Corticosteroid treatment is associated with increased filamentous fungal burden in allergic fungal disease

Marcin G. Fraczek, PhD,[a,b,d] Livingstone Chishimba, PhD,[b]* Rob M. Niven, MD,[b] Mike Bromley, PhD,[a] Angela Simpson, MD, PhD,[b] Lucy Smyth, PhD,[e] David W. Denning, FRCP,[a,b,c] and Paul Bowyer, PhD[a,b] *Manchester and Salford, United Kingdom*

Background: Allergic diseases caused by fungi are common. The best understood conditions are allergic bronchopulmonary aspergillosis and severe asthma with fungal sensitization. Our knowledge of the fungal microbiome (mycobiome) is limited to a few studies involving healthy individuals, asthmatics, and smokers. No study has yet examined the mycobiome in fungal lung disease.

Objectives: The main aim of this study was to determine the mycobiome in lungs of individuals with well-characterized fungal disease. A secondary objective was to determine possible effects of treatment on the mycobiome.

Methods: After bronchoscopy, ribosomal internal transcribed spacer region 1 DNA was amplified and sequenced and fungal load determined by real-time PCR. Clinical and treatment variables were correlated with the main species identified. Bronchopulmonary aspergillosis (n = 16), severe asthma with fungal sensitization (n = 16), severe asthma not sensitized to fungi (n = 9), mild asthma patients (n = 7), and 10 healthy control subjects were studied.

Results: The mycobiome was highly varied with severe asthmatics carrying higher loads of fungus. Healthy individuals had low fungal loads, mostly poorly characterized *Malasezziales*. The most common fungus in asthmatics was *Aspergillus fumigatus* complex and this taxon accounted for the increased burden of fungus in the high-level samples. Corticosteroid treatment was significantly associated with increased fungal load ($P < .01$).

Conclusions: The mycobiome is highly variable. Highest loads of fungus are observed in severe asthmatics and the most common fungus is *Aspergillus fumigatus* complex. Individuals receiving steroid therapy had significantly higher levels of *Aspergillus* and total fungus in their bronchoalveolar lavage.

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Key words: *Lung mycobiome,* Aspergillus*, steroid, antifungal, allergic bronchopulmonary aspergillosis, asthma*

Fungal diseases are a serious health problem worldwide.[1] A common form of fungal disease is caused by allergic reactions to fungi that complicate existing respiratory pathology. Severe asthma is frequently linked with fungal sensitization.[2] Recognized asthma endotypes include allergic bronchopulmonary mycosis,[3] which includes allergic bronchopulmonary aspergillosis (ABPA).[4] A recently proposed phenotype of severe asthma is severe asthma with fungal sensitization (SAFS).[5] All are serious airway diseases whose pathogenesis is only partially understood. ABPA affects between 2% and 5% of adult asthmatics and SAFS may account for 40% of severe asthmatics so the health care and economic impact of fungal allergy is significant. It is currently assumed that ABPA and SAFS are directly linked to fungal colonization of airways and made worse by increased fungal burden; however, existing culture-based tests are inadequate to determine level and composition of fungal communities in the lungs of affected individuals.[6,7]

Most of the microbiome studies performed to date have focused on gastrointestinal tract, oral, skin, and vaginal bacterial microenvironments.[3,8,9] The data available on the lung mycobiome (reviewed in Tipton et al[10]) is limited to samples from individuals that did not have well-characterized fungal disease.

From [a]the Manchester Fungal Infection Group, Division of Infection Immunity and Respiratory Medicine, School of Biological Sciences, University of Manchester; [b]the Manchester Academic Health Science Centre and [c]the National Aspergillosis Centre, Manchester University NHS Foundation Trust (Wythenshawe); [d]the School of Biological Sciences, Manchester Institute of Biotechnology, University of Manchester; and [e]the School of Environment and Life Sciences, University of Salford.

*Livingstone Chishimba, PhD, is currently at Cardiff and Vale University Hospitals Health Board, Cardiff, United Kingdom.

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Corresponding author: Paul Bowyer, PhD, The University of Manchester, Manchester Fungal Infection Group, Institute of Inflammation and Repair, Core Technology Facility, Manchester, UK. E-mail: paul.bowyer@manchester.ac.uk.

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Abbreviations used
ABPA: Allergic bronchopulmonary aspergillosis
BAL: Bronchoalveolar lavage
ICS: Inhaled corticosteroid
ITS: Ribosomal internal transcribed spacer region
OCS: Oral corticosteroid
RT-PCR: Real-time PCR
SA: Severe asthma without fungal sensitization
SAFS: Severe asthma with fungal sensitization

Commonly reported fungal respiratory infections in immunocompromised patients are caused by fungi such as *Aspergillus* spp, *Candida* spp, *Mucorales* or *Cryptococcus* spp.[1] Endemic pathogens such as *Histoplasma* spp, *Coccidioides* spp, and *Blastomyces* spp may also cause diseases in otherwise healthy individuals.[11] *Aspergillus fumigatus* causes life-threatening invasive aspergillosis, chronic pulmonary aspergillosis, *Aspergillus*-associated asthma, and allergic diseases such as ABPA and SAFS.[1,12-14] ABPA is characterized by exaggerated T_H2 $CD4^+$ allergic inflammatory response to *A fumigatus* allergens,[15] including proteases[16] in the bronchial airway of atopic asthma and cystic fibrosis patients. Other fungi are implicated in SAFS and allergic bronchopulmonary mycosis.[3,8] However, there are limited data describing the range of fungal species that are present, their relative and absolute abundances, and how antimicrobial or corticosteroid therapies impact these microbial communities. Corticosteroid treatment is a known risk factor for development of invasive aspergillosis,[9,17,18] but it is also a common treatment to alleviate symptoms of asthma.[19]

