# 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. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)**

***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

**TABLE I.** Patient and healthy control subject demographics

| | ABPA (n = 16) | SAFS (n = 16) | SA (n = 9) | Mild asthma (n = 7) | Normal healthy control (n = 10) | *P* value (ANOVA) |
|---|---|---|---|---|---|---|
| Characteristic | | | | | | |
| Age (y), median (range) | 60 (25-75) | 51.5 (30-68) | 49 (19-65) | 24 (22-62) | 29 (22-58) | .02 |
| Male, n (%) | 11 (68.7) | 5 (37.5) | 4 (44.4) | 6 (85.7) | 5 (50) | .463 |
| BMI (kg/m$^2$), mean ± SD | 27.8 ± 4.9 | 27.2 ± 5.3 | 29.1 ± 6.5 | 26.6 ± 3.81 | 23.3 ± 7.4 | .845 |
| $FEV_1$%, mean ± SD | 64.5 ± 22.2 | 72.8 ± 20.9 | 58.1 ± 16.9 | 95.5 ± 12 | 98 ± 12.9 | <.001 |
| Duration of asthma (y), mean ± SD | 42.8 ± 18.8 | 40.6 ± 17.2 | 31.9 ± 17.5 | 22 ± 15.6 | 0 | .08 |
| Bronchiectasis, n (%) | 16 (100) | 12 (81.3) | 6 (66.7) | 0 | 0 | <.001 |
| Obesity, n (%) | 3 (20) | 3 (18.7) | 3 (33.3) | 0 | 2 (20) | <.582 |
| Atopy, n (%) | 11 (68.8) | 12 (75) | 4 (44.4) | 1 (14.3) | 0 | <.001 |
| Rhinitis/sinusitis, n (%) | 11 (68.8) | 10 (62.5) | 3 (33.3) | 1 (14.3) | 0 | .002 |
| Nasal polyps, n (%) | 5 (31.3) | 6 (37.5) | 2 (22.2) | 0 | 0 | .102 |
| Total IgE (kU/L), median (range) | 1025 (210-9300) | 205 (53.9-680) | 49.2 (14-500) | 96.3 (30-580) | 49.6 (7.6-660) | <.001 |
| Blood eosinophils, median (range) | 0.180 (0.2-1.1) | 0.38 (0.1-0.63) | 0.22 (0.1-0.27) | 0.04 (0-0.08) | 0.15 (0.11-0.24) | .111 |
| Antifungal therapy | | | | | | |
| Current, n (%) | 7 (43.8) | 10 (62.5) | 0 | 0 | 0 | <.001 |
| Previous, n (%) | 5 (31.3) | 2 (12.3) | 0 | 0 | 0 | <.001 |
| Never, n (%) | 4 (25) | 4 (25) | 9 (100) | 7 (100) | 10 (100) | .97 |
| Azithromycin, n (%) | 6 (37.5) | 7 (43.8) | 6 (66.7) | 0 | 0 | .007 |
| Oral corticosteroids, n (%) | 10 (62.5) | 10 (62.5) | 9 (100) | 0 | 0 | <.001 |

bronchoscopy samples were obtained from 5 patients and DNA extraction was unsuccessful in 1. Therefore, 58 patients were included in this study: ABPA, n = 16; SAFS, n = 16; severe asthma without fungal sensitization (SA), n = 9; mild asthma, n = 7; and healthy control subjects, n = 10 (Table I and Table E2). ABPA, SAFS, and SA patients were recruited from a cohort of patients attending the above clinics. Mild asthmatic and healthy control subjects were recruited from among members of staff, general public, and students from the University of Manchester. No individual in the study had cystic fibrosis. All patients gave written informed consent to participate in the study, which was approved by the local Research Ethics Committee (REC reference no 11/NW/0175).

