Received: 4 November 2019 | Revised: 5 August 2020 | Accepted: 8 August 2020

DOI: 10.1111/cea.13722

WILEY

ORIGINAL ARTICLE

The airway fungal microbiome in asthma

Eva-Maria Rick[1] | Kerry F. Woolnough[2] | Paul J. Seear[1] | Abbie Fairs[1] | Jack Satchwell[1] | Matthew Richardson[2] | William R. Monteiro[2] | Michelle Craner[2] | Michelle Bourne[2] | Andrew J. Wardlaw[1,2] | Catherine H. Pashley[1]

[1]Department of Respiratory Sciences, Institute for Lung Health, Leicester Biomedical Research Centre – Respiratory, University of Leicester, Leicester, UK

[2]Institute for Lung Health, Leicester Biomedical Research Centre – Respiratory, Glenfield Hospital, University Hospitals of Leicester, Leicester, UK

Correspondence
Catherine H. Pashley, Department of Respiratory Sciences, University of Leicester, University Road, Leicester LE1 7RH, UK.
Email: chp5@le.ac.uk

Funding information
NIHR Leicester Biomedical Research Centre; Midlands Asthma and Allergy Research Association (MAARA); Asthma UK, Grant/Award Number: AUK-PHD-2013-250; Henry Smith Charity; Academy of Medical Sciences, Grant/Award Number: SBF003\1125; Wellcome Trust; Government Department of Business, Energy and Industrial Strategy; British Heart Foundation

Abstract

Background: Fungal involvement in asthma is associated with severe disease. The full spectrum of fungal species in asthma is not well described and is derived largely from insensitive culture techniques.

Objectives: To use high-throughput sequencing to describe the airway mycobiota in asthmatics with and without fungal sensitization and healthy controls; to compare samples representing different airway compartments; to determine whether the mycobiota was influenced by the fungal composition of outdoor air; and to compare findings with clinically relevant outcomes.

Methods: We amplified the internal transcribed spacer region 2 of the nuclear ribosomal operon to identify the fungal species present. Ninety-seven subjects were recruited and provided sputum (83 asthmatics; 14 healthy subjects), with 29 also undergoing a bronchoscopy. A subset of airway samples were compared with matched outdoor air and mouthwash samples.

Results: Two hundred and six taxa at the species level were identified in sputum, most at low relative abundance. *Aspergillus fumigatus*, *Candida albicans* and *Mycosphaerella tassiana* had the highest relative abundances and were the most prevalent species across all subjects. The airway mycobiota consisted of a complex community with high diversity between individuals. Notable shifts in the balance of fungi detected in the lung were associated with asthma status, asthma duration and biomarkers of inflammation. *Aspergillus tubingensis*, a member of the *Aspergillus niger* species complex, was most prevalent from bronchoscopic protected brush samples and significantly associated with a low sputum neutrophilia. *Cryptococcus pseudolongus*, from the *Cryptococcus humicola* species complex, was more abundant from bronchoscopy samples than sputum, and differentially more abundant in asthma than health.

Conclusions and Clinical Relevance: The airway mycobiota was dominated by a relatively small number of species, but was distinct from the oropharyngeal mycobiota and air samples. Members of the *A. niger* and *C. humicola* species complexes may play unexpected roles in the pathogenesis of asthma.

Wardlaw and Pashley joint senior authors.

KEYWORDS
fungal sensitisation, fungal colonisation, high-throughput sequencing, mycobiome

1 | INTRODUCTION

Asthma is a common, heterogeneous, chronic inflammatory disorder of the airways. It causes considerable morbidity and avoidable mortality. Up to 70% of severe asthmatics are IgE sensitized to fungi compared with ~10% of mild-to-moderate asthmatics and 5% of the general population.[1,2] Thermotolerant fungi such as *Aspergillus fumigatus*, unlike mesophilic species such as *Cladosporium* and *Alternaria* spp. which act predominantly as allergens, are able to non-invasively colonize the bronchial tree causing a variety of clinical presentations grouped under the term allergic fungal airway disease (AFAD).[3-5] AFAD includes all patients where IgE sensitization to thermotolerant filamentous fungi is complicating their asthma in contrast to allergic bronchopulmonary aspergillosis (ABPA) and severe asthma with fungal sensitization (SAFS) which exclude many patients with fungal-related asthma based on relatively arbitrary criteria, particularly total IgE which has recently been shown to be less suitable than *A. fumigatus*-specific IgE as a biomarker of fungal allergy and lung damage.[6] Over a prolonged period, fungal colonization in association with IgE sensitization appears to cause lung damage with bronchiectasis, lung fibrosis and fixed airflow obstruction.[6,7]

Methods for culturing fungi from sputum are often insensitive, favouring faster growing species that can be cultured on generic media.[8,9] *Aspergillus fumigatus* is the fungus most readily isolated from respiratory samples of patients with asthma. However, using a more sensitive culture method twenty-seven different filamentous fungal taxa were detected in sputum from asthmatics.[10] High-throughput sequencing (HTS) has been used successfully to comprehensively define the microbiological component of an ecological niche. Although most human studies have focused on the community of microbes (microbiota) in the gastrointestinal tract, interest has expanded to include studies of the airways including asthmatics.[11] Bacterial microbiota studies in asthma have suggested a discordance between health and asthma.[12,13] Studies of fungi (mycobiota) lag behind those of bacteria and have focused on the gut and other body sites more than the lungs.[14] Nevertheless, specific mycobiota have been identified in the lungs[15,16] and there is growing recognition that the lung mycobiota has a significant impact on the clinical outcome of chronic respiratory diseases[17] with particular progress having been made in cystic fibrosis[18,19] and bronchiectasis.[20,21] Relatively, little is known about the lung mycobiota in asthma.[22,23] The aim of this study was to characterize the airway mycobiota in asthmatics with and without fungal sensitization, to correlate findings with clinical outcomes and to compare it with the mycobiota of healthy controls and concurrent regional outdoor air samples.

2 | METHODS

2.1 | Subjects

Patients with asthma were recruited from respiratory and allergy clinics at Glenfield hospital, Leicester, UK between 2013 and 2017. The inclusion criteria included age ≥18 years, with a physician diagnosis of asthma and objective evidence of variable airflow obstruction. Full details on inclusion and exclusion criteria and clinical characterization are provided in Appendix S1. Healthy volunteers served as controls and included members of the public and staff at Glenfield hospital. The study was approved by an appropriately constituted National Health Service ethics committee (REACT IRAS 159074:CRN 159074 and ABPA UHL 10111). All subjects gave their written informed consent.

2.2 | Allergy testing

Atopy was assessed using skin prick tests to common allergens, and IgE fungal sensitization assessed using skin prick tests and blood immunology to a panel of eight fungi (*Alternaria alternata*, *A. fumigatus*, *Candida albicans*, *Cladosporium herbarum*, *Malassezia* spp., *Mucor racemosus*, *Penicillium chrysogenum* and *Trichophyton rubrum*). A positive test was defined as a weal of ≥3 mm above negative control and specific IgE ≥ 0.35 kU/L. Details of the allergens and fungi tested are provided in Appendix S1.

2.3 | Sputum and bronchoscopic samples

Briefly, sputum was obtained either spontaneously or induced,[24,25] with sputum plugs separated from saliva and homogenized. A subset of subjects who were willing to consent to undertake the procedure underwent bronchoscopy in accordance with recent British Thoracic Society guidelines,[26] providing up to three samples for analysis; bronchial wash, bronchoalveolar lavage (BAL) and protected brush samples. Towards the end of the study, we obtained ethical approval to obtain a mouthwash sample prior to producing sputum from a subset of subjects. Further details are available in Appendix S1.

Aliquots of 100 μL of sputum homogenate, concentrated mouthwash, BAL, bronchial wash and brush samples were used for culture and DNA extraction. Fungal culture was performed as previously described.[7,9,10] Total genomic DNA was extracted using a DNeasy plant mini kit (Qiagen) with a modified protocol including bead-beating.[27,28]

2.4 | Air samples

Outdoor air samples collected 12 m above ground and representative of a wide geographic area,[29] corresponding to patient sampling dates were obtained for a subset of patients. More than 23K Litres of air was sampled into a microcentrifuge tube over a 24-hour period from midnight and DNA extracted as before.[27] Further details are available in Appendix S1.

2.5 | Sequencing

The internal transcribed spacer region 2 (ITS2) of the nuclear ribosomal operon was amplified using barcoded ITS3 and ITS4 primers[30] using a dual index nested PCR approach. Full details including PCR cycling conditions, amplicon cleaning and quantification, and control samples (DNA extraction kit, PCR reagents, sample processing reagents and two fungal mock communities) are provided in Appendix S1. The products were pooled and submitted to the Centre for Genomic Research, University of Liverpool, for paired-end sequencing (2 × 250 bp) on the Illumina MiSeq platform. Sequence data, including those from controls and mock communities, were deposited in the European Nucleotide Archive; study accession number PRJEB31078.

2.6 | Data handling, statistical analysis, bioinformatic processing and data analysis

Study data were collected and managed using a research electronic data capture tool,[31] and statistical analysis performed using PRISM version 7.02 (GraphPad Software). Sequence processing incorporated recommended quality-filtering guidelines.[32] Further processing was implemented using the Quantitative Insights Into Microbial Ecology (QIIME) open-source bioinformatics pipeline version 1.9.1[33] and QIIME2.2019.4.[34] Taxonomy was assigned using the UNITE (User-friendly Nordic ITS Ectomycorrhiza) fungal ITS database. Three alpha diversity indices were used: Chao1 index, a measure of species richness useful for low abundance data sets; observed OTUs, a measure of the number of distinct OTUs; and Shannon index, a measure of richness and evenness. Beta diversity was calculated using the non-phylogenetic Bray-Curtis dissimilarity. To assess the statistical significance of compositional differences, two complementary approaches for differential abundance testing were used: analysis of composition of microbes (ANCOM)[35] and balance trees analysis,[36] sometimes referred to as gneiss analysis. Full details are provided in Appendix S1.

3 | RESULTS

3.1 | Subjects

Sputum samples were obtained from 83 people with asthma (54 IgE sensitized to fungi), and 14 healthy controls (Table 1). The three groups were well matched for sex and age. For asthma, the fungal-sensitized group had earlier onset asthma ($P = .003$), longer asthma duration ($P < .001$), received lower levels of asthma treatment ($P = .041$) and required less oral steroids ($P = .025$), trended towards impaired post-bronchodilator FEV_1 ($P = .077$) and had increased immunological markers of fungal sensitization. Sputum fungal culture rates were low, with no significant difference between the three groups. Approximately three-quarters of the fungal-sensitized group were sensitized to *A. fumigatus* (Table 1).

