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Host–microbe interactions in distal airways: relevance to chronic airway diseases

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ABSTRACT This article is the summary of a workshop, which took place in November 2013, on the roles of microorganisms in chronic respiratory diseases. Until recently, it was assumed that lower airways were sterile in healthy individuals. However, it has long been acknowledged that microorganisms could be identified in distal airway secretions from patients with various respiratory diseases, including cystic fibrosis (CF) and non-CF bronchiectasis, chronic obstructive pulmonary disease, asthma and other chronic airway diseases (e.g. post-transplantation bronchiolitis obliterans). These microorganisms were sometimes considered as infectious agents that triggered host immune responses and contributed to disease onset and/or progression; alternatively, microorganisms were often considered as colonisers, which were considered unlikely to play roles in disease pathophysiology. These concepts were developed at a time when the identification of microorganisms relied on culture-based methods. Importantly, the majority of microorganisms cannot be cultured using conventional methods, and the use of novel culture-independent methods that rely on the identification of microorganism genomes has revealed that healthy distal airways display a complex flora called the airway microbiota. The present article reviews some aspects of current literature on host–microbe (mostly bacteria and viruses) interactions in healthy and diseased airways, with a special focus on distal airways.



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Introduction

This review is the summary of the fourth of a series of workshops exploring the roles of distal airways in chronic airway diseases, including asthma [1], chronic obstructive pulmonary disease (COPD) [2] and other

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respiratory diseases [3]. The focus of this workshop, which took place in Avignon, France, in November 2013, was the roles of microorganisms in chronic respiratory diseases, with a special interest on distal airways.

Inhaled air is contaminated with pollutants, particles and microorganisms (e.g. viruses, bacteria and fungi) that enter the airways during breathing. Until recently, it was assumed that lower airways were sterile in healthy individuals, although it was recognised that upper airways could be colonised by microorganisms. However, it has long been acknowledged that microorganisms could be identified in distal airway secretions from patients with various respiratory diseases, including cystic fibrosis (CF) and non-CF bronchiectasis, COPD, asthma and other chronic airway diseases (e.g. post-transplantation bronchiolitis obliterans). These microorganisms were sometimes considered as infectious agents that triggered host immune responses and contributed to disease onset and/or progression; alternatively, microorganisms were often considered as colonisers, which were considered unlikely to play roles in disease pathophysiology. Importantly, these concepts were developed at a time when the identification of microorganisms exclusively relied on culture-based methods. It is now clear that the majority of microorganisms cannot be cultured using conventional methods, and the use of novel culture-independent methods that rely on the identification of microorganism genomes has revealed that healthy distal airways display a complex flora called the airway microbiota. Recent studies have focused on changes in the airway microbiota under various pathophysiological conditions. The objective of the present article was to review some aspects of current literature on host–microbe (mostly bacteria and viruses) interactions in healthy and diseased airways, with a special focus on distal airways.

Importance of distal airways in chronic airway diseases

Distal airways, which are usually defined as non-cartilaginous bronchioles with an internal diameter <2 mm, are considered an important component of chronic airway diseases, including asthma [1], COPD [2], CF [4] and chronic bronchiolitis [3]. Thus, distal airways are the main site of airflow limitation in asthma [5], COPD [6, 7] and post-transplantation bronchiolitis obliterans [8], and these airways appears particularly vulnerable to inhaled pollutants (e.g. cigarette smoke) [7, 9].

Although reviewing immune mechanisms in the lung is beyond the scope of this article, there is evidence of innate and adaptive immune mechanisms in distal airways [1–3]. For example, chronic airway diseases are characterised by recruitment of phagocytes (e.g. neutrophils and macrophages) in distal airways and alveoli, and lymphoid follicles have been described in distal airways of COPD patients with severe airflow limitation [10]. These latter structures have been suggested to reflect viral or bacterial infection in distal airways [11]. Importantly, bronchiolar and alveolar epithelium and lung macrophages express pathogen-recognition receptors (e.g. Toll-like receptors) that are necessary to recognise specific pathogen-associated molecular patterns for developing an immune response. Although little data exists on differential response to pathogens in patients with various airway diseases, there is evidence that alveolar macrophages of exacerbation-prone COPD patients are more refractory to cytokine induction by common pathogens (e.g. nontypeable *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* and *Moraxella catarrhalis*), presumably due to diminished Toll-like receptor-2 and -4 signalling [12]. These data suggest that the immune response to specific microbes could be impaired in disease due to host-specific factors.

From gut to lung microbiome

The Human Microbiome Project was launched in 2007 to study microbial inhabitants of the human body [13]. At that time, it was realised that human biology may depend, in a large part, on interaction with microbes that live on human mucosal surfaces including skin, mouth and throat, vagina, nostrils and gastrointestinal tracts [13]. Because the human lung was believed to be sterile, it was not included in the original Human Microbiome Project [14, 15], although it was later realised that microbial communities also existed in the lung [16]. As a result, knowledge in this field has evolved more rapidly in the gastrointestinal tract than in the lung, and much has to be learnt from studies performed in the gastrointestinal tract.