## Mycobiome composition of BAL samples

Results from QIIME closed reference BLAST workflows using ITS1 overlapped sequences are shown in Fig 1, *A-C*. *A fumigatus* complex is predominant in lung samples. The load of fungus was estimated using RT-PCR and correlates closely with read count from the raw QIIME analysis (Fig 1, *A*, and Fig E1 in this article's Online Repository at www.jacionline.org). Loads are very variable across samples (Fig 1, *A* and *B*). Healthy individuals all have low fungal burden and are characterized by a high proportion of Basidiomycete DNA (Fig 1, *B*), which is assigned by QIIME to unknown *Malasezziales*. BLAST results for these sequences are most consistent with *M restricta* or a closely related species. Levels of fungus are highly variable between individuals but the highest levels of fungus are observed in severe asthmatics (see Fig E2 in this article's Online Repository at www.jacionline.org). When fungal loads are higher (>2-fold average) than those observed in healthy individuals, the increased load is always due to additional burden of *A fumigatus* complex (Fig E2). *A fumigatus* complex (Fig 1, *B*) forms the largest proportion of fungi in the disease groups.[29] ITS1 is not useful for precise assignment of species within the *A fumigatus* complex and so assignation of species in this clade is represented as *A fumigatus* species complex only. Reverse BLAST+ analysis using formatted fastq files as databases confirmed species identification.

## Diverse *A fumigatus* complex sequences are observed in individual lungs

To further analyze the diversity of *A fumigatus* complex sequences in our samples, all *A fumigatus* sequences were extracted from the fastq files and filtered to remove sequences with Phred scores <30 to minimize any possibility that sequencing errors gave rise to sequence diversity. Typical results are shown as phylogenetic trees in Fig 2. A core set of identical ITS sequences representing between 22% and 61% of the total retrieved sequences was found in each sample. These identical sequences sit at the center of the radial cladograms shown. Each diverse ITS sequence is shown as a branch on the cladogram with phylogenetic distance indicated by the scale. For all samples, the branches consist of only 1 diverse ITS sequence.

## Comparison of the lung mycobiome by disease and treatment

The secondary objective in this study was to determine whether different therapy could be associated with the mycobiome composition of the lung. To determine whether particular therapy or phenotype was associated with altered mycobiome, we compared groups of individuals in this study by disease and treatment type. The groups are complex with considerable potential for confounding treatments or conditions. For example, many individuals receiving antifungal therapy are also receiving steroid therapy. We estimated fungal burden using QIIME fungal composition (Fig 1) adjusted using fungal load as determined by total fungal RT-PCR, as well as by total fungal RT-PCR alone and *Aspergillus*-specific RT-PCR.[7]

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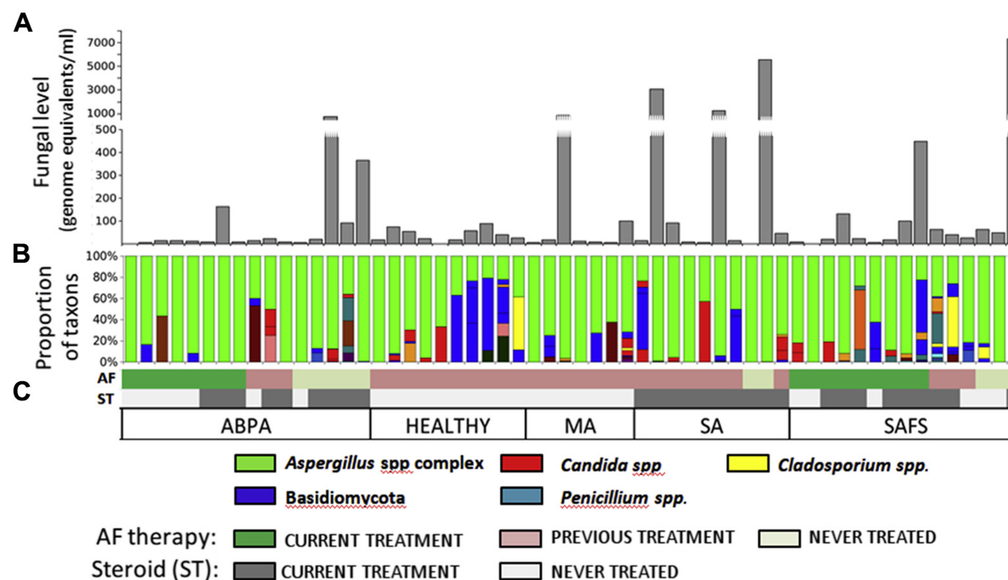