3.2 | Fungal ITS2 amplicons

Fungal amplicons were generated from the majority of samples, although success rates were notably lower for bronchial brush samples, particularly from healthy subjects (Table 1). Forty-five air samples were obtained and sequenced; 28 matched dates when sputum from 31 subjects was obtained and 18 matched days on which 19 bronchoscopies were performed. The average number of high-quality fungal reads remaining after quality control was 123106 per sample (range 5085-543347, Table S1) with the rarefaction curves reaching a plateau, indicating that almost all the mycobiota present were detected (Figure S1).

3.3 | Sputum airway mycobiota

Fungal sequences clustered into 830 operational taxonomic units (OTUs), which after removal of very rare (<0.005% relative abundance) OTUs represented 206 taxa at the species level, of which 154 were named species (Table S1). The sputum mycobiota was dominated by three species: *A. fumigatus*, *C. albicans* and *Mycosphaerella tassiana* (Figure 1A), although, the relative abundance per individual was highly variable. *C. albicans* was the most prevalent fungus detected, being present in all samples, whilst *A fumigatus* had the highest average relative abundance (Table S2). There were no significant differences in alpha diversity between asthma and health, although there was a trend towards increased alpha diversity in sputum obtained from people with asthma. Grouping samples from asthmatics according to whether the individual was sensitized to fungi also showed no difference in alpha diversity (Table S3, Figure 1B). Neither beta diversity nor fungal taxa detected were significantly different grouping samples according to asthma status or sensitization (Tables S4 and 2, Figure 1C).

Culture positivity likely indicates a higher burden of fungus; therefore, we investigated whether culture of yeasts or *A. fumigatus* from sputum influenced the mycobiota. Whilst there was no significant effect on alpha diversity, PERMANOVA analysis indicated significant differences in beta-diversity grouping samples as yeast culture positive or negative, or *A. fumigatus* culture positive or negative, with or without healthy controls (Table S4). The principal coordinate analysis (PCoA) plots showed most *A. fumigatus* culture-positive samples clustered around the sphere representing the *A. fumigatus* sequences, and a large group of yeast only culture-positive samples cluster around the *C. albicans* sequence sphere (Figure S2A,B).

TABLE 1 Demographic and clinical characteristics of the 97 study subjects

	Subjects with Asthma (n = 83)			Control, no asthma	3 groups
	Fungal sensitized (n = 54)	Non-fungal sensitized (n = 29)	*P*-value	(n = 14)	*P*-value
Demographics					
Male[a]	31 (57)	17 (59)	>.9999	7 (50)	.8562
Age in years[b]	58.89 (23-81)	58.86 (32-82)	.9934	56 (23-72)	.7776
Age of asthma onset, y[c]	24 (3-52)	45 (35-55.5)	**.0025**	—	—
Asthma duration[c]	30 (7.5-54.5)	9 (2.25-22.25)	**.0004**	—	—
GINA treatment					
GINA 1-3[a]	10 (19)	5 (17)	**.0409**		
GINA 4[a]	32 (59)	10 (34)			
GINA 5[a]	12 (22)	14 (48)			
Spirometry					
FEV_1% of predicted, PB[d,f]	76.3 (26.31)	86.41 (20.73)	.0769	114.4 (14.83) (n = 12)	**<.0001**
FEV_1/FVC ratio PB[d,f]	65.33 (14.24)	69.82 (10.84)	.1422	79.16 (4.232)	**.0012**
FeNO ppb[c,f]	26 (19-42.75) (n = 44)	34.5 (21-61) (n = 24)	.4651	21 (14.25-27.75) (n = 10)	.1538
Smoking and steroid history					
Never-smokers[a]	36 (67)	13 (45)	.0643	9 (64)	.1437
Smoking (pack years)—ex or current[d,f]	8.778 (7.442) (n = 18)	12.58 (9.63) (n = 16)	.2044	12.91 (18.16) (n = 5)	.4932
Requiring oral steroids[a]	12 (22)	14 (48)	**.0245**	—	—
Dose of ICS- ICS-BM μg/pt/d[d]	1084 (476.8)	1041 (524.1)	.7099	—	—
Leucocyte counts and sputum analysis					
Eosinophil count in blood ($\times 10^{-9}$/L)[c]	0.82 (0.44-1.388)	0.81 (0.29-1.575)	.6366	0.135 (0.0875-0.225)	**<.0001**
Sputum Neutrophil percent[d,f]	67.53 (23.67) (n = 35)	61.65 (22.56) (n = 22)	.3564	78.38 (21.03) (n = 9)	.1894
Sputum Eosinophil percent[c,f]	0.75 (2.5-10.5) (n = 35)	2 (6.95-20.13) (n = 22)	.2039	0 (0-1.25) (n = 9)	**.0008**
Sputum culture positive					
Yeast[a,f]	33 (65) (n = 51)	19 (68) (n = 28)	.8097	7 (50)	.5063
A fumigatus[a,f]	8 (16) (n = 51)	3 (11) (n = 28)	.7377	0 (0)	.2672
Other filamentous fungi[a,f]	3 (0.06) (n = 51)	1 (0.04) (n = 28)	>.9999	0 (0)	.6141
Immunoglobulins and radiology					
Serum total IgE IU/mL[c]	516 (174-1870)	52.9 (32.05-115)	**<.0001**	53.4 (9.9-156.5)	**<.0001**
Atopic[a,f,g]	30 (63) (n = 48)	12 (46) (n = 26)	.2218	6 (43)	.2548
Aspergillus fumigatus sIgG >40 mg/L[a]	34 (63)	5 (17)	**<.0001**	4 (30)	**.0001**
A fumigatus sIgE >0.35 kU/L[a]	40 (74)	0 (0)	**<.0001**	0 (0)	**<.0001**
Number with bronchiectasis[a,f]	18 (34) (n = 53)	7 (25) (n = 28)	.4579	0	**.0365**
Samples analysed by HTS					
Sputum HTS data[a]	53 (98)	26 (90)	.1203	13 (93)	.2327
Bronchoscopy[e]	12 (100)	9 (89)	.4286	8 (63)	.0563
Bronchial wash[e]	12 (100)	9 (78)	.1714	8 (50)	**.0256**
Bronchoalveolar lavage[e]	9 (89)	9 (89)	>.9999	8 (63)	.2889
Protected brush[e]	11 (45)	9 (67)	.4059	8 (38)	.4514
Mouthwash[e]	10 (90)	1 (100)	>.9999	1 (0)	.0633

Notes: P values were calculated with an independent *t* test or ANOVA for parametric values, Fisher's exact test or chi-square test for comparison of proportions and the Mann-Whitney *U* test or Kruskal-Wallis test for comparison of non-parametric for two groups or three groups respectively. Significant *P*-value's ($P < .05$) are given in bold.

Data presented as [a]number (percent of subjects), [b]mean (range), [c]median (interquartile range), [d]mean (standard deviation), [e]number of samples obtained (% successfully amplified), [f]data missing for some patients, number data available for indicated, [g]assessed by skin prick test ≥3 mm to common allergens (grass, tree pollen, cat, dog, house dust mite).

Abbreviations: FeNO ppb, fractional exhaled nitric oxide, parts per billion; FEV1, forced expiratory volume during the first second; FVC, forced vital capacity; HTS, high-throughput sequencing; ICS-BM, inhaled corticosteroid, beclomethasone equivalent; IgE, immunoglobulin E; PB, post-bronchodilator; sIgE, specific immunoglobulin E; sIgG, specific immunoglobulin G.

