

Fungal sensitisation, remodelling and the mycobiome in asthma

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ABSTRACT

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Fungal sensitisation and Allergic Bronchopulmonary Aspergillosis (ABPA) are seen more commonly in severe asthma. Asthmatics with ABPA / SAFS have differing disease trajectories and degrees of disease severity. In view of this uncertainty, this thesis has focused on the role that fungal allergy and the fungal microbiome (mycobiome) have on aspects of airway physiology, inflammation and remodelling.

This thesis suggests that asthmatics with neutrophilic airway inflammation and sensitisation to *A. fumigatus* are at greater risk of a reduction in lung function and the development of radiological abnormalities, whether they fulfil the diagnostic classification for ABPA or not. IgE sensitisation to thermotolerant filamentous fungi, but not total IgE, was associated with a reduction in post bronchodilator FEV₁ (independent of atopic status), bronchiectasis, tree-in-bud and collapse ± consolidation in moderate to severe asthma. This suggests that current diagnostic classifications do not capture all of those at risk of adverse outcomes due to fungal sensitisation.

No features of airway remodelling were found in asthmatics sensitised to fungi. An association was however found between intraepithelial mast cell infiltration, an increase in fungal load and fungal sensitisation.

A 'core' mycobiome of 12 species, belonging to the Ascomycota and Basidiomycota phyla, were identified in all compartments of the lung. *A. fumigatus* was the most abundant fungus, present in healthy controls and asthmatics. Certain fungi were seen to have a protective effect with a reduction in reticular basement membrane thickness and epithelial integrity associated with *Cryptococcus pseudolongus*, whilst others such as *Aspergillus striatus* was associated with increased airway smooth muscle mass.

A new phenotypic group could not be accurately described. This thesis does, however, show that asthmatics sensitised to thermotolerant filamentous fungi are at a greater risk of adverse outcomes and are characterised by neutrophilic airway inflammation and a high fungal load.

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My thanks also to the patients recruited from the asthma clinics at Glenfield Hospital, who donated samples and biopsy tissue for us to process, analyse and draw conclusions.

The statisticians: Matthew Richardson and Christopher Newby gave me invaluable advice on conducting multivariate analysis and handling large databases.

The unit's laboratory staff helped me to learn basic laboratory techniques to enable me to process clinical specimens obtained for the study. Will Monteiro and Abbie Fairs taught me the necessary techniques and steps involved in processing the samples for bronchoscopy. In the immunohistochemistry laboratory Gillian and Fiona Symon guided me through the techniques involved in the immunohistochemical staining process and Aarti taught me how to analyse the biopsy sections.

My thanks to my academic supervisors: Professor Andrew Wardlaw and Dr Catherine Pashley. I have found their support and guidance invaluable whilst planning, conducting and navigating myself through the research journey.

Most of all I thank my family for the time they have given me, their patience, support and love.

STATEMENT OF PERSONAL CONTRIBUTION TO BODY OF WORK CONDUCTED BY THE RESEARCH GROUP

Study 1: Post-bronchodilator FEV₁, lung function decline and radiological abnormalities in asthmatics sensitised to fungi

Data for this study was collected from patients attending the Glenfield Difficult Asthma Clinic. The research nurses sought consent from patients to be included in this study. I planned the study and constructed the research database. I analysed the database using univariate and multivariate statistical techniques. Myself collected the data for the lung function aspect of this study and the most appropriate model for analysing the data was devised by a statistician: Dr Matthew Richardson. I interpreted the results and wrote the published manuscript.

Study 2: Remodelling changes in asthmatics sensitised to fungi.

I wrote the study protocol, obtained ethical approval and was involved in sample processing. I assisted with bronchoscopies. One of the laboratory technicians (Gillian) embedded and cut the biopsies for me and taught me how to stain using the DAKO EnVision™ FLEX system for immunohistochemical staining. I stained the slides and scanned the slides at the appropriate magnification onto the microscope software. I mapped the morphological areas and performed all cell counts. An independent scientist, Dr Fiona Symon, verified the counts. I conducted all of the analysis and data interpretation.

Study 3: Description of the mycobiome in asthma.

I wrote all study documentation: study design, protocols and patient information sheets. I applied for and obtained ethical approval for the study. I created an electronic data storage package on the REDcap system for cross-site data input for both the clinical and scientific team. I ensured it met all research and information governance criteria. I was in charge of study recruitment and was part of a team of

research staff that gained consent for the study and conducted the clinical measurements for patient characterisation. An experienced laboratory technician (William Monteiro) performed all cell counts. I consented and assisted with bronchoscopies. Abbie Fairs, Eva Rick and myself processed the samples obtained. Eva Rick and Dr Catherine Pashley performed the DNA extractions from the samples and processed the DNA for high throughput sequencing. Eva Rick performed the qPCR analysis and Dr Catherine Pashley performed the bioinformatics and biostatistical analyses. I was responsible for the interpretation of the results that were obtained from the bronchoscopy samples.

PUBLICATIONS

Original publications:

Woolnough KF, Richardson M, Newby C, Craner M, Bourne M, Monteiro W, Siddiqui S, Bradding P, Pashley CH, Wardlaw AJ. The relationship between biomarkers of fungal allergy and lung damage in asthma. *Clin Exp Allergy* 2017; 47(1):48-56

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Chapter 1: Introduction

1.1 Asthma

Asthma affects 5.4 million people and 1 in 12 adults in the UK (1). A disproportionate amount of the annual NHS healthcare expenditure on asthma is spent on the 5-10% of those with severe asthma (~1 billion pounds); despite this around 50% of this group are still classified as having uncontrolled asthma and 5-10% deemed refractory to treatment (1, 2). Severe asthma is defined by the European Respiratory Society and the American Thoracic Society as ‘asthma that requires treatment with high dose inhaled corticosteroids plus a second controller medication and/or systemic corticosteroids to prevent it from becoming “uncontrolled” or that remains “uncontrolled” despite this therapy’ (3). Severe disease is associated with a greater degree of impairment at work, greater healthcare utilization and a substantial loss of productivity (4). Thus, there is a need to characterise patients better so that we can identify those at the severe end of the asthma spectrum that are more at risk of adverse outcomes.

The use of cluster analysis to identify phenotypes of asthma, has allowed us to group asthmatics according to their clinical (such as atopy) and inflammatory profile (eosinophilic/neutrophilic/mixed granulocytic/ paucigranulocytic). These phenotypes have then been used to stratify patients into different treatment groups and allowed more targeted and effective management of this heterogeneous condition (5). However, it has previously been shown that markers of atopy, blood eosinophils and exhaled nitric oxide (that help to characterise these phenotypes) do not fully differentiate mild from severe disease (2). Thus, it is crucially important for us to look at all of the pathophysiological mechanisms involved to help identify the different characteristics / biomarkers that affect disease outcomes so that we can target treatment from effectively.

Sensitisation to fungi is seen frequently in moderate to severe asthma and has been associated with several adverse outcomes. Environmental moulds and in particular the non-thermotolerant fungi ((unable to grow at 37°C) which include *Alternaria alternata* and *Cladosporium herbarum*) act as aeroallergens and have previously been linked to life threatening asthma and intensive care admissions,

asthma deaths and exacerbations requiring hospital admission (6-9). Although many of these studies relating spore concentration and sensitisation status to fatal asthma are now quite old the literature does still suggest a link between sensitisation status and spore concentration with an increase of exacerbations and healthcare utilisation (10-12). Thermotolerant fungi, such as *Aspergillus fumigatus*, have the ability to act as both aeroallergens and as colonisers of the airway. Sensitisation to *A. fumigatus* is linked to reduced lung function and is part of the diagnostic criteria for Allergic Bronchopulmonary Aspergillosis ((ABPA) table 1), which occurs in 0.7-3.5% of asthmatics (13, 14). However, other than specific IgE, it is unclear how these parameters of fungal sensitisation are related to adverse asthma outcomes – in terms of lung damage, airway inflammation and airway remodelling. The first study in this thesis will focus on this aspect, looking at how immunological markers of fungal sensitisation are related to adverse outcomes in asthma.

1.2 Phenotypes and endotypes

Asthma is a multidimensional disease. Many endogenous, environmental and behavioural factors affect disease control. These are reflected in the phenotypes and endotypes of asthma that have been identified.

Endotyping is a way of categorising patients into groups that have similar clinical characteristics and pathophysiology; whereas phenotypes create homogenous categories that are grouped together by common traits (15). These phenotypic traits are observable and reproducible and have been used to identify groups of asthmatics that would benefit from immunotherapy and different treatment regimens (16).

Anderson et al. first described endotypes as “subtypes of a condition linked by a distinct functional or pathophysiological mechanism” (17). In addition, Lotvall et al proposed that an endotype should fulfil at least five parameters (defined as: clinical characteristics, biomarkers, lung physiology, genetics, histopathology, epidemiology, and treatment response) (18). Identification of these endotypes and elucidation of their underlying pathophysiological mechanisms will allow new biomarkers / therapeutic targets to be identified and developed, such as those

driven by interleukin 18 and 33, the interleukin-17-regulatory T-cell axis and via epidermal growth factor receptor co-amplification (17). Much more information is required to link genetics, pathophysiology, biomarkers and clinical characteristics together to establish endotypic classifications of asthma before they can be used in routine clinical practice.

1.2.1 The use of cluster analysis to identify phenotypes in asthma.

Cluster analysis has been used in several studies to phenotype patients into groups that have common clinical and inflammatory profiles. Haldar et al described 4 distinct asthma phenotypes in both primary and secondary care: (i) early onset eosinophilic asthma with airways dysfunction, (ii) female, non-eosinophilic, but with a symptom predominant presentation, (iii) early onset asthma with minimal eosinophilic disease, and (iv) late onset eosinophilic asthma (19). Classifying patients by these eosinophilic profiles has also allowed corticosteroid therapy to be down titrated in the inflammation-predominant groups with no consequent loss of asthma control (20).

The Severe Asthma Research Programme (SARP) identified five asthma phenotypes, categorised by clinical characteristics, measures of lung function, corticosteroid dosage and health care utilisation (21). Further studies using model based clustering methodology, such as that by Siroux et al., have also sub-divided asthma into phenotypic groups based on clinical characteristics. This again grouped patients by early/late onset disease, atopy and severity of disease (measured by medication usage) (22). These studies show the reproducibility of clustering and phenotypes using different cohorts of asthmatics.

1.2.2 The use of phenotypes to guide asthma management.

Woodruff et al. have examined the molecular phenotypes of asthma (23). Respiratory epithelial gene expression of POSTN, CLCA1 and SEPINB2 was induced by IL-13 to identify those that had a Th2 or a non-Th2 molecular phenotype. The Th2 phenotype had a greater degree of airway hyperresponsiveness, eosinophilia, thickening of the reticular basement membrane and epithelial mucin gene expression (23).

Baines et al. validated six sputum biomarkers: Charcot-Leydon crustal protein (CLC); carboxypeptidase A3 (CPA3); deoxyribonuclease I-like 3 (DNASE 1L3); alkaline phosphatase tissue-nonspecific isozyme (ALPL); chemokine receptor 2 (CXCR2). These biomarkers were used to identify a gene expression signature that was able to distinguish between inflammatory phenotypes (eosinophilic, neutrophilic and paucigranulocytic) (24). In this study inhaled corticosteroids (ICS) suppressed CLC, CPA3 and DNASE1L3 in eosinophilic asthma and thus brought together both inflammatory and gene expression phenotypes that were able to predict treatment response (24). Several studies have shown improvement in outcomes when treatments are based on molecular and cellular phenotypes. Bahkta et al. combined qPCR expression of periostin, CLCA1 and SeptinB2 (previously recognised as a high-Th2 molecular phenotype signature) into a single metric (25). This molecular phenotype, using qPCR, was able to predict improvements in FEV₁ with ICS over an eight week period (AUC 0.87), over that of exhaled nitric oxide and blood eosinophils (25).

1.2.3 Cluster-derived asthma phenotypes and genetic linkage.

A genetic association study with cluster-derived asthma phenotypes demonstrated associations with four single nucleotide polymorphisms ((SNPs) rs9851461, rs9842772, rs988812 and rs1051124) on chromosome 3 and adult onset non-atopic asthma (26). It also showed associations between mild late onset non-atopic asthma and several SNPs on chromosome 6 (rs2579931), chromosome 7 (rs10264996, rs10259042, rs10230811 and rs1716296), chromosome 8 (rs7834760, rs13272108) and chromosome 11 (rs4576815, rs7938647) (26).

Cluster analysis using clinical characteristics, biomarkers and genetic linkage analysis in these studies has confirmed the need to sub-group patients to tailor therapy more effectively. What is not clear currently in the literature is whether classifying patients into existing diagnostic groups (Allergic Bronchopulmonary Aspergillosis/Mycosis (ABPA/M) and Severe Asthma with Fungal Sensitisation (SAFS) (table 1)), appropriately picks out those at risk of adverse outcomes. Clustering techniques will be used in the first observational study in this body of work to identify whether clustering by the parameters (eosinophils, total IgE, specific IgE to fungi and *A. fumigatus* IgG) used in these diagnostic classifications in

an asthma population that is both sensitised and not sensitised to fungi will help to distinguish between groups that are phenotypically different in terms of clinical characteristics. In addition, cases of ABPA/M, SAFS and outcome measures (such as radiological abnormalities and fixed airflow obstruction) will be mapped to these clustered groups to assess whether the extent of the immunological response (in terms of specific IgE, total IgE, specific IgG and eosinophilia) is associated with more adverse radiological and lung function outcomes.

1.3 Pathogenesis of asthma

1.3.1 Introduction

Airway inflammation in asthma is dependent on many genetic, immune and environmental factors. Both an eosinophilic (Th2-high) and a neutrophilic (non-eosinophilic Th2-low) molecular phenotype have been described (23); however, the immunopathology of asthma is complex resulting from an interplay between multiple pathways, involving Th2, Th1, Th17 and ILC2 cell-induced mechanisms, amongst others (see figure 1.1). The airway epithelial cells and dendritic cells play a key role in directing T cell responses towards one of these pathways, influenced by the co-stimulatory molecules and cytokines present within their environment (27). Fungi are able to become pathogenic and cause uncontrolled allergic disease due to defects in the innate immune mechanism in airways disease or secondary to immunosuppression. Asthma causes a reduction in the mucociliary escalator, mucus hypersecretion and a disruption in epithelial integrity (28-30). These allow fungi to inhabit the airways for a longer period of time, evade the alveolar macrophages and cause allergic disease.

1.3.2 Epithelium

The epithelium is a key component of the inflammatory response seen in asthma. Cytotoxic injury from environmental factors can promote the release of pro-inflammatory cytokines and chemokines (figure 1.1). These enhance the activity of antigen presenting cells and direct the inflammatory process towards an eosinophilic and/or a neutrophilic picture. The cell wall of *A. fumigatus* contains β -glucans. These induce IL-6 production from airway epithelial cells, whilst proteases cause disruption in the epithelial cell barrier (31, 32). The disruption of

the epithelial barrier in this way is, thus, an important virulence factor of *A.fumigatus* spores and hyphae.

1.3.3 Dendritic cells

Dendritic cells (DCs) act as antigen presenting cells for fungal proteases and fungal cell wall components. They can promote either a Th1 or a Th2 allergic response. Their activation causes differentiation of naïve T-cells into distinct T helper cell subsets (Th1, Th2 or Th1/Th17), dependant on the nature of dendritic cell activation and the expression of co-stimulatory molecules, resulting in either a neutrophilic, eosinophilic or mixed neutrophilic/eosinophilic phenotype (27). Innate activation of DCs occurs through antigen binding to the Toll-like receptor 4 ((TLR4) see below) and presenting it to the T cell receptor via MHC class II presentation (33). This is mediated by the production of TSLP (thymic stromal lymphopoietin), IL-25, IL-33, CCL2 and CCL20 (33). These epithelial derived mediators drive the production of IL-5 and IL-13 to elicit a Th2 response (34). TSLP, IL-33 and IL-25 are also necessary for the proliferation and survival of ILC2 cells; whilst mast cell derived prostaglandin D2 (PGD2) stimulate ILC2 cytokine production through its CRTh2 receptor (35). PGD2 also acts to increase the production of IL-4, IL-5 and IL-13 from Th2 cells, eosinophils and basophils by their CRTh2 receptor, thus favouring a Th2 phenotype (36, 37).

1.3.4 Toll-like receptors

Toll-like receptors (TLR) are antigen sensitive pattern recognition receptors that can activate both the innate and the adaptive arms of the immune response (38). Exogenous factors, such as fungal cell wall components, activate TLRs by acting as pathogen-associated molecular patterns (PAMPs), whilst endogenous products (caused by oxidative stress of fungal proteases on epithelial cells) activate TLRs by acting as Danger-associated molecular patterns (DAMPs) (39).

TLR2 and TLR4 are found in epithelial and dendritic cells of asthmatics; they can recognise both bacterial lipopolysaccharides (TLR2) and allergens (TLR4) (39). Activation of TLR4 stimulates the release of IL-25, IL-33 and TSLP polarizes naïve lymphocytes towards becoming Th2 cells (33). In addition, polymorphisms in TLR-9 (allele C on T-1237C) have been shown to reduce its protective function and occur at increased frequency in those with ABPA (40).

1.3.5 Eosinophils

Eosinophils are the major effector cells of early onset atopic disease, as well as late onset non-allergic asthma. They are dependent on the cytokines and chemokines produced from Th2 (IL-4, IL-5, IL-13) and ILC2 cells for their maturation, recruitment and survival (35, 41). A Th2 response can be initiated by both an immune (IgE) mediated pathway and by an innate mechanism. An innate response can occur through activation of the epithelial, dendritic and innate lymphoid cells (42). Fungi activate both of these pathways. Fungal proteases and parts of the fungal cell wall are recognised epitopes for IgE that initiate a Th2 response and recruitment of eosinophils. Innate activation occurs through recognition of fungal cell wall components (exposed during germination) by pattern recognition receptors (PRR) on epithelial cells and damage to epithelial barrier by proteases. Subsequent recruitment and degranulation of eosinophils and mast cells release cysteinyl leukotrienes (CysLT). These act to induce airway smooth muscle contraction, increase vascular permeability and induce mucus production (43). The major basic proteins released by eosinophils contain preformed enzymatic and nonenzymatic cationic proteins. These proteins directly cause airway epithelial damage, hyperreactivity, smooth muscle hyperplasia and subepithelial fibrosis (44).

1.3.6 Neutrophils

Engulfment of fungi by alveolar macrophages leads to neutrophil recruitment. Neutrophils are able to attack hyphae that have evaded the alveolar macrophages and are key in preventing invasive disease. Neutrophilic airway inflammation is a recognised feature of asthmatics sensitised to *A. fumigatus* and is also associated with glucocorticoid resistance in severe asthma and reduced postbronchodilator FEV₁ (PBF₁) (14, 45). It is driven by a Th17 pathway (46). Naïve (Th0) T cell activation and differentiation into Th17 cells occurs through the binding of CD40 and CD40L in the presence of co-stimulation with CD86 on dendritic cells and the influence of IL-1 β , IL-6 and TGF β (27, 47). The maintenance of Th17 activation is dependent on the release of IL-23 from dendritic cells and macrophages (27). The subsequent continued production of IL-17A activates the airway epithelial cells to release the neutrophil chemoattractants and priming molecules: IL-6, IL-8 and GM-CSF (48, 49). These cytokines have been linked to markers of airway remodelling,

as shown by the effects of IL-17A. IL-17A has been shown to increase migration, proliferation and survival of airway smooth muscle cells, as well as increasing mucin MUC5B gene expression and induction of CCL28 expression (B-cell chemokine) by airway epithelial cells (50).

Thus, the immunopathology of severe asthma is complex and reliant on the interplay between eosinophils, mast cells, neutrophils, activated ILC2 and airway epithelial cells, as well as macrophages and dendritic cells. Patients with asthma may have a predominant phenotype, which may respond differently to immunomodulatory treatments, but it is only when we fully understand the molecular pathways behind these phenotypes of asthma that we will be able to target treatments more effectively to avoid remodelling changes from occurring. It is increasingly recognised that different clinical phenotypes co-exist in asthma, due to activation of different T-cell subpopulations at differing times along the inflammatory cascade (51). This may account for the predominant neutrophilic picture on induced sputum seen on those sensitised to *A. fumigatus*, despite the well-established relationship of peripheral blood eosinophilia in the diagnosis of ABPA (52, 53).

Total IgE, peripheral blood eosinophilia, specific IgE and IgG to *A. fumigatus* are used to diagnose ABPA (see table 1). Measures of these humeral responses to fungi are the only routine tests performed in clinical practice in an attempt to screen for ABPA and in an aid to monitor disease activity. The use of these parameters suggests a multifactorial pathogenesis. A raised IgG level to a specific fungus signifies aTh1 response; whilst a positive skin prick test, a raised specific IgE and total IgE titre are indicative of a Th2 response. This body of work will examine the relationship between these parameters of fungal sensitisation and markers of airway inflammation and remodelling in asthma.

1.4 ABPA

1.4.1 Introduction

Allergic Bronchopulmonary Aspergillosis / Mycosis (ABPA/ABPM) is a condition that was first described in the 1950's, with several case series establishing a set of criteria for its diagnosis (54-56). It was originally described in asthmatics with

eosinophilic airway inflammation who were sensitised to *A. fumigatus* and presented with fleeting parenchymal infiltrates and progressive lung damage characterised by fixed airflow obstruction, proximal bronchiectasis and lung fibrosis (54). The prevalence of ABPA in asthma is stated to be 12.9% (95% CI 7.9-18.9%) by a systematic review and meta-analysis of 21 eligible studies, whilst the global burden of ABPA has been estimated to exceed 4.8 million people using the Global Initiative for Asthma (GINA) report (57, 58).

A review by Knutsen et al in 2012 presented a set of criteria that was felt necessary to be present for a diagnosis of ABPA in asthma. These were: (i) a deterioration in lung function, (ii) a positive skin test result to an *Aspergillus* species, (iii) serum total IgE ≥ 1000 ng/mL (416 IU/mL), (iv) raised specific IgE and IgG antibodies to the *Aspergillus* species, and (v) the presence of infiltrates on a chest x-ray (13). However, features such as bronchiectasis affecting the central airways, the presence of eosinophilia in the peripheral blood, precipitating antibodies to the *Aspergillus* species and mucus plugs were not part of the core criteria but seen as additional features to a diagnosis of ABPA (13). This was further revised by the International Society of Human and Animal Mycology (ISHAM) to include a total IgE above 1000IU/ml, a raised specific IgE to *A. fumigatus* and at least two of the following: a peripheral blood eosinophilia $>0.5 \times 10^9/l$ in steroid naïve patients, a raised IgG to *A. fumigatus* or abnormal radiology consistent with ABPA in patients with asthma or cystic fibrosis (59).

1.4.2 Allergic Bronchopulmonary Mycosis (ABPM)

ABPM is diagnosed when the criteria for ABPA is met in the absence of sensitisation to *A. fumigatus*, but in the presence of another fungi identified in airway specimens or by sensitisation. Several fungi have been associated with this condition, including *Alternaria alternata*, *Candida albicans*, *Cryptococcus neoformans*, *Fusarium vasinfectum* and *Penicillium* species (60-64).

1.4.3 Severe Asthma and Fungal Sensitisation (SAFS)

Severe Asthma and Fungal Sensitisation (SAFS) is an additional diagnostic group that has been introduced in order to classify those with severe asthma who do not meet the total IgE cut off value of >1000 IU/ml and evaluate treatment outcomes to anti-fungal therapy (65).

1.4.4 Sensitisation to *A. fumigatus*

Asthmatics that are sensitised to *A. fumigatus* have a greater degree of fixed airflow obstruction compared to those that are not sensitised (52). In addition, radiological abnormalities, such as bronchiectasis and upper lobe fibrosis, do not affect all that are diagnosed with ABPA nor those that are sensitised to *A. fumigatus*. A spectrum of disease exists in those that are sensitised to fungi, with those with a heightened immunological response seeming to be at one end of this spectrum.

1.4.5 Conclusion

It is, thus, unclear what relationship exists between these adverse features and the existing parameters that are used to diagnose ABPA. Until this relationship is examined further, relying on these diagnostic criteria to treat patients risks exposing individuals to known side effects with unknown benefits.

1.5 Biomarkers

1.5.1 Introduction

Biomarkers are defined by the National Institute for Health (NIH) working group as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' (66). Biomarkers have many uses. They are used to (i) **assess disease susceptibility** (e.g. HLA-DRB1 alleles are associated with ABPA susceptibility in cystic fibrosis (67)), (ii) **demonstrate exposure** (e.g. as stated by the World Health Organisation report on 'Biomarkers and Risk Assessment: Concepts and Principles' (68)), (iii) **aid diagnosis** (e.g. as seen in ABPA (59)), (iv) as a **prognostic marker**, and (v) are key in the **monitoring and titration of treatment** in certain conditions (e.g. sputum eosinophil counts are used to guide corticosteroid titration (20)). Biomarkers are used in asthma to characterise airway inflammation, to assess disease severity and eligibility for treatment.

The validity of biomarkers is often assumed, particularly when part of an established diagnostic criterion (69). The diagnosis of ABPA in asthmatics is dependent on several parameters (total IgE, IgE *A. fumigatus*, IgG *A. fumigatus*, blood eosinophils). These parameters are currently used as diagnostic biomarkers

upon which decisions are made to commence high dose oral corticosteroids and antifungal agents. It is, however, unclear as to how these biomarkers relate to disease outcomes. The 1977 Rosenberg-Patterson criteria and the recently revised ISHAM (International Society for Human and Animal Mycology) working group criteria for ABPA is reliant on the use of these parameters as part of its obligatory criteria and two within the supportive criteria (see table 1.1) (55, 59).

1.5.2 Specific IgE to *A. fumigatus*

Specific IgE to *A. fumigatus* has been linked to a reduction in post-bronchodilator FEV₁ and has been part of the diagnostic classification for ABPA since it was first described (52, 70). It has not been identified as a factor for lung function decline and its use quantifiably, as a predictor of outcomes, is yet to be fully established.

1.5.3 Total IgE

Total IgE is used quantifiably to differentiate between ABPA and SAFS (53, 71). Greater declines in IgE (44%) do map initial clinical response to steroids, although this has only been demonstrated in those with a very high initial total IgE (≥ 2500 IU/mL), and has not been shown to predict clinical outcomes (72). Differences in diagnostic thresholds exist for the use of total IgE in the diagnosis of ABPA. In a survey of the AAAAI members 44.9% used ≥ 417 kU/L and 42% used ≥ 1000 kU/L as the cut off for a diagnosis of ABPA (73). Thus, as a marker of disease activity, or severity, precise levels seem to be of limited value.

1.5.4 IgG to *A. fumigatus*

Specific IgG to *A. fumigatus* may reflect environmental exposure or colonisation, although its value in differentiating those with or without sensitisation to *A. fumigatus* over the use of specific IgE is questionable. Very low sensitivity levels have been calculated using latent class analysis for the use of *A. fumigatus* IgG (42.7%) and for peripheral blood eosinophilia (25.5%) in the diagnosis of ABPA (74).

1.5.5 Eosinophilia

Eosinophilia seems to be well established as a marker of asthma and fungal allergy, it is not associated with abnormalities in lung function or immunological markers of fungal allergy and thus seems to be of limited use in the diagnosis of fungal conditions such as ABPA (75). In addition, sputum eosinophil levels are often

lower in those with fungal allergy suggesting a mixed Th2/Th1-17 immune response (52).

1.5.6 The use of the existing ABPA criteria in predicting outcomes

There is thus a need to re-evaluate the use of these parameters in the diagnosis of ABPA to justify their continued use. Agarwal et al performed a latent class analysis in order to estimate the efficacy of the various components used in the diagnosis of ABPA(74). Total IgE was found to have a high sensitivity (97.1%) but low specificity (37.7%), whereas peripheral blood eosinophilia (sensitivity 29.5%, specificity 93.1%) and *Aspergillus* precipitins (sensitivity 42.7%, specificity 91.7%) were found to have low sensitivities but high specificities(74). The only components that performed well were those that identified sensitisation to *A. fumigatus* (sensitivity 94.7-100%, specificity 69.3-79.7%) and bronchiectasis (sensitivity 91.9%, specificity 80.9%)(74).

1.5.7 What this study will add

The observational study in this body of work will examine whether these parameters (total IgE, IgE *A. fumigatus*, IgG *A. fumigatus*, eosinophilia), that currently form the basis of a diagnosis of ABPA, appropriately identify those at risk of adverse outcomes (in terms of lung function and lung function decline, airway inflammation and radiological abnormalities) and thus assess the use of these parameters as diagnostic biomarkers for internal validity i.e. assess the extent of correlation to clinical endpoints.

1.6 Fungal sensitisation in asthma & components

1.6.1 Introduction

Specific IgE and skin prick tests to fungal allergens are used to measure sensitisation status and are part of the diagnostic criteria for both ABPA and SAFS. Discordance exists between these tests, although cross-reactivity between different fungal genera does account for some of this discordance (76-78). The majority of fungal extracts that are used to produce specific IgEs use crude extracts that have been shown to cross-react both between and within species (79). Allergen extracts, that are used in the production of the reagents for skin prick testing and specific IgE, are a mixture of extracted non-allergenic and allergenic

proteins, polysaccharides and lipids (80). A high degree of allergen content variability has been seen with extracts of *A. fumigatus* both within and between manufacturers (81).

The mechanism for radiological abnormalities associated with allergic fungal airway disease is uncertain but may relate to chronic low grade colonization of the airways with thermotolerant fungi leading to a persistent allergic stimulus promoting viscid mucus secretion and chronic small airway obstruction (82).

1.6.2 Testing of fungal sensitisation and the use on components.

Filamentous fungi such as *A. fumigatus* contain many proteins and carbohydrates that act as allergens. Allergy testing contain many crude extracts from non-allergenic and allergenic proteins, polysaccharides and lipids, which cross-react both between and within species (77, 78) (79) (80). It is possible however that it is not the overall IgE response to *A. fumigatus* extracts that determines whether adverse features occur in this subtype of asthma, but the specific allergen to which the IgE is directed. For example some fungal allergens cross-react with human proteins potentially causing an autoreactive allergic response that could increase the risk of tissue damage.

1.6.3 Molecular based allergy diagnostics

Molecular-based allergy (MA) diagnostics benefit from being standardized, have a low degree of variability and have been developed from either recombinant or purified native molecules (80). Asp F1-13, Asp F15-18, Asp F22 & 23, Asp F27-29 and Asp F34 have all been recognised and approved by the WHO-IUIS Allergen Nomenclature Subcommittee as allergens of *A. fumigatus* (83). However, only rAsp f1-4 and rAsp f6 are commercially available with the ImmunoCAP® system from Phadia (see table 1.2). Asp F1, 2 and 4 are species-specific major allergens, whilst Asp F3 and Asp F6 are homologous proteins seen in other genera (84-88). A number of studies have demonstrated the utility of *A. fumigatus* components in the diagnostic separation between ABPA and sensitisation to *A. fumigatus*, although some of these studies do seem to show conflicting results as to which components are the most specific. rAsp F3, rAspF4 and rAsp F6 have been demonstrated to be specific markers for ABPA in both cystic fibrosis and asthma (87, 89-92); whilst rAsp F1 and 2 have also been shown as diagnostic markers of ABPA with AUC

values of 0.75 and 0.78 respectively in a Japanese asthma population (93). In addition, rAsp F1-3 and rAsp F6 components have been quantifiably linked to flares compared to remission episodes of ABPA (90). Although these studies have shown that components can be used to achieve diagnostic separation between ABPA and those sensitised to *A. fumigatus*, it is still unclear in what way these components are related to adverse outcome measures in terms of a reduction in FEV₁ and radiological abnormalities.