**FIG 1.** Mycobiome composition of BAL from individuals with allergic disease. **A,** Level of fungus in BAL as assessed by total fungal RT-PCR. Cycle threshold values were converted into genome equivalents by calibration against known samples. **B,** Fungal composition of each sample. The color key for the most common taxons is given below the diagram. **C,** Patient information for each sample. Samples are grouped by disease: ABPA; healthy control subjects; MA, mild asthmatics; SA; SAFS. Antifungal (*AF*) and steroid treatment is indicated by the bars above the disease type with key below the diagram.

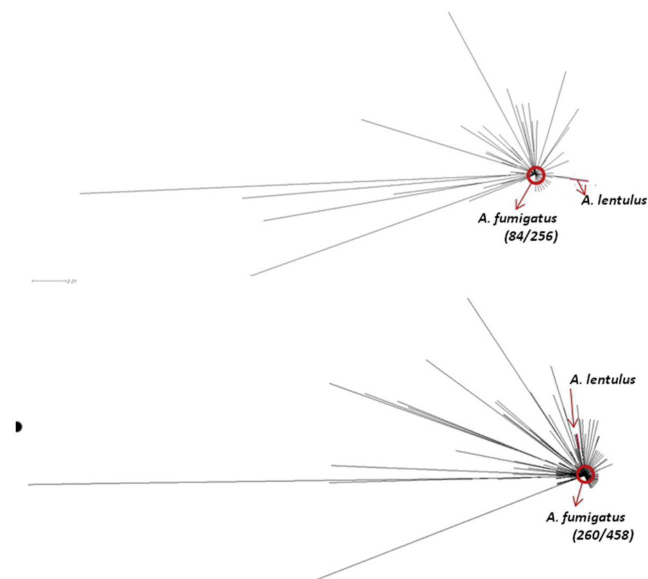


**FIG 2.** Variation in the *A fumigatus* population in individual samples. Two typical samples are shown for patient 34 (healthy control) and 35 (SA). *A fumigatus*-specific sequences were extracted from fastq files by BLAST+, then aligned using MUSCLE trimmed in ALIVIEW and trees constructed using PhyML. Typical *A fumigatus sensu stricto* and *A lentulus* sequences were included in the analysis and are indicated in the figure. Each tree shows a core set of *A fumigatus* sequences with 84 of 256 sequences for patient 34 and 260 of 458 for patient 35 being identical to the *A fumigatus* sequence. Trees are constructed using ITS1 sequence to demonstrate isolate variation, but they are unlikely to represent true speciation.

To determine whether individuals receiving oral itraconazole had lower loads of fungi in their lung samples, patients were initially grouped into those derived from individuals currently on itraconazole therapy (current group, n = 17, including 8 with ABPA and 9 with SAFS), those who had previously been on itraconazole therapy (previous group, n = 10, including 5 with ABPA, 3 with SAFS, and 2 with SA), and those who had never been given itraconazole (never group, n = 31, including 3 with ABPA, 3 with SAFS, 8 with SA, 7 with mild asthma, and 10 who were healthy control subjects) (Fig 3). Antifungal therapy consisted of oral itraconazole, dosed to achieve therapeutic levels as determined by repetitive itraconazole blood measurements. Results based on RT-PCR were used to determine whether this finding was robust. Our results show that fungal load is comparable between those currently on therapy and those who have never been treated. In comparison, loads of fungus in individuals with fungal disease who had stopped taking antifungal therapy are higher. The highest burden of fungus is observed in individuals with a history of itraconazole treatment but who were not on itraconazole therapy when sampled (previous group). The lowest fungal burden was observed in individuals never treated with itraconazole (never group).

