The role of *Aspergillus fumigatus* and other thermotolerant moulds in asthma

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Abstract

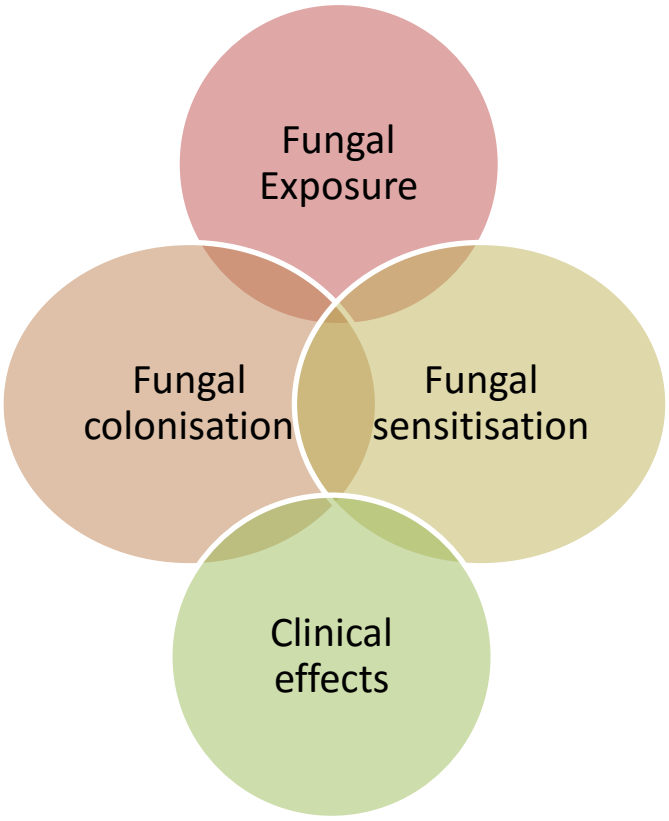
The fungal kingdom contains well over a million species, of which about 80,000 have been named and approximately 600 species cause some form of human disease. Fungal spores are ubiquitous in the airborne environment and inhaled daily often in large numbers though seldom lead to disease. Atopy is a common clinical feature of asthma highlighting the genetic and environment interactions that give rise to clinical phenotypes. Allergy towards fungi is recognised to play an important role in asthma and fungal sensitisation in asthma is associated with an increased risk of multiple hospital and ITU admissions. It is thought that fungal allergy arises from a disproportionate Th2 response to fungal allergens present in spores and hyphae. The risk of IgE sensitisation to fungi may be increased by the capacity of some thermotolerant fungi (typified by *Aspergillus fumigatus*), to colonise the airways, with an extreme though unusual form exemplified by the condition allergic bronchopulmonary aspergillosis (ABPA). This relationship however is imperfectly understood and lesser displays of fungal allergy in severe asthma are increasingly recognised.

The aim was to characterise the relationship between fungal colonisation as defined by a positive sputum culture, fungal sensitisation and the clinical features of mainly moderate-severe asthma. Secondly, this information would guide a placebo controlled trial targeting airway colonisation with Voriconazole.

Over 25 species of filamentous fungi were cultured from asthmatics sputum with the flora dominated by *Aspergillus fumigatus*. There was a correlation between IgE sensitisation to *A. fumigatus* and evidence of lung damage defined by fixed airflow obstruction and bronchiectasis. There was a demonstrable association between lung damage and fungal colonisation.

A three-month randomised trial of oral Voriconazole in patients with asthma and *Aspergillus* sensitisation failed to improve asthma control or reduce exacerbations.

The place of Voriconazole in patients with fungal associated asthma remains to be established.


Fungal
Exposure
Fungal
colonisation
Fungal
sensitisation
Clinical
effects

Authors statement

I confirm that the work submitted here is my own, except where work which has formed part of jointly-authored publications has been included. My contribution and that of other authors to this work has been explicitly indicated below.

Acknowledgements

First and foremost, I would like to thank Michelle Bourne, my research assistant, whose hard work and easy bedside manner with patients ensured not only the success of the study but her encouragement and support will leave me eternally and wholeheartedly indebted.

Suffice to say that the concept and execution of these projects required the cooperation and assistance of many especially from William Monteiro and Abbie Fairs at the Institute for Lung Health laboratory in sample processing.

I am grateful to Asthma UK and the Midlands Allergy and Aerobiology research association, MAARA, for providing an educational grant to help initially undertake this work.

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I am especially grateful to my supervisor Andy Wardlaw for his unending support and inspiration.

Lastly and on a personal note I would like to thank Pfizer for both their courage and financial support in exploring this topic.

STATEMENT OF WORK PERSONALLY PERFORMED

Study 1: IgE Sensitisation to *Aspergillus fumigatus* Is Associated with Reduced Lung Function in Asthma

Dr Kugathasan Mutalithas obtained the initial ethics approval and started initial recruitment of patients attending general respiratory outpatient clinics. Sputum processing and cell count measurements were performed by William Monteiro while Abbie Fairs, Joseph Morley and Catherine Pashley were involved in preparation of fungal culture media, and identification of filamentous fungi in Chapters 1, 2 and 3. I participated in laboratory work culturing fungi from over 10 patients some involving PCR studies. I also performed the clinical assessments including lung function and allergy testing of many of the patients. I obtained the subsequent ethics approval and recruitment, conducted the analysis from a bespoke database I designed and prepared the first draft of the publication that was jointly co-authored.

Study 2: Isolation of filamentous fungi from sputum in asthma is associated with reduced post-bronchodilator FEV_1

I conducted the recruitment, assessment, analysis and initial draft of the manuscript published.

Study 3: Effectiveness of voriconazole in the treatment of *Aspergillus* associated asthma

I co-designed, co-wrote and obtained Ethics and Medicines and Healthcare Products Regulatory Agency approval for this study.
I personally designed and created a secure database for electronic data storage as well as the study related paperwork. I was responsible for all subject recruitment to the study and obtained the consent of all subjects to the study. I performed clinical assessments of subjects at their study visits and at the time of unscheduled visits for exacerbations. I personally performed approximately 30% of all clinical measurements at scheduled visits. I was responsible for analysis and interpretation of all study data.

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1. Overview

There has been a global rise in asthma prevalence [1] in association with atopic disorders. Asthma is a condition characterised by variable airflow obstruction and airway inflammation in response to a variety of stimuli with manifesting symptoms of cough, wheeze and breathlessness.

Atopy is a common feature of asthma and highlights the genetic and environment interactions that contribute to the many clinical phenotypes [2, 3].

Airborne filamentous fungi are recognised to play an important role as environmental allergens [4] and can induce a range of bronchopulmonary disorders [5].

The fungal kingdom contains well over a million species, of which about 80 000 have been named while approximately 600 species cause some form of human disease.

Amongst known bio-aerosols, fungal spores and fragments represent one of the most ubiquitous in the airborne environment.

Fungi inhabit many environments including soil, water and dead matter. They can withstand wide extremes of temperature surpassed only by bacteria (Raspor and Zucan 2006). As a group of organisms, their eukaryotic lineage is equal in numbers to animals and exceeds plants.

Many fungi are inhaled by man daily although filtered at various regions of the respiratory tract depending on particulate size.

1.1 Filtering airway anatomy and physiology in relation to fungi

The nasopharynx traps most particles greater than 20 μm in size (Gregory, 1973). The nasal hairs initially carry out large particle filtration before smaller sizes are deposited by impaction in the nasal passage due to changes in airflow direction within the nose. The mucus lining the nasal passage further helps in their retention by trapping the particles following deposition of particulate matter. Only the largest fungal spores are deposited here in the upper respiratory tract.

The lower respiratory tract, comprising of the conducting passages beyond the larynx, is subject to the deposition of smaller particle sizes varying between 2 μm and 20 μm. This takes place resulting from the increasing air resistance that arises from the dividing bronchi and resulting drop in air speed. Indoor fungi especially *Aspergillus* and *Penicillium* spores with their small spore sizes, 2-3 microns, are therefore optimally placed to bypass most of these filtering mechanisms and reach the medium and small airways. Airway temperature averages between 32.5±0.5°C in the upper trachea to 33±0.5°C in the sub segmental bronchi [6].

The interplay between the pathogenic properties of the fungi and the host response to the presence of fungi in the airways is central to mediating the pattern of disease that develops. This includes;

- rhinitis
- asthma
- hypersensitivity pneumonitis
- allergic bronchopulmonary mycoses (ABPM).

Allergy to fungi is thought to arise from an exaggerated Th2 response to fungal proteins with exposure either from inhaled spores or airway colonisation.

Over 80 genera of fungi have been associated with fungal allergy [7]. Testing this number of allergenic fungi in any panel of skin test reagents is not feasible not least because limited reagents are available but as well the cost involved. Testing has widely been based on the range of known allergic fungi in environmental air sampling and a consensus that skin-test panels should include, at the minimum, *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum* and *Penicillium chrysogenum* individually than in a mixed mould panel. These fungi are frequently present in both the indoor and outdoor airborne environment year round [8]. Trichophyton, though not airborne, is thought to also to play an important role in asthma[9].

It is increasingly recognised that fungi play a significant role in airways disease beyond what has traditionally been perceived as only an infrequent and exaggerated response in ABPA and its feared consequences of airway damage and respiratory failure [5]. Mindful that airway colonisation could be a source of on-going exposure, antifungal therapy has been trialled with varying success in a range of fungal-associated airways disease [literature table 2] though without defining the lower respiratory tract mycobiome in these various conditions before or after treatment. Traditionally isolating fungi from sputum has been challenging either due to the small volumes obtained from the healthy airway and the consequent need to obtain samples by invasive methods using bronchoscopic examinations, or methods intrinsic in culturing fungi. The techniques of culturing fungi have changed very little over the past half century.

The relationship between colonisation, sensitisation, airway inflammation and airflow obstruction therefore remains unclear.

This thesis will explore the role of fungal colonisation and IgE sensitisation in patients with asthma, employing an enhanced and focused culture technique in isolating *Aspergillus*

fumigatus, the most prevalent of indoor fungi and detailing other fungi present. It will provide evidence of a link to persistent airflow limitation and further explore the benefit of eradicating fungi colonising the airway, using Voriconazole, on asthma control, quality of life and exacerbations.

1.2 Fungi and the aerospora

Atmospheric particulate matter can harmfully affect human health as well as impacting on the both climate and precipitation.

The human airway mucosa is sensitive to air pollution, specifically the fine particles of the airborne environment that include mineral dusts, gases and organic matter. A large proportion of this aerosol particulate mass is comprised of fungal spores.[10]

Fungal fragments derived from fragmented conidia or hyphae, which are not culturable, are of a similarly large proportion of airborne biomass and seen in even higher concentrations than conidia of individual allergenic fungi [11].

Overall, fungi, their spores and fungal fragments are the most ubiquitous microbes in the airborne environment.

As humans, we breathe more than 20,000 times a day, inhaling on average between 10-20 m^3 of air, of which fungal spores range from less than 100 to more than 10^5 spores/m^3. Such exposure however seldom results in disease. Both epithelial and secretory cells normally protect against inhaled spores through a mucous barrier in the intact respiratory tract. This starts from the nose. Over 40% of nasal brush samples in a study of healthy volunteers demonstrated recoverable fungus [12].

The normal airway barrier of the lower respiratory tract with trapped substances is cleared through the lining mucociliary escalator serving the conducting airways with coughing as a

back-up system when this mucociliary clearance fails [13]. This clearance has been shown to be impaired by a reduction in ciliary beat frequency on epithelial cells in the presence of *Aspergillus fumigatus* and *Alternaria alternata[14]*. Alveolar macrophages finally provide the last defence against conidia[15, 16] capable of ingesting and lysing conidia primarily using hydrogen peroxide with neutrophils acting against products of germination[17]. These processes can occur even in the absence of opsonins or activation. Both oxidative mechanisms and defensins appear to be involved. Steroids suppress this defence, in part likely by their suppression of the oxidative burst and possibly by decreasing cellular mobilization [18].

Levels of fungal spore exposure naturally vary with the environment. Indoor levels, though overall constant, are usually lower than that outdoor but may be increased in damp buildings by unintended fungal growth. (Flannigan, Samson, Miller, 2001). Outdoor levels also vary with the season of the year and climate. Fungal spores are significantly increased during thunderstorms and have been linked to worsening asthma symptoms [19].

Most humans spend the majority of their time indoors. Here, *Cladosporium* spp, *Penicillium, Aspergillus* and *Alternaria* constitute most the fungal species present in most homes.[20]

Importantly though, relatively few species of fungi are both small enough to penetrate the lower airways and grow at body temperature. While members of both the *Aspergillus* and *Penicillium* genera display these abilities, the commonest fungus causing disease in man is *Aspergillus fumigatus*.

1.2.1 Aspergillus *fumigatus*

A. fumigatus is one of over 180 species of the genus *Aspergillus*. The name is derived from the Latin word *asperge* (to scatter). It is from the fungal family Trichocomaceae. It can be

readily cultured between 20 to 52°C, though optimally grows at 37°C, close to body temperature.

Its spores cannot be distinguished from other species of *Aspergillus* and *Penicillium* by morphology alone, but it has a characteristic morphology in culture that makes it relatively easy to identify. *A. fumigatus* causes a range of disease thought to arise from the interaction between the fungus and host response. The presence of *A. fumigatus* mycelia trapped in luminal mucus that is thought to release allergens that are processed by antigen-presenting cells bearing HLA-DR2 or -DR5 and presented to T cells within the Broncho alveolar lymphoid tissue (BALT). *A. fumigatus* is the main causative agent in ABPA. It is known to colonise lung cavities giving rise to a fungal ball (Aspergilloma), causes a necrotising pneumonia (chronic pulmonary aspergillosis) and is an invasive pathogen in immunocompromised individuals.

These diseases are thought to evolve from colonisation of the respiratory airway leading to an allergic response with airway inflammation, hypersecretion of mucus and secondary obstruction of the airway. Subsequent cycles of inflammation, infection and tissue damage eventually lead to bronchiectasis and in severe cases respiratory failure.

1.2.2 Fungal spores and fragment size

Epidemiologic studies on particulate matter (PM) concentrations have focused on PM10 (particulate matter < 10 pm in aerodynamic diameter) because of their ability to enter the respiratory tract. Fungal spore size and morphology vary widely. They also depend on climatic conditions. Below are 5 of the most common airborne allergenic fungi [21]. Of note, fungal fragments are released in much higher numbers (up to 320 times) than the spores [11].

Fungi	Spore size	Concentrations released at Fragment size 0.3 µm[22]
Aspergillus fumigatus	2–3.5*µm*	11 to 320 times higher than those for spores of *A. versicolor*
Penicillium chrysogenum	2.5–2.5 *µm*	7 to 270 times higher than those for spores of *P. melinii*.
*Cladosporium**	3–11 × 2–5 *µm*	17 to 170 times higher than those for spores of *C. cladosporioides*,
*Alternaria alternate**	18–83 × 7–18 *µm*	N/A
Botrytis	6-7 x 10-12 µm	N/A

*Outdoor fungi

1.3 Asthma, atopy, fungal sensitisation and Allergic bronchopulmonary aspergillosis

Asthma is widely regarded as a chronic inflammatory respiratory disease manifesting in variable airflow obstruction and symptoms of cough, wheeze, and dyspnoea. This definition continues to undergo refinement in concert with greater understanding of its pathophysiology, immunology, and pharmacology. Pathologically, the infiltration of airway tissues with increased numbers of eosinophils, a hallmark of allergic disease, is also seen in asthma[23]. While asthma is a heterogeneous disease with variable presentation, response to treatment and prognosis most cases of asthma are thought to be caused by an exaggerated Th2 lymphocyte response to inhaled proteins from non-pathogenic sources such as house dust mite faeces, pollens, animal danders and fungal spores [24]. The reason why some people develop Th2 responses to aeroallergens is complex with levels of exposure, a strong familial predisposition, a general bias towards Th2 immune responses in industrialised environments and a loss of immune regulation thought to be at the heart of the diathesis.

1.3.1 Definition

This allergic response to a variety of environmental antigens[25] has seen efforts focus at both identification of these and measures at their avoidance. This is central in the management of Farmers lung due to primarily thermophilic *Actinomyces* species. Unfortunately, however, to-date, most studies of allergen avoidance have demonstrated only a modest effect when specific measures have been employed against specific allergens[26, 27]. In distinction to other common allergens such as house dust mite, dog, and cat dander, some fungi easily grow in the airway mucosal surfaces and are capable of dynamic allergen expression during the phases of fungal growth. But while fungal exposure is universal, sensitisation and disease are not.

Asthma affects about 300 million people worldwide with a 15.3% prevalence in England [28]. The mainstay of asthma therapy is inhaled corticosteroids and long acting β2 - adrenoceptor agonists (LABAs) with which most patients achieve good disease control. These therapies, however, unfortunately fail to afford protection in a significant proportion of patients with more severe disease including a tendency for severe exacerbations.

5-10% of these patients are labelled as having severe asthma defined as "asthma which requires treatment with high dose inhaled corticosteroids plus a second controller (and/or systemic corticosteroids) to prevent it from becoming 'uncontrolled' or which remains 'uncontrolled' despite this therapy" [29].

Difficult-to-treat asthma may also be characterised by poor symptom control, persistent airflow obstruction, and or recurrent exacerbations, including fatal or near-fatal episodes, despite high medication requirements to maintain disease control, which itself too often complicates the illness[30]. The wide range of clinical presentations and response to therapy in severe asthma further reflects the heterogeneous nature of asthma in general [31, 32].

1.3.2 Characteristics of disease

Historically, attempts at characterising the heterogeneity in asthma have been made based on specific features such as the age of onset, nature of airflow obstruction, pattern of exacerbations, or inflammatory profile.[2, 33] recently confirmed in cluster analysis models[3]. In an attempt to further address the multiple dimensions of the disease, phenotypes and endotypes of asthma have highlighted the importance of atopy in asthma though unfortunately to-date, have also failed to fully assess fungal atopy in part due to limitations in testing and an under-appreciation of its importance[3, 34].

Various medical associations are unequivocal in recognising the importance of fungi as sensitisers and ability to exacerbate allergic asthma (American College of Occupational and Environmental Medicine[35], European Academy of Allergy and Clinical Immunology [36], Institute of Medicine, American Academy of Allergy, Asthma and Clinical Immunology and American College of Medical Toxicology[37]).
Amongst those with persistent asthma requiring specialist referral, 20–25% have skin-test reactivity to *Aspergillus* or other fungi [38]. Additionally such patients appear to be at increased risk of hospital and ITU admissions [39].

Yet the true prevalence of fungal sensitisation may still be underestimated due to sole sensitisation testing [40] and a lack of appreciation of fungal allergy[41].

1.3.3 Range of fungal exposure

Studies in fungal allergy have broadly been based on the range of known allergic fungi in environmental air sampling that are frequently present in both the indoor and outdoor airborne environment year round [8] and most skin-test panels are restricted to, *Alternaria*

alternata, *Aspergillus fumigatus*, *Cladosporium herbarum* and *Penicillium chrysogenum*.

There is however a broad clinical spectrum in hypersensitivity responses to fungi in patients with asthma. Allergic bronchopulmonary aspergillosis (ABPA), represents one such extreme of a florid hypersensitivity expression primarily occurring in patients with asthma (1%–2% of asthma patients) but also with cystic fibrosis (CF; 1%–15% of CF patients) [42]. It also provides an ideal model of the complex interaction between airborne fungi in the environment, the respiratory tract and immune response in particular to antigens released by *Aspergillus fumigatus* thought to result in permanent airway damage long-term.

1.3.4 Definition of ABPA

ABPA itself has undergone many revisions of its definition since its first description in 1952 by Hinson et al. [43] in concert with developments in immunology and the discovery of IgE in the 1960's.

Three patients with recurrent episodes of wheezy bronchitis, fever, sputum production, peripheral blood eosinophilia and chest X-ray shadowing were originally described. *Aspergillus fumigatus* was isolated from their sputa. Demonstration of fungi and allergy have since formed part of the diagnostic criteria, however, in part due to technical difficulties in culture, the importance of this causal relationship has been overlooked instead leading to increasing reliance upon the demonstrable host response manifested in an elevated total and specific IgE.

After its original description, there continues to be no universally agreed criteria of ABPA (literature table 1). Although only an estimated 8% of asthma patients fulfil The Rosenberg-Patterson or Greenberger criteria [44], it remains one of the most widely accepted benchmark

reliant upon criteria demonstrating mainly allergy and damage as measured by total IgE ≥ 417KU/ml, elevated specific IgE, *Aspergillus* IgG and radiological changes of bronchiectasis.

Similar response mechanisms underlying asthma and bronchiectasis are thought to occur with T-helper type 2 lymphocytes (Th2) releasing IL-5 and orchestrating the eosinophilic and mast inflammatory infiltration while IL-8 gives rise to migration of neutrophils into the airways. Additionally, proteolytic enzymes and mycotoxins released by the fungi lead to airway damage and mucus plugging.

Literature Table 1-Diagnostic criteria used in allergic bronchopulmonary aspergillosis description and studies.

	Patients	Asthma	Blood eosinophilia	Sputum Eosinophil	Radiographic	Fungal culture	Total IgE	SPT	*Aspergillus* IgE	*Aspergillus* IgG	*Aspergillus* Precipitants
Hinson et al. 1952[43]	3	May be	Yes >1		Evidence of collapse and consolidation in different parts of the lungs,	purulent sputum containing characteristic "plugs " and *A. fumigatus*	Not available	no	Not available	Not available	Not available
Henderson et al. 1968[45]	22	yes	Yes >0.5	no	transient lung shadows in different sites,	sputum containing 'abundant fungus'	Not available	yes	Not available	Not available	supportive
Pepys et al 1977[46]	111	yes	yes	yes	Previous episode of transient shadow in chest radiograph with blood eosinophilia	Supporting evidence	Not available	yes	Not available	Not available	Supporting evidence
Rosenberg-Patterson 1977[44]	20	yes	yes	no	History of pulmonary infiltrates (transient or fixed) Central bronchiectasis	Supportive With history of expectorated brown plugs	Elevated (Mean values>1000 IU)	yes	no	Not available	yes
Stevens 2000[47]	28	yes	yes	no	Pulmonary infiltrates; any of the following central bronchiectasis, ring markings, band or glove shadows, upper lobe fibrosis	no	>400 IU or >250 IU with evidence of fluctuation with disease activity	Yes	no	no	
Wark 2003[48]	29	yes	no	no	Central bronchiectasis on HRCT- ABPA-CB Those without- ABPA-S	no	>1000 IU	yes	yes	yes	no
Greenberger 1991 2002[49]	19	yes	No*	no	ABPA –CB Central bronchiectasis (inner two thirds of chest CT field) ABPA-S No radiological abnormality Chest roentgenographic infiltrates*	no	Total serum IgE concentration >417 kU/L (1000 ng/mL)	yes	yes	+/-	Supporting evidence
Agarwal 2009[50]	126	yes			Pulmonary infiltrates (fixed/transient); and proximal or central bronchiectasis seen on high-resolution CT (HRCT) scan	no	elevated total IgE level (*ie*, > 1,000 IU/mL)	yes	yes		yes

*not essential criteria/minor

A peripheral blood eosinophilia and *A. fumigatus* cultured in sputum are not included the unusually stringent criteria set out by Greenberger and colleagues [49] which are to a degree arbitrary and not based on any clear-cut relationship with either severity of disease, the amount of *Aspergillus* colonising the airways or the response to anti-fungal therapy .
Despite this, additional classifications have continued to be described in the effort to earlier address and prevent the significant damage from long-term on-going disease. These various subtypes such as ABPA-S (without bronchiectasis) have largely been viewed as milder forms while the spectrum of fungal allergy is increasingly being appreciated.
In recent years, the term severe asthma with fungal sensitisation (SAFS) has been coined by Denning et al.[5] to describe people with severe asthma who are IgE sensitised to one or more fungi but don't fulfill the criteria for ABPA in particular with a total IgE of <1000 IU/L which has been adopted by some authorities rather than the lower level proposed by Patterson. The clinical and diagnostic manifestations of SAFS are thought to arise from an allergic response to multiple antigens expressed by *A. fumigatus* and possibly other fungi, colonizing the bronchial mucus without however the florid TH_2 response found in ABPA.

