Detection of filamentous fungi in the homes and airways of patients with asthma

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Abstract

Asthma is a heterogeneous condition characterised by variable airflow obstruction, airway inflammation and hyper-responsiveness. Fungal sensitisation has been associated with asthma severity; and airways colonisation by the pathogenic fungus *Aspergillus fumigatus* has been associated with a progressive lung function decline in allergic bronchopulmonary aspergillosis. Interest in the home environment as a source of fungal exposure is increasing; however, there are still no accepted guidelines or standardised methods for the quantification of indoor fungal levels.

We sought to i) investigate typical airborne fungal spore concentrations in homes and to compare exposure levels in asthma patients grouped according to either *A. fumigatus* sensitisation or sputum culture; ii) fully characterise the fungal biota capable of colonising the airways in patients with asthma; and iii) define the clinical characteristics of fungal colonisation.

Aspergillus/*Penicillium*-type conidia exhibited indoor predominance and independence of season, and were highest in old, terraced, non-insulated properties. *A. fumigatus* was the predominant fungus isolated from sputum and IgE sensitisation to *A. fumigatus* was associated with reduced post-bronchodilator FEV_1 in patients with asthma. Sputum culture of filamentous fungi was also associated with reduced lung function, with predominant fungi comprising *Aspergillus* and *Penicillium* species; notably *Penicillium piceum* and species of *Aspergillus* section *Nigri.* Higher levels of airborne *A. fumigatus* were detected in homes of asthmatics with a positive sputum culture for *A. fumigatus.*

In conclusion, sensitisation to *A. fumigatus* and airways colonisation by fungi are associated with reduced lung function in moderate to severe asthma; and this study provides a direct link between home exposure and airways colonisation.

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Declaration of joint efforts

My work was central to the development and production of the final work; however, a number of colleagues were involved in the production of data and subsequently co-authored publications cited. Clinical data was collected by Dr Joshua Agbetile, Dr Dhananjay Desai, Dr Kugathasan Mutalithas and Professor Andrew Wardlaw at Glenfield Hospital. Specifically, Joshua Agbetile and I originally jointly prepared Tables 4.1-3 and 5.2-3.

William Monteiro contributed to sputum differential cell counts produced for Chapters 4 and 5, members of the sputum team were involved in initial inoculations of sputum culture plates and processing of sputum, Dr Catherine Pashley helped in collection of home sampling data in Chapter 6, Richard Edwards helped to produce aeroallergen sampling materials, and Joseph Morley and Catherine Pashley were involved in preparation of fungal culture media, and identification of filamentous fungi in Chapters 4 and 5.

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Abbreviations

ΔC_T	Delta cycle threshold
ΔRn	Ratio of reporter dye to passive reference dye
A. alternata	*Alternaria alternata*
A. flavus	*Aspergillus flavus*
A. fumigatus	*Aspergillus fumigatus*
A. niger	*Aspergillus niger*
A. terreus	*Aspergillus terreus*
A. versicolor	*Aspergillus versicolor*
ABPA	Allergic bronchopulmonary aspergillosis
ABPM	Allergic bronchopulmonary mycoses
ANOVA	Analysis of variance
Asp/Pen-type	*Aspergillus/Penicillium*-type
B. cinerea	*Botrytis cinerea*
BSA	Bovine serum albumin
C. herbarum	*Cladosporium herbarum*
CD4+	Cluster of differentiation 4 positive
CHIT-1	Chitotriosidase-1
Cox1	Cytochrome c oxidase subunit 1
C_T	Cycle threshold
CF	Cystic fibrosis
DMEM	Dulbecco's modified eagles medium
DMEM-10% FNPS	DMEM plus 10% FBS, non-essential amino acids, penicillin and streptomycin
DMSO	Dimethyl sulfoxide

DNA	Deoxyribose nucleic acid
DPX	Distyrene, plasticiser and xylene mountant
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EF-α	Elongation factor alpha
EPA	Environmental Protection Agency
ERMI	Environmental Relative Mouldiness Index
FAM	FAMRA
FBS	Foetal bovine serum
FcεR1	Fc (fragment, crystallisable) epsilon receptor-1
FEV_1	Forced expiratory volume of air in the first second
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
HBSS	Hank's balanced salt solution
HPA	Health Protection Agency
IQR	Interquartile range
Ig	Immunoglobulin
ITS	Internal transcribed spacer region
LSU	Large subunit
MAARA	Midlands Asthma and Allergy Research Association
MCDL	Mean conidia detection limit
MSQPCR	Mould-specific quantitative polymerase chain reaction
P. chrysogenum	*Penicillium chrysogenum*
P. piceum	*Penicillium piceum*
P. marneffei	*Penicillium marneffei*
P. variotii	*Paecilomyces variotii*
PCR	Polymerase chain reaction

PDA	Potato dextrose agar
PGC	Potato dextrose agar with gentamicin and chloramphenicol
PGCF	Potato dextrose agar with gentamicin, chloramphenicol and fluconazole
PNACL	Protein nucleic acid laboratory
RNase	Ribonuclease
Q1	Lower quartile
Q3	Upper quartile
qPCR	Quantitative polymerase chain reaction
RPM	Revolutions per minute
SAFS	Severe asthma with fungal sensitisation
SEM	Standard error of the mean
SPT	Skin prick test
TAE	Tris-acetate-EDTA
TAM	TAMRA
Th2	T helper lymphocyte type 2
vs.	*versus*

List of publications and prizes which have arisen from this thesis

Original research articles

Agbetile J*, **Fairs A***, Desai D, Hargadon B, Bourne M, Mutalithas K, Edwards RE, Morley JP, Monteiro WR, Kulkarni NS, Green RH, Bradding P, Brightling CE, Pavord ID, Wardlaw AJ, Pashley CH. (2012). Isolation of filamentous fungi from sputum in asthma is associated with reduced post-bronchodilator FEV_1. *Clin Exp Allergy* 42(5) 782-791. *Joint first authors

Pashley CH, **Fairs A**, Morley JP, Tailor S, Agbetile J, Bafadehel M, Brightling CE, Wardlaw AJ. (2012). Routine processing procedures for isolating filamentous fungi from respiratory sputum samples may underestimate fungal prevalence. *Med Mycol* 50(4) 433-438.

Pashley, CH, **Fairs A**, Free RC, Wardlaw AJ. (2012). DNA analysis of outdoor air reveals a high degree of fungal diversity, temporal variability, and genera not seen by spore morphology. *Fungal Biol* 116(2): 214-224.

Fairs, A*, Agbetile, J*, Hargadon, B, Bourne M, Monteiro WR, Brightling CE, Bradding P, Green R, Mutalithas K, Desai D, Pavord ID, Wardlaw AJ, Pashley CH. 2010. IgE sensitisation to *Aspergillus fumigatus* is associated with reduced lung function in asthma. *Am J Respir Crit Care Med* 182(11): 1362-1368. *Joint first authors

Fairs A, Wardlaw AJ, Thompson JR, Pashley CH. 2010. Guidelines on ambient intramural airborne fungal spores. *J Investig Allergol Clin Immunol* 20(6): 490-498

Review article

Knutsen AP, Bush RK, Demain JG, Denning DW, Dixit A, Fairs A, Greenberger PA, Kariuki B, Kita H, Kurup VP, Moss RB, Niven RM, Pashley CH, Slavin RG, Vijay HM, Wardlaw AJ. 2012. Fungi and allergic lower respiratory tract diseases. *J Allergy Clin Immunol* 129(2): 280-291

Prizes

Winner of the Barry Kay Award at the British Society for Allergy and Clinical Immunology, June 2009

Winner of the best posters prize at the 4th Advances against Aspergillosis Conference, February 2010

1 Introduction

1.1 The role of fungi in asthma and allergy

Allergic disease is a rising epidemic in the Western world, affecting around one in three people in the UK [1]; the chronic nature of allergic disease and associations with asthma placing considerable demand on the healthcare industry. In Europe, approximately 17% of people are allergic to grass pollen [2], the most common cause of respiratory allergy. Fungal allergy is less common, with around 3.3% of people allergic to *Alternaria,* and 1.7% allergic to *Cladosporium* [2]; however, rates of fungal allergy (or sensitisation) are much higher in severe asthma, with two thirds to three quarters of people with severe asthma being sensitised to one or more fungi [3, 4]. The limited number of available skin-prick test reagents (allergy test preparations from specific fungi) means that fungal allergy is likely to be under-diagnosed since allergy to only a fraction of fungi can be tested for. Furthermore, despite being important aeroallergens, fungi are understudied with regard to their impact on human respiratory health.

The prevalence and morbidity of asthma is increasing, affecting around 300 million people worldwide [5, 6]. Asthma is a heterogeneous chronic inflammatory disease of the airway which is characterised by variable airflow obstruction, airway inflammation and hyper-responsiveness. It is usually associated with eosinophilia and driven by T helper (type 2; Th2) allergen-specific lymphocytes [7-9], leading to the characteristic symptoms of asthma: cough, wheeze and shortness of breath. It is thought that around 5-10% of people with asthma have a severe form of the disease [10] (although higher proportions up to 21% have been reported [11]), and the top 5% in terms of disease

severity account for around half of the total cost to the healthcare industry [12, 13]. It is also these patients who are more likely to be affected by fungal allergy.

Indoor and outdoor fungal aeroallergens are important causative agents of asthma and allergy and interest is particularly increasing in allergies caused by exposures in the home environment, estimated to affect around 12 million people in the UK, with nearly one third of self-reported allergies triggered by mould exposure [1]. In addition to being causative agents of allergy, some fungi are facultative thermophiles; which means that whilst these fungi thrive at high temperatures, they are also able to grow at lower temperatures. This important property of select fungi means that some species are able to grow at human body temperature and may therefore be important opportunistic pathogens of humans.

Filamentous fungi can elicit effects on health in a number of ways: through infection, ranging in severity from superficial cutaneous infections to life-threatening invasive mycoses; through the production of toxic metabolites, causing toxic reactions 6-8 hours after ingestion; or through allergic reactions, following either ingestion or inhalation of fungi [14]. One of the most widely studied and pathogenic opportunistic fungal pathogens is *Aspergillus fumigatus*, which is responsible for fungal infections ranging in severity from minor cutaneous infections to life threatening invasive mycoses.

Comprehensive analysis of the biology, aerobiology and health effects of fungi is required in order to fully understand the role of fungi in airways disease.

1.2 Introduction to the Kingdom Fungi

Fungi are eukaryotic microorganisms which are ubiquitous in nature and can be broadly categorised into mushrooms, yeasts and filamentous fungi (moulds). It is estimated that there may be around 1.5 million species of fungi, of which a mere 74-120,000 have

been described [15, 16]. Fungi have a fundamental role in ecological processes through the degradation of decaying organic matter [17]; therefore fungal exposure is inevitable almost everywhere except artificially sterile environments [17]. Fungi are of great economic importance, and are used in the production of alcoholic beverages, production of antibiotics, enzymes, preservatives, vitamins and amino acids, production of foodstuffs such as bread, cheese and single-celled protein, and are also used in the production of herbicides and pesticides [15].

The currently accepted classification of fungi includes one subkingdom, seven phyla, ten subphyla, 35 classes, 12 subclasses and 129 orders [18]. The seven phyla of the Kingdom Fungi are Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Microsporidia, Glomeromycota, Basidiomycota and Ascomycota.

1.2.1 Growth and reproduction mechanisms of fungi

Fungi reproduce through either sexual or asexual production of spores. In outdoor air, the predominant fungal spores present are from the phyla Ascomycota and Basidiomycota, which together comprise the Dikarya and represent around 98% of fungal species so far described [19]. Some fungi reproduce entirely through asexual means, some by sexual reproduction, and others using both methods. The asexual form of a fungus is termed the anamorph; the sexual form is termed the teleomorph; and the term holomorph is used to describe the fungus as a whole, including the anamorphic and teleomorphic stages of the life cycle.

The most studied fungal spores with regard to allergy are anamorphs of fungi from the Ascomycota (including moulds) which produce conidia (asexually produced spores), which analyses of total airborne spore concentrations by microscopy have suggested may comprise 30-60% of the outdoor airborne fungal biota [14]. Clinically important anamorphs from the Ascomycota include *Alternaria, Aspergillus, Penicillium, Botrytis,*

Cladosporium, Fusarium and *Epicoccum.* Teleomorphic spores from the Ascomycota and Basidiomycota comprise the majority of the remainder of the outdoor fungal biota [14], amongst low levels of spores from other fungi.

1.2.2 Sexual reproduction in the Dikarya

Production of teleomorphic spores from fungi within the Dikarya involves the growth of either simplistic or elaborate fruiting bodies. The phylum Ascomycota contains the largest number of described species in the fungal kingdom [19], with over 64,000 so far described [20]. Teleomorphs of the Ascomycota, termed ascomycetes, produce meiospores called ascospores within a sac termed an ascus. Fusion of haploid cells or gametangia, is followed by meiosis and mitosis, and production of eight ascospores around the eight nuclei produced within the ascus [19, 21, 22] (Figure 1.1A). Violent release of ascospores is caused when the ascus, which is a turgid cell, bursts at maturity, propelling the ascospores a distance variable between species from 2-400 mm [21, 23]. The phylum Basidiomycota contains over 31,000 species [20]. Fusion of haploid nuclei in teleomorphs of Basidiomycota, termed basidiomycetes, forms a diploid nucleus which divides by meiosis inside a club-shaped structure called a basidium. In contrast to the Ascomycota, there is no mitosis phase and the nuclei pass into four rapidly developing basidiospores (Figure 1.1B), produced asymmetrically by one or more sterigmata, which are projections from the basidium (sporophore) [21, 24]. The sporophores of the Basidiomycota are stalked fruiting bodies termed basidiocarps, some of which are commonly referred to as mushrooms, and may have intricate morphologies such as gills, folds, spines or pores on which the basidiospores are presented [24].

Discharge of basidiospores occurs by the excretion of a drop of fluid at the point of attachment between the spore and the sporophore, which causes detachment of the spore

on contact with convection currents [24]. Basidiospores are generally consistent in their size and spherical morphology, in contrast to ascospores which vary considerably in morphology between species [23]. Furthermore, the distance basidiospores are discharged from the basidium is much shorter than in ascomycetes, ranging from 0.1-0.2 mm [21].

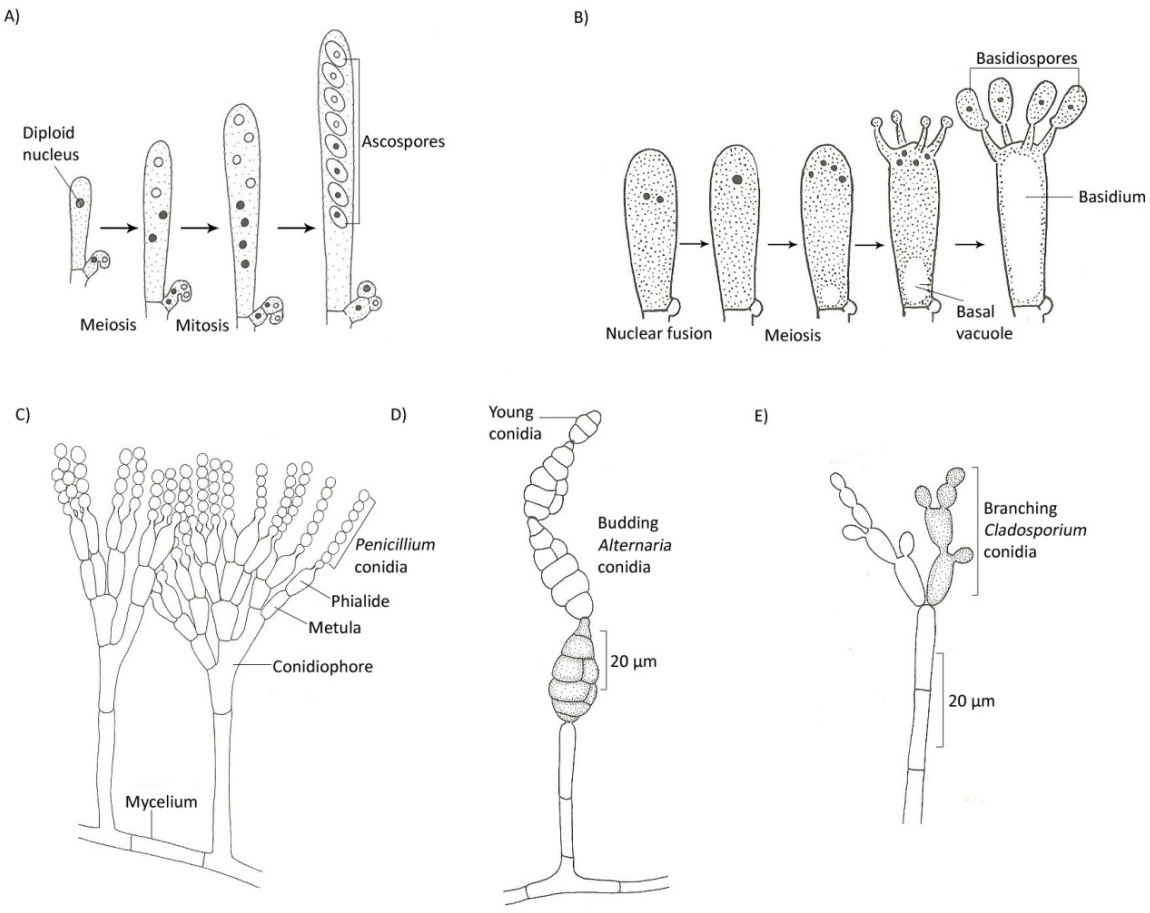
1.2.3 Asexual reproduction in the Dikarya

Asexual reproduction in fungi is achieved through the growth of pycnidium (sacs containing conidia) or external conidiophores, which vary considerably according to species. In anamorphs of the Ascomycota, branching multinuclear filaments termed hyphae grow by extension through the terminal cell and cytoplasm displacement. The hyphae intertwine to produce a mass of compact hyphae termed mycelium and, under favourable conditions, aerial hyphae extend from the mycelium into a wide variety of conidiophores, bearing spores termed conidia [22]. Production of conidia by anamorphs of the Ascomycota varies considerably in different genera. For example, *Botrytis* conidia are produced sequentially, and one at a time, from the terminal ends of branches of conidiophores. In *Aspergillus* and *Penicillium,* conidia are produced from the phialides of distinctive conidiophores. The sequentially-produced conidia result in long chains, with the most recently produced conidium attached to the phialide (Figure 1.1C). *Geotrichum candidum* produces conidia termed arthroconidia through the formation of cross-walls and subsequent fragmentation of hyphae. The common aeroallergens *Alternaria* and *Cladosporium* produce conidia by budding, termed 'blastoconidia'. The terminal ends of hyphae produce conidia, which reproduce by budding to form a chain with the first-produced conidium at the base (Figure 1.1D). Whilst *Alternaria* blastoconidia are produced in single chains, *Cladosporium* forms branching chains (Figure 1.1E) [21].

Many of the Basidiomycota reproduce predominantly by sexual means; however some also have an asexual stage in their life cycle [21]. Some of the Dikarya have a yeast form, where reproduction occurs through production of ballistoconidia, as exhibited by the common environmental fungi *Tilletiopsis* and *Sporobolomyces*, both from the Basidiomycota*;* or by fission or budding, as exhibited by the opportunistic pathogens *Cryptococcus* and *Candida* from the Basidiomycota and Ascomycota, respectively [25].