The human gastrointestinal tract hosts more than 100 trillion bacteria and archaea, which together make up the gut microbiota. The human gut microbiota can be considered an organ within an organ that co-evolved with humans to achieve a symbiotic relationship leading to physiological homeostasis. The human host provides a nutrient-rich environment and the microbiota provides indispensable functions that humans cannot exert themselves. Since most of the bacteria inhabiting the gut are uncultivable, their functions cannot be inferred from composition data. Knowing which microbes are there is not sufficient. Meta-omics has been developed to answer essential questions such as “What is the genetic potential of the non-cultured bacterial fraction of the gut microbiota?” and “What are these microbes really doing?”

Advances in cultivation-independent methods based on analysis of the sequence of the bacterial 16S ribosomal small subunit rRNA gene (which is present in bacterial but not in mammalian genome) quickly expanded our knowledge about the diversity of the microbial ecosystems. First applied to the human gut

in the late 1990s [17], the actual number of gastrointestinal tract phylotypes (molecular species) detected using molecular techniques has far outnumbered the cultivated gut species. From more than 1200 microbes described, only 12% were recovered by application of both molecular and cultivation-based approaches, while the vast majority (~75%) were only detected through 16S rRNA gene sequencing [18]. Chaotic in the early stages of human life [19], the sequential assembly of the human gut microbiota leads to bacterial communities that remain stable over time in healthy conditions in the absence of external perturbations [20]. The average total number of bacterial species was estimated to be close to 1000 per individual, whereas 10 000–40 000 are predicted for the whole microbiota population [21].

While culture-independent approaches, mainly based on the 16S rRNA gene, provided a better description of the human gut microbiota and the wide diversity of their 100 trillion inhabitants (*i.e.* the microbiota), recently developed meta-omics allowed the microbiome to be described, originally defined as the totality of microbes, their genetic elements (genomes) and environmental interactions in a defined environment. In this sense, the human microbiome would represent the collection of microorganisms associated with the human body, and their collective genomes would constitute a metagenome. However, the term microbiome is now commonly used to refer to the collective genomes present in members of a given microbiota.

Within the field of meta-omics, metagenomics refers to the genomic analysis applied to all the microorganisms of a microbial ecosystem without previous identification. It encompasses culture-independent studies of the structures and functions of microbial communities and their interactions with the habitats they occupy to understand their biological diversity [22]. Metagenomics provided the evidence that the human gut microbiome contains more than 100 times the number of genes encoded by our own genome [23]. Moreover, human populations can be clustered into three main groups, *i.e.* enterotypes, based on their microbiome. These enterotypes are characterised by dominant genera and their co-occurring phylogenetic groups that significantly separated the population into three distinct clusters [24].

Remarkably, shifts in the bacterial make-up of the human gut microbiota have been associated with digestive tract dysfunctions such as inflammatory bowel disease (IBD), irritable bowel syndrome and obesity. 10 years ago, the concept of dysbiosis or unbalanced composition of the intestinal microbiota was introduced in the research field of IBD [25]. Even though a tremendous number of specific bacteria have been shown to be modulated in the IBD microbiota [26], no relevant specific triggering agent has been highlighted, emphasising a clear role of this broad dysbiosis in bacterial communities. A decrease in commensals and symbiotic bacteria belonging to the Firmicutes phylum and associated with an increase in pathobionts (or opportunistic pathogens), mostly from the Proteobacteria phylum, seem to be present in most of these inflammatory conditions. Moreover, a decrease in microbial diversity has repeatedly been described in IBD patients in terms of both bacteria and bacterial genes richness. If this is true for IBD, a similar dysbiosis associated with a drastic loss of diversity may be crucial in other pathologies, such as obesity or metabolic syndrome [27].

The discovery of the lung microbiome has led to a growing number of studies over the past 5 years, and understanding the roles of the lung microbiome in health and disease is now the subject of many studies that have been summarised in recent perspective articles [16, 28, 29]. Although progresses have been made regarding methodological issue (*e.g.* techniques used for sampling) and descriptions of the main characteristics of the lung microbiome in health and some chronic airway diseases, much is still to be learnt regarding the factors associated with changes in microbial communities and the relevance of these changes to specific host response and disease mechanisms. Figure 1 highlights the possible host–microbe interactions and their relevance to chronic airways disease.

Host-bacteria interactions in chronic airway diseases

In the next section, we examine the relevance of bacteria to chronic airway disease summarising knowledge obtained using both culture-dependent and culture-independent methods.