1.7 Fungi and the fungal microbiome (mycobiome).

1.7.1 Fungi

Studies on global fungal diversity have estimated that there are between 1.5 and 3 million species (94) and as many as 10 phyla present in the fungal kingdom (95) (see figure 1.2 (reproduced from Heitman (96))). The ability of fungi to inhabit different environments often dictates their ability to act as commensal organisms, allergens or pathogens. Most mesophilic fungi, such as *A. alternata* and *C. herbarum*, (cultured at 18-22°C, but unable to grow at body temperatures) are aeroallergens (82). Thermotolerant fungi (with representatives from all major fungal lineages including the Ascomycota and Basidiomycota phyla) grow at 37°C and are able to both colonise the airway and act as aeroallergens (82). The filamentous fungi *A. fumigatus* and the yeast *C. albicans* are the most commonly encountered fungi in this group.

1.7.2 Pathogenicity of fungi

Fungi are ubiquitous to our environment and are found in many parts of the human body, kept in check by our host defences. *A. fumigatus* is able to cause disease in hosts where there is disruption in the innate immune system and the epithelial barrier, predisposing patients with airway diseases to chronic pulmonary aspergillosis and ABPA (such as those with asthma, cystic fibrosis and COPD), or in the immunocompromised host where diseases such as invasive aspergillosis are seen more commonly (97, 98). Asthma has been associated with both a disruption in the epithelial barrier and an increase in the expression of toll-like receptors (particularly TLR2 & 4) by epithelial cells, which increases susceptibility to fungi pathogenicity (99).

1.7.3 The use of culture, qPCR and HTS in detecting fungi

The pathogenic role of fungi is currently reliant on their detection from culture and quantitative PCR (qPCR). However, this approach is biased towards those fungi which can be cultured and where primers have been developed. Thus, the role of fungi in disease and health has been difficult to fully appreciate with the use of these techniques alone. Selective culture media has increased the number of fungi that are able to be identified from respiratory samples, however, the yield from culture is still relatively low and biased towards those fungi that grow easily (100). Alternative techniques such as qPCR are limited by the number of assays that have been developed for specific fungi and cannot evaluate fungal burden or diversity (101). Molecular techniques such as high throughput sequencing (HTS) provide a culture independent platform with the ability of mapping niche specific microbiomes. This technology has been used extensively in evaluating the diversity of the gastrointestinal microbiome in both health and disease and has started to reveal differences in the diversity of the mycobiome in those with cystic fibrosis and in lung transplant recipients (102-106). Studies in asthma have shown fungal diversity to be increased in ABPA and an increase in the relative abundance of *Aspergillus* compared to mild asthmatics (107); however, it is currently unclear what role different fungi have in the different compartments of the lung and also how it is related to different clinical characteristics in asthma.

1.7.4 What this study will add to the current understanding of the mycobiome in asthma

The aim of the mycobiome component of this thesis is to examine the diversity of fungi that are present in the different compartments of the lung in healthy individuals and in asthmatics, and subsequently evaluate the relationship between the mycobiome and clinical characteristics in asthma.

1.8 Lung function and lung function decline in asthma.

The rate of lung function decline is greater in asthma. In males this is 50ml/year, compared to a healthy control population of 35ml/year, independent of age and atopic status (108). In addition, a 15-year follow-up study has provided further evidence that asthma is associated with a greater decline in FEV₁ in both genders, irrespective of smoking status ($p < 0.001$) (109). Newby et al demonstrated a decline of -25.7mL/year in post-bronchodilator FEV₁ in severe asthma, with a

more accelerated rate of decline (-40.9mL/year) noted in those with a highly fluctuant eosinophilia level (110). In addition, exacerbation rate has also been linked to excess in lung function decline. Those with frequent exacerbations have been shown to have an excess of 16.9mL/year decline in FEV₁, over those with well-controlled asthma (111).

1.8.1 Lung function and lung function decline in asthmatics sensitised to *A. fumigatus*

Asthmatics that are sensitised to *A. fumigatus* have a reduced post-bronchodilator FEV₁ (52). Thus, it would be expected that these asthmatics also have an accelerated rate of lung function decline, compared to a matched population. It is currently unclear whether this is the case or not. Previous studies suggest that changes in airway inflammation and/or remodelling changes have a central role to play in lung function decline in asthma. Airway eosinophilia, exacerbation rates, and airway wall CD8+ T-cell infiltration have all been shown to be associated with lung function decline (110-112). In addition, genetic polymorphisms have also been significantly associated with an excess decline in lung function, such as the S₂ allele of ADAM33 ($p < 0.05$) (113). The first part of this thesis will examine how the parameters used to indicate fungal sensitisation are related to lung function decline in asthma.

1.9 Radiological abnormalities in asthmatics sensitised to fungi.

High-resolution computer tomography (HRCT) scans enable us to visualise morphological abnormalities affecting the large and small airways. Radiological abnormalities have been detected in 71.9% of asthmatics (114). Bronchial wall thickening is a prominent feature of asthma and is seen in 62% of those attending a difficult asthma clinic (115). Comparator studies show asthmatics to have a 1.48mm thicker bronchial wall than those with chronic obstructive pulmonary disease and a 2.34mm thicker bronchial wall than healthy controls ($p < 0.001$) (116). Bronchiectasis is seen as a feature of airway remodelling and has been reported in 36% of asthmatics (117).

Radiological abnormalities have also been associated with deranged lung function. Bronchiectasis, mosaic attenuation and air trapping are more common in asthmatics with moderate to severe airflow obstruction and in those with lower

FEF₂₅₋₇₅ percentages (116). The use of quantitative computer tomography (QCT)-derived morphometry and densitometry has also shown a relationship between a reduction in lung function, airway remodelling and radiological abnormalities. Airway wall thickness and percentage wall area have been positively correlated with epithelial thickness, whilst airway smooth muscle percentage is correlated with mean luminal area (118, 119). QCT has also shown that features such as air trapping are associated with increased vascularity, impairment in lung function and proximal airway narrowing (percentage wall area) (118, 120).

1.9.1 Radiological abnormalities in asthmatics with ABPA

Bronchiectasis, centrilobular nodules and mucoid impaction occur more commonly in patients with ABPA, compared to those with asthma (121). Bronchiectasis is seen as more diffuse, affecting a greater number of lobes (≥ 5 lobes) and characteristically of a more central distribution than those with idiopathic bronchiectasis, although when used as a diagnostic feature this has limited sensitivity (37%) (121, 122). In addition, Menzies et al demonstrated a greater degree of airflow limitation and an increased hazard ratio (2.01 (95% CI 1.26-3.22), $p=0.005$) of developing bronchiectasis associated with sensitisation to *A. fumigatus* (123).

Features of mucoid impaction, specifically the presence of high attenuation mucus (HAM) are related to frequent relapses of ABPA (odds ratio 7.38 (95%CI 3.21-17)), are associated with higher immunological markers and are seen more frequently in patients with ABPA (121, 124-126). The appearance of tree-in-bud abnormalities (centrilobular nodules with or without linear opacities) is also noted to be present in those with ABPA. It is seen on 86% of HRCT scans performed on patients with confirmed ABPA, whilst fleeting shadows has previously been reported in up to 66% of patients (127, 128). Interstitial fibrosis that has a predilection for the upper lobes is characteristic of ABPA but its frequency in patients sensitised to fungi remains unreported.

The frequency of these radiological abnormalities is well documented in asthma; however, few studies to date have investigated the relationship of these abnormalities with markers of inflammation and fungal sensitisation. Although,

there is a mounting degree of research correlating quantitative CT indices with asthma severity and some aspects of airway remodelling, these indices have not been validated as surrogate markers for all aspects of airway remodelling. Therefore, rather than relying on quantitative CT indices as an outcome variable, existing radiological markers of established airway inflammation, obstruction and damage will be used as outcome variables. The cross-sectional study (study 1, chapter 4) will report the presence of these radiological abnormalities in asthmatics with and without sensitisation to fungi and the associations of these abnormalities with the parameters used to diagnose patients with ABPA and characterise those with asthma.

1.10 Airway inflammation and remodelling in asthma

Airway remodelling is known to occur in some individuals with asthma. It is a complex process involving a variety of mechanisms and is dependent on the host response to various physical, infective and inflammatory stimuli (129, 130). The spectrum of clinical presentation and the resultant presence or absence of structural and remodelling changes reflect the heterogeneity of asthma and the variety of mechanisms involved in this process (130). *In vitro*, *in vivo* murine and human studies have provided evidence for the involvement of a variety of inflammatory pathways in the remodelling process involving eosinophils, neutrophils, epithelial cells, mast cells and myofibroblasts (129, 131). However, the exact underlying aetiology and cellular mechanisms involved in the process of airway remodelling remain unclear.

Smooth muscle hypertrophy, subepithelial collagen deposition and fibrosis, mucus gland hyperplasia, vascular remodelling and epithelial damage are the histopathological hallmarks of airway remodelling (132). Airway smooth muscle cells cause bronchoconstriction, but also have an immunomodulatory role, in which expression of chemokines and cytokines are linked to submucosal inflammation (133). An increase in airway smooth muscle mass has been associated with airway hyperresponsiveness, asthma severity and an increased likelihood of dying from asthma, particularly when these changes are most marked in the larger airways (134-136).

1.10.1 Mast cell infiltration of airway smooth muscle

Activated mast cell infiltration of the airway smooth muscle (ASM) bundle is noted to be a pro-inflammatory feature of asthma, as indicated by increased levels of IL-4 and IL-13 secretion by the ASM bundle and also by immunohistochemistry studies (137, 138). The presence of mast cells in the ASM bundle is associated with airway smooth muscle proliferation and a more contractile phenotype, as indicated by an increase in ASM TGF- β 1 secretion and α -smooth muscle actin (139).

Mast cells are an important source of vascular endothelial growth factor (VEGF) and immunohistochemistry studies have demonstrated an association between vascular area and the number of VEGF positive mast cells (140). Angiogenesis (promoted by VEGF) causes infiltration of inflammatory cells and increased airway wall oedema (141). This is thought to play a central role in the pathophysiology of asthma and airway remodelling.

1.10.2 Subepithelial fibrosis

Subepithelial fibrosis results from increased deposition of collagens I, III and V, lumican and biglycan at the level of the *lamina reticularis* (142, 143). This causes thickening of the reticular basement membrane (RBM). RBM thickness is associated with an increase in the numbers of mast cells in bronchoalveolar lavage fluid, an increase in sputum eosinophil counts and bronchial hyper-reactivity (144). Thus, supporting the relationship between airway inflammation, structural changes and airway physiology (144). In addition, the presence of both mast cells and eosinophils in endobronchial biopsies have been associated with increased laminin and tenascin deposition, and thereby RBM thickness, further suggesting a link between these inflammatory cells and airway remodelling in asthma (145).

1.10.3 Eosinophils

Eosinophils produce many pro-fibrogenic molecules and cytokines. Eosinophilic cationic proteins induce both the fibroblast release of TGF- β from fibroblasts and the release of matrix metalloproteinases and platelet-derived growth factor from epithelial cells (146, 147). These suggest potential mechanisms in which eosinophils promote structural changes to the airway in asthmatics.

1.10.4 Airway epithelium

Sputum and endobronchial biopsy studies have shown evidence of epithelial shedding as a consistent feature of asthma (148, 149). In addition, mucus gland hyperplasia in the epithelial layer results in overproduction of mucous and mucous plugging in the peripheral airways. This has been linked to both asthma severity and fatal asthma (136, 150).

Although, some of these remodelling changes (such as airway smooth muscle hypertrophy) have been linked to asthma severity and a decline in lung function (151), we do not know how these changes differ in those asthmatics that are sensitised to fungi compared to those that are not sensitised to fungi. The second study in this thesis aims to explore this area further by comparing endobronchial biopsies from asthmatics that are sensitised to fungi compared to those that are not sensitised to fungi, when matched for disease severity.

1.11 Statistics

Exploratory data analysis uses statistical techniques to look for associations and is important in generating hypotheses. It does not allow the attribution of causality, but is very useful when analysing a large dataset and in creating more homogenous groups. This body of work will necessitate a variety of statistical techniques. Univariate and multivariate statistical analysis will be used to look for associations and significant differences between population groups. Modelling techniques will be used to analyse differences in lung function decline data.

Factor and cluster analysis are multivariate statistical techniques that allow individuals to be classified into groups on the basis of certain chosen variables. This type of classification was chosen so that we could elicit whether individuals grouped by certain immunological variables differed in terms of their clinical characteristics and outcome measures.

1.11.1 Factor analysis

The main objective of factor analysis is to reduce a set of variables into a core group by analysing the interrelationships of the variables with one another, based on shared variance. There are two techniques available: Exploratory Factor Analysis (explores the dataset for patterns) and Confirmatory Factor Analysis

(used to confirm hypotheses) (152). Exploratory Factor Analysis will be used in this project.

In essence, factor analysis utilises techniques used in regression and correlation, whilst taking into account the variance of the variable as it relates to all the other variables, to evaluate these interrelationships. These components add to the 'weight' of the variable to allow similar variables to be grouped together into factors. Factor loading scores are determined by the 'weight' of the variable – when measured graphically as vectors this is seen as the cosine of the angle between a variable and a factor (which in part reflects its correlation coefficient) (152). The highest factor loading scores of these variables are then used to determine which of the variables should be used to represent each domain (153). In addition to this process certain variables that are felt to be better clinical discriminators may also be used, instead of, or in addition to, those with the highest factor loading scores as the input variables in order to perform cluster analysis.

Factor rotation (oblique or orthogonal) is used to ensure that the variables differentially and maximally load onto one factor thus preventing ambiguity (154). Oblique rotation permits the factors to correlate, whilst orthogonal rotation maintains factor independence. Varimax is an orthogonal type of rotation that optimally loads variables with a higher factor loading score, thus this method of rotation was chosen due to its higher discriminatory ability (152).

1.11.2 Cluster analysis

Cluster analysis uses algorithms to differentiate subjects into groups on the basis of a set of input variables. It is a multivariate technique, performed on parametric data, and is an objective way of classifying individuals based on equally weighted variables, helping to reduce selection bias (155, 156). Delineating these subgroups (or clusters) can be useful in uncovering different patterns of disease, clinical presentations and be helpful in identifying those more at risk of adverse outcomes. To enable clearer cluster patterns to be generated a prior exploratory factor analysis is often performed to reduce the number of input variables into a core set.

1.11.3 Different types of cluster analysis

Many different methods of cluster analysis exist. Clustering can be either nested (hierarchical, which allows subclusters) or un-nested (partitioned, where overlapping clusters are identified). Each of these methods utilise different algorithms to differentiate clusters. This may be on the basis of distance or density, or by means of properties or objective function (where each cluster shares the same property/function) (155). The three most commonly used clustering algorithms:

- K-means
- Hierarchical
- Density-based clustering

K-means is a partitioning clustering technique, where a centroid (central point) is used to separate clusters into a predetermined number of groups. Hierarchical clustering yields nested clusters, which are visualised as a dendrogram. Ward's method produces a hierarchical analogue of k-means, and is often used in the initial stages of a K-means cluster analysis to determine the number of clusters present. Density-based cluster analysis includes any core point within a specified radius to cluster groups together and eliminates outliers as noise (155).

1.11.4 Hierarchical versus k-means cluster analysis

Hierarchical clustering is based on (Euclidean) distance, whilst k-means cluster analysis uses a single mean vector to differentiate between clusters (154, 156). Thus, k-means allows for more defined clusters to be characterised, whilst hierarchical clustering maybe be more subjective depending on the visual interpretation of the dendrogram and the chosen cut-off level used for the number of clusters present. Problems may occur with k-means cluster analysis when clusters differ significantly in terms of size, density or when the shape of the clusters are non-globular (155).

Cluster analysis will be used in analysis of the large observational study (study 1) to establish whether patients who are sensitised to fungi can be grouped and see whether they differ in terms of clinical characteristics and outcome variables.

1.11.3 Linear modelling

Linear mixed-effects models have previously been used to analyse lung function decline data in asthmatics (110). This type of analysis is very similar to a regression analysis in that it is used to evaluate the relationship between a response variable and independent variables, but the addition of a mixed model allows repeated measure to be inputted from a longitudinal dataset (157). Random and fixed effects models can both be used, where the explanatory variables are treated as if they arose from random (vary over time e.g. FEV₁) or non-random (fixed, that do not vary over time e.g. height) causes. Components of both are present in the linear mixed-effects model. This model was therefore used to analyse the effects of different sensitisation patterns on serial measures of post-bronchodilator FEV₁.

1.11.4 Power calculations

Power effect size

A power calculation is used to determine that the study sample size is sufficient to detect genuine differences between groups. Thus, it limits the chance of type II errors occurring (158). To calculate the sample size requires several pre-requisites. These include: power, effect size, an estimate of the variability of the data and the significance level (159).

Minimal important difference variability

The power of a study is conventionally set to at least 80%, thus, limiting the chance that the study will detect a difference when none exists (158). The size of the difference between two variables- the effect size, can be measured in several ways (for example by using Cohen's d; odds ratios; Pearson's r correlation coefficient); whilst the minimal important difference is multidimensional and is usually determined by consensus opinion amongst experts (158).

Measures of variability (e.g. standard deviation / confidence intervals) from existing studies with similar outcome points are used and are important in reducing the margin or error (160).

Significance level

The significance level that the study is set to also affects the sample size, with lower significance levels reducing type I statistical errors but increasing sample size (158). A positive result at a $\leq 5\%$ significance level will reliably reject the null hypothesis in 95% of the population studied and this is commonly used in many studies.

Tables & figures

Table 1.1 Diagnostic classification allergic bronchopulmonary aspergillosis ((ABPA) as defined by the Rosenberg-Patterson criteria and the International Society for Human and Animal Mycology (ISHAM) working group) and Severe Asthma with Fungal Sensitivity (SAFS) (55, 59, 71).

Rosenberg-Patterson criteria	ISHAM criteria	SAFS
Primary criteria (1-6 suggestive, all 7 definite): 1. Episodic bronchial obstruction 2. Peripheral eosinophilia 3. Positive immediate skin test to <i>Aspergillus</i> 4. Positive precipitin test to <i>Aspergillus</i> 5. Increased total serum IgE 6. History of transient or fixed lung infiltrates 7. Proximal bronchiectasis Secondary (supportive) criteria: 1. Brown plugs/flecks in sputum 2. Positive late skin test to <i>Aspergillus</i>	Primary criteria (obligatory) 1. Asthma or cystic fibrosis 2. Total IgE > 1000 IU/mL 3. Positive skin prick test or IgE to <i>A. fumigatus</i> Supportive criteria (≥2): a) Positive IgG or precipitins to <i>A. fumigatus</i> b) Raised eosinophil count c) Radiological features of ABPA	Primary criteria: 1. Severe, poorly controlled, asthma 2. Positive skin prick test to fungi or IgE to any fungi >0.4kU/L 3. Total IgE <1000 kU/L

Table 1.2 Commercially available recombinant components for *A. fumigatus*, produced by Phadia (ImmunoCAP® rAsp f1-4, rAsp f6) (161).

<i>A. fumigatus</i> components	Biological function
rAsp f1	Ribonuclease – inhibits eukaryotic protein synthesis. Produced after germination (162).
rAsp f2	Fibrinogen binding protein
rAsp f3	Peroxisomal protein - inactivates reactive oxygen species and protects the fungi from immune attack.
rAsp f4	Unknown
rAsp f6	Magnesium superoxide dismutase

Figure 1.1 The role of Th1, Th2 and Th17 pathways in the pathophysiology of airway inflammation in asthma. Fungi stimulate the inflammatory process via proteolytic allergens, presenting to the alveolar macrophages and by PAMPs/DAMPs recognition. In addition, damage to the airway epithelium occurs directly from proteolytic fungal enzymes and other environmental stimuli. This initiates an innate immune response by activating ILC2 cells, alveolar macrophages and invariant natural killer T cells. The release of airway epithelium derived TSLP induces dendritic cell (DC) maturation (upregulating co-stimulatory molecules and MHC II), whilst IL-25 and IL-33 stimulate ILC2 cells to produce Th2 cytokines. The cytokine milieu present during the presentation of antigen from the DC to a naïve T cell (Th0) directs the immune response towards a Th2, Th1 or a Th17 pathway with subsequent activation of effector cells (basophils, mast cells, eosinophils and neutrophils). In addition an adaptive immune response can occur from the presentation of antigen to memory B cells and the binding of allergens to specific IgE on mast cells. The cytokines, growth factors and mediators produced by these pathways and effector cells lead to the chronic inflammatory and remodelling changes seen in asthma. **Abbreviations:** LPS = lipopolysaccharides; PAMPs/DAMPs = pathogen-associated / damage-associated molecular patterns; PGD2 = prostaglandin D2; TSLP = thymic stromal lymphopoietin; DC = dendritic cell; TGF β = transforming growth factor β ; IFN γ = interferon γ ; GM-CSF = granulocyte macrophage colony-stimulating factor; TNF α = tumour necrosis factor α ; MMP = matrix metalloproteinase; NE=neutrophil elastase; ROS = reactive oxygen species; ASM = airway smooth muscle.

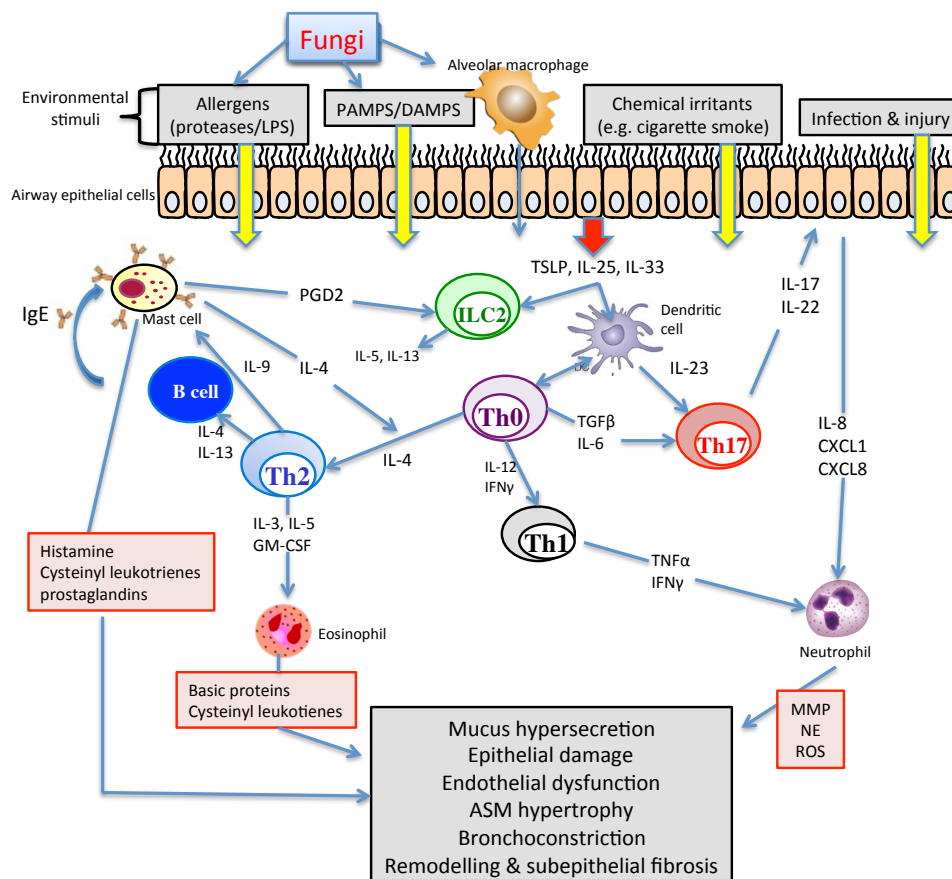
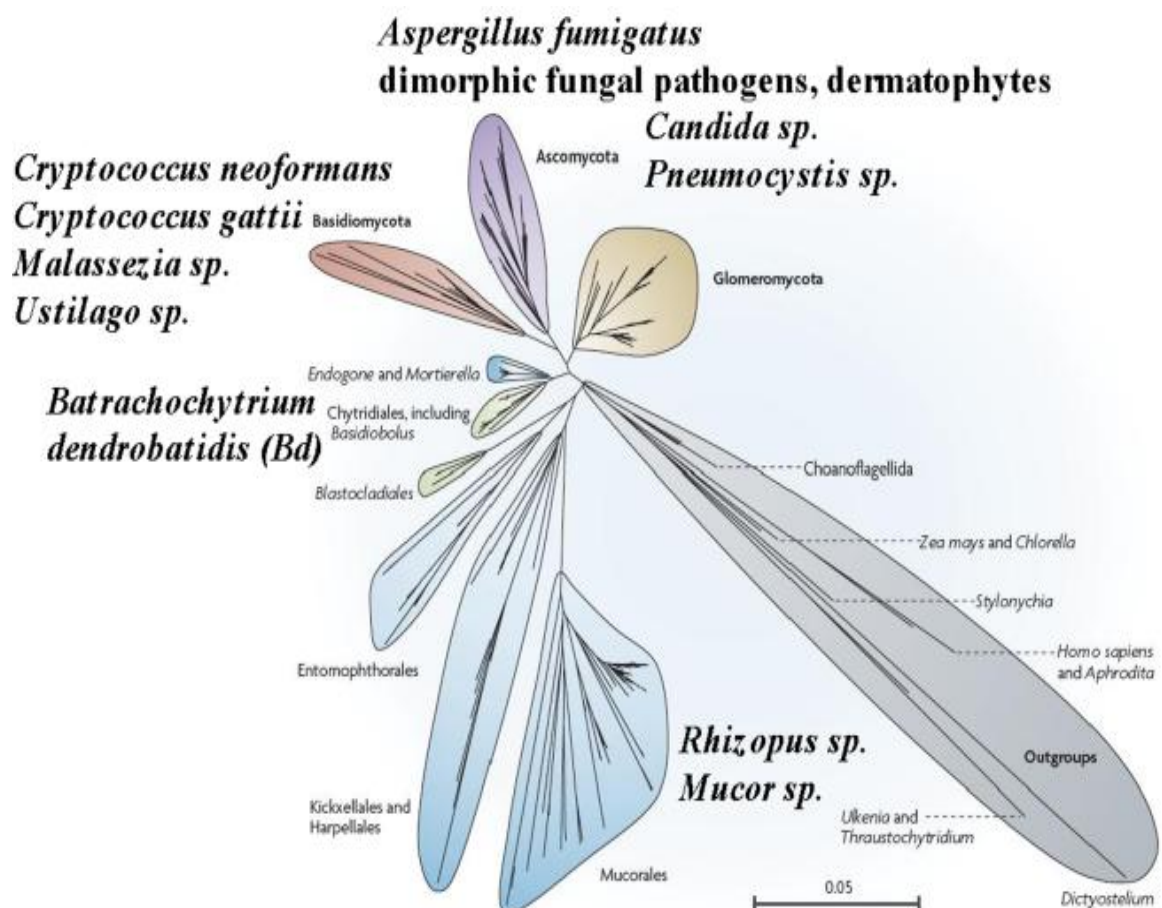


Figure 1.2 Microbial pathogens present within the fungal kingdom (from Heitman J. (96)). Fungi are eukaryotic organisms and have similar biosynthetic pathways to mammalian cells. They obtain energy from organic matter either as saprophytes, parasites or as mutualists. There are an estimated 1.5-3 million species within the fungal kingdom and the majority are microscopic. There are two subkingdoms: the Microporidia and Dikarya subkingdom. The Dikarya subkingdom contains the Ascomycota and Basidiomycota phyla. A further 7 phyla are recognised. Most of the species that are encountered clinically belong to either the Ascomycota and the Basidiomycota phyla.



Chapter 2: Hypotheses

HYPOTHESES

- ❖ I hypothesise that sensitisation status itself is more useful than the diagnostic term of ABPA for identifying those at risk of declining lung function and radiological abnormalities.
- ❖ I hypothesise that a reduction in post-bronchodilator FEV₁ is associated with sensitisation to thermotolerant yeasts, non-thermotolerant fungi and thermotolerant fungi, independent of atopic status.
- ❖ I hypothesise that the frequency of radiological abnormalities (namely bronchiectasis, bronchial wall thickening, tree-in-bud, mucus plugging, lobar collapse and fibrosis) is increased in asthmatics sensitised to fungi compared to those that were not sensitised to fungi.
- ❖ I hypothesise that the use of principle component analysis and cluster analysis to identify immunological and inflammatory variables will characterise different phenotypes of asthmatics, which are unique to those that are sensitised to fungi. This will demonstrate that a spectrum of disease exists and that those diagnosed with ABPA are not a homogenous group.
- ❖ I hypothesise that sensitisation status to thermotolerant fungi are important in identifying those at risk of a decline in post-bronchodilator FEV₁ over time.
- ❖ I hypothesise that remodelling changes, specifically an increase in airway smooth muscle hypertrophy and in reticular basement membrane

thickness, will be seen in asthmatics that are sensitised to thermotolerant fungi.

- ❖ I hypothesise that the use of high throughput sequencing to characterise the fungal microbiome will show predominance towards the fungi that subjects are sensitised to and that a specific signature will arise in those that have a greater degree of remodelling changes.

Chapter 3: Methods

3. 1 INTRODUCTION

An observational study (chapter 4) was undertaken to capture a large population of asthma patients that are sensitised to fungi, along with a control group not sensitised to fungi. The objective was to examine the relationship between serum, blood and sputum biomarkers of fungal sensitisation and outcome data (reduction in post-bronchodilator (PB) FEV₁ with different sensitisations pattern, PBF_{FEV}₁ decline and radiological markers of lung damage). A second prospective study (chapter 5 and 6) was performed to examine the relationship between sensitisation to fungi in asthma and remodelling changes using immunohistochemistry. This study was also performed to map the mycobiome in different compartment of the lung and relate this with clinical characteristics in healthy individuals and asthma.

The methods used in the clinical characterisation and the laboratory sample processing methods are described in this chapter. Study specific methodology is described separately in each of the following chapters (chapters 4, 5 and 6).

3. 2 CLINICAL CHARACTERISATION

Asthma Control Questionnaire

The Asthma Control Questionnaire (ACQ) was originally developed and validated by Juniper and colleagues (163). It is a validated measure of the adequacy of asthma control, with strong discriminative and evaluative properties (163). The components of the questionnaire are shown in table 3.1, and the score interpretation is described in table 3.2. The ACQ score is associated with measures of bronchial hyperresponsiveness and sputum eosinophilia, and is able to predict future risk of exacerbations (164, 165). Use of the ACQ is recommended by international guidance to assess asthma control and as an outcome measure in clinical trials (166, 167). Four versions of the ACQ exist: the original 7-item ACQ and the three shortened versions. These are the ACQ-5, ACQ-6a and ACQ-6b. ACQ-7 is the original version and contains all of the components in the table. Three additional shortened versions have also been developed: ACQ-5 (omits measures

of FEV₁ and the short-acting beta 2 agonist (SABA) question), ACQ-6a (omits only the SABA component) and ACQ-6b (omits FEV₁ assessment). Wyrwich et al demonstrated good validation, agreement and correlation across all four these versions (Cronbach alphas ≥ 0.82 ; test-retest intraclass correlation coefficients ≥ 0.75 ; $r \geq 0.97$), although ACQ-6a had better concordance with GINA and NHBLI criteria for classifying asthma control (168, 169). In addition, the ACQ-6a score consistently demonstrated strong concordance values with the original ACQ-7 Juniper questionnaire (kappa values 0.84 – 0.92) (168). It is for this reason that the ACQ-6a was chosen as a method to evaluate asthma control.