Figure 1.1. Reproduction in the Dikarya

In the Ascomycota, sexual reproduction through meiosis and mitosis produces groups of (typically) eight ascospores (A). In the Basidiomycota, sexual reproduction leads to the production of four basidiospores borne from a basidium though meiosis (B). Asexual reproduction, such as in the ascomycetes *Penicillium* (C), *Alternaria* (D) and *Cladosporium* (E), leads to the production of chains of spores termed conidia. Adapted from Ingold and Hudson, 1993. Chapman & Hall: London.



1.2.4 Fungal nomenclature

Naming of fungi and identification of new species was traditionally based on morphological characterisation by microscopy and culture methods. Later, protein and enzymatic methods were used in further classifications and most recently, taxonomic

classifications are based on DNA-based methods [26]. Sexual and asexual states in fungi were established by the mid-nineteenth century, based on co-occurrence in nature and fungal culture observations; however sexual and asexual forms of fungi had already been given different taxonomic names in the literature prior to these connections being made. Therefore, differential naming of asexual and sexual states of the same fungi continued for ascomycetes and basidiomycetes, governed by the International Code of Botanical Nomenclature [27], with rules becoming very complicated. The general rule is that if there is a sexual state known for a particular species, the species should adopt the generic name based on the sexual state. Where a sexual state is not known for a particular fungus, the species can be named based on the asexual form. However, these rules are often disobeyed due to common use in the literature and unwillingness of mycologists to lose accreditation for the naming of a particular strain. For example, *Aspergillus nidulans* has a known sexual state and should be referred to as *Emericella nidulans* but is commonly referred to in the literature as *A. nidulans* [27]. *Cladosporium herbarum* is the anamorph (asexual form) of *Davidiella tassiana* (the teleomorph, or sexual form) [28], for which the current name is now *Mycosphaerella tassiana* (www.indexfungorum.org) and should be referenced as such; however, the asexual form (*C. herbarum*) continues to reported in the literature. Current steps are being taken towards the development of nomenclature so that each fungal species is represented by a single name [29]; however, many clinically significant fungi are reported using the anamorph, even when a known teleomorph exists. The genus *Aspergillus* contains species of known clinical relevance; the most pathogenic being *A. fumigatus.* Until recently, *A. fumigatus* was regarded as an entirely asexual fungus; however, sexual characteristics have now been demonstrated, with the teleomorph called *Neosartorya fumigata* [30, 31]. Approximately one third of *Aspergillus* species have a known sexual

stage in their life cycle [32], where fusion of hyphal gametangia leads to the production of ascospores by meiosis and mitosis. A sac called a cleistothecium is produced, enclosing many asci containing eight (in the majority of cases) sexual ascospores, which are released when the cleistothecium bursts [21, 32]. Teleomorph genera of *Aspergillus* are *Sclercleisia*, *Warcupiella*, *Emericella*, *Eurotium*, *Neosartorya*, *Petromyces*, *Chaetosartorya*, *Dichlanea*, *Fenellia* and *Hemicarpenteles* [33].

1.3 Introduction to allergy and allergic asthma

Allergic disease is caused by a hypersensitive inflammatory response of the immune system to external stimuli; such as allergens from pollens, pet dander, fungal spores, dust mite, foodstuffs or medications; with reproducible reactions to allergens at doses which are tolerated by non-sensitised people. These reactions are mediated by immunoglobulin (Ig) E, IgG, or allergen-specific lymphocytes [34]. IgE is an important antibody of the immune system which can become elevated in response to allergens, leading to hypersensitivity reactions on repeated exposure. Type 1 (immediate) hypersensitivity is mediated by interactions between allergens and IgE on mast cells and basophils [14] which, due to their elevated presence within mucosal tissues, are amongst the first cells of the immune system to interact with inhaled or ingested pathogens [35].

Allergic asthma and hay fever, which can be triggered by allergens from pollens or fungal spores, are mediated by Type 1 hypersensitivity reactions which can occur in two (early and late) phases; and an allergic reaction may comprise one or both phases [14]. The early phase is caused by mast cell degranulation and release of histamine and pre-formed mediators within minutes of allergen exposure, manifesting as sneezing, nasal discharge or bronchoconstriction (where the airways tighten due to smooth muscle

contraction). The early phase triggers a Th2 mediated inflammatory response, leading to the recruitment and activation of inflammatory mediators, resulting in the late phase of an allergic reaction 3-4 hours after initial exposure and manifesting as nasal congestion, asthma or urticaria [14, 35, 36]. Sensitisation to the allergen is maintained through replenishment of IgE by B cells [35]; therefore, future exposures to the same allergen can trigger further hypersensitivity reactions. In the UK alone, it is estimated that around 30% of adults and 39% of children have at least one atopic condition [37] (sustained elevated IgE to one or more allergens [14]), with an estimated cost to the National Health Service (NHS) at £1 billion per year [37].

Type 2, non-IgE-mediated allergy can be mediated by IgG [34], the most abundant serum immunoglobulin, although it is much less likely that reactions will be immediate [38]. Serum IgG antibodies to allergens can be detected in the absence of allergy symptoms due to the fundamental role of IgG in the secondary immune response, reflecting environmental exposure; however elevated serum IgG can also be reflective of chronic exposure to environmental allergens [34] and IgG to *A. fumigatus* is important in allergic bronchopulmonary aspergillosis (ABPA) [39], a florid hypersensitivity reaction to colonisation of the airways by fungi, predominantly caused by *A. fumigatus*. The question remains as to the relative importance of IgG in allergic disease.

1.3.1 Pathophysiology of asthma and airway hyper-responsiveness

Asthma is characterised by a cascade of events within the lower airway which cause structural changes and obstruction of the airway lumen due to inflammation and mucus hypersecretion. In addition to the inflammatory influx of eosinophils, CD4+ lymphocytes and mast cell degranulation triggered by allergen exposure, structural changes to the airway are caused by activation of epithelium and smooth muscle, sub-

epithelial membrane thickening and fibrosis, and smooth muscle hypertrophy (enlargement of cells) and hyperplasia (increased number of cells) [7, 40, 41]. Activation of epithelium involves cell desquamation, morphological changes and release of pro-inflammatory cytokines [42]. Increased intracellular calcium in smooth muscle leads to binding of phosphorylated myosin to actin causing smooth muscle contraction [43]). These processes lead to the characteristic airway narrowing, increased contractility and elevated production of mucus observed in asthma, manifesting as cough, wheeze and shortness of breath.

Historically, the term 'asthma' was used to describe symptoms of breathlessness. In the modern day, the term is used to describe airway hyper-responsiveness and variable airway obstruction [44]. The term airway hyper-responsiveness describes elevated spontaneous contractility of the airways in response to exogenous or endogenous stimuli, with increased airways irritability and mucus secretion [45] and can be assessed clinically by direct challenge using the bronchoconstrictor histamine or methacholine, or through indirect challenge, such as exercise or cold air exposure [46]. Transient periods of airway hyper-responsiveness and variable airflow obstruction can be relieved by treatment with bronchodilators [44]. In the majority of cases, asthma can be well controlled using combinations of bronchodilators and anti-inflammatory treatments, and in such cases is considered a mild form of the disease. People with severe asthma and regular exacerbations of symptoms requiring hospital admissions are more likely to be fungal sensitised than those with better controlled asthma [3].

It has recently been argued that there is considerable overlap between asthma and other airways diseases, and that airways disease should be considered in terms of an A to E classification for phenotype-directed management: A) bronchodilator-responsive **A**irway hyper-responsiveness and variable airway obstruction; B) corticosteroid-

responsive but bronchodilator-resistant neutrophilic or eosinophilic **B**ronchitis characterised by exacerbations; C) **C**ough reflex hypersensitivity; D) fixed airflow obstruction non-responsive to corticosteroid or bronchodilator treatment due to persistent inflammation and airway **D**amage; and E) **E**xtrapulmonary factors [44].

1.3.2 Demographics of asthma

Asthma affects approximately 5.4 million people in the UK, with around 1500 asthma-related deaths per year in the UK alone [12] and is the most common medical condition in children [47]. Atopy is very important with regard to the management of asthma and is associated with the vast majority of asthma cases [48].

The health cost burden of asthma is highly significant, with medication for asthma costing a total of around £500 million per year in the UK alone, without taking into account lost working days costing an estimated £1 billion and patient care at around £10 billion [13].

1.4 Fungi as allergens

Inhalation of either fungal spores or hyphal fragments [17] can cause respiratory allergy in 20-30% of all atopic individuals, with the major allergic manifestations including asthma, rhinitis, allergic bronchopulmonary mycoses and hypersensitivity pneumonitis [49, 50]. Manifestations of fungal allergy affect the nose or lungs, depending on site of exposure [36].

The prevalence and severity of allergy is highest in early adulthood, declining with age thereafter [51] and whilst an estimated 10% of the population may be sensitised to fungi, only half show clinical symptoms [17]. Fungal sensitisation has been associated

with asthma severity [3], although whether this relationship is causal remains unknown [12].

More than 80 genera of fungi are known to cause type 1 hypersensitivity reactions, with around 150 allergenic fungal proteins so far described [52]. Inhalation of larger fungal spores greater than 10 μm in size, such as those of *Alternaria*, are typically deposited in the nasopharynx, triggering the nasal and ocular symptoms associated with hay fever [14]. Smaller spores, which comprise the majority and include *Aspergillus* and *Penicillium*, are 2-10μm in size, and can easily penetrate the lower airway causing allergic asthma [14, 53]. Fungi differ from other allergens, such as those found in cat dander or pollens, in that spores of select fungi of respirable size which can grow at 37°C have the potential to actively germinate and colonise the lower airway [12].

1.4.1 Production of mycotoxins

In addition to the ability of some thermotolerant fungi to colonise the human airway, another unique property of some fungi in comparison to other inhaled allergens is the production of mycotoxins, which may potentiate their allergenic effect [12]. Mycotoxins are secondary metabolite by-products of fungal metabolism which tend to be cytotoxic, providing a competitive advantage over other organisms by inhibition of growth [54], and can be toxic to humans through inhalation, or ingestion of contaminated foodstuffs. Mycotoxin exposure has been associated with a variety of toxic health effects in humans including damage to the heart, gastrointestinal tract, kidneys, liver and central nervous system, in addition to immunosuppressive effects [55]; and whilst not produced by all fungi, a number of *Aspergillus* and *Penicillium* species have been shown to produce mycotoxins [56]. Aflatoxin, for example, is produced by *Aspergillus flavus* and *Aspergillus parasticus* and is considered the most carcinogenic natural substance discovered to date [55, 57]. Another important

mycotoxin is gliotoxin, which is produced by several fungal species including *A. fumigatus*, and is known to have immunosuppressive effects [57], such as impairment of phagocytosis by neutrophils [58] and reduction in epithelial ciliary beat frequency [59]. Mycotoxins produced by fungi can be found throughout the fungal colony; in spores, hyphae and even in the surrounding substrate on which the fungus is growing [17].

1.4.2 Diagnosing fungal allergy

The simplest diagnostic tool for diagnosing fungal allergy is the use of skin-prick tests (SPTs), which studies suggest should include as a minimum: *A. fumigatus, Penicillium chrysogenum, Alternaria alternata, Cladosporium herbarum, Epicoccum nigrum* and *Fusarium* (or *Gibberella*) species. However, there is a lack of commercial availability of fungal extracts for testing, particularly with regard to basidiospores (from mushroom fungi), for which there are none. [14] This means that it is likely that many fungal allergies go undiagnosed. Further limitations of SPTs causing difficulties in diagnosing allergy include inconsistencies in the preparation of fungal extracts; for example, using either fungal mycelia (the branching hyphae in asexually reproducing fungi) or spores for production. Inter-strain variability could also lead to extracts with altered protein composition, resulting in poorly standardised allergy testing solutions [52].

In addition to SPTs, fungal allergy can also be diagnosed using the ImmunoCAP system® for allergen-specific IgE (Pharmacia Diagnostics), which is a fluorescence-enzyme immunoassay which has the added benefit of enabling quantitative analysis of allergen-specific IgE or IgG levels in serum or plasma [60]. Radioallergosorbent (RAST) tests can also be used to measure allergen-specific IgE or IgG in serum by radio-labelling allergen-specific IgE or IgG bound to an allergen of interest. A recent study suggested that both SPTs and specific IgE measurement by the ImmunoCAP

system should be used in diagnoses of allergy, due to discordance in test results in around a quarter of severe asthma patients [4].

1.4.3 Growth characteristics of the opportunistic pathogenic fungus *Aspergillus fumigatus*

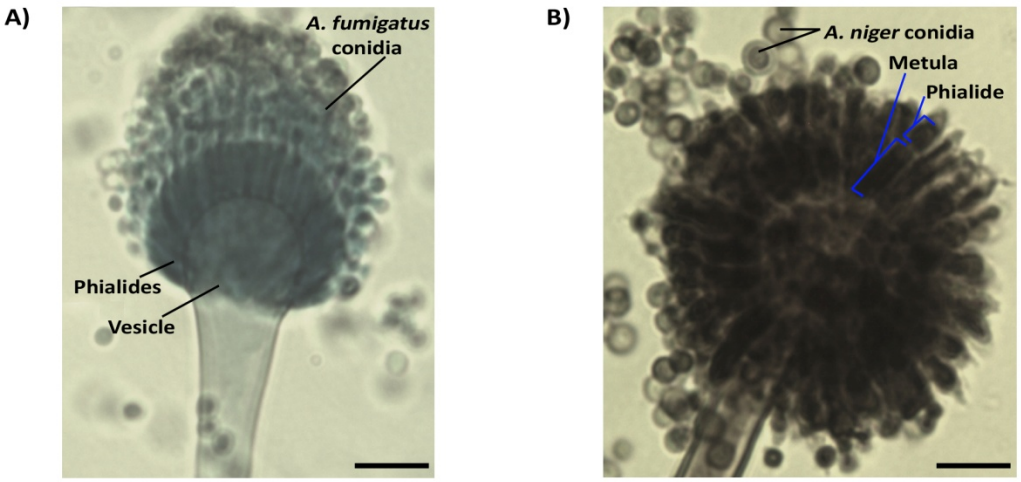
The most pathogenic and widely studied species of *Aspergillus* is *A. fumigatus*, which is distinctive from other species of *Aspergillus* in both macro and micro-morphology. *A. fumigatus* hyphae are hyaline, septate, branch at 45°C and are 7-10 µm in length [61, 62], leading to conidiophores which bear conidia. *A. fumigatus* conidiophores can reach 300 µm in length, leading to flattened club-shaped vesicles (up to 30 µm in diameter) which have no metulae and a single layer of phialides (6-8 x 2-3 µm) on the upper half to upper third of the vesicle, bearing *A. fumigatus* conidia approximately 2-3.5 µm in size [63] (Figure 1.2A). Metulae are terminal branches of the conidiophore, present in most other species of *Aspergillus,* such as *Aspergillus niger* (Figure 1.2B) which support conidia-producing phialides. Clinical isolates of *A. fumigatus* from humans have been shown to vary considerably in morphology in comparison to standard laboratory cultures, with differences in size and shape of conidia or phialides, and occasionally having branched conidiophores [63, 64].

The thermotolerance of *A. fumigatus* is central to its survival; as a pathogen in the human, mammal or avian host and as a degrading organism of compost piles, which can reach up to 70°C [65]. *A. fumigatus* thrives in warm and humid environments, and studies have shown optimum germination of spores at 25°C and optimal mycelial growth at 37-43°C, although it can still grow at temperatures up to 55ºC and survive temperatures up to 75ºC. Moisture is also important, and levels of 99% relative humidity are optimal for conidia formation [55, 61]. The pathogenicity of *A. fumigatus* is correlated with its superior ability to grow at 37°C in comparison to other species of

Aspergillus [66]; however, other species of *Aspergillus* and other filamentous fungi have also been reported in clinical samples [64].

Figure 1.2. Conidiophores of *A. fumigatus* and *A. niger*

Photomicrographs at x630 magnification, stained with polyvinyl lactophenol cotton blue, showing conidiophores of *A. fumigatus* with a single layer of phialides an absence of metulae, and chains of conidia produced on the upper half of the vesicle (A); and *A. niger*, with phialides and metulae around the circumference of the vesicle, producing black conidia (B). Scale bars represent 10 µm.



1.4.4 Non-*fumigatus* species of *Aspergillus* and other filamentous fungi as potential pathogens

Detection of filamentous fungi other than *A. fumigatus* in clinical samples is less common and the clinical importance poorly understood; however, clinical isolates previously reported include *Aspergillus glaucus*, *A. niger*, *A. nidulans*, *A. flavus*, *Pseudallescheria boydii*, *Penicillium* and *Paecilomyces* species, typically with co-culture of *A. fumigatus* [64, 67]. Species of *Aspergillus* and zygomycetes can cause invasive infections in immunocompromised people [68] and *A. terreus* [68], *A. flavus*,

A. niger [69], *Penicillium citrinum* [70], *Penicillium piceum* [71] and *Penicillium marneffei* [72] may be important emerging pathogens. *Candida* and *Cryptococcus* are yeasts which are known opportunistic pathogens [72]. Whilst *Cryptococcus* is typically associated with infection of immunocompromised individuals (although *Cryptococcus gattii* is an emerging pathogen of immunocompetent people), *Candida* is regularly isolated from clinical samples, and is known to cause similar disease to ABPA. Detection of yeast in clinical samples is beyond the realm of this thesis and will not be discussed further.

1.4.5 Allergic bronchopulmonary mycoses

In airways disease, such as asthma or CF, mucoid impaction and impaired mucus clearance from the lower airway provides a favourable environment for opportunistic thermotolerant fungi, such as *A. fumigatus*. *Aspergillus* may grow in mucus of the sinuses causing allergic *Aspergillus* sinusitis; or in mucus of the bronchi causing ABPA [62]. Allergic bronchopulmonary mycoses (ABPM) are florid hypersensitivity reactions to colonisation of the airways by filamentous fungi which lead to progressive pulmonary destruction. ABPA is the most widely studied and well described of ABPM, and is predominantly caused by *A. fumigatus* [62]. Of human infections caused by species of *Aspergillus*, 95% are thought to be explained by *A. fumigatus, A. niger* and *A. flavus* [61].

1.4.6 Severe asthma with fungal sensitisation

Severe asthma with fungal sensitisation (SAFS) is a term coined by Denning *et* al (2006) [12] to describe adults with severe asthma and IgE sensitisation to one or more fungi who do not fulfil all of the diagnostic criteria for ABPA. It is possible that sensitisation to fungi could transpire through either environmental exposure to fungi or

through colonisation of the airways [12]. However, for a diagnosis of SAFS the defining feature is severe asthma and IgE sensitisation to one or more fungal allergens; without evidence of pulmonary infiltrates, bronchiectasis or eosinophilia, precipitins to *Aspergillus*, raised specific IgG to *Aspergillus* antigens, elevated total IgE (>1000 IU/ml) or airways colonisation [12].

1.4.7 Demographics of ABPA and severe asthma with fungal sensitisation

ABPA is thought to affect around 40,000 people in the UK alone [73]; however, the prevalence of ABPA is a contentious topic, probably due to the difficulties in giving a definitive diagnosis of the disease and differences in the severity of asthma of patient cohorts examined. A 2003 review of the literature on ABPA suggested that 1-2% of asthmatics and 1-15% of CF (CF) patients may be affected by ABPA [50], although a recent meta analysis suggested that 12.9% of asthma patients; a much higher proportion than reported previously, may be affected by the disease [74]. Up to three quarters of people with severe asthma may have SAFS [3, 4, 11, 75], and current estimates suggest that SAFS could affect as many as 2.2 million people in the USA and a similar number in Europe [12].

