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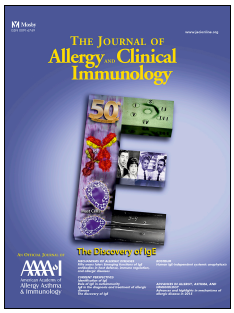

Corticosteroid treatment is associated with increased filamentous fungal burden in allergic fungal disease

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Corticosteroid treatment is associated with increased filamentous fungal burden in 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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ABSTRACT

Background

Allergic diseases caused by fungi are common. The best understood conditions are allergic bronchopulmonaryaspergillosis (ABPA) and severe asthma with fungal sensitisation (SAFS). 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 characterised fungal disease. A secondary objective was to determine possible effects of treatment on the mycobiome.

Methods

After bronchoscopy, ITS1 DNA was amplified and sequenced and fungal load determined by RT-PCR. Clinical and treatment variables were correlated with the main species identified. ABPA (n=16), SAFS (n=16), severe asthma not sensitised to fungi, (n=9), mild asthma patients(n=7) and 10 healthy controls were studied.

Results

The mycobiome was highly varied with severe asthmatics carrying higher loads of fungus. Healthy individuals had low fungal loads, mostly poorly characterised 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 ($p<0.01$).

Conclusions

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

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Capsule summary: This article reports the first analysis of fungal communities in lungs of individuals with fungal disease. Effects of steroid and antifungal drugs suggest complex patterns of fungal burden with potential implications for treatment.

Key words: Lung mycobiome, Aspergillus, steroid, antifungal, ABPA, asthma.

Abbreviations:

ITS: Ribosomal internal transcribed spacer region

RT-PCR: real time PCR

qPCR: quantitative PCR

CF: cystic fibrosis

BTS: British Thoracic Society

CTAB: cetyltriammonium bromide

ICS: inhaled corticosteroid

OCS: oral corticosteroid

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INTRODUCTION

Fungal diseases are a serious health problem worldwide (1). A common form of fungal disease is caused by allergic reactions to fungi that complicates existing respiratory pathology. Severe asthma is frequently linked with fungal sensitisation (2). Recognised asthma endotypes include allergic bronchopulmonary mycosis (ABPM) (3), which includes allergic bronchopulmonaryaspergillosis (ABPA) (4). A recently proposed phenotype of severe asthma is severe asthma with fungal sensitisation (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 healthcare and economic impact of fungal allergy is significant. It is currently assumed that ABPA and SAFS are directly linked to fungal colonisation 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 gastro-intestinal tract, oral, skin and vaginal bacterial microenvironment (3, 15, 16). The data available on the lung mycobiome (reviewed in (8))is limited to samples from individuals that did not have well characterised fungal disease.

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 (9). *Aspergillusfumigatus*causes life-threatening invasive aspergillosis, chronic pulmonary aspergillosis, *Aspergillus*-associated asthma and allergic diseases such as ABPA and SAFS (1,10–12). ABPA is characterised by exaggerated Th2 CD4+ allergic inflammatory response to *A. fumigatus* allergens (13), including proteases (14) in the bronchial airway of atopic asthma and CF patients. Other fungi are implicated in SAFS and allergic bronchopulmonary mycosis (3,15). 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(16–18) but is also a common treatment to alleviate symptoms of asthma (19).

In order 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 ITS1 sequencing, RT-PCR and standard microbiological culture methods. Additionally we examined whether particular treatments were associated with altered mycobiomes in these individuals.

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METHODS

Sample collection and processing.

Patients were identified using the exclusion and inclusion criteria defined in suppl. methods. Briefly, 64 individuals were identified with 58 agreeing to bronchoscopy (Table 1 and suppl. table 1). Bronchoalveolar lavage (BAL) samples were collected using local guidelines and BTS standard procedures (34). 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 as described below. Dilution of endogenous lung fluids in the lavage medium was estimated as described (35) using a Sigma urea assay kit (Sigma – Aldrich, UK).

DNA extraction, PCR and Illumina sequencing.

BAL samples were processed immediately after they were obtained, without storage then DNA extraction was carried out immediately using a CTAB method previously described (36). Controls 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 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 using appropriate QIIME workflows (37-39). 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 compared to assess potential confounding. Minimum group size after stratification was 3. Alpha diversity metrics were calculated for fungi using an Excel spreadsheet. Numbers of fungal otus and diversity statistics are shown in supplementary table 2. Pairwise comparison of specific species within datasets was assessed using T–test or Mann Whitney in cases where deviation from normal distribution was observed. Statistics were adjusted for multiple sampling using Benjamini-Hochberg correction as appropriate. Dunn's comparison test and logistical regression was 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 OTU (organised taxonomic unit) picking workflow.

Quantification of fungal burden using RT-PCR.

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Overall fungal burden was determined using RT-PCR with Aspergillus 18S specific or pan-fungal ITS1-2 region primers as previously described (7,40,41).

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+ (42) with empirically defined parameters E value < $1E^{-31}$, 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 (20). To reduce the possibility of mis-assignation from sequence error, fastq files were first filtered using TrimmomaticSE to remove end sequences with phred 33 scores lower than 30 and any sequences containing 3 base windows with quality lower than phred 30. Where appropriate, sequences were aligned using MUSCLE (43) trimmed using ALIVIEW (44) and used to make neighbour – joining trees using PHYML in Seaview v4.5.4 (45).

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RESULTS

Recruitment of patients to the study.

This was an observational study conducted between November 2011 and November 2013 at the National Aspergillosis Centre (NAC) and the North West Severe Asthma Centre based at the University Hospital of South Manchester. Sixty four adults (31 female and 33 male) aged 22-75 years old were enrolled. All current or previous smokers had <10 pack years. No bronchoscopy samples were obtained from five patients and DNA extraction was unsuccessful in one. Therefore 58 patients were included in this study (ABPA n=16, SAFS n=16, severe asthma without fungal sensitisation (SA) n=9, mild asthma (MA) n=7 and healthy controls (HC) n=10) (Table 1 and Online Supplement Table E2). ABPA, SAFS and SA patients were recruited from a cohort of patients attending the above clinics. MA and HC 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 a 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 Figure 1A-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 (Figure 1 A) (Online Supplement Figure E1). Loads are very variable across samples (Figure 1A and 1B). Healthy individuals all have low fungal burden and are characterised by a high proportion of Basidiomycete DNA (Figure 1B) 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 (Online Supplement Figure E2). 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 (Supp Figure 2). *A. fumigatus* complex (Figure 1B) forms the largest proportion of fungi in the disease groups (20). 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 analyse 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 lower than 30 to minimise any possibility that sequencing errors gave rise to sequence diversity. Typical results are shown as phylogenetic trees in Figure 2. A core

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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 centre 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 one 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 (Figure 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).

In order 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 ABPA, 9 SAFS), those who had previously been on itraconazole therapy (PREVIOUS group; n=10 including 5 ABPA, 3 SAFS, 2 SA) and those who had never been given itraconazole (NEVER group; n= 31 including 3 ABPA, 3 SAFS, 8 SA, 7 MA, 10 HC) (Figure 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, load 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=0.018) with the other comparisons narrowly failing to reach significance (p values between 0.1 and 0.05). This trend occurs in all groups and sub-groups 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=0.79 and p=0.059, respectively) but comparison of CURRENT and PREVIOUS groups is significant (p= 0.03).