Individuals in the previous group had higher fungal loads than individuals in the never group or those currently receiving antifungal therapy (current group). The difference is significant for only the previous–current comparison ($P = .018$) with the other comparisons narrowly failing to reach significance ($P$ values between .1 and .05). This trend occurs in all groups and subgroups tested, when potentially confounding factors such as steroid therapy, disease type, sex, etc, are considered. For comparison of *A fumigatus* load, differences between current and never groups or never and previous groups are not significant ($P = .79$ and $P = .059$, respectively) but comparison of current and previous groups is significant ($P = .03$).

Preliminary analysis of QIIME data suggested potential differences in fungal load between corticosteroid treatment and

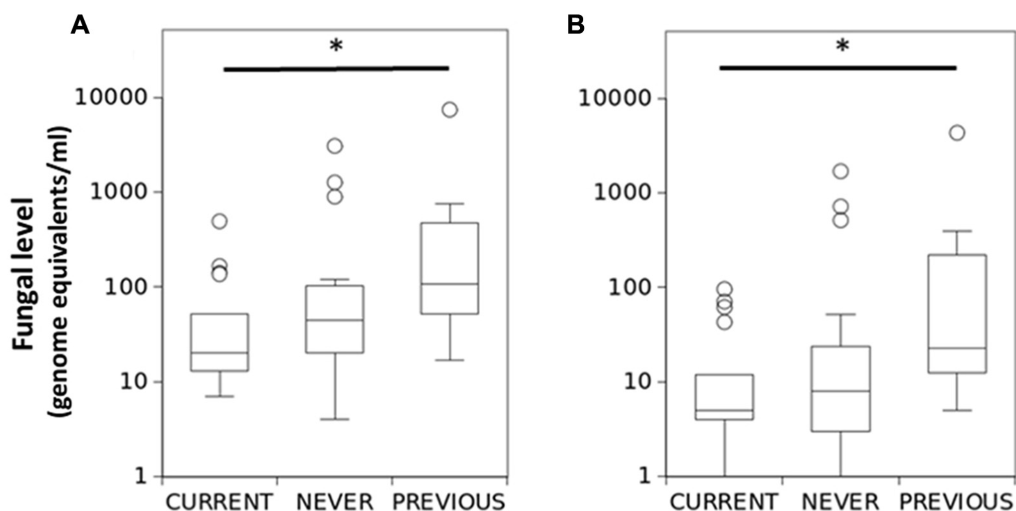


**FIG 3.** Effects of antifungal therapy on fungal load in BAL. Per-individual loads of fungus and *A fumigatus* grouped by antifungal treatment status. **A,** Read count-estimated loads of total fungus grouped by antifungal treatment. **B,** Read count-estimated loads of *A fumigatus* complex spp grouped by antifungal treatment: CURRENT, individuals currently receiving antifungal therapy; NEVER, individuals that have never received antifungal therapy; PREVIOUS, individuals that have previously received antifungal therapy. *$P < .05$.

untreated groups. All SA, SAFS, and ABPA patients were on continuous inhaled corticosteroids (ICS). All SA and SAFS patients were treated with a combination of ICS and oral corticosteroid (OCS). Some patients with ABPA received a combination of continuous ICS plus intermittent OCS therapy. No mild asthma or normal healthy control subjects received steroids (OCS or ICS). A significant difference between therapeutic groups was observed for corticosteroid treatment (Fig 4, *A* and *B*). We initially observed that individuals with ABPA that were receiving corticosteroid therapy showed significantly higher total fungal loads than individuals not receiving corticosteroid therapy (see Fig E3 in this article's Online Repository at www.jacionline.org). The most significant difference between groups was found when considering antifungal therapy with or without corticosteroid treatment (Fig 4, *C* and *D*). The other variables in our dataset did not appear to further confound the data analysis. Fungal burden is slightly lower in individuals receiving antifungal treatment but not corticosteroid therapy when compared with individuals who have never received antifungal therapy. However, fungal burden is significantly higher in individuals receiving corticosteroid treatment independent of antifungal therapy.