1.4.8 Pathophysiology of airway colonisation

In ABPA, *Aspergillus* grows in a non-invasive manner; the patients affected are immunocompetent and the aspergillosis is not systemic, unlike in invasive aspergillosis which affects immunocompromised patients [62]. The pathogenesis of *Aspergillus* with regard to ABPA is driven by the individual's immunologic response [76]. Colonisation of the airways without penetration of surrounding tissues can occur without detrimental effects on health in the absence of allergy to the colonising fungus [17] and it is likely

that in healthy individuals, many incidences of fungal respiratory infection pass without being detected and rapidly resolve [77]. What determines the susceptibility of the subset of people with chronic lung disease to ABPA and the mechanisms involved are incompletely understood [59, 78]. Fungi preferentially grow as a biofilm, where multicellular communities form an extracellular matrix binding together a network of hyphae embedded on the bronchial epithelium, which provides increased resistance to host defences and antifungal treatments [79-81].

A proposed model of the pathogenesis of ABPA is summarised in Figure 1.3. Germination of conidia within the mucus of damaged airways produces branching mycelia, which express antigens and proteases causing activation of airway epithelial cells involving cell desquamation, morphological changes and release of pro-inflammatory cytokines, and may penetrate the bronchial mucosa [42, 50, 62, 82]. In some individuals, this stimulates a Th2-mediated hypersensitivity response of the airway involving pro-inflammatory cytokines and growth factors, T cells, IgE and IgG antibodies [82, 83]. IgE bound to *A. fumigatus* antigens binds to the high affinity IgE receptor FcεR1 on mast cells, stimulating mast cell degranulation and release of histamine and chemokines which promote eosinophil proliferation. Binding of *A. fumigatus* antigens to eosinophils by IgA, IgG and IgE causes the release of inflammatory mediators, leading to airway remodelling [50]. The process is further perpetuated by protease interaction with airway epithelium and fibroblasts, further promoting release of inflammatory mediators [82]. Airway sensitisation to *A. fumigatus* is then maintained through replenishment of IgE by B cells [35].

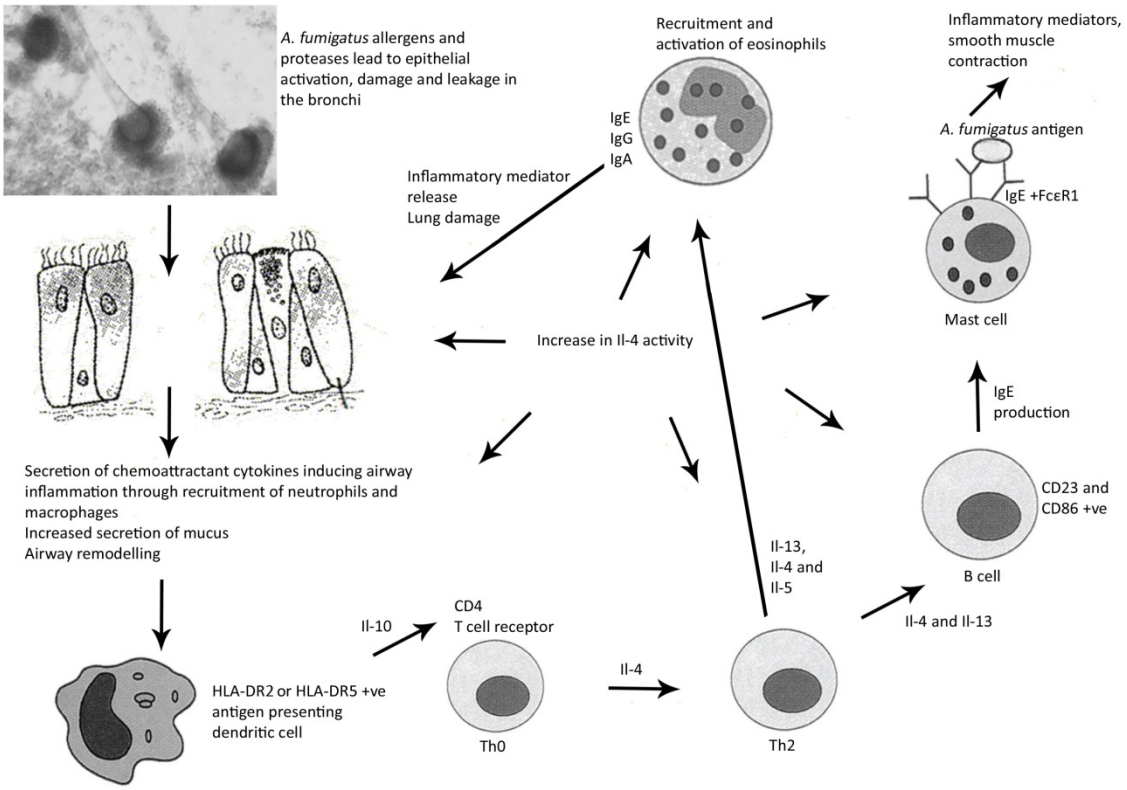
Mycotoxins, enzymes, volatile organic compounds and allergenic proteins produced by fungi lead to continuing damage of the airways [12]. Allergenic proteins such as proteases and glycosidases are excreted by fungi and can impact the host directly,

whereas other enzymes and oxidative stress response proteins may enable germination of the fungi in the hostile environment of the bronchi. Some of these proteins bear a striking resemblance to human counterparts, which may enable their interaction with cells of the immune system and in the event of airways colonisation, it is likely that the infected person is exposed to many of these allergens concurrently [12].

If left untreated, recurrent periods of inflammatory influx of eosinophils and neutrophils, bronchial obstruction and mucoid impaction leads to progressive airways damage in ABPA and irreversible widening of the airways, termed bronchiectasis, the mechanism of which is poorly understood [49, 62]. It has been hypothesised that the airway environment in bronchiectasis may favour fungal colonisation, which could lead to the development of sensitisation through continued exposure to allergens of the colonising fungus [12]. Few studies have examined the longevity of airway colonisation; however, long term airway colonisation in CF patients has been shown to be dominated by few genotypes of *A. fumigatus*, often with a common dominant genotype, in comparison to recently colonised patients who tend to be colonised by multiple genotypes [84].

Figure 1.3. Th2-mediated immunopathophysiology of *A. fumigatus* colonisation in ABPA

Inhaled *A. fumigatus* conidia germinate within the bronchi, secreting a variety of proteins which are processed by dendritic cells possessing human leukocyte antigen receptors HLA-DR2 or HLA-DR5, causing differentiation of T-helper Th0 cells to Th2 via the T cell receptor and CD4 (cluster of differentiation 4), stimulating the production of Il-4, Il-5 and Il-13 cytokines. This stimulates the activation of eosinophils (causing eosinophilic airway inflammation) and production of IgE by B cells. Il-4 also promotes cell surface expression of CD23 (low affinity IgE receptor) and CD86 on B cells, increasing IgE synthesis. Cross-linking of IgE, IgG and IgA bound to eosinophils triggers release of pro-inflammatory mediators. IgE binds to the high affinity IgE receptor FcεR1 on mast cells. When IgE is cross-linked by *A. fumigatus*, this stimulates mast cell degranulation and release of mediators inducing smooth muscle contraction [50, 85].



1.4.9 Host immunity to *A. fumigatus*

People inhale hundreds of ubiquitous *A. fumigatus* conidia every day, without clinical implications in healthy individuals, and what determines host susceptibility to fungal colonisation is unclear [59.]. A variety of mechanisms have been suggested, ranging from factors influencing the skew towards a Th2 mediated response, and factors affecting the host's ability to clear invading fungal pathogens. Conidia which are able to evade the mucociliary clearance mechanisms, and the first line of defence of the immune system provided by bronchoalveolar macrophages and neutrophils, germinate to produce mycelia containing β-glucans, chitin and galactomannan in the cell wall [86, 87]. Dectin-1 is a β-glucan receptor expressed by bronchoalveolar macrophages, which is important in the recognition and clearance of *A. fumigatus* from the lung [88]. Impaired production of pro-inflammatory cytokines and neutrophil recruitment, impaired clearance and killing of *A. fumigatus* conidia has been shown in mice lacking dectin-1 [89].

Risk factors suggested for the development of ABPA have included over activity or increased number of CD4+ T cells, HLA-DR2 and HLA-DR5 restriction and elevated sensitivity to stimulation by Il-4 [50, 90]. The genotype -1082GG, in the promoter region for Il-10, has also been associated with increased *A. fumigatus* colonisation and ABPA in CF patients homozygous for ΔF508 (a mutation in the CF transmembrane conductance regulator) [90, 91]. A small study of six children with SAFS showed all to have a heterozygous 24 base pair duplication in the chitotriosidase-1 (CHIT-1) gene, which has been associated with a lack of chitotriosidase activity; an enzyme which targets chitin-containing fungi, contributing to host defense to inhaled fungal pathogens [92-94].

1.4.10 Availability and limitations of diagnostic tools for ABPA

ABPA is commonly diagnosed based on the presence of five primary criteria: i) asthma; ii) elevated serum IgE (>417 IU/mL or >1000 ng/mL); iii) elevated *A. fumigatus*-IgE and/or *A. fumigatus*-IgG; iv) a positive SPT to *Aspergillus*; and v) presence of central (or proximal) bronchiectasis [50, 62]. Secondary diagnostic criteria for ABPA include pulmonary and sputum eosinophilia and a positive sputum culture for *A. fumigatus* [83, 95]. A definitive diagnosis of ABPA is often difficult due to variability of test results and many patients with fungal-sensitised asthma presenting in clinic fulfil some, but not all of the diagnostic criteria. Subclassifications of ABPA patients are applied in some circumstances; those who fulfil all of the diagnostic criteria for ABPA are termed ABPA-central bronchiectasis patients, and those who fulfil all of the criteria for ABPA, but where central bronchiectasis is absent, are termed ABPA-seropositive [96].

1.4.11 Sputum as a diagnostic tool for fungal airways colonisation

The advent of non-invasive techniques in inducing sputum for airway sampling [97] has enabled new opportunities in phenotyping inflammatory airway diseases. However, detection of *A. fumigatus* in sputum is only utilised as a minor diagnostic criterion for ABPA since isolation from respiratory specimens is often rare in routine mycology laboratories; a previous study suggested *A. fumigatus* culture rates of only 36% in proven or probable aspergillosis [98]. Alternative methods to culture for detection of fungi in sputum include microscopic identification of *Aspergillus* hyphae in sputum (also possible in nasal swabs) using either periodic-acid-Schiff, Grocott's, or Gomori methanamine silver stains [62, 83]; amplification of species-specific fungal DNA by polymerase chain reaction (PCR) [99]; or by enzyme-linked immunosorbent assay (ELISA) targeting galactomannan [100], a soluble antigen released from the hyphal cell

wall during growth [101]. Identification of fungi in sputum should aid an early diagnosis of ABPA and would provide evidence supporting the initiation of treatment to prevent progressive lung damage associated with the disease [76].

1.4.12 Identification of fungi in clinical samples

Whilst *A. fumigatus* is most commonly associated with ABPA, numerous other species of fungi have been implicated in causing similar or identical disease [14]; however, there has been little research to determine the pathogenicity of other fungi detected in sputum of asthmatics. Diagnostic tools which could quickly confirm a fungal infection and identify the causative species or strain are preferable in order to enable identification of the most appropriate course of treatment [72]. Fungi can be identified from clinical samples by fungal culture and identification by morphology, or by using biochemical, immunological or molecular methods [102].

Fungal culture and morphological identification is the cheapest and simplest method, but is time consuming and requires skilled knowledge of fungal morphology. Biochemical methods are also time-consuming and have the added problem of variability in metabolites produced among members of the same fungal species. Immunological methods have great potential, but have only been developed for a limited number of fungal taxa and present issues with cross-reactions between fungi. Molecular methods have the advantage that the fungal culture period is not always necessary, but are expensive and have limitations such as the variable potential for sequence identification of a contaminating fungus [102]. However, differentiation of important clinical fungal isolates has been shown to be possible by PCR reactions, using primers targeting the internal transcribed spacer regions of the nuclear ribosomal operon [103]. Nuclear ribosomal DNA (rDNA) consists of tandem repeats of 18S (small subunit: SSU), 5.8S and 28S (Large subunit: LSU, including the D1/D2 variable

domains), two non-coding internal transcribed spacer (ITS1 and ITS2) regions and two external transcribed spacer regions (ETS), with each repeat separated by non-transcribed transgenic spacer regions (Figure 1.4) [104-106]. SSU rDNA evolves slowly and therefore only enables differentiation between distantly related fungi [107]. The ITS region evolves fastest and therefore enables species-specific differentiation of many fungi including closely related clinically important species of *Aspergillus*, and is also more variable than the LSU region [108, 109]. The ITS region has become the default marker of choice due to its superiority in species differentiation of filamentous fungi, with the LSU region preferred as a marker for identification of yeasts [26]. However, species-specific identification is not always possible with ITS, and protein-coding targets such as β-tubulin, calmodulin, and elongation factor 1α (*EF-1α*) have also been used to distinguish between closely related species [110]. Mitochondrial cytochrome oxidase subunit 1 (*cox1*), a barcoding target for animals, has been investigated for universal amplification of fungi; however, problems with targeting this gene include the variable presence of introns, slow evolution and therefore insufficient variation to enable distinguishing between different species of fungi, and the presence of multiple copies of the gene [26, 111].

Figure 1.4. Schematic diagram of the nuclear ribosomal operon

The fungal nuclear ribosomal operon contains repeats of small subunit (SSU; 18S), 5.8S and large subunit (LSU; 28S) rDNA, with two internal and external transcribed spacer regions (ITS and ETS respectively) [105, 107].

1.4.13 Available antifungal treatments

The beneficial effects of antifungal therapy in ABPA [112, 113] and SAFS [114] have been shown in clinical trials; however, the toxicity of treatments and rising resistance to currently available antifungal agents are cause for concern [115-118], highlighting the need for more accurate detection and diagnosis methods. Around 60% of ABPA patients have been shown to benefit from antifungal therapy [12]; however, available antifungal agents are limited.

Current antifungal treatments are classed as triazoles, polyenes (including amphotericin B) and echinocandins (including caspofungin) [116]. Itraconazole, voriconazole and posaconazole are all antimycotic azoles which have been implemented clinically in the eradication of fungal infections, and act by disrupting the biosynthetic pathway for ergosterol, a component of fungal cell membranes [119]. Polyenes also target ergosterol sites in the fungal cell membrane, causing leakage of contents; whereas echinocandins target synthesis of (1→3) β -D glucan in the cell wall, altering the ability of the fungus to grow and multiply [116]. Treatment of ABPA and SAFS with itraconazole has shown promising results in patient trials [113, 120], with number of exacerbations of symptoms reduced [113, 120] and improvements in asthma-related quality of life [114]. There are, however, problems associated with prescription of antifungal treatments, including toxicity of antifungal agents and detrimental interactions with other medications. Furthermore, whilst *A. fumigatus* is usually susceptible to antifungal therapy, a rise in resistant strains is being reported, which could be attributable to suboptimal treatment of aspergilloma patients, or misuse of environmental azole fungicides [115-118]. A recent study demonstrated the development of azole resistance in two patients, likely due to suboptimal treatment with itraconazole [119], highlighting

the need for better understanding of fungal colonisation of the airways, in order to provide optimal treatment for patients and limit cases of azole resistance.

1.5 Introduction to Aerobiology

1.5.1 History of aerobiology

Aerobiology as a term was first used in the 1930s to define the study of the transport of airborne particles in the indoor (intramural) and outdoor (extramural) environment [121], but dates back to the work of Micheli in the early 18th century, whose observations showed that fungi reproduce by spores or 'seeds', which he concluded to be distributed through the air based on contamination of control fruit samples [24]. Louis Pasteur was another pioneer of aerobiology, originally termed atmospheric micrography [122], and demonstrated the existence and abundance of airborne spores using a filter pump to collect air samples into gun-cotton (nitrocellulose), which he dissolved in alcohol-ether solution and examined by microscopy [24].

Aerobiological research progressed significantly in the twentieth century. The 1930s-50s saw the development of new sampling strategies and aerobiological analyses conducted around the world [122], notably the development of the Hirst automatic volumetric spore trap in 1952 [123], on which the commercially available Burkard volumetric spore trap is based, used in many studies of aerobiology in the modern day. More recent years have seen the development of national aerobiological monitoring networks and multi-centre collaborative research [122], with many modern studies still using traditional methods for sampling and recording aeroallergens.

1.5.2 Fungal aerobiology

Grass pollen is the most common cause of seasonal rhinitis (commonly referred to as hay fever) and is also the most studied aeroallergen. The grass pollen season runs from May to August in the UK, with peak counts observed in June-July; whilst the main allergenic fungal season runs from May until late September. Since the main fungal season overlaps the grass pollen season, fungal allergy can often be dismissed by sufferers and clinicians as seasonal rhinitis due to pollen, despite symptoms extending beyond the pollen season into early autumn [124].

The ubiquitous nature of fungi means that an individual may be exposed to hundreds of thousands of fungal spores in a single day. The mode and frequency of fungal exposure are critical elements in the development of fungal allergy [50] and following sensitisation, allergen avoidance and management of symptoms can be facilitated through aerobiological monitoring of airborne fungal spore levels.

Fungal aeroallergens counted routinely include *Cladosporium, Alternaria, Botrytis, Ganoderma, Ustilago, Leptosphaeria, Didymella, Aspergillus/Penicillium (Asp/Pen)*-type, with generalised groups of hyaline (transparent) and coloured basidiospores and ascospores also monitored daily. *Asp/Pen*-type fungi contain numerous clinically important species; however, it is not possible to distinguish between *Aspergillus* and *Penicillium* fungi based on conidia morphology alone. Furthermore, it is possible that conidia with similar morphology from other fungi, such as *Paecilomyces* and *Wallemia,* could be recorded as '*Asp/Pen*-type'. Basidiospores are grouped together in fungal spore counts due to difficulties in differentiating between different basidiospore morphologies; particularly the hyaline variety. The main seasons, morphologies and potential growth substrates of some of the most abundant indoor and outdoor airborne fungal spore types are summarised in Table 1.1 and Figure 1.5. The taxonomic

distribution of outdoor airborne fungal aeroallergens counted routinely is summarised in Figure 1.6.

Table 1.1. Common airborne fungal spores monitored routinely in the UK

Summary of the main fungal seasons and peak airborne spore counts recorded for common airborne fungal taxa, in addition to size and morphology of spores, and potential growth substrates. Unless cited, data presented is from Derby aerobiology records (Midlands Asthma and Allergy Research Association, MAARA).