1.4 Persistent airflow obstruction, airway inflammation and airway damage

Although asthma is traditionally defined by variable airflow obstruction and a return to normal lung function with optimal treatment, many patients, particularly those with more severe asthma and despite optimal therapy are left with significant irreversible airflow obstruction.
Though no standard definition of this exists, this persistent airflow obstruction as measured by the post-bronchodilator forced expiratory volume in one second (FEV_1) is an important

recognised facet of defining refractory asthma[30], clinically similar to that seen in moderate-severe COPD with dynamic hyperinflation limiting exercise performance. Estimates of 16% - 23% of patients with asthma have been reported to have persistent airflow limitation when defined as an FEV_1 <80% and <9% reversibility[51, 52].

Exacerbations are key events seen in both conditions contributing to much of the morbidity seen, together with the associated accelerated decline in lung function that follows[53, 54].

1.4.1 Airway inflammation

Eosinophilic airway inflammation is recognised to importantly relate to asthma exacerbations [55] and steroid responsiveness [56] while neutrophilic [57, 58] airway inflammation- a common feature of smoking related chronic obstructive pulmonary disease, has been correlated to limited steroid responsiveness [59].

Both eosinophilic and neutrophilic inflammation are independently associated with abnormalities of FEV_1 in asthma [60] as does gender[61], smoking[62], (20-30% of asthma are current smokers), and genetics factors with ADAM 33 polymorphisms [63].

Fungal colonisation has been associated cross-sectionally with impaired lung function in patients with cystic fibrosis where a positive culture of *Aspergillus fumigatus* was also found with an increased risk of hospitalisation [64]. In relation to mould and dampness in the home, the longitudinal European Community Respiratory Health Survey ECRHS II noted that women with dampness at home had an additional decline in forced expiratory volume in 1 second (FEV1) of −2.25 ml/year (95% CI −4.25 to −0.25), with a significant trend in increased lung function decline in relation to the dampness score (p=0.03). This was found independent of self-assessed mould levels and limited fungal atopy testing solely to specific IgE *Cladosporium [65]*.

1.4.2 Airway remodelling

The pathological basis for persistent airflow limitation is thought to result from both structural and functional changes referred to as airway remodelling. This arises from epithelial damage, sub-basement membrane thickening, and smooth muscle hypertrophy of both the large and small airway walls [66].

Using high-resolution computed tomography (HRCT), thickening of the right upper lobe apical segmental bronchus (RB1) has been shown to correlate with airflow limitation [67]. Other abnormalities have been reported in 80% of subjects with severe asthma and often coexisted with bronchial wall thickening (62%), bronchiectasis (40%), and emphysema (8%)[68]. Similar findings have been associated with APBA [69], with bronchiectasis being traditionally an essential diagnostic criteria. In early descriptions, two of the five clinical stages in ABPA, glucocorticosteroid-dependant and end-stage fibrotic ABPA were associated with a decline in lung function and respiratory failure [70, 71].

What remains clear is that there continues to be an unmet need in understanding the aetiology, complexity and phenotypic heterogeneity of airway disease [72] and persistent airflow obstruction and bronchiectasis at the very least represent airway damage in response to a variety of factors that cannot be reversed.

1.5 Critical review of current treatments for asthma and fungal allergy

Asthma costs the NHS an estimated £1 billion a year of which around 80% of the spending on treating those with asthma is spent on the 20% with the severest symptoms [73].

Little doubt remains to efficacy of inhaled and systemic glucocorticoids in reducing both the blood and airway eosinophilia present in asthma with consequent improvements in symptoms, lung function,[74, 75] and attenuation in its decline.[76]. Despite the additional use of long acting bronchodilators, theophylline, anti-IgE therapy, patients with severe asthma continue to experience poor symptom control, a poorer quality of life consequent days off school or work and consequent financial loss.

There are a number of possible reasons for this limitation in the efficacy of traditional therapies, including 1) a failure of traditional guidelines to reflect patients' own priorities of asthma control; 2) inappropriate timing of the introduction of treatments or the use of inadequate doses; 3) a poor understanding of different asthma subgroups, which due to their distinct pathophysiology have different pharmacological responses; 4) poor adherence to therapy [77]. The concept of refractory asthma is important because this group of patients is likely to be the group for which novel approaches, including specific targeted therapies, are likely to be particularly needed.

Antifungal therapy in asthma potentially offers an additional therapeutic strategy in the armamentarium against asthma in those with evidence of fungal involvement either through IgE sensitisation or colonisation. There have however been very few good quality clinical trials of anti-fungals in allergic airways disease. A recent Cochrane review identified only three controlled trials with a robust study design in ABPA [78]despite this being a potentially

invaluable form of treatment.

It is likely that *A.fumigatus* colonises airways damaged by chronic inflammation. This concept would be consistent with the high incidence of ABPA in cystic fibrosis [42]. It is assumed that fungal colonisation and not invasive disease, then leads to further lung damage and frequent exacerbations of asthma with the possibility of progressive lung damage.

1.6 Steroid effect on fungi

The earliest therapies against fungal associated airways disease relate to ABPA. Predating the widespread use of inhaled glucocorticosteroids [2]and despite its high potential toxicity and frequent relapse on discontinuation, treatment for ABPA with oral steroids eventually emerged from mainly uncontrolled published series and small case trials [79-81] of patients on prolonged oral steroids used with the aim of controlling symptoms guided by immunologic and radiological parameters. Both fungi and humans are eukaryotes unlike bacteria and are similar at the cell biological level. Fungal cell membranes however contain ergosterol absent in those of animals and humans. There are at least four classes for antifungal drug therapies used in clinical practice- Polyenes (including Nystatin), Azoles (Imidazole, triazole, and thiazole) Allylamines and Echinocandins. All work targeting fungal cell wall membrane formation or stability. Azole antifungal drugs which are the most widely used generally inhibit the cytochrome P450 enzyme lanosterol 14 α-demethylase; the necessary enzyme that converts lanosterol to ergosterol. The precise mechanisms of antifungal action in asthma however remain unknown. Though it is generally accepted to have some anti-microbial effect and in mediating a modified immunological response, concerns remain about the azole–corticosteroid interaction as seen in earlier trials with adrenal suppression.

Itraconazole, Voriconazole and Posaconazole, which all belonging to the triazole class, have been utilised in treating invasive fungal disease [82, 83] and have paralleled their use in trials for allergic airways disease in an effort to eradicate colonisation [46].

1.7 Itraconazole

1.7.1 Treatment of ABPA

Earlier antifungal therapy in ABPA proved initially disappointing. Neither nebulised Nysatin nor Ketoconazole (a first-generation oral azole antifungal) demonstrated any clinical benefit[84, 85]. In comparison itraconazole has fewer side-effects and a wider spectrum of activity than Ketoconazole.

Initial case studies using itraconazole suggested some benefit though the numbers were overall small. The earliest case report by Denning et al.[86] used itraconazole in 6 patients (3 with cystic fibrosis and ABPA) demonstrating benefit in all patients including clearing of *A.fumigatus* from the sputum in three of four patients demonstrably colonised.

Subsequently itraconazole was used in a multi-centre (13 centres) trial randomised controlled trial, involving 55 patients with ABPA. Over a 16-week period, patients in the active group received 200mg twice daily during which steroid reduction was attempted. This was followed by an open study for a further 16 weeks on 200mg itraconazole daily.

While there was an overall improvement in total number of criteria examined, there was no significant improvement in any one criteria [47]. Another randomised single centre study also used itraconazole 400mg daily for sixteen weeks [48] in 29 subjects with the primary outcome measuring the response of induced sputum eosinophil counts in which there was a significant fall.

There was also a significant reduction in severe exacerbations with a mean of 0 exacerbations in the treatment group and 1.5 in the placebo group ($P<0.03$) which was a marked effect given the relatively short period of study and the small numbers of subjects. Severe exacerbations are closely related to eosinophilic airway inflammation [55], and the striking reduction in exacerbations seen in the paper by Wark et al would be consistent with the reduction in eosinophilic inflammation seen in the study.

1.7.2 Severe asthma with fungal sensitisation

The Fungal Asthma Sensitization Trial (FAST)[87] studied patients with severe asthma who were sensitised by a skin prick or radioallergosorbent testing to one or more fungal allergens and did not fulfill the criteria for allergic bronchopulmonary mycosis. Treatment with oral itraconazole 200 mg twice daily/placebo for 32 weeks resulted in clinically significant improvements of asthma quality of life scores as well as rhinitis and morning peak flows. Although active against some species of *Aspergillus*, itraconazole is not active against all the fungal species that the human airway is constantly subjected to. It's microbiological activity is further limited by variable absorption, antimicrobial resistance in Aspergillus species [88] and the need for monitoring[89] in contrast to newer triazoles such as Voriconazole have better oral bioavailability.

1.8 Voriconazole

In a collection of *A. fumigatus* clinical isolates taken from over 100 patients between 1945 and 1998, three isolates (recovered from a lung transplant patient) were itraconazole resistant; all three were voriconazole susceptible [90]. Voriconazole has since become the new standard of care for the treatment of invasive aspergillosis. It has, in contrast to itraconazole a structurally-dissimilar triazole, superior oral bioavailability (up to 96%) and an extended

spectrum of antifungal activity against opportunistic fungal pathogens. There have been case reports and uncontrolled series suggesting its benefits [91] however to date, no randomised trial has examined its utility or use in clinical practice. One problem with any antifungal is that it is unlikely that improvement in lung function can be achieved if fixed airflow obstruction is already present. To-date however, no trial has assessed the effect of antifungal treatment on eradicating fungi from the asthmatic airway in non-invasive disease bringing to question the mechanisms of efficacy in treating ABPA or any fungal related airways disease.

In summary, despite recognition of fungal allergy, the plausible mechanisms of action and growing body of evidence suggesting benefit of antifungal drug therapy, there have to-date been few trials defining the endotype of fungal associated asthma. The optimum antibiotic duration remains unclear as does the antimicrobial effect itself of the drug on airway fungal culture.

Literature Table 2

Year	Antifungal	Trial type	Duration	Disease	Primary Endpoint	Number of patients	On oral prednisolone	Outcome	Benefit	Ref
1990	Natamycin 5mg	RCT, Placebo controlled	12 months	ABPA	Assess the steroid sparing potential of natamycin.	25	yes	Median prednisolone dose reduction each group natamycin 2.25 mg Vs. placebo 2.5 mg).	no	[92]
1987	Ketoconazole 400mg/day	open trial of nine cases	12 months	ABPA		9		All relapsed during treatment with radiological changes or isolation of *A fumigatus* from sputum	no	[85]
1987	Ketoconazole	RCT, Placebo controlled	12 months	ABPA (Rosenberg)		7	yes	lower symptom scores than placebo, Fall in Serum *Aspergillus* IgG	yes	[84]
2000	itraconazole 200mg BD	RCT multicentre, placebo controlled	4 months	ABPA (Stevens)	Reduction of at least 50 percent in the corticosteroid dose, a decrease of at least 25 percent in the serum IgE concentration, and one of the following: an improvement of at least 25 percent in exercise tolerance or pulmonary-function tests or resolution or absence of pulmonary infiltrates.	55	yes	46% vs 19% improvement overall, but failed to reach statistical significance for each of the outcomes when examined separately.	yes	[47]
2003	itraconazole 200mg BD	RCT	4 months	ABPA (Wark et al.)	Reduction in eosinophilic airway inflammation	29	35%	Decrease in sputum eosinophils of 35% per week, with no decrease seen in the placebo arm (P < .01).	yes	[48]
1991	itraconazole 200mg BD	Uncontrolled	3.9 months	ABPA Patterson and Greenberger)		6 (3 with Cystic fibrosis)	66%	Improvement in lung function, Fall in Total IgE	?yes	[86]
2009	itraconazole 200mg BD	RCT	32 weeks	SAFS	Asthma Quality of Life Questionnaire (AQLQ) score	58	7%	Improvement in AQLQ, rhinitis and peak flows	yes	[87]

Hypothesis

- Fungal colonisation and allergy both play an important role in asthma and the associated airflow obstruction.
- Voriconazole will be effective at lowering or eradicating fungal colonisation and lead to consequent improvements in asthma quality of life measures as well as a reduction in airway inflammation.

Aims

- To assess the validity of non-invasive sampling of the airway and enhanced fungal culture techniques in assessing fungal colonisation
- To define the role of airway colonisation and its relationship to fungal allergy in the asthma population.
- To detail the filamentous fungal biota detected in the lower airway in asthma
- To define the clinical characteristics of *Aspergillus* -associated asthma
- To conduct a randomised placebo controlled trial to assess the effectiveness of Voriconazole in *Aspergillus* associated airways disease.
- To present novel data on the natural history of fungal airways disease and exacerbations

2. Methods

These methods apply to the three chapters. Further details are given in each chapter as required.

2.1 Patient and Control recruitment

2.1.1 Control subjects

Healthy volunteers were also recruited through local advertisements in the hospital or newspaper.

2.1.2 Selection of patients

We studied two populations with asthma- those with and without evidence of fungal allergy. Patients with a diagnosis of asthma were invited to participate in the research studies. They were identified when they attended any respiratory clinic by respiratory physicians or though advertisements from 2007 -2011.

2.1.3 Inclusion criteria

This included an age ≥18 years, a diagnosis of asthma based on clinical grounds by an experienced physician supported with either evidence of airflow obstruction on pre-bronchodilator FEV_1, historical evidence of >12% variability in their FEV_1, a history of significant bronchodilator reversibility to 200 μg of inhaled salbutamol after 15 mins and or evidence of hyper-responsiveness on methacholine challenge with PC20 <8mg/ml.
18 healthy volunteers served as controls and included members of the public and staff at Glenfield hospital.

2.1.4 Exclusion criteria

This was limited to pregnancy, a diagnosis of chronic obstructive pulmonary disease, a medical condition that would increase the likelihood of an adverse reaction to Voriconazole and treatment with an anti-fungal agent in the twelve months prior to entry into the study.

2.2 Lung function testing

2.2.1 Pre & Post broncho-dilator FEV_1

Spirometry was performed using a dry bellow wedge spirometer (Vitalograph, Buckinghamshire, UK) as the best of successive readings within 100 mls. Reversibility was then measured as % change in FEV_1 15 min after nebulised 200μg Salbutamol.

2.3 Sputum induction, collection and processing

Subjects were initially administered Salbutamol 200 mcg by inhalation, 10-30 minutes before the procedure to minimise bronchoconstriction also recording the post bronchodilator FEV_1 measurement obtained prior to starting the induction.

3, 4, and 5% saline was inhaled in sequence for 5 minutes via an ultrasonic nebulizer (Medix, Harlow, UK; output 0.9 ml/min; mass median diameter, 5.5 μm). After each inhalation subjects blew their noses and rinsed their mouths to reduce nasal and oral contamination before they expectorated into a sterile pot.

The procedure was stopped if the FEV_1 measured after each inhalation fell by greater than 20%; if the fall in FEV_1 was between 10-20%, induction was repeated at the same concentration of saline.

The sterile pot was emptied onto a petri dish and sputum plugs selected from it and gathered into a large condensed mass by small circular movements using blunt forceps.

Sputum plugs were then used in two parts- one for cytospins to obtain a differential inflammatory cell count and the second for mycological culture.

2.3.1 Processing

To obtain the total and differential cell count, the sputa was weighed and four times its weight in 0.1% dithiothreitol (DTT, Sigma, Poole, UK), freshly diluted from a stock solution of 1% using Dulbecco's phosphate buffered saline (D-PBS, Sigma, Poole, UK, cat no: D-8662), was added. (e.g. 4mL DTT per gram of selected sputum). The sputa was then dispersed by gentle aspiration into a Pasteur pipette, vortexed for 15 seconds then placed a rocking bench, Spiromix for 15 mins. An equal volume of D-PBS was added (i.e. If 2 ml of 0.1% DTT was added to sputum, now add 2 ml D-PBS) and vortexed for a further 15 seconds. This solution was passed through a 48 p.m nylon gauze (kindly supplied by Dr lan Pavord) placed in a funnel, pre-wet with D-PBS. The filtrate was collected in a clean 15ml centrifuge tube with volume of this cell suspension noted.

The total cell count and cell viability was assessed using a Neubauer haemocytometer.
The haemocytometer was flooded with 10µL of the filtrate mixed with 10µL of 0.4% trypan blue (Sigma, Poole, Dorset) and all cells were counted in the four corner squares of the haemocytometer to include viable, non-viable and squamous cells
The total number of cells and total cell counts were calculated using the following formula:

- Total number of cells ($x10^6$) = [mean number of cells counted/square × 2 × filtrate volume (mL)] / 100
- Total cell count ($x10^6$/g sputum) = [mean number of cells counted/square × 2 × filtrate volume (mL)] / 100 × selected sputum weight

The differential cell count was obtained by again diluting the sputa to a concentration of $0.5x10^6$cells/ml for cytospin preparation and stained with Romanowski stain. The cells were counted and expressed as a percentage of at least 400 inflammatory cells.

Mycological culture was performed by inoculating aliquots of an approximately 170 mg (± 80 mg) of undiluted sputum plug onto a potato dextrose agar (PDA) containing 16 μg/ml chloramphenicol, 4 μg/ml gentamicin and 5μg/ml fluconazole. Plates were incubated at 37°C and frequently inspected for up to 7 days. Subcultures of filamentous fungi were produced and *A. fumigatus* colonies identified based on criteria of their macroscopic and microscopic morphology.

Genetic testing was additionally used to corroborate or identify indeterminate species. This was performed sequencing either the large subunit (LSU) or the internal transcribed spacer (ITS) region 1 of the nuclear ribosomal operon, amplified by PCR. A DNeasy plant mini kit (Qiagen, West Sussex, UK) was used to extract total genomic DNA from pure fungal subcultures following manufacturer's instructions after a bead-beating step (BioSpec mini bead beater, Bartlesville, OK, USA). Sequences were determined using BigDye-Terminator v3.1 chemistry with 3730 sequencers (Applied Biosystems). Sequence data was manually inspected and trimmed, with closest taxonomic match determined by comparison with known sequences in GenBank (March 2010) using the BLASTN database search method.

2.4 Atopy assessment

2.4.1 Skin prick testing- Mould panel

Skin prick testing was performed to a panel of common aeroallergens and an extended fungal panel. A positive response, defined as a weal 3 mm equal to or larger than negative control (saline) for one or more allergen constituted allergy.

The 4 common UK aerollergens tested were: cat fur, dog dander, grass pollen and *Dermatophagoides pteronyssinus.* The fungal panel included *A. fumigatus*, *Alternaria alternata*, *Botrytis cinerea*, *Cladosporium herbarum* and *Penicillium chrysogenum* (Alk-Abello, Denmark).

2.4.2 Radioabsorbant immunoassay

Total IgE, *A. fumigatus*-IgE (assay detection limits 0.01-100 kU/L, normal reference range 0–0.34) and *A. fumigatus*-IgG levels were measured using the ImmunoCAP® 250 system (Phadia, UK). IgE Sensitisation to *A. fumigatus* was defined as present when *A. fumigatus*-IgE >0.35 kU / L according to manufactures instruction. The IgG group was based solely on an elevated *A. fumigatus*-IgG >40 mg / L to the exclusion of any specific IgE sensitisation (*A. fumigatus*-IgE >0.35 kU / L or positive skin prick test to *A. fumigatus)*

2.5 Quantitative asthma symptom scores

2.5.1 Asthma control questionaire

This is a measure comprised of 6 questions regarding various aspects of asthma symptoms and a seventh measure is of airflow obstruction expressed as a percentage of the predicted FEV_1 which in turn is scaled on a 0-7-point scale. Responses to each of the questions are also logged on a 0-6-point scale reflecting control for the preceding 2 weeks. The score is an

average of the 7 measures. Scores of greater than 1.57 indicate suboptimal control of asthma symptoms and a change of 0.5 points between visits is clinically significant.

Analysis was then performed modifying the ACQ using the five symptom domains and excluding lung function and the frequency of beta agonist which can be confounding due to the effects of behaviour.

2.5.2 Visual Analogue Scale Asthma symptoms

Subjects were asked to plot their perceived control for each symptom of cough, wheeze or breathlessness over the preceding 2-weeks on the scale. Patients graded their asthma symptoms and severity using a horizontal 100 mm visual analogue scale (VAS) from no symptom (0 mm) to the worst ever symptom (100 mm). This scale has previously been the most responsive outcome measure in asthma studies.
Analyses were performed for each symptom independently and for a composite score that was the arithmetic mean of scores for all three symptoms.

2.5.3 Visual analogue scale for Nasal Polyps

This validated 100 mm horizontal visual scale measured symptoms associated with nasal polyps across 5 domains: sense of smell, nasal secretion, pressure over sinuses, nasal obstruction, and headache. Composite scores were calculated as the arithmetic mean of scores for all domains.

2.5.4 Juniper Asthma Quality of Life Questionnaire (AQLQ)

Functional impairments of patients with asthma were measured using a 32-item validated questionnaire.

Each of the 32 items was scored between 1 and 7. Higher scores indicate better quality of life. The scores for the 32 items are representative of 4 domains: symptoms (12 items); activities (11 items); emotion (5 items); and environment (4 items). A composite score is calculated from the arithmetic mean of the domain scores themselves a mean of the items for that domain.

2.6 Data handling and Statistical analysis

All data were entered electronically into a bespoke secure Access database (Microsoft, Redmond, WA) and analysed using GraphPad (Version 5; GraphPad Software Inc, CA, USA) Microsoft Excel and PASW for Windows (Version 18.0; SPSS, Inc., Chicago, IL).

Parametric data was expressed as means ± standard error of the mean (SEM) and analysed using student's t-test, analysis of variance (ANOVA) using Bonferroni post-test for multiple comparisons and linear regression. Non-parametric data was expressed as medians with interquartile ranges (IQR) and analysed using Mann-Whitney, chi-squared and Dunn-corrected Kruskal-Wallis tests.

Chapter 3: The significance of isolating *Aspergillus fumigatus* from the asthmatic airway

3.1 Introduction

Colonisation of the human airway by fungi, predominantly by *Aspergillus fumigatus*, has been demonstrated in both subjects with and without asthma [93], however up to 8% of patients with asthma [94] and 13% of those with cystic fibrosis [95] have been reported to demonstrate a florid hypersensitivity reaction to this, defined as Allergic bronchopulmonary aspergillosis (ABPA). *A. fumigatus* has been cultured from the sputum of these patients. It affects around 40,000 people in the United Kingdom.

Aspergillus spp. are ubiquitous within the indoor and outdoor environment, particularly in soil, decaying vegetation, and water-damaged building materials [49] as well as being airborne. Inhalation of *A. fumigatus* spores can lead to colonisation and, in damaged airways with retained mucus, germination within the bronchial tree through the production of hyphae. In some individuals, this can stimulate a T-helper (Th) type 2–mediated inflammatory response involving $CD4^+$ T cells, IgE, and IgG antibodies [96]. The resultant recurrent airway inflammation, bronchial obstruction and mucoid impaction [97] that follow lead to the development of bronchiectasis and eventually fibrosis as described in ABPA. Detection of *A. fumigatus* in respiratory samples however is only used as a minor diagnostic criterion for ABPA, as isolation of *Aspergillus* from respiratory specimens is unusual using traditional culture methods. Additionally, the significance of this is unknown in patients with asthma who do not fulfil the stringent diagnostic criteria for ABPA.

In an effort to address underlying fungal airway colonisation thought to be providing the continued allergenic stimulus in ABPA, oral antifungals, in contrast to steroids that have been the mainstay of treatment, have consequently been employed. itraconazole, an oral

antifungal, has shown some benefit in this regard with two randomised placebo controlled trials. In a multicentre trial of 55 patients over 16 weeks, improved outcomes in a composite end-point of a reduction in corticosteroid dose, serum IgE concentration, improved exercise tolerance, pulmonary-function tests, or resolution of pulmonary infiltrates were achieved [47]. None of the end-points individually however showed any significant improvement. The second study using itraconazole observed a reduction in sputum eosinophils, fewer exacerbations requiring corticosteroid treatment and reduced serum IgE in a study of 29 patients [48].

