The respiratory microbiome in idiopathic pulmonary fibrosis

Richard J. Hewitt[1]*, Philip L. Molyneaux[1,2]*

[1]National Heart and Lung Institute, Imperial College, London, UK; [2]Royal 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

© Annals of Translational Medicine. All rights reserved.

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 (MVH 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).

© Annals of Translational Medicine. All rights reserved.

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

© Annals of Translational Medicine. All rights reserved.

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

1. Raghu G, Collard HR, Egan JJ, et al. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. Am J Respir Crit Care Med 2011;183:788-824.
2. King TE Jr, Pardo A, Selman M. Idiopathic pulmonary fibrosis. Lancet 2011;378:1949-61.
3. Navaratnam V, Fleming KM, West J, et al. The rising incidence of idiopathic pulmonary fibrosis in the U.K. Thorax 2011;66:462-7.
4. Maher TM, Wells AU, Laurent GJ. Idiopathic pulmonary fibrosis: multiple causes and multiple mechanisms? Eur Respir J 2007;30:835-9.

© Annals of Translational Medicine. All rights reserved.

5. Molyneaux PL, Maher TM. The role of infection in the pathogenesis of idiopathic pulmonary fibrosis. Eur Respir Rev 2013;22:376-81.
6. Fingerlin TE, Murphy E, Zhang W, et al. Genome-wide association study identifies multiple susceptibility loci for pulmonary fibrosis. Nat Genet 2013;45:613-20.
7. Nakano Y, Yang IV, Walts AD, et al. MUC5B Promoter Variant rs35705950 Affects MUC5B Expression in the Distal Airways in Idiopathic Pulmonary Fibrosis. Am J Respir Crit Care Med 2016;193:464-6.
8. Seibold MA, Wise AL, Speer MC, et al. A common MUC5B promoter polymorphism and pulmonary fibrosis. N Engl J Med 2011;364:1503-12.
9. Stock CJ, Sato H, Fonseca C, et al. Mucin 5B promoter polymorphism is associated with idiopathic pulmonary fibrosis but not with development of lung fibrosis in systemic sclerosis or sarcoidosis. Thorax 2013;68:436-41.
10. Noth I, Zhang Y, Ma SF, et al. Genetic variants associated with idiopathic pulmonary fibrosis susceptibility and mortality: a genome-wide association study. Lancet Respir Med 2013;1:309-17.
11. Yang IV, Luna LG, Cotter J, et al. The peripheral blood transcriptome identifies the presence and extent of disease in idiopathic pulmonary fibrosis. PLoS One 2012;7:e37708.
12. Idiopathic Pulmonary Fibrosis Clinical Research Network, Raghu G, Anstrom KJ, et al. Prednisone, azathioprine, and N-acetylcysteine for pulmonary fibrosis. N Engl J Med 2012;366:1968-77.
13. Moore BB, Moore TA. Viruses in Idiopathic Pulmonary Fibrosis. Etiology and Exacerbation. Ann Am Thorac Soc 2015;12 Suppl 2:S186-92.
14. Tang YW, Johnson JE, Browning PJ, et al. Herpesvirus DNA is consistently detected in lungs of patients with idiopathic pulmonary fibrosis. J Clin Microbiol 2003;41:2633-40.
15. Lawson WE, Crossno PF, Polosukhin VV, et al. Endoplasmic reticulum stress in alveolar epithelial cells is prominent in IPF: association with altered surfactant protein processing and herpesvirus infection. Am J Physiol Lung Cell Mol Physiol 2008;294:L1119-26.
16. Kropski JA, Pritchett JM, Zoz DF, et al. Extensive phenotyping of individuals at risk for familial interstitial pneumonia reveals clues to the pathogenesis of interstitial lung disease. Am J Respir Crit Care Med 2015;191:417-26.
17. Lok SS, Haider Y, Howell D, et al. Murine gammaherpes virus as a cofactor in the development of pulmonary fibrosis in bleomycin resistant mice. Eur Respir J 2002;20:1228-32.
18. Han MK, Zhou Y, Murray S, et al. Lung microbiome and disease progression in idiopathic pulmonary fibrosis: an analysis of the COMET study. Lancet Respir Med 2014;2:548-56.
19. Richter AG, Stockley RA, Harper L, et al. Pulmonary infection in Wegener granulomatosis and idiopathic pulmonary fibrosis. Thorax 2009;64:692-7.
20. Shulgina L, Cahn AP, Chilvers ER, et al. Treating idiopathic pulmonary fibrosis with the addition of co-trimoxazole: a randomised controlled trial. Thorax 2013;68:155-62.
21. Dickson RP, Erb-Downward JR, Martinez FJ, et al. The Microbiome and the Respiratory Tract. Annu Rev Physiol 2016;78:481-504.
22. Clarridge JE 3rd. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 2004;17:840-62, table of contents.
23. Dickson RP, Erb-Downward JR, Prescott HC, et al. Analysis of culture-dependent versus culture-independent techniques for identification of bacteria in clinically obtained bronchoalveolar lavage fluid. J Clin Microbiol 2014;52:3605-13.
24. Hilty M, Burke C, Pedro H, et al. Disordered microbial communities in asthmatic airways. PLoS One 2010;5:e8578.
25. Molyneaux PL, Mallia P, Cox MJ, et al. Outgrowth of the bacterial airway microbiome after rhinovirus exacerbation of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2013;188:1224-31.
26. Dickson RP, Singer BH, Newstead MW, et al. Enrichment of the lung microbiome with gut bacteria in sepsis and the acute respiratory distress syndrome. Nat Microbiol 2016;1:16113.
27. Dickson RP, Erb-Downward JR, Huffnagle GB. The role of the bacterial microbiome in lung disease. Expert Rev Respir Med 2013;7:245-57.
28. Singanayagam A, Ritchie AI, Johnston SL. Role of microbiome in the pathophysiology and disease course of asthma. Curr Opin Pulm Med 2017;23:41-7.
29. Huang YJ, Erb-Downward JR, Dickson RP, et al. Understanding the role of the microbiome in chronic obstructive pulmonary disease: principles, challenges, and future directions. Transl Res 2017;179:71-83.
30. Zhang Q, Cox M, Liang Z, et al. Airway Microbiota in Severe Asthma and Relationship to Asthma Severity and Phenotypes. PLoS One 2016;11:e0152724.