Fungal genus	Spore size	Morphology	Main season	Seasonal peaks [a]	Risk factors/highest concentrations	Examples of known sources
Cladosporium	5-40 x 3-13 µm [125]	Variable: unicellular/septate; ovoid/barrel/ columnar	May-September	40-000-70,000	Thunderstorms [126]	Bathrooms, window frames [127], wallpaper [128], food and textiles [129] cereals [130]
Alternaria	18-83 × 7-18 µm [131]	Multicellular/ unicellular; barrel	June-October [132]	320-4292 [132]	Harvesting, dry weather	Soil and plants [132] cereal crops [130]
Sporobolomyces	5-6 x 2-2.5 µm [133]	Tear drop	June-August	29,000-112,000	Thunderstorms, rain	Leaves of plants [24]
Tilletiopsis	0.5-4 x 6-12 µm	Crescent /u-shaped	May-October	9-75,000	Rain	Fruit, e.g. apples [134]
Didymella	4-10 x 12-25 µm [130]	Symmetrical; one septate	June to September [135]	30,000-39,000- [135, 136]	Thunderstorms, rain	Crops, wheat, barley [137]

Aspergillus/ Penicillium	2-10 µm	Often present in chains: oval	No seasonal variation	630-5,600	Home renovation [128], indoor contamination, composting sites	Soil, decaying vegetation, water-damaged building materials [62], wall plaster, wallpaper [138], paper, fabric, [129] carpets, mattresses, window frames, insulation materials, soil of indoor plants [128] bird excrement [62, 139].
Leptosphaeria	3-5 x15-29 µm	Septate ascospore. One cell distinguishably larger	April-November	460-4,200	Harvesting infected *Brassica*	*Brassica* crops [140]
Botrytis	6-7 x 10-12 µm	Globose	June-October	2-1,200	Greenhouses	Wine grapes, soil, plants [141]
Ustilago	4.1-11.6 x 3.7-9.8 µm [142]	Round/oval	June-July	1,400-12,100	Summer months	Cereals, plants and grasses [142]
Ganoderma	6.5-13 x 5-9 µm [143]	Oval	May-October	230-330	Woodland in autumn	Hardwood and conifers [144]

[a] Spores per cubic metre of air

Figure 1.5. **Common UK fungal aeroallergens**

Photomicrographs at x630 magnification showing spores of *Alternaria*, *Sporobolomyces*, *Didymella*, *Tilletiopsis* and (hyaline) basidiospores (A), *Didymella* (left) and *Leptosphaeria* (right) (B), *Ganoderma*, (C) *Cladosporium*, (D) *Asp/Pen*-type, (E) *Ustilago* (F), and *Botrytis* (G), stained with polyvinyl lactophenol cotton blue. Scale bars represent 10 µm.

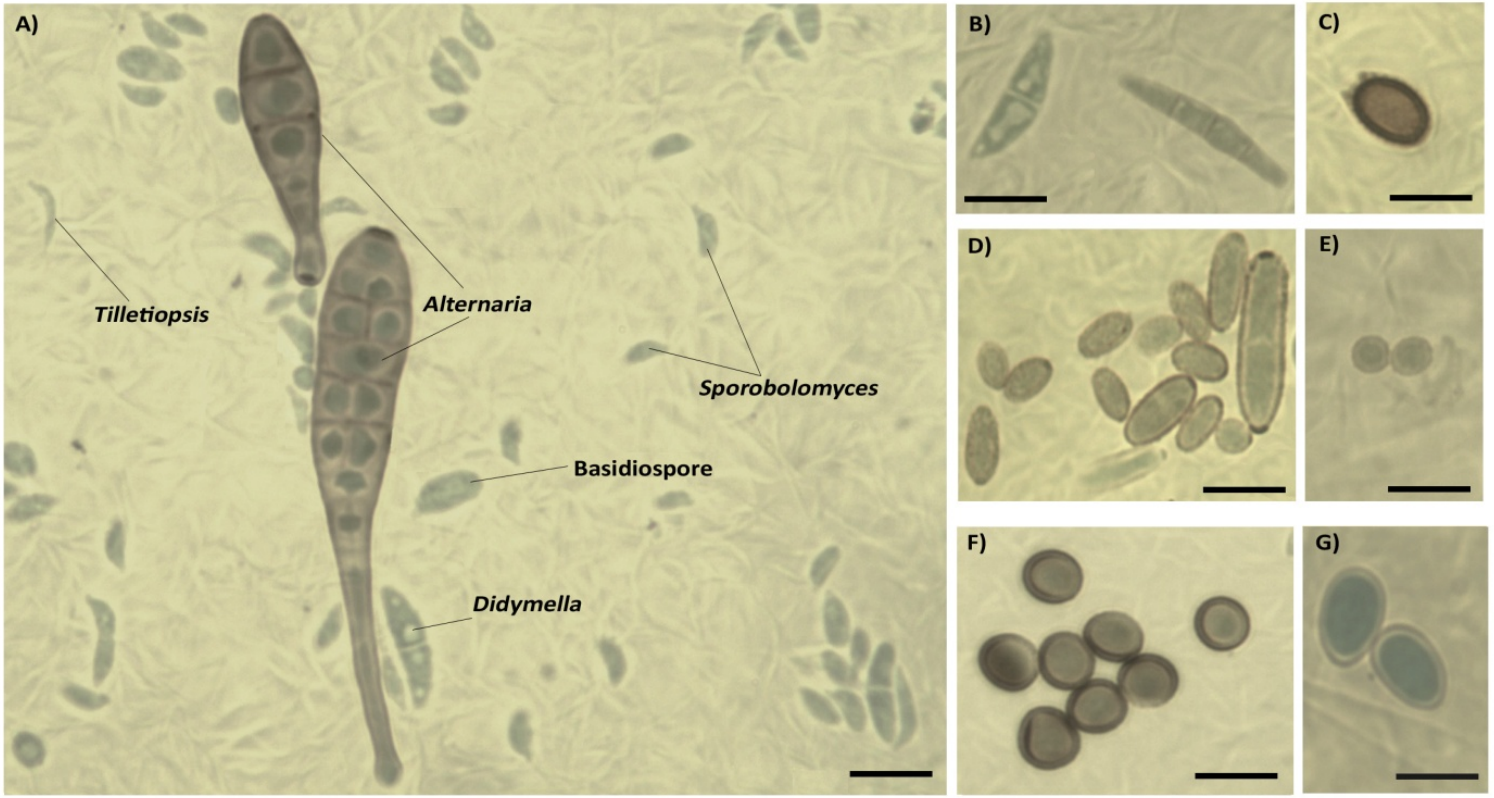
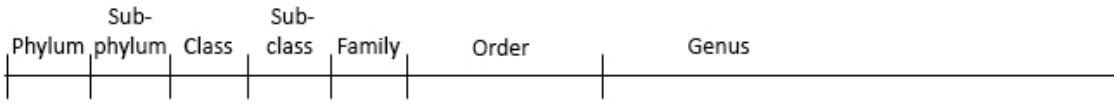


Figure 1.6. Taxonomic distribution of the main fungal aeroallergens

Diagram illustrating the taxonomic distribution of fungal aeroallergens which can be recognised to the level of genus, or closely related genera, and are counted routinely in the East Midlands.



Ascomycota
- **Pezizomycotina**
  - **Dothideomycetes**
    - **Pleosporomycetidae**
      - **Pleosporales**
        - **Pleosporaceae** – *Alternaria, Epicoccum, Drechslera, Stemphylium*
        - **Incertae sedis** – *Didymella*
        - L**eptosphaeriaceae** – *Leptosphaeria*
    - **Dothideomycetidae**
      - **Capnodiales**
        - **Davidiellaceae** - *Cladosporium*
  - **Eurotiomycetes**
    - **Eurotiomycetidae**
      - **Eutotiales**
        - **Trichocomaceae** – *Aspergillus, Penicillium, Paecilomyces*
  - **Leotiomycetes**
    - **Leotiomycetidae**
      - **Helotiales**
        - **Sclerotiniaceae** - *Botrytis*

Basidiomycota
- **Puccinomycotina**
  - **Microbotryomycetes**
    - **Incertae sedis**
      - **Sporidiobolales**
        - **Incertae sedis** - *Sporobolomyces*
- **Ustilaginomycotina**
  - **Exobasidiomycetes**
    - **Exobasidiomycetidae**
      - **Incertae sedis**
        - **Incertae sedis** – *Tilletiopsis*
  - **Ustilaginomycetes**
    - **Ustilaginomycetidae**
      - **Ustilaginales**
        - **Ustilaginaceae** – *Ustilago*
- **Agaricomycotina**
  - **Agaricomycetes**
    - **Incertae sedis**
      - **Polyporales**
        - **Ganodermataceae** – *Ganoderma*

1.5.3 Important fungal aeroallergens in the UK

The most important and most studied outdoor fungal aeroallergens in the UK are *Alternaria* and *Cladosporium* (Figure 1.5A and D); the only fungal spores of those counted routinely for which there are published allergenic thresholds and available SPT solutions. Thresholds of 3,000 spores per cubic metre of air for *Cladosporium* and 100 spores per cubic metre for *Alternaria* are expected to trigger symptoms of allergy in sensitised individuals [145]. *Cladosporium* is an important aeroallergen, often dominating the outdoor airborne fungal biota worldwide [14]. Other fungal spores counted routinely, including *Didymella* and *Sporobolomyces*, can be identified to the level of genus by microscopy and, whilst understudied, can reach high concentrations in outdoor air and have been implicated in allergy; however, investigations are hampered by the lack of characterised allergens. *Asp/Pen*-type conidia, whilst consistently present in outdoor air, do not show seasonal peaks, but can have an impact on asthma and allergy due to indoor sources (Table 1.1).

1.5.4 Fungal spore dispersal and meteorological associations with airborne fungi

All fungi require an external organic growth source, with plant material being the primary source of airborne fungal spores. Factors affecting outdoor concentrations of airborne fungal spores include changes in meteorological conditions, changes in plant ecology over time and geographical location, and the use of fungi as a means of pest control in agriculture [53].

Mechanisms of fungal spore production and dispersal vary considerably between fungi; however the presence of water is crucial. Fungal spores can generally be separated into wet weather and dry weather spores. Ascospores and a large number of basidiospores

(spores from the teleomorphs of Ascomycota and Basidiomycota respectively) require the surface on which they are situated to be wet in order for dispersal to occur (due to movement of turgid cells); although the actual degree of wetness does not affect the magnitude of release. Initial periods of precipitation facilitate spore dispersal, until spore depletion becomes a limiting factor prior to replenishment of stocks [146]. Active release of ascomycetes and lower basidiomycetes therefore occurs during and after rainfall, with minimal effects of wind speed and humidity.

In contrast, wind is more important for the discharge of asexually produced conidia. Spores from fungi such as *Alternaria*, *Aspergillus, Cladosporium* and *Penicillium* are predominantly released during dry, warm, and windy conditions [147]. Distance travelled by a given spore is dependent on wind speed (and direction) and differential deposition rates; for example, non-spherical spores such as *Alternaria* fall slower and can be carried further by the wind than spherical spores, such as basidiospores. Similarly, spores released in clusters fall faster than single spores. Fungal spore detachment is dependent on how the spore is presented and the strength of attachment; therefore local wind speed and height of presentation will determine downwind dispersal [148]. Wind velocity required for detachment varies between fungi; for example, a minimum wind speed of one metre per second is required for detachment of *Cladosporium* and 0.5 metre per second for *Aspergillus* and *Penicillium* [149]. Generally, higher wind speed and drier air will result in enhanced spore liberation. Light is another important factor in the discharge of many asexually-produced conidia [148] and duration of sporulation is largely determined by temperature, humidity and available moisture; and is therefore, greatly influenced by time and season [121].

1.5.5 Health associations with outdoor fungal spore concentrations

Outdoor fungal spore concentrations have been associated with a 2.2% increase in the number of asthma visits [150], increased asthma severity and reduced lung function; particularly in fungal-sensitised patients [3, 151]. However, studies investigating fungal exposure and its implications are complicated by the highly variable nature of fungal concentrations, in addition to difficulties in recording accurate measurements [12]. The local environment is a critical factor in airborne fungal concentrations; for example rural areas, woodlands and farms will have elevated levels of airborne fungal spores. This means that clinicians are faced with extreme difficulties when attempting to ascertain the effect of fungal exposure on human health [152].

Asthma deaths in the UK peak during the period of July to September, which coincides with the main season for *Alternaria* [12, 13], an important aeroallergen which is also associated with thunderstorm-associated asthma [130]. Higher wind speeds and increased humidity during thunderstorms are thought to increase fungal spore production and dispersal [153, 154] and increased levels of *Alternaria*, *Cladosporium* and *Didymella* concentrations have been associated with asthma visits [130].

Airborne basidiospore concentrations have been associated with increased hospital admissions and emergency visits in children during the months of June to October, when fungal spore concentrations are highest [155] and outdoor concentrations have also been associated with asthma symptom scores in both adults and children [151].

Tentative associations have been made between *Didymella* and *Sporobolomyces* concentrations and respiratory allergy [124, 138] and *Didymella* is known to share antigens with *Alternaria* [130]. However, associations between *Didymella*, *Sporobolomyces*, *Tilletiopsis* and allergy are understudied and poorly understood due to the lack of available SPT solutions.

1.5.6 Indoor aerobiology

Indoor concentrations of fungi are typically lower than outdoors unless an indoor source is present [133]. Fungal spores can enter properties passively through a variety of means: through open windows and doors; carriage inside on occupants' shoes and clothing; or entrance through ventilation, heating and air conditioning systems. Fungi can also have indoor sources: as contaminants of building materials, wall or floor coverings, indoor plants, or foodstuffs. There are primarily three adverse effects of fungal contamination of buildings: 1) causing damage to buildings; 2) detrimental effect on living conditions through unpleasant smell or visible presence; and 3) implications on health, although this is a contentious topic [156]. Without wind currents, larger spores tend to settle quickly and it is therefore fungi with smaller spores, including species of *Aspergillus* and *Penicillium*, which are found to be airborne indoor [157]. Indoor fungal concentrations are also highly dependent on geographical location, which determines outdoor fungal diversity and concentrations; with local building construction methods influencing indoor fungal biota [158].

1.5.7 Associations of home characteristics with levels of fungi

The casual association between the home environment and ill health has been the subject of research since before the Second World War [159]. A number of housing characteristics have been identified by previous studies as predictors for elevated indoor fungal growth, including increased relative humidity, temperature, presence of cats, old carpets, highly insulated windows, central heating and wooden board flooring [53]. Indoor bioaerosols are removed from the air by replacement with fresh air from outside; therefore properties with reduced ventilation are susceptible to higher concentrations of bioaerosols [156].

Indoor fungal spore production and dispersal is dependent on many factors, including air flow and moisture content [156]. Whilst it is commonly accepted that increased relative humidity is required for active fungal growth, the water activity (vapour pressure relative to pure water at a given temperature) of a growth substrate is most important [14]. The level of relative humidity required for fungal growth on indoor substrates appears to be a contentious topic. Recommendations suggest maintenance of relative humidity levels between 40-60% [160]; however, levels over 40% has been suggested as sufficient for encouraging fungal growth [17], whilst other studies suggest higher threshold levels ranging from 70-85% [14, 53]. Allergy UK recommends maintenance of relative humidity below 50% [1]. Areas of condensation within properties are also known to encourage fungal growth [161], such as often found in poorly ventilated bathrooms or kitchens.

The likelihood of finding a particular fungus colonising a particular substrate is highly dependent on the water activity required for growth. The water activity (a_w) is defined as the ratio of vapour pressure of a substrate to that of pure water at the same temperature and pressure [162]. Therefore, the fungal biota found on a particular substrate would be expected to change with increasing moisture content. 'Primary colonisers' such as *Penicillium chrysogenum* and *Aspergillus versicolor* have low minimum water activities (a_w <0.8) and can colonise substrates which are relatively dry. 'Secondary colonisers' such as *Aspergillus flavus,* and *Cladosporium cladosporioides* have intermediate minimum water activities (a_w 0.80-0.90) and 'tertiary colonisers' such as *Alternaria alternata, Aspergillus fumigatus* and *Sporobolomyces* have high water activities (a_w > 0.90) and are, therefore, moisture indicator fungi [162].

Visible mould growth is often taken as an indicator of contamination within a property; however, non-visible mould growth concealed within wall cavities or building materials

can also negatively affect indoor air quality [163]. Certain activities cause substantial increases in indoor fungal spore levels; for example, renovation of buildings can disturb dust reservoirs of fungi and greatly increase airborne exposure levels [160]. Building construction and design will also have a major impact on indoor levels of fungi. In recent years, adaptations in the building construction industry have been driven towards facilitating the development of more energy-efficient housing [164] and use of cellulose-based materials, which are easily digested by fungi, are now commonly used as building materials [53]. Increased insulation in properties has led to cold areas in basements and attics, which are then prone to condensation and fungal contamination. In conjunction with these changes in building design, lifestyle changes have also occurred, such as increased use of showers and reduced ventilation, leading to moisture accumulation in properties, thus favouring mould growth [127]. Plaster, concrete and wood have been shown to be most commonly contaminated by fungi in water-damaged buildings; other substrates include paint, wall paper, chip board and brick [165]. A recent international study of water-damaged building materials showed that *Penicillium* species predominated, followed by *Aspergillus* and *Cladosporium* species, amongst less common isolates. The most common species isolated were *Penicillium corylophilum, Aspergillus versicolor* and *P. chrysogenum*. Wood or plywood substrates were shown to be more prone to fungal contamination than stone or plaster [166].

1.5.8 Health associations with indoor mould

Interest in the health effects of indoor fungal exposure is increasing, and was particularly publicised through studies evaluating the aftermath of hurricane Katrina in New Orleans (2005), where airborne fungal spore concentrations in flood-damaged homes reached 630,000 spores per cubic metre of air during the cleanup operation

(culturable fungi reached similar levels with levels of up to 515,000 colony forming units (CFUs) per cubic metre of air). Predominant fungi found in flood damaged homes were species of common indoor genera *Penicillium, Paecilomyces* and *Aspergillus* [167].

Home dampness is well recognised as a risk factor for respiratory illness and higher rates of asthma and respiratory symptoms have been shown in people living in damp housing, with higher airborne fungal spore concentrations shown in houses where damp was reported [168]. Whilst home dampness is known to exacerbate respiratory conditions, whether the relationship is causal, or that home dampness leads to exacerbations of pre-existing conditions is currently unclear [169]. However, the evidence of an association between indoor dampness and mould growth and adverse effects on health is substantiated by research demonstrating improvement of respiratory symptoms and reduced exacerbations of asthma following remediation of moisture sources and fungal contamination in patient homes [170, 171].

A number of studies have attempted to investigate relationships of specific fungal types with the development or exacerbation of allergies and respiratory disease. Exposure to *Penicillium* species has been associated with 'sick building syndrome', which is a broad ranging term used to describe illness within buildings including headaches, difficulty breathing and flu-like symptoms [157]. Indoor exposure to *Cladosporium* and *Penicillium* during the winter has been associated with a greater prevalence of fungal allergies in children, with *Penicillium* exposure also identified as a risk factor for asthma [161], cough and wheeze [172]. Elevated *Cladosporium* and *Aspergillus* concentrations have been associated with increased risk of allergies in children [173]; however, there is also conflicting evidence of a protective effect of *Cladosporium* exposure with regard to sensitisation to aeroallergens. Basidiospore exposure has been

associated with symptoms of rhinitis in children and *Asp/Pen*-type spore exposure has been associated with atopy [174].

1.5.9 Identifying fungal exposure thresholds and acceptable limits

Airborne fungal spore and allergen load remains poorly characterised with regard to indoor air, and it is still not possible to provide guidelines on acceptable levels of fungal exposure within domestic residences due to inconsistencies in study protocols, data reported, and validations of health outcomes [175, 176]. Furthermore, there are no standardised protocols for either the measurement or interpretation of indoor fungal levels [156].

Identifying exposure thresholds for eliciting an allergic reaction requires detailed analysis of the relationship between fungal exposure and respiratory symptoms in a dose-responsive manner, and requires objective measures of fungal levels within properties [177, 178]. Knowledge of concurrent indoor and outdoor fungal concentrations, the full range of fungal taxa present and fungal-specific exposure thresholds for eliciting an allergic response in sensitised individuals must then be applied to studies evaluating the health impacts of exposure to fungi [175].