Spirometry

Spirometry was undertaken according to standardised methods using a dry bellows spirometer (Vitalograph Ltd, Maids Moreton, UK)(170). The best of three acceptable post-bronchodilator (twenty minutes post inhalation of 400µg of salbutamol) manoeuvres to achieve an FEV₁ and FVC within 0.150L were recorded (as percentage predicted). Criteria for an acceptable blow to measure FEV₁ and FVC was taken as an effort that was from maximal inspiration, a smooth continuous exhalation and one in which a plateau was reached in the volume-time curve (exhalation for ≥ 6 seconds with a volume change of <0.025 L for ≥ 1 second) to fulfil the end of test criteria (170).

Methacholine Challenge testing

Measurement of airway hyperresponsiveness was assessed by performing a methacholine challenge testing (PC₂₀) in subjects with a FEV₁ greater than 1 litre in accordance with published guidelines (171). A two-minute tidal breathing protocol was used administering 0.9% saline and then incremental concentrations of methacholine (0.03-16mg/ml) delivered via a nebuliser. Each incremental dose was delivered five minutes apart with an FEV₁ measured at 30 and 90 seconds post nebuliser. The percentage drop in FEV₁ from baseline was calculated, if this exceeded 20% the protocol was terminated and the prior concentration of methacholine is noted as the result (171).

Sputum Induction

Induced sputum was used to obtain differential cell counts. Sputum induction was carried out in accordance with published methods (172). A baseline FEV₁ is measured prior to administration of 200µg of salbutamol. If a repeat FEV₁ is >60% predicted after 20mins then 7mls of 3% saline is given using an ultrasonic nebuliser, tidal breathing and a nose clip. The FEV₁ is repeated, if a <10% drop is noted then increasing concentrations of saline are used (4% and then 5%), if a 10-20% drop is seen then the same concentration of saline is nebulised post-expectoration. If a >20% drop in FEV₁ occurs then the procedure is terminated. To reduce the risk of contamination of the induced sputum with upper airway contents the mouth is rinsed with water and swallowed and the nose is blown prior to the procedure (172).

Measures of atopy / sensitisation

Atopy is defined by the American Academy of Allergy, Asthma and Immunology (AAAAI) as the genetic tendency to develop allergic disease. A heightened immunological response and clinical worsening of disease upon exposure to an allergen manifest it.

Atopic status was based on either a positive skin prick test (>3mm above negative control; ALK-Abelló, Høsholm, Denmark) or a positive specific IgE (>0.35kU/L) to *Dermatophagoides pteronyssinus*, dog, cat, grass pollen or tree pollen. Serum specific IgE to *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Candida albicans*, *Alternaria alternata* and *Cladosporium herbarum* was used to designate sensitisation to fungi (specific IgE >0.35kU/L; UniCAP250 system (Pharmacia, Milton Keynes, United Kingdom)).

The term 'fungal sensitisation' is used to define a group who have any positive specific IgE to fungi or positive skin prick test result to fungi. Asthmatics in this thesis that are specifically sensitised to certain fungi, such as *A. fumigatus*, will be stated as such. Those that are sensitised to a group of fungi, such as the thermotolerant group (see methods section, chapter 4), will be stated specifically.

3.3 BRONCHOSCOPY

The use of bronchoscopy to obtain airway specimens such, as bronchoalveolar lavage (BAL) samples and endobronchial biopsies (EBB), is deemed a safe and acceptable research technique in both healthy volunteers and asthmatics with an FEV₁ >70% predicted (173, 174). Fibreoptic bronchoscopy was performed in the morning, as a day procedure, in accordance with national guidelines under conscious sedation with midazolam (175). Asthmatics received a pre-medication of 2.5-5mg of nebulised salbutamol. Topical lignocaine was used to anaesthetise the nose (2% gel), oropharynx (10% spray), vocal cords and the tracheobronchial tree (2% solution administered via the bronchoscope). A 20ml wash was obtained during bronchoscopy using warmed 0.9% saline; BAL was performed by using sequential 60ml aliquots of warmed 0.9% saline instilled into the right middle lobe up to a maximum of 180mls. Five endobronchial biopsies were taken from the subsegmental carinae of the right middle or lower lobes. Subjects were observed for a period of 2 hours following bronchoscopy and were discharged once safe swallowing had returned.

3.4 RADIOLOGY

Helical thin section computer tomography (CT) scans of the whole thorax (including both inspiratory and expiratory phases) were obtained using a Siemens Somaton Definition AS plus spiral scanner (Siemens Healthcare, Knoxville, TN). Scans were performed in a caudio-cranial direction using 1mm thickness slices at 0.7mm intervals.

The clinical radiology report was scored (blinded to any of the immunological data) for the presence or absence of bronchiectasis, bronchial wall thickness, air trapping, tree-in-bud, mucoid impaction, collapse/ consolidation or fibrosis (see table 3.3 for definitions). Fleeting shadows were recognised by their presence on serial chest x-rays.

3.5 LABORATORY SAMPLE PROCESSING

Sputum processing

In the second, prospective, study induced sputum was spilt for fungal DNA extraction and differential cell counts in a class II laminar flow hood. Sputum processing is performed in accordance with published literature (176). A mucolytic agent allows separation of the cellular plugs from saliva in order to reduce squamous cell contamination. This cellular plug is then placed in 0.1% dithiothreitol (4 x the weight of the sputum plug) and vortexed. An equal volume of the phosphate buffered saline (PBS) is added prior to filtration (48µm nylon gauze (pre-wet with PBS)) and centrifuged for 10 minutes. 10µl of 0.4% trypan blue is added to 10µl of the pellet, flooded onto a haemocytometer and the following calculated: % leukocyte viability, % squamous cells and total cell count. Cytospins are then prepared in order to perform differential cell counts with a Zeiss Axioscope 40 at 40x objective lens magnification.

Bronchoscopy samples

Figure 3.1 illustrates the processing steps for the bronchoalveolar lavage (BAL), brushes and wash samples obtained at bronchoscopy.

Biopsy processing and immunohistochemistry

The endobronchial biopsies are embedded in glycolmethacrylate (GMA) using acetone fixation (figure 3.2). This allowed for better epitope preservation, reduced tissue shrinkage (GMA is a water-miscible resin), whilst the physical support of the polymerised resin would maintain the tissue morphology of the small biopsies and allowed for cutting at 2µm (177).

Biopsy fixation is performed by immediate placement of the biopsy in acetone (containing protease inhibitors) at -20°C for 12 hours (178). This is then replaced with acetone at room temperature for 15 minutes. Following fixation, processing occurs by placing the biopsy in methyl benzoate for 15 minutes and then 5% methyl benzoate in GMA for 2 hours at 4°C. This step is then repeated. The embedding process is further described in figure 5. The GMA blocks were cut into 2µm sections and floated for 60 seconds in 0.2% ammonia solution prior to placing them onto a slide.

The DAKO EnVision™ FLEX system (DakoK8023) was used for the immunohistochemistry (figure 6). This polymeric, immunoenzymatic, method of staining was chosen because of the ability of the dextran polymer backbone to bind enzyme molecules (horseradish peroxidase) to an antibody (179). This reduces background staining and the number of steps in comparison to convention techniques (179). A peroxidase blocking agent was applied to each section for 10 minutes (to block endogenous enzymes) prior to applying the primary mouse and rabbit antibodies for 60 minutes at room temperature (figure 3.3). The primary antibodies used were: (i) smooth muscle actin, (ii) mast cell tryptase, (iii) major basic protein, (iv) neutrophil elastase. IgG1 and antibody diluent were used as the negative controls. The optimum antibody titres are listed in table 3.4. Three wash buffer cycles were performed before and after adding the horse radish peroxidase (HRP) to each section for 30 minutes. The HRP substrate, 3,3' diaminobenzidine

tetrahydrochloride (DAB), was applied for predetermined reaction times (see table 3.4), producing a brown stain.

3.6 BIOPSY ANALYSIS

Digital images of the biopsy sections were acquired on a Carl Zeiss Axio Imager z2 (with AxioCam HRC) microscope at x20 magnification. Slides and images were stored under the subject's study number to enable blinding. Zeiss Zen Imaging software (Zen Pro 2012) was used to map the morphology of the biopsy sections. The total area and the different morphological areas (epithelium, smooth muscle and lamina propria) were mapped on two non-contiguous sections taken >10µm apart and an average of each tissue compartment area were calculated. All non-structural areas (including blood vessels) were mapped and subtracted from these area measurements. Measurements were made in mm² and performed on slides stained with (i) smooth muscle actin and (ii) haematoxylin and eosin (H&E). Nucleated mast cells, neutrophils and eosinophils were counted at x40 magnification in each of these areas and expressed per mm². Reticular basement membrane thickness (RBMT) was measured on intact epithelium at x40 magnification. 50 measurements were taken 20µm apart and the average value of these and of the two non-contiguous sections were taken as the RBMT for that biopsy.

3.7 HIGH THROUGHPUT SEQUENCING METHODOLOGY

High throughput sequencing (HTS) was used to evaluate the abundance of fungi in different compartments of the airway. DNA extraction was performed on the pellet component of centrifuged samples: (i) endobronchial wash, (ii) protected brushings and (iii) bronchoalveolar lavage. Barcoded Internal Transcriber (ITS) region 3 and 4 primers(180) were selected to amplify the ITS2 region of the nuclear ribosomal operon, using a dual index nested PCR approach. Further details of the study specific methodology used are described in the Mycobiome chapter (chapter 6).

3.8 STATISTICAL METHODS

Power calculation and sample size

(i) STUDY 1: Post-bronchodilator FEV₁, lung function decline and radiological abnormalities in asthmatics sensitised to fungi (Chapter 4).

The power calculation was based on previous studies that compared sensitization and culture results to *A. fumigatus* and post-bronchodilator FEV₁ (% predicted) as the primary outcome measure (14, 52). The power calculation assumed that the data was parametric and was based on a 25% Standard Deviation (SD). It was powered at the 80% level and was calculated with the statistics package 'R'. A 4 group ANOVA calculated on an n=300, based on a 24% SD to detect a 5% difference between groups and powered at the 80% level.

(ii) STUDY 2: REMODELLING CHANGES IN ASTHMATICS SENSITISED TO FUNGI AND THE FUNGAL MICROBIOME IN ASTHMA (chapter 5).

We could not do power calculations for exacerbation frequency due to the nonparametric nature of this data. No data exists on pathological and radiological markers of airway damage in this cohort of patients and we were thus unable to do power calculations based on these variables.

The sample size necessary to detect differences in inflammatory cell counts and remodeling changes were based on a study by Richmond et al (181). A sample size of n=14 was sufficient to achieve 80% power and a significance level of 5%. To evaluate differences between groups an n=20 per group would be required to allow for a 20% dropout rate.

(iii) Study 3: DESCRIPTION OF THE MYCOBIOME IN ASTHMA (chapter 6).

The mycobiome study is a preliminary study using specific primers to evaluate the abundance of fungi in the different compartments of the lung and thus a power calculation could not be performed.

Univariate analysis

Data was analysed using SPSS for Windows (version 22; SPSS, Inc., Chicago IL), GraphPad (version 6; GraphPad software Inc., La Jolla, CA) and SAS version 9.4 (www.sas.com). Descriptive statistics were expressed as mean (standard deviation (SD) and 95% confidence intervals (95% CI)) or median (interquartile range). Two group analyses were analysed via an independent t-test and a Bonferroni-corrected one-way analysis of variance for multiple groups. Two group analyses were performed using the Mann-Whitney test and the Dunn-corrected Kruskal-Wallis test was used for multiple group comparisons for non-parametric data.

Correcting for multiple comparisons

A Bonferroni correction was used to reduce the chance of type I errors due to multiple comparisons between variables.

Multivariate analysis

Data underwent logarithmic and z-transformation in order to perform multivariate analyses and to remove problems with unequal variance during cluster analysis. Principle component analysis was performed as a means of performing a factor analysis to identify the highest loading immunological variables. This also reduced the number of immunological variables into a core set. A varimax rotation was used in the generation of the factor analysis and a cut off value of >0.75 was applied to determine the factors that would represent each domain.

Cluster analysis was performed in order to map clinical characteristics to immunological variables and to see how these variables clustered together. The SPSS two-step algorithm was used to estimate the number of clusters within the studied population (182). This estimate was used to specify the number of clusters present in a K-means cluster analysis, which was used as the principle clustering technique.

Linear modelling used in analysis of lung function decline

Longitudinal post-bronchodilator FEV₁ measurements were collected over a 3-5 year period at a stable visit (6 weeks post exacerbation) to calculate lung function

decline. Linear modelling allowed the input of these repeated measures to be used in a 'regression type' analysis. Information based criteria was used to assess the quality of the model with regards to 'fit'. Akaike information criterion (AIC) and its corrected version (AICc), and Bayesian Information Criteria (BIC) are part of this evaluation criteria and use -2LogLikelihood to evaluate model fit (table 3.5) (183).

A generalised linear model was run first (fixed effect model). Transformed variables were used to improve model fit. A random effect for time and a random intercept to produce a mixed effects model improved model fit further (see table 3.5). Thus the model used in the lung function decline analysis was a mixed effects model with components that were both fixed (measures of sensitisation, sputum neutrophil count, sputum eosinophil count and blood eosinophil count) and random (time and random intercept).

Imputation was performed for any missing continuous variable data. This was performed five times to produce five data sets. The average values for the estimates and standard errors were then calculated.

Tables & figures

Table 3.1 Components of the Asthma Control Questionnaire (ACQ) (163, 169)

Components of ACQ	Scoring
Symptom scores	
Night time awakenings	0-6
Symptom severity upon waking	0-6
Activity limitation	0-6
Shortness of breath due to asthma	0-6
Wheezing in the last 7 days	0-6
Short acting beta agonist use in last 7 days	0 (none) 1 (1-2 puffs most days) 2 (3-4 puffs most days) 3 (5-8 puffs most days) 4 (9-12 puffs most days) 5 (13-16 puffs most days) 6 (>16 puffs most days)
FEV₁ % predicted	0 (>95% predicted) - 6 (< 50% predicted)

Table 3.2 Interpretation of the Asthma Control Questionnaire (ACQ)(184). A single cut off point of 1.00 has been identified between those well-controlled (<1.0) and those not well-controlled (>1.00); however, more optimal cut-off points of 0.75 (negative predictive value = 0.85) and 1.50 (positive predictive value=0.88) are more accurate in classifying asthmatics into either well-controlled or inadequately controlled groups (184).

ACQ score	Asthma control	Predictive value
≤ 0.75	Well controlled	Negative predictive value = 0.85
$> 0.75 - < 1.5$	Borderline	
≥ 1.5	Uncontrolled	Positive predictive value = 0.88

Table 3.3 Definitions of radiological abnormalities (185-187).

Radiological Abnormality	Definition
Bronchiectasis	- Bronchoarterial ratio > 1.5 - Lack of tapering in peripheral ½ of the lung, >2cm distal to bifurcation - Bronchial dilatation within 1cm of costal/mediastinal pleura
Bronchial Wall thickening	- >0.5 x diameter of adjacent pulmonary artery - 2x thickness of normal adjacent bronchi - Internal diameter of lumen<80% of total outside diameter
Mucoid Impaction	- Tubular / Y shaped structures in 2nd order bronchi - Branching or rounded opacities on cross-sectional imaging
Tree-in-bud	Dilated, mucus-filled bronchioles
Air trapping	Retention of excess air in all/part of the lung, mainly during expiration, due to either: (i) partial/complete airway obstruction (ii) local abnormalities in pulmonary compliance
Collapse	Resorption atelectasis due inspissated secretions, resulting in mottled intraluminal densities
Consolidation	Increase in pulmonary parenchymal attenuation that obscures vessel margins and airways. The lumen may be visible giving rise to air bronchograms
Fleeting shadows	Migratory pneumonitis with transient, recurrent, alveolar patchy segmental / lobar infiltrates
Fibrosis	- Septal lines, intralobular reticular peribronchovascular interstitial thickening, subpleural interstitial thickening - Honeycombing - Traction bronchiectasis - Predilection for upper lobes

Table 3.4 Primary mouse monoclonal antibody titres (*with Dako antibody diluent S3022; **negative controls). The antibody concentrations had already been optimised to give the dilution and development times in the table. IgG1 was diluted to give a working concentration to match the antibody with the highest working concentration in the panel and was more concentrated than the mast cell tryptase.

Antibody	Dilution*	Chromogen development time (minutes)	Catalogue number	Supplier
Antibody diluent**	None	3:30	S3022	Dako
IgG1**	1:77	3:30	X0931	Dako
Smooth muscle actin (SMA)	1:2000	3	M0851	Dako
Mast cell Tryptase	None	3	IR640	Dako
Major basic protein (MBP)	1:75	3	MON6008	Monosan (Newmarket)
Neutrophil elastase (NE)	1:1000	3:30	M0752	Dako

Table 3.5 Information based criteria to assess model ‘fit’ of the generalised linear model and the linear mixed model for lung function decline data. Akaike information criterion (AIC) and its corrected version (AICc), and Bayesian Information Criteria (BIC) use -2LogLikelihood to evaluate model fit. *with time as a fixed variable; **with a random effect for time and a random intercept. The values for AIC, AICc and BIC are much smaller with the mixed model, indicating that the mixed model fits the data better than the fixed effects model.

	Generalised Linear Model*	Linear mixed model**
-2 Res Log Likelihood	788.8	-187.5
AIC	802.8	-181.5
AICc	802.9	-181.5
BIC	835.6	-171.4

Figure 3.1 Bronchoscopy sample processing (EB = endobronchial).

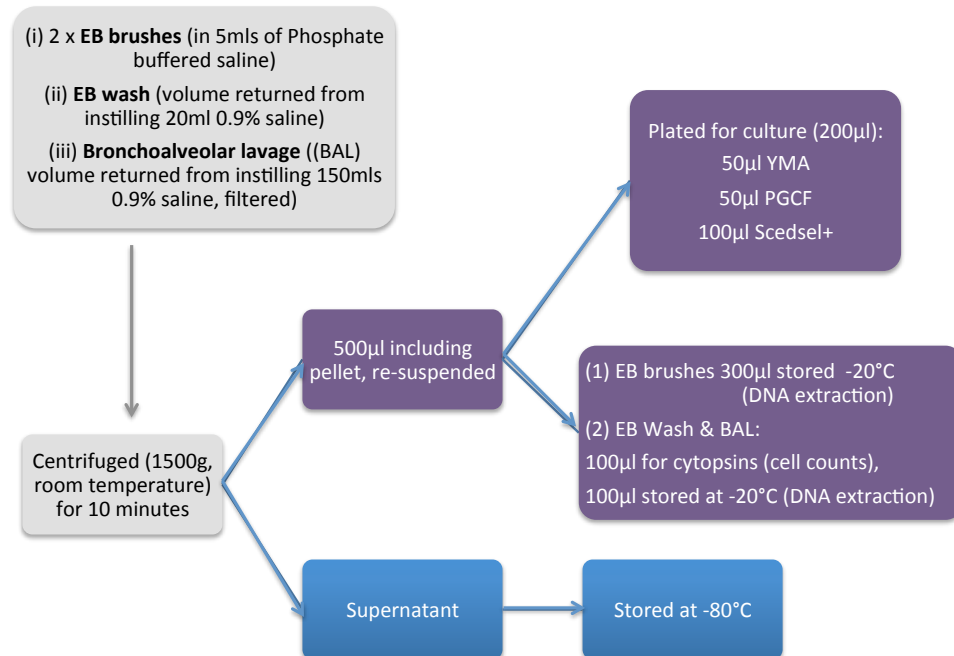


Figure 3.2 Biopsy processing. EBB = endobronchial biopsies; *proximal airways are >2mm in diameter; #35mg benzoyl peroxide dissolved in 5mls of GMA solution A + 125µl GMA solution B prior to use).

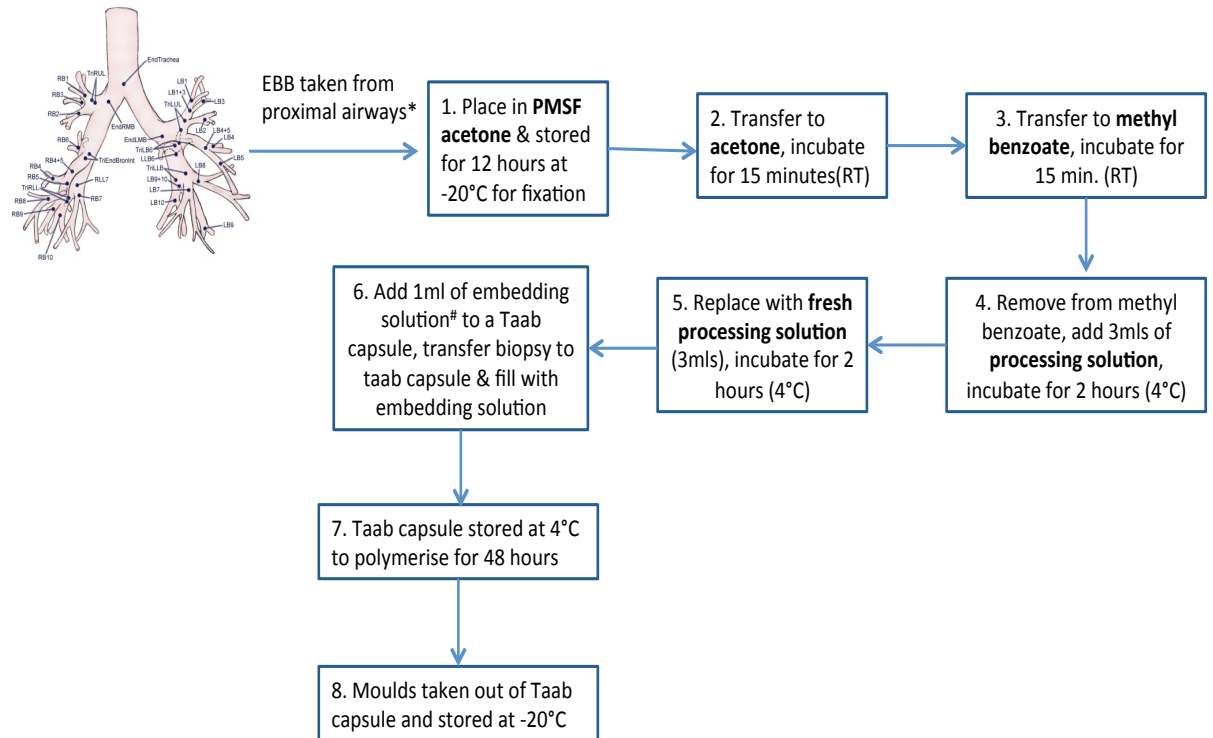
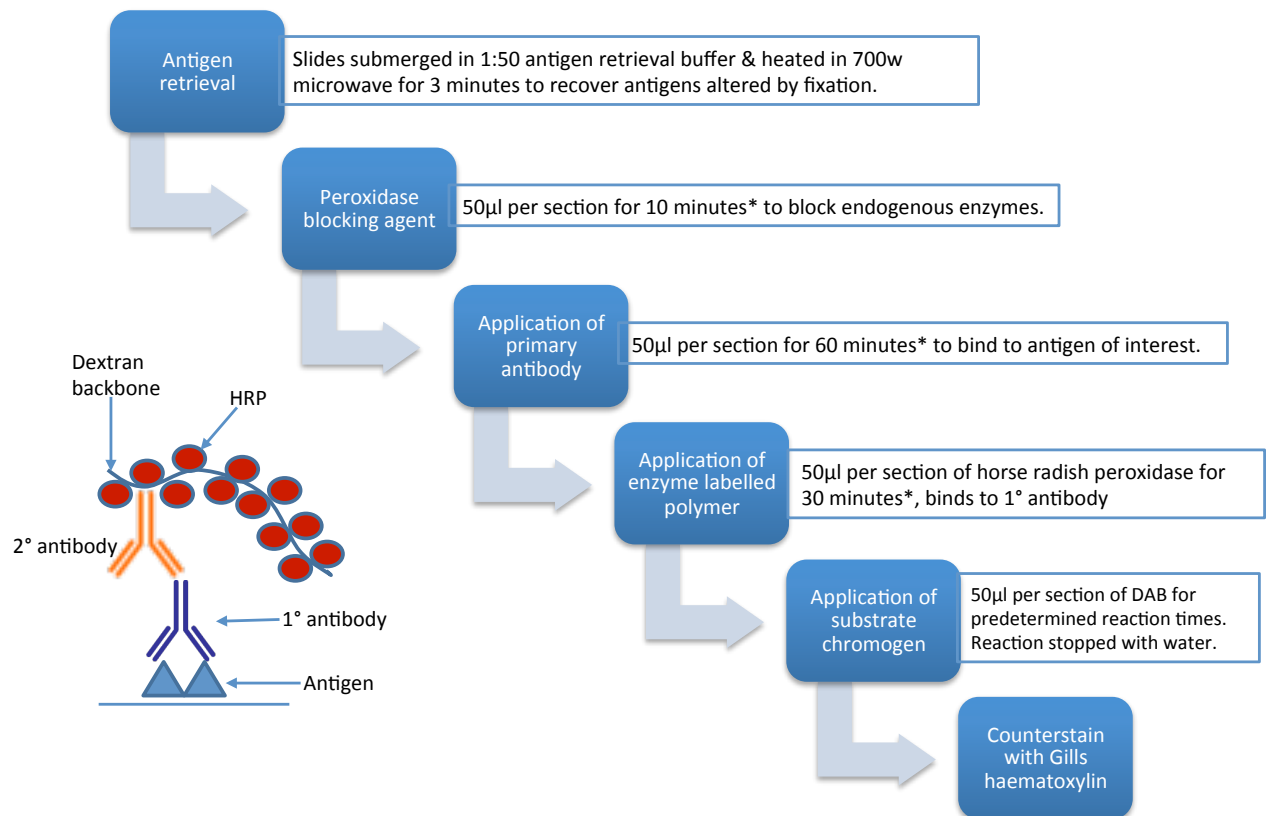


Figure 3.3 Immunohistochemistry protocol (*wash with envision buff x3 following the incubation period). Chromogen is applied to visualise the antibody/antigen complex, counterstaining then allows visualisation of cell nuclei & tissue architecture.



Chapter 4: Post-bronchodilator FEV₁, lung function decline and radiological abnormalities in asthmatics sensitised to fungi (study 1)

4.1 Introduction

Allergy to fungi is associated with asthma, particularly at the severe end of the spectrum (71). The filamentous fungus *A. fumigatus* and the yeast *C. albicans* are both thermotolerant and are able to colonise the airways, whilst non-thermotolerant fungi that rarely grow at 37°C, such as *Alternaria alternata* and *Cladosporium herbarum*, primarily exacerbate asthma by acting as aeroallergens. While the health effects of these non-thermotolerant fungi are predictable and directly related to outdoor spore concentrations, the effects of thermotolerant fungi are more complex and heterogeneous (82). The most well described syndrome associated with fungal allergy is allergic bronchopulmonary aspergillosis (ABPA) (or mycosis [ABPM], when other fungal genera are considered to be causal).

The prevalence of ABPA in asthma has been estimated at 2.5% (range 0.72-3.5%), with a global burden of disease in this population group exceeding 4.8 million (58). Variable presentations and clinical courses are seen and there is limited evidence that the criteria used to define ABPA is able to appropriately select those at risk of disease progression or identify those who will respond to treatment. Total IgE has been given considerable prominence in the diagnosis of ABPA (total IgE > 1000 IU/l) and Severe Asthma with Fungal Sensitisation ((SAFS) total IgE < 1000 IU/l) (65). The ISHAM working group have revised the guidelines criteria for the diagnosis of ABPA and its management (59). However, the management of those patients that fail to fulfil the criteria for ABPA is not fully understood. A continuous spectrum of disease is seen in fungal allergy, including those with ABPA. Patients have a variable clinical course, with some requiring antifungals and having recurrent exacerbations and others not. It remains unclear how the parameters (specific IgE and IgG to *A. fumigatus*, total IgE, peripheral blood eosinophilia) that are used to diagnose ABPA relate to specific disease outcomes

and whether they clearly identify those with fungal allergy that are at risk of disease progression.

An association has already been demonstrated between IgE sensitisation to *A. fumigatus*, fixed airflow obstruction and bronchiectasis (52, 123). In addition, other radiological abnormalities including fleeting shadows, high attenuation mucus and lung fibrosis are a consistent feature of the clinical descriptions of fungal allergy in asthma (124, 127, 188). However, although these studies suggest that sensitisation status is linked to disease severity we do not know how all of the parameters used to diagnose patients with ABPA are linked to these outcome variables and whether different phenotypic groups are seen when we take this into consideration.

This chapter describes the cross-sectional study that was conducted to determine the relationship between total IgE, IgE to thermotolerant, nonthermotolerant fungi and yeasts, IgG *A. fumigatus* and eosinophilia to the following outcome measures: post-bronchodilator FEV₁, decline in post-bronchodilator FEV₁ over a 3-5 year period and radiological evidence of structural abnormalities. It also describes how patients can be classified into different phenotypic groups by the use of these variables and how these are related to outcome measures.

4.2 Methods

Subjects

Data was collected from patients attending secondary and tertiary asthma clinics at Glenfield Hospital (Leicester, UK) from January 2011 to August 2015. Eligibility was based on a clinical diagnosis of asthma and evidence of variable airflow obstruction with either a positive methacholine challenge test (20% drop in FEV₁ caused by <8mg/ml concentration of methacholine) or a >12% variability in FEV₁ spontaneously between clinic visits or following 200 µg of inhaled salbutamol. Asthma severity was graded using the Global Initiative for Asthma (GINA) 2012 criteria (www.ginasthma.org). Patients with a greater than 20 pack year smoking history were excluded from the study, as were patients receiving anti-fungal or immunomodulatory treatment (such as omalizumab), with the exception of glucocorticoids.

Subjects were grouped as being sensitised to fungi if they had a positive specific IgE ($>0.35\text{kU/L}$; UniCAP250 system (Pharmacia, Milton Keynes, United Kingdom)) to any of the following fungal panel: *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Candida albicans*, *Alternaria alternata* and *Cladosporium herbarum*. *Aspergillus* and *Penicillium* species were treated as thermotolerant filamentous fungi (many of these species readily grow at 37°C), and *A. alternata* and *P. Cladosporium* as non-thermotolerant filamentous fungi (most species will not grow at 37°C). Patterns of fungal sensitisation were grouped as follows: Group 1 = thermotolerant filamentous fungi (*A. fumigatus*, *P. chrysogenum*), Group 2 = thermotolerant yeasts (*C. albicans*), Group 3 = non-thermotolerant fungi (*A. alternata*, *C. herbarum*); Group 4 = not sensitised to fungi.

Atopic status was based on either a positive skin prick test ($>3\text{mm}$; ALK-Abelló, Høsholm, Denmark) or a positive specific IgE ($>0.35\text{kU/L}$) to *Dermatophagoides pteronyssinus*, dog, cat, grass pollen or tree pollen. Skin prick test (SPT) data of common aeroallergens is regarded as the gold standard for measurement of atopic status but it is necessary to perform both SPT and specific IgE for the diagnosis of fungal allergy, due to the discordance between the two methods (76, 189). Subjects that had a specific IgE to *A. fumigatus* value $>0.35\text{kU/L}$ also underwent molecular-based allergy diagnostic testing to the components: rAspF1-4 and rAspF6 (Phadia ImmunoCAP system).