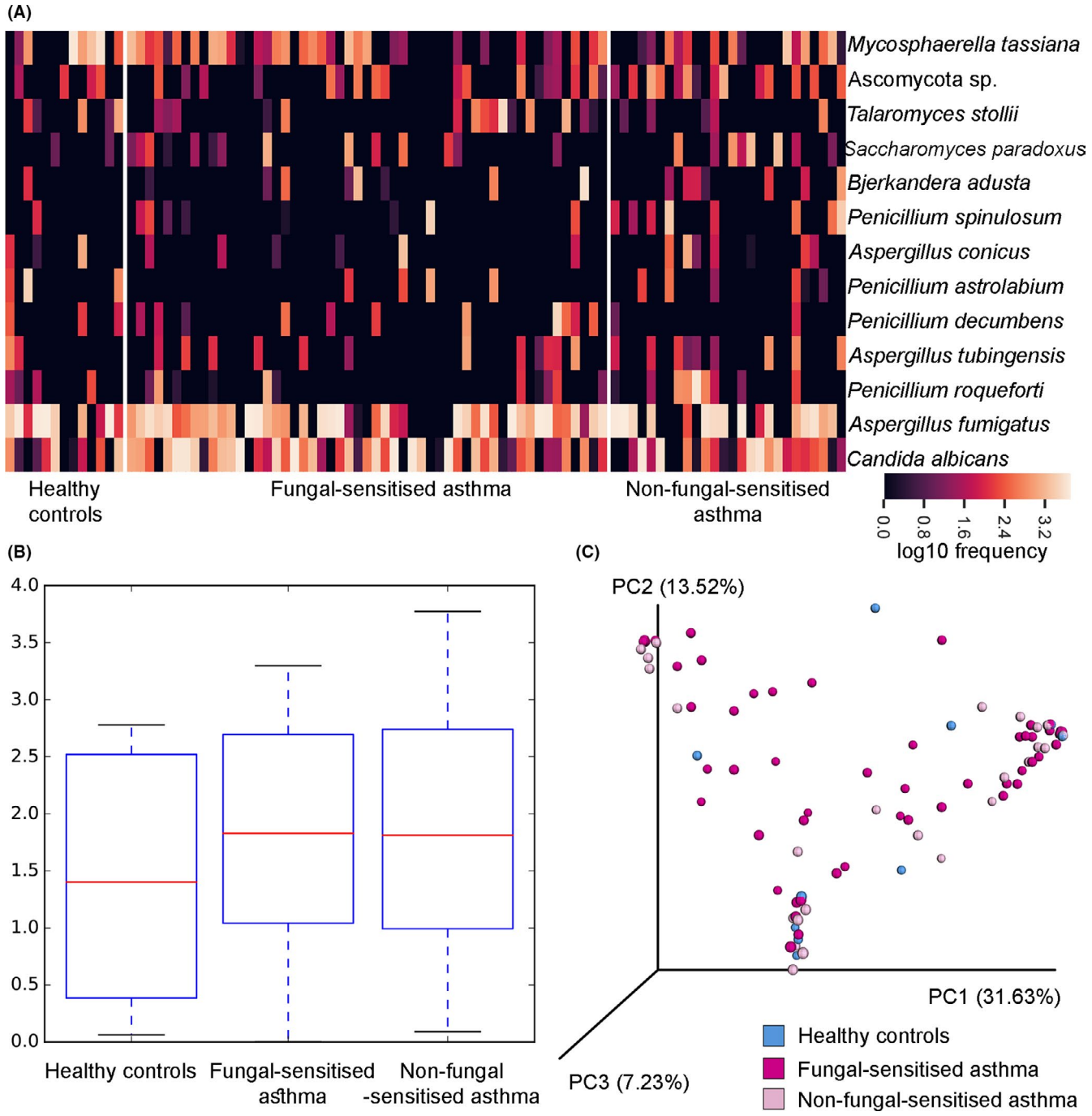

FIGURE 1 Sputum samples grouped into healthy controls (n = 13), fungal-sensitized asthmatics (n = 53) and non-fungal-sensitized asthmatics (n = 26). A, Heatmap showing the relative abundance of fungal ITS2 sequence data between samples. The data were rarefied then transformed using ln(x + 1) due to zeros in the data set. Only taxa with a relative abundance ≥1% are shown. The relative abundance is indicated by colour, with the lighter shades representing the more abundant sequences. B, Box plot of Shannon alpha diversity indices, showing no significant differences in alpha diversity between groups. C, Principal coordinate analysis plots based on the Bray-Curtis distance values, showing no grouping of samples by asthma status or sensitization

ANCOM and balance trees analysis confirmed that yeast culture was highly associated with increased relative abundance of *C. albicans* sequences (Table 2, Figure S3A). Similarly, balance trees analysis found a cluster of four species comprising *A. fumigatus*, *Aspergillus tubingensis*, *Penicillium roqueforti* and an unidentified Ascomycota associated with the culture of *A. fumigatus* (Table 2, Figure S3B).

3.4 | Comparing lung compartments

Twenty-nine subjects underwent a bronchoscopy, resulting in ITS2 sequence data from 23 bronchial washes, 21 BAL and 14 brush samples, as detailed in Table S1. Consistent with sputum, *C. albicans*, *M. tassiana* and *A. fumigatus* were amongst the most prevalent and relatively abundant fungi detected from all sample types; however,

TABLE 2 Fungal taxa that were differentially abundant between sampled grouped by categorical clinical characteristics, analysed by either analysis of composition of microbiomes (ANCOM) or balance trees analysis (gneiss)

		ANCOM			Gneiss			
Category	**Variables (n)**	**Taxa**	***W***	**Clr**	**Balance**	**Covariate**	**FDR corrected *P*-value***	**Taxa**
Asthma and healthy controls								
Asthma	Yes/no	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Fungal sensitization	Healthy control/fungal sensitized/not-fungal sensitized	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Sputum culture yeast	Yes/no	*Candida albicans*	23	−3.86	y6	Culture yeast yes	5.36e-18	*C. albicans*
Sputum culture *Aspergillus fumigatus*	Yes/no	n/s	n/s	n/s	y0 (4 taxa)	Culture *A. fumigatus* yes	7.61e-4	*A. fumigatus*, *Aspergillus tubingensis*, *Penicillium roqueforti*, Ascomycota sp.
					y6	Culture *A. fumigatus* no	6.99e-5	*C. albicans*
IgG *A.fumigatus*	Yes/no	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Smoking status	Never/current/exsmoker	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Peripheral blood eosinophil level	High/moderate/low	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Sputum eosinophils	High/low	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Sputum neutrophils	High/low	Ascomycota sp.	16	3.47	y0	Sputum neutrophil category_low	7.90e-4	*A.tubingensis*, *P. roqueforti*, Ascomycota sp.
Inflammatory phenotype	Eosinophilic/neutrophilic/paucigranulocytic/mixed granulocytic	*A. tubingensis*	7	6.52	y0	n/s	n/s	n/s
		Ascomycota sp.	3	5.07				
		Saccharomyces paradoxus	2	3.38				
		Malassezia globosa	2	2.31				
Asthma only								
Fungal sensitization	Fungal sensitized/not-fungal sensitized	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Sputum culture yeast	Yes/no	*C. albicans*	23	−3.56	y8	Culture yeast yes	9.41e-15	*C. albicans*
Sputum culture *A.fumigatus*	Yes/no	n/s	n/s	n/s	y0 (3 taxa)	Culture *A. fumigatus* yes	2.1e-4	*A. fumigatus*, *A. tubingensis*, Ascomycota sp.
					y8	Culture *A. fumigatus* no	1.63e-5	*C. albicans*
IgG *A. fumigatus*	Yes/no	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Bronchiectasis	Yes/no	n/s	n/s	n/s	n/s	n/s	n/s	n/s
GINA class	1-3, 4, 5	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Oral steroids	Yes/no	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Inhaled steroid dose	High, medium, none-low	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Smoking status	Never, current, exsmoker	n/s	n/s	n/s	n/s	n/s	n/s	n/s

(Continues)

TABLE 2 (Continued)

Category	Variables (n)	ANCOM			Gneiss			
		Taxa	W	Clr	Balance	Covariate	FDR corrected *P*-value*	Taxa
Peripheral blood eosinophil level	High, moderate, low	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Sputum eosinophils	High/low	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Sputum neutrophils	High/low	Ascomycota sp.	4	−2.81	n/s	n/s	n/s	n/s
		A. tubingensis	4	−2.39				
		M. globosa	3	1.42				
		S. paradoxus	2	1.96				
		Penicillium aethiopicum	2	0.96				
		Candida dubliniensis	2	1.01				
Inflammatory phenotypes	Eosinophilic/neutrophilic/paucigranulocytic/mixed granulocytic	*A. tubingensis*	13	9.87	y0	Paucigranulocytic	2.63e-2	*A. tubingensis*, *P. roqueforti*, Ascomycota sp.

Abbreviation: n/s, not significant.

*P values were calculated by performing regression analysis on each balance and corrected by the Bonferroni FDR method for multiple comparisons.

other fungi were also prominent from the bronchoscopy-derived samples, such as *A. tubingensis*. The fungus with the highest relative abundance remained *A. fumigatus* (Figure 2A, Table S5), and however, *C. albicans* was no longer the most prevalent.

There was no significant difference in alpha diversity between the sample types (Figure 2B, Table S6). In contrast, there were significant differences in beta diversity between the brush and wash and brush and sputum (Figure 2C, Table S7), although significance was lost when correcting for multiple samples and only brush samples showed clustering on a PCoA plot (Figure 2D).

As with sputum, grouping by culture of yeast resulted in significant differences in beta diversity (pseudo-F = 4.52, P = .003, Figure S2C). There was no significant effect on beta-diversity grouping samples according to whether they came from healthy controls or asthmatics; however, there were significant differences when samples were grouped according to the patient they were obtained from (PERMANOVA pseudo-F = 3.02, P = .001, Figure S2D).

ANCOM analysis revealed four differentially abundant species (Table S8, Figure 4A), *C. albicans* (higher relative abundance in sputum), and *A. tubingensis*, *Hyphodontia radula* and *Cryptococcus pseudolongus* (higher relative abundance in bronchoscopy samples). Balance tree analysis, whilst not statistically significant, grouped *A. tubingensis*, *C. pseudolongus*, *A. fumigatus*, *H. radula*, *Aspergillus striatus*, and an unidentified Ascomycota. The box plot of log-ratios suggested that the composition of species from brush samples were most divergent from sputum (Figure 4A-iii).

Fungi detected from bronchoscopy-derived samples were found to be differentially abundant between asthma and health, with or without corresponding sputum samples. An unidentified fungus was most significantly different; however, without sputum samples ANCOM determined *Cladosporium sphaerospermum*, *H. radula* and *C. pseudolongus* were significantly different (Table S9, Figure S5). The unidentified fungus was found to have a higher mean relative abundance in healthy control samples, whereas the other three were higher from asthmatics. Balance trees analysis did not find any significant differences; however, the box plot suggested a trend towards differences and the heatmap revealed a cluster of six fungi with sputum and seven without that may explain some of the compositional variability (Table S9, Figure S5). *H. radula* and *C. pseudolongus* were present in both clusters.

Given the variability of the mycobiota and the significant effect of the individual, we used a paired analysis matching samples by subject. Significant differences in Shannon diversity index were observed between health and asthma pairing sputum samples with BAL or wash, Figure S4.

3.5 | Influence of the oral cavity

Mouthwash samples were dominated by yeast species. Whilst the most prevalent fungi were consistent with sputum, the top three being *C. albicans*, *M.tassiana* and *A. fumigatus*, the oral mycobiota was