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Preliminary analysis of QIIME data suggested potential differences in fungal load between corticosteroid treatment and untreated groups. All severe asthma, SAFS, ABPA were on continuous ICS. All severe asthma and SAFS patients were treated with a combination of ICS and OCS. Some patients with ABPA received a combination of continuous ICS plus intermittent OCS therapy. No mild asthma or normal healthy subjects received steroids (OCS or ICS).A significant difference between therapeutic groups was observed for corticosteroid treatment (Figure 4A and 4B). We initially observed that individuals with ABPA that were receiving corticosteroid therapy showed significantly higher total fungal loads than individuals not receiving corticosteroid therapy (Online Supplement Figure E3). The most significant difference between groups was found when considering antifungal therapy with or without corticosteroid treatment (Figure 4C and 4D). 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 to individuals who have never received antifungal therapy. However, fungal burden is significantly higher in individuals receiving corticosteroid treatment independent of antifungal therapy.

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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 level 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 asthmatics or healthy controls 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 to previous studies (21–24). Additionally we manually removed fungal speciesidentified as arising from reagent contamination. In our study we did not observe *Wallemia* spp. contamination (25). This study was limited by the numbers of patients that could feasibly be bronchoscoped and the high variability of fungal level 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 but more importantly by careful selection of individuals using narrow treatment and/or endotype criteria. Lavage aliquots from several washes were combined in this study in order to maximise recovery of fungal DNA and the resulting data is likely to represent the combined bronchial and bronchoalveolarfungal 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 (8,25)*.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 OTU 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 completely informative locus for exact speciation within the *A. fumigatus* complex and therefore precise taxonomic interpretation of these results is not feasible (20). Our ITS primer set does not amplify *Pneumocystis jirovecii* but it is likely to be present in some individuals.

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Fungal burdens in the lungs of individuals with asthma or severe forms of asthma are comparable to those in individuals with well characterised allergic fungal disease. Fungal loads are highly variable between individuals with very high loads of fungus occurring only in those with ABPA, 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 immunopathological 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 (Figure 2) suggested that infection or colonisation 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 fungifrom 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 (26,27). Alternatively, the excess mucus could 'protect' *A. fumigatus* from phagocytic attack or differential adhesion to epithelial cells (28). In any scenario, at least some of the inhaled spores probably germinate forming mycelia that produce allergens in the airways (7,29). 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 (30).

Inhaled corticosteroids are commonly administered to asthmatics. We found that individuals receiving corticosteroids without antifungal therapy had higher total fungal loads in the lungs. This result mirrors that seen in COPD patients based on sensitive culture techniques and high dose inhaled corticosteroids (31). Corticosteroids impair monocyte, macrophage and neutrophil phagocytosis of *A. fumigatus*(32). 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 minimising 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 (33), so the potential for immune stimulation in the airways is high. ABPA is generally treated with steroid therapy

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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 lung 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 antifungals 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 oral corticosteroids, 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 steroid may encourage fungal growth in the lung 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 debate on the use of corticosteroids in the therapy of allergic fungal disease.

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POTENTIAL CONFLICTS OF INTEREST

Dr Bowyer is a co - founder of Alergenetica SL, an immunotherapeutics company and Syngenics Ltd., a food diagnostics company and has current grant support from Medical Research Council, Fungal Infection Trust, EU Framework 7 and National Institute of Health.

Dr Denning holds Founder shares in F2G Ltd a University of Manchester spin-out antifungal discovery company, in Novocyt which markets the Myconostica real-time molecular assays and has current grant support from the National Institute of Health Research, Medical Research Council, Global Action Fund for Fungal Infections and the Fungal Infection Trust. He acts or has recently acted as a consultant to Astellas, Sigma Tau, Basilea, Scynexis, Cidara, Biosergen, Quintilles, Pulmatrix, Zambon and Pulmocide. In the last 3 years, he has been paid for talks on behalf of Astellas, Dynamiker, Gilead, Merck and Pfizer. He is also a member of the Infectious Disease Society of America Aspergillosis Guidelines and European Society for Clinical Microbiology and Infectious Diseases Aspergillosis Guidelines groups.

The other authors have declared that no conflict of interest exists.

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Figure legends:

Figure 1.Mycobiome composition of BAL from individuals with allergic disease.(A) Level of fungus in BAL as assessed by total fungal RT-PCR. Ct values were converted into genome equivalents by calibration against known samples. (B) Fungal composition of each sample. The colour key for the most common taxons is given below the diagram. (C) Patient information for each sample. Samples are grouped by disease: ABPA, allergic bronchopulmonaryaspergillosis; HEALTHY, healthy controls; MA, mild asthmatics; SA, severe asthmatics with no fungal sensitisation; SAFS, severe asthmatics with fungal sensitisation. Antifungal (AF) and steroid treatment is indicated by the bars above the disease type with key below the diagram

Figure 2.Variation in the *A. fumigatus* population in individual samples. Two typical samples are shown for patient 34 (healthy control) and 35 (severe asthma). *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. fumigatussensustrictu*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 84/256 sequences for patient 34 and 260/458 for patient 35 being identical to the *A. fumigatus* sequence. Trees are constructed using ITS1 sequence to demonstrate isolate variation but are unlikely to represent true speciation.

Figure 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 load 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.

Figure 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)**qPCRdetermined loads of fungus grouped by both antifungal treatment (1: current itraconazole treatment with no steroid treatment, 2: current itraconazole treatment with current steroid treatment, 3:previousitraconazole treatment with current steroid treatment, 4: previousitraconazole treatment with no steroid treatment, 5: no itraconazole treatment with no steroid treatment and 6: noitraconazole treatment with no steroid treatment)). **(D)**qPCR determined loads of *A. fumigatus* 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). All individuals with previous antifungal therapy have SAFS or ABPA. *, p value <0.05; **, p value <0.01.

Table 1.Patient and healthy control demographics. ABPA, Allergic bronchopulmonaryaspergillosis; SAFS, Severe asthma with fungal sensitization; SA, severe asthma without fungal sensitisation; tIgE, total IgE, SD, standard deviation; FEV1, forced expiratory volume in the first second; FVC, forced vital capacity; % pred, percentage of the predicted value.