Using a focused culture method towards detecting *A. fumigatus* in sputum and sputum induction negating the need for invasive airway sampling, I aimed to detail and define the relationship between the clinical and laboratory features of AFAA. The most striking and unanticipated observation was an association between *A. fumigatus*–IgE sensitisation and evidence of fixed airflow obstruction in association with neutrophilic airway inflammation.

3.2 Methods

3.2.1 Subjects

Patients with asthma were recruited consecutively from respiratory and allergy clinics at Glenfield Hospital (Leicester, UK) from August 2007 to April 2009. Inclusion required a clear clinical history of asthma, with either: airflow obstruction on prebronchodilator FEV_1 and historical evidence of greater than 12% variability in their FEV; or history of asthma with a greater than 12% improvement in FEV_1 15 minutes after 200 μg inhaled salbutamol and/or a provocative concentration of methacholine required to cause a 20% fall in FEV_1 of less than 8 mg/ml at the time of recruitment. Exclusion criteria included a main respiratory diagnosis other

than asthma or inability to produce sputum. Healthy subjects were recruited from staff at Glenfield Hospital. Subject groups were:

- (1) *A. fumigatus*–IgE (>0.35 kU/L or positive SPT to *A. fumigatus* wheal, 3 mm in diameter), irrespective of Aspergillus IgG status;
- (*2*) *A. fumigatus*–IgG positive only (>40 mg/L), IgE negative;
- (*3*) Non-sensitised asthma (negative *A. fumigatus*–IgE and *A. fumigatus*–IgG <40 mg/L); and
- (*4*) healthy subjects.

Asthma severity was assessed using the Global Initiative for Asthma (GINA)

3.2.2 Clinical Assessment

Demographic and clinical data were collected and included: sex, age at asthma onset, asthma duration, smoking history, physiological parameters of lung function using spirometry, sputum differential counts using induction with hypertonic saline, airways hyper-responsiveness using methacholine challenge testing, high resolution CT chest for radiological evidence of bronchiectasis, and a treatment history of prescribed inhaled and systemic corticosteroid therapy. Allergy testing was also performed to common aeroallergens. CF genotype was requested from the local clinical genetics service.

3.3 RESULTS

Patient Demographic Data

TABLE 1: STUDY COHORT CHARACTERISTICS

	Healthy	Asthma *Af*-IgE (+/- *Af*-IgG)	Asthma *Af*-IgG only	Non-sensitised asthma
Subjects, n	14	40	13	26
Male	9	19	5	11
Age, mean (SEM)	33(±2.5)	58(±2.0)†	58(±4.9)†	53(±2.6)†
Never smokers	11	19	7	13
Ex-smokers	3	17	6	13
Current Smokers	0	4	0	0
Age of asthma onset, years*		24 (3-44)	30 (11-63)	39 (23-50)
Duration of asthma, years*		27 (15-45)‡	18 (7-38)	10 (5-28)
Requiring Maintenance oral steroids, n (%)		12 (30%)	6 (46%)	11 (42%)
Prednisolone oral dose (in those on maintenance), median (mg)*		10	10	10
ICS dose, µg day^{-1}*		1000 (800-1000)	700 (400-950)	800 (800-1000)

Definitions of abbreviations: SEM = standard error of the mean; ICS = inhaled corticosteroid
* Median with interquartile range shown in parentheses.
† *p < 0.0001 versus healthy controls by ANOVA*
‡ *p < 0.05 versus non-sensitised asthma by Kruskal-Wallis test*

STUDY COHORT CHARACTERISTICS

TABLE 2: AIRWAY INFLAMMATION AND FUNGAL CULTURE ACCORDING TO *Af* SENSITISATION

	Healthy	Asthma+*Af*-IgE (+/- IgG)	Asthma *Af*-IgG only	Non-sensitised asthma
Sputum culture of *Af*, n (%)	1 (7.1)	25 (62.5)*	5 (38.5)	8 (30.8)
Sputum eosinophils, %†	0 (0-0.8)	2.1 (0.5-6.1)	1.2 (0.5-6.3)	8.7 (0.6-16.6)
Sputum neutrophils, %†	50.7 (29.5-62.8)	80.9 (50.1-94.1)‡	79.5 (50.1-87.5)	49.5 (21.2-71.4)
Blood eosinophil x 10^{9}†	0.1 (0.1-0.4)	1.0 (0.7-1.7)	0.8 (0.6-1.2)	1.0 (0.5-1.5)
Total IgE IU/ml†	36.9 (7.2-52.0)	791 (359.3-2415.0)§	74.5 (36.9-141)	150.0 (82.08-320.0)
Af-IgE >0.35 kU/L, n (%)	0	39 (98)	0	0
Af-IgG >40 mg/L, n (%)	2 (14)	20 (48)	13 (100)	0
Atopy, (%)‖	21	55	38	54
Positive SPT *Af*, n (%)	0	26 (65)	0	0
Sensitisation to other fungi,%	7.1	24.4	0.0	0.0
CF genotype mutations				
Not detected	-	37	13	23
Heterozygous	-	3	0	1

Definitions of abbreviations: Af = Aspergillus fumigatus, GM = geometric mean, SPT = skin prick test
* $p < 0.05$ versus non-sensitised asthmatics by chi squared analysis †Median with interquartile range in parentheses ‡$P < 0.01$ versus nonsensitised asthma by Kruskal-Wallis test. §$P < 0.001$ versus IgG-only and

nonsensitised patients with asthma by Kruskal-Wallis test. ‖Positive SPT test with a wheal diameter greater than 3 mm on exposure to common aeroallergens, excluding *A. fumigatus.*

TABLE 3: AIRWAY DAMAGE ACCORDING TO AF SENSITISATION

	Normal	Asthma *Af*-IgE (+/- IgG)	Asthma *Af*-IgG (only)	Non-sensitised asthma
FEV_1 post BD[*]	109(2)	68(5)[†]	77(7)	88(5)
FEV_1/FVC post BD[*‡]	85(5)	64(2)	64(5)	75 (2)[‡]
FEV_1 post BD with positive *Af* culture[*]	-	69.30 (27.95)	68.60 (21.80)	86.50 (17.83)
Bronchiectasis, n (%)	-	27(68)[§]	5(38)	9(35)

Definition of abbreviations: Af = Aspergillus fumigatus, FEV_1 = percent predicted forced expiratory volume in the first second; FEV_1/FVC = percent predicted FEV_1/forced volume vital capacity; BD = bronchodilator

[*] *Mean values with standard error of the mean in parentheses*

[†] *$p < 0.05$ versus non-sensitised asthmatics by ANOVA*

[‡] *$p < 0.01$ versus asthma Af-IgE and Af-IgG by ANOVA*

[§] *$p < 0.05$ by chi-squared analysis*

TABLE 4: MULTI-LINEAR REGRESSION ANALYSIS PREDICTING POST BRONCHODILATOR FEV1 (% OF PREDICTED) IN SUBJECTS WITH ASTHMA

$R^2 = 0.493$	Unstandardised coefficients	Standardised coefficients	
p = 0.001	B (SEM)	Beta	p
Af primary culture	-14.577 (7.037)	-0.278	0.046
Sputum eosinophils, %*	-13.945 (5.891)	-0.340	0.024
Af sensitisation (SPT or *Af*-IgE)	-18.570 (7.327)	-0.356	0.016
Sputum neutrophils, %	-0.382 (0.151)	-0.393	0.016
Af-IgG	-0.036 (0.102)	-0.048	0.724
Bronchiectasis	9.849 (7.066)	0.188	0.172
Duration of asthma	-0.280 (0.169)	-0.209	0.105

Definitions of abbreviations: SEM = standard error of the mean *Af = Aspergillus fumigatus,* SPT = skin prick test

* Normalised by log transformation.

Clinical and demographic data are shown in Table 1. Healthy subjects were significantly younger ($P < 0.0001$) and had a higher ratio of never smokers to subjects with a smoking history but age, gender and smoking history were otherwise well matched amongst patients with asthma. The majority of participants were no longer smokers.

3.3.1 Airway inflammation and fungal culture according to *Aspergillus fumigatus* sensitisation.

The repeatability of culturing sputum was assessed in 17 patients (8 culture-negative on the first visit). Sputum was obtained on 2 occasions within 6 months in clinically stable patients. Of the nine patients with *A. fumigatus* cultured in sputa, seven remained positive, and seven of the eight culture-negative patients remained negative. This had a kappa value of 0.648 (0.184), regarded as showing substantial agreement [98].

Culture rates of *A.* fumigatus were significantly different across groups ($P = 0.004$). *A. fumigatus*–IgE–sensitised patients with asthma (63%) had significantly higher rates of *A. fumigatus* cultured from sputum compared to non-sensitised patients with asthma (31%) ($P < 0.05$; Table 2). 1 in 13 healthy subjects isolated Aspergillus fumigatus (7.7%). There was no significant difference between *A. fumigatus*–IgG-only–sensitised patients with asthma and *A. fumigatus*–IgE–sensitised patients with asthma or non-sensitised patients with asthma.

3.3.2 Airway Inflammation according to *A. fumigatus* sensitisation

Compared to non-sensitised patients with asthma, differential cell counts of sputum neutrophils were significantly higher in patients with *A. fumigatus*–IgE sensitisation ($P < 0.01$; Table 2)

Elevated neutrophils were also shown in non-sensitised asthma in comparison to healthy subjects ($P < 0.01$). Sputum eosinophils were significantly higher in *A. fumigatus*–IgE–sensitised ($P < 0.01$) and non-sensitised patients with asthma ($P < 0.05$) in comparison to healthy subjects, but did not differ within the asthma groups, concordant with peripheral blood eosinophils, which did not differ significantly between the groups.

Nearly all patients with *A. fumigatus*–IgE–sensitised patients with asthma had elevated *A. fumigatus*–IgE greater than 0.35kU/L by CAP, with 65% having a positive skin prick test to *A. fumigatus* (Table 2). *A. fumigatus*–IgE–sensitised patients with asthma demonstrated significantly higher total serum IgE (IU/ml) than non-sensitised patients with asthma and *A. fumigatus*–IgG-only–sensitised patients with asthma ($P < 0.001$). A total of 48% of *A. fumigatus*–IgE–sensitised patients also had *A. fumigatus*–IgG sensitisation, and 24.4% of *A. fumigatus*–IgE–sensitised patients had positive SPT to other fungi. Mutations in the CF gene (Δf508) were found in four patients, all of whom were heterozygous (Table 2). Four patients with elevated *A. fumigatus*–IgE fulfilled all the major criteria for ABPA.

3.3.3 Regression Analysis of Predictor Variables with Measurements of Lung Function

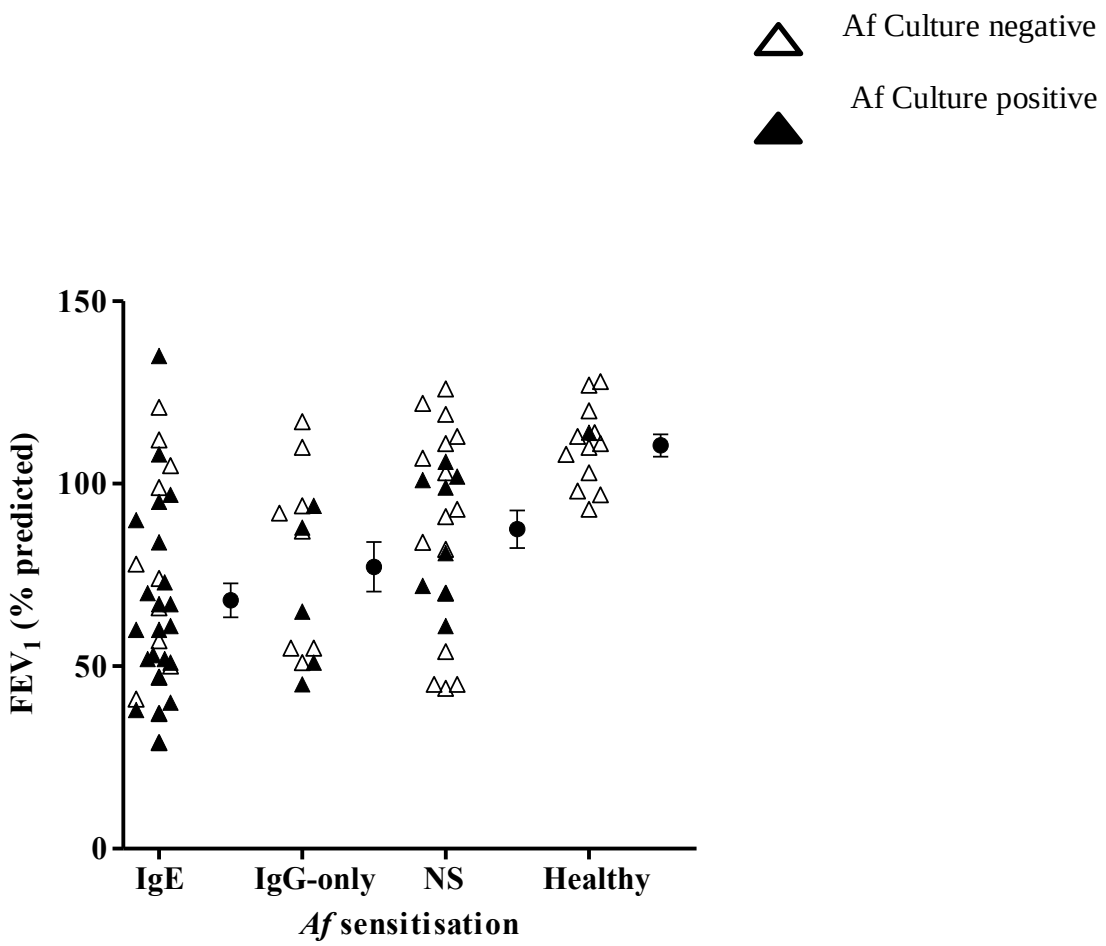
In a multilinear model of lung function, *A. fumigatus*–IgE sensitisation and sputum neutrophil differential cell count were the predictive factors with the greatest statistical significance ($P = 0.016$; Table 4), further supported by positive sputum culture of *A. fumigatus* ($P = 0.046$) and eosinophil differential cell count ($P = 0.024$; Table 4). Although asthma duration and *A. fumigatus*–IgE sensitisation were also shown to be closely correlated, ($r_s = 0.307$; $P = 0.011$) the duration of asthma did not significantly affect the clinical outcome when adjusted for other clinical explanatory variables in the model (Table 4). Removal of the four patients with ABPA,

due concerns to the extent of bronchiectasis itself causing fixed airflow obstruction, further strengthened the model (from $r^2 = 0.493$ [Table 4] to $r^2 = 0.636$) *A. fumigatus*–IgE sensitisation, sputum neutrophil differential cell count, positive sputum culture, and eosinophil differential cell count remain predictors of lung function; however, duration of asthma became a supporting factor (from $P = 0.105$ [Table 4] to $P = 0.04$), with bronchiectasis approaching significance ($P = 0.05$).

3.3.4 Lung Function According to *A. fumigatus* Sensitisation and Culture

Reduced FEV_1 (% predicted, post-bronchodilator) was observed in *A. fumigatus*–IgE–sensitised patients in comparison to non-sensitised patients with asthma, independent of *A. fumigatus* sputum culture ($P < 0.05$; Table 3, Figure 1). *A. fumigatus*–IgE–sensitised patients also showed significantly lower airway reversibility ($P < 0.05$), reduced FEV_1 in the presence of *A. fumigatus* sputum culture ($P < 0.05$), and significantly higher rates of bronchiectasis ($P < 0.05$) in comparison to non-sensitised patients with asthma (Table 3).

Figure 1.



Geometric mean (SEM) FEV1 (% predicted, **postbronchodilator**) according to Aspergillus fumigatus sensitisation in the three asthma groups: A. fumigatus–IgE–sensitised ± A. fumigatus–IgG sensitisation (IgE), A. fumigatus–IgG-only–sensitised (IgG only), and nonsensitised patients with asthma (NS), in comparison to healthy control subjects, in sputum culture-positive (closed symbols) and -negative (open symbols) subjects. Patients with classical allergic bronchopulmonary aspergillosis are represented by squares; all other subjects are represented by triangles. *P < 0.05 versus nonsensitised subjects with asthma by analysis of variance.

3. 4 Discussion

This study provides insight to the relationship between airborne fungi cultured from sputum, specifically *Aspergillus fumigatus*, sensitisation as a presumed response, as well as a reduction in lung function parameters in patients with asthma.

Here, the employment of hypertonic saline to induce sputum, a process demonstrated to be useful in obtaining sputum to quantify and treat airway inflammation in asthma [55], enhances the security of obtaining good quality specimens (sputum plugs) from the lower airway as well as avoiding environmental contamination, mindful of the ubiquity of fungal elements in the aerospora. Comparatively, the standard method in processing sputum mycological investigations potentially underestimates the prevalence of fungi present in the airway [99, 100]. Unlike the standard method, a more focused approach with 10 μl of undiluted homogenized sputum/dithiothreitol mix was inoculated onto the plates from sputum plugs selected from specimens. The plates were observed for up to 5 days.

The repeatability of this processing method, which showed substantial agreement, was based on 17 patients where 2 samples were obtained within 6 months; an interval relevant to retesting after an intervention such as antifungal treatment. This suggests that this method is reasonably robust, considering the inherent variability in the amount and quality of sputum obtained by induction.

The clinical syndrome of allergic bronchopulmonary aspergillosis once had central to its diagnosis, a requirement of culturing *A.fumigatus* from the sputa of patients but this was felt to be insufficiently sensitive or specific as a marker of ABPA and was removed from the standard criteria [43].

Subsequent to this came the understanding that the isolation of fungi was more prevalent in

patients with asthma at 8.4%[46]. Why some patients are susceptible to colonisation with *A. fumigatus* is not known [49]; but the potential for the involvement of immune-genetic factors has been suggested and they are clearly involved in the hypersensitivity response [101-104]. This study confirms this finding in demonstrating not only the increased rate of fungal colonisation in patients with asthma with an improved culture technique, but also its relationship to sensitisation with *A. fumigatus* noting that a positive sputum culture of *A. fumigatus* was strongly linked to *A. fumigatus*–IgE sensitisation. A high rate of positive culture was noted in the *A. fumigatus*–IgG-only and non-sensitised patients with asthma. This being a cross sectional study however is unable to address an alternative possibility that these patients are sensitised prior to their exposure to fungi or whether sensitisation is an inheritable trait.

In fact, *A. fumigatus*–IgG-only–sensitised patients with asthma had closer colonisation rates to the non- sensitised patients with asthma. However, both the *A. fumigatus*– IgG-only–sensitised and non-sensitised patients with asthma showed relatively high rates of *A. fumigatus* colonisation, at over 30% (13 patients) This was specific in the sense that healthy subjects had a low rate of culture, although one caveat is the younger age of the control subjects. Most of the patients (54%) were classed as having refractory asthma; 32 (42%) patients were GINA-5, 38 (48%) were GINA-4, 7 (9%) were GINA-3, and 2 (2%) were GINA-2 but there appeared to be no significant difference in prescription of oral or inhaled corticosteroids in the distribution of the three asthma groups of sensitisation. It would be interesting to know the *A. fumigatus* culture rates in patients with only mild asthma.

A number of studies have associated fungal sensitisation with asthma severity [40, 105, 106] and, specifically, *A. fumigatus* sensitisation and ABPA have been associated with progressive lung function decline in CF, likely due, in part, to coinciding *Pseudomonas aeruginosa* infection [95, 107, 108].

In this study, it is noted that a longer history of asthma duration was significantly linked to patients with asthma and *A. fumigatus*–IgE–sensitisation ($P < 0.05$) compared to those non-sensitised. Atopy is a risk factor for asthma and the premise that continuous allergen exposure may be responsible for more severe disease is intriguing.

Patients with asthma who were not sensitised had better lung function than those sensitised therefore being the first cross-sectional study to show an association specifically between *A. fumigatus*–IgE sensitisation and reduced lung function in asthma. Although there was no formal steroid trial, patients were stable and optimally treated suggesting that the post-bronchodilator FEV_1 reflects fixed airflow obstruction; a common finding in asthma, particularly in moderate to severe phenotypes where it affects around 23% of patients [52]. The cause of fixed airflow obstruction in asthma remains unknown, although there are association with an accelerated rate of decline in lung function over time in those with longer duration of asthma[109], smoking[62], neutrophilic airways inflammation[57] [110]. It is therefore of interest that we found airway neutrophils to be increased in the *A. fumigatus*–IgE–sensitised group. It is possible that *A. fumigatus* is simply a commensal in culture-positive non-sensitised patients with asthma, but it is equally possible that *A. fumigatus* is responsible for driving the inflammatory response especially as fungal culture was also independently associated with reduced FEV1 in the multivariate model. An obvious limitation to this is that there was no measurement of fungal colonisation accounted for in the model.

In contrast, other studies have shown that adult-onset non-allergic asthma progresses more rapidly to severe remodelling [111] while the results of the ENFUMOSA study showed that severe asthma is associated with lower prevalence of atopy, as well as family history of asthma, than mild disease [112]. Regardless, these are not epidemiological studies and it is not possible to say from this study whether the association between *A. fumigatus*–IgE

sensitisation and reduced lung function is causal. It is possible that sensitisation is related to long-term colonisation of the airways in asthma, which may occur preferentially in damaged airways though conversely atopic tendencies maybe inherited contributing to both fixed airflow obstruction in asthma as well as fungal atopy[113]. There remains however a paucity of evidence excluding the interplay environmental factors in the demonstration of atopy regardless of genetic predispositions.

Though it is not apparent why, the type of sensitisation response appears to dictate the reduction in lung function. There was no significant difference in lung function between *A. fumigatus*–IgG-only–sensitised patients with asthma and the other asthma groups, which may partly be due to low numbers within this group. However, raised *A. fumigatus*–IgG alone does not appear to be a particularly good marker of either colonization or reduced lung function, and may simply reflect previous or high levels of exposure to *A. fumigatus* spores though data supporting this to data has been limited [114]. Data from patients with COPD and *A. fumigatus* would also support this [115]

There was no significant difference in the relationship between *A. fumigatus*–IgE sensitisation and age in the asthma population and no significant difference in age at onset were found between the asthma groups.

Characteristic of ABPA is a raised serum eosinophils, a hypersensitivity response, used in its diagnostic criteria. itraconazole has been shown to reduce eosinophilic airway inflammation in subjects with ABPA [48] though an increased sputum neutrophilia, airway obstruction, and increased levels of IL-8 have also been associated with ABPA [116, 117].

The data from the current study shows elevated levels of neutrophils in *A. fumigatus*–IgE–sensitised patients in comparison to non-sensitised patients with asthma, suggesting a Th1- or Th17-mediated immune response. The multivariate analysis also brought out a relationship between sputum eosinophils and FEV_1, showing, specifically, that airway inflammation, determined through sputum differential cell counts, should be taken into consideration in the clinical characterisation of AFAA.

In summary, this study shows a strong relationship between detection of *A. fumigatus* in sputum and A. fumigatus–IgE sensitisation, in addition to a strong inverse relationship between *A. fumigatus*–IgE sensitisation and lung function. Moreover, *A. fumigatus*–IgE sensitisation, airway inflammation, and *A. fumigatus* culture from sputum can be used collectively to model lung function in *Aspergillus fumigatus* associated-asthma. Using this data is important in assessing the utility of antifungal agents in these patients, particularly with regard to sputum culture indicating airways colonisation.