All subjects within the study gave written informed consent for the use of the clinical data for research purposes (REC reference 13/EM/0287).

Clinical assessment

Demographic details (age, sex, BMI) and clinical data (age of asthma diagnosis, asthma duration, smoking status and pack year history, Asthma Control Questionnaire-6 scores (163), methacholine challenge test results and GINA status) were collected during a stable visit (at least six weeks following an exacerbation). In addition, each subject was classified by their clinical asthma phenotype (early-onset atopic, obese non-eosinophilic, early-onset symptom predominant, late onset inflammation predominant)(19). Spirometry was undertaken according to standardised methods by a trained physiologist using a

dry bellows spirometer (Vitalograph Ltd, Maids Moreton, UK)(170). Post-bronchodilator FEV₁ was recorded as percentage predicted. A minimum of 3 post-bronchodilator FEV₁ values per year, obtained at a stable visit (6 weeks post exacerbation), was recorded over a 3-5 year period, to calculate lung function decline.

Sputum induction was carried out in accordance with published methods (172). Induced sputum was used for cytopins to obtain differential cell counts but was not collected for fungal culture.

Radiological imaging

High resolution computerised tomography (HRCT) scans were undertaken in those with severe and/or difficult to treat asthma in accordance with International ERS/ATS guidelines on definition, evaluation, and treatment of severe asthma (3). Scans of the whole thorax (including both inspiratory and expiratory phases) were obtained using a Siemens Somaton Definition AS plus spiral scanner (Siemens Healthcare, Knoxville, TN). Scans were performed in a caudio-cranial direction using 1mm thickness slices at 0.7mm intervals.

The clinical radiology report was scored (blinded to any of the immunological data) for the presence or absence of bronchiectasis, bronchial wall thickness, air trapping, tree-in-bud, mucoid impaction, collapse/consolidation or fibrosis. Fleeting shadows were recognised by their presence on serial chest x-rays.

Data handling (see section 3.8)

Lung function decline data was calculated using a mixed effects linear model. The model was run with both continuous and categorical sensitisation data. $\log(\text{PBF}_{\text{FEV}_1})$ was the dependent variable used for the model. The independent variables were: age, BMI, gender, sputum neutrophils, highest peripheral blood eosinophils, total IgE, IgG *A. fumigatus*, IgE *A. fumigatus*, IgE *P. chrysogenum*, IgE *C. albicans*, IgE *A. alternata*, IgE *C. herbarum*. The categorical sensitisation data used for the model were as follows: atopic status and sensitisation grouping (Group 1 = thermotolerant filamentous fungi (*A. fumigatus*, *P. chrysogenum*), Group 2 = thermotolerant yeasts (*C. albicans*), Group 3 = non-thermotolerant fungi (*A.*

alternata, *C. herbarum*) and Group 4 = not sensitised to fungi). An interaction term of time_years*sensitisation indicator was also included.

A correlation matrix of the immunological variables used in the factor analysis is presented in table 4.3. The clinical importance of these immunological variables and factor loading scores from a principle component analysis (table 4.8) was used to reduce the number of these immunological variables prior to performing cluster analysis.

4.3 Results

Descriptive Clinical Data

The study consisted of 431 asthmatics. 329 (76.3%) subjects were sensitised to one or more fungi and 102 (23.7%) were not sensitised to any fungi. These two groups were well matched in terms of age, gender, smoking status, pack year history and GINA score (see table 4.1 & 4.2). Those sensitised to one or more fungi were seen to have early onset, atopic asthma (52.9%) or late onset eosinophilic asthma (37.1%); whilst those that were not sensitised to any fungi had a slightly greater BMI ($p<0.05$) and had predominantly late onset eosinophilic disease (67.6%). 31.3% (n=103) of the group were sensitised to one or more fungi and 49% (n=50) of the group not sensitised to fungi were taking maintenance oral prednisolone. 69.4% (n=299) of the cohort had a thoracic CT scan, of these 222 were sensitised to fungi (67% of those with fungal sensitisation) and 77 (75% of those without fungal sensitisation) were not sensitised to fungi.

Effect of fungal sensitisation and atopic status on lung function

A reduction in post-bronchodilator FEV₁ (PBFEV₁) was seen in those sensitised to the thermotolerant filamentous fungi (group 1: 73.1% predicted (SD 23.2; 95% CI 70.2-76)) compared to (i) those that were sensitised to thermotolerant yeasts (group 2: 77% predicted (SD 20.28; 95% CI 71.2-82.9), $p=0.263$), (ii) subjects sensitised to non-thermotolerant fungi (group3: 85.2 % predicted (SD 19.68; 95% CI 76.7-93.7), $p<0.05$), and (iv) those that were not sensitised to any fungi (group 4: 81.8% predicted (SD 21.45; 95% CI 78.5-87.2), $p<0.001$) (see figure 4.1). In addition, IgE sensitisation to *A. fumigatus*, independent of atopic status, was

associated with a significantly lower PBF_{EV1} compared to those that were just atopic (71.9% predicted (SD 23.98; 95% CI 67-77) vs 83.7% predicted (SD 19.76; 79.1-88.2), $p=0.005$) (figure 4.1).

Measured quantitatively there were weak correlations between the PBF_{EV1} and sIgE to *A. fumigatus* ($r -0.254$, $p<0.001$), sIgE to *P. chrysogenum* ($r -0.198$, $p<0.001$), sIgE to *C. albicans* ($r -0.112$, $p<0.05$) and total IgE ($r -0.117$, $p<0.05$). Post-bronchodilator FEV₁ was also weakly correlated with sputum neutrophil count ($r -0.203$, $p<0.001$) and duration of asthma ($r -0.249$, $p<0.001$), which may suggest that the nature of this relationship maybe non-linear. No significant correlation was seen with sIgG to *A. fumigatus*, sputum/blood eosinophil count or any of the other immunological variables (see correlation matrix in table 4.3).

Components

85 asthmatics that were sensitised to *A. fumigatus* had serum tested for the presence of rAspF1-4 and rAspF6. 57.8% (n=44) were male, 91.8% (n=78) had a GINA score ≥ 3 and the majority had either early onset atopic disease (50.6%, n=43) or late onset eosinophilic asthma (40%, n=34). There was no difference in the components when separated by GINA score, phenotype or atopic status that reached statistical significance.

37.6% (n=32) had a diagnosis of ABPA (as defined by the ISHAM criteria (59)), 21.2% (n=18) had a diagnosis of SAFS, whilst the majority (41.2%, n=35) were sensitised to *A. fumigatus* and did not fit either of these diagnostic classifications. The differences between the components in these groups were statistically significant (see table 4.4).

rAsp F6 was the only component to correlate well with total IgE ($r=0.798$, $p<0.001$). Although significant at the <0.05 level the other components were only weakly correlated total IgE, *A. fumigatus* IgE, peripheral blood eosinophil count and asthma duration.

No correlations were seen between rAsp F1, rAsp2, rAsp 3, rAsp F4 and rAsp F6 and PBF_{EV1}. Bronchiectasis was the only radiological abnormality where a raised level of Asp F3 (4.4 (0.81-12.9) vs 0.34 (0.07-3.91), $p=0.008$) and rAsp F4 (0.47

(0.03-3.16) vrs 0.09 (0.01-0.35), $p=0.033$) was associated its presence (figure 4.2). No other statistical differences were seen between the components and the frequency of the radiological abnormalities studied (tree-in-bud, bronchial wall thickening, mucoid impaction, collapse/consolidation, air trapping, fleeting shadows and fibrosis). Receiver operating curves did demonstrate an AUC value of 0.71 for rAsp F4 in predicting the development of fleeting shadows.

Lung function decline in asthmatics sensitised to fungi

48.7% (n=210) of the cohort had 3-5 years PBF_{EV1} measurements. 100% of these subjects had ≥ 3 years of serial PBF_{EV1} values, 46.7 % (n=98) had ≥ 4 years and 20.5% (n=43) had ≥ 5 years of lung function data.

The mixed effects model showed that atopic status, blood eosinophilia and sputum eosinophilia were **not** associated with a decline in PBF_{EV1} over time (table 4.5 and 4.6). It did, however, demonstrate a statistically significant reduction in PBF_{EV1} over time in those that were categorised as being into group 1 (sensitised to *A. fumigatus* and *P. chrysogenum*) compared to non-sensitised individuals or any other sensitisation pattern (table 4.6, $p<0.05$). A stronger signal was seen in the same model when individuals that were both atopic and sensitised to *A. fumigatus* were compared to atopic and sensitisation status alone (table 4.5).

When PBF_{EV1} (litres) is taken as a function of sensitisation to *A. fumigatus* IgE and decline is measured in millilitres per year we see that the effect for time is highly significant ($p<0.0001$, table 4.7). The regression coefficient for time in years is -0.05468. Thus, as time increases lung function declines in those that are sensitised to *A. fumigatus* ($p<0.05$, table 4.7). The regression coefficient demonstrates that asthmatics that are sensitised to *A. fumigatus* have an accelerated decline in lung function – a reduction of 103mls in post-bronchodilator FEV₁ per year compared to a decline of 81mls per year in those not sensitised to *A. fumigatus*, which is approaching significance ($p=0.063$) at the 5% level (table 4.7).

Radiological abnormalities & serum biomarkers of fungal sensitisation

The presence of bronchiectasis, tree-in-bud and collapse /consolidation were significantly increased in the group sensitised to fungi compared to those that

were not sensitised to fungi ($p<0.05$; figure 4.3). Group 1 (subjects sensitised to thermotolerant filamentous fungi) had an increased prevalence of bronchiectasis ($p<0.001$), tree-in-bud ($p<0.05$) and collapse/consolidation ($p<0.05$) compared to any of the other groups (figure 4.3). In addition, an increase in level of specific IgE to *A. fumigatus* was seen in association with several of the radiological abnormalities (table 4.9). A raised total IgE was observed in the presence of tree-in-bud ($p=0.007$) and fleeting shadows ($p<0.001$). *A. fumigatus* IgE was associated with the development of bronchiectasis ($p<0.001$), tree-in-bud ($p<0.001$), fleeting shadows ($p<0.001$), collapse / consolidation ($p=0.002$) and fibrosis ($p=0.023$). IgG to *A. fumigatus* was increased in bronchiectasis ($p=0.003$), tree-in-bud abnormalities ($p=0.024$) and fleeting shadows ($p=0.014$). A higher prevalence of radiological abnormalities was seen in those sensitised to the thermotolerant filamentous fungi (group 1). However, sensitisation to the thermotolerant yeasts (group 2) or the non-thermotolerant fungi (group 3) was not associated with any of the radiological abnormalities (figure 4.3).

Blood eosinophilia was the only parameter to be related to any of the radiological abnormalities. A raised blood eosinophil count was associated with the presence of tree-in-bud (1.85 (SD 2.95; 95%CI 0.93-2.79) versus 0.99 (SD 1.06; 95%CI 0.86-1.12) $\times 10^9/L$; $p<0.001$) and fibrosis (1.7 (SD 3.38; 95% CI 0.41-2.99) versus 1.04 (SD 1.11; 95%CI 0.91-1.18) $\times 10^9/L$; $p=0.024$). Sputum eosinophilia and neutrophilia were not associated with any radiological abnormalities.

Receiver operating characteristic (ROC) curves

Receiver operating characteristic (ROC) curves and area under the curve (AUC) values were calculated for each radiological abnormality using total IgE, specific IgE to fungi, IgG to *A. fumigatus* and markers of airway inflammation. Only two of the immunological biomarkers were associated with statistically significant AUC values. *A. fumigatus* IgE had an AUC value of 0.77 ($p=0.001$), giving a sensitivity of 81.5% and specificity of 63.3%, when a cut off value of 1.88kUA/L was used for the development of bronchiectasis. *A. fumigatus* IgG produced an AUC value of 0.79 ($p<0.001$), a sensitivity of 77.8% and a specificity of 60% when a cut off value of 26.39mg/L was used for the development of bronchiectasis. None of the other

immunological variables had statistically significant AUC values >0.75 for any of the remaining radiological abnormalities.

Cluster membership of asthmatics sensitised to fungi as a predictor of outcomes

Principle component analysis demonstrated the highest loading immunological variables to be: Total IgE, *A. fumigatus* IgE, *A. fumigatus* IgG and sputum eosinophil count (table 4.8). These variables were used in a K-means cluster analysis to reveal three cluster populations. The baseline characteristics of these clusters are shown in table 4.10. These cluster population were matched in terms of age, gender, BMI, GINA and ACQ6 scores. The asthma phenotypes among these clusters were very similar, with the majority in each cluster having either early-onset atopic disease (cluster 1: 47.1%, cluster 2: 42.2%, cluster 3: 43.6%) or late onset inflammatory asthma (cluster 1: 47.1%, cluster 2: 45.1%, cluster 3: 43.9%). Cluster 1 was slightly younger in asthma onset with a longer duration of disease compared to the other clusters. Cluster 1 was also more neutrophilic, whilst cluster 2 was more eosinophilic than any of the other clusters. A similar proportion of atopy was seen between all clusters. Cluster 1 had higher values of total IgE, specific IgE to fungi and IgG to *A. fumigatus* compared to the other two clusters. Cluster 1 also had a greater loss of lung function and a higher rate of radiological abnormalities. Patients classified as having ABPA (based on the ISHAM criteria) were distributed across all 3 groups with a similar number seen in clusters 1 and 3, but a higher proportion of case being present in cluster 1 (table 4.10). Asthmatics meeting the SAFS classification were only present in clusters 2 and 3.

4.4 Discussion

Previous studies have demonstrated an association between sensitisation to, and culture of, *A. fumigatus* to a reduction in airway reversibility, lower lung capacity and an increased frequency of bronchiectasis (14, 52). It was unclear, however, whether this relationship was limited to the thermotolerant filamentous fungi or also occurred with the non-thermotolerant fungi, such as *Alternaria alternata* and *Cladosporium herbarum*, that have been linked the persistence and severity of

asthma (13). This large cross sectional study has demonstrated that the association between fungal sensitisation and a reduction in $PBFEV_1$ is limited to the thermotolerant filamentous fungi *A. fumigatus* and *P. chrysogenum* and is not associated with the thermotolerant yeast *C. albicans* or any of the non-thermotolerant fungi. Similarly, the relationship between airflow obstruction and sensitisation to *A. fumigatus* was not simply a function of atopy as patients who were atopic without fungal sensitisation had well maintained lung function.

Atopy and age of asthma diagnosis strongly distinguished the sensitised and non-sensitised groups in the first section of the analyses but were not seen to be strong differentiating variables in the multivariate analysis. The variables used in the cluster analysis were based on clinical criteria used in the diagnosis of ABPA and the factor loading scores and therefore did not split the cohort purely on sensitisation status but by the height of the immunological response recorded by these variables. The cluster analysis was felt to give a better reflection of the spectrum of disease present and also explains why atopy and age of onset were not strong differentiating variable and were similar across all three clusters.

The link between sensitisation to *A. fumigatus* and adverse outcome measures in asthma

Lung function decline measured over a 3-5 year period in this cohort suggested an accelerated decline in lung function in those sensitised to *A. fumigatus*, with neutrophilic airway inflammation. *A. fumigatus* sensitisation was also seen to be a marker for bronchiectasis, although with limited specificity. This study provides further support of an association between IgE sensitisation to colonising filamentous fungi and adverse features of asthma; as opposed to IgE sensitisation being simply an incidental biomarker or a secondary phenomenon. A criticism as to the role sensitisation status has in the development of bronchiectasis and a reduction in post-bronchodilator $FEEV_1$ has been its relationship to early onset of disease and asthma duration. Refractory disease, atopy and fungal sensitisation are seen more frequently in early onset disease. These adverse outcomes may be expected to be a function of time since diagnosis so that those with long standing asthma would have a greater impairment in lung function than those with new

onset asthma. This study has found that fact that poor lung function tracks with fungal sensitisation and not atopy or early onset disease alone. This supports the hypothesis of fungal sensitisation being directly related to adverse outcomes in asthma rather than being attributable to disease duration or atopy.

Patients in this study with IgE sensitisation to *A. fumigatus* exhibited a number of radiological abnormalities including fleeting lung shadows, collapse and consolidation, lung fibrosis and tree-in-bud changes. The first three are recognised features of fungal allergy, but the link to tree-in-bud abnormalities is less well recognised, though it is noted in other studies (127). Fleeting shadows, although now unusual, are a classical hallmark of ABPA. There was a strikingly high total IgE in patients with this abnormality. Muroid impaction and high attenuation mucus is a recognised feature of ABPA (124). It is likely that all of the radiological abnormalities described are caused by the process and consequences of chronic airway obstruction by viscous mucus.

An eosinophilia is frequently associated with fungal allergy (190). Blood eosinophilia generally correlates well with sputum eosinophilia with an AUC of 0.83 (95% CI 0.78-0.87) and represent an eosinophilic asthma phenotype with uncontrolled airway inflammation (191, 192). However, Fairs et al have previously shown that patients sensitised to *A.fumigatus* had a mixed eosinophil and neutrophil differential sputum profile which may be due to colonisation of the airways with *A.fumigatus*, leading to a more infective profile (52). This observational study only found an association between blood, but not sputum eosinophils. The sputum eosinophil count is expressed as a differential; a high percentage of neutrophils would thus cause a lower percentage of eosinophils. This confounder is the most likely reason why the radiological abnormalities were not closely related to sputum eosinophilia. In addition the patient's highest peripheral blood eosinophil count was recorded, which was done more frequently than induced sputum, to give a better representation of the duration of 'unchecked' underlying airway inflammation.

The importance of the total IgE is a key component for the diagnosis of ABPA. High levels of total IgE were seen with (the unusual) fleeting shadows and to a lesser

extent tree-in-bud shadowing, but total IgE was not associated with a reduction in PBF_{EV1} or any other radiological abnormalities. Total IgE does not therefore appear to be a particularly useful for predicting those at risk of bronchiectasis or a reduction in PBF_{EV1}. In contrast we did find an association between radiological evidence of raised *A. fumigatus* specific IgG and bronchiectasis, tree-in-bud changes and fleeting shadows, although the difference in concentrations between those with abnormalities and those without was not marked and the mean value fell below the normal cut off of 40mg/L. An association with specific IgG to *A. fumigatus* and radiological abnormalities was also demonstrated in the ROC curve for bronchiectasis. However at optimum values the specificity of both *A. fumigatus* IgE and IgG for the presence of bronchiectasis was not particularly strong.

Cluster analysis was used to determine whether ABPA could be objectively identified in our cohort with the use of the parameters used in the diagnosis of ABPA as the input variables. Three clusters were revealed, which apart from a slight difference in length of time since asthma was diagnosed, did not show any significant demographic differences. The largest cluster (cluster 3) had no striking immunological signature. Cluster two was identified by a very high sputum eosinophilia and may equate to the 'hypereosinophilic' asthmatics identified in a previous cluster analysis (19). Cluster 1 which represented about 10% of the fungal-sensitised asthmatics had a florid immunological signature with a very high total IgE, specific IgG and IgE to *A. fumigatus*, and polysensitisation to fungal allergens. They also had significantly higher rates of radiological abnormalities than the other two clusters. This group therefore equates to the patients that would be identified by the ABPA criteria, although it is important to stress that there is overlap with the other two groups with no clear distinction between them in terms of total IgE or *A. fumigatus* specific IgG or IgE. There are however, a small number of asthmatics that develop a florid immunological response to fungi and are at high risk of a decline in lung function and in developing structural abnormalities.

Sensitisation to *A. fumigatus* is one of the main criteria for the diagnosis of ABPA, which is known to be more prevalent in severe asthma (13). ABPA remains a heterogeneous condition with a variety of clinical presentations. Of the various

criteria used to diagnose ABPA, only measures of *A. fumigatus* sensitisation have been found to be sensitive and specific enough to be used for its diagnosis (74). This study has evaluated the relationship between all of the parameters used in the diagnosis of ABPA and the adverse features that are associated with it in an attempt to define a more homogenous group of asthmatics. This is seen in cluster 1. This cluster had a florid immunological response to fungal allergens and a high rate of radiological abnormalities and a reduction in $PBFEV_1$, but did not contain all of those that met the ABPA classification based on the current ISHAM criteria. Cluster 3 contained a similar number (although a lower proportion) of asthmatics that fitted the ABPA criteria. This group also had a lower rate of radiological abnormalities associated with lung damage. Thus whilst it is clear that ABPA does represent a distinct clinical entity, it does not appear useful in capturing all of those that are at risk of developing adverse outcomes. This study would suggest that all patients sensitised to fungi are at an increased risk of developing an accelerated decline in lung function and the development of bronchiectasis compared to non-sensitised asthmatics and, although those with a florid immunological response may increase this risk, it is not just restricted to those that meet the ABPA criteria.

Concluding remarks

This study has demonstrated a significant association between IgE sensitisation to colonising thermotolerant filamentous fungi and a reduction in post-bronchodilator FEV_1 and a number of radiological abnormalities in moderate to severe asthma. Analysis of the parameters used in the diagnosis of ABPA/ABPM has shown that total IgE is neither a sensitive or specific biomarker for the development of bronchiectasis in asthma. In addition this study has also identified a small subset of patients with fungal allergy and asthma who have a florid immunological response to fungal allergens with polysensitisation, high levels of total IgE and *A. fumigatus*-specific IgG and IgE, poor lung function and high rates of radiological abnormalities. These patients equate to those described as having ABPA, although it is important to emphasise that no specific criteria could be usefully used to identify them. Taken together, these two observations suggest that IgE sensitisation to thermotolerant filamentous fungi is a risk factor for the

development of a reduction in PBF_{FEV1}, an accelerated decline in lung function and bronchiectasis in asthma irrespective of whether the criteria for ABPA are present.

Limitations

This study was an cross-sectional study and is only able to show associations and cannot demonstrate any causal link between any of the immunological and inflammatory variables and the outcome variables studied. It is still unclear why such a spectrum of disease severity exists in fungal allergy. This piece of work does not explain why some asthmatics with a florid immunological response have a poor disease trajectory, whilst others with the same immunological response seem relatively unaffected. This may, in part, explain why the lung function decline data did not show a direct link with lung function decline and sensitisation to the thermotolerant filamentous fungi despite there being a known association with a reduction postbronchodilator FEV₁.

It should be noted that this study was open to selection bias as it was intentionally aimed to recruit those sensitised to fungi and may unintentionally identify a larger group that was sensitised to thermotolerant fungi. The lung function decline data, was performed during a stable visit, however, patients were not characterized again during these visits (e.g. induced sputum cell counts were not performed). This may open this data up to confounding by these unmeasured variables.

Tables & figures

Table 4.1 Baseline clinical characteristics of asthmatics sensitised to fungi and those not sensitised to fungi. *mean (SD, 95%CI), #median (IQR)

Characteristic	Not sensitised to fungi (n=102, 23.7%)	Sensitised to fungi (n=329, 76.3%)	Significance level (p value)
Age (years)*	52.8 (16.42; 43.7-58.7)	50.9 (18.35; 43.2-57.1)	0.346
Male, n (%)	47 (48.1%)	168 (39%)	0.379
BMI (kg/m ²)*	29.9 (6.6; 26.2-33.5)	28.4 (6.5; 26.8-32.7)	0.489
Ex/current smokers, n (%)	35 (34.3)	88 (26.7)	0.18
Smoking pack years (years)*	8.2 (5.6; 5.1-10.1)	8.5 (6.19; 6-10.6)	0.9
Age of asthma diagnosis (years)#	39 (21-50)	7 (2-36)	<0.001
Duration of asthma (years)*	16.3 (15.6; 9.5-22.6)	31.9 (20.5; 21.8-38.9)	<0.001
GINA $\geq$ 3, n (%)	94 (92.2%)	299 (90.9%)	0.545
ACQ 6 score*	2.21 (1.4; 1.7-3.1)	2.29 (1.3; 2.1-3.1)	0.721
Atopy, n (%) (non-fungal allergens)	31 (30.4)	198 (60.2)	<0.001

Table 4.2 Clinical characteristics of the asthma cohort for which lung function decline data was collected (NFS = no fungal sensitisation; FS = fungal sensitisation present). *mean (SEM); #median (IQR)

	NFS (16.2%, n=34)	FS (83.8%, n=176)	P value
Age (years)*	58.4 (2.56)	55.2 (1.19)	0.273
Male (n,%)	12 (35.3%)	94 (53.4%)	0.052
BMI (kg/m ²)*	29.5 (1.04)	28.9 (0.44)	0.565
GINA ≥3			
Blood eosinophils*	1.03 (0.12)	0.98 (0.07)	0.746
Sputum eosinophils#	8.15 (0.50- 30.50)	6.50 (1.25-16.5)	0.710
Sputum neutrophils#	77.78 (51.75- 89.75)	85.63 (66.38-94.63)	0.104

Table 4.3 Correlation matrix of the immunological variables, sputum cell counts and blood eosinophils. All variables are log converted with the exception of sputum neutrophils. r = pearson's correlation coefficient. P = P value. $AF = A. fumigatus$, $PC = P. chrysogenum$, $CA = C. albicans$, $AA = A. alternata$, $CH = C. herbarum$, eosin = eosinophils, neut = neutrophils. Significant correlations highlighted in red.

		total IgE	AF IgE	AF IgG	PC IgE	CA IgE	AA IgE	CH IgE	sputum eosin	sputum neut	Blood eosin
total IgE	r	1	.616	.112	.671	.703	.412	.594	.025	.005	.099
	P		.000	.038	.000	.000	.000	.000	.667	.930	.042
AF IgE	r	.616	1	.236	.840	.588	.540	.557	.010	.177	.081
	P	.000		.000	.000	.000	.000	.000	.862	.003	.100
AF IgG	r	.112	.236	1	.210	.121	.236	.081	-.007	.064	-.023
	P	.038	.000		.000	.035	.006	.362	.916	.314	.669
PC IgE	r	.671	.840	.210	1	.669	.621	.676	-.069	.124	.072
	P	.000	.000	.000		.000	.000	.000	.263	.046	.161
CA IgE	r	.703	.588	.121	.669	1	.559	.659	-.091	.028	-.032
	P	.000	.000	.035	.000		.000	.000	.136	.656	.539
AA IgE	r	.412	.540	.236	.621	.559	1	.789	-.056	.046	-.015
	P	.000	.000	.006	.000	.000		.000	.581	.659	.851
CH IgE	r	.594	.557	.081	.676	.659	.789	1	.050	-.018	-.090
	P	.000	.000	.362	.000	.000	.000		.628	.864	.276
sputum eosin	r	.025	.010	-.007	-.069	-.091	-.056	.050	1	-.281	.129
	P	.667	.862	.916	.263	.136	.581	.628		.000	.025
sputum neut	r	.005	.177	.064	.124	.028	.046	-.018	-.281	1	-.047
	P	.930	.003	.314	.046	.656	.659	.864	.000		.420
Blood eosin	r	.099	.081	-.023	.072	-.032	-.015	-.090	.129	-.047	1
	P	.042	.100	.669	.161	.539	.851	.276	.025	.420	

Table 4.4 Differences in components between those with ABPA, SAFS and those sensitized to *A. fumigatus*. *median (interquartile range); ** AUC value.

	ABPA (n=32)	SAFS (n=18)	Senitised to <i>A.fumigatus</i> (n=35)	P value
rAsp F1	3.58 (0.27-15.95)*	0.14 (0.13-1.05)*	0.54 (0.17-5.15)*	0.002
	0.68**	0.25**	0.50**	
rAsp F2	2.32 (0.26-9.72)*	0.13 (0.05-0.66)*	0.34 (0.07-2.11)*	0.001
	0.73**	0.29**	0.42**	
rAsp F3	7.04 (1.04-14.65)*	0.33 (0.01-1.58)*	1.06 (0.1-9.79)*	0.002
	0.68**	0.27**	0.49**	
rAsp F4	1.33 (0.15-7.39)*	0.02 (0.01-0.07)*	0.12 (0.02-0.85)*	<0.001
	0.74**	0.24**	0.44**	
rAsp F6	0.20 (0.02-2.12)*	0.01 (0.01-0.01)*	0.04 (0.01-0.12)*	<0.001
	0.71**	0.23**	0.49**	

Table 4.5 The effects of atopic status, serum eosinophils and sputum eosinophils on lung function decline (mixed effects model). Atopic status, serum eosinophilia and sputum eosinophilia were not associated with a decline in FEV₁ over time. However, it did demonstrate a significant reduction in FEV₁ over time in asthmatics with neutrophilic airway inflammation that were both atopic and sensitised to *A. fumigatus* (p<0.001).

Effect/variable		Estimate	Standard Error	DF	t Value	Pr > t
Intercept		-0.00785	0.2987	135	-0.03	0.9791
Age (years)		-0.01277	0.001198	135	-10.66	<.0001
Height (cm)		0.007852	0.001540	135	5.10	<.0001
Weight (kg)		0.004366	0.000963	135	4.53	<.0001
Gender	Female	0.05903	0.04268	135	1.38	0.1689
Time(years)		-0.02713	0.008906	395	-3.05	0.0025
Sensitisation status to <i>A. fumigatus</i> and atopic status	Sensitised to <i>A. fumigatus</i> and atopic	-0.2207	0.05093	135	-4.33	<.0001
	Not sensitised to <i>A. fumigatus</i> , but atopic	-0.1689	0.06252	135	-2.70	0.0078
	Sensitised to <i>A. fumigatus</i> , but not atopic	-0.07977	0.05236	135	-1.52	0.1300
Sputum eosinophil count (%)		-0.00022	0.000958	135	-0.23	0.8195
Sputum neutrophil count (%)		-0.00167	0.000801	135	-2.09	0.0384
Blood eosinophil count (x10 ⁹ /L)		0.02196	0.01683	135	1.30	0.1942

Table 4.6 The effects of sensitisation status on lung function decline (mixed effects model). The model demonstrates a statistically significant reduction in FEV₁ over time in those that were categorised as being into group 1 (sensitised to *A. fumigatus* and *P. chrysogenum*) compared to non-sensitised individuals or any other sensitisation pattern (p<0.05).

Effect (variable)	Category	Estimate	Standard Error	DF	t Value	Pr > t
Intercept		-0.7147	0.2623	201	-2.73	0.0070
Age (years)		-0.01349	0.000969	201	-13.92	<0.0001
Height (cm)		0.01123	0.001455	201	7.72	<0.0001
Weight (kg)		0.004242	0.000798	201	5.31	<0.0001
Gender	Female	0.04371	0.03549	201	1.23	0.2195
Time (years)		-0.03713	0.007469	564	-4.97	<0.0001
Atopic status	Non Atopic	0.02931	0.03097	201	0.95	0.3452
Sensitisation to fungi	<i>A. fumigatus</i> & <i>P. chrysogenum</i>	-0.09430	0.04582	201	-2.06	0.0409
	<i>C. albicans</i>	-0.00749	0.05979	201	-0.13	0.9005
Sensitisation status	Sensitised to other fungi	-0.05074	0.07985	201	-0.64	0.5259

Table 4.7 Mixed effects lung function decline model with post-bronchodilator FEV₁ (PBF₁) (litres) taken as a function of IgE sensitisation to *A. fumigatus*. Decline is measured in millilitres per year, the effect for time is significant (p<0.001). The regression coefficient for time in years is -0.05468. Thus, as time increases lung function declines in those that are sensitised to *A. fumigatus* (p<0.05). The regression coefficient demonstrates that asthmatics that are sensitised to *A. fumigatus* have an accelerated decline in lung function – a reduction of 103mls in post-bronchodilator FEV₁ per year compared to a decline of 81mls per year in those not sensitised to *A. fumigatus*, which is approaching significance (p=0.063) at the 5% level. SE = standard error

Effect/variable	Estimate	SE	DF	t value	Pr > t	alpha	Lower	Upper
Intercept	0.6701	0.0275	208	24.39	<0.0001	0.05	0.6160	0.7243
Time (years)	-0.0547	0.0094	563	-5.82	<0.0001	0.05	-0.0731	-0.0362
Sensitisation to <i>A. fumigatus</i>	0.1179	0.0509	208	2.32	0.0216	0.05	0.0175	0.2182
time_years*AF_Sens	0.0168	0.0238	563	0.71	0.4791	0.05	-0.0299	0.0635

Table 4.8 Factor loading scores from principal component analysis.

