The respiratory microbiome in idiopathic pulmonary fibrosis

Richard J. Hewitt1*, Philip L. Molyneaux1,2*

1National Heart and Lung Institute, Imperial College, London, UK; 2Royal Brompton Hospital, London, UK

Contributions: (I) Conception and design: All authors; (II) Administrative support: All authors; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

*These authors contributed equally to this work.

Correspondence to: Dr. Philip L Molyneaux. Fibrosis Research Group, Inflammation, Repair & Development Section, National Heart and Lung Institute, Sir Alexander Fleming Building, Imperial College, London SW7 2BB, UK. Email: p.molyneaux@imperial.ac.uk.

Abstract: Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal disease of unknown cause. Current evidence suggests that it arises in genetically susceptible individuals as a consequence of an aberrant wound-healing response following repetitive alveolar injury. Overt respiratory infection and immunosuppression carry a high mortality, while polymorphisms in genes related to epithelial integrity and host defence predispose to IPF. Recent advances in sequencing technologies have allowed the use of molecular microbial technologies to characterise the respiratory microbiota in patients with IPF. Studies have suggested that changes in the overall bacterial burden are related to disease progression and highlighted significant differences between the microbiota in IPF subjects and healthy controls. Indeed differences in the microbiota between IPF patients may differentiate those with stable compared to progressive disease. As our understanding of the IPF microbiome evolves, along with refinement and advances in sampling and sequencing methodologies we may be able to use microbial signatures as a biomarker to guide prognostication and even treatment stratification in this devastating disease.

Keywords: Idiopathic pulmonary fibrosis (IPF); microbiome; infection; 16S

Submitted Oct 26, 2016. Accepted for publication Nov 30, 2016
doi: 10.21037/atm.2017.01.56

View this article at: http://dx.doi.org/10.21037/atm.2017.01.56

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive, fibrosing interstitial lung disease which causes debilitating breathlessness, an inexorable decline in lung function and ultimately respiratory failure (1). It is a disease linked to ageing with a median age at diagnosis of 66 years, and carries a grave prognosis with an average survival of 2.5 to 3.5 years (2). In the UK, there are over 5,000 new diagnoses of IPF annually (3). A diagnosis of IPF requires a constellation of typical features on high resolution CT (HRCT) and/or histology demonstrating the characteristic pattern of usual interstitial pneumonia (1).

With no single or dominant causative factor yet identified and treatment options which merely slow decline, there remains considerable international research effort to improve our understanding of the pathogenic mechanisms which contribute to IPF. An established hypothesis is that repeated micro injury to alveolar epithelial cells (AEC) by unknown environmental triggers (e.g., cigarette smoke, gastric micro aspiration, particulate dust, viral infections) in genetically susceptible individuals results in an exaggerated wound-healing response (4). The ensuing deposition of excess extracellular matrix and formation of fibroblastic foci causes irreversible damage to the architecture of the lung with loss of alveolar structure, impaired gas exchange and ultimately results in respiratory failure (2). This presumptive model of development suggests a role in IPF for both host and environmental factors, with, in all likelihood, interactions between the two (5).

Studies which have explored genetic susceptibility to IPF have found increased risk with genetic variants involved in regulation of innate host response (6). Specific examples
include a single nucleotide polymorphism (SNP) in the promoter region of mucin 5B gene (MUC5B) (rs35705950) encoding a key component of airway mucus, and a SNP in the toll-interacting protein (TOLLIP) gene (rs5743890) encoding an adaptor protein which modulates signalling through toll-like receptors (TLRs) (7-10).

A study of the peripheral blood transcriptome from 130 IPF patients demonstrated up-regulation of four genes involved in immune defence including alpha-defensins compared with controls (11). These studies suggest genetic susceptibility in innate immune defence may play a role in the pathogenesis of IPF, and lend support to the concept that infection, through its interaction with the host immune system, may contribute to the sequence of events that result in fibrosis. This review will explore our current knowledge of the role played by the respiratory microbiome in IPF and highlight areas of controversy and future research priorities.

**Why should we think about infection in IPF?**

Immunosuppression with prednisolone and azathioprine, in combination with N-acetylcysteine (NAC) increased the risk of death and hospitalisation compared with placebo in IPF patients (12). From this it can be inferred that immune dysfunction plays a role in IPF disease progression, and provides further suggestion of a potential aetiological role for infection. Viruses have long been suspected of playing a role in the pathogenesis of IPF, and there is growing evidence, obtained both from human tissue and animal models, to support a mechanistic role for airborne viruses in the initiation and progression of IPF (13). The most consistent association of viral infection with IPF has been that of the human herpes viruses (HHV) which include CMV, EBV, HHV-7 and HHV-8. Tang and colleagues demonstrated that one or more HHV were found by PCR of lung tissue in 97% of IPF patients compared with only 36% of controls (P<0.0001) (14). Immunohistochemistry and dual fluorescence microscopy studies of lung tissue sections from IPF patients, demonstrated the presence of herpesvirus antigens in AECs lining areas of fibrotic lung, that were also positive for markers of endoplasmic reticulum (ER) stress (15). Interestingly, a study of asymptomatic, “at-risk” first-degree relatives of patients with familial interstitial pneumonia (FIP), found elevated levels of herpesvirus DNA in lavage fluid, compared with normal control subjects. Furthermore, immunohistochemistry identified markers of ER stress in AECs from transbronchial biopsies of “at-risk” patients, closely associated with herpesvirus antigens (16).