Cystic fibrosis

CF is a genetic disease characterised by abnormal ion transport, leading to a multi-organ disease predominantly involving the lungs with prominent airway inflammation, leading to bronchiectasis and chronic respiratory failure [30]. Chronic airway infection with specific Gram-negative (*e.g.* *Pseudomonas aeruginosa* and/or *Burkholderia cepacia*) and Gram-positive bacteria (*e.g.*, *Staphylococcus aureus*) are well-recognised features of CF lung disease [30, 31], explaining the major interest in host–microbe interaction in this disease. At the time of lung transplantation in CF patients, studies of lung explants have revealed that most distal airways show structural abnormalities with mucous plugs [4] containing bacteria macrocolonies [32, 33]. However, in earlier stages of the disease, microbiological data were mostly obtained using bacterial culture of sputum, which are easy to obtain in older children and adult CF patients but do not specifically reflect distal airways.

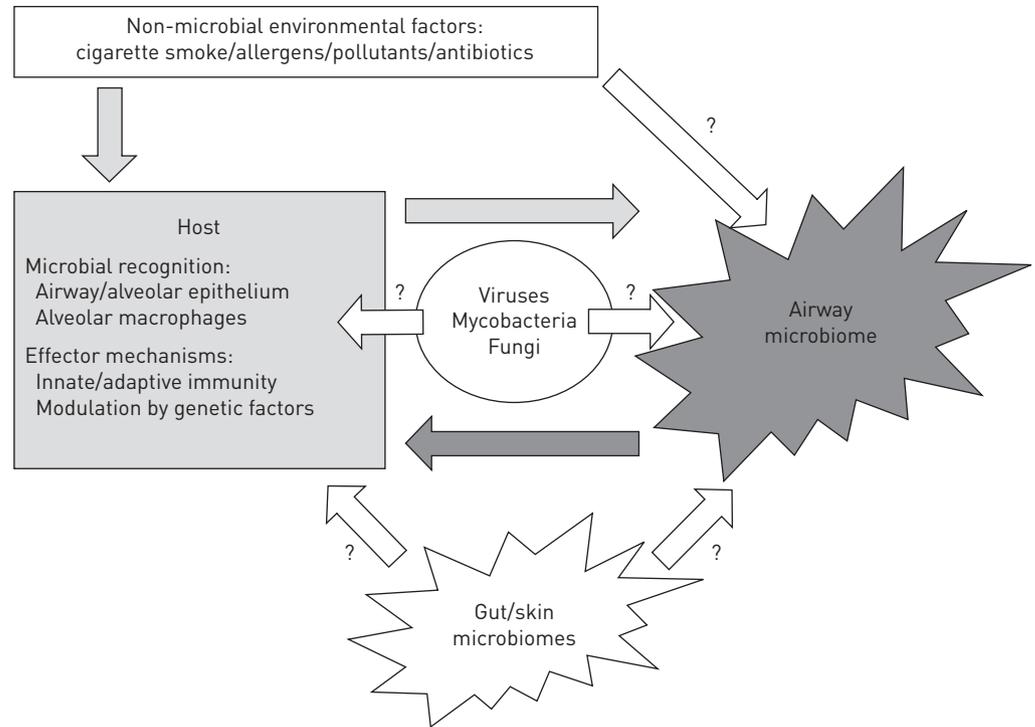


FIGURE 1 Schematic representation of host–microbe interactions in airways. Bacterial airway microbiome is composed of multiple and interacting bacterial communities. Bacteria may interact with other microbes (e.g. mycobacteria, viruses and fungi) and with the host, modulating immune responses. These interactions can be modulated by the effects of environmental factors on the host and/or the microbiome. The role of other bacterial communities, including gut and skin microbiome, in modulating airway diseases is also emerging.

Distal airways in CF neonates, in whom lung structure and function were considered normal at birth, were considered sterile when no pathogen was cultured in upper airway samples [34]. Studies that used cultures of bronchoalveolar lavage (BAL) in young children diagnosed by newborn screening revealed that bacterial infection (e.g. by *S. aureus*, *P. aeruginosa* or *H. influenzae*) in distal airways often occurred early in life in CF children [35–37]. In the AREST-CF (Australian Respiratory Early Surveillance Team for Cystic Fibrosis) study, detection of proinflammatory bacteria (e.g. *P. aeruginosa*, *S. aureus*, *H. influenzae* and *S. pneumoniae*) in BAL fluid in the first 2 years of life was associated with a clinically significant reduction in lung function measured by forced expiratory volume in 0.75 s (FEV_{0.75}) [38]. The roles of bacteria cultured in CF airways appear different among different bacteria, although most bacteria cultured in usual aerobic conditions have been associated with significant clinical impact. Chronic airway infection with *P. aeruginosa* and/or *B. cepacia* [30] is clearly associated with increased airway inflammation, faster lung function decline and poorer prognosis, whereas the impact of other Gram-negative pathogens, including *Stenotrophomonas maltophilia* [39, 40] or *Achromobacter xylosoxidans* [41–43], is more controversial. Chronic *S. aureus* airway infection has been independently associated with haemoptysis in CF patients [44] and recent studies showed that positive sputum cultures for small colony variants (slow-growing antibiotic-resistant mutants) of *S. aureus* [45] or methicillin-resistant *S. aureus* [46] were independently associated with disease progression in CF patients. Positive cultures for *S. aureus* in the BAL fluid of young CF children was associated with a greater rate of decline in lung function [47], further indicating the role of *S. aureus* infection in CF distal airways. Finally, distal airway infection with *P. aeruginosa*, *S. aureus* or other microbes (mixed oral flora and *A. fumigatus*) was associated with increased IL-8 levels and neutrophil elastase activity in the BAL fluid of young children with CF [48]. Neutrophil elastase activity in BAL fluid is associated with early bronchiectasis in CF children [49].