Chapter 4 Isolation of filamentous fungi from sputum in asthma is associated with reduced post-bronchodilator FEV_1

4.1 Introduction

Many inhaled occupational fungal antigens are associated with an IgG-mediated response or type III hypersensitivity reaction; 'farmer's lung' (*Thermoactinomyces vulgaris, Micropolyspora faeni*) and 'malt worker's lung' (*A. clavatus*) are some of a few clinical syndromes recognised under the term extrinsic allergic alveolitis.

Aside from *Aspergillus*, other fungal genera have been associated with clinical and radiological features similar to those of ABPA including *Penicillium, Candida, Curvularia, Drechslera, Fusarium, Geotrichum, Helminthosporium, Schizophyllum* and *Stemphylium[96, 118, 119]* and while *Aspergillus fumigatus* is the most renowned of fungi in the aerospora to give rise to airway allergy as exemplified by ABPA, *A. niger, A. flavus, A. nidulans, A. glaucus and A. oryzae [96]* too have been implicated.

There is evidence suggesting that patients with severe asthma are more likely to be atopic to fungal allergens than patients with milder disease while not having the syndrome of ABPA. A broad diagnostic label 'severe asthma with fungal sensitisation (SAFS) has recently been applied to this group [5] that are characterised by presence of sensitisation to one of a panel of environmental fungi, with *A. fumigatus* atopy most commonly detected, followed by *Candida albicans* and *Penicillium notatum*. The beneficial effect of a course of itraconazole in these patients further supports the role that colonisation may play a part here as well [87].

In the previous chapter, it was noted that a significant number of other fungi were being cultured, either alone or in the presence of others particularly in those patients who were not sensitised to *A. fumigatus*.

Ultimately the range of fungi that may colonize the airways in asthma is unknown. Unlike in Cystic fibrosis [120, 121], there is a paucity of data on the range of fungal colonisation in patients with asthma. Studies to date in asthma have focused mainly on patients suspected of having ABPA, and primarily reported only culture of *A. fumigatus*.

The purpose of this study was therefore to fully characterize the fungal biota cultured from asthmatic sputum and again examine the relationship between fungal culture and clinical features of asthma.

4.2 Methods

The same basic populations of patients with asthma were studied with further patients recruited to the study over a 3-year period during 2008–2010 from the difficult asthma clinic in Glenfield General Hospital, Leicester, United Kingdom.
The same methods were also employed in categorising and describing the patients included. Additional methods at identifying a range of fungi were employed.

4.3 Fungal culture and identification

Selected sputum plugs were plated directly onto fungal-specific culture media as previously described in Chapter 1 and incubated at 37°C for up to 7 days. This incubating temperature facilitated best the growth of Aspergillus species. Fungi were identified based on

macroscopic and microscopic morphology[122]. Species identity was validated by sequencing either the large subunit [123] or the internal transcribed spacer region 1 [124] of the nuclear ribosomal operon, using PCR conditions as previously described [123]. Total genomic DNA was extracted from pure subcultures using the DNeasy plant mini kit (Qiagen, West Sussex, UK) following manufacturer's instructions, with the inclusion of a bead-beating step (BioSpec mini bead beater, Bartlesville, OK, USA). Sequences were determined using BigDye-Terminator v3.1 chemistry with 3730 sequencers (Applied Biosystems, Warrington, UK). Sequence data were manually inspected and trimmed, with closest taxonomic match determined by comparison with known sequences in GenBank (March 2010) using the $BLAST_N$ database search method.

Statistical analysis was performed using methods described in the main methods section (2.6)

4.4 Results

TABLE 5 Demographic data

	Asthma patients (n=126)			Healthy controls (n=18)†	Comparing three groups
	No fungi cultured (n=58)	Any fungi (n=68)	P value		P value
Age in years (range)	55 (21-84)	58 (24-83)	0.23	40 (21-67)	<0.001
Smoking history (pack years) ‡	0(0-3)	0(0-10)	0.51	0 (0-3)	0.44
Gender (male) %	41%	52%	0.21	50%	0.42
Serum total IgE kU/L‡	159 (43-494)*	207 (89- 717)*	0.08	30.9 (9.2-50.4)	<0.001
Atopic ∫	53%	61%	0.44	17%	0.01
Age of asthma onset, years ‡	34 (9.5-47.25)	25 (5.25-46)	0.69	-	
Duration of asthma, years ‡	22 (10.75-42.5)	23 (7-41.5)	0.89	-	
FEV_1% of predicted, post bronchodilator	82.8 (24.8)*	70.8 (25.4)*	<0.01	111.6%*	<0.001*
Volume change post bronchodilator, (ml) ‡	100 (50-250)	50 (0-150)	0.01		
Fungal sensitisation, (any %) ***Aspergillus fumigatus* (positive/n)** ***Penicillium chrysogenum*(positive/n)** *Botrytis cinerea* (positive/n) *Alternaria alternate* (positive/n) *Cladosporium herbarum* (positive/n)	38% 18/58 5/37 3/32 6/39 7/38	56% 34/68 17/48 7/40 10/57 12/56	0.08 0.01 0.03 0.50 1 0.80	0.17% 0 0 0 1 0	<0.01
GINA treatment GINA 5 GINA 4	 38% 55%	 44% 49%	 0.71	 -	
Inhaled corticosteroid dose in micrograms **	1600 (800-2000)	2000 (800-2000)	0.04	-	
Number with bronchiectasis (%)	17(35%)	31(46%)	0.06	-	
Total cell count x 10^3mg of sputum	3.151	3.451	0.91	-	
Sputum neutrophil (%)‡‡ (95% C.I)	58.09 (48.8-69.2)	51.65 (42.5-62.8)	0.47	-	
Sputum eosinophil (%)‡‡(95% C.I)	2.52 (1.5-4.2)	2.09 (1.4-3.2)	0.61	-	

† Three subjects had positive fungal cultures. ‡median (IQR), ‡‡geometric mean ∫Assessed by SPT ≥3mm or Specific IgE common aeroallergens
**Beclometasone Diproprionate equivalent *Post-test comparison p <0.05

Figure 2 Lung function and culture of fungi in patients with Asthma

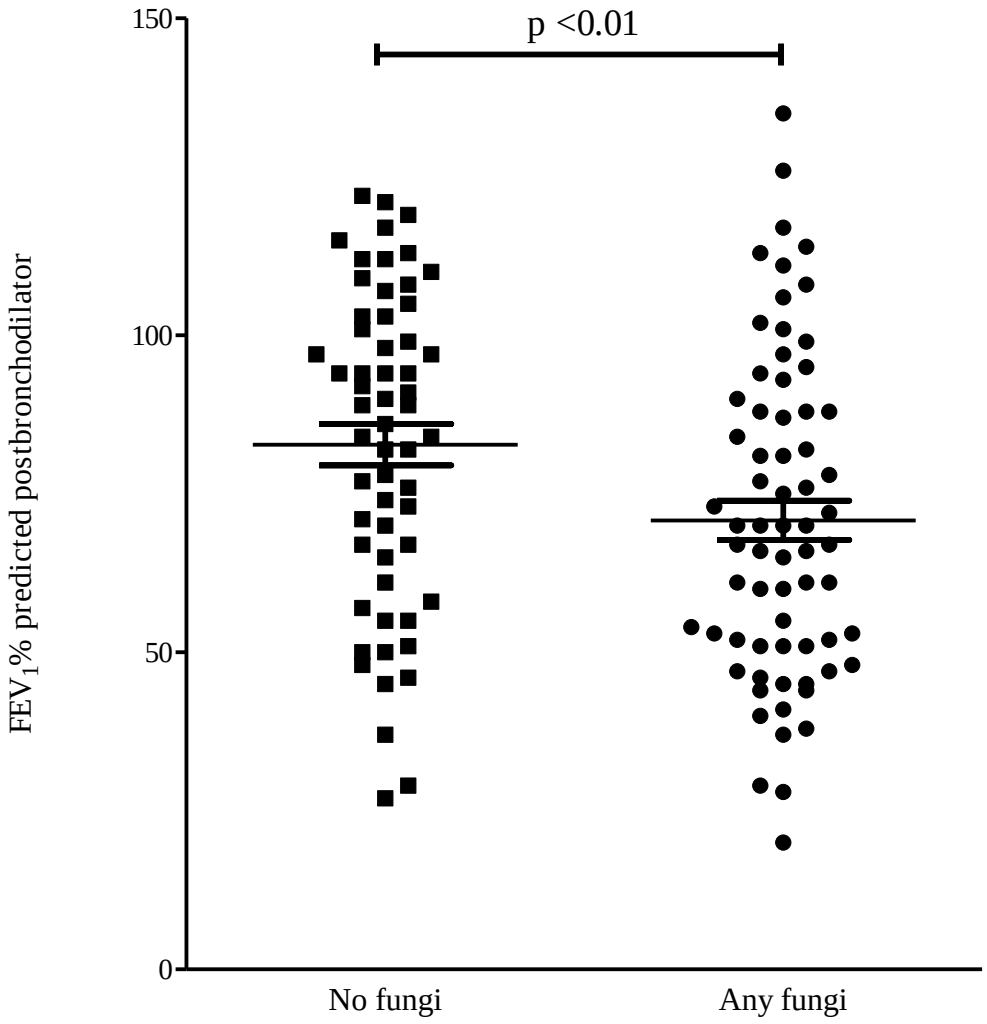
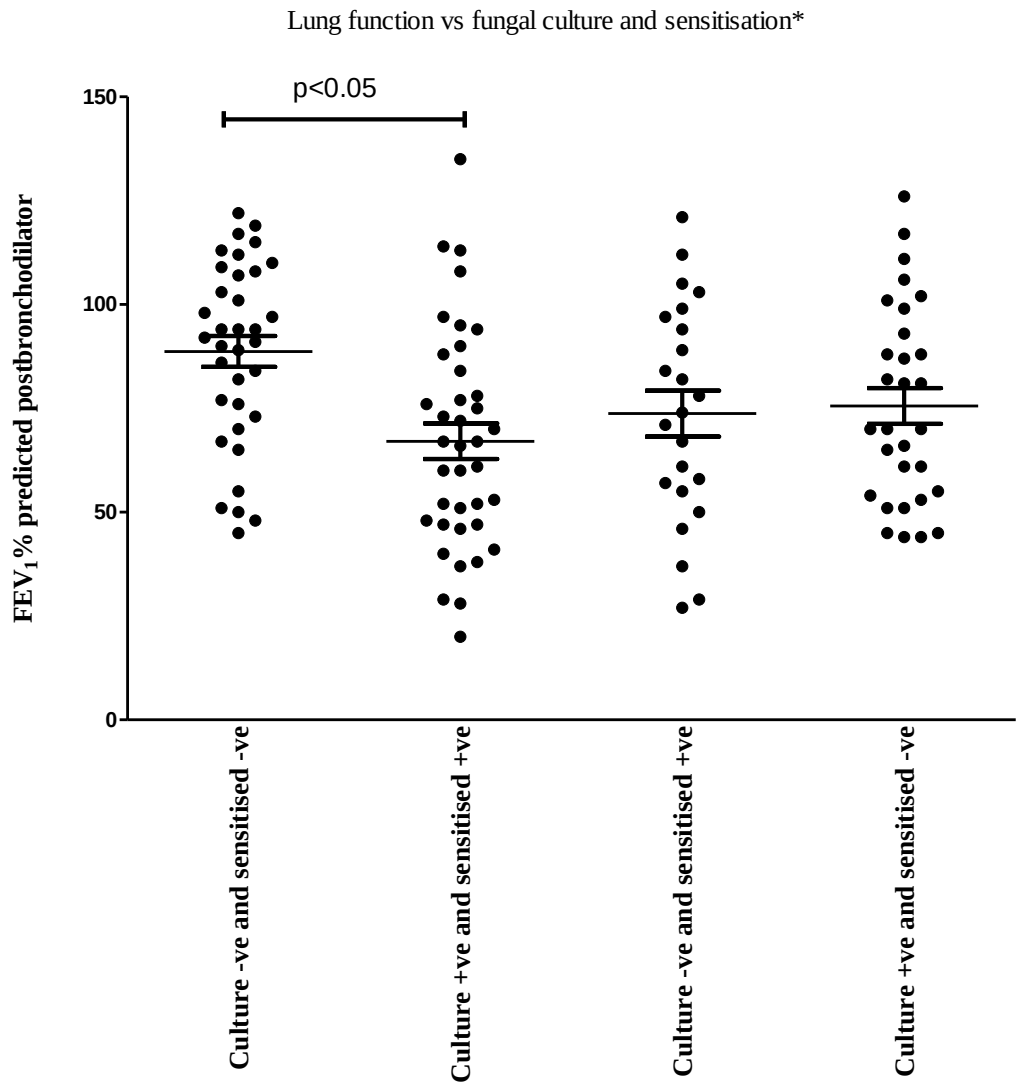
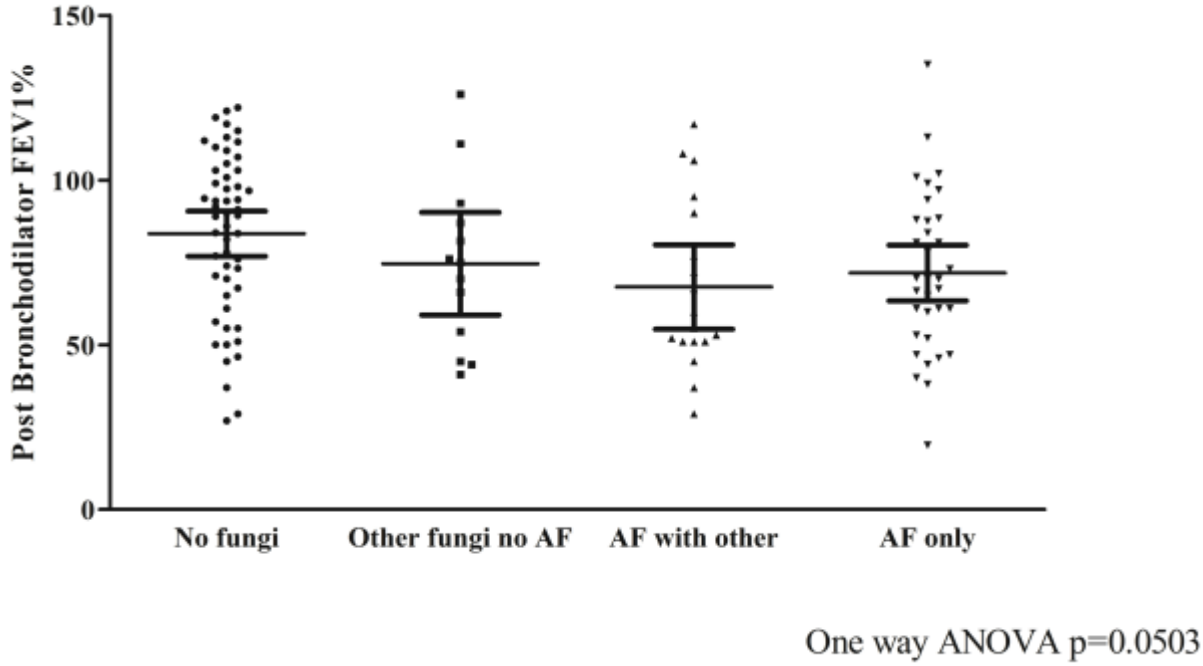


Figure 3



*sensitisation to any fungi of the fungal panel tested

Figure 4



Lung function in relation to fungi

Table 6 . Identification and incidence of filamentous fungi cultured from the sputum of asthmatics and healthy controls. Isolates were either the only filamentous fungi cultured (mono), grew in co-culture with *A. fumigatus* (co-Af), or in co-culture with other filamentous fungi listed but no *A. fumigatus* (co-other).

Class	Genus	Species/taxonomic identifier	Asthmatic (n=126)			Healthy control (n=18)		
			Mono	co-Af	co-other	Mono	co-Af	co-other
Eurotiomycetes	*Aspergillus*	*A. fumigatus*	37		18	1		
		A. fischeri var. *glaber*		1				
		A. niger complex	2	4	1			
		A. terreus		1				
		A. ustus			1			
		section Flavi (species undetermined)		2				
		section Nidulantes (species undetermined)	1	1				
		species undetermined		1				
	Penicillium	*P. brasilianum*	1					1
		P. capsulatum,			1			
		P. chrysogenum or *P. gladioli*	1					
		P. citrinum	1					
		P. citrinum or *P. griseofulvum*			1			
		P. diversum		1				
		P. verruculosum	1					
		P. marneffei	1					
		P. simplicissimum or brasilianum						1
		P. piceum	1	3	3			
		P. pinophilum		1				
		subgenus *Penicillium* (species undetermined)		2				
		species undetermined		1				
	Paecilomyces	*P. variotii*		1				
		Thermoascus crustaceus (*Paecilomyces* teleomorph)		2				
	Gymnascella	*G. citrina*			1			
Zygomycota (class undetermined)	*Rhizomucor miehei*	*R. miehei*		1				
	genus undetermined	species undetermined		1				

Agaricomycetes	*Coprinus*	*C. xanthothrix*			1
	genus undetermined	species undetermined	1		
Sordariomycetes	*Chaetomium*	*C. bostrychodes*		1	
	genus undetermined	species undetermined	1		
No filamentous fungal growth			58	15	

4.4.1 Demographic characteristics

The demographic characteristics of the subjects with asthma and the healthy controls are shown in Table 5. The mean (± SD) age of subjects with asthma was 56 ± 13.4 years, with median (IQR) age of onset 33 (7–46.3) years. The asthma cohort was matched with the healthy volunteers with respect to smoking history and gender. Asthma subjects were, however, significantly older with higher rates of atopy than controls. Forty-eight percent of asthma subjects had evidence of IgE sensitisation to commercial extracts of at least one of a panel of five fungi, 22% to two or more fungi.

Ninety-four percent of asthma subjects required Global Initiative for Asthma (GINA 4) or greater treatment and within the population, there was a significant degree of fixed airflow obstruction as evidenced by impaired post-bronchodilator FEV_1. Forty-two percent of subjects with asthma scanned had bronchiectasis, although only four patients met criteria diagnostic of allergic bronchopulmonary aspergillosis.

4.4.2 Fungal culture and sensitisation

A significantly higher rate of fungal culture was detected in the sputum of subjects with asthma (54%) compared with healthy controls (17%, $P < 0.01$). Within the group of culture-positive asthmatics, A. fumigatus was the sole fungus isolated from 54% (37/68) of subjects; over a quarter (27%) cultured both A. fumigatus and at least one other fungus; and nearly a fifth (19%) cultured at least one fungus without co-culture of *A. fumigatus*

A total of 97 fungal cultures representing 27 different taxa of filamentous fungi were obtained from 68 asthmatic sputa. The majority (92%) were from the genera *Aspergillus* and *Penicillium*, with A. fumigatus representing 57% of isolates (Table 6). In addition to *A. fumigatus*, a further 18 different *Aspergillus* or *Penicillium* species or groups of closely related species were detected, eight of which represented nine isolates where A. fumigatus was not co-cultured.

4.5 Discussion

This is the first study that has systematically detailed the fungal biota in sputum from a large number of subjects with asthma. There are three important notable and novel observations: firstly, in moderate-severe asthma, filamentous fungi other than *Aspergillus fumigatus* are commonly isolated from the sputa of over half the subjects on stable visits; secondly that most of the cultured fungi are from the allergenic fungal genera *Aspergillus* and *Penicillium* with *A. fumigatus* being the single most common species; and thirdly, that a positive sputum fungal culture [figure 2] is associated with impaired bronchodilator FEV_1, supporting the hypothesis that fungal colonisation of the airways causes the development of fixed airflow obstruction in asthma.

In normal clinical practice, isolation of fungi from routinely processed sputum is unusual in patients with asthma even in those subjects who fulfil all the criteria for ABPA. On those occasions when there is a positive report, it is invariably *A. fumigatus.* Part of the reason is that sputum is not routinely analysed for fungi in asthma where adequate spontaneous samples are infrequently produced. Furthermore, in those infrequent cases where microbiological analysis is sought, samples are usually only sent when there is sputum purulence and bacterial infection is being considered. Fungal culture is mainly considered when ABPA is suspected although culture of fungi is not a major diagnostic criterion in any of the most widely used studies [44, 49, 125].

Based on this data, a full appreciation of the potential role of filamentous fungi in asthma requires a focused approach to fungal culture. In support of this, a further study was undertaken comparing the way in which sputum is routinely processed in the national health service (NHS) clinical microbiology laboratory[122] compared to this research based technique using a different culture medium and quantity of sputum inoculated onto the media

[100]. This demonstrated fewer culture isolates overall with the standard technique, mainly because the sample was made up to be relatively very diluted.

In the current study, there was a high rate of positive culture in subjects with asthma. Culture specificity was demonstrated in that only three out of eighteen healthy controls (17%) cultured fungi in their sputum. Sputum samples from healthy controls and asthma subjects were treated identically and laboratory staff were blinded to the subjects' medical status ruling out environmental contamination as the cause of the difference in culture rates observed. Although a positive sputum culture may indicate colonisation of the airways suggesting the fungus is growing non-invasively in the bronchial lining fluid, it could be obtained from the upper airway or the result of germination of an inhaled spore [11]. Removal of saliva and selection of sputum plugs, combined with a marked reduction in positive culture rates from healthy subjects, suggests that it is unlikely that the fungi cultured are coming from the upper airway.

There was a difference in age between the healthy controls and asthmatics so one cannot exclude the possibility that rates of culture increase with lung age. It is however unlikely to explain the difference seen nor is there a plausible biological explanation of age alone predicting a positive culture. Although there were high rates of positive culture it is possible that molecular techniques such as the polymerase chain reaction (PCR) would result in even higher positive rates of detection and this approach needs to be compared with our culture method.

Repeatability of *A. fumigatus* culture has been shown to be reasonably good [Chapter 3]. A caveat of the culture approach is that one cannot readily quantitate the amount of fungi in a sputum sample and quantitative PCR (QPCR) may be an advantage in this respect; however, QPCR is only able to detect the fungus to which specific primers have been designed.

Most of the fungi cultured were from the *Aspergillus* and *Penicillium* genera. Many mechanisms enable these fungi to colonise the human airway; a small spore size permits them to bypass the filtering system of the upper airways and continue deposition in the distal small airways; and the thermotolerant growth properties many members have, allows them to grow at body temperature in the airways.

In the context of ABPA and CF other species of *Aspergillus* (most commonly *A. niger* and *A. flavus)* have been reported in a minority of patients. *Penicillium* spp. have been reported in as many as 9% of CF patients [126] but are not routinely distinguished in species and are often regarded as a contaminant[127]. In this study, 13% of subjects with asthma cultured one or more species of *Penicillium*, 7% in the absence of co-culture with *A. fumigatus*. *P. piceum* was the most common species of *Penicillium* cultured from people with asthma in the study, and is a member of the *P. marneffei* complex[128], an emerging opportunistic human pathogen[129]. Fungi from the genera *Paecilomyces, Rhizomucor, Coprinus* and *Chaetomium,* from which were isolated species in this study, have been described in case reports of pathogenic infection [130], mucormycosis [131], pulmonary infection [132], and invasive mycotic infections [133] respectively. This suggests that isolates from these fungal genera should not necessarily be disregarded as being clinically insignificant.

The airborne concentration levels of *A.fumigatus* are known to be higher in homes of those subjects isolating the fungus from sputum [114], and a similar trend was shown with *Aspergillus/Penicillium*-type concentrations analysed by microscopy. This suggests that the home environment should be strongly considered as a potential source of fungal exposure predisposing people with asthma to airways colonisation.

There were also a significant number of subjects that had more than one fungus isolated from their sputum, suggesting either heavy exposure or, perhaps potentially, a defect in host defence against fungi making them susceptible to colonization by a range of fungi not

typically seen in the home environment [11, 134]. Such defences involve a combination of innate and adaptive immunity[135] and the extent to which there is deficiency in any of these pathways in some people with asthma is unknown. The development of IgE sensitisation in those not currently sensitised but colonised is worthy of further study.