These studies provide a plausible mechanistic link by which viral infection may provoke AEC stress and dysfunction could lead to an abnormal wound-healing, fibrotic response. In support of this concept, a murine model studying murine gammaherpes virus 68 (MHV 68), a virus closely related to EBV, showed that bleomycin resistant mice infected with MHV 68 then exposed to bleomycin, had significantly more fibrosis than those treated with bleomycin alone (17).

There are significantly fewer studies evaluating the role of bacteria in IPF and this may correspond to difficulties using culture-dependent microbiological techniques to prove the presence of bacteria; it is estimated 70% of bacteria from mucosal surfaces cannot be cultured (18). The first study to evaluate the microbiological colonisation of the lower airways in a small cohort of 22 IPF patients using quantitative culture methods, found pathogens in 36% IPF patients but nothing in the lavage of control patients. Intriguingly the IPF patients had no clinical evidence of infection in the 4 weeks prior to bronchoscopy and were not immunosuppressed (19).

Bacterial infection has been indirectly implicated to IPF disease progression and mortality in a double-blind placebo-controlled trial evaluating outcomes in 181 patients with fibrotic idiopathic interstitial pneumonia (IIP), the majority of cases IPF, randomised to receive 960 mg co-trimoxazole or placebo twice daily for 12 months (20). Whilst it failed to reach it’s primary outcome and there were a large number of drop-outs, post hoc analysis suggested that, in treatment adherent subjects, co-trimoxazole led to a reduction in infections and mortality. This apparent survival benefit could not be accounted for by a difference in pulmonary function measures.

**The lung microbiome**

The epithelial surfaces of the respiratory tract, previously thought to be sterile, have been shown using culture-independent techniques to accommodate dynamic microbial communities (21). Molecular sequencing of the variable regions of the bacterial 16s-rRNA gene can now be used to identify bacterial species; in research studies of the microbiome, clusters of bacteria sharing similar gene sequences are classified into operational taxonomic units (OTUs) which can be compared to 16s rRNA reference databases (22). High-throughput bacterial 16s-rRNA sequencing has been shown to identify bacterial DNA in 95.7% BAL specimens compared conventional culture techniques which detected bacteria in 39.1% BAL samples (23).
Applying these molecular techniques to characterise the microbial flora of the respiratory tract in patients and healthy controls has highlighted associations which suggest the interaction between the microbiome and host may be relevant to the aetiology and progression of lung disease (24-29). Furthermore, differences have been found in the microbiome in severe asthma compared to non-severe asthma and controls, suggesting that the microbial communities in the airway may influence disease phenotype (30).

The microbiome in IPF

The first exploratory application of a culture-independent molecular technique in IPF studied the microbiome in BAL from 20 patients diagnosed with an IIP, including 17 with IPF, and 2 controls (31). Using 16s-rRNA gene PCR and degenerating gel electrophoresis (DGGE) the study found organisms often associated with the oropharynx as well as uncultured bacterial sequences corresponding to the Streptococcus, Neisseria and Actinobacteria genera. Interestingly, bacterial DNA was not detected in 5 out of 8 patients colonized with pneumocystis jirovecii suggesting this fungus may impair bacterial colonisation of the airways (31).

A small study investigated the upper and lower respiratory tract microbiota in a heterogenous group of 18 ILD patients including 5 with IIP, 6 patients with pneumocystis pneumonia and 9 healthy controls (32). 16s-rRNA gene sequencing of BAL revealed no significant differences in the microbiome between ILD and healthy controls. There was a signal toward lower bacterial diversity in the IIPs but this was not statistically significant. There was a divergence in microbiota from upper to lower airways in 21% patients.

Correlating Outcomes with biochemical Markers to Estimate Time-progression in idiopathic pulmonary fibrosis (COMET), a multicentre cohort study, retrospectively analysed baseline BAL samples to provide insight into the potential role played by the microbiome in IPF disease progression (18). Included were 55 IPF patients with no active infection at the time of screening. At baseline the mean FVC was 70.1% predicted (SD ±17) and DLCO 42.3% predicted (SD ±14). The COMET study followed-up participants prospectively at 16 weeks intervals up to 80 weeks so provided longitudinal outcome data (33). DNA from BAL samples underwent 454 pyrosequencing of the 16s-rRNA gene to identify OTUs, the most abundant of which were Prevotella sp, Veillonella sp and Cronobacter sp (18). Cox regression and principle component analysis identified an association between disease progression and the relative abundance of both a Streptococcus and Staphylococcus OTU (18). Dichotomising patients into those with high and low numbers of these bacteria, Han and colleagues were able to show clear survival differences between groups. Despite this less than 50% of subjects had either or both of these above the statistically modelled levels associated with risk of disease or progression, meaning neither finding can be used to fully explain disease pathogenesis or progression.