Because anaerobic conditions exist in mucous plugs in CF airways [32], TUNNEY *et al.* [50] hypothesised that anaerobic bacteria, which are not detected by routine aerobic culture methods, could contribute to the pathophysiology of CF lung disease. These authors found that anaerobic species (*Prevotella*, *Veillonella*, *Propionibacterium* and *Actinomyces*) were cultured in large numbers from the sputum of adults with CF and the BAL fluid of children with CF [50]. Colonisation with *P. aeruginosa* significantly increased the likelihood that anaerobic bacteria would be present in the sputum, thus suggesting interspecies interactions. This latter suggestion was sustained by the fact that all of the anaerobic isolates tested were

susceptible to meropenem, a common antibiotic used in the treatment of *P. aeruginosa* exacerbation where it shows some superiority over other antipseudomonal antibiotics [50, 51]. However, the role of anaerobic bacteria in CF lung disease remains to be established.

Recently, studies have relied on culture-independent methods to examine bacterial contents in CF airways [52]. An important aspect in studies assessing the microbiome in CF patients relates to variability of results in samples obtained in different airway compartments. Sputum may be difficult to obtain in children and paediatric microbiome studies are often based on oral swabs [53, 54]. GODDARD *et al.* [55] performed molecular identification of microbiome in oral swabs, sputum and pulmonary explants of CF patients: oral swabs and sputum were discordant in terms of diversity and composition, whereas sputum analysis identified prominent lung pathogens found in pulmonary explant analysis. End-stage CF lungs are heterogeneous in terms of tissue damage, suggesting the possibility of topographical variations in microbiota composition. GODDARD *et al.* [55] analysed lung microbiota in pulmonary explants from different pulmonary lobes, and found an absence of topographical significant change in microbiota composition in 10 patients with end-stage CF lung disease.

The CF microbiome is a complex and dynamic bacterial community [54, 56, 57]. The microbiome is less diverse and much richer in patients with severe CF compared to patients with severe COPD [58]. With the use of culture-independent methods for microbial detection [52, 59], emerging pathogens are becoming of interest. Dynamic microbial composition, richness and diversity in CF airways is now better characterised [53, 54, 57, 60–63]. The composition of the CF microbiome can be altered due to clinical (*e.g.* age and FEV₁ decline) and environmental factors (*e.g.* presence of *P. aeruginosa*, antibiotic exposure, *CFTR* genotype and gut microbiome) [53, 54, 57, 60–63]. Initially, the airways of children with CF display a rich and diverse microbiome and over time and disease progression the bacterial community decreases in terms of diversity, often displaying a predominant pathogen (*e.g.* *P. aeruginosa* and/or *B. cepacia*) [53, 54, 57, 60].

Experimental studies are only beginning to explore the role of microbiome in CF lung disease. A novel CF neonate pig model showed earlier and richer bronchial colonisation and a decrease in bacterial clearance [64, 65], suggesting a predisposition for airway microbiome dysbiosis in CF lungs. Exposure of *Drosophila* to various airway microbiota (obtained from sputum in 44 CF patients) showed three different profiles of fly survival: 1) a synergistic effect of both CF microbiota and *P. aeruginosa* with a diminution of fly survival compared to flies exposed to *P. aeruginosa* alone; 2) no effect of CF microbiota with or without *P. aeruginosa*; and 3) a protective effect of CF microbiota when associated to *P. aeruginosa* [66]. This study suggests a clear impact of bacterial interactions in microbiota which may exhibit protective or deleterious effects on the host.

The role of antibiotics on airway microbiome is controversial with studies indicating that airway microbiome is highly resilient with a return to basal composition soon after exacerbation and intravenous antibiotics [60, 61], whereas other studies describe a significant change in microbiome composition (decreased diversity) linked to antibiotic exposure [53, 67]. These contradictory findings could be related to a threshold in antibiotic exposure after which the microbiome loses its resilience capacity, a phenomenon which has already been described in the gut microbiome [68]. Overall, antibiotic treatments for recurrent exacerbations have been proven to have a favourable impact on the life expectancy of a CF patient [69]. Nevertheless, understanding the long-term impact of modified microbiome composition due to recurrent antibiotic treatment and/or studying the effect of targeted antibiotic treatments against prominent pathogens as well as non-cultivable bacteria could help in developing novel therapeutic strategies.