© Annals of Translational Medicine. All rights reserved.

31. Friaza V, la Horra Cd, Rodríguez-Domínguez MJ, et al. Metagenomic analysis of bronchoalveolar lavage samples from patients with idiopathic interstitial pneumonia and its antagonic relation with Pneumocystis jirovecii colonization. J Microbiol Methods 2010;82:98-101.
32. Garzoni C, Brugger SD, Qi W, et al. Microbial communities in the respiratory tract of patients with interstitial lung disease. Thorax 2013;68:1150-6.
33. Naik PK, Bozyk PD, Bentley JK, et al. Periostin promotes fibrosis and predicts progression in patients with idiopathic pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 2012;303:L1046-56.
34. Molyneaux PL, Cox MJ, Willis-Owen SA, et al. The role of bacteria in the pathogenesis and progression of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2014;190:906-13.
35. Morris A, Gibson K, Collman RG. The lung microbiome in idiopathic pulmonary fibrosis. What does it mean and what should we do about it? Am J Respir Crit Care Med 2014;190:850-2.
36. Salter SJ, Cox MJ, Turek EM, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol 2014;12:87.
37. Willner D, Daly J, Whiley D, et al. Comparison of DNA extraction methods for microbial community profiling with an application to pediatric bronchoalveolar lavage samples. PLoS One 2012;7:e34605.
38. Cox MJ, Moffatt MF, Cookson WO. Outside In: Sequencing the Lung Microbiome. Am J Respir Crit Care Med 2015;192:403-4.
39. Clooney AG, Fouhy F, Sleator RD, et al. Comparing Apples and Oranges?: Next Generation Sequencing and Its Impact on Microbiome Analysis. PLoS One 2016;11:e0148028.
40. Metzker ML. Sequencing technologies - the next generation. Nat Rev Genet 2010;11:31-46.
41. Sim K, Cox MJ, Wopereis H, et al. Improved detection of bifidobacteria with optimised 16S rRNA-gene based pyrosequencing. PLoS One 2012;7:e32543.
42. O'Dwyer DN, Dickson RP, Moore BB. The Lung Microbiome, Immunity, and the Pathogenesis of Chronic Lung Disease. J Immunol 2016;196:4839-47.
43. Willner D, Haynes MR, Furlan M, et al. Spatial distribution of microbial communities in the cystic fibrosis lung. ISME J 2012;6:471-4.

Cite this article as: Hewitt RJ, Molyneaux PL. The respiratory microbiome in idiopathic pulmonary fibrosis. Ann Transl Med 2017;5(12):250. doi: 10.21037/atm.2017.01.56

© Annals of Translational Medicine. All rights reserved.