Rotated Component Matrix				
	Component			
	1	2	3	4
Total IgE	.760	.345	.004	-.015
<i>A. fumigatus</i> IgE	.567	.074	.607	-.256
<i>A. fumigatus</i> IgG	.094	.274	.807	-.023
<i>C. albicans</i> IgE	.831	.021	.105	-.072
<i>A. alternata</i> IgE	.828	-.124	.257	-.013
Sputum eosinophils	-.027	.772	.053	.041
Sputum neutrophils	-.155	-.427	.550	.471
Blood eosinophils	.062	.753	.126	-.003

Table 4.9 The relationship between presence / absence of radiological abnormalities and total IgE, specific IgE to *A. fumigatus* and IgG to *A. fumigatus*.

#median (interquartile range)

		Total IgE (kU/L)[#]	<i>A. fumigatus</i> IgE (kUA/L)[#]	<i>A. fumigatus</i> IgG (mg/L)[#]
Bronchiectasis	Present	578 (152-1582)	3.3 (0.23-26.5)	35.00 (19.25-62.00)
	Not present	406 (116-1535)	0.21 (0.04-1.34)	23.05 (12.80-47.50)
	P value	0.257	<0.001	0.003
Tree in bud	Present	987 (260-2680)	8.53 (0.58-24.10)	39.75 (18.90-79.50)
	Not present	406 (132-1336)	0.48 (0.05-5.36)	27.20 (14.50-52.85)
	P value	0.007	<0.001	0.024
Fleeting shadows	Present	2324 (1395-5000)	32.15 (16.3-75.6)	74.10 (27.7-181.0)
	Not present	440 (134-1336)	0.55 (0.06-6.51)	27.60 (14.65-54.65)
	P value	<0.001	<0.001	0.014
Collapse / consolidation	Present	574 (154-1787)	1.34 (0.12-16.6)	30.60 (15.40-57.60)
	Not present	442 (132-1453)	0.42 (0.05-5.53)	28.00 (14.60-53.50)
	P value	0.450	0.002	0.173
Fibrosis	Present	872 (147-1787)	1.68 (0.49-36.50)	40.60 (21.30-71.70)
	Not present	427 (136-1535)	0.53 (0.06-6.59)	27.20 (14.70-53.50)
	P value	0.205	0.023	0.065

Table 4.10 Cluster populations and their relationship to immunological biomarkers, lung function and radiological abnormalities. This identified three clusters of asthmatics: (i) an eosinophilic group with a lower frequency of fungal sensitisation, and better-preserved lung function; (ii) a neutrophilic group with a higher degree of immunological response to fungi, lower PBF_{FEV1} values and a higher frequency of radiological abnormalities was also seen; (iii) a neutrophilic group with high rates of sensitisation to *A. fumigatus*, but with only a modestly raised level of total IgE, a lower frequency of radiological abnormalities and relatively well-preserved lung function.

*mean (SD, 95%CI); #median (interquartile range).

Postbronchodilator FEV₁ (PBF_{FEV1}).

	Cluster 1 (n=34)	Cluster 2 (n=71)	Cluster 3 (n=326)	P value
<u>Demographics</u>				
Age (years)*	57.77 (15; 54.2-73.9)	51.78 (19.9; 41.6-58.6)	50.62 (17.7; 48.6-54.5)	0.084
gender (n, % male)	15 (44.1%)	36 (50.7%)	164 (50.3%)	0.781
BMI (kg/m²)*	27.1 (7.3; 24.6- 29)	29.2 (7; 25.4- 30.2)	28.8 (6.4; 29.1-31.5)	0.264
Age of asthma diagnosis (years)#	6.0 (1-60)	18 (1-69)	19 (1-62)	0.528
Asthma duration (years)*	37.84 (23.4; 12.6-58.5)	28.68 (21; 13.6-28.1)	26.9 (19.9; 20.5-27.8)	0.014
GINA ≥3 (n, %)	34 (100%)	65 (91.5%)	294 (90.2%)	0.586
Maintenance oral steroids (n, %)	9 (26.5%)	25 (35.2%)	109 (33.4%)	0.586
ACQ6 score*	1.87 (1.1; 0.7- 2.7)	2.15 (1.4; 1.3- 2.5)	2.31 (1.3; 2- 2.5)	0.512
Atopy (n, %)	19 (55.9%)	35 (49.3%)	175 (52.8%)	0.755
<u>Markers of airway inflammation</u>				

Blood eosinophil count (x10⁹/L)*	1.28 (0.9; 0.7-2)	0.98 (1; 0.8-1.7)	2.31 (1.4; 0.8-1.1)	0.419
Sputum neutrophils (%)*	80.37 (21.5; 52.7-99.4)	53.58 (31.3; 41.2-68.1)	75.73 (22.1; 74.6-82.1)	<0.001
Sputum eosinophils (%)#	8.25 (1.25-40.5)	58.25 (26.5-93.75)	4.25 (0-31)	<0.001

Fungal biomarkers

Total IgE (kU/L)#	4669 (2023-5000)	279 (42-1791)	436 (32-5000)	<0.001
sIgE <i>A. fumigatus</i> (kUA/L)#	66.05 (27.8-100)	0.22 (0.01-24.9)	0.49 (0.02-21.6)	<0.001
sIgG <i>A. fumigatus</i> (mg/L)#	63.7 (12.3-200)	55.5 (5.4-200)	21.4 (4.1-87.4)	<0.001
sIgE <i>P. chrysogenum</i> (kUA/L)#	9.47 (1.6-30.7)	0.1 (0-4.7)	0.17 (0-6.09)	<0.001
sIgE <i>C. albicans</i> (kUA/L)#	5.44 (0.72-43.6)	0.24 (0.01-3.82)	0.45 (0-9.57)	<0.001
sIgE <i>A. alternata</i> (kUA/L)#	3.85 (0.22-23.75)	0.3 (0.03-8.64)	0.29 (0.02-8.2)	<0.001
sIgE <i>C. herbarum</i> (kUA/L)#	3.1 (0.31-13.74)	0.14 (0.01-4.98)	0.24 (0-16.2)	<0.001
Sensitised to fungi (n, %)	33 (97.1%)	48 (67.6%)	248 (76.1%)	0.004

Cases ABPA & SAFS

ABPA (ISHAM criteria)(n(%))	27 (79.4%)	7 (9.9%)	29 (8.9%)	<0.001
SAFS (n (%))	0	13 (18.3%)	82 (25.2%)	<0.001

Lung function

PBFEV₁ (% predicted)*	67.04 (23.1; 40.5-88.2)	76.14 (23; 64.3-85.8)	77.25 (22.3; 69.7-77.1)	0.043
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Radiological

<u>abnormalities (n, %)</u>				
Bronchiectasis	23 (79.3%)	17 (35.1%)	89 (40.1%)	<0.001
Tree-in-bud	10 (34.5%)	7 (14.6%)	24 (10.8%)	0.002
Bronchial wall thickening	16 (55.2%)	18 (37.5%)	106 (47.7%)	0.278
Mucoid impaction	9 (31%)	12 (25%)	36 (16.2%)	0.084
Collapse/consolidation	14 (48.3%)	19 (39.6%)	60 (27%)	0.026
Air trapping	11 (37.9%)	15 (31.2%)	56 (25.2%)	0.286
Fleeting shadows	6 (20.7%)	1 (2.1%)	3 (1.3%)	<0.001
Fibrosis	7 (24.1%)	5 (10.4%)	17 (7.7%)	0.018

Figure 4.1. a. Post-bronchodilator FEV₁ (% predicted (means; SD, 95%CI)) compared between different sensitisation patterns. Those sensitised to *A. fumigatus* and *P.chrysogenum* (group 1; mean FEV₁ 73.04% (23.2; 70.2-76)) were found to have a lower FEV₁ compared to asthmatics not sensitised to fungi (group 4; mean FEV₁ 81.8% (21.5; 78.5-87.2), $p < 0.001$). Sensitisation to *C. albicans* (group 2; mean FEV₁ 77.03% (20.3; 71.2-82.9)) and sensitisation to *A. alternata* and *C. herbarum* (group 3; mean FEV₁ 85.2% (19.7; 76.7-93.7)) were not linked to a reduced FEV₁ compared to group 4 ($p > 0.05$).

b. Post-bronchodilator FEV₁ (% predicted (means; SD, 95%CI) in subjects grouped as being (i) atopic and sensitised to *A. fumigatus* (n=147), (ii) atopic but not sensitised to *A. fumigatus* (n=80), (iii) sensitised to *A. fumigatus* but not atopic (n=95), (iv) not sensitised to *A. fumigatus* nor atopic (n=104). Sensitisation to *A.fumigatus* (AF), but not atopy was associated with a significantly lower FEV₁ compared to those that were just atopic (71.9% (24; 67-77) vrs 83.7% (19.8; 79.1-88.2)), $p < 0.05$).

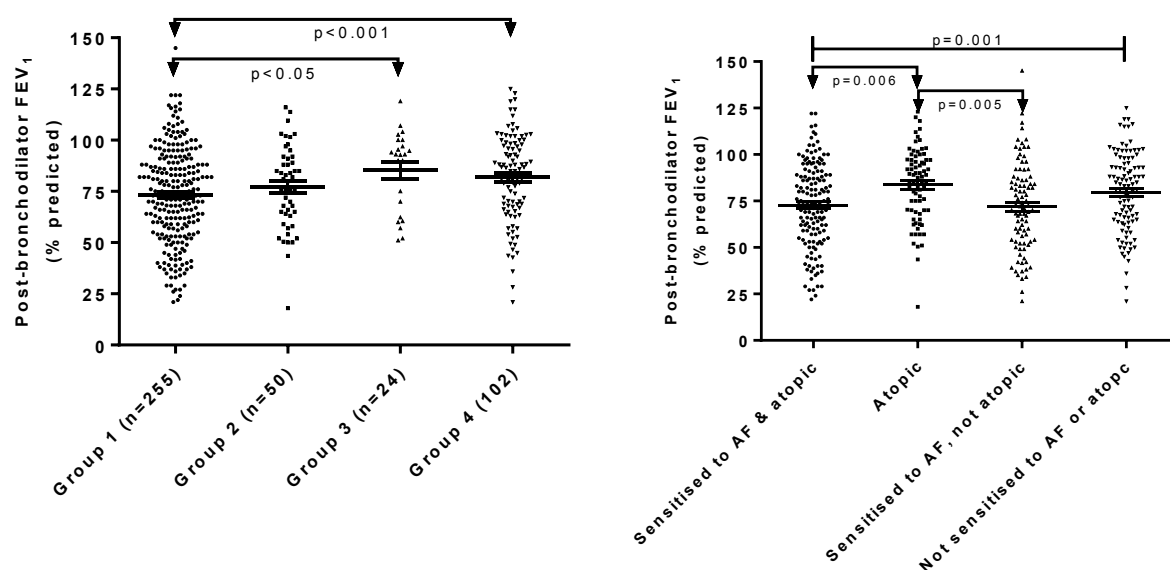


Figure 4.2 rAsp f 3 and rAsp f 4 in those with and without bronchiectasis. A raised level of rAsp f 3 (4.4 (0.81-12.9)KU/L vrs 0.34 (0.07-3.91)KU/L, $p=0.008$) and rAsp f 4 (0.47 (0.03-3.16)KU/L vrs 0.09 (0.01-0.35)KU/L, $p=0.033$) was associated with the presence of bronchiectasis. The current cut off value for rAsp f 3 and rAsp f 4 is 0.35 KU/L.

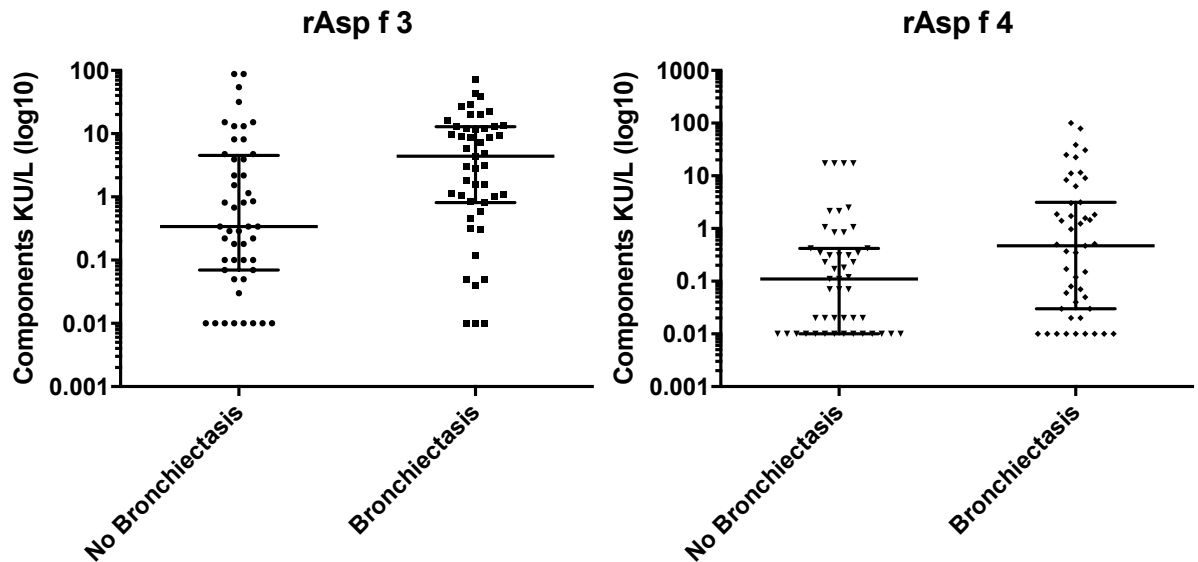
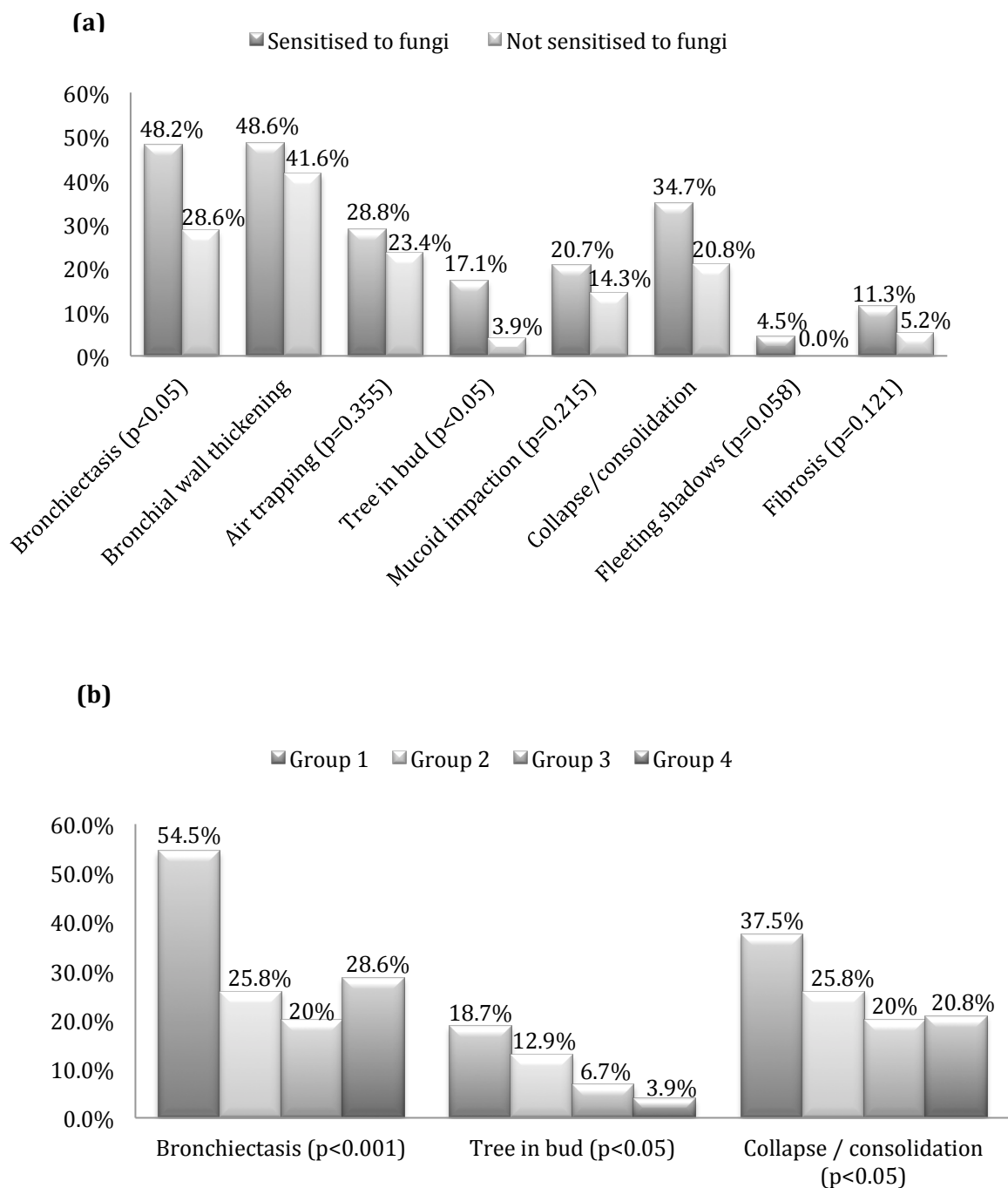


Figure 4.3 Frequency of radiological abnormalities in different sensitisation groups. An increased frequency of radiological abnormalities was seen (i) an asthma population sensitised to fungi compared to one that is not sensitised to fungi, and (ii) according to different sensitisation status (group 1 = sensitised to *A. fumigatus* and *P. chrysogenum*; group 2 = sensitised to *C. albicans*; group 3 = sensitised to non-thermotolerant fungi; group 4 = not sensitised to fungi).



Chapter 5: Airway remodelling in asthmatics sensitised to fungi (study 2)

5.1 Introduction

Airway remodelling affects both the large and small airways of asthmatics. It has been associated with disease severity and duration, airway hyperresponsiveness, airflow limitation and a reduction in lung function (193, 194). Features that characterise airway remodelling in asthma include: loss of epithelial integrity, subepithelial fibrosis, basement membrane thickening, goblet cell hyperplasia and increased airway smooth muscle mass (193).

There are many aspects to airway remodelling. All have been linked to different clinical features of asthma. The degree of epithelial shedding and basement membrane thickness is correlated with bronchial hyperresponsiveness (195). Asthma severity has been directly related to subepithelial fibrosis, thickening of the basement membrane, mucous gland hyperplasia and airway smooth muscle hypertrophy (136, 196-198).

Eosinophils, neutrophils and mast cells are known to have a key role in airway remodelling. The chemokines, cytokines and growth factors released from these cells, and the structural cells of the airway, are important drivers of this process. TGF- β is a profibrotic cytokine produced by a wide variety of structural and inflammatory cells (199, 200). Eosinophils are a major source of TGF- β in the asthmatic airway (199). TGF- β promotes fibroblast differentiation, proliferation of airway smooth muscle and expression of matrix metalloproteinases (200). Neutrophil production of matrix metalloproteinase-9 (MMP-9) in response to allergen exposure may also be implicated in the remodelling process (201). In addition, release of tryptase by activated mast cells induces fibroblast proliferation dependant upon the activation of protease-activated receptor-2 (PAR-2) (202). Thus, the presence of these cells in the asthmatic airway are important when evaluating patients that may be at risk of remodelling.

A number of studies have shown the importance of airway remodelling and its association with adverse outcomes in asthma (119, 136). There is an increasing need to identify the clinical and inflammatory phenotypes of asthma, as well as

those that are affected by remodelling changes so that treatment (particularly immunomodulatory ones) can be targeted more effectively (203).

Sensitisation to *A. fumigatus* is associated with a reduction in FEV₁(52). This has been reinforced by this body of work, which also suggests that this is limited to the thermotolerant filamentous fungi and may be linked to lung function decline irrespective of atopic status and the other parameters used to diagnose ABPA. It is, however, unclear at present whether asthmatics that are sensitised to thermotolerant filamentous fungi, yeasts or non-thermotolerant fungi are more at risk to a greater degree of airway remodelling compared to non-sensitised individuals. It is also unclear whether polysensitisation and atopy has an effect on airway remodelling. The aim of this study was to evaluate whether airway remodelling occurs to a greater degree in those sensitised to fungi, and whether this is affected by sensitisation pattern, polysensitisation and atopy.

5.2 Methods

Biopsy specimens were analysed from the “Establishing what relationship exists between fungal colonisation and allergy and the clinical, radiological and pathological characteristics of lung disease” (Research Ethics Committee (REC) approval reference: 14/WM/1099) and from “Gene expression profiling in mild and severe asthma, eosinophilic bronchitis and normal controls” (REC reference: 04/Q2502/74). Demographic details (age, gender, BMI) and clinical data (age of asthma diagnosis, asthma duration, GINA status and sputum cell counts) were collected during a stable visit (six weeks post exacerbation). Spirometry was undertaken according to standardised methods by a trained physiologist using a dry bellows spirometer (Vitalograph Ltd, Maids Moreton, UK). Post-bronchodilator FEV₁ was recorded as percentage predicted. High Resolution computerised tomography (HRCT) scans were undertaken of the whole thorax (including both inspiratory and expiratory phases) using a Siemens Somaton Definition AS plus spiral scanner (Siemens Healthcare, Knoxville, TN). Scans were performed in a caudo-cranial direction using 1mm thickness slices at 0.7mm intervals. Healthy subjects did not complete the ACQ, nor were they asked to undergo a HRCT scan.

Subjects underwent a fiberoptic bronchoscopy under conscious sedation according to published guidelines (204). Biopsies were taken under direct vision from the proximal airways (from the segmental carina of the tertiary bronchi). Acetone fixation was used to embed the biopsies in GMA prior to cutting at 2µm. The DAKO EnVision™ FLEX system (DAKOK8023) method of staining was used with the following antibodies: (i) smooth muscle actin, (ii) mast cell tryptase, (iii) major basic protein, (iv) neutrophil elastase. IgG1 and antibody diluent were used as the negative controls. Two non-contiguous sections >10µm apart were used to map the different morphological areas (epithelium, smooth muscle and lamina propria). Nucleated mast cells, neutrophils and eosinophils were counted at x40 magnification in each of these areas and expressed per mm². Counts were taken to be valid and representative if the morphological area in the biopsy was greater than 0.1mm².

Reticular basement membrane thickness (RBMT) was measured on intact epithelium at x40 magnification. 50 measurements were taken 20µm apart and the average value of these and of the two non-contiguous sections were taken as the RBMT for that biopsy. The cohort was separated into groups based on those sensitised to a fungal panel and those not sensitised to fungi. A separate analysis of the differences in cell counts between the compartments and the presence of any morphological differences in reticular basement membrane thickness, percentage of intact epithelium and proportion of airway smooth muscle present was also analysed on the basis of sensitisation to different thermotolerant fungi: (i) thermotolerant filamentous fungi (*A. fumigatus*, *P. chrysogenum*), (ii) thermotolerant yeast (*C. albicans*), (iii) the non-thermotolerant fungi (*A. alternate*, *C. herbarum*) and (v) non-sensitised asthmatics. Repeat counts were performed at 30 days to assess intra-rater reliability. In addition, another independent scientist performed cell counts in order calculate Inter-rater reliability.

Statistical analysis

The Shapiro-Wilk test was used to assess the normality of data. Morphological data and cell counts were all shown to be non-parametric. Descriptive data are expressed as medians and inter-quartile ranges. Comparisons between two

groups were made using the Mann-Whitney U test, whilst the one-way analysis of variance Kuskall-Wallis test was used to compare multiple groups. The Bonferroni correction was used to adjust for multiple comparisons. The Spearman rank correlation method was used to assess whether the cell counts were associated with total IgE, specific IgE to thermotolerant and non-thermotolerant fungi. All significance tests were two-tailed, with a threshold P value of less than 0.05 considered to indicate statistical significance.

5.3 Results

Biopsy specimens were analysed from 9 healthy controls and 39 asthma patients. 21 of these asthma subjects were sensitised to fungi and 18 were not sensitised to fungi. These were matched for asthma severity, smoking exposure, BMI and gender (see table 5.1). No significant differences were seen between those sensitised to fungi and those not sensitised to fungi in the differential cell counts of induced sputum and in broncho-alveolar lavage samples ($p>0.05$; table 5.2).

The biopsy cell counts in each morphological area were verified independently and on separate occasions. The cell count intra-class correlation coefficient for the intra-rater reliability was 0.905 (95%CI 0.816-0.948; $p<0.001$). The inter-rater reliability was 0.849 (95% CI 0.773-0.899, $p<0.001$).

The overall median area and interquartile range for each of the morphological areas were as follows: 0.19 (0.12-0.37) mm² of intact airway epithelium, 0.26 (0.11-0.39) mm² of airway smooth muscle and 1.25 (0.79-1.6) mm² of lamina propria present. Counts were used from each of the morphological areas where the total area in the biopsy was $>0.1\text{mm}^2$. 21 asthmatics (11 that were sensitised to fungi and 10 not sensitised to fungi) had an intact area of epithelium $>0.1\text{mm}^2$ and 36 asthmatics (19 sensitised to fungi, 17 not sensitised to fungi) had biopsies with $>0.1\text{mm}^2$ of airway smooth muscle present. The total area of lamina propria was $>0.1\text{mm}^2$ in all of the biopsies.

Morphological analysis of these biopsies found no statistical differences between those sensitised to fungi and those not sensitised to fungi in terms of reticular basement membrane thickness, proportion of intact epithelium and percentage of airway smooth muscle present (table 5.3).

The number of nucleated mast cells were increased in both the epithelial compartment and the lamina propria (submucosa) in those sensitised to fungi compared to asthmatics not sensitised to fungi (17.86 (7.8-34.6)/mm² compared to 0 (0-2.3)/mm² in the epithelium and 10.92 (7.02-20.75)/mm² in the lamina propria compared to 7.37 (5.62-11.05)/mm² in the lamina propria of non-sensitised asthmatics, $p < 0.05$; figure 5.1). There were no significant differences in the median number of mast cells per mm² of airway smooth muscle between the groups (2.73 (0-6.2)/mm² of airway smooth muscle in those sensitised to fungi compared to 0 (0-2.24)/mm² of airway smooth muscle in asthmatics not sensitised to fungi, $p > 0.05$), nor were there any significant differences in eosinophil or neutrophil counts in the airway epithelium, airway smooth muscle or the lamina propria between these groups ($p > 0.05$, table 5.3).

There were no statistical difference between the number of mast cells, eosinophils or neutrophil per mm² of epithelium, airway smooth muscle or in the lamina propria when the cohort was separated by sensitisation to the different thermotolerant fungi (thermotolerant filamentous fungi (*A. fumigatus*, *P. chrysogenum*), versus the thermotolerant yeast (*C. albicans*)) compared to non-sensitised asthmatics ($p > 0.05$). No individuals in the cohort were sensitised only to non-thermotolerant fungi (*A. alternate*, *C. herbarum*). There was also no difference in reticular basement membrane thickness, proportion of intact epithelium or percentage of airway smooth muscle that reached statistical significance between any of these groups ($p > 0.05$). In addition, when a diagnosis of ABPA was taken into consideration, no statistical differences were seen in any of the remodelling parameters measured nor in the inflammatory cell counts in the different compartments ($p > 0.05$).

There were no statistical differences in inflammatory cell counts nor with regards to RBMT, percentage of intact epithelium and proportion of airway smooth muscle present in asthmatics that were atopic compared to those that were not atopic ($p > 0.05$). In addition, there no statistical differences seen in the morphological areas or in the cell counts in the different compartments when polysensitisation was taken into account ($p > 0.05$).

A moderate correlation was seen between the number of mast cells per mm² of intact respiratory epithelium and total IgE (Spearman's correlation coefficient (r_s) = 0.636, $p < 0.05$), as well as specific IgE to *A. fumigatus* (r_s = 0.675, $p < 0.05$). Weak correlations were seen between total IgE and mast cells per mm² of lamina propria (r_s = 0.409, $p < 0.05$); specific IgE to *A. fumigatus* and neutrophils per mm² of epithelium (r_s = 0.48, $p < 0.05$), mast cells per mm² of lamina propria (r_s = 0.414, $p < 0.05$) and eosinophils per mm² of lamina propria (r_s = 0.381, $p < 0.05$). A weak correlation was also seen between total IgE and reticular basement membrane thickness (r_s = 0.379, $p < 0.05$). No significant correlations were observed with regards to percentage of intact epithelium and percentage of airway smooth muscle present and total IgE, IgE to *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Candida albicans*, *Alternaria alternate* and *Cladosporium herbarum*.

Several significant associations were seen between sputum and BAL cell counts and the inflammatory and remodelling parameters measured. A moderate positive correlation existed between the percentage of intact epithelium present and BAL eosinophils (r_s =0.536, $p=0.039$); the number of mast cells present in the epithelium and the peripheral blood eosinophil count (r_s =0.518; $p=0.048$). Further weak correlations were seen in the percentage of airway smooth muscle present and sputum eosinophils (r_s =0.43; $p=0.025$); sputum eosinophils and mast cells in the lamina propria (r_s =0.372; $p=0.028$), in addition to a weak correlation between the number of mast cells in the lamina propria and the peripheral blood eosinophil count (r_s =0.372; $p=0.028$).

No significant correlations were seen between eosinophils, neutrophils and mast cells in the lamina propria compared to BMI, asthma duration, inhaled corticosteroid dose, post-bronchodilator FEV₁ or methacholine challenge test results in asthmatics sensitised to fungi compared to non-sensitised. Mast cell numbers in the lamina propria were seen to moderately correlate with ACQ 6 scores (correlation coefficient 0.591, $p < 0.05$), although neutrophils and eosinophils did not. No significant correlations were seen with annual exacerbation rates or RBMT and these variables.

A significant association was seen between some of the radiological abnormalities (bronchiectasis, tree in bud, bronchial wall thickening, mucoid impaction, collapse/consolidation, air trapping, fleeting shadows and fibrosis) and the markers of remodelling and inflammatory cell influx measured by immunohistochemistry. Bronchial wall thickening was associated with the percentage of intact epithelium present ($p=0.009$), the numbers of mast cells present within the airway smooth muscle was associated with the presence of collapse/consolidation ($p=0.048$) and air trapping was associated with the number of mast cells present in the lamina propria ($p=0.04$).