1.6 Sampling strategies

Sampling methods employed to monitor indoor fungal levels are dependent on a number of factors including location, cost and type of analysis. Sampling may concentrate on air or surface (including dust) samples, or both. A wide variety of sampling devices are available for analysis; traditionally using microscopy or culture-based methods for morphological identification, and more recently by immunological, biochemical or molecular techniques. Whilst not enabling discrimination between

clinically important fungi, fungal concentrations can be estimated by measuring levels of ergosterol (from fungal membranes) by mass spectrometry and (1→3) β -D glucan (a component of the fungal cell wall) by ELISA [179, 180], and species-specific analysis is possible through the use of PCR or quantitative PCR (qPCR).

Air samples represent transient periods of fungal concentrations present in the air which may be inhaled. The efficiency of air sample collection can be affected by fluctuations in relative humidity due to changes in fungal spore size; high humidity can cause fungal spores to swell, whereas low humidity can cause spores to contract. In contrast, dust samples are taken as measure of longitudinal fungal concentrations present in reservoirs which could be disturbed and aerosolised leading to respiratory exposure; however home and flooring alterations can be a confounding factor in the accurate evaluation of levels.

In mould-contaminated homes, investigators often do a 'walk through' of the property prior to sampling, in order to identify areas of visible mould contamination. Fungal growth can potentially cause structural damage; however if an effect of exposure on health is being considered, it must be hypothesised that the fungi are travelling from their growth substrate to areas where occupants are experiencing symptoms [156].

1.6.1 Air Samples

Types of air sampler include slit samplers which draw air onto a sticky surface on a microscope slide or single-use cassette; slit, hole, or multi-stage samplers which draw air onto agar; cyclone samplers with draw air into microcentrifuge tubes; samplers collecting air samples into liquid (liquid impingers); and filtration samplers collecting air samples onto filters of various pore sizes and materials. In addition, air samplers may be static or portable [156]. Fungal concentrations in intramural air samples are

highly reliant on conditions at the time of sampling and must be interpreted with caution.

1.6.1.1 Viable sampling: Impaction and culture

Viable air sample collection utilises air suction devices which deposit air onto culture plates, and many studies of indoor fungal contamination have utilised culture as a means of quantification and identification, reporting concentrations as CFUs per cubic metre of air. Collection and analysis of culture-based samples costs much less than samples analysed by more modern, molecular-based methods. Multiple stage impactor samplers such as the six-stage Andersen viable impactor enable separation of spores by size; however, single stage samplers are often used for sampling fungi, with comparable results for estimating total fungal levels [181]. Limitations of culture-based techniques include short sample duration, labour and duration-intensive analysis, biased identification due to preferences in growth media, and detection only of viable spores [156]. In heavily contaminated areas, sampling is restricted to just a few minutes or less, since overloading of plates with fungal colonies will prevent quantification [182]. Desiccation of agar over time is another time-limiting step of culture-based techniques, limiting sampling periods to around five minutes; and short sampling times will decrease the likelihood of detecting intermittent fungal spore concentrations. Viable sample collection does facilitate analysis of metabolite and antigen production [156]; however, viable sampling can be laborious (due to incubation times for fungal growth) and gives a less accurate indication of airborne fungal concentrations than visual methods [156], as not all fungi can be readily grown in culture.

1.6.1.2 Cyclone samplers, liquid impingement and filtration sampling

Cyclone samplers can be used to collect air samples through establishment of a vortex [182] into either dry microcentrifuge tubes or those containing liquid [182-185]. Collection into liquid can improve collection efficiency but limits sampling times to around five hours due to evaporation [184]. Cyclone samplers can have poor efficiency in collecting small particles less than 10 µm in size [156]; however, the Burkard cyclone sampler is a commercial example of a dry cyclone sampler, which enables collection of dry samples into a microcentrifuge tube with high efficiency of collection of particles down to 1 µm in size [182]. The Burkard cyclone sampler has been shown to be more efficient than the Burkard volumetric spore trap at collecting particles less than 2.5 µm in size (19.9% in comparison to 9.5% of total particles collected by the volumetric spore trap) [186].

Liquid impingement samplers and filter sampling can be used with culture-based techniques; however, the same problems remain as with traditional culture-based techniques, although longer sampling periods up to a maximum of six hours is possible [187]. Samples collected into liquid can be diluted prior to fungal culture, which eliminates saturation of plates; however, issues with liquid impingers include low flow rates (and high expense in models designed to combat flow rate problems), evaporation of liquid and loss of sample due to re-aerosolisation. Both techniques have the inherent problem of loss of viability over time when used for culture-based purposes and may have increased detection limits [188]. The potential of liquid impingement samplers for quantification of fungi by flow cytometry has been reported but is not widely used [189]; however, there is also potential for samples collected into liquid to be analysed by microscopy, immunological, biochemical or molecular methods [182, 190].

A variety of filtration samplers are available with variable flow rates, materials and pore sizes of filters used. Fungi collected onto filters can be analysed by microscopy (although this is more difficult the sample must first be extracted from the filter), culture, biochemical, immunological or molecular methods; however for viable analyses, the duration of sampling is limited to 10 minutes due to loss of viability of collected spores due to dehydration [190, 191].

1.6.1.3 Personal sampling

Whilst static samplers can give an overall indication of fungal exposure in specific environments, assessing an individual's personal exposure to fungi, including home, outdoor, occupational or pastime exposures is now possible following the development of personal samplers. Various types of personal sampler have been developed utilising methods employed for static sampling devices and with comparable efficiencies, including filter samplers [192], one-stage or two-stage samplers collecting airborne material into microcentrifuge tubes [184, 185], and liquid collection samplers [193].

1.6.1.4 Continuous sampling and analysis by microscopy

Indoor fungal spore concentrations are highly dependent on environmental conditions and can be greatly increased by periods of activity [184]. If fungal concentrations are variable, continuous sampling (which is not possible using culture-based techniques) is preferred in order to improve detection rates; however, in the unlikely event that fungal spore concentrations within a room are at equilibrium, a short sample may suffice [156]. An alternative method of fungal quantification to culture-based methods is the collection of air samples onto a sticky surface, which is analysed by microscopy using standardised techniques [132, 194].

Continuous sampling methods have a number of advantages over culture-based techniques. Firstly, continuous sampling enables quantification of the total fungal biota without the bias of viability [133, 195, 196] and fungal spores may not need to be viable to elicit effects on health [197]. Samples of longer duration and the possibility of time-dependent analyses to detect intermittent fluctuations in concentrations are further advantages. A disadvantage of continuous sampling is that the resultant air sample trace requires skilled analysis and may be prone to misidentification, with identification of important fungal aeroallergens only possible to the level of genus. Smaller spores may also be obscured by the background matrix used for fungal spore entrapment, with high levels of variability when concentrations are low [198], due to the extrapolation of raw counts required to provide meaningful data. The Air-O-Cell® cassette is a commercially-available disposable sampler based on the same methodology but only enables short sampling periods (up to ten minutes), producing a small trace on a coverslip within the commercially-available cassette, which is attached to an air pump. It is recommended that for non-viable sampling, total fungal spore counts should be provided with lower detection limits and comparative outdoor data from which indoor to outdoor ratios can be derived [156].

1.6.2 Surface sampling

An alternative to air sampling is surface (and dust) sampling. Fungal concentrations in air and dust are both taken to reflect current exposure levels of fungi. Surface sampling can be useful within a property since it enables the investigator to easily ascertain whether areas of obvious discoloration are due to fungal contamination or residual dirt or water stains. Surface sampling can be performed using ‘tape-lift’ sampling whereby the sticky side of clear tape is used to sample the fungi and is then placed sample side down onto a microscope slide, stained and examined. Fungal taxa can then be identified

to the level of genus. An alternative is ‘swab sampling’, where a sample of the contaminated area is taken using a cotton swab, which is transported to the laboratory and used to inoculate culture plates. An advantage of the swab-culture method is that species identification is possible; however, the process is much more time-consuming, with inherent difficulties such as differences in preferential growth media among different fungal species [156].

1.6.3 Dust sampling

The collection of dust samples can be used in conjunction with, or as an alternative to air samples, and can provide an indication of long-term personal exposure to fungi without inconveniencing subjects as long-term air sampling might. Advantages of dust analysis include the ability to detect fungal by-products and proteins, lack of discrimination against non-viable spores [156] and samples should not be affected by transient fluctuations in spore concentrations which can affect the integrity of short-term air samples [199]. However, numerous dust samples from many different surfaces would be required for complete analysis [156]. Differences in end-point analyses of dust samples are also important: traditionally, dust samples were analysed by culture of diluted dust samples, however, viable spore counts from culture-based analyses have been shown to only loosely correlate with dust ergosterol levels, for example [199]. Disproportionate measures of some fungi using dust and air sampling methods [129] is also of concern when attempting to interpret results of indoor air investigations. The lack of a strong relationship between indoor air and dust samples suggests that where possible, both types of sampling should be conducted [196].

1.6.4 Development of molecular methods for detection and quantification of fungi

In recent years, molecular methods have been utilised for the quantification of fungi in air and dust samples and since viability is irrelevant for DNA extraction and amplification, air samples collected for PCR analysis can be collected over long periods [200]. Fungal primer targets for PCR and qPCR reported previously have included mitochondrial genes [200], 18S ribosomal RNA (rRNA) [201], 5.8S, 18S and 28S rRNA [202].

In 1999, Haugland *et al.* published a report quantifying *Stachybotrys chartarum* conidia in air samples, an organism with wide-ranging health associations, by qPCR utilising primers targeting ITS rDNA, demonstrating only one-fold disparity to concentrations determined by microscopic analysis. The method works through use of an internal control organism, *G. candidum*, which is spiked into samples for analysis at known and constant concentrations, acting as a control for DNA extraction and amplification efficiency [203].

In 2002, Haugland *et al*, as part of the United States Environmental Protection Agency (EPA), patented a mould-specific qPCR (MSQPCR) technique enabling quantification of 130 major indoor fungi [204]. One of the problems associated with the enumeration of fungi by traditional microscopic means is the inability to distinguish between *Aspergillus*, *Penicillium* and *Paecilomyces* by spore morphology alone and consequently these spores are grouped together. There are a number of problems associated with this approach, not least the limitations associated with attributing clinical outcomes of exposure. The importance of species-specific analysis of environmental samples was highlighted in species and group level analysis of dust samples using the EPA MSQPCR assay, which showed that dust samples comparable in

absolute numbers for *Asp/Pen*-type fungi could be differentially dominated by non-hazardous species or species which produce potentially hazardous mycotoxins [205]. Good agreement between concentrations of conidia quantified using a generic *Aspergillus, Penicillium* and *Paecilomyces variotii* (PenAsp) assay, and the sum of the species-specific assays suggests that predominant indoor species were accounted for in the 62 individual target assays used to design the generic assay [205].

1.6.5 DNA extraction from fungi

Accurate analysis of fungal spore concentrations by MSQPCR first requires efficient fungal DNA extraction from samples, which is inhibited by the cell walls of fungi. A comparison of six different methods of DNA extraction from *A. fumigatus* and *Candida albicans,* specifically with regard to sample preparation, showed that enzymatic lysis was the optimum technique for extraction of *C. albicans* DNA and highest yields of *A. fumigatus* DNA was extracted following oscillation with glass beads [206]. One minute bead-beating of samples using glass beads has also previously been shown to be most effective in extracting DNA from a diverse subset of fungi, in comparison to other methods such as sonication, pestle grinding with liquid nitrogen or at ambient temperature, and freeze-thawing for cell lysis [207], although the efficiency of DNA extraction and recovery from larger fungal conidia was shown to be significantly greater than smaller conidia [207]. Roe *et al.* (2001) found that DNA extraction efficiency and yield was further impeded by the addition of dust samples, as opposed to conidia alone; however, the use of lower quantities of dust (10 mg in comparison to 40 or 70 mg) improved DNA extraction efficiency [208].

1.7 Summary

Airborne fungal spores are very important causative agents of asthma and allergy; however, little is known regarding normal ranges for concentrations of indoor fungal spores in the UK, and exposure thresholds for evoking allergic reactions. *A. fumigatus* is associated with lung function decline in ABPA; however, sputum culture of *A. fumigatus* is only utilised as a minor criterion for diagnosis of the disease due to poor recovery rates. Furthermore, the role of *A. fumigatus* in asthma in the absence of ABPA and CF is poorly understood. Whilst *A. fumigatus* is the most pathogenic fungus associated with colonisation in airways disease and invasive infections, a number of other fungi, including other species of *Aspergillus* and *Penicillium*, have been isolated from clinical samples. *Aspergillus* and *Penicillium* are important indoor fungi; however, little is known regarding relationships between exposure levels and the clinical characteristics of asthma and allergy.

1.7.1 Hypotheses and aims

Hypothesis 1

Indoor concentrations of airborne fungal spores in non-complaint properties will be positively associated with concurrent outdoor concentrations of fungi

Specific aims

- To determine baseline airborne fungal spore concentrations in non-complaint (no moisture/mould-related problems) residential properties, to provide guidelines on 'typical' ranges
- To investigate the contribution of outdoor fungal spore concentrations and effect of season on indoor levels
- To identify home characteristics predictive of elevated airborne fungal spore concentrations

Hypothesis 2

Fungal allergy, as a consequence of colonisation, plays an important role in exacerbating airway inflammation and reducing lung function in asthma

Specific aims

- To use sputum as a diagnostic tool to determine the prevalence of *A. fumigatus* colonisation of the airways in asthma
- To determine the relationship between *A. fumigatus* sensitisation and airways colonisation
- To define the clinical characteristics of *A. fumigatus*-associated asthma
- To fully characterise and determine the clinical significance of the filamentous fungal biota detected in the lower airway in asthma

Hypothesis 3

Fungal allergy and airway colonisation is related to elevated exposure in the home environment

Specific aims

- To investigate airborne and dustborne levels of fungi in the homes of asthmatics with and without fungal sensitisation, using traditional microscopy and molecular (MSQPCR) methods for fungal quantification
- To investigate home exposure levels of fungi with regard to fungal sensitisation and detection in sputum

2 Materials and methods

All suppliers for reagents, equipment and software used for these studies are presented in the appendix, section 8.1.

2.1 Environmental sampling

2.1.1 Air sampling

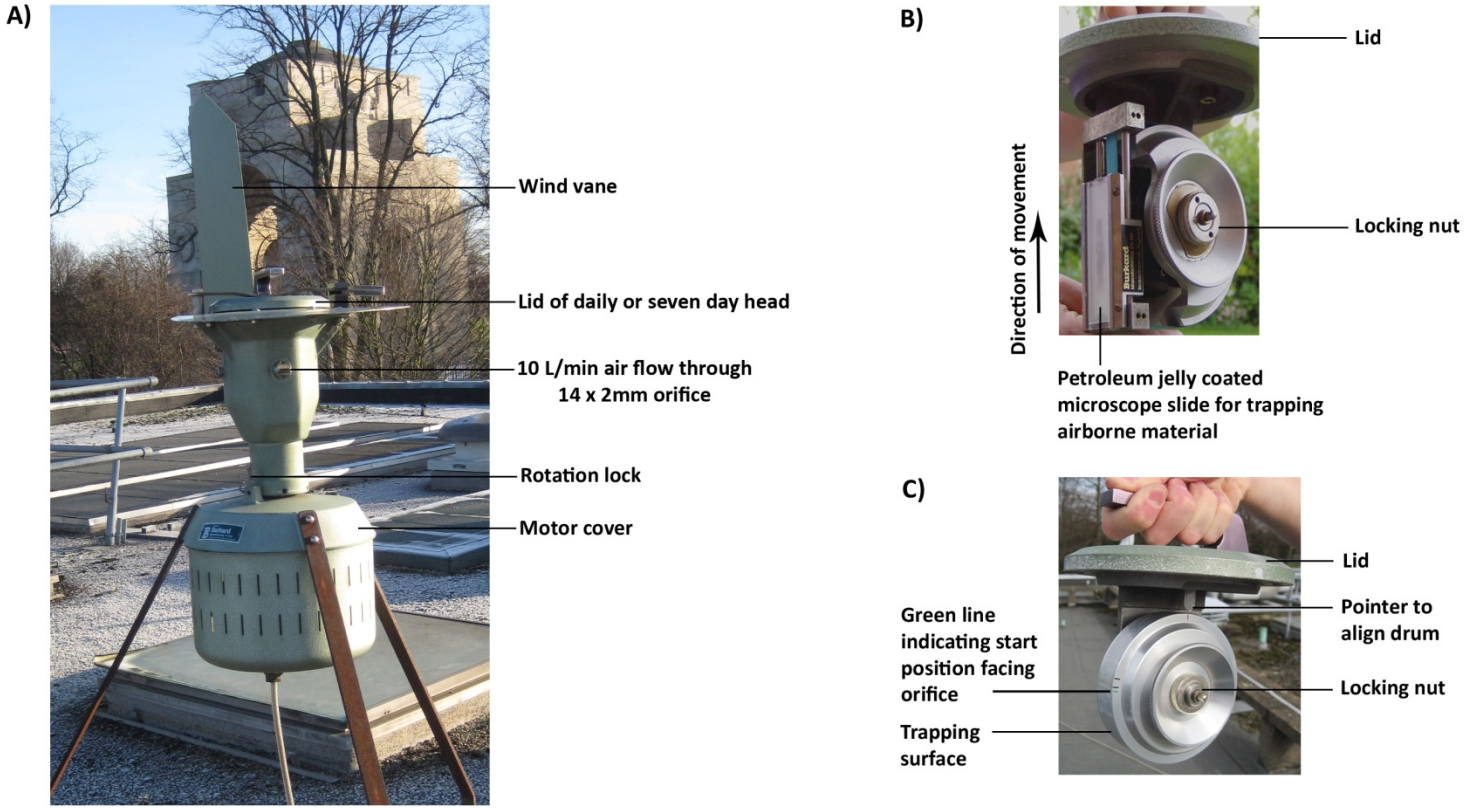
Air samples were collected via impaction onto an adhesive surface. Microscope slides were used for daily outdoor aeroallergen capture and indoor samples, and seven day drums were prepared for weekly outdoor aeroallergen analysis.

2.1.1.1 Collection of outdoor air samples

Outdoor air samples were collected using the Burkard seven day recording volumetric spore trap (Figure 2.1) located on the roof of a University of Leicester building, 1 km south of the city centre, 12 m above the ground and 60 m above sea level, which has recently been shown to be sufficient to provide aeroallergen data for a 41 km area [209]. The Burkard spore trap has two interchangeable heads enabling daily or weekly aeroallergen analysis from air samples collected at a constant flow rate of 10 L/minute. The sampler has a rain shield and a wind vane ensuring that the sampler is facing into the wind for sample collection. During the peak pollen season, samples were collected daily from 24 hour samples impacted onto vertical slides at 2 mm/hour. For the remaining season, air samples were impacted onto a seven day drum, which rotates continuously, also at 2 mm/hour (Figure 2.1).

Figure 2.1. Outdoor aeroallergen sampling

Photographs showing the location of the Burkard seven day recording volumetric spore trap (A), and the operating mechanisms for the daily head (B), and weekly head (C).