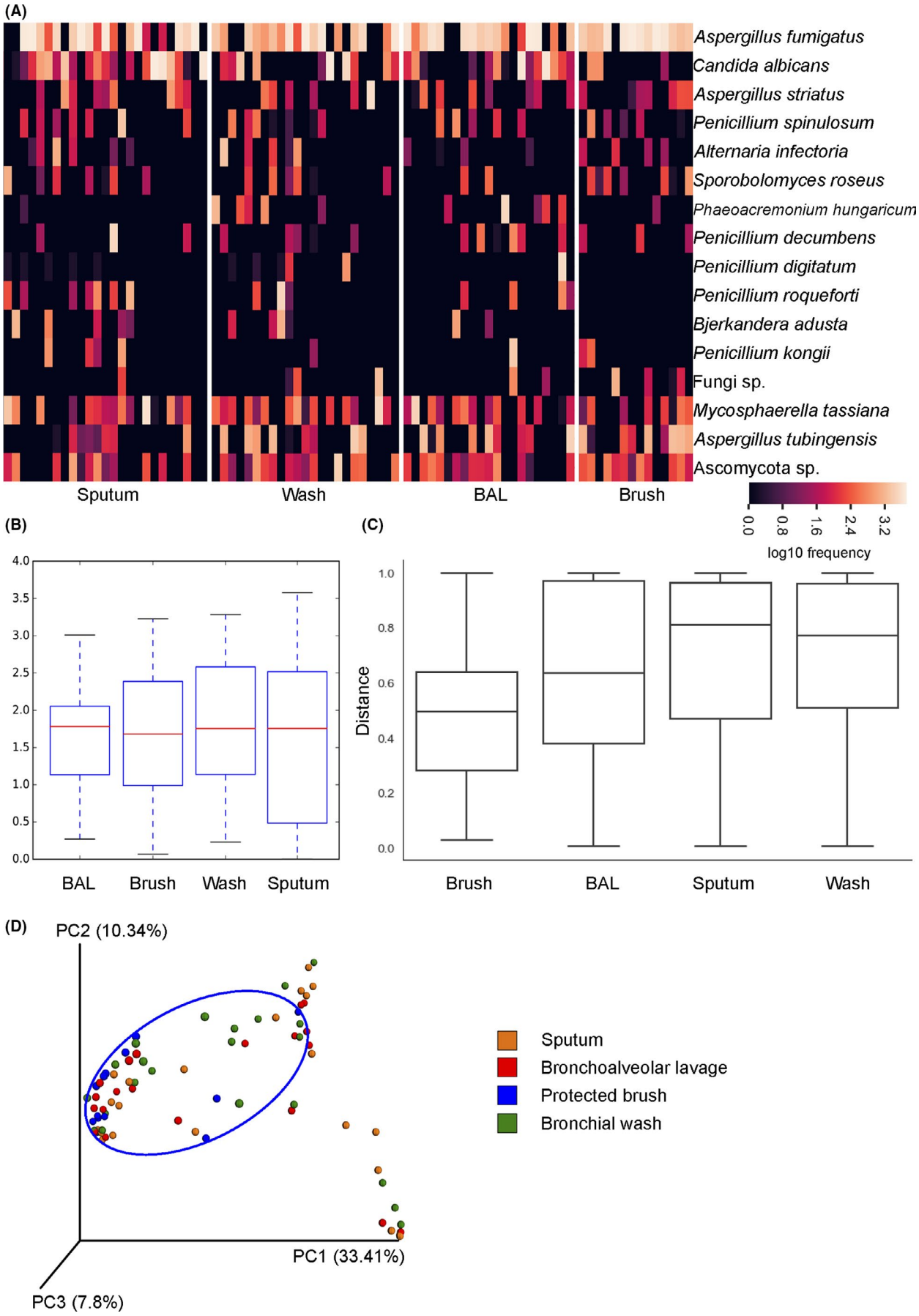
(A)
Aspergillus fumigatus
Candida albicans
Aspergillus striatus
Penicillium spinulosum
Alternaria infectoria
Sporobolomyces roseus
Phaeoacremonium hungaricum
Penicillium decumbens
Penicillium digitatum
Penicillium roqueforti
Bjerkandera adusta
Penicillium kongii
Fungi sp.
Mycosphaerella tassiana
Aspergillus tubingensis
Ascomycota sp.
Sputum
Wash
BAL
Brush
0.0
0.8
1.6
2.4
3.2
log10 frequency
(B)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
BAL
Brush
Wash
Sputum
(C)
Distance
1.0
0.8
0.6
0.4
0.2
0.0
Brush
BAL
Sputum
Wash
(D)
PC2 (10.34%)
PC1 (33.41%)
PC3 (7.8%)
Sputum
Bronchoalveolar lavage
Protected brush
Bronchial wash

FIGURE 2 Samples from patients who underwent a bronchoscopy representing sputum samples (n = 25), bronchial wash (n = 23), bronchoalveolar lavage (BAL) (n = 21) and protected brush samples (n = 14). A, Heatmap showing the relative abundance of fungal ITS2 sequence data between samples. The data were rarefied then transformed using ln(x + 1) due to zeros in the data set. Only taxa with a relative abundance ≥1% are shown. The relative abundance is indicated by colour, with the lighter shades representing the more abundant sequences. B, Box plot of Shannon alpha diversity indices, showing no significant differences in alpha diversity between groups. C, Box plots of Bray-Curtis dissimilarity showing distance from brush (brush to sputum, P = .013, q = 0.078, brush to wash P = .035, q = 0.105). D, Principal coordinate analysis plots based on the Bray-Curtis distance values suggesting weak clustering of protected brush samples

distinct in terms of fungal abundance. The eight most abundant taxa comprised seven named yeast species and an unidentified fungus, the top three being *C. albicans*, *Candida smithsonii* and *Candida tropicalis*, with mean relative abundances of 72%, 6.5% and 5.5%, respectively (Figure S6A, Table S10). No filamentous fungi were detected with a mean relative abundance >1%.

Alpha diversity was lower from mouthwash than sputum samples; particularly using the Shannon index, although did not reach statistical significance (Table S6, Figure S6B, P = .061). There was no significant difference in beta-diversity measures (Table S7) between mouthwash and sputum and no differentially abundant taxa detected (Table S8). PCoA plots showed no clustering by sample type or patient ID (Figure S6C,D).

3.6 | Impact of outdoor air

Mycosphaerella tassiana dominated air samples, being the most prevalent and abundant fungus detected (Table S11, Figure 3A). In contrast, neither *A. fumigatus* nor *C. albicans* featured amongst the abundant airborne fungi. Air samples exhibited far higher alpha diversity, and far greater beta diversity when compared to clinical samples obtained on the same day (Tables S6 and S7, Figure 3B,C). The PCoA plot showed air samples cluster together around spheres representing the coordinate's sequences of *M tassiana* and Leptosphaeriaceae sp centred, and close to that of *Sporobolomyces roseus* (Figure 3D). A small number of clinical samples clustered with air samples, obtained both from people with asthma and healthy controls (Figure 3D) and representing all clinical sample types, albeit mostly sputa (Figure 3E).

Both ANCOM and balance trees analysis detected taxa that were significantly highly divergent between the air and clinical samples, with four species, *A. fumigatus*, *C. albicans*, *A. tubingensis* and *C. pseudolongus*, being highlighted by both analyses (Table S8, Figure 4B). These species have high relative abundances in each of the clinical sample groups (Tables S2 and S5), whereas were not abundant in outdoor air samples (Table S10). Further details can be found in Appendix S1.

3.7 | Relationship between mycobiota and clinical outcomes

To determine whether any clinical characteristics were associated with the lung mycobiota, we performed analyses with and without healthy controls, unless asthma specific. Asthma duration had no association with measures of alpha or beta diversity (Tables S3 and S4); however, the balance trees analysis suggested the fungal community composition changes significantly with time since diagnosis (Figure 5G). GINA class, bronchiectasis, post-bronchodilator FEV_1, smoking status, levels of fractional exhaled nitric oxide (FeNO), use of oral steroids and dose of inhaled steroid had no significant associations with diversity metrics, or associations with differentially abundant taxa (Tables 2, S3 and S4). However, there was a trend towards increased beta diversity between people with asthma not taking or on low doses of inhaled steroids compared to those taking high doses (Figure S7E). Balance trees analysis suggested groups of fungi changed with increasing levels of FeNO (Figure 5H).

Commonly used serological biomarkers for studying fungal allergy include total IgE and *A. fumigatus*-specific IgE and IgG. No effect on diversity measures was seen with total IgE or *A. fumigatus*-specific IgE (Tables S3 and S4); however, treating the two measures as continuous variables, there appeared to be statistically significant shifts in the mycobiota with increasing levels, both with and without the inclusion of healthy controls (Table S12, Figure 5A,B). Combining sputum samples, alpha diversity was significantly lower with all three metrics for individuals positive for *A fumigatus* IgG (IgG > 40 mg/L) compared to negative. Furthermore, the Shannon index suggested a statistically significant decrease in alpha diversity with increasing amounts of serum *A fumigatus* IgG. (Table S3, Figure S7). The relationship was also observed when excluding healthy controls; however, significance was lower and only significant with the Shannon index. Beta diversity was also significantly different according to *A. fumigatus* IgG status, with and without healthy controls (Table S4), but no taxa were found to be differentially abundant (Table 2). For samples from asthmatics, there was a statistically significant shift in the mycobiota as levels of *A. fumigatus* IgG increased (Table S12, Figure 5E), with *A. fumigatus* being amongst the associated group of fungi.

To investigate whether the mycobiota was influenced by biomarkers of inflammation, patients were grouped according to sputum inflammatory phenotypes. Peripheral blood eosinophilia was not associated with differences in alpha diversity (Table S3), and however, there were significant beta-diversity differences between samples from people with low levels ($<0.2 \times 10^{-9}$/L) compared to high ($>0.4 \times 10^{-9}$/L, Table S4, Figure S7). Balance trees analysis suggested significant shifts in balances between groups of fungi for people with asthma associated with increasing blood eosinophil counts (Table S12, Figure 5F). Using sputum cell differential data, we first compared individuals who were eosinophilic (sputum eosinophils ≥3%) against non-eosinophilic, and individuals who were neutrophilic (sputum neutrophils ≥61%) against non-neutrophilic. We then grouped patients by their inflammatory phenotype[37]; classifying patients as neutrophilic,

eosinophilic, mixed granulocytic (≥61% neutrophils and ≥3% eosinophils) or paucigranulocytic (<61% neutrophils and <3% eosinophils). Sputum inflammatory data were only available for a subset of subjects, comprising 18, 14, 17 and 5 people with asthma, or 24, 14, 17 and 7 combined with healthy controls, for neutrophilic, eosinophilic, mixed granulocytic and paucigranulocytic phenotypes