5.4 Discussion

The results of this study suggest an association between mast cell infiltration in the epithelium and lamina propria and fungal sensitisation in asthma.

EPITHELIUM

Epithelial desquamation is known characteristic of chronic asthma. It is, however, unknown whether this is a specific feature of certain asthma phenotypes or more common in patients sensitised to fungi. Studies to date have looked at murine models sensitised to *A. fumigatus*. These have demonstrated epithelial shedding, an increased epithelial thickness and an increase in EGFR expression in response to live challenges with conidia to be features of chronic exposure to *A. fumigatus* (205, 206). In addition, Kauffman et al found epithelial desquamation to be more frequent in individuals exposed to the proteases of *A. fumigatus* and *A. alternata*. These proteases were also able to induce the production of the pro-inflammatory cytokines IL-6 and IL-8 from airway derived epithelial cell lines (207). However, in the biopsies of the cohort of asthmatics studied no difference was found in the percentage of intact epithelium present between those sensitised to fungi and asthmatics not sensitised to fungi. In addition there was no statistical difference between asthmatics sensitised to *A. fumigatus*, those with ABPA (although only two patients fitted the ISHAM diagnostic criteria for ABPA) and non-sensitised asthmatics, nor was there a correlation between the percentage of intact epithelium and specific IgE to *A. fumigatus*. An association was, however, found between specific IgE to *A. fumigatus* and total IgE with an increased number of

intraepithelial mast cells. A statistically significant increase in the number of mast cells within the epithelium was also found in those sensitised to fungi compared to those not sensitised to fungi. This would support a significant immunological relationship between exposure to fungi, in particular *A. fumigatus*, and the influx of mast cells.

An association between specific IgE to *A. fumigatus* and total serum IgE with the number of mast cells within the epithelium is supported by previous studies that have shown the vital role that monomeric IgE has in initiating and maintaining mast cell activation. Monomeric IgE was found to initiate calcium influx of cultured human lung mast cells (HLMC), with subsequent stimulation of histamine release, arachnidonic acid metabolism and cytokine synthesis in a dose dependent manner. This study also found evidence of persistent activation with continued exposure (208).

Epithelial cells express many factors that act to recruit, activate and prolong the survival of mast cells. Stem cell factor (SCF) is an important chemotactic factor for both mast cells and eosinophils. Epithelial expression of SCF is induced by IL-13 and is up-regulated in asthma (209). In addition, intraepithelial mast cell density is significantly increased in those with a 'Th2-high' asthma phenotype (210). Epithelial derived Thymic stromal lymphopoietin (TSLP) is induced by mast cells that have been activated by the FcεRI mechanism (211). Providing an important link between the epithelium and mast cell activation by specific IgE and the Th2 inflammatory process.

Mast cells are key effector cells of Th2 inflammation and their localisation in the airway is closely associated with their protease phenotype. The mast cells expressing tryptase and carboxypeptidase A3, but low levels of chymase, are found to localise to the epithelium, be associated with eosinophilic asthma and predict corticosteroid responsiveness (212, 213). This study's finding of an increased number of intraepithelial mast cells in patients sensitised to fungi only looked at tryptase positive mast cells and thus it was not possible to distinguish which mast cell phenotype was present in the population of cells identified. However, a moderate correlation was found between the peripheral blood eosinophil count

and mast cell numbers within the epithelium, as well as, a positive correlation between epithelial percentage and BAL eosinophilia, suggesting a link between mast cells, Th2 inflammatory phenotype, eosinophilic inflammation and epithelial integrity.

Mucus plugging, fleeting shadows (recurrent pulmonary infiltrates), tree-in-bud abnormalities, consolidation and segmental/lobar collapse are recognised radiological features of ABPA that have been attributed to excess mucus production (59, 214, 215). The mast cell mediators: histamine, prostanoids and leukotrienes are known to induce mucus secretion (216). IL-13 stimulates MUC5AC protein secretion and has been linked to goblet cell hyperplasia, whilst selective blockade of trypsin has been shown to reduce mucus secretion, goblet cell hyperplasia and eosinophilic airway inflammation in a mouse model (217, 218). In addition, a link between amphiregulin expression on mast cells via activation of FcεRI is known to induce epithelial mucin gene expression and has also been linked to mucus hypersecretion (219). Significant correlations have also previously been made between mucus plugging and mast cell density in mucous glands within the epithelium in post-mortem studies (220). This would suggest a significant link between intraepithelial mast cell numbers and mucous production. Thus, although the presence of mucous glands was not examined in this study, the increased numbers of intraepithelial mast cells is significant in this cohort of asthmatics that are sensitised to fungi and may describe a distinct clinical phenotype. This warrants further study.

Lamina propria

Mast cells are increased in the lamina propria of asthmatics and whilst their numbers have been shown to reduce with corticosteroids treatment, it is still higher in those with severe asthma (212, 221). Thus the presence of mast cells is not surprising and their increased number in the asthmatic cohort sensitised to fungi compared to those not sensitised is in keeping with an association between IgE-bound mast cells, eosinophilic inflammation and a Th2 process.

The two populations were well matched in terms of inhaled and oral corticosteroid dose, therefore, corticosteroid exposure did not account for the differences in the

number of mast cells in the lamina propria. This would suggest that the differences in mast cell populations are specific to the underlying pathological process.

The association between mast cells in the lamina propria and higher ACQ6 scores may be explained by the autocrine mediators (leukotriene C4, prostaglandin D2 and histamine) released by mast cells. These mediators are known to cause mucosal oedema and bronchoconstriction, which may cause higher ACQ6 scores.

Airway Smooth Muscle

Mast cell infiltration of the airway smooth muscle is known to be associated with bronchial hyperresponsiveness and asthma severity (137). This is described as being a common feature of all asthma phenotypes (216). However, mast cell numbers were not increased in the airway smooth muscle of asthmatics sensitised to fungi compared to those not sensitised and not increased in asthmatics overall compared to healthy controls ($p=0.094$). This may be a reflection of the cohort's exposure to both inhaled and systemic corticosteroids suppressing mast cell localisation within the airway smooth muscle. Mast cell sputum signature gene expression have shown a reduction in mast cell biomarkers in patients treated with corticosteroids and this effect may explain the low numbers of mast cells that we encountered in the airway smooth muscle (222).

Conclusion

The immunopathological features of asthma have been the focus of much attention in trying to delineate the different phenotypes of asthma. This study gives a preliminary overview of the key findings that are more specific to asthmatics that are sensitised to fungi. The associations that this study has found, suggest that mast cell recruitment and activation is an area of study that warrants further investigation. Mast cells interact with many of the cells involved in both the inflammatory and the remodelling process including epithelial cells, dendritic cells, naïve T cells, B cells and fibroblasts. In particular their location, phenotype and density are linked to disease severity and may have implications for targeting treatments to different clinical phenotypes (216).

This study did not show any evidence of tissue infiltration by eosinophils or neutrophils in any of the compartments. The majority of asthmatics were on high dose inhaled corticosteroid or regular systemic corticosteroid therapy. This may account for the low numbers of tissue eosinophils. The subjects were also biopsied whilst their asthma was stable (at least 6 weeks post exacerbation) and this may account for the lower numbers of neutrophils than was expected, given the results of previous murine studies (see below).

Human studies have consistently shown subepithelial fibrosis, epithelial shedding/desquamation, and an increase in both airway smooth muscle mass (ASM) and reticular basement membrane thickness (RBMT) to be key features of remodelling that occur in asthma (223). Clinical findings have demonstrated asthma severity and fixed airflow obstruction to be linked to an increase in airway smooth muscle mass and subepithelial fibrosis with treatments such as bronchial thermoplasty and monoclonal antibodies currently under evaluation to target these areas and improve disease control (224).

This study analysed the proportion of intact airway epithelium, the percentage of airway smooth muscle present and the thickness of the reticular basement membrane to evaluate whether asthmatics that were sensitised to fungi had a greater degree of airway remodelling. This revealed no evidence of remodelling, based on the parameters studied, in the biopsies of asthmatics studied. The cohort was well matched in terms of asthma severity and this could account for these results. However, increased collagen deposition and goblet cell metaplasia are recognised features of airway remodelling in murine models of fungal asthma (225). Indeed, several murine models of allergic fungal asthma (which have undergone chronic inhalation of *Aspergillus conida* to mimic ubiquitous exposure) have looked at airway responses, immunological mechanisms and evidence of remodelling in this subtype of asthma. These studies have revealed a mixed (protease driven) Th1 and (allergen induced) Th2 response (226). The presence of both an eosinophilia and an influx of neutrophils into the airways have also been found, in addition to goblet cell metaplasia and an upregulation of EGFR expression by epithelial cells (205, 206). A significant disadvantage of this study is that these features of airway remodelling were not examined in the biopsies. It

thus may be that there are elements of remodelling present, other than the ones studied, which are more characteristic of this asthma phenotype and more specific to asthmatics that are sensitised to fungi. This requires further investigation. If these changes are present in this subtype of asthma (as they are in murine models) this may give more weight to the argument that this entity of asthma does in fact describe a distinct clinical phenotype and also a specific immunopathological endotype.

Another aspect of the immunopathology of asthma that requires further study is the impact different proteases have on the epithelial barrier and the influx of inflammatory cells. This study did not look at this aspect, but the lack of a change in epithelial integrity may reflect different exposure levels to different fungi and their proteases. An analysis of *A. fumigatus* proteases Asp F5 and Asp F13 showed significant airway hyperreactivity and an increase in airway inflammation. However, it seems that epithelial denudation and induction of areas of hyperplasia are a result of the interplay of several of the *aspergillus* proteases, as these changes were more evident in the wild type murine model rather than those exposed to single proteases (32). This requires further mechanistic studies.

The lack of associations found with clinical endpoints such as exacerbation rates, reductions in lung function and the frequency of radiological abnormalities may be due to the different populations of mast cells present and also their state of activation. This study did not examine which phenotypes of mast cells were present nor whether they were in a state of activation. This needs further evaluation.

There are flaws with immunohistochemistry that could have also influenced the results of this study. Although the pathological role of the epithelium is well described in asthma (227, 228), assessing the degree of epithelial shedding in endobronchial biopsies may not be a sensitive measure of it. The loss of epithelial integrity has been questioned. It has been questioned whether this is a feature of biopsy size and quality rather than a pathological hallmark of airway remodelling in asthma (229). Thus there are many factors that could have influenced the

results of this study and further follow-on studies are required before any definitive conclusions can be made.

Summary statement

The immunopathology of allergic asthma driven by fungi is complex and multifaceted. The prominence of mast cells in the epithelium and lamina propria is an interesting finding that requires further study. This study did not show any evidence of remodelling in those sensitised to fungi compared to those not sensitised to fungi and this may be for several reasons, as already discussed. Further studies are required to fully examine all of the aspects of remodelling in these patients to evaluate whether there are specific differences in this subset of asthmatics. Thus, whilst murine models have demonstrated evidence of an activated epithelium, collagen deposition and goblet cell hyperplasia, this is yet to be proven in human studies.

Figures and tables

Table 5.1 Clinical characteristics of the cohort, where biopsy specimens were obtained. *mean (95%CI), **median (interquartile range)

	NFS (n=18)	FS (n=21)	Control (n=9)	P value
Age*	52.2 (45.5-59)	46.8 (40.7-54.9)	46.7 (34.2-59.2)	0.582
Gender (% male)	58.3	50	75	0.638
BMI*	30.65 (28.3-33)	28.44 (26.2-30.6)	25.54 (20.4-30.7)	0.07
Smokers (%)	9.1	5.3	1.1	0.227
Pack year history**	11 (6-19)	12.5 (10-15)	9 (3.7-14)	0.867
GINA score (%≥3)	100	94.7	0	0.573

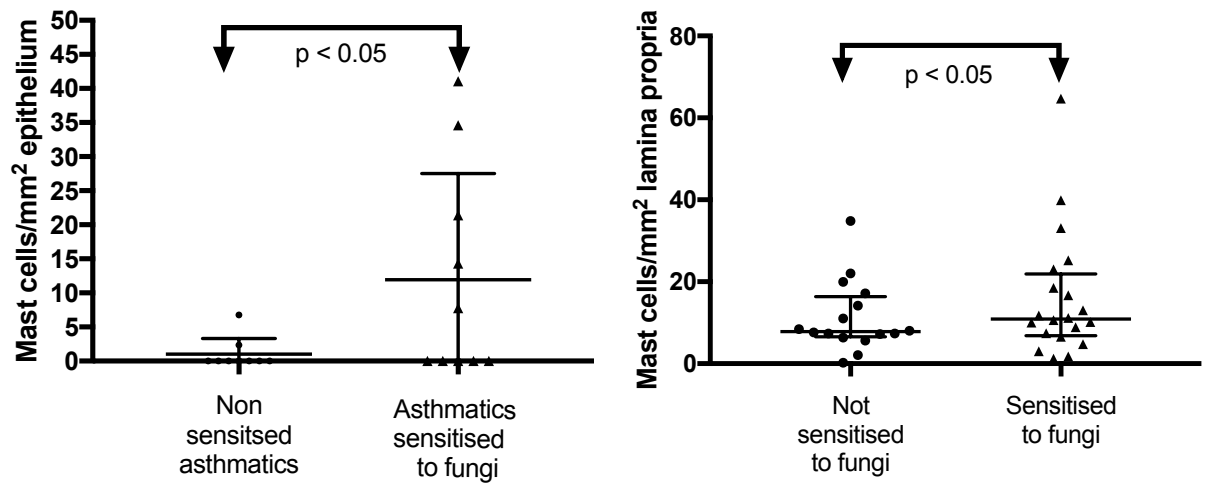
Table 5.2 Peripheral blood eosinophil count, induced sputum and broncho-alveolar lavage (BAL) differential cell counts in those sensitised to fungi compared to those not sensitised to fungi. *mean (95%CI), **median (interquartile range).

	NFS (n=18)	FS (n=21)	P value
Peripheral blood eosinophil count (x10⁻⁹/L)**	0.32 (0.18-0.6)	0.36 (0.21-0.56)	0.076
Sputum eosinophils (%)**	2.5 (0.25-14.5)	6 (0.5-14.75)	0.309
Sputum neutrophils (%)**	74.7 (66.5-84.5)	61.06 (45.8-87.25)	0.573
BAL eosinophils (%)**	4.6 (1.75-6)	1.75 (0.75-3.25)	0.081
BAL neutrophils (%)**	14.55 (8.5-73)	22 (13.5-48.5)	0.818
BAL lymphocytes (%)*	2.86 (0.6-5.1)	1.25 (0.3-2.2)	0.123

Table 5.3 Comparison of inflammatory cell counts and remodelling changes in asthmatics sensitised to fungi (FS group), compared to asthmatics not sensitised to fungi (NFS group). Values reported as medians (interquartile range).

	NFS (n=18)	FS (n=21)	Control (n=9)	P value
Reticular Basement Membrane Thickness (μm)	9.83 (8.07-11.73)	10.59 (9.42-11.93)	8.26 (7.7-9.09)	0.068
Percentage of intact epithelial area (%)	12.26 (10.29-23.53)	10.54 (7.03-14.91)	9.48 (5.11-16.52)	0.471
Percentage ASM area (%)	10.14 (6.88-16.54)	16.81 (7.82-24.66)	4.4 (1.59-10.65)	0.225
Mast cells/mm^2 epithelium	0 (0-2.36)	17.86 (7.78-34.62)	0 (0-4.24)	0.046
Mast cells/mm^2 ASM	0 (0-2.24)	2.73 (0-6.2)	0 (0-0)	0.11
Mast cells/mm^2 submucosa	7.37 (5.62-11.05)	10.92 (7.02-20.75)	2.03 (0.85-4.67)	0.014
Eosinophils/mm^2 epithelium	0 (0-0)	0 (0-0)	0 (0-0)	1
Eosinophils/mm^2 ASM	0 (0-0)	0 (0-0)	0 (0-0)	1
Eosinophils/mm^2 submucosa	5.81 (1.04-11.43)	5.1 (2.45-12.59)	0.73 (0.32-4.16)	0.234
Neutrophils/mm^2 epithelium	0 (0-0)	0.78 (0-5.35)	0 (0-0.82)	0.49
Neutrophils/mm^2 ASM	0 (0-0)	0 (0-0)	0 (0-0)	1
Neutrophils/mm^2 submucosa	7.65 (2.81-17.33)	9.21 (2.97-15.15)	1.85 (1.77-5.93)	0.23

Figure 5.1 Mast cell numbers per mm² of epithelium and lamina propria in asthmatics sensitised and not sensitised to fungi.



Chapter 6: Description of the mycobiome in asthma (study 3).

6.1 Introduction

Molecular techniques such as high throughput sequencing (HTS) have revealed the presence and importance of non-cultivable organisms (230). These technologies have allowed the microbiomes of different niches in the human body to be mapped, revealing important links between differences in the microbiome, the development of allergy and disease states (231). This has been done extensively with the bacterial microbiome throughout mucosal surfaces, especially in the gastrointestinal tract (231, 232). However, limited information is available regarding the fungal part of the microbiome – the mycobiome. Previous chapters in this thesis have examined whether the immunological, physiological, radiological and histological end-points, including those involved in the various diagnostic criteria, used to diagnose and classify fungal sensitisation have any link to adverse features in asthma. Immunohistochemistry has been used to elucidate whether there are any specific features of remodelling and inflammatory cell infiltrate that are specific to asthmatics sensitised to fungi. This chapter will now examine how the fungal microbiome differs throughout the respiratory tract and whether there are any differences in the mycobiome specific to asthma and whether there are any aspects of the mycobiome associated with adverse clinical features.

Fungi activate both the adaptive and the innate immune system. Chitins, glucans and mannans act as pathogen-associated molecular patterns (PAMPS) for the innate immune system, activating the epithelium and the inflammatory cascade (233). In addition, fungi are known triggers of atopic asthma and are linked to a reduction in lung function in IgE sensitised asthmatics (52). Thus eliciting the constituents of the lung mycobiome are critical in understanding the pathogenicity of fungi in disease, their role in immune modulation and also in maintaining lung health. The advent of high throughput sequencing has now enabled the diversity of the mycobiome to be investigated in some detail.

Culture

Culture methods for fungi are not standardised, often insensitive and biased towards faster growing fungi that grow well on specific culture mediums, although their importance remains in confirming strains and susceptibility testing towards antifungals (234). The national standard for mycology culture was (developed by the health protection authority (HPA) (policy number BSOP57)) not optimised specifically for fungal growth and has a yield of <10% (235). However, significant improvements in yield have been seen with recent developments in methodology. The use of higher volumes of undiluted respiratory specimens (as seen with the Mycology Reference Centre Manchester (MRCM) method) have improved yield (specifically of *A. fumigatus*) compared to standard methodology, although this still only ranges from 20-75% for sputum, bronchial aspirates and bronchoalveolar lavage (BAL) samples in an aspergillosis population (236). Increasing the inoculation quantity, in addition to inoculating extracted sputum plugs (rather the use of whole saliva) directly onto potato dextrose agar plates containing chloramphenicol/gentamicin/fluconazole (PGCF) rather than Sabouraud dextrose agar (SDA) containing just chloramphenicol has also improved yield (100). However, even with these improvements in yield, the clinical relevance of isolating specific fungi from culture is still questioned. The isolation of filamentous fungi and its association with a reduction in lung function may be a reflection of colonisation in severe disease rather than an effect of colonisation in itself (14, 234). It is currently not known whether fungi, not able to be grown on culture medium, have an unrecognised role in disease pathogenicity. It is, therefore, difficult to gain a true understanding of the role fungi have in asthma with the use of culture alone.

Molecular technology

Nucleic acid amplification tests have been used to increase specificity and yield. Current European guidelines recommend the use of quantitative polymerase chain reaction (qPCR) in conjunction with microscopy, histopathology and culture for the diagnosis of *Aspergillus* disease and pulmonary invasive aspergillosis (237). However, qPCR tests have been shown to have superior sensitivity and specificity over that of culture for the diagnosis of fungal disease (238). Denis et al demonstrated 100% sensitivity, a specificity of 81% with BioEvolution PCR and a

specificity of 71% with MycoGENIE PCR in detecting *A. fumigatus* (239). In addition, Grancini et al showed 90% sensitivity and 97% specificity with the use of qPCR (240). Many different qPCR protocols have been used, mainly for diagnosing invasive fungal infection in immunosuppressed individuals, where the likely pathogen is known, is cultivatable and species-specific primers are chosen. However, there are limitations with qPCR in assessing fungal burden and prevalence. For us to understand the role of fungi in humans we need techniques that enable us to map the fungal mycobiome of a healthy lung and compare this to the changes that occur in diseased states. Mapping the lung's mycobiome requires molecular technology to identify both cultivatable and non-cultivatable organisms. The development of "next-generation" sequencing, and HTS technology, allows us to do this. HTS produces millions of sequences concurrently through sequencing multiple sites in parallel (241). This clonal amplification followed by cyclical rounds of parallel sequencing reduces time and cost (242).

Limitations due to cost, mostly due to the older Sanger DNA sequencing platforms have gradually been overcome with the development of new platforms that are more efficient and cost effective, enabling more widespread use of the technology (242).

The use of HTS has enabled the bacterial diversity of microbiomes in different niches to be characterised (231). The bacterial microbiome of the lung has been studied in some detail, with increasing importance being shown of the relationship between immune tolerance, airway inflammation and microbial dysbiosis in the lung (243). Examination of the fungal microbiome lags behind that of bacteria. Fungi are present in lower abundance compared to bacteria and are more sensitive to DNA isolation methods, leading to lower recovery rates (244, 245). Thus, approaches that lead to a higher quality of sequence data are preferred. A dual-indexing approach, similar to that used for 16S rRNA bacterial gene sequencing, on the Illumina MiSeq platform was chosen in this study to avoid problems with low sequence diversity (246).

The Illumina MiSeq HTS platform produces the largest number of reads following quality filtering compared to Ion Torrent PGM, and Roche 454 GS FLX Titanium

platforms during analysis of the gut microbiome, and allowed for a greater depth of coverage and greater alpha diversity during sputum analysis of cystic fibrosis patients using 16S sequences (247, 248). In addition, the Illumina MiSeq platform has been shown to overcome problems with low sequence diversity and is cost-effective in comparison to other platforms (246). Quality filtering is important during sequence data analysis to remove erroneous reads that would overestimate diversity, especially when using the Illumina MiSeq platform (249). Bioinformatics processing with the Quantitative Insights Into Microbial Ecology (QIIME) pipeline, and the use of published guidelines, ensures sufficient quality processing to enable higher-quality sequence reads, a decrease in the Operational Taxonomic Unit (OTU) threshold and an increase in both alpha and beta diversity (248, 249).

Fungal sequence databases have been generated using primers that amplify the common internal transcribed (ITS) region of the nuclear ribosomal repeat unit: spanning ITS-1, 5.8S and ITS-2 (250). This locus, between the small rRNA subunit of 18S and the large rRNA subunit of 28s, has been used to generate universal primers for amplicon sequencing. The ITS region was chosen due to its high level of preservation and synapomorphic variability (an area unique to a particular phyla and sometimes species specific) between fungi (251). This region also has a more specific barcode gap between inter- and intraspecific variation for fungal identification (250). In addition, HTS protocols are able to sequence these amplicons (ITS1, ITS2) that identify fungi and allow taxonomic classification (252).

The microbiome and the mycobiome

Niche-specific microbiota have been of great interest in asthma. Identification of the different phenotypes of asthma have recognised those with an infective phenotype to respond better to targeted antibiotic therapy, although this has been disputed by other studies (253, 254). Studies looking at the bacterial microbiome in asthma have shown differences between health and disease, but it may also be the case that it also differs by asthma phenotype. HTS on induced sputum samples targeting the 16s rRNA sequences revealed greater bacterial diversity and a predominance of Proteobacteria (which include the known pathogenic bacteria: *Haemophilus*, *Neisseria* and *Moraxella*) in asthma compared to a predominance of

Bacteroides in healthy controls (255). The composition of the bacterial microbiome also varies in asthmatics that are atopic, have increased airway hyperresponsiveness, in severe asthma and following inhaled corticosteroid treatment (256-258). Oral corticosteroid (OCS) dose has been shown to affect relative abundance of bacteria within the microbiome, with a decrease in Bacteroidetes and Fusobacteria and an increase in Proteobacteria associated with OCS use in asthmatics (259). In addition, a significant decrease in the relative abundance of Firmicutes, Bacteroides and Actinobacteria has been demonstrated in asthmatics with a lower FEV₁, as well as a reduction in alpha diversity and an eosinophilic phenotype (259). The composition of the airway bacterial microbiome has not, however, been associated with neutrophilic airway inflammation (260). Thus, a dysbiosis has been demonstrated in the microbial composition of the lung in asthma. The understanding of the mycobiome, in comparison to that of the bacterial microbiome, is much less complete so it therefore follows that a dysbiosis in the mycobiome may also exist.

Previously fungi (such as *Cryptococcus neoformans*, *Pneumocystis* and *Candida*) were only thought to be pathogenic in the lung of immunocompromised individuals, whilst others (*Aspergillus*) were felt to represent colonisation as a result of environmental exposure and only become pathogenic in diseased states, such as in ABPA (261). The fungal component of the microbiome has to be recognised as a key component in health and disease and has started to be explored in much more detail, with most studies until recently focusing on the mycobiome of the gut. These studies have shown a fungal dysbiosis and an increase in fungal diversity to be associated with several diseases such as mucosal inflammation in inflammatory bowel disease (predominance of *Candida* spp., *Gibberella moniliformis*, *Alternaria brassicicola*, and *Cryptococcus neoformans*) and colorectal adenomas (102, 103). Studies examining the mycobiome of the lung have now emerged in asthma, cystic fibrosis (CF) and lung transplant recipients (104-106, 262, 263). In contrast to the gut mycobiome, a reduction in fungal diversity appears to be a common feature amongst diseased states in the lung and is also highly variable between individuals with the same disease states (263, 264).

Studies to date have shown the mycobiome of healthy individuals to be dominated by environmental fungi of the Ascomycota phyla, in particular those of the *Davidiellaceae* family including *Cladosporium cladosporoides*, *Eremothecium sinicaudum*, *Eurotium*, *Penicillium* and *Aspergillus*, as well as *Candida* (104, 106, 262). *Aspergillus* and *Penicillium* have been reported in asthmatics, healthy individuals and in cystic fibrosis (104, 262, 265). These environmental fungal genera seem to represent a core mycobiome that is unaffected by disease states. However, it is unclear as to whether the results of studies relying on spontaneous or induced sputum specimens represent a true picture of the lower airways or are biased due to contamination from the upper airways.

In cystic fibrosis a reduction in fungal diversity has been shown to be associated with disease severity and a decrease in lung function (104, 266). *Ascomycota* and *Basidiomycota* phyla predominate. *Saccharomycetes* and the polymorphic yeasts *Candida albicans* and *C. dubliniensis* dominated in sputum samples performed over several time points (267). However, this same study also showed a significant degree of change in the sputum fungal community over time, suggesting either a transient colonization of environmental fungi or differing degrees of environmental contamination. Willger et al showed *Candida* to be the most dominant (in abundance and prevalence) genus in all sputum samples analysed, with relative stability in the fungal mycobiota during and after completion of antibiotic treatment (105).

The use of culture independent molecular technology, and the subsequent expansion of genomic fungal databases, has revealed a diverse fungal community. Its contents in different biological niches are yet to be fully established, as is the role of fungi and the mycobiome in immune modulation and host inflammation in the lung. This study intends to examine the mycobiome in the different compartments of the lung to evaluate whether specimen type affects the composition of the mycobiome. It also intends to compare the mycobiome of severe asthmatics and healthy controls to explore whether the fungal load and the components of the mycobiome are associated with adverse clinical features (ACQ6 scores, reduction in postbronchodilator FEV₁, airway hyperresponsiveness, oral and inhaled corticosteroid dose), particular phenotypes of asthma, markers of

airway inflammation (FeNO, sputum cell counts) and immunohistochemical evidence of airway inflammation and remodelling (endobronchial biopsy).

Compartments

The different compartments of the lung have been felt to impact on the interpretation of culture results, particularly in invasive aspergillosis (IA). Previously, in the absence of histopathology, isolation in BAL was considered diagnostic with other respiratory samples felt to be either inferior with a lower sensitivity or more likely to represent colonisation, although recent studies using genotyping have contested this view (268). Studies of the bacterial microbiome suggest there are significant differences in the relative abundance and the beta diversity of the microbial communities in the different compartments of the lung, suggesting that this may be the same for fungi (269).

Mapping the mycobiome of the different compartments of the lung poses many challenges, particularly that of contamination from the upper to the lower airway during sampling. Thus it is also important to consider the oropharynx and nasopharynx when trying to establish the fungal mycobiome of the lower airway. The oral mycobiome diversity or composition has not been shown to alter according to oral health (270). *Candida* and *Aspergillus* were found to be the most abundant genera in healthy individuals with a median relative abundance of 21% and 44% respectively. Other common genera that were found in >80% were *Penicillium*, *Schizophyllum*, *Rhodotorula* and *Gibberella* (270). A previous study by Ghannoun et al found fifteen genera (including *Candida*, *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, *Aspergillus*, *Fusarium* and *Cryptococcus*) to be present in 20% of healthy individuals and represent the core mycobiome of the oral cavity (271). Charlson et al examined the mycobiome of the oropharynx and the lung in healthy individuals and lung transplant patients using ITS1/ITS2 primers. The oral wash ITS amplification contained environmental fungi, in addition to, *Candida* and *Aspergillus* and thus was very similar to that reported by Ghannoun et al; whereas the oral mycobiome of lung transplant patients was dominated by *Candida* (106). The yield from the Bronchoalveolar lavage (BAL) was minimal in healthy volunteers with few fungal reads comprising

environmental fungi (such as *Cladosporium*), which were also seen in the oral wash, and a low abundance of *Aspergillus* (106). Lung transplant recipients had high levels of ITS amplification, dominated by either *Candida* or *Aspergillus*. In cases where *Candida* was found in high abundance this was also found in the oral wash, whereas this was not the case for *Aspergillus* (106). This would, therefore, suggest that the mycobiome differs significantly between health and disease, particularly in the presence of immunosuppression and that there are significant differences between the mycobiome of the upper and lower airway. This study aimed to evaluate whether difference in the mycobiome existed between different airway compartments in asthma and healthy volunteers.

6.2 Methods

This chapter reports on a subset of asthmatics that were recruited to the mycobiome project. These were those that underwent a fiberoptic bronchoscopy, based on my involvement in their recruitment, characterisation and processing of the samples taken at bronchoscopy. The main aim of this aspect of the project was to examine the differences in the mycobiome between the different compartments of the lung.

Recruitment and clinical characterisation

Patients were recruited from tertiary asthma and fungal allergy clinics at Glenfield Hospital, Leicester U.K. Healthy volunteers were recruited from advertisement within the Glenfield Hospital and via the Leicester Respiratory Biomedical Research Unit website (<http://www.leicsrespiratorybru.nihr.ac.uk>). Demographic details (age, gender, BMI, smoking history), clinical data (age of asthma diagnosis, asthma duration, oral and inhaled corticosteroid therapy (beclometasone dipropionate hydrofluoralkane equivalent), ACQ6 scores, GINA status and sputum cell counts), spirometry (PBF_{EV1} (percentage predicted)), methacholine provocation test (PC₂₀) and fractional exhaled nitric oxide (FeNO) results were recorded.