The retrospective nature of the microbiome work in the COMET study meant that there was some variability in bronchoscopic sampling. However, the authors allayed concerns regarding the potential for upper airway contamination of samples by demonstrating the OTUs of interest in lung biopsy samples. Protected sterile brushes sampled the microbiome in the proximal and distal airways of two lung explants highlighting regional differences in the relative abundance of OTUs, but nevertheless validating some of the bronchoscopy findings, in particular the presence of the Streptococcus OTU of interest (18). To date there has been no characterisation of the lung tissue microbiome in IPF, a disease of the lung parenchyma.

The largest study published to date, investigated 65 well-defined IPF patients and 44 controls which included 27 healthy controls and 17 patients with moderate COPD (34). In the IPF group the mean DLCO was 44.7% (SD ±13%) predicted and FVC 76.5% (SD ±18) predicted. DNA was extracted from BAL samples taken from the right middle lobe and using 16s rRNA sequencing allowed clustering into OTUs. The first notable finding was of a twofold higher bacterial load (quantified by 16s rRNA gene/mL BAL fluid) in IPF BAL compared with control subjects (P<0.0001). Secondly, there was a significant association between patients with higher BAL bacterial load and disease progression at 6 months (defined by a decline in FVC by 10%) compared with controls (P=0.02). Furthermore, it was possible to stratify patients into tertiles according to bacterial burden in order to predict mortality risk; the hazard ratio for those in the top tertile was 4.59 (95% CI 1.05–20). After logistic regression analysis the abundance of Veillonella, Neisseria, Streptococcus and Haemophilus spp. all remained significantly associated with IPF. Providing a mechanistic link between bacterial burden and a SNP known to be relevant in IPF, the study found that patients carrying a minor allele at the MUC5B promoter SNP rs35705950 had a lower bacterial burden (P=0.01).

In contrast to previous findings, this study provides...
evidence that it is bacterial burden rather than specific microbial communities that predict prognosis (18,34). The presence of both patient and sample control groups in this study adds validity to the results. A criticism of the study was lack of upper respiratory tract samples for comparison, to exclude changes in the composition of the microbiome related to aspiration (35).

Limitations & future questions

Han and colleagues were restricted to defining the progression-related bacteria as *Streptococcus* OTU 1,345 and *Staphylococcus* OTU 1,348 because 16S rRNA sequencing could not be used for species-level identification (18). Further work, in the form of either culture-specific or microbe-specific sequencing, is needed to formally identify these bacteria. Although there were several *Streptococcus* and *Staphylococcus* spp identified in the cohort, only two specific OTUs were associated with disease progression.

There are some general limitations of microbiome research in any lung disease. Given the sensitivity of the molecular technologies employed, an obvious concern in many studies is contamination of samples from the upper respiratory tract when sampling, providing a false representation of the true microbiome (18,34). Reagents and extraction kits are also significant sources of contamination and become particularly important with low biomass samples like those generated from the respiratory tract (36,37). Bronchoscopy is not the only stage that contamination can be introduced in studies of the microbiome (38). Significant variation has also been found when comparing microbiome data from the same patient samples using different sequencer platforms and methodologies (39). The most significant are the biases introduced by primer design, which may select for or against particular bacteria, resulting in some bacterial species not being detected (40,41).

More specifically, whilst IPF microbiome studies have been able to derive bacterial species and burden using high throughput molecular technologies, they have not been able to establish a causal, mechanistic link to disease process or progression. It remains unclear whether the changes to the lung microbiome reported in the IPF studies are instrumental to the disease pathogenesis, or are the consequence of an underlying immune defence defect in this group of patients. Furthermore, these studies do not enlighten us to the interaction between each of the bacterial colonies which is likely to also be relevant (42).

Sequencing DNA from a BAL sample provides a “snapshot” in time of the microbial diversity of the lower airways but does not evaluate the dynamic changes that may be occurring longitudinally. Serial bronchoscopies for this purpose would not be practical so less invasive methods of monitoring the lower airway microbiome over time will need consideration. Additionally, taking a BAL from one lobe of the lung may not be the representative of the microbiome in other lobes, particularly considering the histological hallmark of IPF, UIP, demonstrates spatial heterogeneity with areas of fibrosis adjacent to normal parenchyma (2). In ex-planted lungs from a cystic fibrosis transplant patient, 16s rDNA sequencing of tissue sections revealed regional differences in microbial communities within the lung (43).

It is feasible that as our understanding of the IPF microbiome evolves, and sampling and sequencing methodologies are refined, the composition of a patient’s microbiome may act as a biomarker to guide prognostication and treatment stratification. Given the findings of a trial evaluating co-trimoxazole in IPF, one of the key questions for future IPF studies will be whether specific microbiome “signatures” in patients should be targeted with prophylactic antibiotics to improve survival.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References