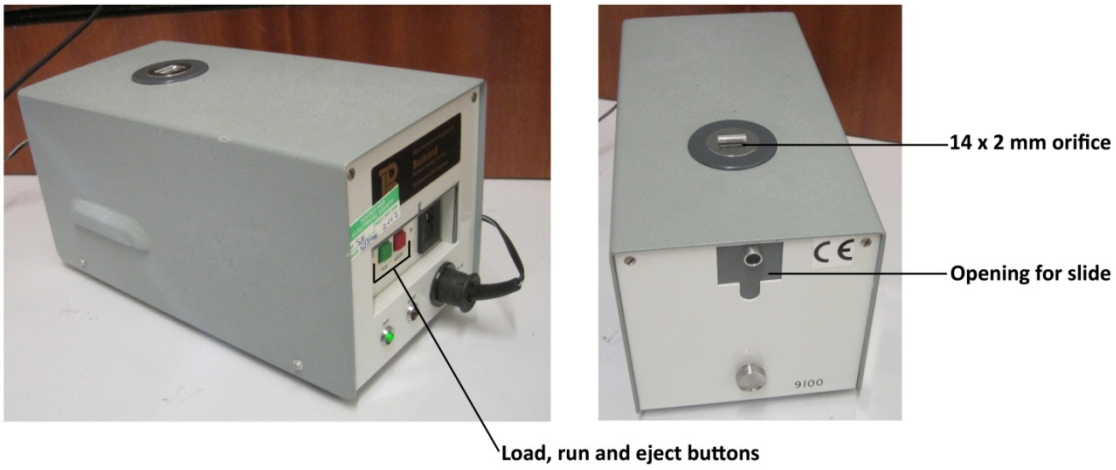
2.1.1.2 Collection of indoor air samples

Air samplers impacted onto slides

Indoor air samples were collected using Burkard continuous recording air samplers (Figure 2.2) placed approximately 0.5-1.0 m from the floor. These samplers draw air through a 2 mm x 14 mm orifice on the top of the sampler at a constant flow rate of 10 L/minute, impacting air onto Vaseline-coated slides moving at a constant speed of 2 mm/hour for a period of 24 hours, producing a 14 x 48 mm trace on each slide.

Figure 2.2. Continuous recording air sampler for indoor sampling

Microscope slides coated with petroleum jelly were loaded into samplers, with the coated surface facing the orifice. Air was drawn through the orifice at 10 L/minute onto the surface of each slide over a period of 24 hours.

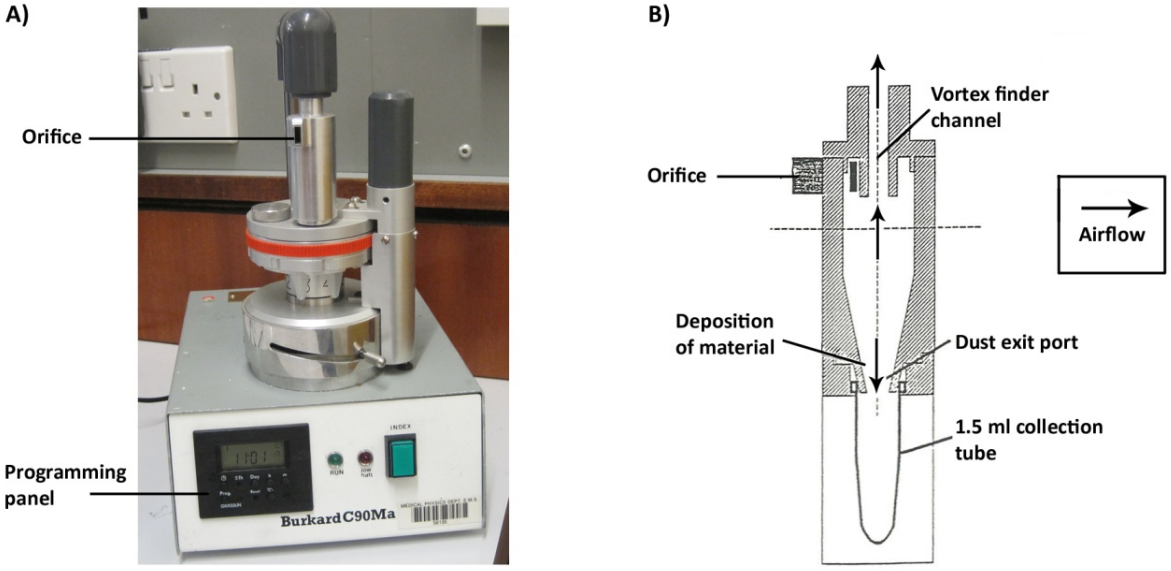


Air samples collected into vials

Air samples were collected into 1.5 ml vials for DNA extraction, using the Burkard multi-vial cyclone sampler (Figure 2.3A), which was placed adjacent to the slide sampler. The air flow at 16.5 L/minute through the vortex finder channel (Figure 2.3B) generates a reverse phase cyclone airstream, which deposits airborne particulates through the dust exit port into the collection vial due to the reduction in airflow at this point.

Figure 2.3. Multi-vial cyclone sampler for indoor sampling

Photograph (A) and operating mechanism (B) of the Burkard multi-vial cyclone sampler. Air was collected at 16.5 L/minute into 1.5 ml collection tubes via a cyclone airstream. Operating mechanism modified from [186].



Preparation of slides for daily aeroallergen analysis

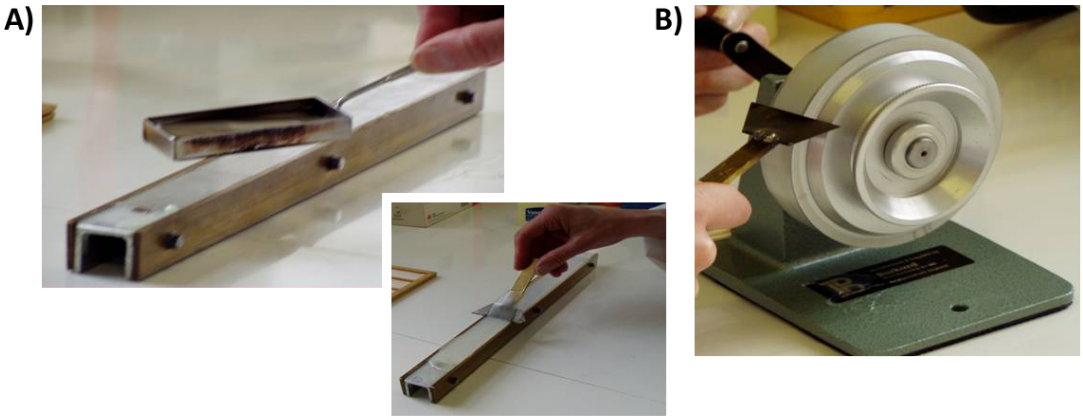
Clear microscope slides were used for the preparation of daily slides for indoor air samples and daily outdoor air samples during the height of the pollen and fungal season (May – September). Petroleum jelly was melted using a blow torch and poured onto slides held in batches of four on a purposefully-built jig (Figure 2.4A). Once cooled, excess petroleum jelly was levelled off using a blade, followed by flaming briefly with the blow torch in order to create a perfectly smooth finish. Petroleum jelly was removed from the four edges of each slide using a notched blade.

Preparation of drums for weekly outdoor aeroallergen analysis

Seven day drums were placed on a purposefully-built jig. Melinex tape was wrapped firmly around the entire circumference of the drum and secured using double-sided tape. The drum was then rotated through molten petroleum jelly. Once cooled, excess petroleum jelly was removed using a blade around the circumference of the drum (Figure 2.4B), followed by flaming using the blow torch to create a completely smooth finish.

Figure 2.4. Preparation of petroleum jelly-coated slides and drums for aeroallergen sampling

Air sample slides were prepared by pouring molten Vaseline onto four slides placed in a purposefully-built jig and levelled off with a blade (A) before flaming with a blow torch to smooth. Melinex tape was wrapped around seven day drums, secured with double-sided tape and rotated through molten petroleum jelly, before levelling with a blade (B) and flaming to smooth.



2.1.1.3 Staining samples

Following sampling, seven day drums were prepared for staining by removal of the coated melinex tape, slicing into 24 hour intervals using a scalpel blade, and mounting onto microscope slides using lactophenol. Daily slides required no preparation prior to staining. Air sample slides were stained by inverting onto coverslips with polyvinyl lactophenol cotton blue (a common semi-permanent mycological stain) diluted approximately 1:10 with lactophenol. This stain is an acid dye, which stains chitin in the cell walls of fungi. Lactophenol serves as a mounting fluid; phenol kills any suspended organisms and the lactic acid conserves the fungal structures.

2.1.1.4 Development of a novel data acquisition and export aeroallergen database

Historically, collection of aeroallergen data was achieved using modified typewriters and paper records with calculations performed manually. In order to improve the speed and efficiency of counting, a novel aeroallergen database was designed using Microsoft Access and Excel databases. Historical aeroallergen data from the Midlands Asthma and Allergy Research Association (MAARA) was used to identify the most common airborne fungal spore types, which were then assigned to corresponding computer keyboard keys. A single keystroke represented a count of one for the corresponding spore type. Separate indoor and outdoor fungal spore databases were created by Tony Andrews (University of Leicester), implementing Microsoft Excel macros which incorporated correction factors specific to analyses, minimising the risk of errors in data collection and simplifying analyses.

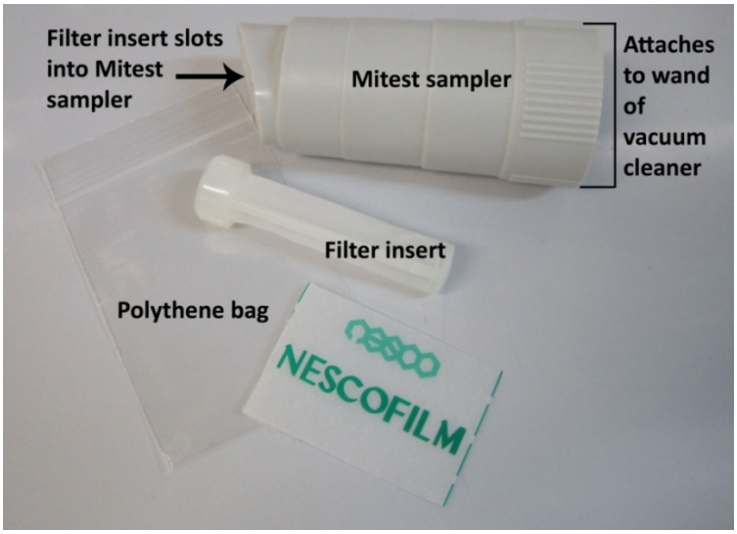
2.1.2 Dust sampling

2.1.2.1 Collection of dust samples

Dust samples were collected following the completion of air samples. A five minute vacuum of the living room floor was conducted as previously described [210]. For carpeted properties, a 1 m^2 area of carpet was marked and vacuumed at a constant rate for five minutes. For living rooms with a hard floor, a one minute vacuum sample was taken from each of four sides of the room, followed by a one minute sample of the remaining accessible floor space. Dust samples were collected using a Victor V9 industrial tub vacuum with a Mitest sampler and filter insert attachment (pore size 40 µm [211], Figure 2.5). Dust samples were collected into the filter insert, sealed with Nescofilm® and placed in a sealed polythene bag.

Figure 2.5. Sampling equipment for dust collection

Dust samples were collected into a filter insert placed inside the Mitest sampler, which attached to the wand of the vacuum cleaner. On completion of five minute samples, filters were sealed with Nescofilm and sealed in a polythene bag.



2.1.2.2 Processing dust samples

Fine dust was collected using 100 mm, 255 µm aperture sieves under aseptic conditions, into a pre-weighed petri dish. Gross weight was measured and net weight of the dust sample calculated in order to give an approximate value for overall 'dustiness' of a property. Five mg (+/- 0.5 mg) dust samples were weighed in duplicate into sterile 2 ml screw cap vials containing 600 mg (± 60 mg) 212-300 µm acid-washed glass beads and samples stored at -20°C until DNA extraction.

2.1.3 Seasonal categorisation

Data was categorised into seasons based on the dates and times of the seasonal solstice or equinox (Table 2.1).

Table 2.1. Classification of seasons during sampling periods

Dates and times of vernal and autumnal equinoxes and summer and winter solstices during the property sampling periods (source: Royal observatory, National Maritime museum, Greenwich. http://www.nmm.ac.uk).

	Dates and times of commencement of each season				
Season	2006	2007	2008	2009	2010
Spring	20 March, 18.25	21 March, 00.07	20 March, 05.48	20 March, 11.43	20 March, 17.32
Summer	21 June, 12.26	21 June, 18.06	20 June, 23.59	21 June, 05.45	21 June, 11.28
Autumn	23 September, 04.03	23 September, 09.51	22 September, 15.44	22 September, 21.18	23 September, 03.09
Winter	22 December, 00.22	22 December, 06.08	21 December, 12.04	21 December, 17.47	21 December, 23.38

2.1.4 Temperature and humidity data

Temperature (°C) and relative humidity data (%) were collected using LogBox-RHT-data loggers, placed immediately adjacent to air samplers during sampling and were pre-programmed to record temperature and relative humidity at 10 minute intervals throughout the 24 hour sampling period. Mean values of temperature and relative humidity for each property were then used in analyses.

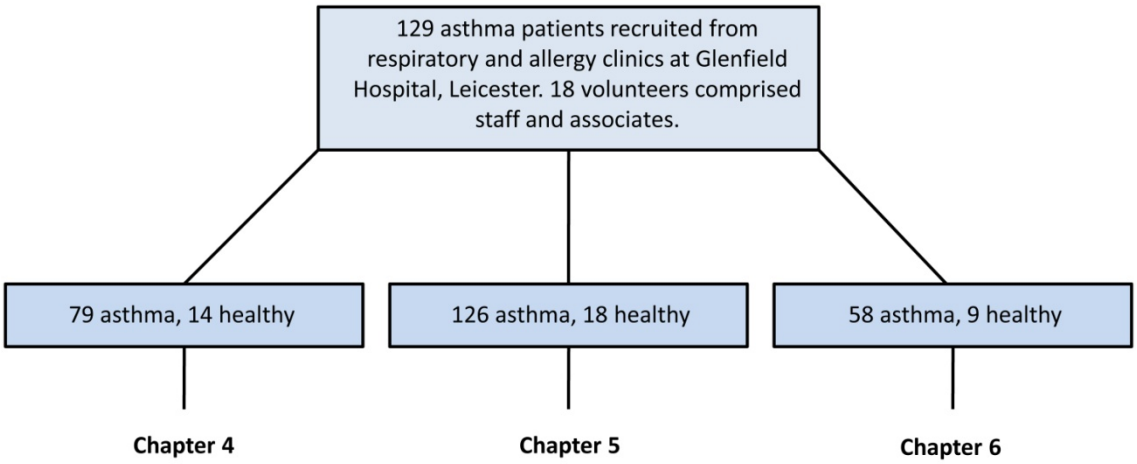
2.2 Clinical sampling

2.2.1 Study cohorts and sample collection

All studies were approved by the Leicestershire and Rutland ethics committee (for reference numbers see appendix, section 8.2), and all subjects gave their written, informed consent to participate (for patient information leaflets and consent forms, see appendix section 8.3 and 8.4). Seventy nine asthma patients from respiratory clinics and 14 healthy subjects from staff and associates were recruited from Glenfield hospital (Leicester, UK) from August 2007-April 2009 to investigate the role of *A. fumigatus* in asthma (Chapter 4, Figure 2.6). Later studies following further recruitment until May 2010 incorporated an extended cohort of 126 asthma patients and 18 healthy subjects (Chapter 5, Figure 2.6). Fifty four asthma patients and nine healthy subjects from Chapter 4, one asthma patient from Chapter 5, and three further asthma patients attending the respiratory and allergy clinics at Glenfield hospital consented to home air sampling (Chapter 6, Figure 2.6).

Figure 2.6. Study participants for Chapters 4-6

Asthma patients were recruited into studies consecutively from asthma clinics at Glenfield Hospital, Leicester. Healthy volunteers comprised staff and associates.



2.2.1.1 Clinical assessment

Sections 2.2.1.1-2.2.1.5 were carried out by colleagues at Glenfield Hospital, Leicester and all clinical assessment data were kindly provided by Professor Andy Wardlaw, Dr Kugathasan Mutalithas, Dr Joshua Agbetile and Dr Dhananjay Desai. Inclusion criteria for the studies were a clinical diagnosis of asthma (evidence of airflow obstruction on pre-bronchodilator FEV_1 (forced expiratory volume of air in the first second of expiration) and/or historical evidence of >12% variability in FEV_1) and evidence of reversible obstruction (bronchodilator reversibility (improvement in FEV_1) 15 minutes after 200 μg inhaled albuterol) and/or airway hyper-responsiveness on methacholine challenge, defined as concentration of methacholine causing a 20% fall in FEV_1 (PC20) <8 mg/ml. Any patients with a respiratory diagnosis other than asthma or who were unable to produce sputum were excluded.

Study participants were seen at a single stable visit, during periods when their asthma was adequately managed and controlled, with no exacerbations of symptoms. Clinical

data collected included gender, age of asthma onset, duration of asthma, physiological parameters of spirometry (Vitalograph Gold Standard), sputum eosinophil and neutrophil differential cell counts, smoking history, radiological evidence of bronchiectasis, atopy, prescribed inhaled and systemic corticosteroid therapy, pulmonary function testing, reversibility and airway hyper-responsiveness data. All patients were recruited under the supervision of Professor Andy Wardlaw, and clinical assessments performed by specialist registrars, consultants and research nurses at Glenfield Hospital, Leicester, UK.

2.2.1.2 Pulmonary function testing and methacholine challenge

All subjects from whom sputum was obtained underwent pulmonary function and methacholine challenge testing. Post-bronchodilator FEV_1 measurements were recorded 15 minutes after 200 μg of inhaled albuterol according to current guidelines [212]. FEV_1 was calculated according to the best successive readings in 100 ml, using a dry bellows spirometer [213, 214]. Airway hyper-responsiveness was assessed by methacholine challenge using the tidal breathing method, whereby doubling measures of nebulised methacholine were administered (0.03-16.00 mg/ml) using a Wright nebuliser, with <8 mg/ml as a cut off [215]. Reversibility was assessed as ≥12% improvement in FEV_1 15 minutes after 200 μg albuterol.

2.2.1.3 Allergy testing

Atopy was assessed using routine SPTs to common aeroallergens including grass pollen, dog and cat fur, *Dermatophagoides pteronyssinus* in addition to a fungal panel consisting of: *A. fumigatus*, *A. alternata*, *Botrytis cinerea*, *C. herbarum* and *P. chrysogenum*. Total IgE, *A. fumigatus*-IgE and *A. fumigatus*-IgG levels were measured using the ImmunoCAP 250 system. Asthma patients were divided into groups: 1) *A.*

fumigatus-IgE sensitised, as defined by a positive SPT to *A. fumigatus* with wheal ≥3mm above negative control or *A. fumigatus*-IgE >0.35 kU/L; 2) IgG-only sensitised with *A. fumigatus*-IgG >40 mg/L; and 3) non-sensitised. Group 4 comprised healthy controls.

2.2.1.4 Bronchiectasis

High-resolution computed tomography of the thorax was performed on asthma patients using a Picker PQS or Siemens sensation 16 scanner. Cross-sectional images were obtained using settings of 1 mm collimation at 10 mm intervals in full inspiration. A diagnosis of bronchiectasis was made based on the radiologist's clinical report, where there was evidence of bronchial dilatation (where the internal bronchial diameter is greater than the accompanying pulmonary artery).