Blood tests (blood eosinophils, total IgE, specific IgE: *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Candida albicans*, *Alternaria alternata*, *Cladosporium herbarum*) and skin prick testing (panel: *Aspergillus fumigatus*, *Penicillium*

chrysogenum, *Candida albicans*, *Alternaria alternata*, *Cladosporium herbarum*, *Dermatophagoides pteronyssinus*, dog, cat, grass pollen and tree pollen) was also performed. All of these results were obtained during a stable visit (minimum of six weeks post exacerbation). The National Health Service Research Ethics Committee approved the study (REACT IRAS 159074:CRN 159074 and ABPA UHL 10111) and informed written consent was obtained from all participants.

Sputum and bronchoscopic samples

Sputum plugs were separated from saliva, which was produced either spontaneously or was induced (as conducted by Pavord et al (172)). This was then homogenised at 37°C with 4x w/v 0.1% DL-dithiothreitol.

Subjects underwent bronchoscopy as previously described and according to the BTS guidelines (272). Samples were obtained in the following order: (i) bronchial wash (20mls of pre-warmed 0.9% saline) of middle lobe bronchus, (ii) bronchoalveolar lavage ((BAL) 120mls of 0.9% saline in 60ml aliquots) performed in the right lower lobe, (iii) protected brush sample taken from the right intermediate bronchus and (iv) endobronchial biopsy at the orifice of the anterior segment of the right lower lobe. The brush was placed in 5mL of Dulbecco's Phosphate-buffered saline and vortexed. The BAL sample was passed through sterile nylon gauze prior to centrifugation. All samples were centrifuged at 1500xg for 10 minutes. 100µl aliquots of the pellet were then used for DNA extraction.

DNA extraction was performed on 100µl aliquots of sputum homogenate, bronchial wash, BAL and protected brush samples. A modified protocol of the DNeasy plant mini kit (Qiagen, West Sussex, UK) including bead-beating was used to aid total fungal genomic DNA extraction (252).

Endbronchial biopsies were obtained, processed and analysed from all subjects undergoing a bronchoscopy as previously described (chapter 5).

Immunohistochemical markers of tissue inflammation (eosinophils, neutrophils and mast cells per mm² of epithelium, airway smooth muscle and lamina propria) and remodelling (percentage of intact airway epithelium, airway smooth muscle mass and reticular basement membrane thickness) were compared to the results

of the HTS data in those where an adequate tissue sample was obtained and who also had successful reads obtained from any of the compartments analysed.

Fungal DNA amplification & sequencing

Fungal DNA was amplified using primers for the fungal internal spacer region 2 (ITS2) of the nuclear ribosomal DNA (rDNA) operon. A dual index nested HTS method, using ITS3 (5'GCATCGATGAAGAACGCAGC 3') and ITS4 (5'TCCTCCGCTTATTGATATGC 3') primers, was used (180). In the first PCR round 20µl reaction mixtures were prepared, including 10µl of the KAPA HiFi HotStart Ready Mix (Kapa Biosystems, USA), 5µl of template DNA and 0.25µl of the barcoded ITS3 and ITS4 primers (10µM each). Cycling consisted of 5 minutes at 95°C, 18 cycles of 20 seconds at 98°C, 20 seconds at 62°C, 30 seconds at 72°C and a further 5 minutes at 72°C. Agencourt AMPure XP beads (Beckman Coulter, UK) or the AxyPrep Mag PCR clean-up kit (Axygen Biosciences, USA) were used to purify the pooled amplicons. These were eluted in 10µl of PCR grade water. 9µl of this was used for the second PCR round, in addition to 10µl KAPA HiFi HotStart Ready Mix and 0.5µl of the forward and reverse barcoded Illumina adaptor incorporated primers (10µM each). Cycling conditions remained the same, with the exception of the annealing temperature (65°C) and the number of cycles, which was reduced to 15.

Products were quantified (using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, UK)), pooled in batches of up to 96 amplicons based on an equal molar concentration (calculated on a 2100 BioAnalyser) of the region of interest. Paired-end sequencing (2x250bp) was subsequently performed using the Illumina MiSeq platform on the pooled products at the Centre for Genomic Research, University of Liverpool.

Quality filtering guidelines were followed during sequence processing (249). Cutadapt 1.9.1 removed primer sequences, retaining only amplicons from correct primer sequences, allowing a maximum primer error rate of 0.1 (273). A minimum of 15 overlapping bases was also set between reads using FLASH 1.2.11 (Fast Length Adjustment of Short reads) to reduce sequencing error (274). In addition, singleton removal and non-fungal sequence removal was undertaken

during sequence processing. The Quantitative Insights Into Microbial Ecology ((QIIME) version 1.9.1) open-source bioinformatics pipeline and additional quality-filtering guidelines were followed during sequence processing (249, 275). The QIIME release (2016-11-20) of the UNITE (User-friendly Nordic ITS Ectomycorrhiza) fungal ITS database was used to assign taxonomy using dynamic clustering thresholds (276, 277). Taxa that were present in less than 25% of the samples and taxa not able to be identified to the species level were excluded from analysis.

Statistical analysis

Data was analysed using SPSS for windows (version 22; SPSS, Inc., Chicago IL) and PRISM (version 7.02; GraphPad Software, San Diego, CA, USA). Parametric data is expressed as means and standard deviation (SD); non-parametric data is expressed as median with interquartile ranges. Unpaired student-t test and Mann-Whitney u test were used to analyse data between two groups, whilst the Bonferroni-corrected ANOVA and Kruskal-Wallis tests were used for multiple group comparison, dependent on the normality of data. Categorical variables are expressed as proportions and analysed using Fishers exact test or the Chi-square test for multiple comparisons.

QIIME bioinformatics software was used to calculate alpha and beta diversity and analyse the results of the HTS data. The alpha (Chao1 index) diversity, alpha rarefaction plots and beta (Bray-Curtis distance) diversity values were calculated using the workflow: *core_diversity_analyses.py*. Alpha diversity of bronchoscopy samples between groups was calculated with a non-parametric t-test using 999 Monte Carlo permutations to account for the non standard distribution of the data. A Bonferroni correction was also used to account for multiple samples. The script: *compare_alpha_diversity.py* was used to generate alpha diversity box plots using the non-parametric t-test, as described. The script: *compute_core_microbiome.py* was used to determine the core mycobiome present in 25% of the samples. Relative abundance Heatmaps was produced using R version 3.4.1 (<http://can.r-project.org/>). Differences in OTU frequencies were analysed using the nonparametric t-test (with Monte Carlo simulation), the Mann-Whitney U test and

the script: *group_significance.py*. The Benjamini-Hochberg corrected Kruskal-Wallis test was used to compare between multiple groups. Correlations between continuous variables and the OTU frequency of fungal taxa were determined by Spearman's rho correlation.

Differences among the species in the bronchoscopy samples were calculated using QIIME and the script: *group_significance.py*. A Chi-square contingency table was used to determine significance with Benjamini, Krieger and Yekutieli corrected p values. This was also used to evaluate any differences in the presence of absence ratios of taxa in asthma versus healthy controls.

6.3 Results

Demographics and clinical characteristics

29 subjects underwent bronchoscopy. 21 of these were asthmatics, of which 12 were sensitised to fungi, and 8 were healthy controls. The demographic details are seen in table 6.1 and the clinical characteristics of the study participants are displayed in table 6.2. The study population was well matched in terms of age, BMI and smoking history ($p>0.05$). Lung function (as measured by PBF_{EV1}) was preserved across all groups. The asthmatic cohort was well matched for asthma severity (according to GINA scores), inhaled corticosteroid dosage and had stable disease (according to ACQ6 and exhaled nitric oxide scores). The asthmatics that were sensitised compared to those that were not sensitised to fungi differed in terms of airway inflammation, asthma duration and oral steroid dosages. The asthmatics sensitised to fungi were more eosinophilic, tended to have an earlier onset of disease and a longer duration of asthma. The non-sensitised group were more comparable to healthy controls with a tendency towards neutrophilic airway inflammation and were on a higher dose of maintenance oral steroid therapy.

HTS output & airway mycobiota in different clinical groups

DNA extraction was performed on the sputum samples and the three specimens obtained at bronchoscopy (bronchial wash, bronchoalveolar lavage (BAL) and protected brush). Fungal amplification was successful in at least one sample type in 63% ($n=5$) of the healthy controls, 89% ($n=8$) of asthmatics not sensitised to

fungi and in 100% (n=12) of asthmatics sensitised to fungi. 14 subjects had fungal amplicons successfully amplified from all three of the bronchoscopic samples. 5 subjects had 3 samples (out of the potential of 4) where fungal amplicons were successfully amplified, 6 individuals had 2 samples that were successfully amplified and 4 subjects had only 1 sample where sufficient reads were obtained.

Overall, DNA amplification success was dependent upon sample type.

Amplification was achieved in 81% of BAL samples (21/26) and 79% of bronchial wash samples (23/29) compared to a 50% recovery rate from the protected brush specimens (14/28) ($p<0.05$). Comparing the different groups (healthy control, asthmatics sensitised and not sensitised to fungi) amplification success was lower from the bronchial wash samples of the healthy volunteers compared to that obtained from either of the asthmatic groups ($p<0.05$). This trend was also seen with samples obtained from BAL and from the protected brush, but failed to reach statistical significance (see table 6.3).

The minimum number of ITS2 fungal sequence reads was 5,085 per sample. This was of an adequate level to allow analysis. Sufficient depth of sampling was achieved for all sample types as shown by the Chao1 alpha diversity rarefaction curve (figure 6.1). Successful fungal ITS2 rDNA sequence data was obtained from 25 sputum samples, 23 bronchial washes, 21 BAL specimens and 14 of the protected brush samples.

Fungal load

Mean fungal load was measured by qPCR analysis as the number of *Cladosporium herbarum* spore equivalents in the sputum, wash, protected brush and BAL samples. A reduction in mean fungal load was seen in those not sensitised to fungi compared to those sensitised to fungi ($7267.0 \pm 9109.7SD$ versus $659.8 \pm 914.4SD$, $p<0.05$). Fungal load was also seen to differ between asthmatics that had early onset asthma compared to those that had late onset eosinophilic asthma in the BAL compartment ($2373.2 \pm 2720.6SD$ versus $150.2 \pm 149.2SD$, $p<0.05$).

A moderate correlation was seen with asthma duration ($r_s=0.578$, $p<0.05$) and BAL fungal load. A moderate inverse correlation with inhaled corticosteroid dosage

($r_s=-0.556$, $p<0.05$) and the protected brush sample fungal load was also seen. Sputum eosinophil counts were inversely correlated with both fungal loads in the sputum ($r_s=-0.730$, $p<0.05$) and the protected brush sample ($r_s=-0.800$, $p<0.05$). No relationship was seen between fungal load in any of the samples and postbronchodilator FEV₁, FeNO, PC₂₀ scores, total IgE and specific IgE to *A. fumigatus*.

OTU frequencies

Overall a total of 830 distinct fungal operational taxonomic units ((OTUs) units of clustered PCR sequences) were identified, representing 206 fungi at the species level.

We examined the differences in the OTU frequencies of fungal species that were present in at least 25% of the samples. Following correction for multiple comparisons no differences were seen between asthmatics and healthy controls in the samples obtained at bronchoscopy (table 6.4). A trend was noted, however, towards lower OTU frequencies in the healthy controls, with the exception of *Mycosphaerella tassiana*. This was seen at a higher OTU frequency in the healthy volunteers ($p=0.049$).

There were significant differences in the OTU frequencies of certain fungi depending on the compartment studied (table 6.5):

Brush sample – had a higher OTU frequency for *A. fumigatus* and *A. tubingensis*.

Samples obtained from lower airway at bronchoscopy – had a higher OTU frequency of *C. pseudolongus* compared to sputum.

Sputum and brush samples – had a higher OTU frequency of *S. roseus* in sputum and brush compared the bronchial wash or BAL samples.

Fungal prevalence by compartment

The most prevalent fungi (number of subjects where the species was detected), present in over half of sputum samples were *Candida albicans* (100%),

Mycosphaerella tassiana (87%) and *Aspergillus fumigatus* (82.6%); whilst *Aspergillus fumigatus* (48.1%) and *Mycosphaerella tassiana* (13.5%) had the highest overall abundance (percentage of reads).

The most prevalent species in the BAL samples were *Mycosphaerella tassiana* (100%), *Aspergillus fumigatus* (95%), *Candida albicans* (95%) and *Aspergillus tubingensis* (70%). *Aspergillus fumigatus* (44.6%), *Candida albicans* (11.2%) and *Aspergillus tubingensis* (7.9%) were the most abundant species overall.

The most prevalent species in the protected brush sample were *Aspergillus fumigatus* (100%), *Aspergillus tubingensis* (93.3%), *Mycosphaerella tassiana* (86.7%), *Candida albicans* (86.7%) and *Cyptococcus pseudolongus* (86.7%). The most abundant species were *Aspergillus fumigatus* (56.7%), *Aspergillus tubingensis* (10.5%) and *Exophiala equine* (6.7%).

The most prevalent species in the wash samples were *Aspergillus fumigatus* (94.7%), *Candida albicans* (94.7%), *mycosphaerella tassiana* (89.5%) and *Aspergillus tubingensis* (63.2%). The most abundant of these species were *Aspergillus fumigatus* (42.4%), *Aspergillus tubingenesis* (9.4%) and *Candida albicans* (4.7%).

Summary of fungal prevalence by sample type and compartment

The sample types selected in this study were chosen to give an indication of the mycobiome in the different airway compartments of the lung. Thus the bronchial wash gives an indication of the central conducting airways, the BAL of the distal small airways and the protected brush of the epithelial compartment. The prevalence of fungi was very similar in the bronchial wash and BAL specimens with the five most prevalent fungi being: *C. albicans*, *M. tassiana*, *A. fumigatus*, *A. striatus* and *A. tubingensis*. These were all present in >56% of samples. In contrast *A. fumigatus*, *A. tubingenesis*, *C. pseudolongus*, *M. tassiana* and *C. albicans* were more prevalent in the protected brush specimens. These were all present in >78% of samples. A greater number of fungi had a prevalence of >50% in the protected brush sample in comparison to the bronchial wash and BAL specimens. Three species differed significantly in prevalence dependant on the compartments

studied. *A. tubingensis*, *C. pseudolongus* were more prevalent in the protected brush samples and *S. roseus* was more prevalent in the sputum and the brush samples.

Relative abundance

The relative abundance of fungi present at the species level in each sample type is depicted in figure 6.2. *A. fumigatus* was the most abundant fungus in all the sample types obtained at bronchoscopy, with its proportion being greatest in the bronchial wash sample. *Aspergillus tubingensis* was the second most abundant species in the protected brush sample, whilst *Candida albicans* was the second most abundant fungus in the BAL and bronchial wash samples. Twelve species of fungi were present in at least 25% of subjects for the sputum, BAL, brush and wash samples and were felt to represent core species of the mycobiome (as depicted in the Venn diagram in figure 6.3).

A difference in the relative abundance of fungi was seen at the species level in those with asthma compared to healthy controls. A higher proportion of *A. tubingensis*, *C. pseudolongus* and *P. decumbens* were seen in asthmatics, whilst a higher abundance of *M. tassiana* was found in healthy controls.

Alpha and beta diversity

Species richness was assessed by alpha diversity. This did not show any significant difference in the diversity of fungi in sputum, bronchial wash and BAL specimens and in the protected brush (figure 6.1). In addition, the taxonomic abundance profiles (beta diversity) between these sample types were not significantly different.

Relationship between the lung mycobiome and clinical characteristics in asthma.

A. infectoria, *A. striatus*, *A. tubingensis*, *C. pseudolongus*, *H. radula*, *P. spinulosum*, *C. dubliniensis* and *M. globosa* were present in one or more specimens obtained from the lower airways and were more prevalent in asthmatics compared to healthy controls, though this failed to reach significance following correction for multiple comparisons. The only species that was significantly more prevalent in asthma

compared to healthy controls was *A. tubingensis* (BAL = 75% versus 25%, $p=0.048$; combined samples = 72% versus 33%, $p=0.02$ (table 6.6)). In the samples where these fungi were detected compared to samples where these were not detected no significant relationship was found between asthma duration, asthma severity, indices of lung function and oral / inhaled steroid dosage ($p>0.05$). A significant inverse relationship was, however, found between percentage of sputum neutrophils and the presence of *A. tubingensis* ($r^2=-0.363$, $p=0.019$) and *C. pseudolongus* ($r^2=-0.347$, $p=0.021$).

Immunohistochemistry & the airway mycobiome

Endobronchial biopsies were taken from 19 subjects who underwent bronchoscopy. Adequate biopsies were obtained from 79% ($n=15$) of these individuals. All of those with an adequate biopsy had at least one specimen where successful DNA amplification was achieved. This cohort comprised of 11 asthmatics (5 sensitised to fungi) and 4 healthy controls.

Fungal load

Significant relationships were seen between fungal load (as measured by quantitative PCR) and mast cell numbers present in the epithelium and lamina propria. A strong inverse correlation was seen between the fungal load in the wash sample and mast cells per mm^2 of the epithelium ($r_s=-0.925$, $p<0.001$). A moderate inverse correlation was seen between the number of mast cells per mm^2 of the lamina propria and the fungal load in the bronchial wash sample ($r_s=-0.673$, $p<0.05$). A moderate positive correlation was seen between reticular basement membrane thickness (RBMT) and sputum fungal load, which just failed to reach statistical significance ($r_s=0.600$, $p=0.051$).

No further associations were seen between fungal load and:

- mast cells, eosinophils and neutrophils in the ASM
- eosinophils or neutrophils in the epithelium or lamina propria
- percentage of intact airway epithelium
- percentage of airway smooth muscle.

Rarefaction was performed based on the sample with the lowest number of sequences so that all samples contained the same number of sequences prior to performing Spearman's correlation between the indices of remodelling (percentage of intact epithelium, percentage of airway smooth muscle (ASM) present and reticular basement membrane thickness (RBMT)) and the amount of fungus in a sample. Adjusted p values, using Fisher's z transformation, were used due to the large number of correlations performed. Unadjusted p values revealed a significant relationship between RBMT and the amount of *Cryptococcus pseudolongus*, *Mycosphaerella tassiana*, *Penicillium spinulosum* and Ascomycota species present in the BAL sample, *Penicillium spinulosum* in the brush sample and Ascomycota species in the wash sample ($p < 0.05$). The percentage of epithelium present was not correlated with any fungus in the BAL, although unadjusted correlation was seen with *Cryptococcus pseudolongus* in the brush sample and *Aspergillus fumigatus* in the wash sample ($p < 0.05$). The percentage of ASM present was correlated with the amount of *Phaeotheca triangularis* present in the BAL fluid and *Aspergillus striatus*, *Saccharomyces paradoxus* and *Leptosphaeriaceae* species present in the wash sample ($p < 0.05$). However, none of these correlations were significant after correction for multiple comparisons ($p < 0.05$) (see table 6.6).

6.4 Discussion

This study demonstrates a noticeable dominance of a small number of fungi in all compartments of the lung in both healthy and asthmatic individuals. *Aspergillus fumigatus* was the most abundant fungi in the sputum, wash, BAL and protected brush samples; whilst *Aspergillus tubingensis* was the second most abundant fungus in the brush and wash samples, and the third most abundant in the BAL fluid. *Candida albicans* and *Mycosphaerella tassiana* were amongst the most prevalent fungi in the samples. However, when the compartments were compared statistically, noticeable differences in prevalence rates were only seen amongst *Aspergillus tubingensis* and *Cryptococcus pseudolongus*, which were much more prevalent in the protected brush samples. In addition *Mycosphaerella tassiana*, *A. tubingensis*, *C. pseudolongus* and *P. decumbens* were the only fungi seen to differ between asthmatics and healthy controls, when p values were adjusted to account of multiple comparisons. No other fungi were seen to be of a higher OTU

frequency, prevalence or abundance between samples that reached statistical significance. This would suggest a dominance of a small number of fungi and stability in the mycobiome across different compartments of lung, with the exception of the epithelial compartment (as designated by the protected brush specimens). The epithelial compartment had a greater number of fungi present in more than 50% of the samples in comparison to the samples representing the proximal conducting (bronchial wash) and distal small (BAL) airways.

Core Mycobiome constituents

The core mycobiome was comprised of 12 species of fungi that were present in at least 25% of subjects. Species from the Ascomycota and the Basidiomycota phyla predominated, particularly those of the *Aspergillus* and *Penicillium* genus. In addition to these filamentous fungi, the yeast *Candida albicans* is also present as a core constituent.

Aspergillus fumigatus is a thermotolerant filamentous fungus. The relative abundance of *A. fumigatus* was high both in asthma and the healthy lung. *A. fumigatus* is known to have a key role in the development of allergic fungal airways disease. It is frequently cultured from patients with cystic fibrosis and asthma, where colonisation is related to disease severity and inflammatory mediators (52, 278). It is, thus, likely to represent a key component of the mycobiome.

Aspergillus tubingensis is a thermotolerant filamentous fungus, a member of the genus *Aspergillus*, section *Nigri* and morphologically indistinguishable from *A. niger* (279). Thus, although this fungus may have previously been misclassified in culture data as *Aspergillus niger*, recent advances in mass spectrometry have revealed its presence in chronic respiratory diseases, but its significance and role in airway inflammation is unclear (279). The notable predominance of *A. fumigatus* and *A. tubingensis* in the respiratory epithelial compartment (brush) is important given the crucial role the epithelium has in the immune response to fungi. This requires further study.

Candida albicans dominates the oral mycobiome both in health and disease (271). This study also demonstrates its presence in the lower airway. Its detection in the protected brush, where contamination from the oropharynx is less likely, as well

as the wash and BAL samples, suggests that it is a key component of the mycobiome of both asthmatic and healthy lungs. *Cryptococcus pseudolongus* belongs to the phyla Basidiomycota, whilst *Mycosphaerella tassiana* are classified with *Cladosporium herbarum* in the Davidiella section of the Ascomycete family. There are known environmental sources and Mycosphaerellaceae has been found in greater abundance in tilled soil (280).

Bittinger et al. extracted DNA from BAL and oropharyngeal wash samples in healthy subjects, HIV positive individuals and lung transplant recipients. This study found an increased presence of *Candida*, *Cryptococcus*, *Cladosporium* and *Aspergillus* present in the BAL samples and oral wash in subjects suffering from more severe lung disease (263). An increased fungal load and predominance of the *Aspergillus* complex has been found in severe asthma on higher doses of corticosteroids in comparison to mild asthma and healthy controls (281). The reasons for the differences encountered by these studies and this one could be attributed to the primers used and also the cohort of patients examined. Both of the studies involving asthmatics patients included those with mild-moderate disease and were also on a spectrum of inhaled and oral corticosteroids. This study included asthmatics mostly at the severe end of the spectrum, all of which were either on moderate to high doses of inhaled steroid.

Fungal diversity, lung compartments and links to airway remodelling

Previous studies examining the mycobiome have shown the presence of fungi to be highly variable between individuals and a lower fungal diversity in disease states compared to healthy controls (282). This study also suggests a degree of variability between individuals (as seen by the moderate variation in beta diversity). This was most noticeable in the epithelial compartment with the increased prevalence of *Aspergillus tubingensis* and *Cryptococcus pseudolongus* compared to the other compartments of the lung.

Instead of supporting a trend towards lower fungal diversity in disease states, as seen with other studies, this study suggests a relative stability in the fungal community between healthy controls and asthmatics individuals. In addition, calculations of alpha and beta diversity in the samples revealed relative stability of

the fungal community between different compartments of the lung, with a core number of species present across the different compartments of the lung and also between healthy controls and asthmatics. This could be for several reasons. Different extraction methodologies have been shown to affect the number of fungi that can be detected by HTS, and an optimised protocol is yet to be established (245). The primers selected in this study could be biased towards identifying a narrower spectrum of fungi and thus may inadvertently demonstrate a lower diversity of fungi in the samples analysed (283). In addition, successful DNA amplification varied between the different samples (sputum 86.2%, bronchial wash 79%, protected brush 50% and BAL 81%). Thus significant associations may have been affected by small sample size.

The fungal load of the bronchial wash samples was inversely correlated to mast cell numbers in both the airway epithelium and in the lamina propria. The reason for this is unclear and this relationship was not supported by the fungal load in the BAL and protected brush samples. Fungal load was not related to any of the indices of airway remodelling, nor were any specific fungi. This may be due to the severity of asthmatics that were recruited and this may become a feature if asthmatics of different severities were recruited.

The uncorrected p values do suggest that certain fungi have a protective effect on aspects of airway remodelling, whilst others are more damaging. The presence of *Cryptococcus pseudolongus* in the brush and BAL samples were associated with an increase in the percentage of intact airway epithelium and a reduction in the RBMT. The presence of *Penicillium spinulosum* was associated with a reduction in RBMT and several fungi were correlated to a decrease in ASM (*Phaeotheca triangularis*, *Saccharomyces paradoxus* and *Leptosphaeriaceae* species). Adverse features of airway remodelling were seen with *A. fumigatus* and *Aspergillus striatus* in the wash sample. These fungi were correlated with epithelial desquamation and an increase in airway smooth muscle.

Neutrophilic airway inflammation & the mycobiome

Neutrophils are known to be an important factor in host defence against invasive fungal infection (284). Thus, the association between sputum neutrophilia and

specific fungi is not surprising, and it may be an important indicator of a more pathogenic role of *Aspergillus tubingensis* and *Cryptococcus pseudolongus* in the lung. This study also demonstrated an increased prevalence of these two fungi in the epithelial compartment of the lung, an area of the lung that is crucial in activating both the innate and adaptive immune system in response to fungal allergens and to protect against invasive infection. Pattern recognition receptors recognise components of the fungal cell wall and are expressed by airway epithelial cells, neutrophils and dendritic cells (285). The subsequent release of inflammatory cytokines by these cells and disruption in epithelial barrier integrity in response to fungal proteases leads to airway inflammation (285, 286). Previous studies have shown fungal dysbiosis to be associated with disease severity in asthma. Fraczek et al. have demonstrated higher fungal loads and an increase in the relative abundance of *Aspergillus fumigatus* complex to be present in severe asthma when compared to mild asthma (281).

There may be a difference in immune function that underlies the increased prevalence of these two fungi, rather than reflecting increased pathogenicity. The increased prevalence of these two fungi was not associated with inhaled corticosteroid dose (although there was no mild asthmatics on lower dosages if inhaled corticosteroid to compare to) suggesting that the alteration in the airway mycobiota composition may not be due to the immunomodulatory effects of corticosteroid treatment but may be a result of altered immune function in certain individuals that may lead to an increase in prevalence of these fungi and neutrophilic airway inflammation.

Limitations of this study

There are a number of challenges in characterising the mycobiome, several of which this study highlights and have already been discussed. DNA extraction methodology, choice of primers and use of specific reference databases may have affected yield, diversity and abundance calculations so that the fungal profile of the different lung compartments in the upper and lower airway may not have been accurately represented.

The heterogeneity in asthma also makes interpretation of results difficult. This study may, therefore, have lacked the power to determine whether the mycobiome does differ in different the different compartments of the asthmatic lung and in different phenotypes of asthma. However, this study does seem to suggest a link with subepithelial and submucosal mast cell infiltration and fungal load. However, due to poor yield the relationship between this and specific fungi is yet to be established.

Future directions

Observational studies such as this one give a snap shot of the lung mycobiome and are important in generating hypotheses. We are yet to establish how the mycobiome changes over time, what interactions occur with other constituents of the microbiome and what changes occur in host immunity in response to a change in the constituents of the mycobiome. This study was performed in asthmatics that were at least six weeks post exacerbation. The mycobiome may change during exacerbations, both infective and non-infective and also in unstable asthma. A further study evaluating the mycobiome before, during and after exacerbations would be interesting to see whether the mycobiome changes during this time.

This study did not evaluate for possible interactions between bacteria and fungi (covariance). Investigating this would help evaluate what bacterial-fungal interactions occur and what effect these may have on the host.

Concluding remarks

Fungi are known to be ubiquitous in our environment and thus, their presence in the lung microbiome is not unsurprising. This study shows an agreement with previous mycobiome studies showing a predominance of Ascomycota and Basidiomycota phyla in the core mycobiome of healthy subjects and asthmatics. However, this study does indicate a difference in the composition and the relative abundance of fungi in the lung compared to previous studies and also a lack of diversity between the healthy controls and asthma, although this could be attributed to the extraction method, primers and sample size of the study introducing bias. It is however, the first study to show a predominance of

Aspergillus tubingensis in the epithelial compartment of the lung and an association with neutrophilic airway inflammation. This study also suggests that certain fungi that are present in the lung may have a protective effect against airway remodelling. This finding requires further study.

Tables and figures

Table 6.1 Demographic details of study participants. *mean (SD), **median (IQR)

	Asthmatics sensitised to fungi (n=12)	Asthmatics not sensitised to fungi (n=9)	Healthy controls (n=8)	p-value
Age (years)*	48.9 (18.2)	58.7 (8)	50.8 (18.8)	0.362
Male, n (%)	2 (16.7)	5 (55.6)	4 (50)	0.492
BMI (kg/m²)*	32 (10.8)	27.1 (4.2)	27 (6)	0.299
Smoking pack years (n=ex/current)*	12.5 (3.5) (n=2)	11.1 (5.8) (n=7)	14.7 (10.1) (n=3)	0.762
Atopy, n (%)	6 (50)	2 (22.2)	3 (37.5)	0.083
Age of asthma onset (years)**	22.5 (3.5-41)	51 (42-53)		0.139
Asthma duration (years)**	26 (2-37)	5 (2-20)		0.606

Table 6.2 Clinical characteristics of study participants. *mean (SD), **median (IQR)

	Asthmatics sensitised to fungi (n=12)	Asthmatics not sensitised to fungi (n=9)	Healthy controls (n=8)	p-value
<u>Disease severity</u>				
GINA ≥ 3 (n, %)	7 (58.3)	8 (88.9%)		0.098
Inhaled corticosteroid dose (ICS-BM $\mu\text{g}/\text{pt}/\text{day}$)*	1100 (667.6)	1288.9 (625.4)		0.556
Maintenance oral steroid (n, %)	1 (8.3)	6 (66.6)		0.178
Maintenance steroid dose (mg)*	0.625 (1.77)	6.75 (6.7)		0.023
ACQ 6 score*	1.1 (1.3)	1.01 (1.02)		0.881
PC20**	6 (1.8-9)	1.7 (0.16-16)	16 (16-16)	0.19
Post-bronchodilator FEV ₁ (% predicted)*	95.2 (11.6)	92.7 (16.6)	109.2 (14.6)	0.406
<u>Airway inflammation</u>				
FeNO (ppb)**	24 (16-71)	31 (23-58)	25 (15-27)	0.381
Sputum eosinophil count (%)**	12.4 (4.6-49.4)	6.4 (2.3-10)	0 (0-0.25)	0.02
Sputum neutrophil count (%)*	54.1 (37.3)	81.2 (14.2)	73.9 (24.2)	0.316
Blood eosinophil count ($\times 10^{-9}/\text{l}$)*	0.62 (0.47)	0.29 (0.29)	0.14 (0.09)	0.026
<u>Immunological biomarkers</u>				
Total IgE IU/ml**	692.5 (470-1485.5)	41 (28.4-76.9)	24.5 (5.5-110)	0.001
IgE <i>Aspergillus fumigatus</i> mg/L**	6.19 (0.37-13.45)	0.02 (0.01-0.04)	0.00 (0-0.01)	<0.001
IgG <i>Aspergillus fumigatus</i> kU/L**	37.5 (19.9-61.9)	15.4 (10.8-28.3)	36.5 (22.6-48.4)	0.116

Table 6.3 Amplification success of samples obtained from bronchoscopy. Table displays number of samples obtained and % successfully amplified in brackets.

