Microbiome in lung explants of idiopathic pulmonary fibrosis: a case–control study in patients with end-stage fibrosis

INTRODUCTION
In the quest for environmental factors leading to the development and progression of idiopathic pulmonary fibrosis (IPF), recent research has implicated the lung microbiome. The microbiome hypothesis was generated by clinical observations of the impact of interventions with plausible effects on lung microbiota, such as antibiotics, immunosuppression and gastric acid suppression. Direct supportive evidence came from two well-designed studies using next-generation sequencing to analyse bronchoalveolar lavages (BALs) from early-stage IPF patients. While these two studies implicated the baseline microbial load or specific taxa composition in IPF progression and associated host responses, we currently lack information about the microbiome in lung tissue or in end-stage IPF.

METHODS
See online supplementary material for detailed methods. Briefly, MiLES-IPF is a case–control study comparing the microbiome composition of tissue specimens taken from lung explants (figure 1).

Figure 1  Design of the MiLES-IPF study. We performed a case–control study with collection of IPF lung tissue samples in the operating room (for lung transplant cases) or the morgue (for patients undergoing rapid autopsy). We included lung explants from 40 patients with IPF: 10 with acute exacerbations, 31 men (78%), mean age 63 years and mean predicted forced vital capacity 46%. Similar processes were performed for control donor lungs (CORE—32 organ donation candidates offering 37 tissue samples) and five cystic fibrosis explants. Under sterile conditions, we resected basilar, subpleural pieces from each lung (weighing ~85 mg on average). In small subsets of patients with IPF, we also obtained matched apical (or middle lobe) tissue samples and performed bronchial washings immediately post-explantation for analysis of the lavage fluid. Tissue samples were flash frozen to −80°C until experiments with DNA extraction, PCR amplification of the hypervariable V4 region of the highly conserved 16S rRNA gene and sequencing with the Illumina MiSeq platform. CORE, Center for Organ Recovery and Education; IPF, idiopathic pulmonary fibrosis; QIIME, Quantitative insights into Microbial Ecology.

Furthermore, objective data on dysbiosis during acute exacerbations (AEIPF) are limited despite conjectures about microbial triggers. For these reasons, we conducted the Microbiome in Lung Explants study in IPF (MiLES-IPF) to define discriminating features of the lung tissue microbiome in IPF and potential microbial perturbations in AEIPF.
Figure 2  Main results of microbiome analyses in MiLES-IPF. (A) Comparison of number of 16S reads between different types of samples. IPF lungs had much lower number of reads compared with CORE and CF lungs (p values <10^{-5}). (B) Principal coordinates analysis of Bray-Curtis dissimilarity distances between different types of samples. Higher distances between samples (depicted by spheres) indicate higher levels of taxonomic dissimilarities. (C) Quantitative PCR (qPCR) of the 16S rRNA gene results with end-point fluorescence (EPF) shown (y axis) for each sample (x axis). Samples are grouped according to types (CF, CORE, IPF, negative controls, positive controls). Each bar indicates a different sample. The cut-off EPF for quantitation of 16S rRNA gene signal is shown with a dashed line. Given that only 36% of CF and CORE samples provided quantifiable 16S qPCR signal despite having high numbers of 16S reads by sequencing (as shown in panel A), the microbial mass present in our tissue samples had to be at the limit of detection of the qPCR protocol (see online supplementary material); however, no IPF or negative control sample produced a quantifiable 16S qPCR signal. (D) Taxonomic composition of individual samples classified by sample type. Each bar represents a patient sample and relative heights of each colour-coded bar indicate relative abundance of corresponding taxa. Cystic fibrosis-related pathogens are coded in red, common oral taxa are coded with variations of blue and skin taxa are coded with variations of brown. CORE, Centre for Organ Recovery and Education; IPF, idiopathic pulmonary fibrosis; CF, cystic fibrosis.
donation candidates deemed unsuitable for transplant via the Center for Organ Recovery and Education (CORE). We also included five controls from patients with cystic fibrosis (CF), a disease with high microbial load in lung tissue. Microbiome experiments and analyses were performed according to well-established protocols and analytical pipelines (online supplementary material).

RESULTS
Of 40 IPF patients, 10 with AEIPF had similar demographic and pulmonary function test characteristics as those with chronic IPF (see online supplementary table S1), but higher prevalence of diffuse alveolar damage on pathology (p<0.05).
To infer relative bacterial loads, we examined the number of high-quality 16S sequences (reads) produced by sequencing of each tissue sample (figure 2A). IPF lungs (both AEIPF and chronic) had an exceedingly low number of reads (183 (244)) in the range of reagent negative controls, a yield that was at least 15-fold smaller compared with CORE or CF lungs (p<10−5 each). Similarly, by quantitative PCR, we were unable to detect any quantifiable signal of 16S rRNA gene copies in all IPF lung samples, whereas we did so in 36% of CORE or CF samples (figure 2C).
Further ecological analyses suggested that the trace microbial signal in IPF samples was accounted for by experimental contamination (background signal). By principal coordinates analysis of Bray-Curtis dissimilarity distances (figure 2B), IPF samples clustered with negative reagent controls and were statistically dissimilar from CORE samples (anosim p<0.001).

To examine for possible spatial heterogeneity of microbial communities in IPF lungs, we compared matched apical and basilar samples for a small available subset of patients (n=3), but found no consistent differences in microbial load or taxonomy (online supplementary figure S7). To assess for potential differences between airway-based and tissue-based samples, we compared another subset of matched post-explant bronchial washings and tissue samples, and identified overall higher number of reads in bronchial washings but limited taxonomic overlap with tissue samples (range of overlapping taxa 29% to 53%) (online supplementary figure S8). As expected, our tissue-based IPF samples had entirely discordant taxonomic composition compared with BAL IPF samples from a previous study (online supplementary figure S9).2

DISCUSSION
Our analyses in a well-phenotyped cohort of lung explants from end-stage IPF patients provided a surprisingly low bacterial signal that was similar to negative control samples. Furthermore, no differences were detected between AEIPF and chronic IPF.

It is possible that the resident microbiota in IPF lungs demonstrated in previous studies arrive in the respiratory tract by micro-aspiration associated with IPF,1 8 and then they remain predominantly in the bronchiectatic airways with impaired clearance and not in the distal fibroed parenchyma.

Despite methodological limitations of our study (ie, cross-sectional design, single sample per explanted lung, unavailability of strictly normal tissue, small sample size), the relevant finding is that parenchymal samples with advanced IPF had effectively no detectable microbiota, in contrast with other types of explant tissue. Our negative findings do not invalidate the microbiome hypothesis in IPF, but make a call for topographically detailed investigations on the spatial heterogeneity and possible airway predominance of host-microbiota interactions in IPF.
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