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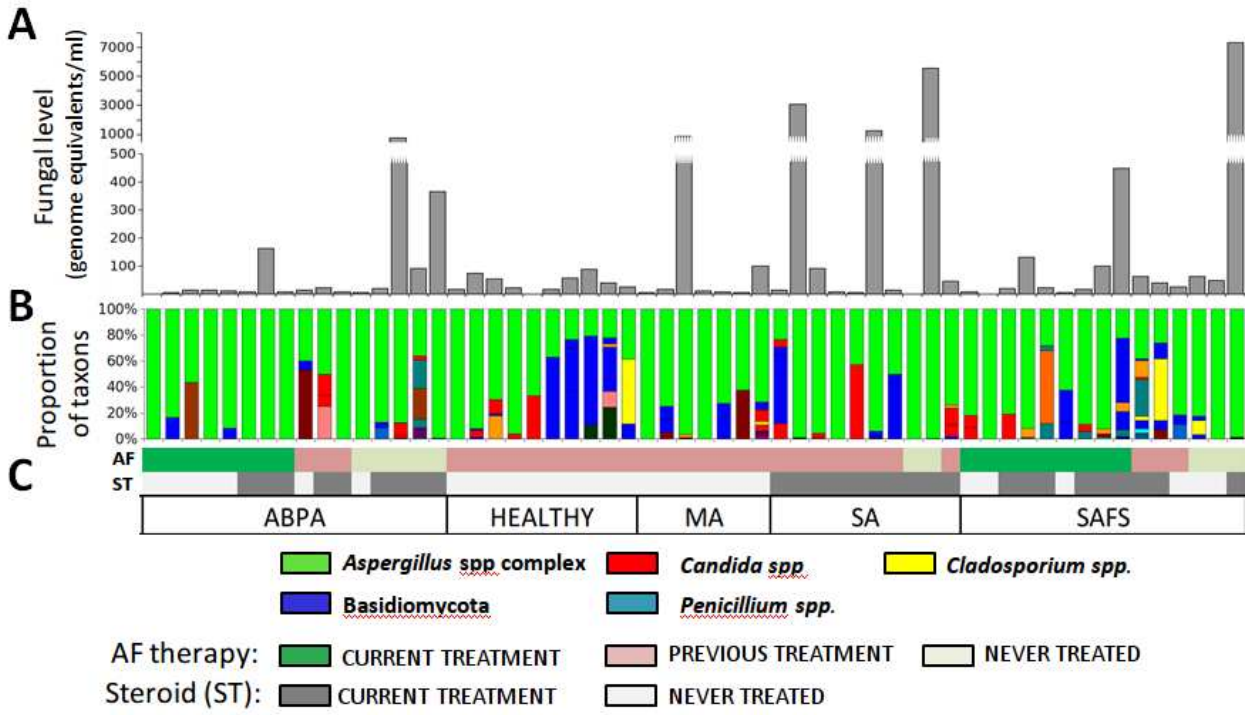
Table 1. Patient and healthy control demographics

		Severe asthma				
Characteristic	ABPA (n=16)	SAFS (n=16)	SA (n=9)	Mild Asthma (n=7)	Normal Healthy (10)	P-value (ANOVA)
Age (yrs.), median (range)	60 (25-75)	51.5 (30-68)	49 (19-65)	24 (22-62)	29 (22-58)	0.02
Gender: Male (%)	11 (68.7)	5 (37.5)	4 (44.4)	6 (85.7)	5 (50)	0.463
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)	0.845
FEV1% ,mean (sd)	64.5 (22.2)	72.8 (20.9)	58.1 (16.9)	95.5 (12)	98 (12.9)	<0.001
Duration of asthma (yrs), mean (sd)	42.8 (18.8)	40.6 (17.2)	31.9 (17.5)	22 (15.6)	0	0.08
Bronchiectasis, n (%)	16 (100)	12 (81.3)	6 (66.7)	0	0	<0.001
Obesity, n (%)	3 (20)	3 (18.7)	3 (33.3)	0	2 (20)	<0.582
Atopy, n (%)	11 (68.8)	12 (75)	4 (44.4)	1 (14.3)	0	<0.001
Rhinitis/sinusitis, n (%)	11 (68.8)	10 (62.5)	3 (33.3)	1 (14.3)	0	0.002
Nasalpolyps, n (%)	5 (31.3)	6 (37.5)	2 (22.2)	0	0	0.102
Total IgE, median (range)ku/l	1025 (210-9300)	205 (53.9-680)	49.2 (14-500)	96.3 (30-580)	49.6 (7.6-660)	P<0.001
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)	0.111
Antifungal therapy:						
Current, n (%)	7 (43.8)	10 (62.5)	0	0	0	<0.001
Previous, n (%)	5 (31.3)	2 (12.3)	0	0	0	<0.001
Never, n (%)	4 (25)	4 (25)	9 (100)	7 (100)	10 (100)	0.97
Azithromycin	6 (37.5)	7 (43.8)	6 (66.7)	0	0	0.007
Oralcorticosteroids	10 (62.5)	10 (62.5)	9 (100)	0	0	<0.001

ABPA=Allergic bronchopulmonaryaspergilosis; SAFS= Severe asthma with fungal sensitization; SANFS=severe asthma without fungal sensitisation; tIgE =total IgE, SD=standard deviation; FEV1=forced expiratory volume in the first second; FVC=forced vital capacity; % pred=percentage of the predicted value



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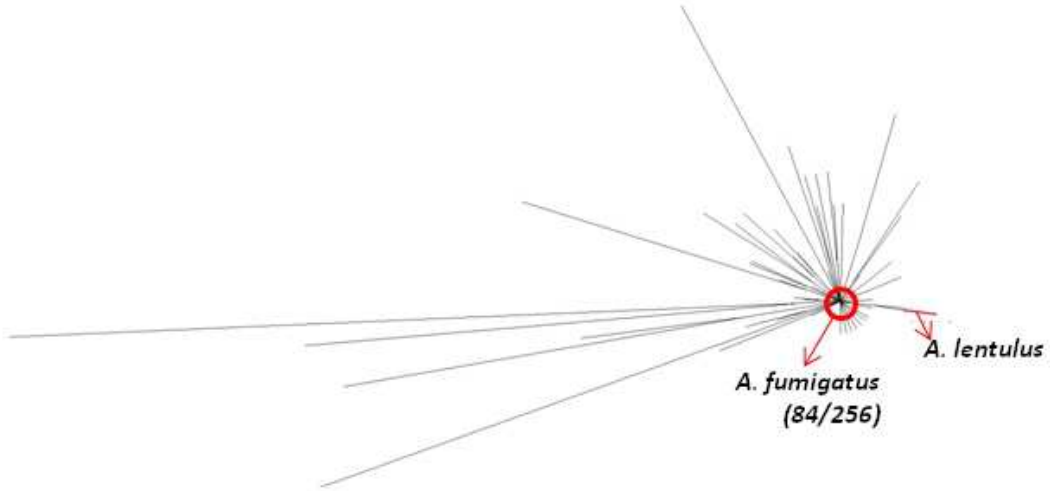