	Asthmatics sensitised to fungi (n=12)	Asthmatics not sensitised to fungi (n=9)	Healthy controls (n=8)	p-value
Bronchial wash	12 (100%)	9 (78%)	8 (50%)	0.0256
Bronchoalveolar lavage	9 (89%)	9 (89%)	8 (63%)	0.2889
Protected endobronchial brush	11 (45%)	9 (67%)	8 (38%)	0.4514

Table 6.4 Operational taxonomic unit (OTU) frequencies of fungal species that differed between the asthma cohort and healthy controls. The Kruskal-Wallis test was used to OTU frequencies, the Benjamini-Hochberg FDR procedure was used to correct for multiple comparisons.

Sample(s)	Fungus	Asthma (mean OTU)	Healthy (mean OTU)	p-value	Adjusted p-value
Sputum	<i>Candida dubliniensis</i>	397	0	0.015	0.306
	<i>Mycosphaerella tassiana</i>	4,435	29,059	0.002	0.049
Bronchoscopy	<i>Aspergillus tubingensis</i>	10,796	8,659	0.018	0.192
	<i>Cryptococcus psuedolongus</i>	1,361	98	0.020	0.192
	<i>Penicillium decumbens</i>	1,426	240	0.042	0.264
	<i>Mycosphaerella tassiana</i>	2,664	14,056	0.010	0.190
BAL, brush & wash	<i>Cryptococcus pseudolongus</i>	1,679	138	0.041	0.333

Table 6.5 Fungal species that differed in either OTU frequency or prevalence between the samples obtained from different lung compartments. The Kruskal-Wallis test was used to compare OTU frequencies, the Benjamini-Hochberg FDR procedure was used to correct for multiple comparisons. A chi-square contingency test analysed prevalence rates, with multiple comparisons corrected for by the Benjamini, Krieger and Yekutieli procedure (with 5% Q-value false-discovery rate).

	Test-Statistic	BAL mean	Brush mean	Wash mean	Sputum mean	Adjusted P-value
<u>OTU frequencies</u>						
<i>Aspergillus fumigatus</i>	9.54	57,596	102,527	40,020	55,482	0.098
<i>Aspergillus tubingensis</i>	10.74	10,821	20,351	13,892	1,123	0.098
<i>Cryptococcus pseudolongus</i>	9.49	1,329	1,450	1,334	502	0.098
<i>Sporobolomyces roseus</i>	9.09	672	3,058	822	2,544	0.098
<u>Prevalence (%)</u>						
<i>Aspergillus fumigatus</i>	1.215	90.5	92.9	82.6	84.0	0.767
<i>Aspergillus tubingensis</i>	12.65	61.9	92.9	47.8	36.0	0.035
<i>Cryptococcus pseudolongus</i>	13.45	47.6	92.9	34.8	40.0	0.035
<i>Sporobolomyces roseus</i>	8.801	28.6	71.4	26.1	40.0	0.135

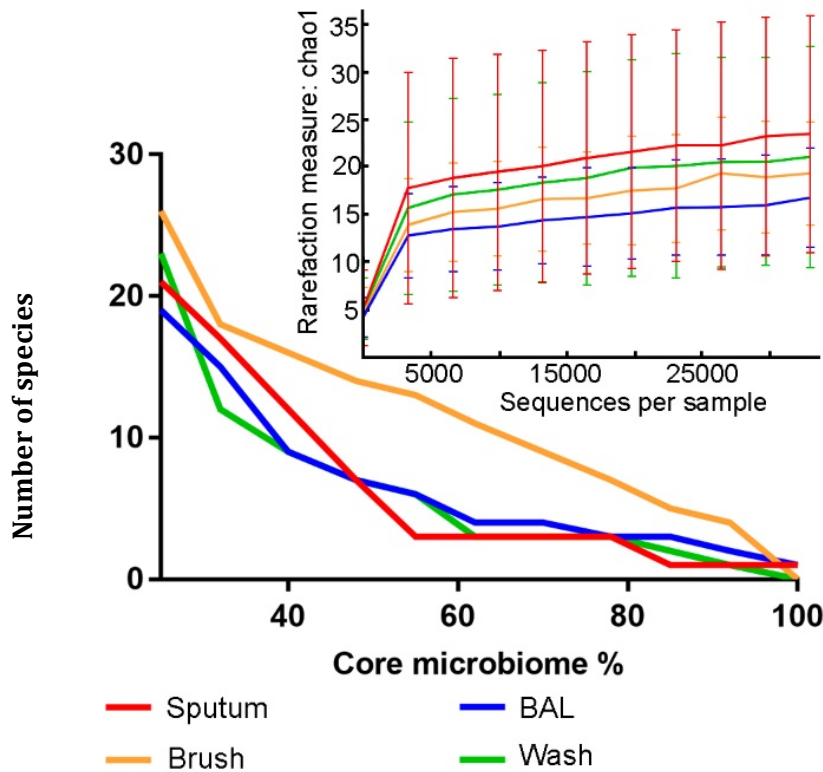
Table 6.6 Correlations (unadjusted and adjusted) between indices of airway remodelling (percentage of intact airway epithelium, percentage of airway smooth muscle (ASM) present and reticular basement membrane thickness (RBMT)) and the amount of fungi present in each sample type (BAL, protected brush, wash). Exploratory data analysis (uncorrected p value) suggests a protective role of certain fungi within the mycobiome, whilst other are associated with markers of airway remodelling.

	Fungi	Spearman's Rho	Unadjusted p value	Adjusted p value
Percentage of intact airway epithelium	<u>BAL</u> – none significant	-	-	-
	<u>Brush</u>			
	<i>Cryptococcus pseudolongus</i>	0.802	0.013	0.417
	<u>Wash</u> <i>Aspergillus fumigatus</i>	-0.627	0.037	0.721
Percentage of ASM	<u>BAL</u> <i>Phaeothea triangularis</i>	-0.669	0.022	0.708
	<u>Brush</u> – none significant	-	-	-
	<u>Wash</u>			
	<i>Aspergillus striatus</i>	0.749	0.006	0.216
	<i>Saccharomyces paradoxus</i>	-0.669	0.022	0.265
	Leptosphaeriaceae species	-0.669	0.022	0.265
RBMT	<u>BAL</u>			
	<i>Cryptococcus pseudolongus</i>	-0.72	0.009	0.303
	<i>Mycosphaerella tassiana</i>	-0.64	0.031	0.341
	<i>Penicillium spinulosum</i>	-0.62	0.04	0.341
	<u>Brush</u>			
	<i>Penicillium spinulosum</i>	-0.77	0.021	0.482
	<u>Wash</u>			
	Ascomycota species	-0.78	0.003	0.120

Figure 6.1 Chao1 alpha diversity. Samples included in the analyses were: BAL (n= 21), bronchial wash (n=23), protected brush (n=14) and sputum (n=25) samples.

- A. Graph depicting the core microbiome showing the number of taxa shared between 25% and 100% of the samples and a rarefaction curve. Insert shows the rarefaction plots using chao1 alpha diversity.
- B. Box plots of Chao1 alpha diversity indices of patients undergoing a bronchoscopy grouped by sample type.

A.



B.

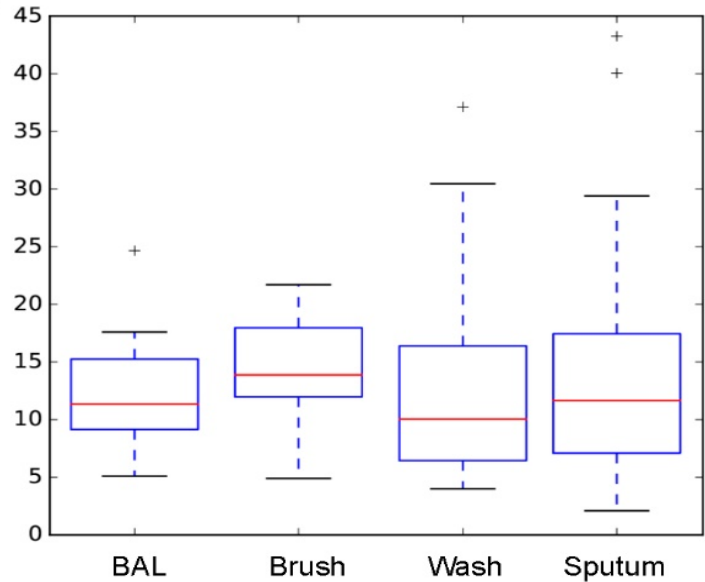
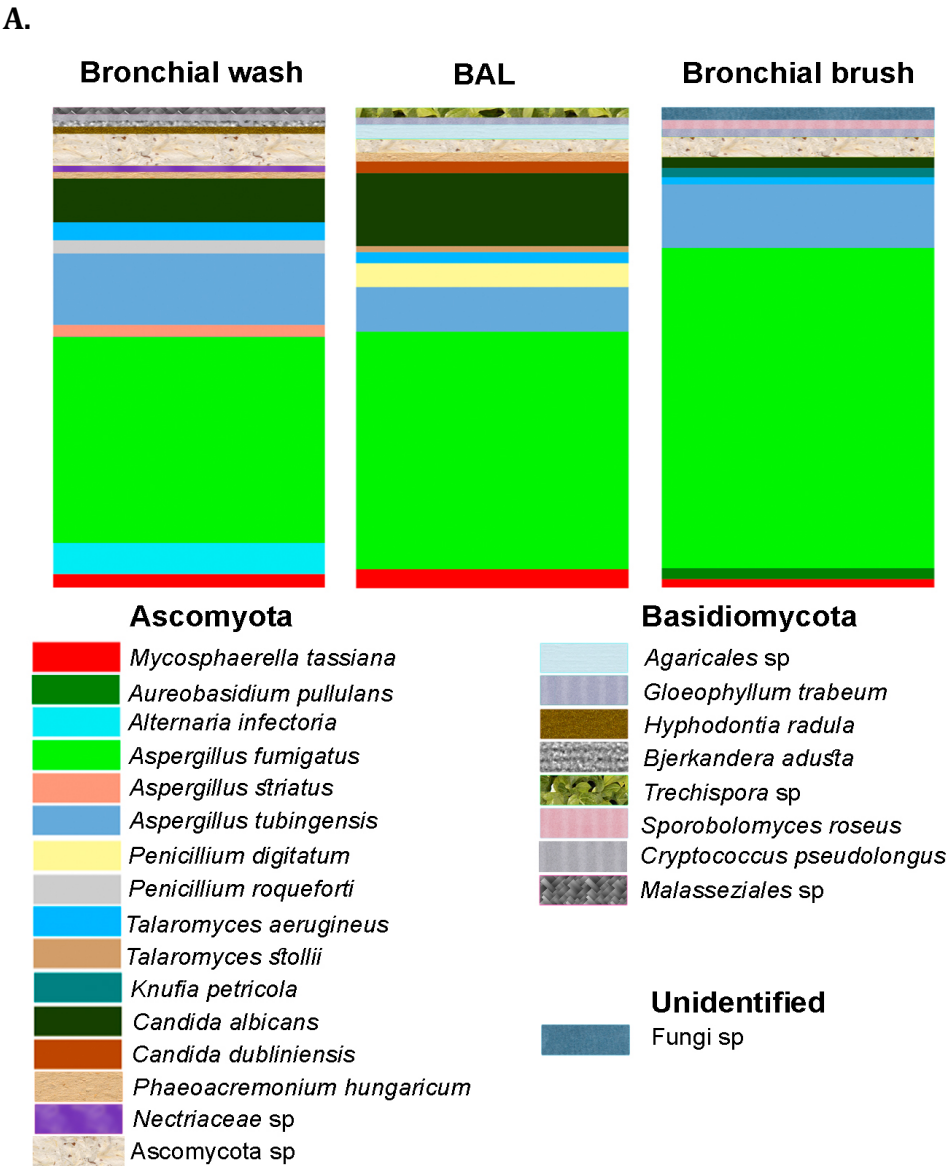
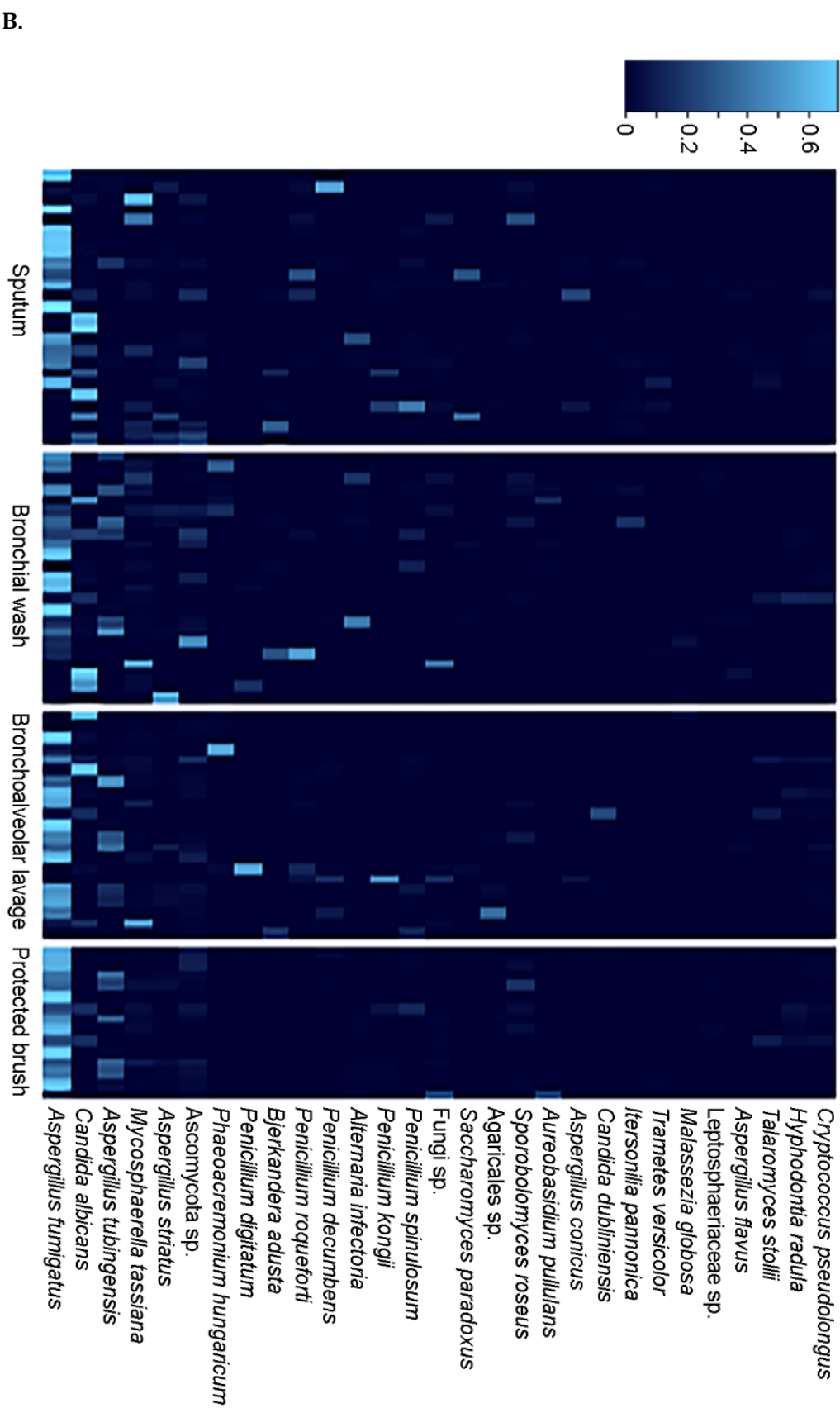


Figure 6.2 Relative abundance of fungi. Samples included in the analyses were: BAL (n= 21), bronchial wash (n=23), protected brush (n=14) and sputum (n=25) samples. Taxa present in $\geq 25\%$ of samples and with a total OTU observation cutoff of 1% in A and 0.1% in B are represented.

- A. Relative abundance (as a percentage) of fungi present at the species level.
 B. Heatmap of the relative abundance of fungal ITS2 sequences between sample types. Transformed data (using $\ln(x+1)$) is shown, compensating for zeros in the dataset.





Chapter 7: Conclusions and further work

7.1 Introduction

This thesis has focused on the role that fungal allergy and the fungal microbiome (mycobiome) have in the clinical manifestations of asthma and asthma severity, without being restricted by existing diagnostic classifications for fungal allergy (ABPA/ABPM/SAFS).

Three studies were conducted. The first used univariate and multivariate analysis on a large cross-sectional cohort of asthmatic patients to evaluate the relationship between immunological and inflammatory variables and markers of disease severity (PBF_{FEV}₁, lung function decline and radiological abnormalities). Cluster analysis was used to categorise patients into more homogenous groups on the basis of these variables. Markers of disease severity were then mapped to these groups to evaluate whether characterising patients in this manner would identify those more at risk of developing adverse clinical features of asthma.

The second study in this thesis used immunohistochemistry to measure recognised parameters of airway remodelling in asthmatics sensitised and not sensitised to fungi.

The final study of this thesis aimed to characterise and examine the role of the mycobiome in asthma. Firstly we aimed to gain a better understanding of the core constituents of the mycobiome in the different compartments of the lung and examine whether any differences were observed between fungal load, prevalence and abundance. We also evaluated whether any of the core mycobiome constituents had a specific relationship with any aspect of airway physiology, inflammation or features of asthma severity and remodelling.

This concluding chapter summarises the outcomes of these studies, their limitations and discusses future directions for research in this area.

7.2 Study 1 – Post-bronchodilator FEV₁, lung function decline and radiological abnormalities in asthmatics sensitised to fungi

Fungal sensitisation, ABPA and SAFS are seen more commonly in severe asthma. The cohort of asthmatics that fulfil these classifications represent a heterogeneous

group that have a spectrum of disease severity. To address this heterogeneity we investigated how the immunological variables used to classify patients as being sensitised to fungi, ABPA or SAFS related to adverse clinical features of asthma. The aim was to define a group with similar adverse features of asthma severity, characterised by specific immunological and inflammatory variables.

A large cross-sectional cohort of asthmatics sensitised to thermotolerant filamentous fungi, non-thermotolerant filamentous fungi and thermotolerant yeasts, and a control group of non-sensitised asthmatics, were recruited.

7.2.1 Relationship between markers of airway inflammation and immunological variables to airway physiology and radiological abnormalities

Univariate analysis showed a reduction in lung function to be limited to subjects IgE sensitised to thermotolerant filamentous fungi, independent of atopic status. Reduced lung function was not associated with sensitisation to the non-thermotolerant fungi or the thermotolerant yeasts. This association was supported by trends in the lung function decline data. Tree in bud, bronchiectasis and collapse $\pm$ consolidation were also seen more frequently in asthmatics that were sensitised to the thermotolerant filamentous fungi and were not associated with sensitisation to other fungi. Only weak correlations were present between any of the immunological variables and PBF_{FEV1}.

Strikingly the total IgE, the level of which is central to the criteria for both ABPA and SAFS, was not closely related to clinical outcomes. This section of the results suggested that the presence of IgE sensitisation to thermotolerant filamentous fungi, but not the magnitude was important in determining those at risk of declining lung function and developing radiological abnormalities.

7.2.2 Cluster analysis

To test whether incorporating a greater number of variables in the analysis would reveal distinct endotypes of asthma I undertook a cluster analysis on the whole population. This identified three clusters of asthmatics:

- (i) An eosinophilic group with a lower frequency of fungal sensitisation, and better-preserved lung function. This cluster most likely relates to the hypereosinophilic

exacerbation prone asthmatics noted in previous such analyses (19).

- (ii) A neutrophilic group (~10% of the subjects that were sensitised to fungi) with a higher total IgE and specific fungal IgE values, lower PBF_{FEV₁} values and a higher frequency of radiological abnormalities was also seen. This second cluster probably represents the patients with florid allergic fungal airway disease described in the case series from the 1970's, which led to the development of the criteria for ABPA (55).
- (iii) A neutrophilic group with high rates of sensitisation to *A. fumigatus*, but with only a modestly raised level of total IgE, a lower frequency of radiological abnormalities and relatively well-preserved lung function.

7.2.3 ABPA and the cluster analysis

Patients classified as having ABPA, based on the ISHAM criteria, were distributed across all 3 groups with similar numbers seen in clusters 1 and 3. Thus, a specific phenotype for fungal allergy could not be identified. The cluster analysis did, however, demonstrate that those with the most heightened immunological response and neutrophilic airway inflammation are at an increased risk of developing radiological abnormalities and a reduction in lung function. This is further supported by the lung function decline data (chapter 4, table 4.5), where a significant reduction in FEV₁ over time associated with neutrophilic airway inflammation and sensitisation to *A. fumigatus*.

7.2.4 Concluding remarks

This study used both clinical data and markers of airway physiology and radiological endpoints in an attempt to produce a more effective way of characterising an asthma endotype linked to fungal allergy. Contrary to previous classifications of ABPA as being characterised as an eosinophilic disease, this study suggests that asthmatics with neutrophilic airway inflammation and those with a higher immunological response to fungi are at greater risk of a decline in lung function and radiological abnormalities, whether they fulfil the diagnostic

classification for ABPA or not. Neutrophilic inflammation in this group would indicate involvement of both innate immune activation and IgE-mediated activation of the acquired immune system. Thus, although this study was not able to fully characterise a specific phenotype or endotype to replace ABPA or SAFS, it does suggest that these diagnostic classification do not capture all those at risk of developing inflammatory and structural changes in the lung as a consequence of fungi.

7.2.5 Future directions

A further longitudinal study is required to characterise groups of asthmatics that are sensitised to thermotolerant filamentous fungi that have a higher frequency of exacerbations and a greater need for inhaled and systemic corticosteroid treatment. This future project might aim to establish whether this cohort of asthmatics are characterised by the same neutrophilic airway inflammation and investigate whether the same heightened immunological response is seen. One further step would then be to investigate whether this cohort of asthmatics is more responsive to antifungal treatments in a clinical trial.

7.2.6 Limitations

This study recruited a cohort of asthmatics with moderate to severe disease and did not recruit those with mild to moderate asthma. Its findings are therefore at risk of selection bias. The results of this study therefore cannot be generalised to include those with mild asthma, and may have been confounded by the recruitment of only those with mainly severe asthma. This is a significant limitation of this study. In addition, the statistics performed were of an exploratory nature and thus would not be able demonstrate any causal links to the outcome variables that were measured.

7.3 Study 2 – Remodelling changes in asthmatics sensitised to fungi.

The second study in this thesis used immunohistochemistry to map features of airway remodelling to immunological variables of fungal allergy and assess whether any specific features of airway remodelling were increased in asthmatics that are sensitised to fungi.

This study did not show that asthmatics that are sensitised to fungi are at an increased risk of airway remodelling. It did, however, demonstrate a significant increase in the number of mast cells in the epithelium and lamina propria of asthmatics sensitised to fungi. In addition, moderate correlations were seen between intraepithelial mast cells and specific IgE to *A. fumigatus* and total IgE. This would support a significant immunological relationship between exposure to fungi, in particular *A. fumigatus*, and the influx of mast cells in asthma.

There are several limitations inherent in this study that may have affected its results. Previous murine studies have demonstrated increased collagen deposition and goblet cell metaplasia to be key features of fungal asthma (225). A significant disadvantage of our study is that these features were not examined. This should be the focus of further studies. The different phenotypes of mast cells present and their state of activation were not evaluated and is also a significant disadvantage in being able to interpret the association between increased intraepithelial and submucosal mast cell numbers with fungal sensitisation. This is an area that requires further study.

Unfortunately, the small size of the study population restricted the ability to compare remodelling features between the different fungal groups (filamentous thermotolerant fungi, non-thermotolerant fungi and thermotolerant yeasts). Although this study was powered to show a difference in the remodelling parameters studied, given the results of the cross-sectional study it may be that remodelling may occur in groups with similar characteristics as those described in cluster 1 of the first study. To characterise patients in this way would require larger study numbers.

7.4 Study 3 – Description of the mycobiome in asthma.

A number of studies have been published on the subject of the mycobiome. Notable differences have been described regarding the core constituents of the oral mycobiome in health and disease. Key questions remained as to whether the constituents of the mycobiome are different in the proximal and distal airway and in the epithelial compartments of the lung, and whether disease severity affect fungal load and the dominance of certain fungi within the mycobiome.

This was a prospective study performed on asthmatics in a stable state.

Bronchial wash specimens (representing the proximal airways), BAL samples (peripheral airways) and a protected brush specimen (epithelial compartment) were examined to elicit whether there were any differences in the mycobiome between these compartments. The core constituents of the mycobiome were then compared to clinical features of asthma.

7.4.1 Core mycobiome

The key constituents of the core mycobiome were dominated by 12 species of fungi from the Ascomycota and Basidiomycota phyla (figure 6.3). Striking similarities were seen in the fungal community in all of the compartments studied. This is reflected in the plateaus in the rarefaction curves and the calculations of alpha and beta diversity. Although the constituents of each of the compartments were similar, differences were seen when the species in these compartments were compared by OTU frequency, prevalence rates and relative abundance.

7.4.2 Mycobiome in the different compartments of the lung

A. fumigatus was the most abundant fungus in all the compartments studied.

Secondary differences were seen between the epithelial compartment and that of the proximal and distal airways with *Aspergillus tubingensis* more abundant in the epithelial compartment compared to *Candida albicans* in the proximal and distal airways. Specimens from the proximal and peripheral airway had several fungi present at similar prevalence rates with *C. albicans* and *M. tassiana* being the most dominant, followed by several members of the *Aspergillus* genus. Most of these fungi were also present in the epithelial compartment, but in a different order of prevalence with *A. fumigatus*, *A. tubingensis* and *C. pseudolongus* being the most dominant fungi present in the epithelial compartment. Further differences were seen in the epithelial samples when compared to the specimens taken from the proximal and peripheral airways. An increase in prevalence of both *A. fumigatus* and *A. tubingensis* was also seen in the respiratory epithelial compartment, in addition to an increase in the OTU frequency of *A. fumigatus* in this compartment. These are important findings given the crucial role the epithelium has in the immune response to fungi. This requires further study.

7.4.3 Mycobiome in health and asthma

Relatively few differences were found between asthmatics and healthy controls. Higher OTU frequencies of *A. tubingensis* were seen in asthmatics, whilst *M. tassiana* had higher OTU frequencies in healthy controls. In comparison to healthy controls, patients with asthma had higher OTU frequencies. In addition, differences in the relative abundance of certain fungi were shown to be specific in differentiating asthma from healthy controls. *A. tubingensis*, *C. pseudolongus* and *P. decumbens* were more abundant in samples from asthmatic individuals than they were in healthy controls, whilst *M. tassiana* was the only fungi to have a higher level of relative abundance in samples from healthy volunteers.

7.4.4 Relationship of clinical characteristics of asthma, fungal load and mycobiome

A few clinical features of asthma demonstrated interesting associations with the results from the HTS data, however whether these are clinically relevant is yet to be established. Fungal load was higher in early onset disease and in asthmatics sensitised to fungi. Fungal load was also inversely correlated with sputum eosinophilia and inhaled corticosteroid dose. However, the presence or absence of certain fungi was not found to be associated with any of the clinical characteristics of asthma that were studied with the exception of sputum neutrophilia, which was found to have a weak inverse correlation with *A. tubingensis* and *C. pseudolongus*. Fungal load was strongly correlated with intraepithelial mast cell infiltration in the proximal airway. In addition, a moderate inverse relationship was seen between fungal load in this compartment and mast cells in the lamina propria.

The uncorrected p values do suggest that certain fungi have a protective effect on aspects of airway remodelling, whilst others are more damaging. The presence of *Cryptococcus pseudolongus* in the brush and BAL samples were associated with an increase in the percentage of intact airway epithelium and a reduction in the RBMT. The presence of *Penicillium spinulosum* was associated with a reduction in RBMT and several fungi were correlated to a decrease in ASM (*Phaeotheca triangularis*, *Saccharomyces paradoxus* and *Leptosphaeriaceae* species). Adverse features of airway remodelling were seen with *A. fumigatus* and *Aspergillus striatus* in the wash sample. These fungi were correlated with epithelial desquamation and an increase in airway smooth muscle.

7.4.5 Concluding remarks

This study showed striking similarities in the constituents of fungi present in the different compartments of the lung. A few 'key players' included members from the *Aspergillus* genus, *Candida*, *Mycophaearella* and *Cryptococcus* dominating these compartments. Preliminary results from the immunohistochemistry data would suggest a protective role of certain fungi in the mycobiome, but this requires further confirmatory studies.

12 species from both the Ascomycota and Basidiomycota phyla represented the core mycobiome. Specific differences did occur between compartments with regard to OTU frequency, relative abundance and prevalence. However, the clinical significance of these specific differences is not clear. Further studies are needed to establish whether the weak inverse correlation between *A. tubingensis* and *C. pseudolongus* and sputum neutrophilia is meaningful in terms of exacerbation rates and whether these constituents remain stable over time.

7.4.6 Limitations

The limitations of this study in terms of primer selection, DNA extraction methodology and sample size have already been discussed. The results may also have been affected by primer amplification bias. Both ITS1 and ITS2 primer set are available. Thus further confirmatory studies are required using both primer sets to establish the reproducibility of our findings.

7.4.6 Future directions

Fungal load was associated with fungal sensitisation, sputum eosinophilia and corticosteroid dose. The clinical significance of this is uncertain. Interventional trials using nebulised antifungals may be an appropriate next step to ascertain whether this would reduce fungal load and have any meaningful impact on sputum eosinophilia and corticosteroid dose.

No longitudinal element was included in this study and would be an important next step to assess the stability of the mycobiome over time, during and following exacerbations. In addition, it is becoming increasingly recognised that there is a need to look at all parts of the microbiome rather than the bacterial or fungal microbiome in isolation. This is due to the influence of bacterial and fungal

interactions and how alterations in one part of the microbiome may have implications for other parts of the microbiome (covariance).

7.5 SUMMARY

The first study in the thesis highlights the issues that exist with the current diagnostic classifications in fungal allergy. The parameters used in these classifications were not shown to be specific or sensitive enough to detect all those at risk of adverse physiological or radiological outcomes. The study also shows lung function decline in patients sensitised to *A. fumigatus* with neutrophilic airway inflammation. This is a novel finding.

Histologically the second study shows an increase in intraepithelial mast cells, an increase in mast cells in the lamina propria and fungal load to be specific features associated with fungal sensitisation. In addition, specific fungi in the mycobiome were associated with a protective effect on aspects of airway remodelling, whilst others may have a more damaging role.

The third study demonstrated 12 species of fungi, belonging to the Ascomycota and Basidiomycota phyla, to be present in all compartments of the lung and was felt to represent the core mycobiome. *A. fumigatus* and *Aspergillus* species were the most dominant fungi, in terms of abundance, in both asthmatics and healthy states throughout the different compartments of the lung. Key differences were, however, seen in the composition of the fungi in the epithelial compartment, with an increased abundance seen of *A. tubingensis* specifically being associated with neutrophilic airway inflammation.

Thus this body of work would suggest that identifying patients with neutrophilic airway inflammation that are sensitised to thermotolerant filamentous fungi and have a high fungal load might identify those at risk of poor outcomes and where antifungal treatment maybe more effectively targeted. Further mechanistic and interventional studies are necessary to support this conclusion.

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