Allergic bronchopulmonary mycoses are associated with IgE sensitisation. Unfortunately, there was not a complete data set for fungal sensitisation, in part due to a lack of immunological testing solutions. However with this caveat, those with a positive culture were significantly more likely to be sensitised to *A. fumigatus* or *P.chrysogenum* (the only species within the *Aspergillus and Penicillium genera* with commercially available skin test reagents). No such relationship between sensitisation and culture was seen for the non-thermophilic *Alternaria, Cladosporium* or *Botrytis* which act as aeroallergens, but do not colonise the airways supporting the idea that colonisation and sensitisation are directly related. As previously noted, sensitisation to *A. fumigatus* alone is associated with an increased rate of positive sputum culture for *A. fumigatus* [15]. Linking sensitisation and culture to the panel of fungi cultured in this study is more problematic due to the lack of reagents for the majority of species identified. Furthermore, the degree of cross-sensitisation between species is not clear [136]. If sensitisation testing with specific IgE was available for all the panel of fungi then the rates of sensitisation in the positive sputum group would be even higher.

The major question arising from this study is the extent to which fungal colonisation is clinically important. Are the fungi commensals commonly found in damaged lungs or do they have a pathogenic role? Very few of the subjects, 4, in the study fulfilled all the criteria for classical ABPA, where there is good evidence for a tissue-damaging role of chronic fungal colonisation with *A. fumigatus*. In support of the idea that the fungi are pathogenic is the finding that there is an association between a positive fungal culture and reduced post-

bronchodilator FEV_1[Figures 3-4]. This extends to the observation of the previous study where IgE sensitisation to *A. fumigatus* was associated with impaired lung function, but where culture for *A. fumigatus* was only weakly associated as part of a multi-variable analysis [15]. The larger numbers of subjects in this study and the extension to cover any filamentous fungus has resulted in a negative association between lung function and culture irrespective of sensitisation status. If a subject is also IgE sensitised to fungal allergens then the effect on lung function appears greater than for culture alone (12% versus 22%), suggesting that both factors are involved in impairment of lung function. There were not enough subjects to explore the effects of a filamentous species other than *A. fumigatus* cultured in isolation with lung function, though there was a trend to lower lung function in this group compared to the no-culture group (figure 4).

The relationship between sputum neutrophilia and lung function has previously been described [57] though this was not apparent in this study. Importantly fungi produce a range of toxins which are potentially tissue damaging. Additionally, where sensitisation is present, they can promote an inflammatory response through allergic mechanisms as well as potentially by an autoimmune like process caused by cross-reaction between fungal and human antigens [137, 138] It is therefore very plausible that chronic colonisation of the airways with a range of fungi could promote IgE sensitisation and in turn lead to chronic airway damage. This work would suggest that identification of airway damage in asthma and the presence of fungi in the airway using optimal techniques is an important priority in asthma management as the outlook could be improved with anti-fungal therapy.

In summary, there are high rates of fungal cultured from sputum with evidence of sensitisation to mainly the *Aspergillus* and *Penicillium* genera in subjects with moderate to severe asthma who generally do not fulfil the criteria for ABPA. A positive sputum culture is

associated with fixed airflow obstruction supporting the hypothesis that this asthma phenotype is caused by chronic fungal airway colonisation.

Chapter 5 Effectiveness of Voriconazole In the Treatment of *Aspergillus fumigatus* Associated Asthma (EVITA^3)

5.1 Introduction

It is well recognised that colonisation of the airways with filamentous fungi (moulds) together with raised specific IgE can occur in asthma (and cystic fibrosis), where it is associated with a distinct syndrome called allergic bronchopulmonary mycosis (ABPM) [139, 140]. The main moulds associated with this condition are *A. fumigatus* and related thermotolerant members of the *Aspergillus* genera causing allergic bronchopulmonary aspergillosis (ABPA) [49, 50]. The classical clinical features of ABPM are fleeting lung shadows, proximal bronchiectasis and a cough productive of viscid mucus. These are associated with laboratory findings of a raised total IgE, a raised fungal specific IgE (and/or a positive SPT), and IgG and a peripheral blood eosinophilia.

Up to 50% of patients with refractory asthma have been reported as being IgE sensitised to fungi [40]. However, most patients who are IgE sensitised to *A. fumigatus* do not fulfil all the criteria for ABPA. They often have levels of total IgE below the accepted ABPA threshold (>410IU/L or 1000ng/ml although some authorities use >1000IU/L) [48, 141-143], concentrations of specific IgG in the normal range, absence of proximal bronchiectasis and no evidence of fleeting shadows. Earlier chapters have shown that approximately ~60% of people with moderate to severe asthma who are IgE sensitised to *A. fumigatus*, but without ABPA, have a positive sputum culture for the mould suggesting airway colonisation is commonly associated with sensitisation [144, 145]. Patients with either sensitisation or a positive sputum culture have a lower post-bronchodilator FEV_1 than matched asthmatics and in patients who have both sensitisation and a positive sputum culture, compared to those who have neither, the mean difference in post-bronchodilator FEV_1 is 20% predicted suggesting a

relationship between both lung damage and fungal allergy and infection [144, 145]. A positive sputum culture and sensitisation have also been associated with increased incidence of bronchiectasis[146].

If, as is thought to be the case in patients with ABPA, persistent colonisation of the bronchial tree with *A. fumigatus* is contributing to the clinical picture in asthmatics with allergy to *A. fumigatus* without ABPA, it raises the question whether treatment with anti-fungal therapy would be of benefit in this group of patients? Most descriptions of the use of anti-fungals in ABPA in asthma and cystic fibrosis have been limited to case reports. There have been two significant placebo controlled studies of anti-fungal treatment for ABPA identified in a Cochrane review both of which reported benefits of itraconazole [47, 48, 78]. The only other randomised study of anti-fungal treatment in asthma was by Denning *et al*. who treated 58 people with SAFS with itraconazole 200mg twice daily for 32 weeks and observed a significant improvement compared in AQLQ [87].

One problem with interpreting studies that have used itraconazole is that it can markedly enhance the effects of both endogenous and exogenous corticosteroids [147]. Thus the improvements seen in the above studies could be due to a pharmacokinetic effect on corticosteroid bioavailability rather than anti-fungal activity. This pharmacokinetic effect has not been reported to occur with voriconazole. It is generally considered that voriconazole is at least as effective as itraconazole in the treatment of invasive infections of *A. fumigatus* and is regarded as first line therapy in many centres. [148]. We proposed a study of voriconazole in people with asthma who were sensitised to *A. fumigatus* was undertaken to determine if this improved their asthma control.

5.2 Methods

5.2.1 Patients

Subjects (all over 18 years), were recruited during 2010 and 2011 mainly from the respiratory clinics at Glenfield Hospital, although ten subjects were referred into the study from other hospitals in the East Midlands, UK.

The inclusion criteria were:

- a clinical diagnosis of asthma with at least historical evidence of variable airflow obstruction (variation in FEV_1 or >12% or Pc20<8mg/ml),
- evidence of IgE sensitisation to *A.fumigatus* (raised specific IgE of >0.35 IU/L or a SPT of >3mm greater that the negative control), and
- at least two severe exacerbations (defined as requiring a minimum of 3 days of high dose oral corticosteroids for their asthma), in the previous 12 months.

Exclusion criteria were pregnancy, a diagnosis of COPD, a medical condition that would increase the likelihood of an adverse reaction to voriconazole and treatment with an anti-fungal agent in the twelve months prior to entry into the study.

5.2.2 Study Design

This was an investigator led, single centre, double blind, placebo controlled, randomised, parallel group study conducted between 2010 and 2012. The funding agency Pfizer provided the drug and placebo but had no role in the accrual or analysis of the data. Ethical approval from the Leicestershire Ethics Committee (UKCRN ID 7763) and the UK Medical and Health Products Regulatory Agency (MHRA: 09/H0402/63) was obtained including cystic fibrosis genotyping. Each patient gave written informed consent. The clinical trials

registration numbers were ISRCTN42366088 and EudraCT 2009-011452-21. The visit schedule together with the investigations undertaken at each visit is shown in supplementary Table 1. At a baseline visit demographic details were collected including smoking history, treatment and exacerbation history. If a HRCT scan had been undertaken for routine clinical purposes the presence or absence of bronchiectasis on the radiology report was recorded. Spirometry was performed, and quality of life measurements recorded. Blood was drawn for total IgE, specific IgE and IgG to *A. fumigatus*, a full blood count and routine biochemistry, serum cortisol and cystic fibrosis genotyping. Those with a significant CF genotype were referred to a Geneticist. Sputum was obtained either spontaneously or by induction for a cell differential and fungal culture. Skin prick test to a panel of aeroallergens including *A. fumigatus* was undertaken. After a run-in period of up to one month to ensure the subjects condition was stable and to allow measurement of the sputum differential and fungal culture which was used for randomisation the subjects were started on treatment at visit two. Treatment was given for three months during which subjects were seen at monthly intervals. They were then seen bimonthly until the end of the study. Investigations were repeated at each visit according to the schedule in Table 1. Voriconazole levels were measured at visits three or four, one or two months after starting treatment. This was with the aim of measuring compliance. Exacerbations were treated either by their personal physician or by the study team and managed according to standard clinical practice. Chronic asthma treatment was not altered during the period of the study. Randomisation was in blocks of three with the use of the minimisation method using the criteria of sputum eosinophil count, the number of exacerbations in the previous twelve months and sputum fungal culture. Voriconazole was given at a dose of 200mg twice daily with the drug and matched placebo provided by Pfizer. The two primary outcome measures were the change in the Juniper asthma quality of life questionnaire (AQLQ) from baseline to the end of the treatment period and the number of

severe exacerbations, defined as above, over the twelve months of the study. A change of 0.5 or more on this 7-point scale is considered clinically the Minimal Important Difference (MID).

Secondary outcomes measures were the modified Juniper asthma control questionnaire (ACQ 6 which excludes FEV_1), a combined visual analogue score (VAS) based on three 100mm visual analogue scales (VAS) which measured symptoms of cough, breathlessness and wheeze , a nasal polyp questionnaire [149], post-bronchodilator FEV_1, sputum eosinophil and neutrophil count, peripheral blood eosinophil count, total IgE and *A. fumigatus* specific IgE and IgG.

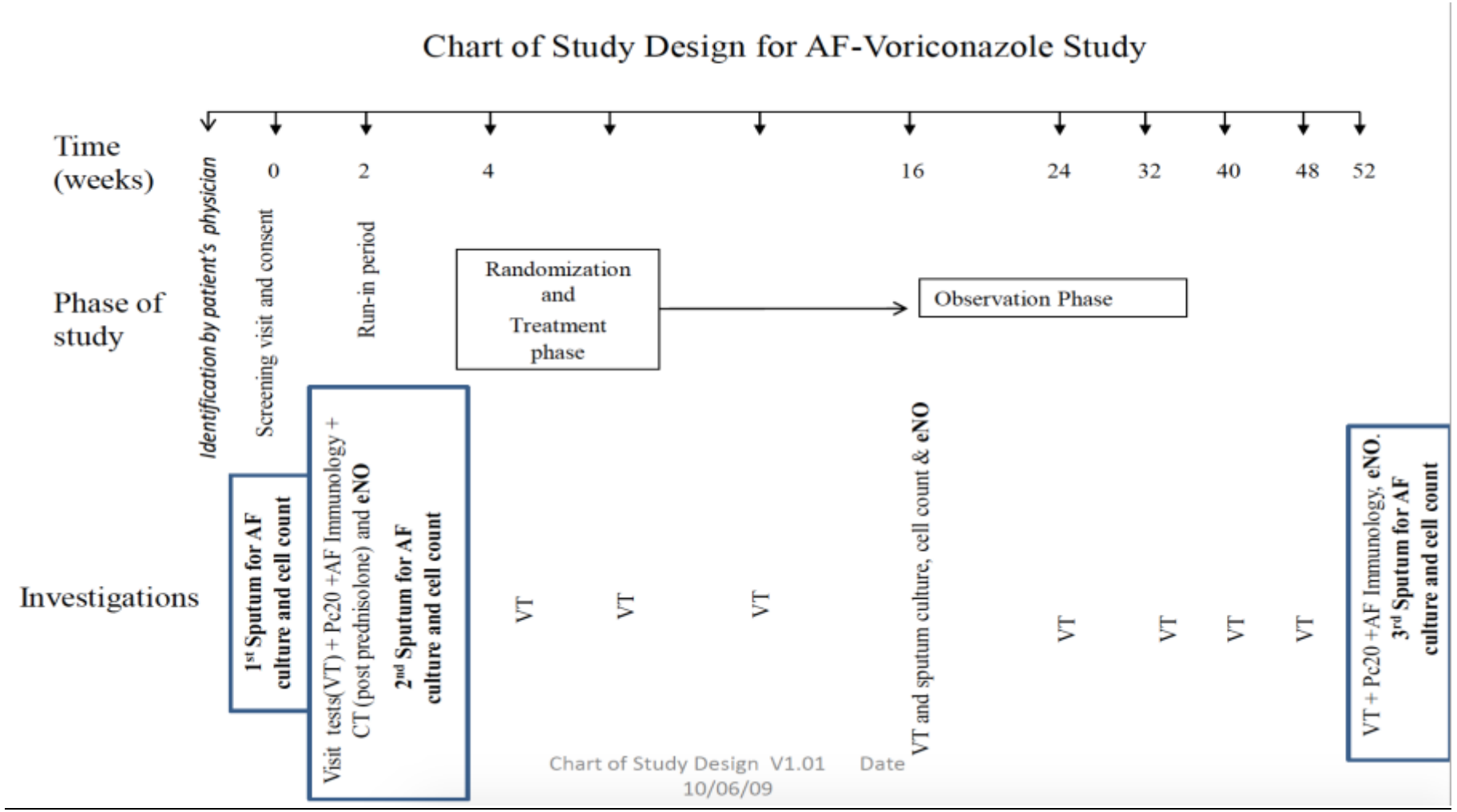


Chart of Study Design for AF-Voriconazole Study
Time (weeks)
0
2
4
16
24
32
40
48
52
Phase of study
Identification by patient's physician
Screening visit and consent
Run-in period
Randomization and Treatment phase
Observation Phase
Investigations
1st Sputum for AF culture and cell count
Visit tests(VT) + Pc20 +AF Immunology + CT (post prednisolone) and eNO
2nd Sputum for AF culture and cell count
VT
VT
VT
VT and sputum culture, cell count & eNO
VT
VT
VT
VT
VT + Pc20 +AF Immunology, eNO. 3rd Sputum for AF culture and cell count
Chart of Study Design V1.01 Date 10/06/09

5.2.3 Investigations.

Clinical investigations and measurement of the sputum differential and fungal culture were undertaken as previously described and detailed in the methods section 2.3 Measurement of the total IgE and specific IgE and IgG were undertaken in the routine University Hospitals of Leicester immunology laboratory using the ImmunoCAP system. Serum for voriconazole levels was sent to the Health Protection Agency mycology reference centre in Bristol.

5.2.4 Statistics

The study was powered on severe exacerbations. 25 patients were required in each group assuming two exacerbations per patient per year in the placebo group and one exacerbation per patient per year in the Voriconazole arm ($\alpha = 0.05$, $\beta=0.02$).

$(\mu_1-\mu_2)^2= \int(\alpha,P)\sigma^2 \times (1/n_1 +1/n_2)$.

The exacerbation data was analysed using negative binomial regression. Those patients who took at least one week of treatment were analysed on an intention to treat basis. For the quality of life data, the mean ACQ6, mean AQLQ and mean VAS are shown. Baseline scores were compared with post-treatment data collected at visit five and error bars represent the standard error of the mean. Within-group data was analysed using paired t-tests; between group comparisons were analysed separately at baseline and visit five using unpaired t-tests. Statistical software packages used for various analyses included PASW statistics 18 and GraphPad Prism, version 4 (GraphPad Software).

5.3 Results

5.3.1 Recruitment

The details of recruitment are shown in the CONSORT diagram (Figure 1). About a third of patients contacted and screened agreed to take part. 65 patients were randomised. Six dropped out before taking any drug because they changed their mind about participating in the study between the screening visit and taking the first study medication and took no further part in the study. 59 patients were therefore entered into the analysis. Of these, three in each group did not complete the course of treatment although four of these continued in the study.

5.3.2 Baseline demographics and investigations

Baseline details of the patient demographics and investigations are shown in Table 2. The active and placebo groups were generally well matched with no significant differences between them, although there was a trend towards more men in the placebo group. The patients in both groups had moderate to severe disease with a requirement for high doses of corticosteroids and a substantial degree of fixed airflow obstruction and bronchiectasis. Two patients fulfilled all the criteria for a diagnosis of ABPA.

5.3.3 Primary outcomes

There was no significant difference in the total number of severe exacerbations or in the number of subjects exacerbating between the two groups over the twelve-month period of the study (Figure 2). The voriconazole group had a mean of 1.16 exacerbations per subject over the twelve months of the study compared to 2.5 in the twelve months prior to the study. There was a mean of 1.4 exacerbations in the placebo group compared to 3.0 in the previous 12

months. This represented a 54% reduction from baseline in each group. 27 patients in the Voriconazole arm had one or more exacerbations compared to 18 patients in the placebo arm. The exacerbation rate was overall showed no significant difference between groups- 1.25 vs 1.52/patient/year; mean difference 0.27; 95% CI 0.24 to 0.31. The AQLQ score improved from a mean of 4.55 at baseline in the voriconazole group to 5.22 at the end of the treatment period (Figure 3A. It then fell back within two months and was 4.85 at the end of the trial. A similar pattern was seen in the placebo group improving from 4.66 at baseline to 5.54 at the end of treatment and 5.13 at the end of the study. There were no statistically significant differences between the voriconazole and placebo groups- change in AQLQ 0.44 vs 0.35, mean difference between groups 0.08; 95% CI 0.07-0.09.

5.3.4 Secondary outcomes

There were no significant differences between the voriconazole and placebo groups in the three other quality of life measures that we used, the ACQ6, VAS and nasal polyp questionnaire (Figure 3A and 3B). These measures demonstrated the same pattern as the AQLQ with an improvement in both groups to the end of the treatment period followed by a rapid return towards baseline. No significant difference was seen between the two groups in post-bronchodilator FEV_1 which did not change from baseline to any significant degree throughout the study in either group (Figure 3C), There were no significant differences between the two groups in the sputum or blood eosinophil count and the sputum neutrophil count (Figure 4), or the total IgE and *A. fumigatus* specific IgE or IgG (Figure 5).

5.3.4.1 Sputum fungal culture

The pattern of sputum culture for A. fumigatus was variable and interpretation was complicated by missing data due to insufficient sputum (Figure 6). Overall 81% of subjects in the placebo group and 84% of subjects in the voriconazole group had at least one positive sputum culture for *A. fumigatus* during the course of the study. When classified into the pattern of response as described in the supplementary appendix twelve subjects had either a definite (seven) or possible (five) response in the voriconazole group with seven treatment failures, compared to three possible responders in the placebo group (there were no definite responders), and eleven treatment failures. This difference was significant ($p< 0.033$ Fishers exact test). There were thirteen subjects in each group where the response could not be ascertained due to a persistently negative culture or missing data. There were no clinical correlations with sputum response.

5.3.4.2 Cystic Fibrosis genetic screening.

Because of the increased incidence of ABPA in cystic fibrosis we screened patients for CF genotype by sending a blood sample to our regional genetics laboratory which screens 90% of the known CF gene associated genetic mutations. Five people were heterozygous for a CF gene, but no subjects were homozygous.

5.3.4.3 **Voriconazole levels**.

Voriconazole levels were measured at visit three or four in 24 patients taking placebo and 26 patients taking voriconazole. All the patients in the active treatment arm had detectable levels of voriconazole (mean level 0.89 [0.2-2.8µg/ml). No voriconazole was detected in the

patients taking placebo. We found no correlation between voriconazole levels and response to voriconazole for any of our outcomes.

5.3.7 Adverse events

The adverse events are listed in Table 3. No serious adverse events were clearly related to voriconazole. The adverse events were largely to be expected from the known side effects of voriconazole many of which are common to all azoles including visual disturbance, photosensitivity, which was one of the major problems for subjects during the summer months, raised transaminases and skin rash. One patient had distressing hair loss though all other side effects resolved on stopping the drug. Three subjects in the voriconazole group were unable to complete the course of treatment because of adverse events, but two of these were able to take it for more than two months.

5.4 Discussion

This is the first report of a randomised controlled study that has investigated the effects of voriconazole in asthma complicated by allergy to *A. fumigatus*. Voriconazole has a similar *in vitro* minimum inhibitory concentration against *A. fumigatus* to itraconazole and posaconazole and a good profile of tissue penetration into the lung tissue and epithelial lining fluid [150]. Its use has generally been restricted to invasive infections or situations where itraconazole treatment has failed, where anecdotally it appeared to have additional benefit [91]. Previous clinical trials of itraconazole in ABPA and SAFS had demonstrated improved quality of life, reduced exacerbations, steroid sparing properties and reduced inflammatory and immune markers [47, 48, 87]. Stevens et al recruited 55 patients with oral steroid dependent ABPA from 13 centres. They were treated for 16 weeks with itraconazole 200mg/twice daily. The primary outcome was the number of responders based on pre-determined major and minor criteria including a reduction in the requirement for oral steroids. There were 13/28 responders in the itraconazole group and 5/27 in the placebo group ($p<0.048$). Wark et al recruited 29 patients with ABPA from a single centre. Subjects were treated with 400mg day of itraconazole for sixteen weeks. The primary outcome was a reduction in sputum eosinophils, which was achieved, as well as secondary outcomes of a significant reduction in total IgE and severe exacerbations. Denning et al recruited 59 patients with severe asthma who were sensitised to at least one of several common allergy-causing fungi including a proportion sensitised to *A. fumigatus*. They excluded people with an IgE of >1000IU/L or a raised fungal IgG. They treated with itraconazole 200mg/twice daily for 32 weeks. 41 patients completed the full course of treatment. They found a significant improvement in their primary outcome of AQLQ at 32 weeks with a modest, but significant reduction in total IgE. The outcomes were based on these studies with a greater

emphasis on detecting a reduction in severe exacerbations because of the link between eosinophilic inflammation (which is associated with fungal allergy), and an exacerbation phenotype [151]. It is not clear why this study found no benefit of anti-fungal treatment compared to the above studies. A similar number of patients were recruited and the treatment dose was the same although of shorter duration, (especially compared to the FAST study). The patients were similar to those recruited by the other groups in terms of the severity of asthma although the patients in the studies by Wark et al and Stevens et al had immunologically more florid disease, particularly with respect to total IgE. The patients in the study by Denning et al had a different pattern of fungal allergy as a basis for recruitment, but this may have been expected to reduce the power of the study because only a proportion had allergy to thermotolerant, potentially colonising fungi.

A fixed dose of voriconazole was used based on the manufacturers guidance and the dose of itraconazole used in the studies quoted above. No attempt was made to adjust the dose based on voriconazole levels, not least because of the difficulty of varying the dose while maintaining a double-blind design. Like Denning et al (although unlike Wark and Stevens et al), measures of voriconazole levels were obtained to provide evidence of compliance in addition to pill counting at each visit, but trough levels were not rigorously measured and there was some missing data. Some patients did have levels below the recommended trough level of ~0.5υg/ml and one cannot exclude the possibility that tissue levels were sub-optimal in those patients. There was however no association between voriconazole levels and response to treatment.

As in previous studies we found high rates of a positive sputum culture for A. fumigatus with 41% of subjects at baseline having a positive sputum and >80% of subjects having at least

one positive sputum over the course of the study. Rates in normal subjects are ~5% in our hands on a single visit, but we do not have normal values for more than two measurements. There was a trend for more positive sputum samples in the voriconazole group at baseline, but as noted above the numbers with at least one positive sputum over the course of the study were well matched between the two groups. In terms of sputum culture there were significantly more responders in the voriconazole group than the placebo group. However, clearance of the sputum was not complete with five out of 24 subjects where there was sputum data having a positive sputum (albeit with only one colony in each case), at visit five. There was also a rapid relapse with 22 out of 31 subjects in the voriconazole group with data having at least one positive sputum in the four post treatment visits. Numbers of subjects with definitive sputum data were too small to make meaningful comparison of the clinical response between sputum responders and non-responders. This presumed relapse rate is not surprising given the duration of therapy which remains unknown. The duration of treatment in the study by Denning et al was 32 weeks which was the point of maximal response in outcome parameters. Fungal culture rates also appeared less during the study.