2.2.1.5 Sputum induction

Sputum was collected either spontaneously or through induction, performed as described previously [213, 216], with stepwise inhalation of 3%, 4% and 5% saline for five minutes via an EasyNEB II ultrasonic nebuliser. Bronchoconstriction was minimised by inhalation of 200 µg albuterol prior to sputum induction. Sputum was expectorated into a sterile container, and stored on ice until processing within two hours.

2.3 Sample processing

2.3.1 Preparation of media for fungal culture

Potato dextrose agar (PDA) was selected as optimal medium for fungal culture based on observations of sporulation of seven allergenic fungal genera [216]. PDA was dissolved

in deionised water (39g/L) and sterilised by autoclaving. Stock solutions of antibiotics were dissolved in recommended solvents, filter-sterilised through 0.2 μm acrodisc filters and stored at -20ºC:

Preparation of culture plates and long-term storage slopes was conducted under aseptic conditions in a class I hood. Autoclaved PDA was cooled to approximately 50°C, and immediately prior to pouring, chloramphenicol and gentamicin antibiotics were added at optimum concentrations for isolation of fungi [217] (Table 2.2). This medium was referred to as PGC, and was used for subcultures of fungi in 9 mm petri dishes, and for 7-10 ml slopes prepared in 30 ml universal containers for long-term storage of isolates. Fluconazole (5 μg/ml, Table 2.2.) was added to PGC medium in preparation of plates for culture of initial sputum samples (PGCF plates) to enhance recovery of *A. fumigatus* from sputum samples through suppression of *Candida* growth [218].

Table 2.2. Preparation of antibiotics for fungal culture media

Antibiotic	Stock concentration	Solvent	Final concentration
Chloramphenicol	34 mg/ml	Ethanol	16 μg/ml
Gentamicin	50 mg/ml	Deionised water	4 μg/ml
Fluconazole	5 mg/ml	Dimethyl sulfoxide (DMSO)	5 μg/ml

2.3.2 Sputum processing and culture

Sputum was manually separated from saliva in a petri dish using forceps under aseptic conditions inside a class II hood and collected into a cellular mass. The resultant sputum plug was then divided into two parts; the first used to produce differential inflammatory cell counts from sputum cytospins, the second was used for fungal culture.

2.3.2.1 Production of sputum cytospins for differential inflammatory cell counts

A volume (ml) of 0.1% dithiothreitol (DTT) four times the weight (mg) of sputum plug was added to a selected sputum plug. DTT is a sulphydryl reagent, which breaks disulphide bonds cross-linking glycoproteins in sputum, causing mucolysis and homogenisation [97, 219]. The sputum-DTT mix was vigorously agitated for 15 seconds followed by slow agitation on ice using a bench rocker for 15 minutes. The homogenate was then neutralised with an equal volume of Dulbecco's phosphate buffered saline and filtered through 48 µm nylon gauze. The volume of filtrate was recorded and 10 µl used in a 1:1 dilution with trypan blue in order to provide a viability count using the exclusion method. 10 µl of the trypan blue-sputum filtrate solution was added to a Neubauer haemocytometer, with viable (gold), non-viable (blue) and squamous cells recorded from four of the large corner and central squares of a single chamber at x200 magnification. Samples were then centrifuged at 2000 rpm for 10 minutes at 4°C and the supernatant removed. The cell pellet was resuspended and the cell suspension was adjusted to 0.5 x 10^6 cells/ml. 75 µl of cell suspension was cytocentrifuged at 450 rpm, for six minutes at room temperature to produce cytospins, which were air dried, followed by Romanowsky staining (appendix section 8.6) and coverslips mounted using DPX (distyrene, plasticiser and xylene mountant).

2.3.2.2 Fungal culture from sputum

Following removal of the first part of sputum samples for preparation of cytospins, the second part of neat sputum plug (approximately 170 mg (± 80 mg)) was inoculated onto PGCF plates and spread across the agar. Plates were sealed using Nescofilm and transferred to a dedicated mycology laboratory, where culture plates were incubated at

37°C, with regular inspection, for up to two weeks. Subcultures of filamentous fungi were produced as necessary according to the rate of spread across the plate to prevent overgrowth of fast-growing colonies resulting in loss of individual isolates.

2.3.3 Fungal culture

All processing of fungi was performed under aseptic conditions in a class II hood. The cabinet was thoroughly cleaned with MicroSol 3+ solution (a sporicidal and fungicidal detergent) at a 1:2 dilution, and left for ten minutes between processing of different sputum samples and different fungal isolates.

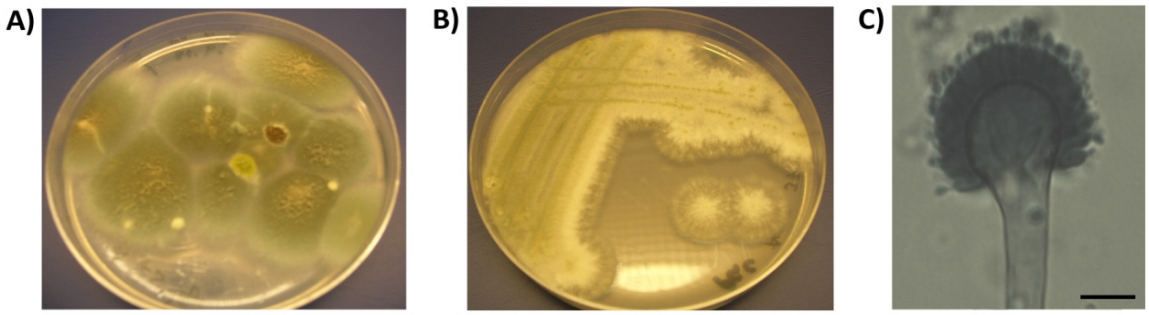
2.3.3.1 Sputum isolates

Individual colonies were subcultured by spread-plating onto PGC plates, using a wire loop to transfer fungal material, with subcultures incubated for up to four weeks at 37°C. Colonial morphologies were recorded by photograph (Figure 2.7B) and under aseptic conditions, small amounts of fungal material was transferred to PGC slopes. Needle-mounts of individual colonies were taken; targeting the protruding growth structures of the fungi (most commonly conidiophores). Needle-mount samples were mounted onto a glass microscope slide with a drop of polyvinyl lactophenol cotton blue, examined briefly under a dissecting microscope at x20 magnification and hyphae teased apart in order to maximise visibility of growth structures, before adding a coverslip. Slope cultures were sealed with Nescofilm, incubated at 37°C until confluent, followed by transfer for long-term storage at 4°C. Needle mounts of filamentous fungi were examined and recorded by microscopy (Figure 2.7C) and, with the exception of *A. fumigatus* and *A. niger*, broadly classified according to colonial and microscopic features as *Aspergillus*, *Penicillium/Paecilomyces*-type, zygomycete or other fungi. For the purposes of this study, yeast cultures were not examined in any detail. It was

possible to accurately identify *A. fumigatus* based on colonial and microscopic features [220] (Figure 2.7C). Non-*A. fumigatus* isolates were prepared for DNA extraction, PCR and sequence identification. Samples of approximately 50 mg were transferred using a wire loop into a 2 ml sterile screw-cap tube containing 600 mg (± 60 mg) sterile acid-washed glass beads and stored at -20°C.

Figure 2.7. Identification of *A. fumigatus* based on macroscopic and microscopic features

Isolates of *A. fumigatus* were identified on original sputum plates (A) and were subcultured to select pure isolates. *A. fumigatus* isolates had characteristic macroscopic colony features, with a cream underside and green suede-like appearance on the top of the colony (B). Needle-mounts of isolates were stained with polyvinyl lactophenol cotton blue and examined by microscopy at x630 magnification to confirm identification of *A. fumigatus*. Conidiophores have a distinctive appearance, with an absence of metulae and a single layer of phialides around the upper third to half of the vesicle. Scale bar represents 10 µm (C).



2.3.3.2 Fungal culture and conidia harvesting from type fungi

A. flavus, *A. fumigatus*, *A. niger*, and *P. chrysogenum* (Table 2.3) were grown to confluence according to our standard technique for PGC plates; with the modification

that fungi were incubated at room temperature until confluent. 0.5% $Tween_{80}$ was prepared in deionised water and filter-sterilised using 0.2 µm acrodisc filters. Conidia were harvested by inoculating fungal plates with 5 ml 0.5% $Tween_{80}$ and disrupting with a sterile cell spreader. The fungal suspension was then removed by pipette and filtered through a sterile Buchner funnel containing Whatman filter of pore size 22 µm to remove fungal hyphal fragments from the conidia suspension. Fungi were resuspended in sterile 0.5% $Tween_{80}$ and counted by haemocytometer, using the average of five small squares to calculate the number of conidia per ml using Equation 2.1:

Equation 2.1

Number of conidia/ml = 1000 (NDn/ASDp)

Where N = total cells counted, Dn = dilution of solution, A = area of microscope counted per square (mm^2), S = number of squares counted, Dp = depth of chamber (0.1 mm). Concentrations were multiplied by 1000 to convert mm^3 to ml.

Fungal conidia concentrations were adjusted to approximately either 1×10^7 or 1×10^8 spores/ml depending on efficiency of fungal spore harvest (Table 2.3), with the exception of *G. candidum*, which was adjusted to 2×10^8 spores/ml. 130 µl aliquots of target fungi were frozen at -80 °C until required.

Table 2.3. Fungal conidia stock concentrations prepared for production of calibration curves of target fungi

Target fungi (strain ID)	Stock conc. (spores/ml)	Forward primer sequence[a]	Reverse primer sequence[a]	Probe sequence[a]	Amplifies[a]
G. candidum (UAMH 7863)	2.0850 x 10^8	GeoF1: 5'-GATATTTCTTGTGAATTGCAGAAGTGA	GeoR1: 5'-TTGATTCGAAATTTTAGAAGAGCAAA	GeoP1: 5'-CAATTCCAAGAGAGAAACAACGCTCAAACAAG	*G. candidum strain* UAMH 7863 (reference strain)
A. fumigatus (NCPF 7097)	1.0015 x 10^8	AfumiF1: 5'-GCCCGCCGTTTCGAC	AfumiR1: 5'-CCGTTGTTGAAAGTTTTAACTGATTAC	AfumiP1: 5'-CCCGCCGAAGACCCCAACATG	*A. fumigatus, Neosartorya fischeri*
A. flavus ATCC 16883	1.0900 x 10^8	AflavF1: 5'-CGAGTGTAGGGTTCCTAGCGA	AflavR1: 5'-CCGGCGGCCATGAAT	AflavP1: 5'-TCCCACCCGTGTTTACTGTACCTTAGTTGCT	*A. flavus/oryzae*
A. niger (ATCC 16888)	1.0100 x 10^7	AnigrF1: 5'-GCCGGAGACCCCAACAC	AnigrR1: 5'-TGTTGAAAGTTTTAACTGATTGCATT	AnigrP1: 5'-AATCAACTCAGACTGCACGCTTTCAGACAG	*Aspergillus niger/awamori/ foetidus/phoenicis*
P. chrysogenum (NCPF 2715)	1.0600 x 10^8	PchryF4-1: 5'-GCCTGTCCGAGCGTCACTT	PchryR8: 5'-CCCCCGGGATCGGAG	5'-CCAACACACAAGCCGTGCTTGAGG	*P. chrysogenum*, svar. 2
Aspergillus/ Penicillium	1.0223 x 10^7	PenAspF1: 5'-CGGAAGGATCATTACTGAGTG	PenAspR1: 5'-GCCCGCCGAAGCAAC	PenAspP1mgb: 5'-CCAACCTCCCACCCGTG	*Penicillium, Aspergillus* and *Paecilomyces variotii*

Abbreviations: ID, identification; conc, concentration; UAMH, The University of Alberta Microfungus Collection and Herbarium; NCPF, National Collection of Pathogenic Fungi; ATCC, The American Type Culture Collection. All probes were labelled with 6-FAM (FAMRA: carboxyfluorescin). All assays except the generic assay were labelled with a TAMRA (rhodomine) quencher. The *Aspergillus/Penicillium* (PenAsp) generic probe was labelled with a black hole quencher.

[a]http://www.epa.gov/microbes/moldtech.htm

Harvesting of conidia for *in vitro* assays

For *in vitro* cell culture assays, *A. fumigatus* conidia were harvested in HBSS to prevent any effects of $Tween_{80}$ in assays and adjusted to concentrations of 1×10^8 conidia/ml.

2.3.4 Isolation and culture of bronchoalveolar macrophages

All cell culture was performed under aseptic conditions in a class II hood.

2.3.4.1 Preparation of media

Antibiotics

Penicillin and streptomycin antibiotics were dissolved in water to concentrations shown in Table 2.4, filter sterilised through 0.2 µm acrodisc filters and stored at -20ºC:

Table 2.4. Antibiotics used in the preparation of macrophage culture media

Antibiotic	Stock concentration	Solvent	Final concentration /500 ml medium
Streptomycin sulphate	100 mg/ml	Deionised water	50 µg/ml
Penicillin G	50 mg/ml	Deionised water	50 U/ml

Culture media

Dulbecco's modified eagle's medium (DMEM) was modified with 2% foetal bovine serum (FBS) for initial washes. Subsequent culture media consisted of DMEM modified with 10% FBS, 1% non-essential amino acids, penicillin (50 U/ml) and streptomycin (50 µg/ml) (DMEM-10% FNPS). All media was stored at 4ºC.

Steroid

Dexamethasone (4 mg/ml) was diluted in Hank's balanced salt solution to stock concentrations of 10^{-7} to 10^{-4} M (40 ng/ml-40 µg/ml) and stored at 4ºC in the dark.

2.3.4.2 Culture of macrophages

Bronchoalveolar macrophages were obtained from patients undergoing upper lung lobectomies. Lung samples were finely sliced, washed in DMEM supplemented with 2% FBS, filtered twice through 100 µm nylon gauze and transferred to a sterile sealed container. Typical volumes of filtrate retrieved ranged from 40-80 ml.

Filtrate was transferred to 50 ml centrifuge tubes and washed twice, through centrifugation at 1400 rpm and resuspension of the cell pellet with HBSS. On the second wash, red blood cells were lysed using approximately 10 ml sterile deionised water for 30 seconds, followed by resuspension with HBSS and recentrifugation at 1100 rpm. The cell pellet was resuspended in 10 ml DMEM-10% FNPS. Viability of macrophages was assessed by the trypan blue exclusion method (as described in section 2.3.2.1, with the modification that 400 cells were counted). Purity of macrophages was assessed by the Kimura stain (see appendix section 8.6), where 10 µl of a 1:10 dilution of cell suspension: Kimura stain was assessed by microscopy at x200 magnification in a haemocytometer and the number of macrophages counted per 5 large squares.

13 mm round glass coverslips were dipped in ethanol and dried inside 24-well plates, followed by seeding of 4 x 10^5 macrophages onto each coverslip in a final medium volume of 1 ml. Cells were incubated overnight at 37°C, 5% CO_2 for macrophage adherence, followed by washing vigorously twice with 1 ml HBSS. 10 µl dexamethasone was added to 990 µl DMEM culture medium at final concentrations ranging from 10^{-9}-10^{-6} M in triplicate wells for each dilution. HBSS without dexamethasone was used as a negative control. Following 24 hours incubation, which

has been shown previously to enable suppression of macrophage activity [221], macrophages were challenged with 4 x 10^6 *A. fumigatus* conidia and incubated for 45 minutes (37°C, 5% $C0_2$). Macrophages were thoroughly washed twice with HBSS to remove extracellular conidia, previous media with or without steroid replaced, and cells incubated for a further 4 hours. Coverslips were washed with HBSS, fixed in methanol for ten minutes and stained with Rapi-Diff.

2.3.5 DNA extractions

All DNA extractions were performed under aseptic conditions in a class II hood, cleaned as described in section 2.3.3. Negative control DNA extractions were performed on new extraction kits, using tubes containing beads only for clinical isolates and dust samples, and using tubes containing beads and 100 µl 0.5% $Tween_{80}$ for fungal conidia preparations and air samples.

The manufacturer's instructions for the Biospec mini-beadbeater-16 suggested filling 2 ml vials at least half full with glass beads; however, previous studies had used 200-300 mg, a much lower quantity [203, 207]. Triplicate DNA extractions from 1.045 x 10^5 and 1.045 x 10^7 *A. fumigatus* conidia were carried out with 300, 600 and 900 mg of glass beads, with DNA measured by Qubit (section 2.3.5.3). Similar yields of DNA were retrieved from DNA extractions using 300 and 600 mg of beads. DNA extractions using 900 mg of beads yielded higher concentrations of DNA when spiked with 1.045 x 10^7 conidia but lower concentrations when spiked with 1.045 x 10^5 than 300 or 600 mg beads. Therefore, all DNA extractions were performed using 600 mg beads.

2.3.5.1 DNA extractions from filamentous fungi

Fungal DNA was extracted using a modification of the Qiagen DNeasy plant mini kit. To minimise the possibility of cross contamination of samples or kit reagents, 400 µl of

lysis buffer and 4 µl ribonuclease (RNase) were mixed vigorously in a 7 ml bijou by vortex in multiples representing the number of extractions being performed at any given time (plus two extra measures to account for loss through pipetting or evaporation of the solution). Individual samples pre-inoculated into 2 ml screw-cap tubes with glass beads were centrifuged at 14,000 rpm for 1 minute to minimise aerosols, followed by addition of 404 µl of the lysis buffer-RNase mix to each tube, vigorously mixing by vortex for 2-3 seconds and incubation on ice until all subsequent samples had been processed. Fungal material was disrupted by 2 minutes bead-beating using a Biospec mini-beadbeater-1 at 4800 oscillations/minute (for extraction of filamentous fungal isolates from sputum only); latterly using a Biospec mini-beadbeater-16 at 3450 oscillations/minute to optimise efficiency of sample processing. Sample tubes were then incubated by water bath at 65°C for 10 minutes, inverting tubes 2-3 times during this period. 130 µl of buffer AP2 was added to each of the samples, agitated by vortex for 2-3 seconds and incubated on ice for 5 minutes, followed by centrifugation at 14,000 rpm for 5 minutes at room temperature. Lysate was then transferred to the QIAshredder Mini spin column and DNA precipitation, binding and elution performed according to the kit instructions. 200 µl eluted DNA for each sample was stored at -20°C prior to analysis.

Calibrator fungi

DNA from fungi used as calibrators in MSQPCR assays was extracted as per individual fungal species with the modification that, prior to adding 404 µl of the lysis buffer-RNase mix, samples were spiked with 10µl *G. candidum* reference organism (2.085 x 10^8 conidia/ml).

2.3.5.2 DNA extractions from environmental samples

Dust samples

DNA from dust samples was extracted as per calibrator fungi in section 2.3.5.1.

Air samples

Preparation of air samples for DNA extraction following methods previously employed [222] showed that a 100 µl wash with 0.5% $Tween_{80}$, 2 minutes vigorous agitation by vortex and transfer into sterile 2 ml screw-cap microcentrifuge tubes containing 600 mg (± 60 mg) glass beads, did not capture all of the fungal material present in the sample tubes. This was demonstrated by both microscopic detection of biological material and subsequent amplification of fungal DNA on repeating the wash, vortex and extraction procedure. Therefore, to enable maximal recovery, DNA extractions were performed in the original 1.5 ml vials and sealed with Nescofilm for the 2 minute beat-beating step. Sterile glass beads and 100 µl 0.5% $Tween_{80}$ were added to each air sample tube, vigorously mixed by vortex for 2 minutes then centrifuged for 1 minute at 14,000 rpm in order to prevent loss of material from inside the lid or cross-contamination of samples through aerosolisation of samples. 404 µl of the lysis buffer-RNase mix and 10µl *G. candidum* reference organism (2.085×10^8 spores/ml) were added to each tube and extractions performed as described above (section 2.3.5.1).

