentially pro-inflammatory microbes such as *Clostridium difficile* have been shown. Breastfeeding has been shown to be protective in asthma development, possibly via breastmilk-derived human oligosaccharides (which enhance the growth of SCFA producers in the gut (146). Such associations support future research into the interplay between diet, gut-and-lung microbiota, and lung physiology in early life and may lead to therapeutic targeting of chronic lung issues, via the gut microbiome.

Conclusion

The paediatric lung microbiota is currently an under investigated area of research. Reduced diversity of gut microbiota appears to be a hallmark of various disorders, however in the lungs the relationship between lung microbiota diversity and chronic lung disease is still unclear. The increased abundance of oral taxa may be hallmarks of respiratory disease. Greater investigation into polymicrobial communities, including fungi and viruses is needed, alongside further explanation of the functional consequences of changes to these communities in health and disease. It is essential future studies include adequate controls. The gut-lung axis and unified airway hypotheses may elucidate the importance of factors rarely considered in paediatric respiratory disease, such as the impact of diet and SCFAs or NP disturbances on the lung microbiome.

A multi-microbiome, multi-omics approach is an ambitious research aim, but to understand the role, and interplay, of bacteria, fungi, viruses, and the human host in paediatric respiratory health and disease, one must consider all aspects of this complex web of interactions.

Future Research Directions

Interactions between microbiota and host in health and disease is complex and studies that integrate multi- site microbiome analyses remain limited. The understanding of oral hygiene and effects on the lung microbiome is unclear in children, although in adults poor oral health is linked to lung disease (132). Future studies should consider the effects of oral microbiota and metabolites on the lung microbiota in paediatrics. Similarly, nasopharyngeal-lung and gut-lung connections have emerged, suggesting integrating samples from extrapulmonary sites and investigating their functional effects may advance our understanding of the relationship between the microbiome and pulmonary disease.

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There are no conflicts of interest.