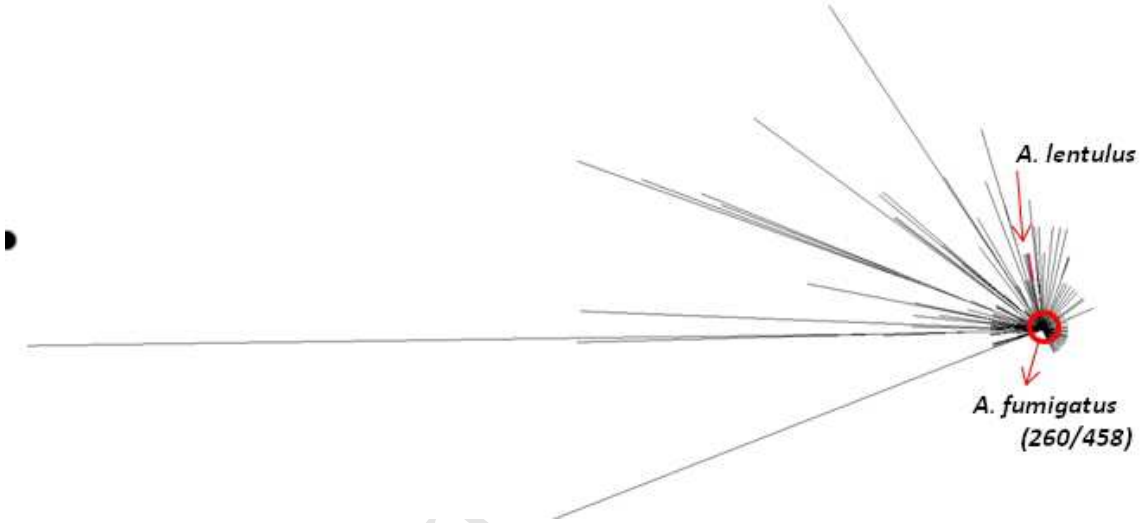

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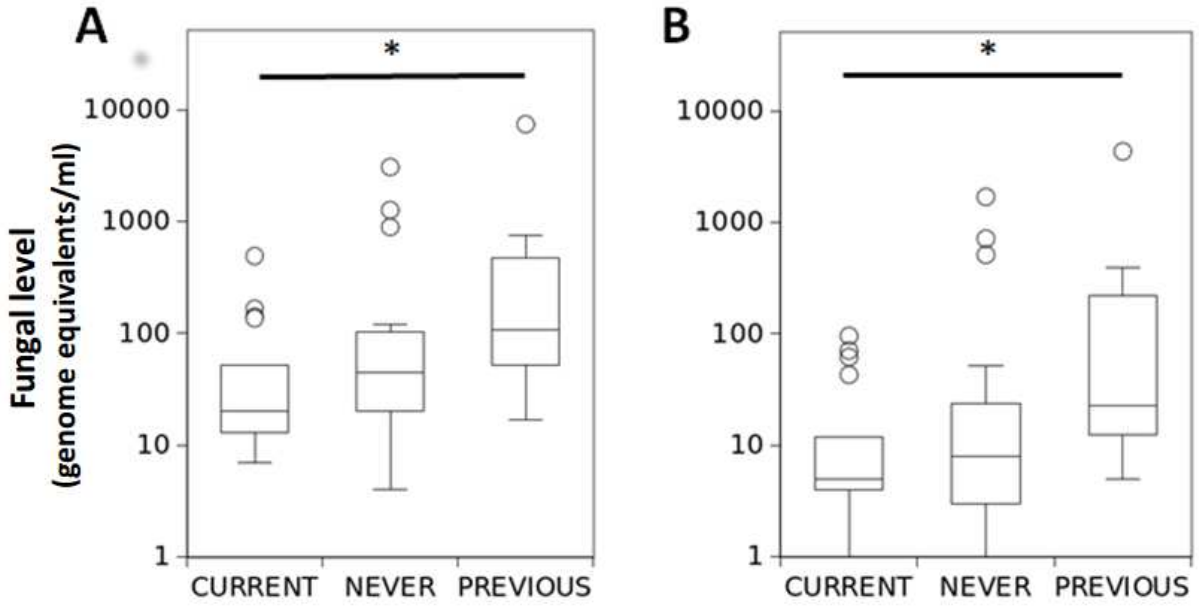

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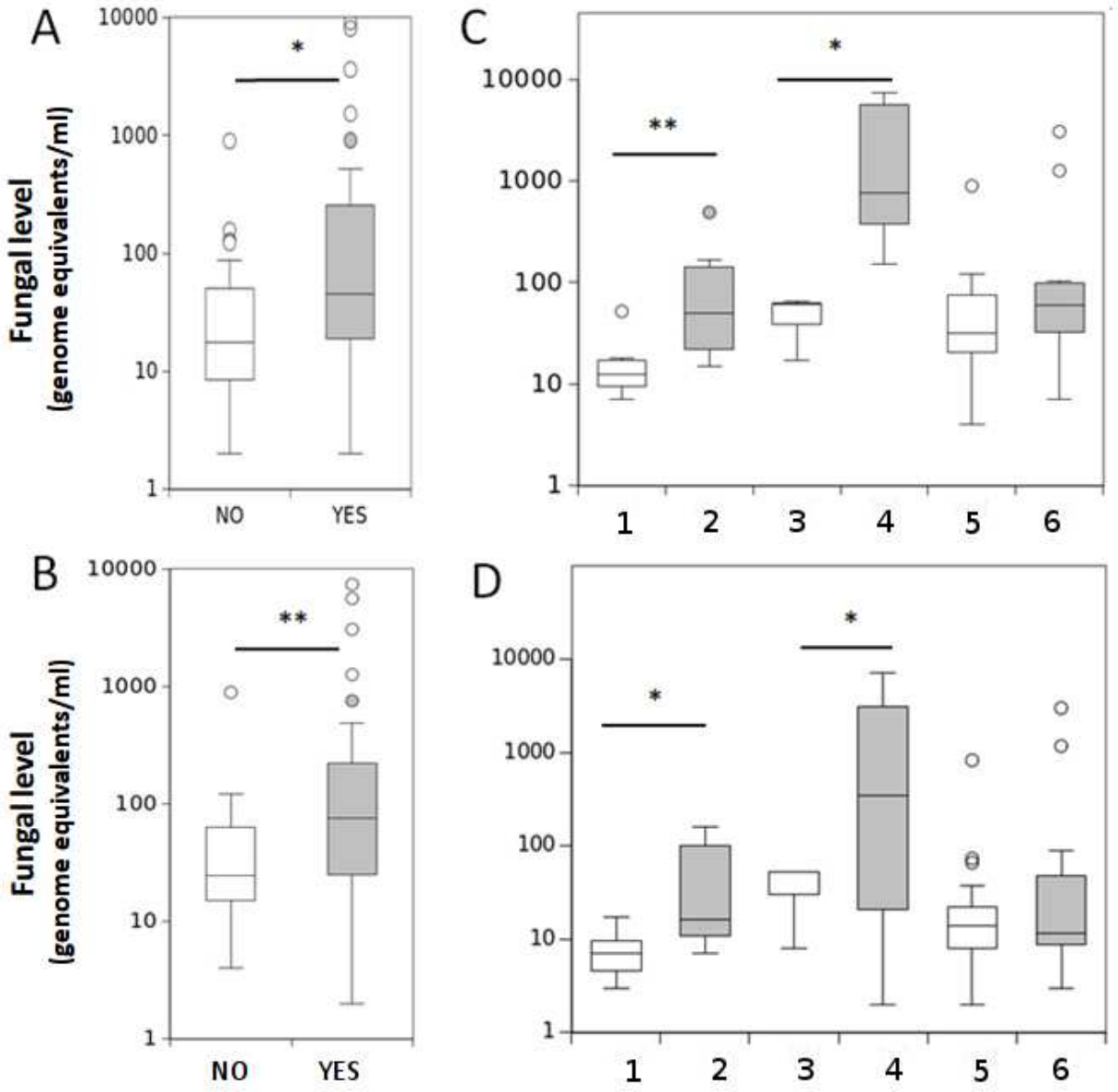
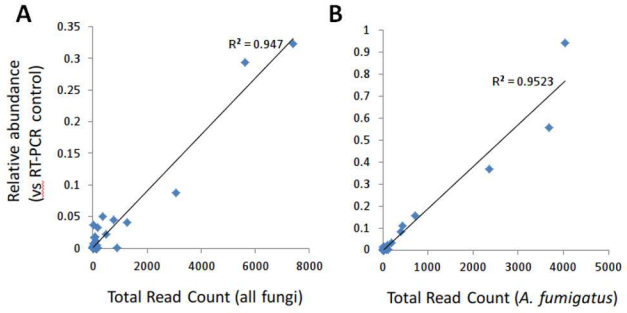