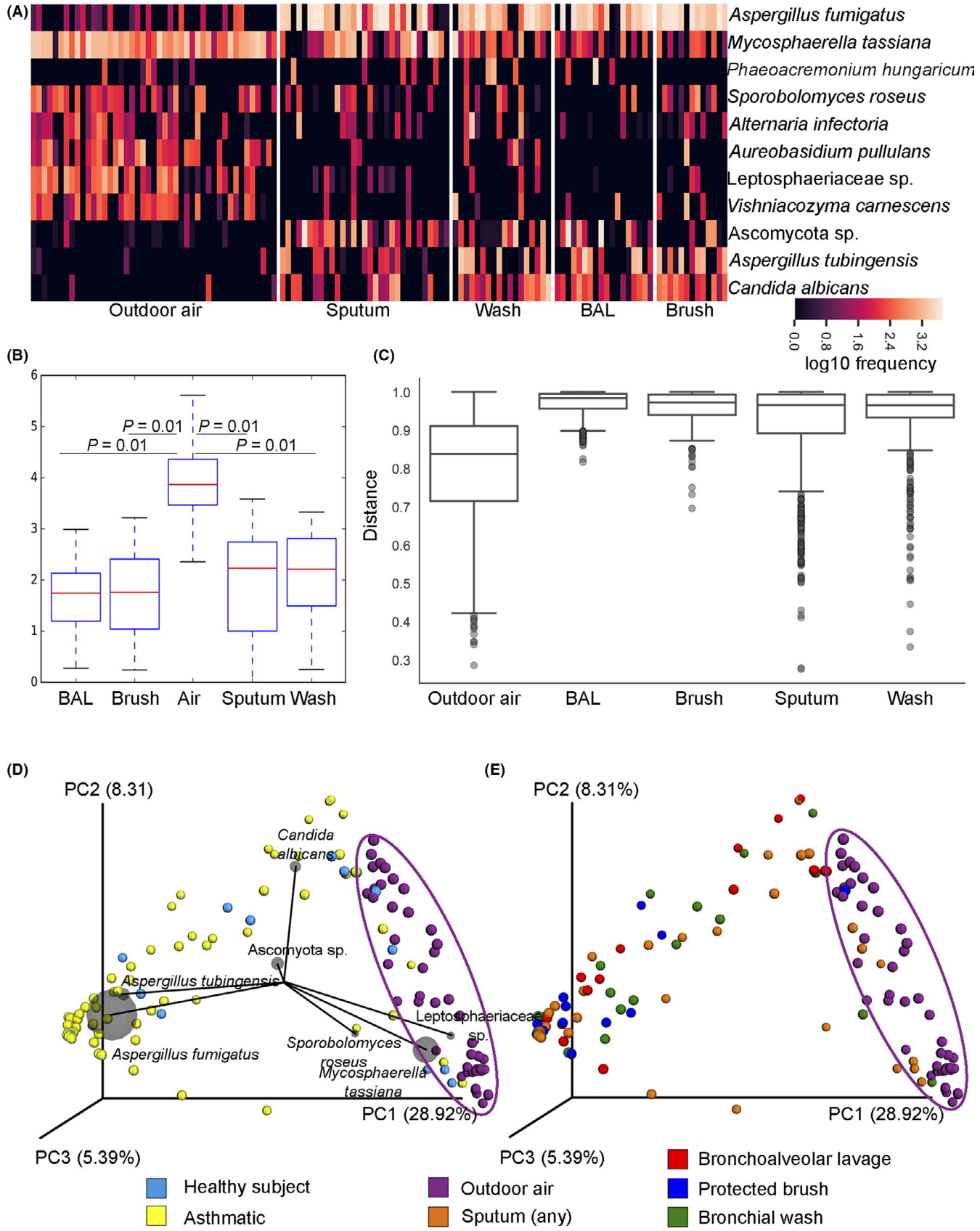

FIGURE 3 Outdoor air samples were analysed from 45 d, matching clinical samples from sputum (n = 31), bronchial wash (n = 18), BAL (n = 18) and protected brush samples (n = 13). A, Heatmap showing the relative abundance of fungal ITS2 sequence data between samples. The data were rarefied then transformed using ln(x + 1) due to zeros in the data set. Only taxa with a relative abundance ≥1% are shown. The relative abundance is indicated by colour, with the lighter shades representing the more abundant sequences. B, Box plot of Shannon alpha diversity indices, showing greater alpha diversity in outdoor air samples than clinical samples. C, Box plots of Bray-Curtis dissimilarity showing distance from outdoor air (outdoor air to brush, sputum, wash and BAL were each $P = .001$, $q = 0.003$, brush to sputum $P = .025$, $q = 0.05$). D, E, Principal coordinate analysis plots based on the Bray-Curtis distance values, the purple ellipse surrounding the air samples; in (D) clinical samples are coloured according to whether they were from healthy controls or people with asthma and in (E) according to sample type. Grey spheres in (C) indicate the coordinates where the main fungal species are centred, with size correlating to relative abundance of the labelled organism

respectively. No association was seen with diversity metrics (Tables S3 and S4). Sputum eosinophils and neutrophils as continuous variables were associated with changes in the balance of groups of fungi in the mycobiota (Table S12, Figure 5C,D). Both ANCOM and balance trees analysis found significantly different abundant species between samples from patients with high versus low neutrophil counts and amongst individuals grouped into the four inflammatory phenotypes. The strongest signal was from *A. tubingensis*, with and without the inclusion of healthy controls, which were negatively associated with neutrophils (Table S12, Figure 6). *Penicillium roqueforti* was often grouped with *A. tubingensis*.

4 | DISCUSSION

This study provides a comprehensive assessment of the airway mycobiota in asthma compared to healthy controls. We also compared the oral mycobiota and aeromycobiota allowing an assessment of those species that were present as a result of inhalation rather than colonization. The analysis of the airway mycobiota revealed a complex community with high diversity between individuals, but distinct shifts in the mycobiota detected dependent on the lung compartment studied and the health status of the individual. This was particularly true of the epithelial lining of the lung, sampled using protected brushes which minimizes risk of oropharyngeal contamination.[38] These samples revealed an unexpected prominence for a number of fungal species including *A. tubingensis* and *C. pseudolongus*. Stimulation of the bronchial epithelium leading to secretion of alarmins such as TSLP and IL-33 is increasingly recognized as critical to the chronic T2 airway inflammation that is a hallmark of asthma.[39] Our data would be consistent with the concept that epithelial colonizing fungi are responsible, at least in part, for this persistent epithelial activation.[40]

Three species dominated the airway mycobiota: *A. fumigatus*, *C. albicans* and *M. tassiana* (previous name *Davidiella tassiana*, the sexual form of *C. herbarum*). Similar findings have been found in cystic fibrosis[17] and asthma,[22,23] although the relative abundance of the three to each other varied between the studies which may reflect differences in subject groups, DNA extraction protocols or primer choices. *M. tassiana* likely represents a group of *Cladosporium* species as the ITS region poorly resolves species within the *herbarum* complex.[41] As a largely mesophilic genera, incapable of growing at body temperature, they are likely to have been present as a result of inhalation of spores from outdoor air rather than representing colonization the airway.[27] Using outdoor air samples as environmental controls, we have shown that the *Cladosporium* and Leptosphaeriaceae detected in the lung mycobiota, but whose spores are routinely detected in air samples,[27] were highly abundant and prevalent from air samples. In contrast, neither *A. fumigatus* nor *C. albicans* were present to any degree in air samples, and due to their thermotolerance, it is likely that they are colonizing the airways even in healthy subjects. *C. albicans* almost universally colonizes the oropharyngeal mucosa[42]; however, whilst *C. albicans* dominated the mouthwash samples, we noted several non-*albicans* yeast species in the mouthwash that were not found in the lower airways. It is clear from this study that *C. albicans* and other yeasts can also be found in substantial amounts in the lower airway. It is possible that there was contamination from oral flora in sputum, BAL and wash samples, but *C. albicans* was also present, albeit at lower levels, in the protected brush specimen where minimal contamination would be anticipated. The increased beta diversity between brush and wash, and between brush and sputum supports this. *A. fumigatus* is a dominant pathogen in fungal lung disease, but it was interesting to discover that it was present in nearly all subjects, including the non-asthmatics. Other studies of the normal airways have also demonstrated the presence of *Aspergillus* as part of the normal airway mycobiota.[43] We have previously shown increased rates of sputum culture for *A. fumigatus* in asthmatics (~50%) compared to healthy subjects (~10%), especially in people with fungal IgE sensitization.[7,10] In this study, we only cultured*A. fumigatus* from asthmatics, although at lower rates than our earlier studies because most of the sample was used for DNA extraction. Fraczek et al[22] in one of few studies of the mycobiota in asthma focussed on overall fungal load in BAL fluid suggesting that corticosteroids could encourage fungal growth, although that observation was confounded by asthma severity and treatment with azoles. In our study, all the asthmatics were on IHC, and although the dose of IHC did not appear to influence the prevalence and relative abundance of the airway mycobiota, there was a trend towards increased beta diversity between the high and low dose groups. The correlation between *A. fumigatus* and *C. albicans* relative abundances with culture outcomes validated our HTS findings.

More than 200 fungal taxa were detected in the airway; however, the majority were only present at low concentrations in a minority of individuals. Due to the compositional nature of HTS data, conventional statistical tools such as the *t* test and ANOVA