Non-CF bronchiectasis

P. aeruginosa and *H. influenzae* are prominent pathogens in patients with non CF-bronchiectasis, and chronic colonisation with *P. aeruginosa* in sputum was associated with poorer prognosis [70]. Recent studies have explored the airway microbiota in patients with non-CF bronchiectasis and a positive correlation between bacterial diversity and FEV₁ was reported [71]. ROGERS *et al.* [72] observed a correlation between microbiota composition and clinical outcome. These authors proposed that patients could be classified into three groups according to the composition of their airway microbiota: 1) *P. aeruginosa* dominated; 2) *H. influenzae* dominated; and 3) other taxa dominated. Patients with *P. aeruginosa*- and *H. influenzae*-dominated communities had significantly worse lung function. Predominance of *P. aeruginosa*, followed by *Veillonella* species, was the best predictor of future exacerbation frequency [72]. Detection of *P. aeruginosa* was associated with poor lung function and exacerbation frequency, irrespective of analytical strategy [72]. The hypothesis that disruption of the lung microbial ecosystem by infection, inflammation and/or antibiotic therapy creates a disturbed, less diversified microbial community with downstream consequences for immune function remains to be further investigated. For example, direct and/or indirect interactions between the predominant species and the wider bacterial community could be implicated in disease outcome [73].

COPD

The role of bacteria in the lower airways of patients with COPD has long been suspected, but has been difficult to establish [74]. To date, most studies have been performed using culture-based techniques with only a few studies being performed using new molecular techniques. Furthermore, many studies relied on sputum examination whereas only a few studies have sampled distal airways using bronchoscopy. SETHI *et al.* [75] studied BAL in 26 ex-smokers with stable COPD, 20 ex-smokers without COPD and 15 healthy nonsmokers. The authors reported that potentially pathogenic bacteria were cultured (≥ 100 colony forming units·mL⁻¹) in approximately one-third of stable COPD patients, whereas potentially pathogenic bacteria were not recovered in ex-smokers without COPD and were cultured in only one of the healthy nonsmokers [75]. Other bronchoscopic studies have also used culture-based techniques and have reported the presence of potentially pathogenic bacteria in the distal airways in ~30% of stable COPD patients [76–78]. The most frequently isolated potentially pathogenic bacteria was *H. influenzae*, but *S. pneumoniae*, *M. catarrhalis* and *P. aeruginosa* were also found in some COPD patients. One study further described intracellular NTHi in proximal airways of COPD patients by performing *in situ* hybridisation and immunofluorescence microscopy in bronchial biopsies [78]. An important finding was that colonised COPD (*i.e.* COPD patients with potentially pathogenic bacteria in distal airways) had greater neutrophil counts and increased concentrations of inflammatory biomarkers (*e.g.* IL-8 and active matrix metalloproteinase-9) compared with non-colonised COPD patients or ex-smokers and nonsmokers, suggesting that bacterial colonisation could contribute to progression of airway disease in COPD [75]. ZHANG *et al.* [79] collected sputum samples at baseline and after 1 year in 46 COPD patients and found that 37% of these patients had bacterial colonisation (defined by $\geq 10^6$ colony forming units·mL⁻¹) at baseline. COPD patients with lower airway bacterial colonisation had increased sputum concentration of IL-8, IL-6 and tumour necrosis factor (TNF)- α compared with COPD patients without colonisation [79]. Interestingly, lower airway bacterial colonisation at baseline was associated with greater decline in lung function and with increased numbers of COPD exacerbations [79]. In a study of 54 COPD patients, PATEL *et al.* [80] further suggested that the presence of potentially pathogenic bacteria and inflammatory biomarkers (IL-6 and IL-8) in sputum were increased in COPD patients with bronchiectasis and were associated with more frequent and more severe COPD exacerbations.

Another major finding came from molecular characterisation of bacterial strains cultured in sputum of COPD patients. SETHI *et al.* [81] reported that culture of a new strain of *H. influenzae*, *M. catarrhalis* or *S. pneumoniae* in sputum was more often associated with the occurrence of COPD exacerbations than when previously isolated strains of these bacteria were cultured. Interestingly, exacerbations associated with new bacterial strains showed increased levels of sputum TNF- α and neutrophil elastase compared with exacerbations associated with pre-existing strains, other pathogens or no pathogens [82].

More recently, studies have focused on describing the airway microbiome in smokers with and without COPD [58, 83–85]. Results of the sequencing in the BAL fluid showed high bacterial diversity; *Prevotella*, *Sphingomonas*, *Pseudomonas*, *Acinetobacter*, *Fusobacterium*, *Megasphaera*, *Veillonella*, *Staphylococcus* and *Streptococcus* constituted the major part of the core microbiome found in healthy subjects and COPD patients [85]. At least two studies suggested that smoking alone did not alter the lung microbiome [83, 84]. One study examined the effect of experimental rhinovirus infection and showed an increase in bacterial burden and a significant outgrowth of *H. influenzae* from the existing microbiota in some, but not all, subjects with COPD [86].