A
B
C
D
Fungal level
(genome equivalents/ml)
10000
1000
100
10
1
NO
YES
*
**
1
2
3
4
5
6



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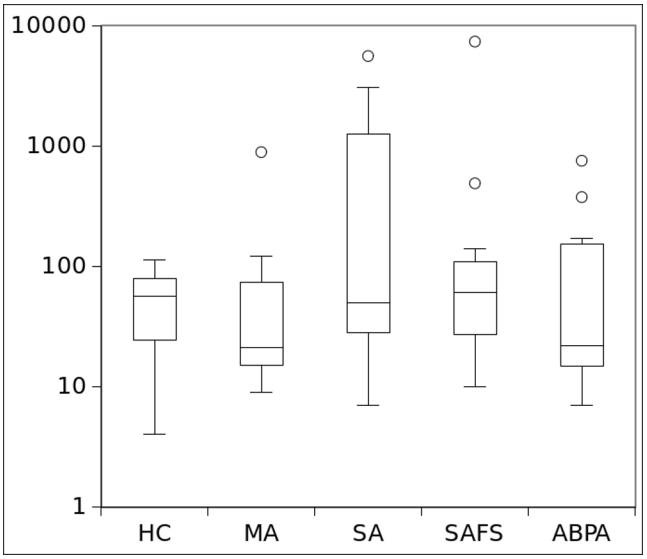

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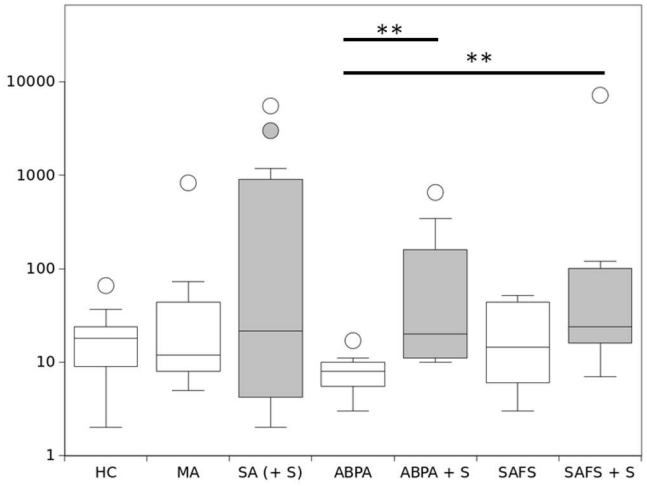

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Online Data Supplement

Corticosteroid treatment is associated with increased filamentous fungal burden in airways

Marcin G. Fraczek[1,2,5], Livingstone Chishimba[2,6], Rob M. Niven[2], Mike Bromley[1], Angela Simpson[2], Lucy Smyth[3], David W. Denning[1,2,4] and Paul Bowyer[1,2]

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Recruitment of patients to the study

Inclusion criteria. The inclusion criteria included: men or women, age (18-70 years old), lifelong non-smokers (<10 pack years) who had a physician diagnosis of asthma (or no asthma for healthy controls), ABPA, or SAFS; had no coexisting inflammatory chronic lung disease (except bronchiectasis in ABPA, SAFS and SA) and had an FEV1 ≥1 L/sec taken within the preceding 3 months. All patients with asthma underwent reversibility testing and confirmed by post-bronchodilator (PBD) increase in FEV1 ≥12% and or ≥200 mL compared with baseline (67) (except in some cases of chronic asthma who might have fixed airflow obstruction (68-69), in the absence of features suggestive of COPD). Patients with symptoms suggestive of asthma but with no evidence of PBD reversibility features of asthma (provided no fixed airflow obstruction) underwent airway hyperresponsiveness test using methacholine challenge test (MCT) (70). A diagnosis of asthma was considered when there was a significant bronchial responsiveness demonstrated by ≥ 20% fall in the FEV1 with methacholine challenge (PC20 or PD20 FEV1) on a 5-breath dosimeter protocol (71-72).

ABPA was defined as the patient's highest serum total IgE of greater than 1000IU/L, raised serum anti-*Aspergillus* IgE (or positive *Aspergillus* skin prick test), a history of asthma, with compatible symptoms of ABPA (expectoration of mucus plugs and difficult to control asthma) (4,11). Central bronchiectasis and a raised *Aspergillus* IgG were not required to make a diagnosis of ABPA, although commonly found. SAFS was diagnosed by a combination of severe asthma, positive IgE or skin prick test to any fungus tested and a total IgE of <1000 IU/L, as previously defined (27).

Subjects also needed to have had stable disease with no recent flare−ups (within 4 weeks) and to have met the safety criteria to have a bronchoscopy including oxygen saturations whilst breathing room air of >90%. Normal healthy subjects had no evidence of any long term lung condition and no chest infection in the preceding 4 weeks. A summary of subject demographics is shown in Table 1 and details of phenotyping are shown in Supp. Table 1.

Exclusion criteria. Patients with a significant smoking history (>10 pack years), doubtful diagnosis, significant respiratory comorbidities (except bronchiectasis) or a history of myocardial infarction within the preceding 6 weeks were excluded from the study. We also excluded patients with conditions that may mimic asthma including COPD, tracheobronchomalacia (TBM), hypersensitivity pneumonitis, pulmonary vasculitis including eosinophilic granulomatosis with polyangiitis (EGPA - formerly known as Churg-Strauss syndrome). Also excluded were those patients with conditions associated with an elevated IgE such as hyper-IgE syndrome or patients on immunosuppressive therapy (except oral corticosteroids) such as methotrexate, anti-TNF

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therapy and anti-IgE therapy (omaluzimub). In addition, those unable to give valid informed consent were excluded.

Sample collection and processing.

Patients were identified using the exclusion and inclusion criteria defined above. Briefly, 64 individuals were identified with 58 agreeing to bronchoscopy (Table 1 and suppl. Table 1). Bronchoalveolar lavage (BAL) samples were collected using local guidelines and BST standard procedures (31). If possible the lavage samples were collected from lobes with significant radiological abnormality. Where there were no radiological abnormalities such as in SAFS, healthy controls (HC) or most asthmatics, the samples were collected from the right lower lobes. A maximum volume of 240 ml (4 x 60 ml) of pre-warm sterile 0.9% saline (NaCl) solution was instilled. The volume instilled was judged by the clinician based on the patients' clinical condition and current oxygen saturation. Recovery of BAL varied between disease groups: for HC recovery was 50-113% (mean 67%), ABPA – 20-76% (mean 51%), mild asthma (MA) 57-81% (mean 69%), severe asthma non-fungal sensitised (SA) 50-84% (mean 69%), SAFS 30-93% (mean 61%). After bronchoscopy all collected samples were immediately placed on ice and transferred to the The BAL was subjected to DNA extraction, PCR and Illumina sequencing as described below. Dilution of endogenous lung fluids in the lavage medium was estimated as described (32) using a Sigma urea assay kit (Sigma – Aldrich, UK).