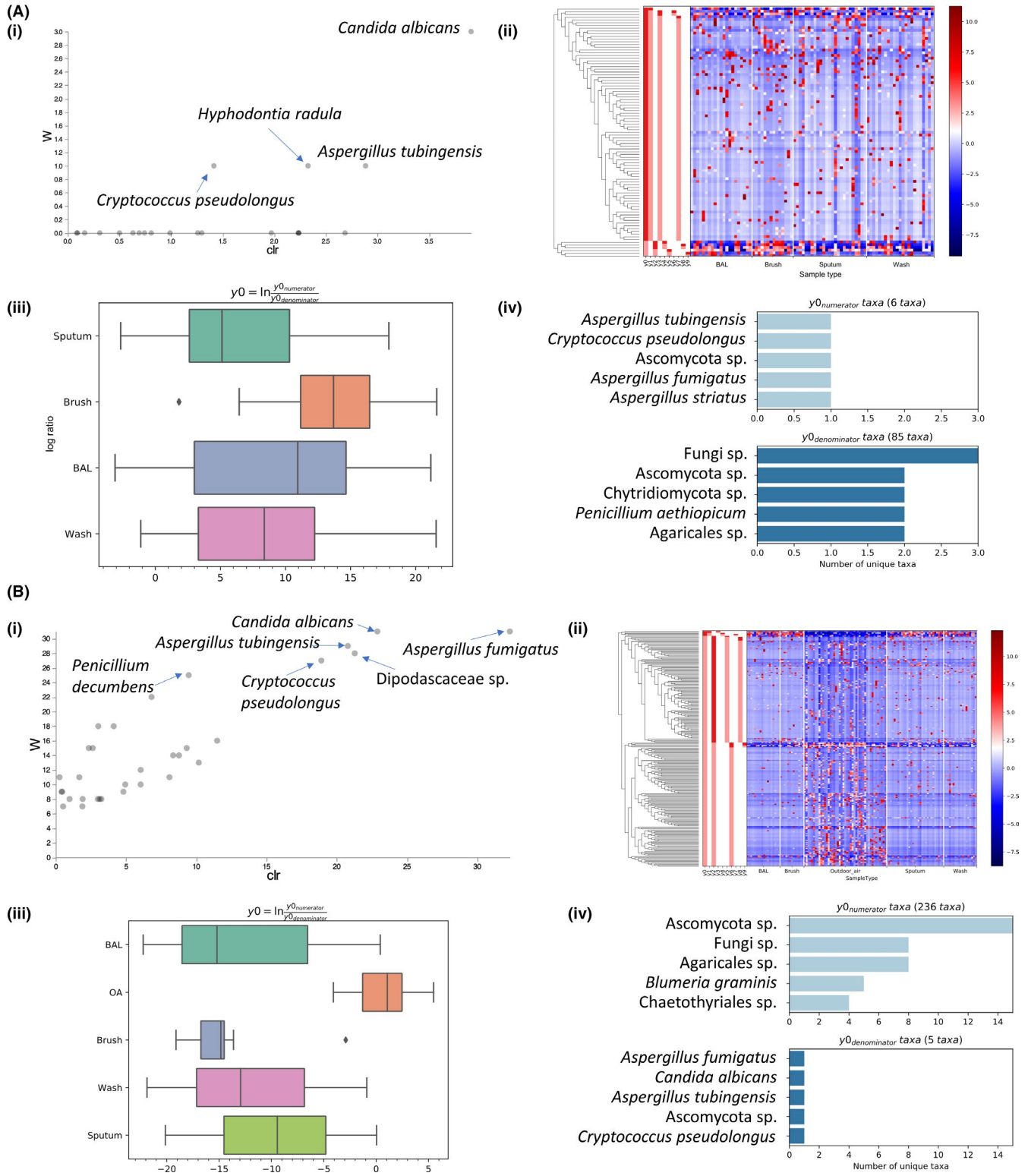

FIGURE 4 A, Bronchoscopy-derived samples with corresponding sputum samples, and B, outdoor air samples and clinical samples obtained on the same day. Showing (i) volcano plot from the analysis of composition of microbiomes (ANCOM) test comparing sample types. The *y*-axis (*W* value) represents the number of times the null hypothesis (no change in abundance between groups) has been rejected, whilst the clr (centre log ratio transformed) value on the *x*-axis represents the mean difference in abundance of a certain taxon between sample types. Only fungi with significantly different relative abundances as determined by ANCOM have been labelled. (ii) Heatmap of fungal species from unsupervised Ward's hierarchical clustering. Balances y0-y9 showing the numerator (pink) and denominator (red) are shown on the left hand side of the heatmap. (iii) Box plot showing the log-ratios of fungal taxa in the statistically significant gneiss balance. (iv) Box plot with the fungal taxa that comprised each side of the balance (numerator in pale blue and denominator in dark blue), indicating the number of unique taxa at the species level for each named taxa. For large groups, the named taxa are those representing the most unique taxa.

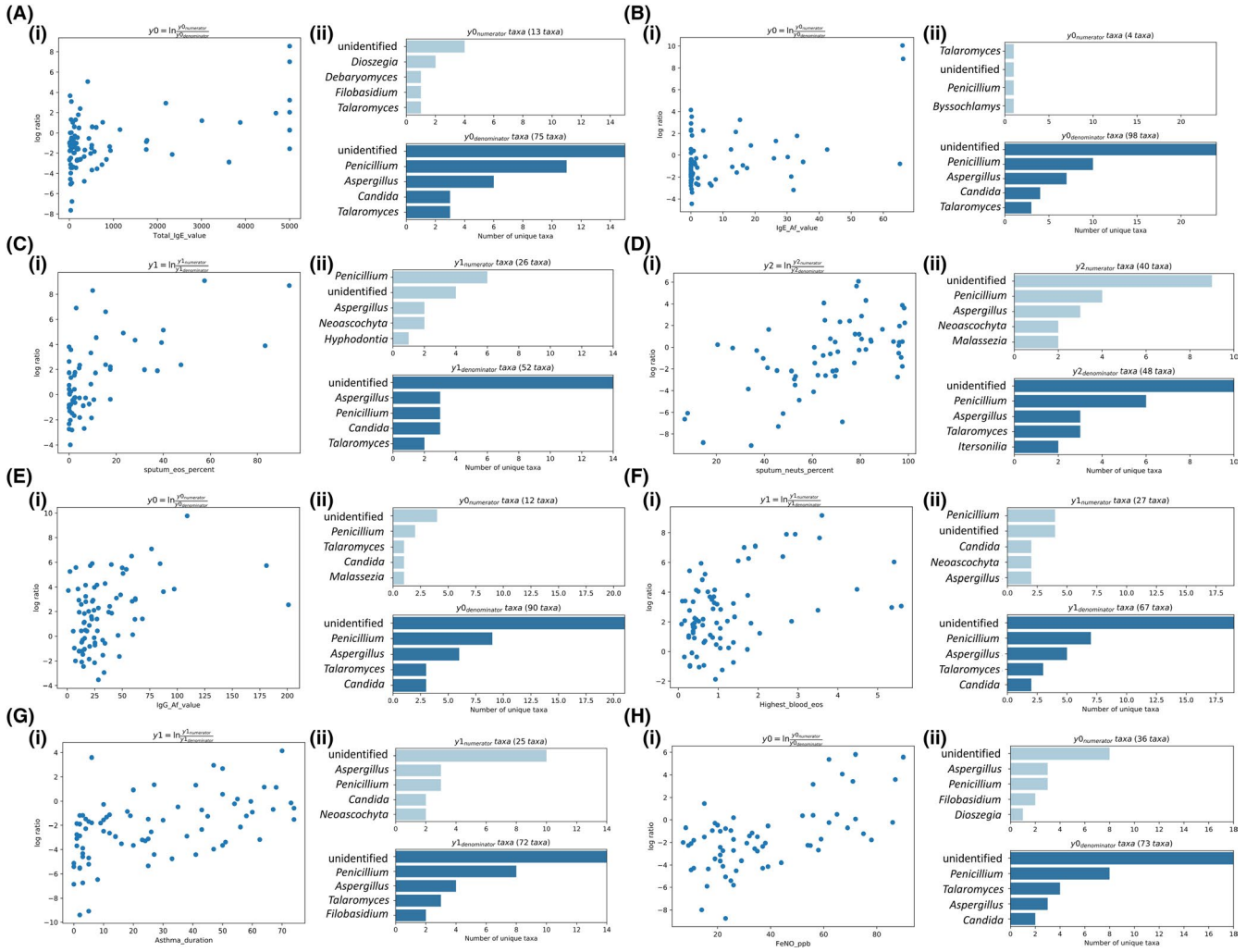

FIGURE 5 Relationships between sputum mycobiota and continuous clinical variables combining samples from people with asthma and healthy controls (A) total IgE (IU/mL), (B) *A. fumigatus* IgE (kU/L), (C) sputum eosinophil percent and (D) sputum neutrophils percent. For only people with asthma showing (E) *A. fumigatus* IgG (mg/L), (F) peripheral blood eosinophil count ($\times 10^{-9}$/L), (G) asthma duration (years) and (H) fractional exhaled nitric oxide, parts per billion. (i) Scatterplot of the numerator/denominator log-ratios relative to the continuous variable. (ii) Box plot with the fungal taxa that comprised each side of the balance (numerator in pale blue and denominator in dark blue) at the level of genus showing the top five genera in terms of number of unique taxa they represent. Full species level detail for numerator given in Table S12

struggle to accurately detect differentially abundant taxa between groups.[44] To account for this compositionality, analysis of composition of microbiomes (ANCOM)[35] and balance trees analysis[36] were used in this study. ANCOM is a powerful method for determining microbial differential abundance, with good control of false-discovery rates.[45] ANCOM does, however, have issues with large numbers of zeros and assumes that less than 25% of taxa are changing in abundance.[36] To limit the possible increase in error, balance tree analysis was used in addition to ANCOM that attempts to calculate changes in the balance (log ratio of abundance) between subcommunities of taxa rather than individuals. These subsets of taxa were constructed either by clustering on how often they co-occur with one another (categorical data) or clustering on a continuous variable if continuous data were investigated. Gradient clustering is more prone to false positives, and hence, caution was used in interpreting implicated fungi. The use of balances enables the identification of taxa whose relative abundances were most significant in explaining compositional variations.

Few bacterial studies have compared diversity scores between asthma and health, and those that have had conflicting findings ranging from decreased[46] to increased alpha diversity in asthmatics,[12] with other studies finding no differences in diversity metrics.[47] Fewer studies have compared the mycobiota, but adult,[22] and paediatric[23] studies have found no significant differences in standard diversity metrics between asthma and health. Using paired samples between sputum and bronchoscopy-derived samples from individuals, we found significant differences in alpha diversity between asthma and health. We found differentially abundant species, including *C. pseudolongus*, between asthma and health when analysing bronchoscopy-derived but not sputum samples, and noted significant shifts in the mycobiota with asthma duration.