## DISCUSSION

To our knowledge, this is the first study to identify the composition of the mycobiome in lungs of individuals with fungal disease. We show differences in fungal load in groups of individuals receiving corticosteroid treatment and antifungal therapy. The lung mycobiome varies substantially between individuals but is always at low levels in healthy individuals and increased load is always due to higher levels of *A fumigatus* complex.

*A fumigatus* complex is the predominant fungus in the lung mycobiome in asthma and allergic fungal disease. Individuals receiving corticosteroid therapy have significantly higher loads of fungus in their lungs than those not receiving steroid therapy irrespective of their underlying disease. None of the mild asthmatic or healthy control subjects were on therapies that are predicted to influence their lung mycobiome or bacterial microbiome, and so these findings probably reflect the normal mycobiome of Caucasians in the North West of England.

In general, our study shows comparable levels and taxa of fungi as were shown in previous studies.[33-36] Additionally we manually removed fungal species identified as arising from reagent contamination. In our study, we did not observe *Wallemia* spp contamination.[37] This study was limited by the numbers of patients that could feasibly be imaged using bronchoscopy and the high variability of fungal levels between samples. Therefore statistical power in some comparisons is low. However, we note that where specific treatments can be separated from confounding factors, significant differences in mycobiome associated with treatment can be observed, even with the limited numbers obtained. Future studies would benefit from either much larger recruitment of individuals with fungal disease or by careful selection of individuals using narrow treatment and/or endotype criteria. Lavage aliquots from several washes were combined in this study to maximize recovery of fungal DNA, and the resulting data are likely to represent the combined bronchial and bronchoalveolar fungal communities.

In addition to *A fumigatus* complex, we found a wide variety of other fungi. Our analysis shows a higher proportion of *Aspergillus* than has been previously reported with lower loads of *Candida*.[10,37] Control experiments using no sample or spiked BAL samples from cough patients did not suggest that the efficiency of PCR and sequence assignation from *C albicans* was lower than that observed with *A fumigatus,* indicating that the observed lower level of *Candida* spp is not caused by PCR or sequencing bias. Manual assignment of fungal taxa from the fastq files suggested that closed-reference organized taxonomic unit workflows using larger ITS databases, containing many more representatives of closely related fungal species, could be a more accurate reflection of species and fungal burden. ITS1 is not a