Asthma

The impact of environmental microbiota on allergic sensitisation leading to asthma onset has been well described [87–91]. Based on data obtained in the PARSIFAL (Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle) [92] and GABRIELA [90] cohorts, it is now clear that children who grow up in a rural environment are less likely to develop asthma or any other atopic- or autoimmune-related disease. In a murine model of asthma (ovalbumin sensitisation), mice raised in a sterile environment were more likely to develop airway hyperresponsiveness in comparison to control mice raised in a normal environment [93]. These studies suggest that exposure to a rich and diverse environment microbiota early in life is a strong protective factor against asthma onset.

Airway microbiota has been studied in the BAL fluid of asthmatic children. HILTY *et al.* [94] suggested that the distal airway microbiota of asthmatic children contained a higher proportion of Proteobacteria (*e.g.* *Haemophilus*, *Neisseria*, *etc.*) and *Staphylococcus* whereas *Bacteroidetes* were increased in healthy children. HUANG *et al.* [95] demonstrated that bronchial epithelial brushings of adult asthmatic patients presented a richer and less diverse microbiota than control subjects; bronchial hyperresponsiveness was negatively correlated to microbiome diversity. In contrast with these data, MARRI *et al.* [96] reported a lower diversity in the induced sputum of nonasthmatic subjects and that Proteobacteria were present in higher proportions

in the sputum of asthmatic patients, whereas *Firmicutes* and *Actinobacteria* were more frequently found in nonasthmatic subjects.

The possible impact of bacterial colonisation on asthma onset and asthma exacerbations has been the focus of many studies. Studies have investigated bacterial colonisation in patients with preschool asthma [97–100] and reported colonisation prevalence ranging from 12.5% to 61%, with the large variations being ascribed to differences in patient characteristics, sampling and threshold in bacterial culture positivity. Bacterial colonisation was also reported in the sputum of older children and adult patients during exacerbations or under basal conditions [101, 102]. In the Copenhagen Prospective Study on Asthma in Childhood (COPSAC) cohort [103], asthma, prevalence of wheezing, exacerbation severity, eosinophil blood count and total serum IgE were measured at 4 years of age. 1-month-old children were cultured for the presence of *H. influenzae*, *M. catarrhalis* and/or *S. pneumoniae* in hypopharyngeal aspiration and 21% had colonisation with one or a combination of these organisms [103]. No correlation was found with *S. aureus* colonisation (61% of patients) in older patients (1 year-old). When analysing immune response profiles in the COPSAC cohort, T-helper cell (Th)1/Th2/Th17 profiles were associated with *H. influenzae* and *M. catarrhalis* colonisation for 1 month, whereas colonisation with *S. aureus* was associated with Th17 profile, suggesting a bacterial colonisation-specific immunomodulation with unknown consequences [104]. Only one retrospective study on the effect of antibiotic treatment in asthma exacerbation of preschool children showed an improvement in severe asthma control in children receiving antibiotic treatment [100]. These findings suggest that, along with the fact that high environmental microbiota diversity is associated with a decrease in the risk of developing asthma, colonisation with selected prominent bronchial bacteria is correlated with a higher risk of asthma. In a recent study, GOLEVA *et al.* [105] studied the effect of airway microbiota composition on corticosteroid response in asthmatic subjects. Corticosteroid-resistant asthmatic patients demonstrated airway expansion of specific Gram-negative bacteria, which induced corticosteroid resistance through transforming growth factor- β activated kinase-1/mitogen-activated protein kinase activation [105], highlighting possible effects of the airway microbiome on response to therapy.

Post-transplantation bronchiolitis obliterans

The outcome of lung transplantation is mainly impaired by infections and the development of bronchiolitis obliterans syndrome (BOS), also called chronic allograft dysfunction [106]. *De novo* airway colonisation with *P. aeruginosa* in transplanted patients is an independent risk factor for BOS development [107, 108]. Studies using culture-independent methods have revealed that lung transplant patients have a specific lung microbiome which differs from pre-transplant lung microbiome and changes over time [109]. WILLNER *et al.* [110] found that recolonisation of the allograft by *P. aeruginosa* in individuals with CF is not associated with BOS (in contrast to findings with *de novo* colonisation) and that re-establishment of pretransplant lung populations in the allograft seems to have a protective effect against BOS. These intriguing and novel findings suggest important roles for bacterial microbiome in the development of BOS involving host-bacterial interactions.