There was a significant improvement in all three measures of quality of life at the end of the treatment period compared to baseline, but this was also seen in the placebo group and the between group differences were not significant. This contrasts with the study by Denning et al where the improvements in AQLQ with itraconazole were similar in magnitude to our study, but there was no placebo effect [87]. Benefits with placebo are common in research studies especially with subjective symptom data due to optimisation of therapy, regression to the mean or psychological effects. These patients were stable when recruited and on optimal therapy. In addition, the benefits of treatment in both groups were transitory. This suggests that psychological factors were the main explanation for the placebo effect in this study.

These factors arose from the frequent patient and physician interaction during the intervention period together with patient expectancy, at the outset, based on preceding trials demonstrating benefit was likely a significant psychological contributor to this response. Comparatively however, the magnitude of the placebo response seen in this trial is not in line with existing literature. [152]. This underlies the importance of objective outcomes in asthma trials and simultaneously the difficulties in studying a naturally variable disease. The patients were generally well matched aside from differences in gender, which seems unlikely to have had a material effect on the outcome of the study. Of the 65 subjects who were consented to take part in the study and were randomised six changed their mind before taking any treatment. Five of these were in the placebo group that skewed the recruitment numbers, but again it seems unlikely this affected the outcome. Although a significant number of subjects reported side effects of the medication only one subject in the voriconazole group took less than two months treatment and only two patients were lost to follow up, both in the placebo arm.

The study was powered to show a 50% reduction in severe exacerbations based on the placebo group having at least two exacerbations over the 12-month period. In the event the rate of exacerbations in the placebo group was only 1.4 which represented a ~50% reduction from the previous 12 months whereas the rate in the voriconazole group was 1.16 which similarly was a ~50% reduction from baseline. Thus, although strictly speaking it was underpowered the lack of even a hint of a difference between the two groups makes it unlikely that larger numbers of subjects would have resulted in a different outcome. The numbers in this study were very similar to those in the FAST study of Denning *et al* where they observed a significant improvement in AQLQ in the itraconazole group. We don't believe therefore that the failure to show any effect on quality of life in our study was due to a lack of power.

One possible explanation for the lack of any clinical or laboratory benefit in our study compared to those that have used itraconazole is that the benefits of itraconazole in those studies was due primarily to a pharmacokinetic effect on corticosteroid bioavailability, especially considering the high doses of inhaled and oral steroids which tend to be used in ABPA and SAFS. This is a well-recognised problem with itraconazole that confounds interpretation of the use of this drug [147, 153]. This pharmacokinetic effect has not been reported with voriconazole and in the subset of subjects where we performed cortisol levels voriconazole had no discernible effect on serum cortisol (data not shown). Denning et al found that half the patients they tested who were taking itraconazole had reduced cortisol levels, but the improvement in AQLQ was no different in these patients compared to those with unchanged cortisol, although numbers were small.

The duration of drug therapy with Voriconazole would seem flawed based on the likelihood of relapse seen and the suggestion microbiological clearance was not optimal at that time. This however should be weighed against both the cost and side-effects of therapy.

In conclusion, this study does not provide any evidence that patients with moderate to severe asthma, who are IgE sensitised to *A. fumigatus*, but do not fulfil all the criteria for ABPA will gain any short to medium term benefit to their asthma control from a 3-month course of voriconazole.

Table 1

Effectiveness of voriconazole in the treatment of *Aspergillus* associated airways disease (EVITA[3]) trial.

ISRCTN42366088

EudraCT	2009-011452-21
MREC N°	09/H0402/63
UKCRN ID	7763

	Screening			Active treatment phase			Observation phase			
Time (week)	-4		0	4	9	13	22	31	40	52
	V1		V2	V3	V4	V5	V6	V7	V8	V9
Screening visit- History & examination, ECG, medication review.										
Sputum										
Cell differential	x			x	x	x	X	x	x	x
Culture	x			x	x	x	X	x	x	x
Blood										
FBC/U&E/LFT/(CRP)	x			x	x	x	X	x		x
Serum cortisol	x				x		X			
Total IgE, Af-IgE, Af- IgG	x					x				x
CD16				x						
Voriconazole levels				x	(x)					
Genetics –CF genotyping and DNA analysis	x									
Lung Function tests										
Spirometry	x			x	x	x	X	x	x	x
FeNO	x			x	x	x	X	x	x	x
Exhaled breath analysis	x					x				
Skin prick testing	x					x				x
Asthma AQLQ, ACQ, VAS Asthma & Nasal polyps, Peak flow diary	x			x	x	x	X	x	x	x

Drug review /randomisation

Table 2: Baseline Measurements

	Voriconazole (N=32)	Placebo (N=27)	P value
Demographics			
Men	38%(12)	63%(17)	0.07
Age (mean [range], years)	59 [27-80]	59 [38-78]	0.90
Age at onset (mean [range], years)	19 [2-60]	20 [2-63]	0.50
Body mass index (mean [range])	27[18-37]	27 [17-36]	0.55
Spirometry			
FEV_1 % of predicted post-bronchodilator	72.6 ±27.7	62.7±20.3	0.13
FEV_1/FVC ratio post-bronchodilator	0.61±0.18	0.60±0.17	0.70
Leucocyte Counts and sputum analysis			
Eosinophil count in blood ($x10^{-9}$/litre) † [S.E]	0.46[0.06]	0.41[0.04]	0.49
Sputum Eosinophil counts (geometric mean [log SD]%)	2.88 [1.19]	4.68[1.04]	0.29
Sputum Neutrophil counts %	66.87 ± 22.17	60.23±23.56	0.31
Sputum cell Total cell count*10^6/mL†	6.84 (4.44-10.55)	4.05(2.41-6.82)	0.11
Sputum culture positive for *Aspergillus* Fumigatus Baseline (n)	50% (16)	30% (8)	0.06
Patient reported outcomes			
Asthma Quality of Life Questionnaire (Baseline [S.E])	4.55[0.25]	4.66[0.28]	0.76
Modified Juniper Asthma Control Questionnaire (ACQ 6)	2.15±0.98	2.27±1.20	0.68
Average VAS score for asthma symptoms	40.00 ± 4.58	41.45 ± 4.02	0.81
Average VAS score for nasal polyps	35.33 ± 19.4	31.41 ± 18.47	0.44
Immunoglobulins and radiology			
Total IgE†(log_{10} SD)	459(3.19)	659(3.12)	0.33

Positive atopic status to common aeroallergens% (n)*	69% (22)	70% (19)	1.00
Baseline Specific IgE to *Aspergillus* fumigatus; RAST †	4.79(2.38-9.65)	5.69 (2.84-13.01)	0.54
Baseline *Aspergillus fumigatus* IgG†	30.8(23.54-42.60)	31.7(21.70-43.80)	0.74
Bronchiectasis present or radiology report of CT scan % (n)	52% (16)	62%(16)	0.59
Smoking and steroid history			
Smoking (pack years) in ex or current smokers	14	11.9	0.68
Never Smokers,% (n)	59 % (19)	63% (17)	0.8
Rescue corticosteroid courses in previous year	2.5 (2-4)	3 (2-5)	0.19
Dose of inhaled corticosteroid-beclomethasone equivalent (median [IQR], μg/pt/day)	2000[900-2000]	2000[400-2000]	0.36
Number on maintenance prednisolone (median dose) % (n)	28% (9)	33% (9)	0.89
Median dose of maintenance Prednisolone [range]	5mg[2.5-10]	5mg[5-10]	0.89

¶s.e †Geometric mean(95%C.I). *house dust mite, cat, dog, grass. **Total IgE ≥410 IU, *Aspergillus* IgE ≥0.35, *Aspergillus* IgG >40mg/ml, proximal bronchiectasis.
p values were calculated with an independent t test for parametric values, Fishers exact test for comparison of proportions and the Mann-Whitney u test for comparison of non-parametric

Figure Legends

Figure 1: Recruitment of subjects

Of the 184 people with asthma and IgE sensitisation approached to take part in the study about half declined to be involved. A minority did not meet the inclusion criteria mainly because they had less than two exacerbations in the previous twelve months. Of the 65 subjects randomised to drug or placebo six (five in the placebo group), changed their mind between visit one and two. Of those who took the treatment and were included in the analysis one subject in the placebo group was lost to follow up between visit two and three and two subjects in the placebo group and three in the voriconazole group did not complete the full course of treatment (although two of these took more than two months of drug).

Figure 2: Severe exacerbations.

There was a linear increase in the number of severe exacerbations in both groups with no treatment effect. The total number of exacerbations in the placebo group were thirty-eight with thirty-seven in the voriconazole group which represented a 54% reduction from baseline in both groups. Five patients in the voriconazole group didn't exacerbate and nine in the placebo group. This difference was not significant.

Figure 3: Patient reported outcomes

There was a significant improvement in the quality of life scores in both groups from baseline during the course of treatment with the voriconazole group increasing from a mean of 4.55 to a maximum of 5.3 and the placebo group from 4.66 to 5.6 ($p<0.001$). There was no significant difference between groups and the improvement was not maintained after the treatment period had finished. A similar pattern was observed for both the asthma control score and the mean VAS score with a significant improvement from baseline in both groups during the treatment period but no significant difference between groups and a return towards

baseline immediately on stopping treatment. This was also seen with nasal reported symptoms.

Figure 4: Blood and sputum eosinophil and neutrophil counts

Patients in both groups had a mild peripheral blood eosinophilia at baseline with no significant difference between groups. There was no change in the peripheral blood eosinophil count over the course of the study. There was a suggestion of a reduction in the blood eosinophil count in the voriconazole group on commencement of treatment with a return to baseline towards of the end of the study however there were no significant differences between the placebo and voriconazole groups. There was a modest reduction in sputum eosinophil counts in the voriconazole group during treatment but this was not different to placebo and no significant changes in sputum neutrophil count were observed

Figure 5: Immunoglobulins

All patients had raised *A. fumigatus* specific IgE at baseline with no difference between groups. There was no change in the specific IgE during the course of the study. Both groups of subjects had a raised IgE at baseline which was not significantly different between groups. There was no significant change in total IgE over the course of the study in either group. Both groups had A. fumigatus specific IgG in the normal range with no difference between groups. There was no significant change in the specific IgG over the course of the study.

Figure 1 CONSORT diagram

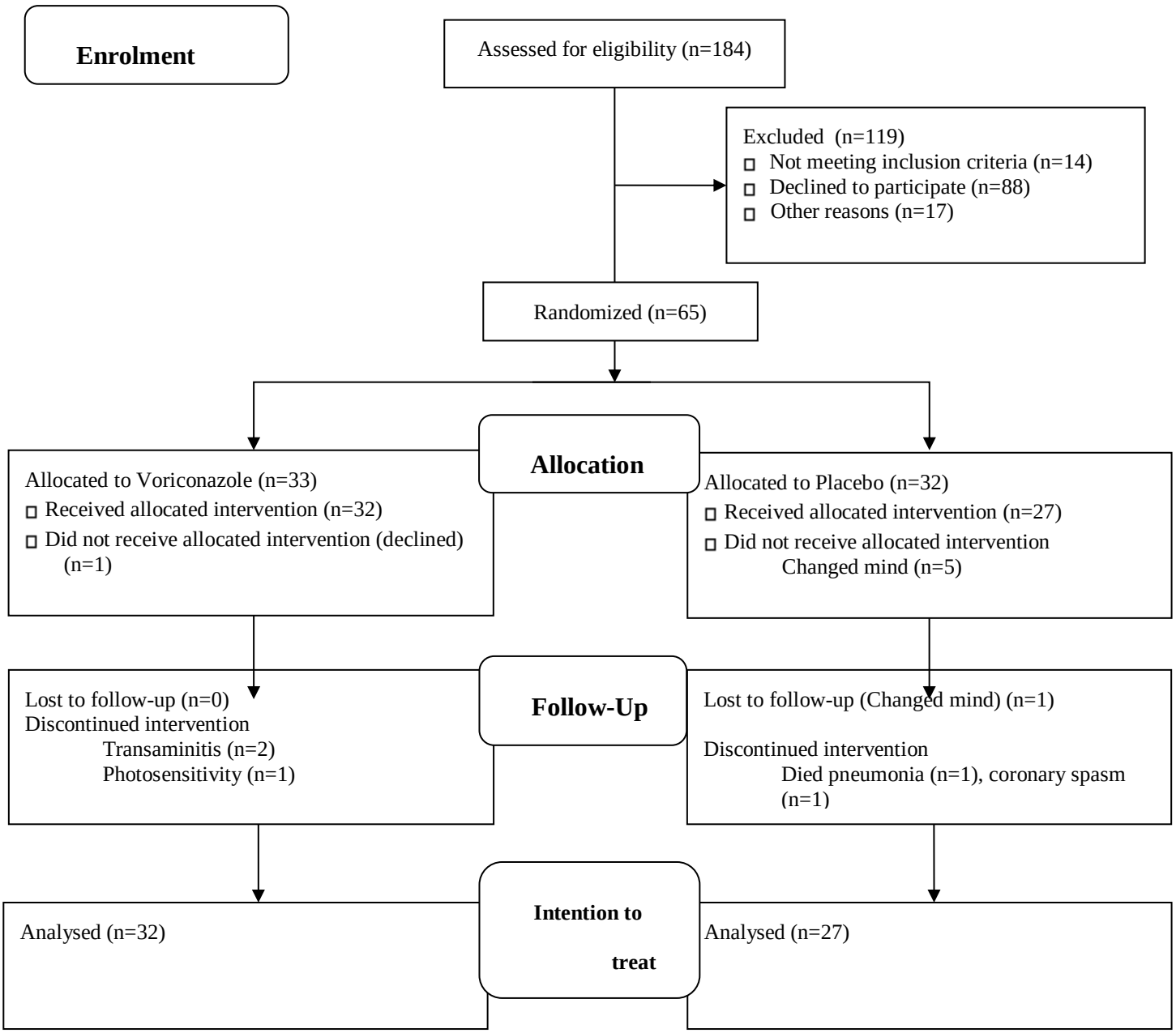


Figure 2

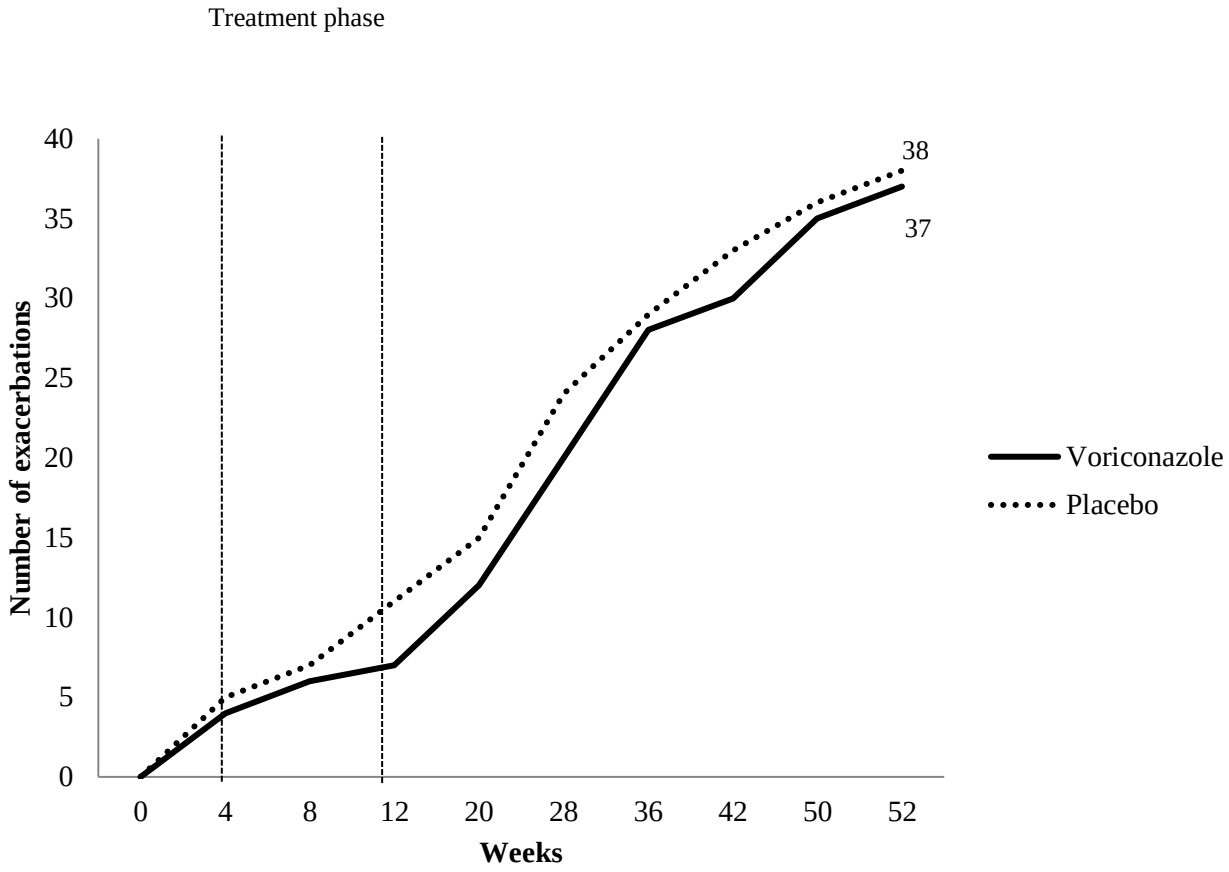

Treatment phase
Number of exacerbations
40
35
30
25
20
15
10
5
0
38
37
0
4
8
12
20
28
36
42
50
52
Weeks
Voriconazole
Placebo

Figure 3A

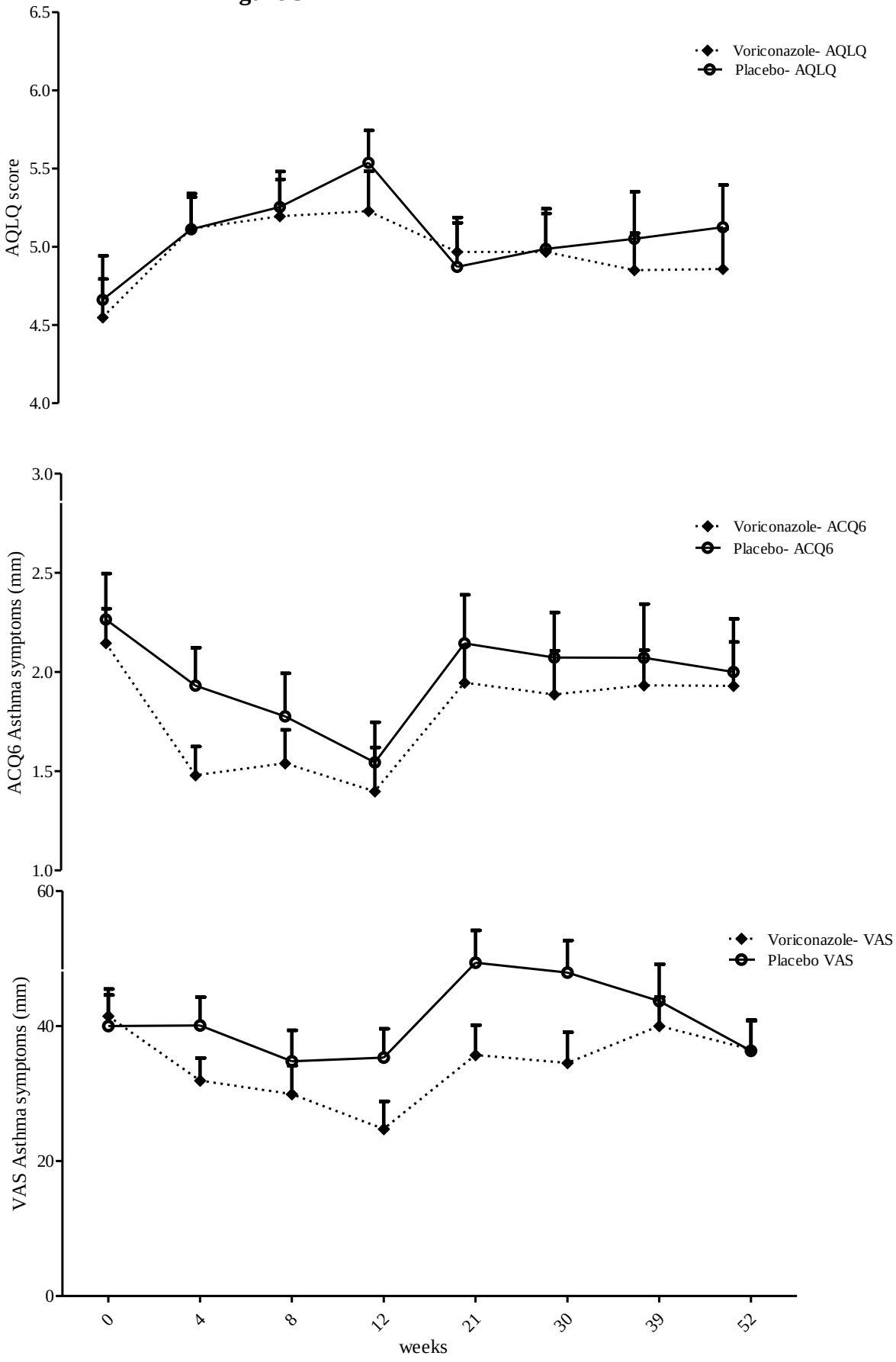

AQLQ score
6.5
6.0
5.5
5.0
4.5
4.0
Voriconazole- AQLQ
Placebo- AQLQ
ACQ6 Asthma symptoms (mm)
3.0
2.5
2.0
1.5
1.0
Voriconazole- ACQ6
Placebo- ACQ6
VAS Asthma symptoms (mm)
60
40
20
0
Voriconazole- VAS
Placebo VAS
0
4
8
12
21
30
39
52
weeks

Figure 3B

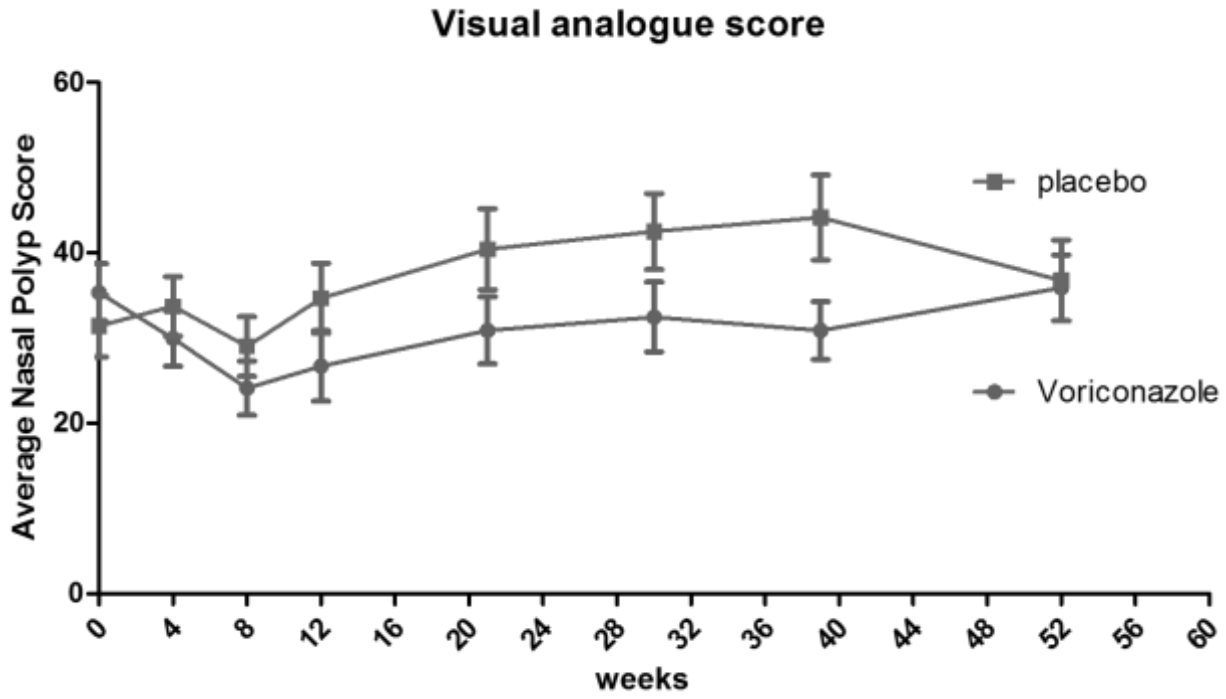

Visual analogue score
Average Nasal Polyp Score
60
40
20
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
weeks
placebo
Voriconazole