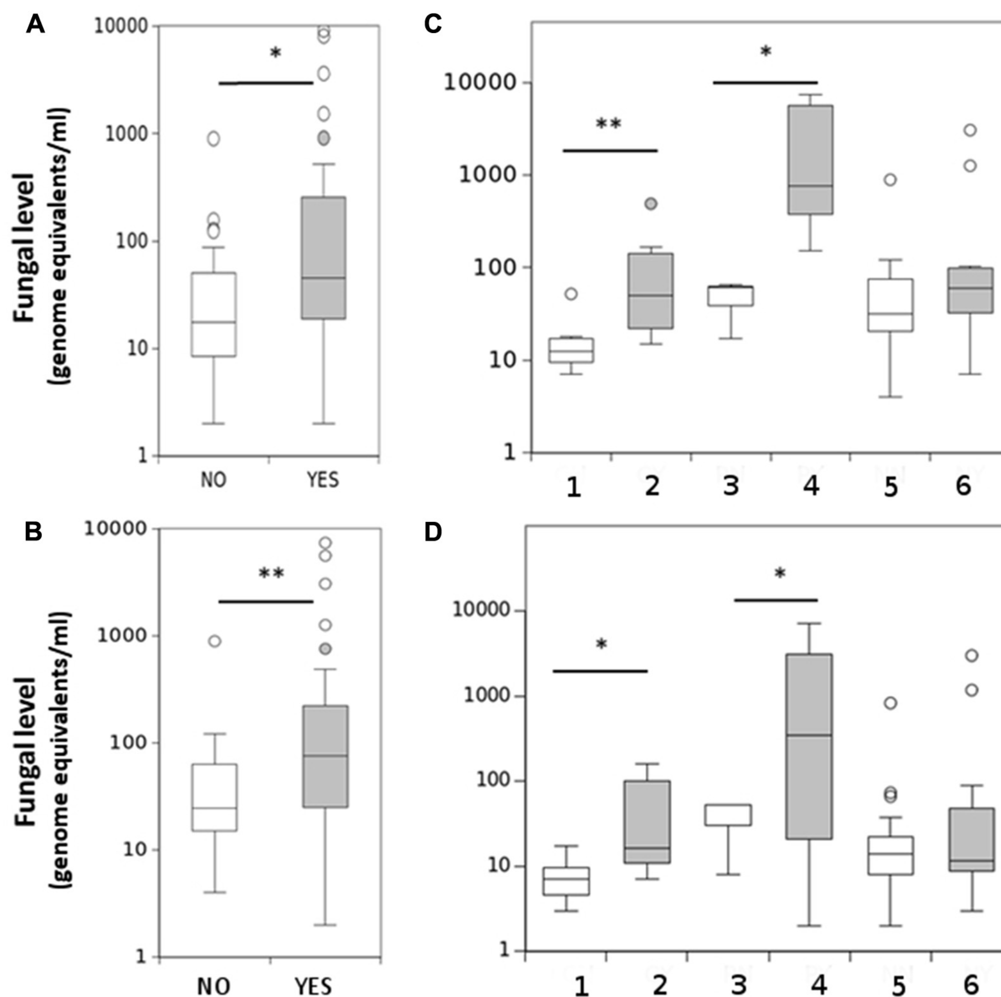


**FIG 4.** Per-individual averaged loads of fungus and *A fumigatus* grouped by antifungal and corticosteroid treatment. **A,** Read count-estimated loads of fungus grouped by corticosteroid treatment. **B,** Read count-estimated load of *A fumigatus* complex spp grouped by corticosteroid treatment. **C,** Quantitative PCR-determined loads of fungus grouped by both antifungal treatment and corticosteroid treatment: 1, current itraconazole treatment with no steroid treatment, 2, current itraconazole treatment with current steroid treatment; 3, previous itraconazole treatment with current steroid treatment; 4, previous itraconazole treatment with no steroid treatment; 5, no itraconazole treatment with no steroid treatment; and 6, no itraconazole treatment with no steroid treatment. **D,** Quantitative PCR determined loads of *A fumigatus* grouped by both antifungal treatment and corticosteroid treatment (groups 1 to 6 as defined in **C**). All individuals with previous antifungal therapy have SAFS or ABPA. *$P < .05$; **$P < .01$.

completely informative locus for exact speciation within the *A fumigatus* complex and therefore precise taxonomic interpretation of these results is not feasible.[29] Our ITS primer set does not amplify *Pneumocystis jirovecii,* but *P jirovecii* is likely to be present in some individuals.

Fungal burdens in the lungs of individuals with asthma or severe forms of asthma are comparable to those in individuals with well-characterized allergic fungal disease. Fungal loads are highly variable between individuals with very high loads of fungus occurring only in those with ABPA, mild asthma, and severe asthma. The difference in fungal load is almost always caused by increased level of *A fumigatus* complex fungi. This might suggest that SAFS is simply a variant form of SA with a disproportionate IgE sensitivity and associated immunopathologic reactions to fungi. The high variability in fungal load means that the differences between disease groups observed are not significant. Alpha-diversity metrics do not suggest differences in composition between these groups.