Non-tuberculous mycobacteria

Airway infection with non-tuberculous mycobacteria (NTM) can develop in patients without previous immunodepression status and/or pulmonary disease. KUBO *et al.* [111] compared lung function and high-resolution computed tomography (CT) scans in 12 women diagnosed with *Mycobacterium avium* pulmonary infection *versus* nine healthy controls. Whereas no difference was observed in FEV₁, *M. avium*-infected patients demonstrated a significant decrease in forced expiratory flow at 25–50% of forced vital capacity and showed significant increases in residual volume (% predicted) and residual volume/total lung capacity (%), indicating lung hyperinflation. Analysis of inspiratory and expiratory CT scans revealed that gas trapping was increased in patients with *M. avium* pulmonary infection [111].

NTM may also occur in patients with previous airway disease, including COPD, asthma or CF. In a case-control study comparing 332 patients with pulmonary NTM infection *versus* 3320 controls [112]. COPD (OR 15.7, 95% CI 11.4–21.5) was described as a risk factor for NTM pulmonary infection; the association was even stronger in COPD patients who received previous or ongoing inhaled corticosteroid treatment (OR 19.6, 95% CI 9.7–39.6) [112]. NTM pulmonary infection was further identified as a risk factor for lung function decline in COPD patients [113]. ANDRÉJAK *et al.* [112] also identified asthma as an independent risk factor for NTM pulmonary infection (OR 7.8, 95% CI 5.2–11.6), and inhaled corticosteroids have also been associated with NTM infection in asthmatic patients [114]. CF appears to be an independent risk factor for NTM pulmonary infection [112]. ESTHER *et al.* [115] followed 1216 CF patients for 8 years, 536 had positive sputum cultures for *Mycobacterium abscessus* (55.6%) and *M. avium* (35.4%). *M. abscessus* infection was an independent risk factor for FEV₁ decline. OLIVIER *et al.* [116] followed 159 patients for 15 months; 60 patients had positive sputum culture for NTM (75% *M. avium*).

No difference was found in FEV₁ decline but CT scan impairments and progression were increased in the NTM infected group.

Hypersensitivity pneumonitis is one of the most frequent causes of small airways diseases and is usually due to the inhalation of organic dust (e.g. mouldy hay or straw) or chemical compounds (e.g. isocyanates) [3]. Hypersensitivity pneumonitis also occurred after exposure to *M. avium* in hot tubs (hot tub lung) [117, 118] or to *Mycobacterium immunogenum* in metal working fluids used in the industrial sector (machine operator's lung) [119]. In both cases of NTM-induced hypersensitivity pneumonitis, patients have no history of previous pulmonary disease. Treatment was based on exposure avoidance without any NTM-specific therapy.

Viruses

Cystic fibrosis

Respiratory viruses are commonly found in CF airways. A recent prospective study among 100 adult CF patients followed up for 12 months, in whom sputum, nose and throat swabs were collected every 2 months and at onset of pulmonary exacerbation, concluded that respiratory viruses were detected at 30.5% of visits and human rhinovirus accounted for 72.5% of viruses [120]. These findings confirm the prevalence of rhinovirus found in previous studies in CF children [121–123]. The effects of seasonal changes on CF exacerbation occurrence remain unclear [124], but viral infections can trigger pulmonary exacerbations in adult and paediatric CF patients. RAMIREZ *et al.* [125] studied the antiviral response gene expression in CF patients with positive viral PCR in sputum and concluded that virus-induced exacerbations were associated with virus-specific immune responses. The relationship between viral infection and disease progression has been described in several studies [126–128], suggesting a possible role for viral infection on bacterial colonisation. JOHANSEN *et al.* [129] described a seasonal distribution of *P. aeruginosa* chronic infection onset with a peak in incidence in November in Danish CF children. In addition, studies have shown that acquisition of *P. aeruginosa* in CF patients is often preceded by a viral respiratory infection [126, 130]. VAN EWYJK *et al.* [131] studied the possible interactions between *P. aeruginosa* and rhinovirus *in vitro* and found that respiratory syncytial virus (RSV) infection of cultured epithelial cells enhanced pseudomonal cell adherence as RSV possibly acts as a coupling between *P. aeruginosa* and epithelial cells. These data suggest a role of specific viral–bacterial interactions in exacerbations of CF lung disease.

Asthma

Understanding the relationship between childhood-onset asthma and viral bronchiolitis is an important aim. In mice, viral infection promotes a Th2 inflammation and proallergic response to allergen exposure [132]. Parainfluenzae type 1 virus induced persistent distal airway lesions (at 3 and 14 months after inoculation) in rodents, which correlated to functional abnormalities [133]. These experimental data suggest that bronchiolitis could be the first step in childhood-onset asthma. Nevertheless, an observational study of a monozygotic twin cohort with discordant history of bronchiolitis showed no difference in terms of frequency of asthma, sensitisation or respiratory function [134].