To establish the composition of the lung mycobiome in allergic fungal disease, we investigated the mycobiome composition in the lungs of healthy individuals or those with ABPA, SAFS, asthma, and severe asthma using ribosomal internal transcribed spacer region 1 (ITS1) sequencing, real-time (RT)-PCR and standard microbiological culture methods. Additionally we examined whether particular treatments were associated with altered mycobiomes in these individuals.

METHODS

Sample collection and processing

Patients were identified using the exclusion and inclusion criteria defined in the supplemental Methods (in this article's Online Repository at www.jacionline.org). Briefly, 64 individuals were identified with 58 agreeing to bronchoscopy (Table I and see Table E1 in this article's Online Repository at www.jacionline.org). Bronchoalveolar lavage (BAL) samples were collected using local guidelines and British Thoracic Society standard procedures.[20] After bronchoscopy, all collected samples were immediately placed on ice and transferred to the laboratory for processing. The BAL was subjected to immediate DNA extraction, then PCR, and Illumina sequencing (Illumina Inc, San Diego, Calif). Dilution of endogenous lung fluids in the lavage medium was estimated as described[21] using a Sigma urea assay kit (Sigma Aldrich, Gillingham, Dorset, United Kingdom [UK]).

DNA extraction, PCR, and Illumina sequencing

BAL samples were processed immediately after they were obtained, without storage, and then DNA extraction was carried out immediately using a cetyl triammonium bromide method previously described.[22] Control samples consisted of sterile water or known dilution series of *A fumigatus* or *C albicans* DNA substituted for BAL and also added to BAL before the initial sample prep and then carried through each step in the procedure before sequencing. Where appropriate, reads associated with potential reagent contaminant fungi were excluded from subsequent analysis.

Statistics

Unless otherwise stated, statistical analysis was performed within QIIME v1.8 (http://qiime.org) using appropriate QIIME workflows.[23-25] Beta diversity for fungal taxa was calculated using Bray-Curtis metrics. Confounding variables were selected *a priori* and included age, weight, sex, corticosteroid treatment, antifungal treatment, azithromycin treatment, and disease. Groups were stratified and differences in effects were compared to assess potential confounding. Minimum group size after stratification was 3. Alpha diversity metrics were calculated for fungi using an Excel spreadsheet (Microsoft, Redmond, Wash). Numbers of fungal otus (organized taxonomic units) and diversity statistics are shown in Table E2 (in this article's Online Repository at www.jacionline.org). Pairwise comparison of specific species within datasets was assessed using *t* test or Mann-Whitney *U* test in cases where deviation from normal distribution was observed. Statistics were adjusted for multiple sampling using Benjamini-Hochberg correction as appropriate. Dunn comparison test and logistical regression were used to identify potential confounding factors in comparison of multiple groups as appropriate.

Analysis of mycobiome

Fungal ITS1 sequences were compared with the UNITE database using the QIIME 1.8 closed-reference organized taxonomic unit picking workflow.

Quantification of fungal burden using RT-PCR

Overall fungal burden was determined using RT-PCR with *Aspergillus* 18S specific or pan-fungal ITS1-2 region primers as previously described.[7,26,27]

Estimation of *A fumigatus* complex clonality and diversity

To estimate clonality and diversity in the sample, we arbitrarily defined identical ITS1 sequences as clonal and ITS1 sequences differing from the most common clonal ITS1 sequence as diverse. It is not certain that this definition will distinguish species within the sample. *Aspergillus* complex sequences were isolated from fastq files using BLAST+[28] with empirically defined parameters *E* value < E-31, match length > 95% based on both the ability of the *A fumigatus* ITS query to identify *A fumigatus* complex sequences in the large ITS database and comparison of resulting sequences isolated from the fastq files with *A fumigatus* complex ITS sequences from ITS databases and from the type sequences as previously defined.[29] To reduce the possibility of misassignation from sequence error, fastq files were first filtered using TrimmomaticSE to remove end sequences with Phred 33 scores <30 and any sequences containing 3 base windows with quality lower than Phred 30. Where appropriate, sequences were aligned using MUSCLE[30] trimmed using ALIVIEW[31] and used to make neighbor-joining trees using PhyML in Seaview v4.5.4.[32] Open source software cited above: BLASTp (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/LATEST/), TrimmomaticSE (http://www.usadellab.org/cms/index.php?page=trimmomatic), MUSCLE (http://www.drive5.com/muscle), ALIVIEW (http://ormbunkar.se/aliview/), and Seaview (http://doua.prabi.fr/software/seaview).

RESULTS

Recruitment of patients to the study

This was an observational study conducted between November 2011 and November 2013 at the National Aspergillosis Centre and the North West Severe Asthma Centre based at the Manchester University NHS Foundation Trust (Wythenshawe). Sixty-four adults (31 female and 33 male) 22 to 75 years of age were enrolled. All current or previous smokers had <10 pack years. No

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