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Quantification of fungal burden using RT-PCR.

Overall fungal burden was determined using RT-PCR with ITS1 and ITS4 primers with SYBR-Green detection in an Applied Biosystems 7500 RT-PCR machine. Each sample was assayed in triplicate using standards containing known quantities of *A. fumigatus* and *C. albicans* DNA. The MycAssay*Aspergillus* kit (Myconostica) and the SmartCycler system (Cepheid, Sunnyvale, CA, USA) were used for *A. fumigatus* complex qPCR essentially as described (13). Negative DNA and known DNA controls as well as internal qPCR controls were also analysed. Data are presented as relative values compared to a 100 ng *A. fumigatus* standard calculated using a $\Delta\Delta CT^2$ method (44). MycAssay*Aspergillus* may also generate positive results for *Penicillium* spp. (45). Genome equivalents were calculated using *A. fumigatus* AF293 as comparator assuming a rDNA copy number of 38 as previously determined for this isolate (1 ng/ml of a 28Mb haploid genome ≈3049 rRNA copies@38 per genome ≈ 108 genomes/ml). This figure is unlikely to be accurate for all species in this study hence genome equivalent measures should be regarded as estimates.

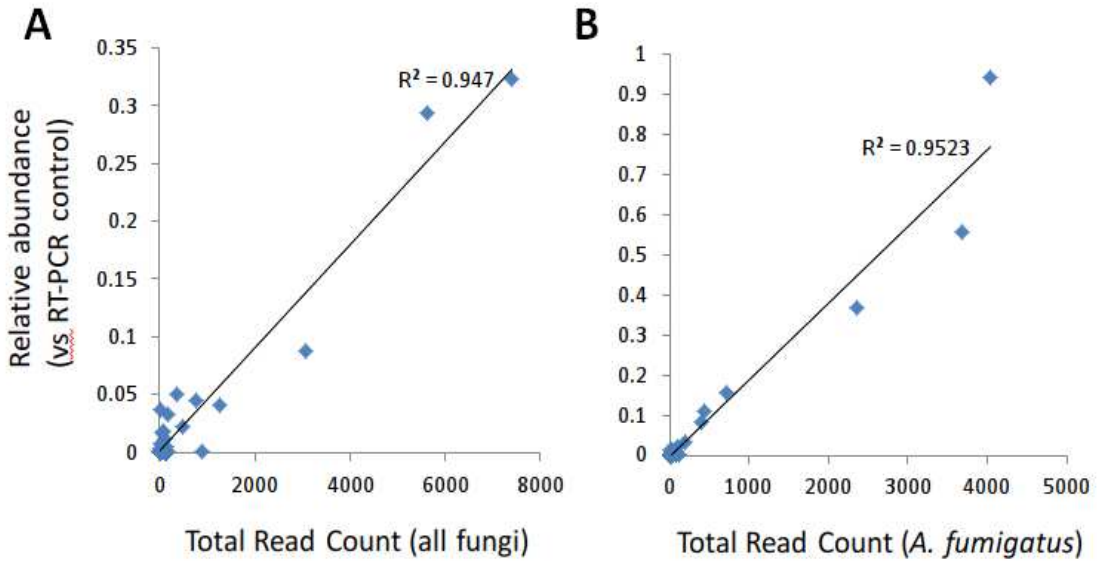

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Figure E1. Correlation between read count estimates and RT-PCR based estimates of *A. fumigatus* level. Total read counts for all fungi (**A**) or all members of the *A. fumigatus* complex plus Penicillia (**B**) were plotted against relative quantity (μg/ml) assessed by comparison ($\Delta\Delta Ct^2$) to a RT-PCR standard consisting of 100 ng *A. fumigatus* DNA. There is good correlation between the RT-PCR using ITS1 and ITS4 primers and total fungal read count (R2 = 0.947) and between *Aspergillus* specific PCR and *Aspergillus* read count (R2 = 0.95). Panel A relative to 200ng total fungal DNA, Panel 2 relative to 50 ng *A. fumigatus* AF293 DNA.

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Fungal mycobiome level in different disease groups.

Average genome equivalent levels from individuals in disease groups were calculated and are shown in Supp. Figure 2. No significant difference between any 2 groups was observed.

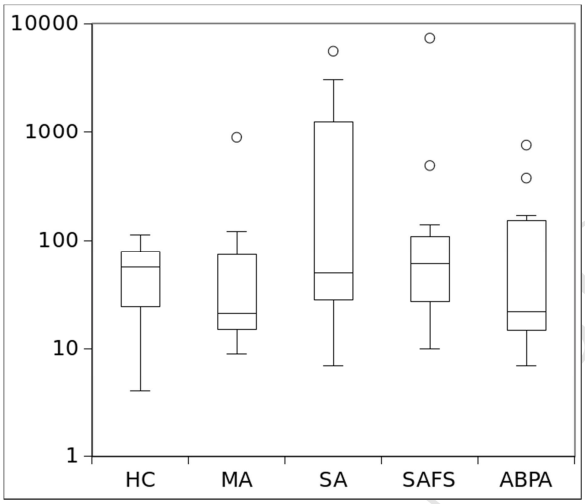

Figure E2.Loads of fungus in lung by disease group. Average genome equivalents/ml for all fungi are plotted against the disease type. No significant differences between groups is observed. HC, healthy control; MA, mild asthma; SA, severe asthma; SAFS, severe asthma with fungal sensitisation; ABPA, allergic bronchopulmonary aspergillosis.

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Effects of steroid on fungal level

We initially examined the effect of steroid treatment on fungal level by comparing average fungal level in different disease groups separated by steroid treatment. Only individuals in the severe asthma (SA), ABPA and SAFS groups received steroid therapy. We noted that effects in this analysis apparently significantly associating steroid therapy with increased fungal level (Figure E3) could be confounded by antifungal therapy leading to the analysis presented in Figure 4.

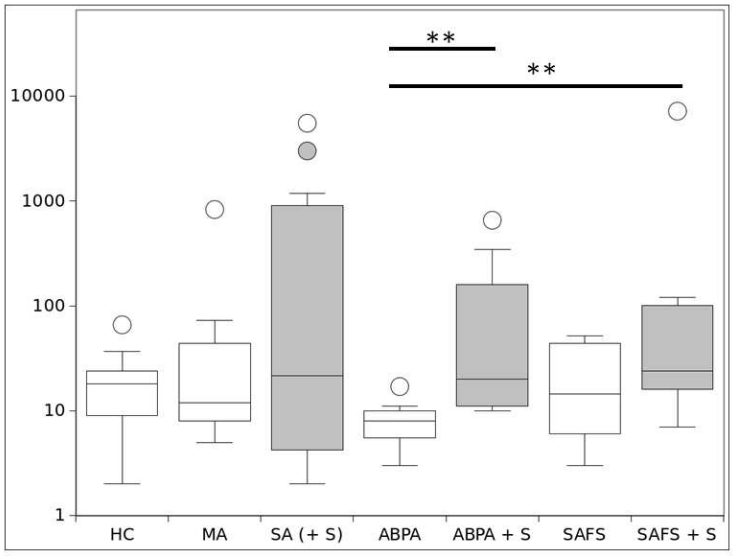

Figure E3.Loads of fungus in lung by disease group considering corticosteroid therapy. Average levels for all fungi (genome equivalents per ml) in each group are plotted against the disease type. HC, healthy control; MA, mild asthma; SA, severe asthma; SAFS, severe asthma with fungal sensitisation; ABPA, allergic bronchopulmonary aspergillosis; **, $p < 0.01$; +S current corticosteroid therapy.