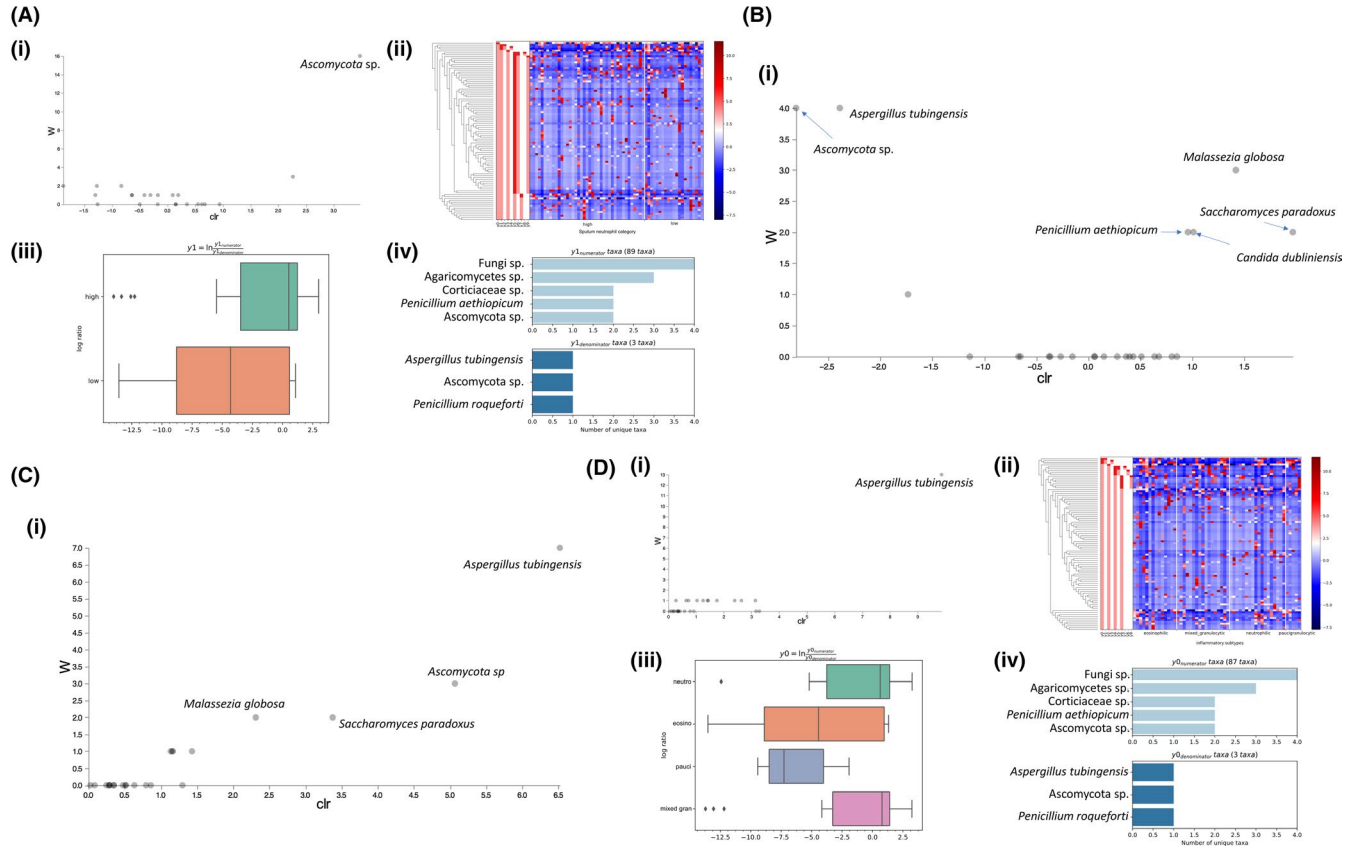

FIGURE 6 Differentially abundant fungal taxa associated with markers of inflammation. A, B, Samples grouped according to sputum neutrophilic high (≥61%) or low for (A) asthma and healthy controls combined or (B) only people with asthma. C, D, Samples grouped according to inflammatory phenotypes, neutrophilic (neutro), eosinophilic (eosino), paucigranulocytic (pauci) or mixed granulocytic (mixed gran) for (C) asthma and healthy controls combined or (D) only people with asthma. Showing (i) volcano plot from the analysis of composition of microbiomes (ANCOM) test comparing samples. The *y*-axis (*W* value) represents the number of times the null hypothesis (no change in abundance between groups) has been rejected, whilst the clr (centre log ratio transformed) value on the *x*-axis represents the mean difference in abundance of a certain taxon between sample types. Only fungi with significantly different relative abundances as determined by ANCOM have been labelled. (ii) Heatmap of fungal species from unsupervised Ward's hierarchical clustering. Balances y0-y9 showing the numerator (pink) and denominator (red) are shown on the left hand side of the heatmap. (iii) Box plot showing the log-ratios of fungal taxa in the statistically significant gneiss balance. (iv) Box plot with the fungal taxa that comprised each side of the balance (numerator in pale blue and denominator in dark blue), indicating the number of unique taxa at the species level for each named taxa. For large groups, the named taxa are those representing the most unique taxa

There has been increasing momentum towards using endotyping as a means of grouping individuals by a distinct pathophysiological mechanism.[48] Stratifying patients according to their underlying inflammation is one such approach, and studies have compared the composition of the bacterial microbiota of the lower airways with inflammatory features such as neutrophilia or eosinophilia with conflicting findings. Sputum neutrophilia but not eosinophilia was correlated with decreased diversity measures in severe asthma[37] and moderate-to-severe asthma,[49] whereas relationships seen with eosinophils in mild asthma.[50] To our knowledge, this is the first study to compare inflammatory phenotypes with the lung mycobiota, finding significant differences with blood eosinophils and sputum differential counts. Beta diversity was significantly different between individuals with low and high blood eosinophil counts, and groups of fungi within the mycobiota were seen to shift with increasing blood eosinophil count. Whilst increases in both sputum eosinophils and neutrophils were seen to cause shifts in the mycobiota, it was associations with neutrophils that were most interesting, with relationships detected with specific fungi. *A. tubingensis* demonstrated the strongest relationship, being negatively associated with sputum neutrophils, possibly by inducing neutrophil apoptosis.[51] *A tubingensis* had a strikingly higher relative abundance and prevalence in the bronchoscopy samples compared to sputum, especially in the epithelial compartment. Both *A. tubingensis* and *P. roqueforti* exhibited a negative relationship with neutrophils, and both were associated with culture of *A. fumigatus*, which is associated with worse lung function.[10] *C. pseudolongus* was also significantly more abundant in bronchoscopy samples, particularly from brush and BAL with a prevalence in brush samples of 93% (data not shown). *C. pseudolongus* was one of the fungi noted to be differentially more abundant in asthma than health. *A. tubingensis* (part of the *A. niger* complex) is increasingly detected from respiratory secretions and is less susceptible to triazole antifungals than *A. fumigatus*,[52] and however, only one nucleotide distinguishes *A. niger* from *A. tubingensis* using

ITS2, so either species could be implicated based on ITS2 HTS data. *C. pseudolongus* is a member of the *Cryptococcus humicola* complex, although it is indistinguishable from fellow complex member *Cryptococcus longus* using ITS2.[53] *C. humicola* is suspected to be a rare cause of non-*neoformans* cryptococcal infection.[54]

Although this study includes more subjects than many comparable respiratory bacterial microbiota studies[55-57] or asthma mycobiota studies,[22,23] we lacked power to measure significant differences in community structure and were unable to effectively adjust for factors such as age or medication usage. There are inherent challenges studying the mycobiota using a PCR-based approach. Non-homogenous lysis of fungal cells during DNA extraction may introduce biases, and however, we utilized a recommended bead-beating approach.[58] We have previously shown our DNA extraction method and primer choice to be highly suitable for capturing a diverse range of fungal diversity[27] and included in this study two mock fungal communities[28] to test the DNA extraction and HTS-PCR protocols and to optimize our bioinformatic pipeline. Recently, our region of choice (ITS2) has been shown to introduce less bias than the more commonly used ITS1 region in both mock communities and sputum samples,[58] supporting our choice of regions. One caveat with either ITS region is that many clinically relevant species are indistinguishable from close relatives using ITS alone[59,60] and therefore are more reliably referred to by the species complex within which they reside. Primers that are universal for the organisms of interest are a requirement of microbiota studies, but will preferentially amplify the most prevalent flora. Our study did not identify *Pneumocystis* which is sometimes identified in respiratory samples. This was not unexpected as there is only one copy of the ITS2 locus in the *Pneumocystis* genome.[61] A similar study utilizing ITS2 for high-throughput-sequencing cystic fibrosis sputum samples had negative HTS results from samples that had been positive using a highly sensitive species-specific nested PCR approach.[17] The sample preparation for HTS is prone to contamination due to multiple processing steps[62] and reagents like those used for DNA extraction.[63] We included PCR, DNA extraction and sample processing reagent controls which were examined alongside the samples they controlled for prior to commencing the main analysis. Whilst choice of primers may influence the findings,[64] as could index hopping whereby incorrect barcode identifiers are incorporated into growing clusters,[65] we have mitigated some of these risks by utilizing a conservative quality-filtering approach[32] and focusing on the more abundant members of the mycobiota.

IgE sensitization to filamentous thermotolerant fungi such as *A. fumigatus* is associated with poor asthma control, fixed airflow obstruction, bronchiectasis and various radiological abnormalities resulting in a pattern of asthma we have termed AFAD.[4,6] Whilst a number of clinical correlations were observed, a larger study utilizing a quantitative approach may be needed to fully address the clinical significance of the fungi detected. This was a cross-sectional study, and one of the next steps should be a longitudinal investigation to establish the stability of the lung mycobiota during stable state and exacerbations in order to interpret structural abnormalities that develop over decades.

In summary, we have shown that the airways are colonized by a community of fungi dominated by *A. fumigatus* and *C. albicans*, which are the main components of the normal airway mycobiota. This community is highly variable; however, notable shifts in the balance of fungi within communities and between compartments of the lung were associated with asthma status, asthma duration and biomarkers of inflammation. In particular, members of the *A. niger* and *C. humicola* species complexes were revealed by this molecular approach to be playing an unexpected potential role in asthma pathogenesis.

ACKNOWLEDGEMENTS

The research was supported by the NIHR Leicester Biomedical Research Centre, the Midlands Asthma and Allergy Research Association (MAARA), Asthma UK (AUK-PHD-2013-250) and the Henry Smith Charity, the Academy of Medical Sciences [SBF003\1125], the Wellcome Trust, the Government Department of Business, Energy and Industrial Strategy and the British Heart Foundation. The views expressed are those of the authors and not necessarily those of the NHS the NIHR or the Department of Health.

DATA AVAILABILITY STATEMENT

Sequence data were deposited in the European Nucleotide Archive; study accession number PRJEB31078.

ORCID

Andrew J. Wardlaw https://orcid.org/0000-0001-6583-0791
Catherine H. Pashley https://orcid.org/0000-0003-4023-3610

REFERENCES

1. Knutsen AP, Bush RK, Demain JG, et al. Fungi and allergic lower respiratory tract diseases. *J Allergy Clin Immunol*. 2012;129:280-291.
2. Denning DW, O'Driscoll BR, Hogaboam CM, et al. The link between fungi and severe asthma: a summary of the evidence. *Eur Respir J*. 2006;27:615-626.
3. Rick E-M, Woolnough K, Pashley CH, Wardlaw AJ. Allergic fungal airway disease. *J Investig Allergol Clin Immunol*. 2016;26:344-354.
4. Woolnough K, Fairs A, Pashley CH, Wardlaw AJ. Allergic fungal airway disease: pathophysiologic and diagnostic considerations. *Curr Opin Pulm Med*. 2015;21:39-47.
5. Wardlaw AJ, Woolnough K, Pashley CH. Lassoing a chimera: the semantics of allergic fungal airway disease. *Clin Exp Allergy*. 2015;45:1746-1749.
6. Woolnough KF, Richardson M, Newby C, et al. The relationship between biomarkers of fungal allergy and lung damage in asthma. *Clin Exp Allergy*. 2017;47:48-56.
7. Fairs A, Agbetile J, Hargadon B, et al. IgE sensitization to *Aspergillus fumigatus* is associated with reduced lung function in asthma. *Am J Respir Crit Care Med*. 2010;182:1362-1368.
8. Fraczek MG, Kirwan MB, Moore CB, et al. Volume dependency for culture of fungi from respiratory secretions and increased sensitivity of *Aspergillus* quantitative PCR. *Mycoses*. 2014;57:69-78.
9. Pashley CH, Fairs A, Morley JP, et al. Routine processing procedures for isolating filamentous fungi from respiratory sputum samples may underestimate fungal prevalence. *Med Mycol*. 2012;50:433-438.
10. Agbetile J, Fairs A, Desai D, et al. Isolation of filamentous fungi from sputum in asthma is associated with reduced post-bronchodilator FEV1. *Clin Exp Allergy*. 2012;42:782-791.