Figure 3C

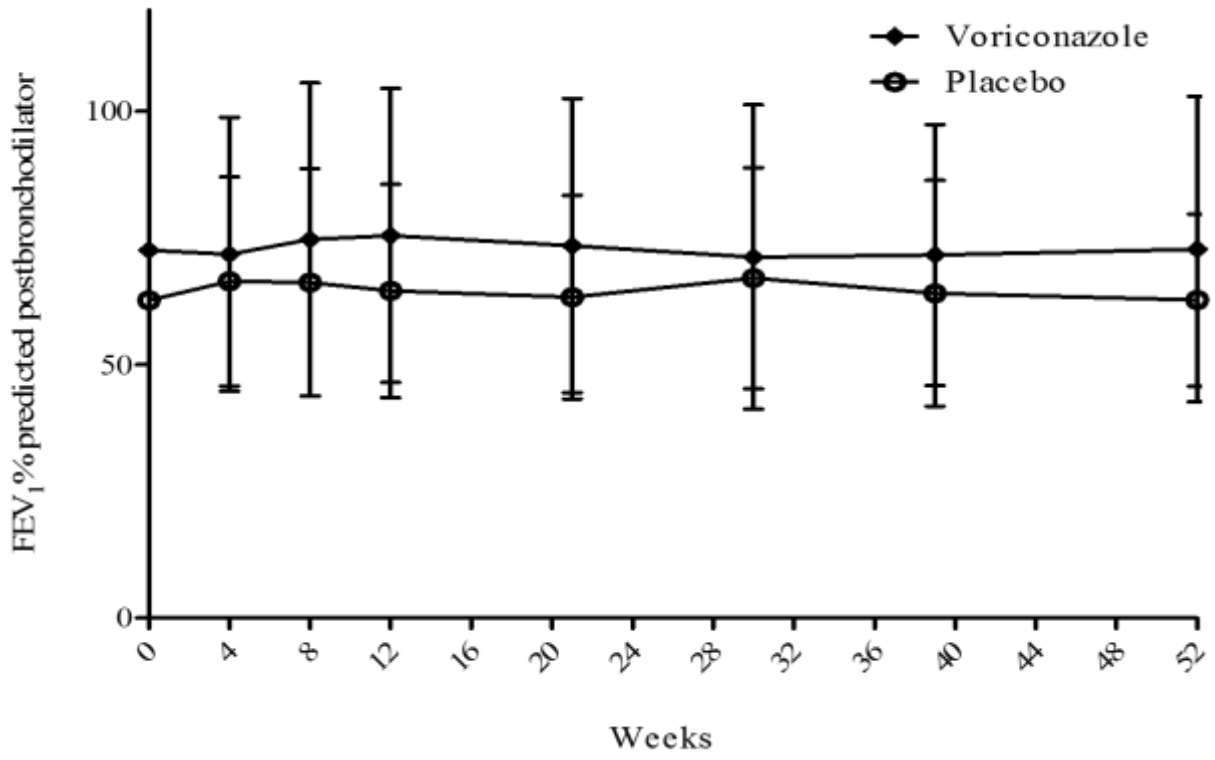

Voriconazole
Placebo
FEV1 % predicted postbronchodilator
100
50
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Weeks

Figure 4

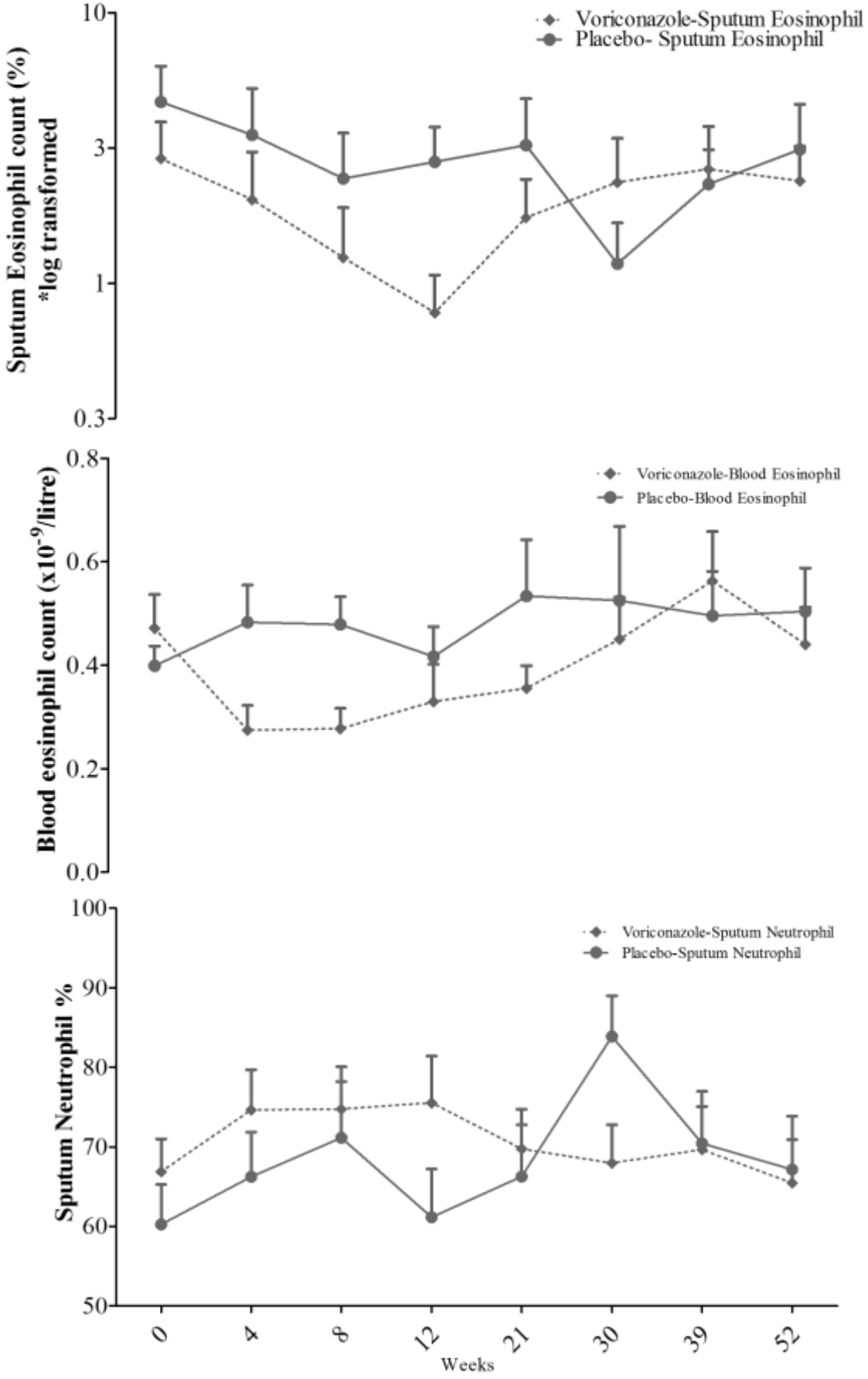

Voriconazole-Sputum Eosinophil
Placebo- Sputum Eosinophil
Sputum Eosinophil count (%)
*log transformed
10
3
1
0.3
Voriconazole-Blood Eosinophil
Placebo-Blood Eosinophil
Blood eosinophil count (x10-9/litre)
0.8
0.6
0.4
0.2
0.0
Voriconazole-Sputum Neutrophil
Placebo-Sputum Neutrophil
Sputum Neutrophil %
100
90
80
70
60
50
0
4
8
12
21
30
39
52
Weeks

Figure 5

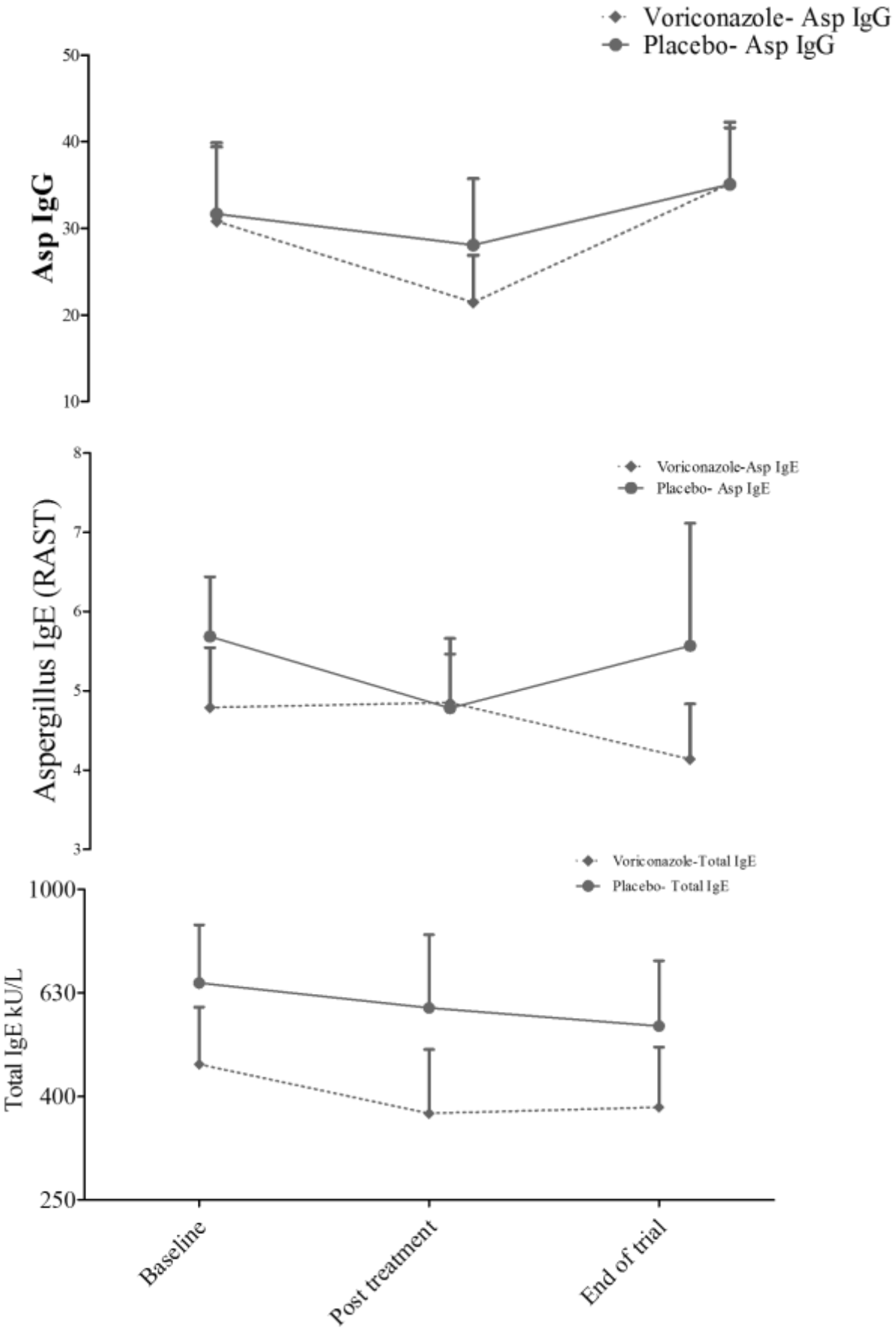

Voriconazole- Asp IgG
Placebo- Asp IgG
Asp IgG
50
40
30
20
10
Voriconazole-Asp IgE
Placebo- Asp IgE
Aspergillus IgE (RAST)
8
7
6
5
4
3
Voriconazole-Total IgE
Placebo- Total IgE
Total IgE kU/L
1000
630
400
250
Baseline
Post treatment
End of trial

Table 3

Reported Adverse Events/Side effects

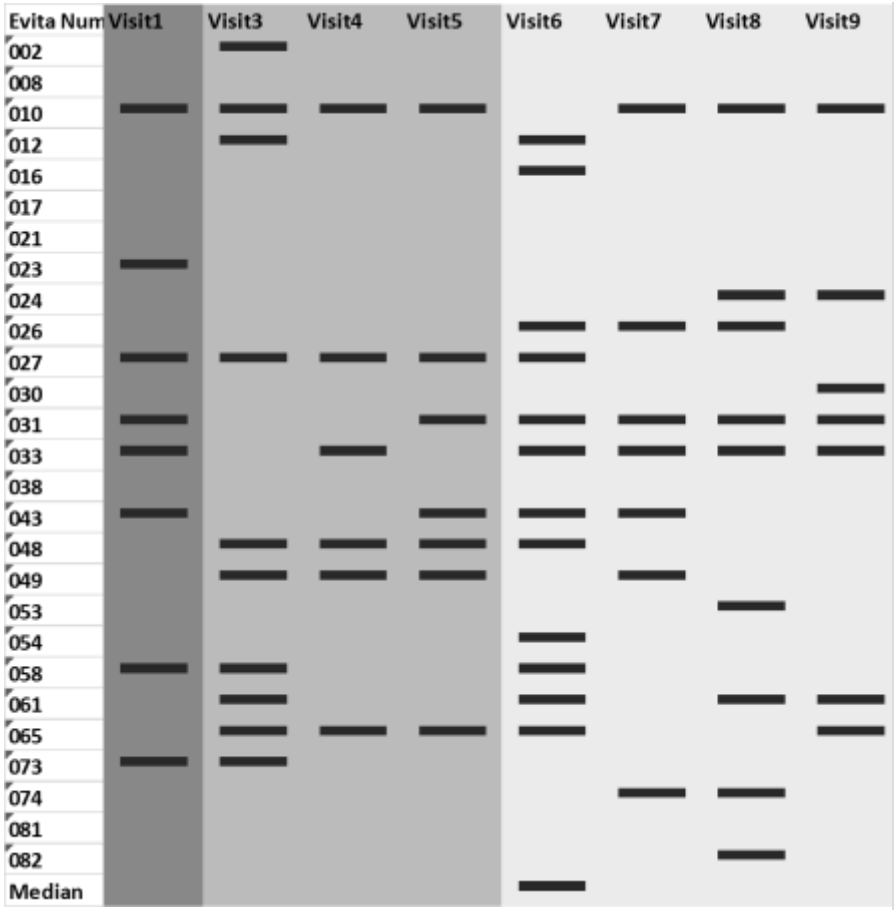
Event	Voriconazole (n= 32)	Placebo (n=27)
Serious adverse events		
Acute Coronary Syndrome	0	1
Hospitalisation for asthma	6	4
Nightmares	1	0
Deep venous thrombosis	1	0
Diabetes mellitus	0	1
Pneumonia	0	1
Adverse events		
Altered visual acuity	9	0
Rash*	6	3
Photosensitivity	14	0
Hair loss	1	0
Eczema	4	2
Otitis media	1	2
Sinusitis	5	0
Headaches	4	3
Dizziness	2	0
Hyper somnolence	0	1
Sleep disturbance	2	0
Upper respiratory tract infection	1	4
Urinary tract infection	0	1
Elevated transaminases	5	0

Musculoskeletal pain	5	2
Ankle oedema	3	1
Abdominal pain	1	1
Diarrhoea	0	2
Gastro-Oesophageal reflux symptoms	1	0
Nausea	0	1
Gingivitis	0	1
Post menstrual bleeding	1	0

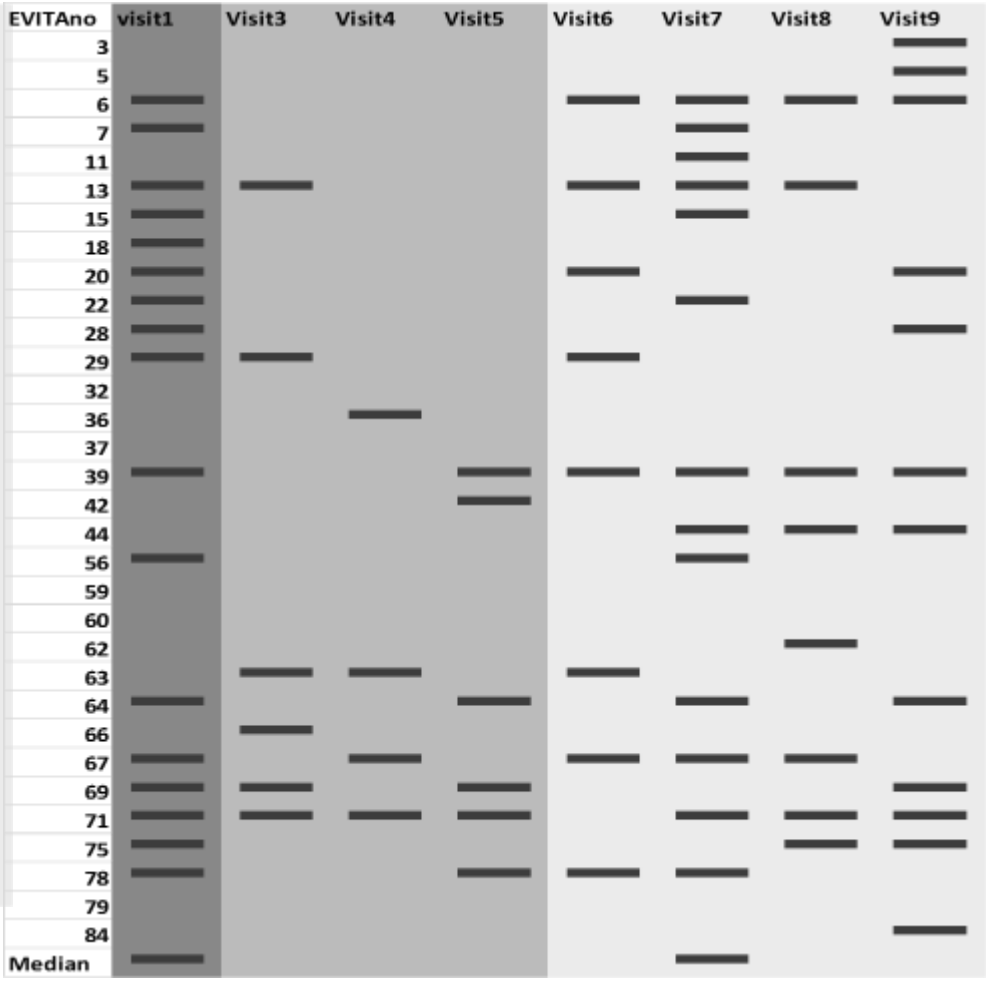
*including Erythema Marginatum,

Figure 6- Culture data

Placebo



Voriconazole



6 Conclusions

6.1 Summary of findings

This thesis has been concerned primarily with understanding further the role of fungi in the clinical expression of asthma.

Asthma is regarded as a chronic inflammatory disorder influenced by environmental stimuli some of which are unknown. Atopic tendencies are also a common feature.

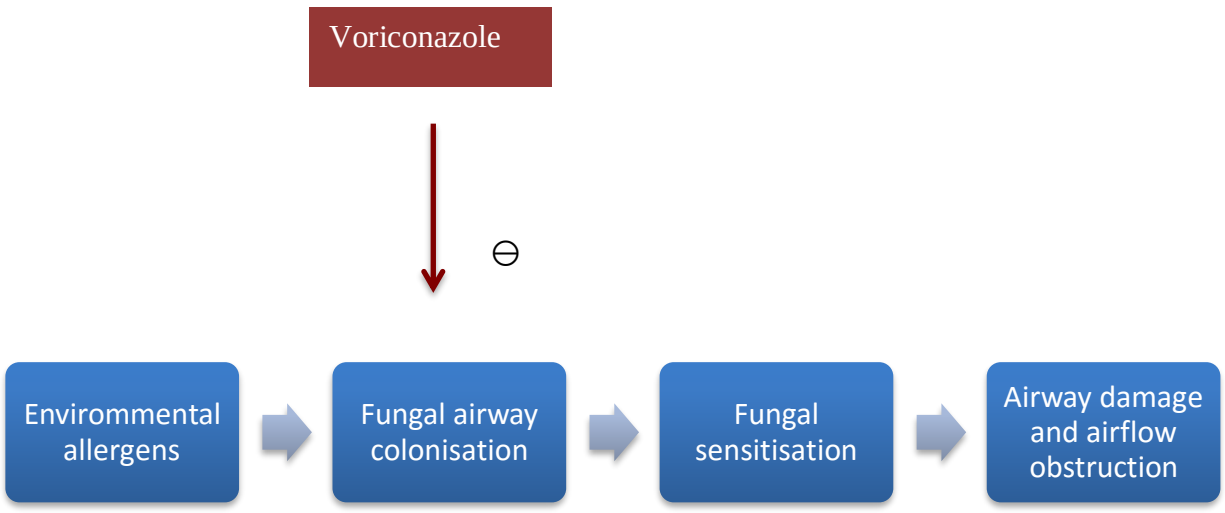
Though only a few fungi are renowned for their allergenicity, their ubiquity in the aerospora could provide potentially ample source of irritation to an atopic host.

In this thesis, I have explored and advanced the understanding of the various paradigms of fungal allergy in asthma. I showed that fungal airway colonisation is important in correlating with demonstrable fungal atopy in asthma further refining the physiology, immunology and fungal microbiology expressions in relation to each other and the result of an intervention in addressing fungal colonisation.

I have made use of sputum induction using hypertonic saline, a non-invasive airway sampling method more recently used in clinical management of asthma and which has been firmly integrated into guidelines as a method for obtaining microbiological samples in diagnosing pulmonary tuberculosis [154] and Pneumocystis *jiroveci* [155]. This thesis further validates this technique in providing a non-invasive method of sampling the lower respiratory tract used in detailing the fungal airway microbiome as well as the subsequent modified technique used in culturing fungi. A range of Fungal species found colonising the airway and measured using a modified culture technique was almost 7 times more frequently present in patients with asthma compared to healthy controls. Similar numbers were also obtained when a cohort of patients with COPD were examined; filamentous fungi were cultured at baseline in 49% (63 out of 128) of COPD subjects, of which 75% (47 out of 63) were *A. fumigatus* [156].

Sensitisation to fungi however appears to demonstrate a stronger association with airflow obstruction as demonstrated in the multivariate analysis in patients with asthma. Sensitisation to IgE specific *A. fumigatus*, irrespective of corresponding filamentous fungal culture, was also associated with lower lung function (FEV_1 39% predicted *versus* 51% predicted; mean difference 11%, 95% CI 3–20%; p=0.01) in those with COPD at stable state. This is a novel finding and supports the spectrum of fungal allergy in asthma not being limited to those with only ABPA where it was originally described.

I explored the use of voriconazole in those with asthma and fungal sensitisation. Unfortunately, fungal sensitisation remained unchanged despite a 3-month course of treatment in this group of patients. Furthermore, the culture rates of *Aspergillus fumigatus* rates appeared to remain unchanged at the end of the study. Disappointingly there were no differences in both Primary and second endpoints leaving the role on this antifungal unclear. While the study is novel, being the first randomized controlled trial of voriconazole, it may have been overly ambitious to hope for fixed airway remodeling to reverse itself in this study.