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DNA extraction, PCR and Illumina sequencing.

In order to determine the microbial composition in the BAL, each sample was subjected to DNA extraction, PCR and IlluminaMiSEQ/Nextera XT sequencing. BAL samples were processed immediately after they were obtained, without storage. After initial processing (above), samples were transferred to high performance 50 ml Falcon tubes (VWR) and centrifuged at 10000 x *g* for 20 min at 4°C. DNA extraction was carried out on both the pellet and supernatant (500 µl) using a CTAB method previously described (33). The DNA was resuspended in sterile dH_2O, quantified using a spectrophotometer and stored at -80°C until used for PCR and sequencing. Only DNA obtained from the pellets was used for sequencing.

PCR was designed to target the fungal ITS1 region(Suppl. Table 1). PCRs were carried out in accordance with the '16S Metagenomic Sequencing Library Preparation' protocol (Illumina) with modifications as described below.Briefly, KAPA HiFiHotstart Polymerase (Peqlab) was used for PCR in a total volume of 25 µl per sample. In the first PCR step 5 µl DNA containing 100 ng of total sample DNA was used with primers targeting the rDNA genes and containing specific Illumina overhang adapters. For fungal PCR, 5 mM of forward and reverse primer was used. Initial touchdown PCR of 5 cycles (95°C for 30 sec, 45-50°C for 30 sec, 72°C for 30 sec) was carried out before 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Following the PCR all 5 reaction mixtures per BAL sample were pooled and purified using Agencourt AMPure XP magnetic beads as per manufacturer's instructions. The quality of the reactions was tested by electrophoresis on a 1.5% agar gel. Subsequently 5 µL of each purified pooled sample was used for indexing PCR using the Nextera XT Index Kit (Illumina) as per manufacturer's instructions and all reactions were bead purified as above. In order to balance the indexed sequencing reactions were pooled, normalised to 10 nM and sequenced using both lanes of an IlluminaMiSeq for 2x250 paired end reads. Controls consisted of sterile water or known dilution series of *A. fumigatus* or *C. albicans* DNA substituted for BAL and also added to BAL in the initial sample prep then carried through each step in the procedure before sequencing. The resulting fastq files were filtered for quality using TrimmomaticSE v0.32 using trailing/leading quality scores of 30 and a 3 bp sliding window phred score of 28. Results were analysed using QIIME v1.8. Read counts were adjusted to normalise for urea level and pre-sequencing dilution so that numbers represented reads from equivalent initial BAL volumes adjusted for bronchoscopy dilution effects using urea level. We make the assumption that BAL recovery does not represent either concentration or dilution of the wash liquid within the lung. Adjustment of data to account for recovery was performed but did not

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alter the significance of the results, hence results presented are adjusted for dilution using the urea assay but not for BAL recovery by volume.

Design of PCR for lung mycobiomes.

PCR of complete fungal ITS (ITS1-5.8S-ITS2) sequences using ITS1 and ITS4 primers did not PCR efficiently from lung samples (only 16/58 samples resulted in visible ITS band on an agarose gel) and test runs suggested that mean read length obtained from a 2x250 bpMiSEQ run after QC (Phred>/=26) was 214 bp, giving sequences that could not be overlapped in most cases. Hence, we chose to sequence the shorter fungal ITS1 target using primers ITS1 and ITS2 giving amplicons short enough in most cases (estimated size range was 150-290 bp) to give overlapping forward and reverse sequence. We note however, that several known fungal target amplicons are longer and may therefore become under-represented in the overlapped sequence set depending on sequence length after quality filtering. For this reason fungal populations were calculated using forward and reverse paired sequences alone and overlapped ITS1 sequences. Minor differences were observed between forward (ITS1 primer) and overlapped sequences whereas reverse (ITS2 primer) sequences gave a slight bias towards Basidiomycete taxons.

Table E1. Primers used to amplify fungal rRNA genes in the first amplification reaction for mycobiome analysis. Each fungal specific primer sequence had an Illumina specific adaptor attached at the 5' end (forward primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, and reverse primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG)

Primer name	Sequence (5'-3')
Fungal ITS1:	
FungITS1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG tccgtaggtgaacctgcgg
FungTS2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG gctgcgttcttcatcgatgc
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

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Statistics.

Unless otherwise stated statistical analysis was performed within QIIME v1.8 using appropriate QIIME workflows. Beta diversity for fungal taxa was calculated using Bray - Curtis metrics. Principle component analysis did not indicate significant association of species to any disease group. Other statistical tests were performed using Graphpad Prism v5.0 or SPSS v20.

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 compared to assess potential confounding. Minimum group size after stratification was 3. Alpha diversity metrics were calculated for fungi using an Excel spreadsheet. Apha diversity statistics are shown in supplementary table 2. Retesting of ITS1 sequences against databases was performed essentially as described in Nilsson *et al*. (39). Pairwise comparison of specific species within datasets was assessed using T–test or Mann Whitney in cases where deviation from normal distribution was observed. Statistics were adjusted using Benjamini-Hochberg correction as appropriate. Dunn's comparison test was used to identify potential confounding factors in comparison of multiple groups as appropriate.

Analysis of mycobiome.

The human mycobiome was initially assessed using open-reference OTU picking in QIIME with the UNITE database as reference. This returned results with very low read counts for fungi in all samples with high proportions of *Candida* spp. Although these results matched the low number of OTUs (10-20) previously observed (41-42) we noted that results obtained from a test dataset (ITS-soils, http://qiime.org/1.9.0/tutorials/fungal_its_analysis.html) using closed-reference workflows gave much higher read counts for fungi with significantly different composition QIIME workflows using closed-reference OTU picking and manual assignation of taxon by BLAST (see below) also gave very similar and higher read count results. Results were checked using FHiTINGS and MEGAN5.6.3 (37-43) which suggested that closed reference workflows using BLAST assignment gave the best results in terms of comparison to manual OTU calling and to alternative methods.

Fungal ITS1 sequences were therefore compared with the UNITE database using the QIIME 1.8 closed-reference OTU (organised taxonomic unit) picking workflow. To confirm that read count results were representative of fungal species in the sample, we used BLAST+ to extract fungal ITS1 sequences from the fastq files after QC and then used BLAST to assign each sequence to a large fungal ITS database. This database consists of 186,000 unique fungal ITS sequences collected in our laboratory between 2010 and 2016. We also included several curated type ITS1 sequences for

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clinical isolates of the *Aspergillus*, *Penicillium*, *Malasezzia* and *Cladosporium* genera in order to ensure that these taxa were accurately identified. BLAST+ parameters were set to exclude partial matches and to output a single result for each input from the fastq. The number of BLAST hits per species was obtained using custom BASH scripts. This analysis showed close agreement with the closed reference workflow. Sequence files were also analysed by reference to the FHiTINGS database (40) again showing close comparison to the results from the QIIME workflow.