11. Huang YJ, Boushey HA. The microbiome in asthma. *J Allergy Clin Immunol*. 2015;135:25-30.
12. Marri PR, Stern DA, Wright AL, et al. Asthma-associated differences in microbial composition of induced sputum. *J Allergy Clin Immunol*. 2013;131:346-352.
13. Huang YJ, Nelson CE, Brodie EL, et al. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J Allergy Clin Immunol*. 2011;127:372-381.
14. Huffnagle GB, Noverr MC. The emerging world of the fungal microbiome. *Trends Microbiol*. 2013;21:334-341.
15. Charlson ES, Diamond JM, Bittinger K, et al. Lung-enriched organisms and aberrant bacterial and fungal respiratory microbiota after lung transplant. *Am J Respir Crit Care Med*. 2012;186:536-545.
16. Cui L, Lucht L, Tipton L, et al. Topographic diversity of the respiratory tract mycobiome and alteration in HIV and lung disease. *Am J Respir Crit Care Med*. 2015;191:932-942.
17. Delhaes L, Monchy S, Fréalle E, et al. The airway microbiota in cystic fibrosis: a complex fungal and bacterial community-implications for therapeutic management. *PLoS One*. 2012;7:e36313.
18. Kramer R, Sauer-Heilborn A, Welte T, et al. Cohort study of airway mycobiome in adult cystic fibrosis patients: differences in community structure between fungi and bacteria reveal predominance of transient fungal elements. *J Clin Microbiol*. 2015;53:2900-2907.
19. Botterel F, Angebault C, Cabaret O, et al. Fungal and bacterial diversity of airway microbiota in adults with cystic fibrosis: concordance between conventional methods and ultra-deep sequencing, and their practical use in the clinical laboratory. *Mycopathologia*. 2018;183:171-183.
20. Mac Aogáin M, Chandrasekaran R, Lim AYH, et al. Immunological corollary of the pulmonary mycobiome in bronchiectasis: the CAMEB study. *Eur Respir J*. 2018;52.
21. Chandrasekaran R, Mac Aogáin M, Chalmers JD, et al. Geographic variation in the aetiology, epidemiology and microbiology of bronchiectasis. *BMC Pulm Med*. 2018;18:1-14.
22. Fraczek MG, Chishimba L, Niven RM, et al. Corticosteroid treatment is associated with increased filamentous fungal burden in allergic fungal disease. *J Allergy Clin Immunol*. 2018;142:407-414.
23. Goldman DL, Chen Z, Shankar V, et al. Lower airway microbiota and mycobiota in children with severe asthma. *J Allergy Clin Immunol*. 2018;141:808-811.
24. Pavord ID, Pizzichini MMM, Pizzichini E, Hargreave FE. The use of induced sputum to investigate airway inflammation. *Thorax*. 1997;52:498-501.
25. Pizzichini E, Pizzichini M, Efthimiadis A, et al. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir J*. 1996;9:1174-1180.
26. Du Rand IA, Blaikley J, Booton R, et al. British Thoracic Society guideline for diagnostic flexible bronchoscopy in adults: accredited by NICE. *Thorax*. 2013;68(Suppl 1):i1-i44.
27. Pashley CH, Fairs A, Free RC, Wardlaw AJ. DNA analysis of outdoor air reveals a high degree of fungal diversity, temporal variability, and genera not seen by spore morphology. *Fungal Biol*. 2012;116:214-224.
28. Tonge DP, Pashley CH, Gant TW. Amplicon–based metagenomic analysis of mixed fungal samples using proton release amplicon sequencing. *PLoS One*. 2014;9:e93849.
29. Pashley CH, Fairs A, Edwards RE, et al. Reproducibility between counts of airborne allergenic pollen from two cities in the East Midlands, UK. *Aerobiologia*. 2009;25:249-263.
30. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. New York, NY: Academic Press; 1990:315-322.
31. Harris PA, Taylor R, Thielke R, et al. Research electronic data capture (REDCap)-A metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform*. 2009;42:377-381.
32. Bokulich NA, Subramanian S, Faith JJ, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods*. 2013;10:57-59.
33. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2010;7:335-336.
34. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37:852-857.
35. Mandal S, Van Treuren W, White RA, et al. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis*. 2015;26:27663.
36. Morton JT, Sanders J, Quinn RA, et al. Balance trees reveal microbial niche differentiation. *Msystems*. 2017;2:e00162-16.
37. Taylor SL, Leong LEX, Choo JM, et al. Inflammatory phenotypes in patients with severe asthma are associated with distinct airway microbiology. *J Allergy Clin Immunol*. 2018;141:94-103.e15.
38. Grønseth R, Drengenes C, Wiker HG, et al. Protected sampling is preferable in bronchoscopic studies of the airway microbiome. *ERJ Open Res*. 2017;3(3): 00019-2017. https://doi.org/10.1183/23120541.00019-2017
39. Wang W, Li Y, Lv Z, et al. Bronchial allergen challenge of patients with atopic asthma triggers an alarmin (IL-33, TSLP, and IL-25) response in the airways epithelium and submucosa. *J Immunol*. 2018;201:2221-2231.
40. Wiesner DL, Klein BS. Lung epithelium: barrier immunity to inhaled fungi and driver of fungal-associated allergic asthma. *Curr Opin Microbiol*. 2017;40:8-13.
41. Bensch K, Groenewald JZ, Braun U, et al. Common but different: the expanding realm of *Cladosporium*. *Stud Mycol*. 2015;82:23-74.
42. Diaz PI, Hong BY, Dupuy AK, Strausbaugh LD. Mining the oral mycobiome: methods, components, and meaning. *Virulence*. 2017;8:313-323.
43. Limon JJ, Skalski JH, Underhill DM. Commensal fungi in health and disease. *Cell Host Microbe*. 2017;22:156-165.
44. Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. Microbiome Datasets Are Compositional: And This Is Not Optional. *Front Microbiol*. 2017;8:2224. https://doi.org/10.3389/fmicb.2017.02224
45. Weiss S, Xu ZZ, Peddada S, et al. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*. 2017;5:27.
46. Denner DR, Sangwan N, Becker JB, et al. Corticosteroid therapy and airflow obstruction influence the bronchial microbiome, which is distinct from that of bronchoalveolar lavage in asthmatic airways. *J Allergy Clin Immunol*. 2016;137:1398-1405.e3.
47. Li N, Qiu R, Yang Z, et al. Sputum microbiota in severe asthma patients: Relationship to eosinophilic inflammation. *Respir Med*. 2017;131:192-198.
48. Lotvall J, Akdis CA, Bacharier LB, et al. Asthma endotypes: A new approach to classification of disease entities within the asthma syndrome. *J Allergy Clin Immunol*. 2011;127:355-360.
49. Simpson JL, Daly J, Baines KJ, et al. Airway dysbiosis: haemophilus influenzae and Tropheryma in poorly controlled asthma. *Eur Respir J*. 2016;47:792-800.
50. Sverrild A, Kiilerich P, Brejnrod A, et al. Eosinophilic airway inflammation in asthmatic patients is associated with an altered airway microbiome. *J Allergy Clin Immunol*. 2017;140:407-417.e11.
51. Watt AP, Courtney J, Moore J, et al. Neutrophil cell death, activation and bacterial infection in cystic fibrosis. *Thorax*. 2005;60:659-664.

52. Gautier M, Normand AC, L'Ollivier C, et al. *Aspergillus tubingensis*: a major filamentous fungus found in the airways of patients with lung disease. *Med Mycol*. 2016;54:459-470.
53. Takashima M, Sugita T, Shinoda T, Nakase T. Reclassification of the *Cryptococcus humicola* complex. *Int J Syst Evol Microbiol*. 2001;51:2199-2210.
54. Bernal-Martinez L, Gomez-Lopez A, Castelli MV, et al. Susceptibility profile of clinical isolates of non-*Cryptococcus neoformans*/non-*Cryptococcus gattii Cryptococcus* species and literature review. *Med Mycol*. 2010;48:90-96.
55. Morris A, Beck JM, Schloss PD, et al. Comparison of the respiratory microbiome in healthy nonsmokers and smokers. *Am J Respir Crit Care Med*. 2013;187:1067-1075.
56. Wang Z, Bafadhel M, Haldar K, et al. Lung microbiome dynamics in COPD exacerbations. *Eur Respir J*. 2016;47:1082-1092.
57. Goleva E, Jackson LP, Harris JK, et al. The effects of airway microbiome on corticosteroid responsiveness in asthma. *Am J Respir Crit Care Med*. 2013;188:1193-1201.
58. Ali N, Mac Aogain M, Morales RF, et al. Optimisation and benchmarking of targeted amplicon sequencing for mycobiome analysis of respiratory specimens. *Int J Mol Sci*. 2019;20:4991.
59. Chen M, Zeng J, De Hoog GS, et al. The 'species complex' issue in clinically relevant fungi: a case study in *Scedosporium apiospermum*. *Fungal Biol*. 2016;120:137-146.
60. Cavalier-Smith T. Kingdoms Protozoa and Chromista and the eozoan root of the eukaryotic tree. *Biol Lett*. 2010;6:342-345.
61. Nahimana A, Francioli P, Blanc DS, et al. Determination of the copy number of the nuclear rDNA and beta-tubulin genes of *Pneumocystis carinii* f. sp hominis using PCR multicompetitors. *J Eukaryot Microbiol*. 2000;47:368-372.
62. Lindahl BD, Nilsson RH, Tedersoo L, et al. Fungal community analysis by high-throughput sequencing of amplified markers – a user's guide. *New Phytol*. 2013;199:288-299.
63. Loeffler J, Hebart H, Bialek R, et al. Contaminations occurring in fungal PCR assays. *J Clin Microbiol*. 1999;37:1200-1202.
64. Bellemain E, Carlsen T, Brochmann C, et al. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC Microbiol*. 2010;10:189.
65. Quince C, Walker AW, Simpson JT, et al. Shotgun metagenomics, from sampling to analysis. *Nat Biotechnol*. 2017;35:833-844.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Rick E-M, Woolnough KF, Seear PJ, et al. The airway fungal microbiome in asthma. *Clin Exp Allergy*. 2020;00:1–17. https://doi.org/10.1111/cea.13722