6.2 Future studies

From these studies, further important questions have arisen such as:

- When does fungal sensitisation occur in relation to those with asthma?
- What other significance do the presence of other fungi found have?
- What is the evidence for causation of fixed airflow obstruction due to fungal sensitisation and at what time is intervention with an antifungal best used to prevent the development of fixed airflow obstruction?

The main weakness in the management of fungal airway colonisation to-date has been an understanding of its natural history in man. Though these findings correlating fungal sensitisation to colonisation have been found in adults it is likely to have implications that fungal colonisation begins as early as in childhood.

Exposure to indoor fungi has been associated with poorer health effects such as cough, wheeze and asthma in children from a variety of settings especially when indirect measures such as visible moulds or mildew on surfaces have been used instead of fungal spore counts for assessment of fungal exposure. This is potentially a better representation of long-term exposure to fungi than direct measurements often during short sampling times.[157, 158].

Fungal sensitisation has also been recognised in children with severe asthma [159, 160] with markers of fungal cytokine responses such as IL 33 detected in BAL samples. It therefore seems feasible that sensitisation to fungi is likely to represent co-existing airway colonisation in children though this is yet to be tested.

Fungal colonisation is likely to be one of the multi-inflammatory stimuli [25] that contribute the development of severe airway disease in asthma. Its association with sensitisation and the finding of fixed reduced airflow obstruction in asthma is most striking. Despite this, a link to causation would require longitudinal studies prior to the development of sensitisation which itself maybe a marker of airway damage.

6.3 Critisims and Limitations of studies

Many issues have been identified and discussed in earlier chapters though a few are noteworthy of mention again.

The aim of the study was focused on exploring the role of *A.f* and other moulds in asthma. The methods here of culturing fungi itself biases identification of some certain fungi, since the majority of fungi cannot be sufficiently detected by current culture methods. The incubating temperature at 37 °C was also preferentially favoured to culturing *A.f*. This assessment of airway colonisation is also unable to quantify the airway fungal burden measured. As yet, is remains unclear whether the burden of fungi found in the airway play a role in contributing to the disease burden.

Though environmental contamination is of some concern and mindful that fungi can be found in the oral rinse of healthy patients, our healthy subjects in comparison to patients with asthma did not demonstrate any deficiency in technique. Further measures were in place to reduce the likelihood of contamination using a nose clip and rinsing the mouth prior to coughing up sputum. The culture technique also incorporated safe-guards to reduce contamination by the removal of sputum plugs [161] obtained from the lower airway from saliva. This technique has however yet to be validated against invasive sampling bronchoscopic methods offering bigger sample yields.

While using sputum induction generally provided adequate samples, sputum volumes obtained during the interventional study with voriconazole were at times insufficient for both cell differential counts and culture especially during the intervention period. The intervention duration is also questionable as to its length. To date this was the shortest duration of any anti-fungal study in asthma. It is therefore not possible to fully address the impact of antifungal use with voriconazole with this duration of therapy.

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Appendix

Presentations/Abstract

Agbetile, J., et al. (2013). "S90 Effectiveness of Voriconazole In the Treatment of *Aspergillus fumigatus* Associated Asthma." Thorax **68**(Suppl 3): A48-A48.

Bafadhel, M., et al. (2011). "S91 *Aspergillus fumigatus* sensitisation in patients with chronic obstructive pulmonary disease." Thorax **66**(Suppl 4): A43-A43.

Agbetile, J., et al. (2010). "S136 Fungal sputum culture in patients with severe asthma is associated with a reduced post bronchodilator FEV1." Thorax **65**(Suppl 4): A61-A62.

Agbetile, J., et al. (2010). "S133 Eosinophilic airway inflammation is associated with FEV1 decline in severe asthma." Thorax **65**(Suppl 4): A61-A61.

European respiratory society 2009
Volume 34, supplement 53, September 2009. European Respiratory Journal: Spoken session
Relationship between *Aspergillus fumigatus* Sensitisation and positive sputum culture in patients with asthma (ERS) Poster **2120**
J Agbetile, C. Pashley, B. Hargadon, M. William, M. Bourne, I. Pavord, A. Wardlaw, A. Fairs

Review Articles

New therapies and management strategies in the treatment of asthma: patient-focused developments
J Agbetile, R Green- Journal if asthma and allergy, 2011

Publications arising from thesis

Chapter 3
Isolation of filamentous fungi from sputum in asthma is associated with reduced post-bronchodilator FEV_1
Agbetile J*, Fairs A*, Desai D, Hargadon B, Bourne M, Mutalithas K, et al
Clin Exp Allergy 42(5): 782 -791.
* Joint first authors

Chapter 4
IgE Sensitisation to *Aspergillus fumigatus* Is Associated with Reduced Lung Function in Asthma
Fairs A,* Agbetile J,* Hargadon B, Bourne M, Monteiro WR, Brightling CE, Bradding P, Green RH, Mutalithas K, Desai D, Pavord ID, Wardlaw AJ, Pashley CH. Am J Respir Crit Care Med 2010;201001-0087OC.
* Joint first authors

Chapter 5
Effectiveness of Voriconazole in the treatment of *Aspergillus fumigatus*–associated asthma (EVITA3 study)

Agbetile J, Bourne M, Fairs A, Hargadon B, Desai D, Broad C, Morley J, Bradding P, Brightling CE, Green R, Haldar P, Pashley C, Pavord I, Wardlaw AJ. *Journal of Allergy and Clinical Immunology July 2014 Volume 134, Issue 1, Pages 33–39*
http://dx.doi.org/10.1016/j.jaci.2013.09.050

Study title: Studies on Aspergillus Lung Disease.

University Hospitals of Leicester
NHS Trust

Glenfield Hospital
Groby Road
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LE3 9QP

Tel: 0116 287 1471
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Minicom: 0116 287 9852

2.1.1 Participant Infor

Study title: Studies on Aspergillus Lung Disease. This is a study that will help us understand how the fungus Aspergillus affects the lungs of patients with asthma, bronchiectasis and cystic fibrosis.

Principal Investigator: Professor Andrew Wardlaw, Professor of Respiratory medicine, Institute of Lung Health, Glenfield Hospital, Leicester. Tel. 0116 287 1471. Ext 3841.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

Aspergillus is a fungus found almost everywhere but especially so in dead plant matter. The spores from this fungus are plentiful and are inhaled by us all every day. People with asthma respond to the inhaled fungal spores differently compared to non-asthmatic people - those with bronchiectasis and or cystic fibrosis. Even within the

group of asthmatics some respond to it differently from others. A small group of asthmatics are seen to be worse affected by this fungus, with poor control of their underlying asthma, and if left untreated can lead to considerable destruction to the lung over a period of time. Why some asthmatics respond to the fungus in this way is of interest. Firstly, it will help us identify and treat those that are affected, and secondly, it will help us further understand the different types of asthma. We suspect that some patients with asthma, bronchiectasis or cystic fibrosis may have abnormalities in their immune responses that could lead to this exaggerated response to this fungus. This study aims to identify those patients who have been exposed to this fungus and then study the nature of their immune response to this fungus.

Why have I been chosen?

You have been or are currently being reviewed at a chest clinic at Glenfield Hospital. Blood tests and/or skin allergy testing that you have had in the past has shown evidence that you may have been exposed to the fungus *aspergillus fungus*. You may be one of approximately 50 who will be approached for this study. You may also be invited to take part in this study even if you have no evidence of exposure to this fungus as part the study will also include a small number of control subjects.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

All those taking part in the study will be invited to attend the clinic to give a sample of blood (approximately 30ml). This visit will be short and in most cases should take no longer than 20 minutes. Blood tests will be examined for signs of fungal allergy and also analyse for genetic variations that may cause added susceptibility to this fungus.

No extra clinic appointments either at the Hospital or with the GP will be required. Subjects will continue to visit their GP for other needs. Some participants may be asked to provide sputum samples during this visit. Where ongoing exposure to the fungal spores is suspected we may provide you with a spore trap to be placed at your home for a period of 24-48hrs.
In those participants who show evidence of chronic aspergillus lung disease small wash samples will be obtained from the lung itself. Any subject who we suspect may not tolerate the procedure will not be asked. This will involve having a Bronchoscopy. Should you decide not to have this procedure you may still take part in the reminder of the study.

Lung wash samples will be obtained using a bronchoscope. Small volumes of saline will be used to wash a small area of the lung and the collected fluid will then be used for lab studies. This procedure is routinely performed for patients with other lung diseases.

- Bronchoscopy: Time will be scheduled for one morning when participants will be invited to attend the hospital. Instruction sheets on where and when to attend will be sent participants prior to the procedure. Further consent will be sought prior to procedure and you can decide not to have the test should you wish not to go ahead. Small amount of sedation will be given prior to the procedure. The procedure itself will last up to 30 minutes. Participants will be allowed home approximately 1-2 hours after the procedure.

What do I have to do?

For most of you this will only be a single visit to hospital to provide a blood and a sputum sample. Clinical information regarding your lungs will be gathered from your medical records. For those who will be having a bronchoscopy, additional instructions will be provided before the test. For this, patients will be asked to refrain from eating and drinking from midnight prior to the day of the test.

What are the possible disadvantages and risks of taking part?

Providing a blood sample will involve a visit to the hospital. Those having the bronchoscopy test will need to spend half at the hospital on the day of the test. There is a small risk of complications associated with the bronchoscopy procedure relating to the sedation used. This includes heart rhythm problems and over sedation. The procedure itself may induce coughing however this would be expected to resolve promptly after the test. This test is routinely performed for patients with lung disease and all those taking having this test will be monitored carefully throughout according to the standard protocols.

What are the possible benefits of taking part?

We will be able to identify whether aspergillus is involved in your asthma, and this could result in you being commenced on the appropriate therapy.

What happens when the research study stops?

You will continue to have regular follow up visits at the chest clinic. Results and any new information gathered will be fed back during your subsequent visits.

What if something goes wrong?

If you are harmed in by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspects of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

Who has reviewed the study?

This study has been reviewed by the Northamptonshire, Leicestershire and Rutland Ethics committee.

Thank you for reading this

Centre Number: :
Study Number:
Patient Identification Number for this trial:

2.1.2 CONSENT FORM

Title of Project: Studies on Aspergillus Lung Disease.

Name of Researcher: **Dr. Das. K. Mutalithas / Prof. A. Wardlaw.**

Please initial box

1. I confirm that I have read and understand the information sheet dated 31/01/2011 version 1.5 for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [company name] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

2.1.3 GP Letter

Date

Dr .....
Address

Dear Dr

Re: Your patient

Study title: Studies on aspergillus Lung Disease

Your patient has recently agreed to participate in the above study which is taking place at Glenfield Hospital. Details of the study are outlined in the enclosed patient information sheet.

If you require any further information please do not hesitate to contact me on (0116) 256 3021.

Yours sincerely

Professor A.J.Wardlaw

Glenfield Hospital

Enc. Patient Information Sheet

Effectiveness of voriconazole in the treatment of Aspergillus associated asthma

EVITA[3]

2.1.4 ACQ7

ASTHMA CONTROL QUESTIONNAIRE

UNITED KINGDOM VERSION

For further information:

Elizabeth Juniper, MCSP, MSc
Professor
20 Marcuse Fields,
Bosham,
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PO18 8NA. UK
Telephone: + 44 (0) 1243 572124
Fax: + 44 (0) 1243 573680
E-mail: juniper@qoltech.co.uk
Web: www.qoltech.co.uk

February 2001

Please answer questions 1 - 6.

Circle the number of the response that best describes how you have been during the past week.

1. On average, during the past week, how often were you **woken by your asthma** during the night?

0 Never
1 Hardly ever
2 A few times
3 Several times
4 Many times
5 A great many times
6 Unable to sleep because of asthma

2. On average, during the past week, how **bad were your asthma symptoms when you woke up** in the morning?

0 No symptoms
1 Very mild symptoms
2 Mild symptoms
3 Moderate symptoms
4 Quite severe symptoms
5 Severe symptoms
6 Very severe symptoms

3. In general, during the past week, how **limited were you in your "daily" activities** because of your asthma?

0 Not limited at all
1 Very slightly limited
2 Slightly limited
3 Moderately limited
4 Very limited
5 Extremely limited
6 Totally limited

4. In general, during the past week, how much **shortness of breath** did you experience because of your asthma?

0 None
1 A very little
2 A little
3 A moderate amount
4 Quite a lot
5 A great deal
6 A very great deal

5.	In general, during the past week, how much of the time did you **wheeze?**	0 Never 1 Hardly any of the time 2 A little of the time 3 A moderate amount of the time 4 A lot of the time 5 Most of the time 6 All the time
6.	On average, during the past week, how many **puffs/inhalations of short-acting bronchodilator** (eg. Ventolin/Bricanyl) have you used each day? *(If you are not sure how to answer this question, please ask for help)*	0 None 1 1 - 2 puffs/inhalations most days 2 3 - 4 puffs/inhalations most days 3 5 - 8 puffs/inhalations most days 4 9 - 12 puffs/inhalations most days 5 13 - 16 puffs/inhalations most days 6 More than 16 puffs/inhalations most days

To be completed by a member of the clinic staff

7.	FEV_1pre-bronchodilator: …… FEV_1predicted:…….. FEV_1%predicted:……. (Record actual values on the dotted lines and score the FEV_1% predicted in the next column)	0 > 95% predicted 1 95 - 90% 2 89 - 80% 3 79 - 70% 4 69 - 60% 5 59 - 50% 6 < 50% predicted

Asthma Diary card

EVITA3
A Study of the effectiveness of voriconazole in aspergillus associated asthma

(version 1 dated 30/09/09)

Asthma Diary Card Visit No. ☐

Daytime Asthma	**Night Time Asthma**
0; Normal 1; Occasional wheeze/breathless/cough 2; Wheeze/ breathless/ cough most of day 3; Asthma very bad: unable to do normal activities at all	0; None 1; Awoke once due to asthma 2; Awoke 2-3 times due to asthma 3; Awake most of the night due to asthma

Please complete the diary card each day

Please do not take any reliever inhalers on the day of your visit

If you have any queries please contact Bev/ Michelle on 0116 2563119 or page Dr Agbetile at Glenfield Hospital

Appointments	**Date**	**Time**
Clinic Visit		
Sputum Test		

Week 3 Date:							
Days off work due to asthma? Course of prednisolone? GP or hospital visits/ contacts?							
Daytime asthma							
Night- time wakening							
Peak Flow **AM** (best of 3)							
PM							
No. of puffs of ventolin or bricanyl in 24 hours							

Name: **Date:**

Week 4 Date:							
Days off work due to asthma? Course of prednisolone? GP or hospital visits/ contacts?							
Daytime asthma							
Night- time wakening							
Peak Flow **AM** (best of 3)							
PM							
No. of puffs of ventolin or bricanyl in 24 hours							

Baseline PEF:
70% of **baseline** PEF:

Last Juniper score:
Juniper score :

No. of severe exacerbations:

No. of days when peak flow< 70% baseline:

Daytime Asthma	**Night Time Asthma**
0; Normal 1; Occasional wheeze/breathless/cough 2; Wheeze/ breathless/ cough most of day 3; Asthma very bad: unable to do normal activities at all	0; None 1; Awoke once due to asthma 2; Awoke 2-3 times due to asthma 3; Awake most of the night due to asthma

Medication and doses:

1)

2)

3)

4)

5)

Week 1 Date:							
Days off work due to asthma? Course of prednisolone? GP or hospital visits/ contacts?							
Daytime asthma							
Night- time wakening							
Peak Flow **AM** (best of 3)							
PM							
No. of puffs of ventolin or bricanyl in 24 hours							

Week 2 Date:							
Days off work due to asthma? Course of prednisolone? GP or hospital visits/ contacts?							
Daytime asthma							
Night- time wakening							
Peak Flow **AM** (best of 3)							
PM							
No. of puffs of ventolin or bricanyl in 24 hours							

Any other health service contact?
e.g. Practise nurse/ NHS Direct/ A&E

If your peak flow falls to ……..(which is 70% of your best) on 2 consecutive days, then you will need a course of steroids. Please contact the research nurses at Glenfield on 0116 2563119 or contact Dr Agbetile on bleep 2686 during office hours, or your G.P. outside these times. If you contact your G.P., please remind them you are in this study.

Asthma symptoms- Visual Analogue Scale

EVITA[3] STUDY

VISUAL ANALOGUE SCORES

Study Number: Date:

Visit Number:

Please mark a cross along the scale to show how severe you feel your symptoms are for each of the symptoms listed.

Best Ever **Worst possible**

Scale

Cough _____

Breathlessnes _____

Wheeze _____

To be completed by research staff

Symptoms	VAS Measurement (mm)
Cough	
Breathlessness	
Wheeze	

Asthma Nasal Polyps- Visual analogue score

Effectiveness of Voriconazole in Aspergillus Associated Asthma
P.I. Professors A.J Wardlaw and I.D. Pavord
Blomqvist et al. Journal Allergy and Clinical Immunology Feb 2001: 224-228

EVITA[3] STUDY

VISUAL ANALOGUE SCORES
FOR NASAL POLYPS

Study Number: Date:

Visit Number:

Please mark a cross along the scale to show how severe you feel your symptoms are for each of the symptoms listed.

Best Ever **Worst possible**

Scale

Sense of Smell _____

Nasal Secretion _____

Pressure over sinuses _____

Nasal obstruction _____

Headache _____

To be completed by research staff

Symptoms	VAS Measurement (mm)
Smell	
Secretion	
Sinus pressure	
Obstruction	
Headache	

Asthma Quality of Life Questionaires

ASTHMA QUALITY OF LIFE QUESTIONNAIRE WITH STANDARDISED ACTIVITIES (AQLQ(S))

SELF-ADMINISTERED
UNITED KINGDOM VERSION

QOL TECHNOLOGIES LTD.

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ASTHMA QUALITY OF LIFE QUESTIONNAIRE (S) PATIENT ID _____
(UNITED KINGDOM)
SELF-ADMINISTERED DATE _____

Please complete **all** questions by circling the number that best describes how you have been during the **last 2 weeks as a result of your asthma.**

HOW LIMITED HAVE YOU BEEN **DURING THE LAST 2 WEEKS** IN THESE ACTIVITIES **AS A RESULT OF YOUR ASTHMA?**

		Totally Limited	Extremely Limited	Very Limited	Moderate Limitation	Some Limitation	A Little Limitation	Not at all Limited
1.	STRENUOUS ACTIVITIES (such as hurrying, exercising, running up stairs, sports)	1	2	3	4	5	6	7
2.	MODERATE ACTIVITIES (such as walking, housework, gardening, shopping, climbing stairs)	1	2	3	4	5	6	7
3.	SOCIAL ACTIVITIES (such as talking, playing with pets/children, visiting friends/relatives)	1	2	3	4	5	6	7
4.	WORK-RELATED ACTIVITIES* (tasks you have to do at work)	1	2	3	4	5	6	7

*If you are not employed or self-employed, these should be tasks you have to do most days.

5.	SLEEPING	1	2	3	4	5	6	7

HOW MUCH **DISCOMFORT OR DISTRESS** HAVE YOU FELT **DURING THE LAST 2 WEEKS**?

		A Very Great Deal	A Great Deal	A Good Deal	Moderate Amount	Some	Very Little	None
6.	How much discomfort or distress have you felt over the last 2 weeks as a result of CHEST TIGHTNESS?	1	2	3	4	5	6	7

IN GENERAL, **HOW MUCH OF THE TIME DURING THE LAST 2 WEEKS** DID YOU:

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	Hardly Any of the Time	None of the Time
7. Feel CONCERNED ABOUT HAVING ASTHMA?	1	2	3	4	5	6	7
8. Feel SHORT OF BREATH as a result of your asthma?	1	2	3	4	5	6	7
9. Experience asthma symptoms as a RESULT OF BEING EXPOSED TO CIGARETTE SMOKE?	1	2	3	4	5	6	7
10. Experience a WHEEZE in your chest?	1	2	3	4	5	6	7
11. Feel you had to AVOID A SITUATION OR ENVIRONMENT BECAUSE OF CIGARETTE SMOKE?	1	2	3	4	5	6	7

HOW MUCH **DISCOMFORT OR DISTRESS** HAVE YOU FELT **DURING THE LAST 2 WEEKS**?

	A Very Great Deal	A Great Deal	A Good Deal	Moderate Amount	Some	Very Little	None
12. How much discomfort or distress have you felt over the last 2 weeks as a result of COUGHING?	1	2	3	4	5	6	7

IN GENERAL, **HOW MUCH OF THE TIME DURING THE LAST 2 WEEKS** DID YOU:

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	Hardly Any of the Time	None of the Time
13. Feel FRUSTRATED as a result of your asthma?	1	2	3	4	5	6	7
14. Experience a feeling of CHEST HEAVINESS?	1	2	3	4	5	6	7

IN GENERAL, **HOW MUCH OF THE TIME DURING THE LAST 2 WEEKS** DID YOU:

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	Hardly Any of the Time	None of the Time
15. Feel CONCERNED ABOUT THE NEED TO USE MEDICATION for your asthma?	1	2	3	4	5	6	7
16. Feel the need to CLEAR YOUR THROAT?	1	2	3	4	5	6	7
17. Experience asthma symptoms as a RESULT OF BEING EXPOSED TO DUST?	1	2	3	4	5	6	7
18. Experience DIFFICULTY BREATHING OUT as a result of your asthma?	1	2	3	4	5	6	7
19. Feel you had to AVOID A SITUATION OR ENVIRONMENT BECAUSE OF DUST?	1	2	3	4	5	6	7
20. WAKE UP IN THE MORNING WITH ASTHMA SYMPTOMS?	1	2	3	4	5	6	7
21. Feel AFRAID OF NOT HAVING YOUR ASTHMA MEDICATION AVAILABLE?	1	2	3	4	5	6	7
22. Feel bothered by HEAVY BREATHING?	1	2	3	4	5	6	7
23. Experience asthma symptoms as a RESULT OF THE WEATHER OR AIR POLLUTION OUTSIDE?	1	2	3	4	5	6	7
24. Were you WOKEN AT NIGHT by your asthma?	1	2	3	4	5	6	7
25. AVOID OR LIMIT GOING OUTSIDE BECAUSE OF THE WEATHER OR AIR POLLUTION?	1	2	3	4	5	6	7
26. Experience asthma symptoms as a RESULT OF BEING EXPOSED TO STRONG SMELLS OR PERFUME?	1	2	3	4	5	6	7

IN GENERAL, **HOW MUCH OF THE TIME DURING THE LAST 2 WEEKS** DID YOU:

	All of the Time	Most of the Time	A Good Bit of the Time	Some of the Time	A Little of the Time	Hardly Any of the Time	None of the Time
27. Feel AFRAID OF GETTING OUT OF BREATH?	1	2	3	4	5	6	7
28. Feel you had to AVOID A SITUATION OR ENVIRONMENT BECAUSE OF STRONG SMELLS OR PERFUME?	1	2	3	4	5	6	7
29. Has your asthma INTERFERED WITH GETTING A GOOD NIGHT'S SLEEP?	1	2	3	4	5	6	7
30. Have a feeling of FIGHTING FOR AIR?	1	2	3	4	5	6	7

HOW LIMITED HAVE YOU BEEN **DURING THE LAST 2 WEEKS**?

	Most Not Done		Several Not Done		Very Few Not Done		No Limitation
31. Think of the OVERALL RANGE OF ACTIVITIES that you would have liked to have done during the last 2 weeks. How much has your range of activities been limited by your asthma?	1	2	3	4	5	6	7

HOW LIMITED HAVE YOU BEEN **DURING THE LAST 2 WEEKS**?

	Totally Limited	Extremely Limited	Very Limited	Moderate Limitation	Some Limitation	A Little Limitation	Not at all Limited
32. Overall, among ALL THE ACTIVITIES that you have done during the last 2 weeks, how limited have you been by your asthma?	1	2	3	4	5	6	7

DOMAIN CODE:

Symptoms: 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 29, 30
Activity Limitation: 1, 2, 3, 4, 5, 11, 19, 25, 28, 31, 32
Emotional Function: 7, 13, 15, 21, 27
Environmental Stimuli: 9, 17, 23, 26