The observation of diverse sequences in the *A fumigatus* complex group (Fig 2) suggested that infection or colonization did not arise from a single inoculum. Assuming that identical ITS1 sequences represent expansion of mycelium from a small inoculum, then the presence of a high proportion of identical ITS sequences in all samples suggests that a substantial proportion of the *A fumigatus* complex in any given sample arises from a single inoculum or a dominant strain. The remaining sequences are diverse with no sequence duplicated and suggest the presence of a wide range of fungi from the *A fumigatus* complex in each diseased persons' lungs. It is possible that lungs of individuals with excess mucus (a characteristic feature of asthma) act as efficient spore traps

and simply accumulate fungal spores from inhaled air and that relative loads of fungus observed simply reflect environmental abundance and the physical dynamics of spore deposition for small sporing fungi. However, it is well known that there are genetic factors that predispose some individuals to ABPA, and these factors may also explain why some individuals have higher fungal burden.[38,39] Alternatively, the excess mucus could "protect" *A fumigatus* from phagocytic attack or differential adhesion to epithelial cells.[40] In any scenario, at least some of the inhaled spores probably germinate, forming mycelia that produce allergens in the airways.[41] Substantial mycelium is commonly observed intermingled with eosinophils and neutrophils in plugs of sputum from asthmatics supporting the hypothesis that at least some of the fungal population could grow from a single inoculum.[42]

Inhaled corticosteroids are commonly administered to asthmatic patients. We found that individuals receiving corticosteroids without antifungal therapy had higher total fungal loads in the lungs. This result mirrors that seen in chronic obstructive pulmonary disease patients based on sensitive culture techniques and high-dose ICS.[43] Corticosteroids impair monocyte, macrophage, and neutrophil phagocytosis of *A fumigatus*.[44] If the unproven assumption that the fungal load of *A fumigatus* reflects allergen load is true, then this has important implications for management of allergy in these individuals. If steroids do increase fungal burden as our results suggest, then minimizing and monitoring of fungal burden may be a useful route for management. We note, however, that in these patients, the increased fungal burden observed is not linked to demonstrably worse disease using the immunological tools we currently have. *A fumigatus* produces over 80 IgE-binding proteins,[45] so the potential for immune stimulation in the airways is high. ABPA is generally treated with steroid therapy with remission of symptoms[5]; however, relapse is frequent on discontinuation. The interaction of steroid treatment with the lung mycobiome in such cases would clarify the effects of steroid treatment on fungal burden. One potential weakness of this study is the lack of information regarding cumulative steroid use in the cohort, if this is important. Increased cumulative use could be a factor determining fungal burden, and correlation of cumulative steroid use and fungal burden is a future objective for this work. A further possibility is that the age of the study participants may also determine fungal burden. We are unable to show any confounding effect for age in our data; however, the numbers of individuals in the study groups are small, and we cannot rule out the possibility that age-related effects may emerge from larger study numbers in the future. One other potential effect of age may be that it is correlates with increased cumulative steroid exposure.

As expected, individuals receiving antifungal therapy had lower loads of fungus in the lungs, and it is striking that the load in individuals who stopped antifungal therapy was significantly higher than in untreated individuals. Individuals with worse asthma linked to fungal allergy in our practice are more often prescribed antifungal medications, and it is of great interest that higher fungal loads in their lungs are associated with cessation of therapy.

The links between increased fungal load and OCSs, or cessation of antifungal therapy are telling. Steroid therapy for fungal allergic disease has a long history of successful use; however, it is possible that overuse of steroids may encourage fungal growth in the lungs, leading to future recurrence of symptoms. A careful sequential analysis of the mycobiome before, during, and after steroid or antifungal therapy would shed light on this issue. Increased sample sizes would improve statistical analysis. Sequential studies following mycobiome composition and level during the course of treatment would clarify our observations and frame the debate on the use of corticosteroids in the therapy of allergic fungal disease.

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**Clinical implications: This is the first study assessing lung fungal populations in fungal disease and to assess mycobiomes in individuals receiving steroids or antifungal therapy. This information may influence therapy in fungal allergic disease.**

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