Wheezing episodes early in life are mostly consequences of viral infection [135], especially with RSV (children aged <1 year) or human rhinovirus (hRV). RSV-related bronchiolitis in children aged <1 year justifies hospital intake in <5% of cases. hRV infection frequency increases with age [136], and hRV A and C are associated with severe respiratory exacerbations [137, 138]. RSV involvement in the origin of asthma has been the focus of several investigations. Presentation of RSV-associated bronchiolitis (severity, recurrence of wheezing and asthma) differs depending on studied populations [139]. RSV bronchiolitis is considered a risk factor for developing asthma in childhood [140, 141], and only one study has described no increase in asthma frequency in 13-year-old children after RSV infection in early life [142]. BACHARIER *et al.* [141] described other factors associated with asthma onset after RSV infection including maternal asthma, airway allergen sensitisation at 3 years of age, recurrent episodes of wheezing before the age of 3 years, and exposure to canine allergens. In the same study, the authors suggested that overexpression of CCL5 in nasal epithelium cells during bronchiolitis episodes could be another predictive factor for asthma during childhood [141]. Finally, in a population of 260 000 children, JAMES *et al.* [143] estimated that 13% of asthma cases were direct consequences of a previous episode of RSV bronchiolitis. RSV-related bronchiolitis is a risk factor for asthma onset in childhood depending on the severity of the episode and frequency is higher in susceptible populations.

hRV infection is involved in recurrent episodes of childhood wheezing and asthma onset; this implication is even more frequent in the case of associated risk factors not linked to the severity of hRV-related bronchiolitis [144–147]. In the Childhood Origins of ASThma (COAST) cohort, which studied 285 neonates with at least one atopic and/or asthmatic parent, the prevalence of wheezing episodes during the first 6 years

of life in relation to history of RSV- and hRV-related bronchiolitis was studied [136, 148]. hRV-related bronchiolitis was identified as a risk factor for onset of wheezing episodes at 3 years of age (OR 10, 95% CI 4.1–26), and this association was maintained at 6 years of age (OR 9.8, 95% CI 4.3–22). Unlike other viruses, hRV-related bronchiolitis seems to be related to a decline in respiratory function [149].

Genetic background involvement in asthma onset after viral infection has been described previously by CARROLL *et al.* [139], who showed that maternal asthma was a risk factor for childhood onset asthma after hRV bronchiolitis. CALISKAN *et al.* [150] described that variants at the 17q21 locus were associated with asthma in children who had hRV wheezing illnesses with expression of two genes at this locus (OR 26.1, 95% CI 5.1–133.0). The risk of developing asthma was significantly higher in comparison to neonates with only hRV infection or with only the variant genotype [150]. In addition, IL-10 polymorphisms have been described to be associated with hRV bronchiolitis-induced asthma [151], suggesting interactions between viruses and immunity maturation leading to childhood-onset asthma.

Early sensitisation to aero-allergens has been described as being associated with HRV bronchiolitis-induced asthma [152, 153]. Innate immunity represented by interferon production has been studied in association with childhood wheezing illnesses, early respiratory tract infections, asthma and viral stimulations (mainly hRV) [154–156]. Decrease of interferon-gamma production by mitogen-stimulated mononuclear cells determined from peripheral blood samples in a subset of 9-month-old healthy infants enrolled in the Tucson Children's Respiratory Study was associated with a significantly higher risk of wheezing between 2 and 13 years of age (relative risk 2.29, 95% CI 1.35–3.89) [154]. The association between impaired basal respiratory function (before any wheezing exacerbation) and the risk of bronchiolitis has been studied previously and the results showed that early obstruction or higher bronchial reactivity was associated with higher risk of hRV-related bronchiolitis [157] or severity of viral exacerbation [158].

Future directions

Advance in technology, including the discovery of the human airway microbiome, open a new era for the understanding of chronic respiratory diseases. Given the importance of distal airways in chronic airway diseases, it appears essential to understand how changes in composition of the airway microbiome are associated with disease onset, exacerbation and progression. Current challenges are related to sampling of the distal airway microbiome without contamination of the oropharyngeal microbiome and/or proximal airway microbiome. To date, most studies have been performed in relatively small numbers of patients and have mostly described the microbial communities in proximal and distal airways, but the role of the airway microbiome in disease is far from understood. Human data will be usefully complemented by experimental models, including animal studies. The role of specific microbes in modulating the effects of other microbes in disease is starting to emerge: for example, *P. aeruginosa* has been suggested to contribute to *S. aureus* eradication in CF airways by stimulating synthesis secretory phospholipase A2-IIA (a host enzyme with bactericidal activity) [159]. Furthermore, most studies have focussed on a single group of microbes (*e.g.* bacteria), whereas interactions between various infectious agents (*e.g.* viruses and bacteria [86] or fungi [160]) are probably important in chronic respiratory diseases. Mycobacteria have also been suggested to act on the immune system with potential therapeutic use in asthmatic patients [161]. Furthermore, the role of the microbial community outside of the lung (*e.g.* gut and skin microbiome) in modulating airway disease is emerging [88, 89]. These discoveries have the potential to change the understanding of host–microbe interactions and their relevance in respiratory disease, which may lead to novel therapeutic approaches of chronic airway disease.

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