Assessment of the main taxons

To check accuracy and specificity of the result, sequences from the most common taxons (>10% of total) were extracted from fastq files, aligned, and then consensus sequence was cross referenced with the NCBI nucleotide database. This method revealed two strong false positive results for *Phoma* and "unknown Dothidiomycete isolate 108" that arise through spurious amplification of bacterial sequences by the fungal ITS primers containing short (12-20 bp) regions of homology to *Phoma* or the Dothidiomycete ITS1 target in the database. These results are excluded from analysis using a BLAST E threshold better than $1E^{-20}$.

Correctness of species and otu assignation was tested by using a reverse BLAST procedure. Briefly each fastq file was quality filtered as described then converted to FASTA. This was used as a database for comparison using the UNITE, SOILS or our bespoke database as query. Results correlated very closely to the forward BLAST procedure used.

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. The most common clonal ITS1 sequences were identical to *A. fumigatus* sensu strictu ITS1 sequence. Diversity as defined may not represent different species within the sample.

Aspergillus complex sequences were isolated from fastq files using BLAST+ with empirically defined parameters 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 (46). To reduce the possibility of diversity arising from sequence error, fastq files were first filtered using TrimmomaticSE to remove end sequences with phred 33 scores lower than 30 and any sequences containing 3 base windows with quality lower than phred 30. Files were then filtered to remove sequences shorter than full length ITS1. This procedure typically removed 30-40% of identified Aspergillus ITS1 sequences. One reference A.

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fumigatus sequence and one A. lentulus ITS1 sequence were manually added to the resulting FASTA file before further analysis for comparison. Sequences were aligned using MUSCLE (47) trimmed using ALIVIEW (48) and used to make neighbour – joining trees using PHYML in Seaview v4.5.4 (49).

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ID no.	date bronc	age	gender	ht	weight (kg)	BMI	smoking st	pack yrs
1	04.11.2011	56	Male	167	69.4	24.9	Previous sn	10
2	18.11.2011	25	Female	168	74.2	26.3	Previous sn	1
3	25.11.2011	68	Male	171	76.4	26.1	Previous sn	4
4	25.11.2011	46	Male	175	92.8	30.3	Never smo	0
5	02.12.2012	30	Female	178	82.4	26	Previous sn	8
6	06.01.2012	63	Male	179	81.2	25.3	Never smo	0
7	06.01.2012	23	Female	175	84	27.4	Never smo	0
8	20.01.2012	44	Male	178	89.8	28.3	Never smo	0
9	20.01.2012	57	Female	169	81.8	28.6	Previous sn	15
10	03.02.2012	24	1	172	100.4	33.94	mild asthm	0
11	03.02.2012	64	Male	186	77.6	22.4	Never smo	0
12	02.03.2012	64	Female	162	112	42.7	Previous sn	7
13	02.03.2012	52	Female	170	89.8	31.1	current sm	8
14	09.03.2012	62	Male	177	91.7	29.3	Never smo	0
15	09.03.2012	75	Male	171	85.8	29.3	Previous sn	1
16	16.03.2012	55	Female	161	66	25.5	Never smo	0
17	23.03.2012	53	Female	165	53.6	19.7	Never smo	0
18	23.03.2012	58	Male	180	90.8	28	Never smo	0
19	30.03.2012	62	Male	181	78	23.8	Never smo	0
20	30.03.2012	67	Female				Never smo	0
21	04.05.2012	50	Female				Never smo	0
22	04.05.2012	48	Female	167	64.2	23	Never smo	0
23	04.05.2012	47	Female	166	109.6	39.8	Previous sn	7
24	11.05.2012	58	Male	177	80	25.5	Never smo	0
25	11.05.2012	19	Female	168	55.2	19.2	Never smo	0
26	18.05.2012	55	Female	160	109.7	42.9	Never smo	0
27	25.05.2012	65	Male	174	93.2	30.8	Never smo	0
28	25.05.2012	67	Male	165	68.2	25.1	Never smo	8
29	01.06.2012	26	Female	177	65.2	20.8	current sm	10
30	01.06.2012	24	Female	164	72	26.8	current sm	0
31	01.06.2012	42	Female	175	91	29.7	Previous sn	10
32	15.06.2012	54	Female	168	77.8	27.6	Never smo	0
33	15.06.2012	55	Female	155	59.2	24.6	Never smo	0
34	22.06.2012	32	Male	171	72.2	24.7	Never smo	0
35	29.06.2012	49	Female	163	59.4	22.4	Never smo	0
36	06.07.2012	60	Male	170	87.8	30.4	Never smo	10
37	06.07.2012	49	Male	183	95	28.4	Previous sn	6
38	03.08.2012	39	Male	178	76.2	24	Previous sn	5
39	24.08.2012	30	Female	168	70.9	25.1	Never smo	0
40	31.08.2012	46	Female	163	107.2	40.4	Previous sn	15
41	30.11.2012	48	Male	172	98.8	33.4	Previous sn	0
42	########	23	Male				Never smo	0

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Patient sample	observed otus	chao1	shannon	berger parker_d
1	5	6.5	1.700439718	0.615384615
10	2	2	0.811278124	0.75
11	8	8	2.014945307	0.583333333
12	5	5.5	1.578897903	0.65
13	30	31.90909091	4.136940716	0.19375
14	3	3	1.298794941	0.625
15	5	5.5	0.796169217	0.828402367
16	5	5.5	1.408222676	0.708333333
17	5	5	1.478047839	0.681818182
18	15	15.14285714	2.611733064	0.515625
19	5	5	1.08715544	0.787878788
2	6	7.5	1.949137865	0.555555556
20	4	4	1.74741304	0.421052632
21	5	5	1.213971684	0.710144928
22	5	5	2.12833105	0.409090909
23	8	9.5	2.220490188	0.488372093
24	4	4	1.536534965	0.558139535
25	2	2	0.468995594	0.9
26	2	2	1	0.5
27	3	3	0.776973436	0.84375
28	3	3	1.040852083	0.75
29	1	1	0	1
3	4	4	0.938742551	0.817307692
30	7	7	1.405929659	0.729166667
31	2	3	1	0.5
32	13	23	0.972529102	0.819607843
33	20	21.42857143	0.619552663	0.876052501
34	19	21.5	0.70459438	0.863686534
35	32	36.5	0.79373888	0.848174387
36	5	5	2.321928095	0.2
37	3	6	1.584962501	0.333333333
38	4	4.5	1.186278124	0.75
39	5	6	1.492271866	0.666666667
4	25	27	3.093317501	0.384615385
40	7	10	1.899721832	0.6
41	2	2	0.886540893	0.695652174
42	3	3	1.298794941	0.625
43	2	2	0.503258335	0.888888889
44	3	3	0.803071727	0.833333333
45	1	1	0	1
46	4	7	1.79248125	0.5
47	4	4	0.955486022	0.811320755
48	4	4	1.321606958	0.705882353
49	8	8	0.823590875	0.840466926
5	9	9.25	0.906011896	0.813823857
50	5	5	1.987773371	0.461538462