2.3.5.3 Quantification of DNA

DNA was quantified using a Qubit® fluorometer and the Quant-iT dsDNA high sensitivity (HS) assay according to manufacturer's instructions. Fluorescent probes bind to double stranded DNA and fluoresce intensely. The concentration of sample DNA is determined through comparative analysis of the fluorescence of reference standards.

1 µl Quant-iT reagent was added to 199 µl Quant-iT reaction buffer for the number of samples to be quantified in addition to two kit standards. 10 µl of each PCR product or kit standard was added to 190 µl of this working solution, mixed by vortex, incubated at room temperature for 2 minutes and quantified using the Qubit fluorometer.

2.4 Sample analysis

2.4.1 Recording and calculating fungal spore concentrations by microscopy

Extensive training on the identification and quantification of fungi from air samples was provided by highly experienced aerobiologists from MAARA, located in Derby (UK), enabling all data collected to be analysed by the same person, thus eliminating inter-personal variation. A single longitudinal traverse of one field width was counted down the centre of each slide, in a standard method described previously [132] at x630 magnification, using an eyepiece graticule of width 156 µm.

The most accurate method for quantification of airborne fungal spores deposited onto a 24 hour trace would be to count the entire 48 x 14 mm trace. However, this would be an incredibly laborious task and a single longitudinal traverse is commonly utilised as an approximation of concentrations. An alternative method is to count twelve transverse traverses; however, comparison of both techniques showed that similar concentrations were detected using both methods, and that whilst slightly higher concentrations were detected using the twelve transverse traverse method, the extra time required in order to produce the spore counts was unlikely to be justified [195].

2.4.1.1 Fungal taxa identified

Common airborne fungal taxa were identified to the level of genus or closely related groups. Seventeen fungal taxa were identified to the level of genus based on distinct spore morphology: *Alternaria, Botrytis, Cladosporium, Didymella, Drechslera, Entomophthora, Epicoccum, Erysiphe, Ganoderma, Leptosphaeria, Pithomyces, Polythrincium, Sporobolomyces, Stemphylium, Tilletiopsis, Torula* and *Ustilago.* Other fungi were categorised into closely related groups due to being undistinguishable by spore morphology alone or to ease counting: ascospores, hyaline basidiospores, coloured basidiospores, rusts and smuts, *Lewia/Pleospora*-type, and *Asp/Pen*-type conidia.

2.4.1.2 Calculation of correction factors

Raw data for fungal spore counts were adjusted according to the correction factor appropriate for the given magnification, calculated using Equation 2.2:

Equation 2.2: Calculation of correction factors for airborne fungal spore concentrations.

Mean fungal spore concentration/m^3 air = x(t/lsw)

x = raw data, t = total area of 24 hour trace (mm^2), l =length of trace (mm), s = volume of air sampled over the 24 hour period (m^3), w = width of counting area (mm). Modified from [194].

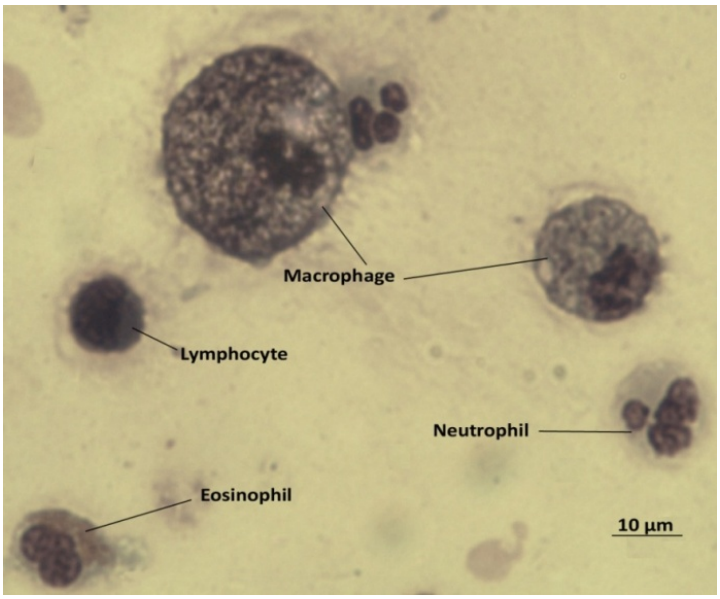
All spore counts were produced on a Zeiss Axioskop 40 microscope, for which the correction factor was calculated as 6.2 when recording full field view at x630 magnification.

2.4.2 Producing inflammatory differential cell counts from sputum cytospins

Sputum cytospins were examined by light microscopy and 400 cells (macrophages, neutrophils, eosinophils, epithelial cells and lymphocytes) from each cytospin were counted in order to provide a differential cell count [97] (Figure 2.8).

Figure 2.8. Identification of inflammatory cells in sputum

Photomicrograph showing a Romanowsky-stained sputum cytospin at a magnification of 630x, illustrating macrophages, neutrophils, eosinophils and lymphocytes.



2.4.3 Determining the phagocytic index of bronchoalveolar macrophages

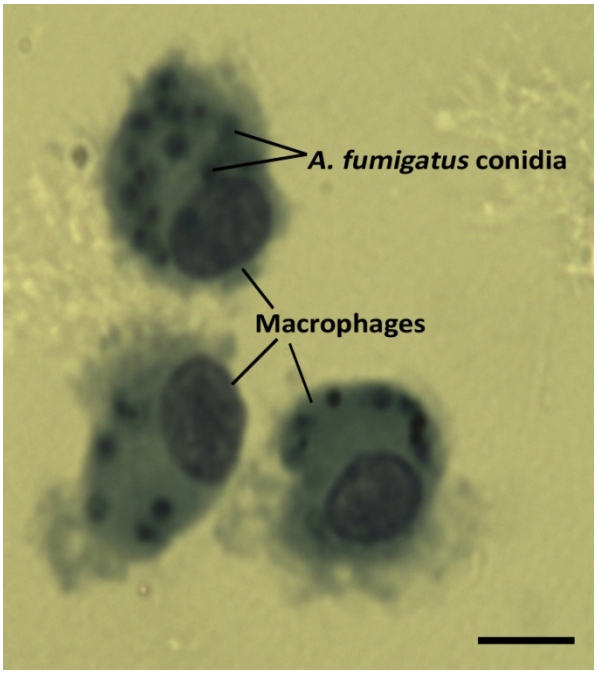
Viability of macrophages was assessed by the trypan blue exclusion method (non-viable cells are permeable to the stain enabling differentiation) by inverting coverslips onto slides with a drop of trypan blue. Proportions of viable and non-viable macrophages were determined from approximately 400 counted per coverslip, for macrophages

incubated with and without *A. fumigatus* conidia, with highest (10^{-6} M) and lowest (10^{-9} M) steroid concentrations.

The phagocytic index was assessed by microscopic analysis of 200 macrophages per coverslip, in triplicate for each treatment and non-treatment control. The number of conidia per macrophage was counted at a magnification of x630 (Figure 2.9), analysed blind to treatment conditions of coverslips.

Figure 2.9. Phagocytosis of *A. fumigatus* conidia by bronchoalveolar macrophages

Photomicrograph of bronchoalveolar macrophages at x630 magnification after 24 hour pre-treatment with dexamethasone, four hours post-phagocytosis of *A. fumigatus*, at a ratio of 10:1 conidia: macrophages. Scale bar represents 10 µm.



2.4.4 DNA-based identification of fungal isolates using the polymerase chain reaction

Fungal DNA was amplified using primers targeting ITS region 1 of the nuclear ribosomal operon, using ITS5 and ITS2 conserved fungal primers; (5') 5'-GGAAGTAAAAGTCGTAACAAGG; and (3') 5'- GCTGCGTTCTTCATCGATGC [107]. Where this was not possible, primers targeting D1/D2 domain of the large subunit (LSU) ribosomal DNA (rDNA) were used: (5') 5'- GAGTCGAGTTGTTTGGGAATGC; and (3') 5'-GGTCCGTGTTTCAAGACGG [223].

The total PCR reaction volume was 25 µl, containing 5µl template DNA, 0.625U Hotstar Taq DNA polymerase, 0.625 pmol forward and reverse primers, 0.25 mM of each deoxynucleotide triphosphate (dNTP), 1 x PCR buffer (with 15 mM $MgCl_2$). Molecular biology grade (0.1 µm filtered) water was used in place of DNA for negative controls. PCR reactions were performed in a Biometra Thermocycler with the following cycling conditions: one cycle at 95°C for 15 minutes, followed by 40 cycles of 20 seconds at 94°C for denaturation of DNA, 20 seconds at 55°C for primer annealing, and 90 seconds at 72°C for DNA extension, with a seven minute extension cycle at 72°C, before cooling to 4°C.

2.4.4.1 PCR product clean up and quantification

PCR product clean up

PCR products were purified using QIAquick PCR clean up kit spin columns and quantified using Quant-iT dsDNA BR assay kits, as for the HS kit (section 2.3.5.3).

Preparation of agarose gels

10X Tris-acetate-EDTA (TAE) buffer was produced by dissolving 48.44g Tris, 3.72g EDTA (ethylenediaminetetraacetic acid), and 12 ml acetic acid in 1 L deionised sterile water, adjusted to a final pH of 8 through addition of acetic acid. 10X TAE was diluted 1:10 with deionised water to prepare a 1X working solution.

5 µl PCR products were run with 1 µl loading dye on 2% (wt/vol) agarose gels, containing 0.5 µl (10 mg/ml) ethidium bromide per 100 ml molten agarose, at 100V for 30 minutes to 1 hour, with a 100 bp reference ladder. Gels were visualised and photographed on an ultra violet trans-illuminator to confirm a single product.

3-10 ng samples of PCR product were then sent to the protein nucleic acid chemistry laboratory (PNACL) at the University of Leicester for DNA sequencing using Applied Biosystems 3730 sequencers. Sequencing was performed in one direction only. Sequencing reactions used the ITS-5 primer for the ITS1 region and LSU-F for the LSU region.

2.4.4.2 Sequence analysis

Returned sequences were visually inspected and trimmed in FinchTV (Version 1.4.0). Edited sequences were then exported as FASTA files prior to alignment searches for closest taxonomic match performed in GenBank (March-April 2010) using the $BLAST_N$ method [224]. The closest taxonomic hit was reported, where possible, to the level of species, using current fungal names according to Index Fungorum (http://www.indexfungorum.org). Where multiple species of the same genus were equally likely, the genus was reported. Where two species were equally likely, both were reported.

2.4.5 Mould-specific qPCR

DNA from pure conidia suspensions (in 0.5% $Tween_{80}$) of calibrator fungi (*A. fumigatus, A. niger, A. flavus, P. chrysogenum*) and mixed conidia suspensions for positive controls (10^5 conidia) were co-extracted with spiked 10 µl *G. candidum* in triplicate as described in section 2.3.5.1. Positive controls for MSQPCR assays were prepared by DNA extractions from mixtures of 10^5 conidia from all target fungi. 100 µl 0.5% $Tween_{80}$ was used as a negative control for the DNA extraction.

2.4.5.1 Thermal cycling conditions

Standard thermal cycling conditions were based on methods described previously [205]. The final volume per reaction was 25 µl, containing; 5 µl of either template DNA or molecular biology grade water as negative control; 1x TaqMan® Universal PCR mastermix which contains a premix of AmpliTaq Gold® DNA polymerase, AmpErase® uracil-N-glycosylase (UNG), dNTPs with deoxyuridine triphosphate (dUTP), passive reference dye, and optimised buffer components; 0.1 mg/ml BSA; 1 µM each primer and 80 nM probe. Mastermixes of MSQPCR reactions were prepared with surplus to account for loss through pipetting or evaporation of the solution. MSQPCR reactions were performed and monitored in the Applied Biosystems FAST 7500 Real-Time PCR instrument as per manufacturer's instructions. Thermal cycling conditions were: 2 minutes at 50°C followed by 10 minutes at 95°C, then 40 cycles of 15 seconds at 95°C for denaturation of template and 1 minutes at 60°C for primer and probe annealing and DNA extension. The initial 2 minute 50°C step enables UNG enzyme activity in the TaqMan® Universal PCR mastermix , which prevents carry over contamination of PCR products by cleaving the uracil base from the phosphodiester backbone of DNA containing uracil (which can generate false positives in reactions),

without affecting DNA containing thymine bases (target DNA) [225]. The 10 minute step at 95°C immediately following the 2 minute 50°C step activates the AmpliTaq Gold enzyme. The 5' to 3' nuclease activity of the enzyme cleaves the probe between the reporter dye and quencher during the polymerisation of DNA when the probe is hybridised to the target DNA strand [226].

2.4.5.2 Cycle threshold (C_T) values

MSQPCR cycle threshold (C_T) values are the point at which fluorescence exceeds background levels. Fluorescence is provided by the reporter dye (6-FAM) label of the probe, which binds to denatured DNA and fluoresces when the quencher of the probe is cleaved during the process of DNA extension. The amount of fluorescence is therefore equivalent to the amount of DNA, quantifying DNA synthesis. The C_T value for the fluorescence emission intensity for MSQPCR is inversely proportional to the amount of DNA present in the sample; higher C_T values, (such as those of 37-40) indicate low levels of DNA, in comparison to low C_T values (such as 20-30), which indicate high concentrations of DNA.

2.4.5.3 Generation of calibration curves

For each target assay and the reference *G. candidum* assay, a minimum of five dilutions from serial dilutions of triplicate samples of DNA (from 1-2 x 10^{6-7} spores depending on the assay; Table 2.3) were analysed in duplicate by MSQPCR. Baseline and C_T thresholds for conidia detection were manually adjusted to optimise the detection limits and accuracy of each assay. C_T values generated by the Applied Biosystems FAST 7500 Real-Time PCR instrument for serial dilutions of calibrator fungi DNA were viewed as SDS documents, followed by exporting and analysis in Microsoft Excel.

Baselines

Baseline ranges were selected as described [227] to include C_T values between 3 and 'x', where 3 was the default lower limit, and 'x' was 2 cycles prior to the start of the amplification curve of the highest concentration of DNA, in logarithmic view (Figure 2.10A).

Thresholds

MSQPCR thresholds were investigated within the exponential (doubling) phase of the amplification plot in logarithmic view. Data from MSQPCR calibration curves was exported to Microsoft Excel in increments of a 0.01 change in threshold fluorescence within the range 0.03-0.1. For each increment, the data was exported and the standard deviation of replicates was compared with the correlation coefficient of the regression line. Threshold values were chosen generating the minimum deviation and highest correlation coefficient of the data, in combination with lowest possible limits of detection.

Calculation of amplification efficiencies and detection limits of assays

For all target fungi assays, average C_T values for co-extracted *G. candidum* in the neat DNA only, were subtracted from C_T values for serial dilutions of target DNA to create $\Delta C_{T, ref.}$ These values were plotted against theoretical Log_{10} (number of conidia) of each dilution and the linear relationship between ΔC_T and Log_{10} (number of conidia) investigated by least-squares regression analysis. (Equation 2.3, Figure 2.10B).

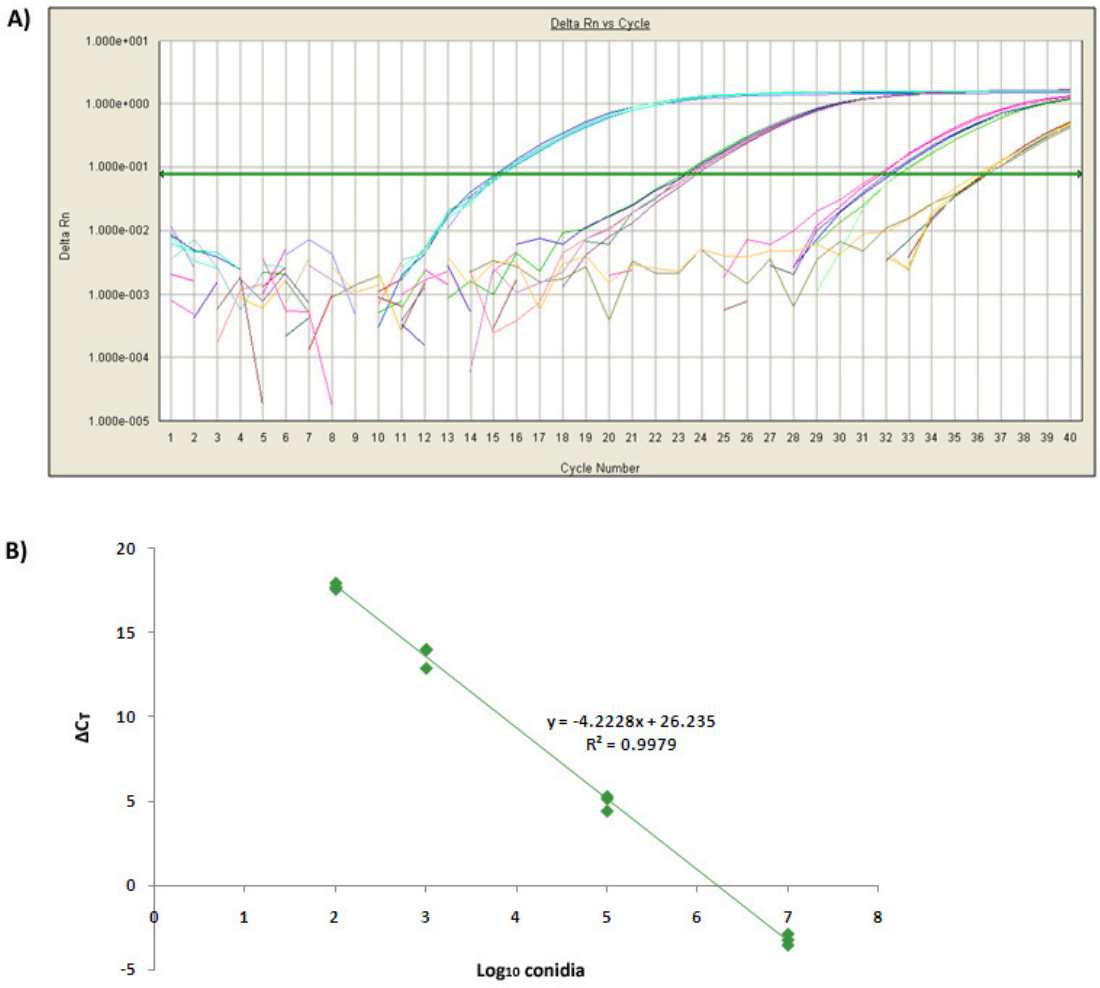
Equation 2.3.

Mean $C_{T,\ target}$ – Mean $C_{T,\ reference}$ = $\Delta C_{T,\ ref}$

Where Mean $C_{T,\ target}$ = mean target C_T at each dilution and Mean $C_{T,\ reference}$ = mean *G. candidum* C_T for the undiluted DNA.

Figure 2.10. Generation of MSQPCR calibration curves

MSQPCR curves from serial dilutions of *A. fumigatus* DNA using the Applied Biosystems Fast 7500 SDS software in logarithmic view (A); and the ΔC_T plot for the *A. fumigatus* assay following subtraction of C_T values for co-extracted *G. candidum*, plotted against log-transformed serial dilutions of conidia (B).



Calculation of theoretical detection limit

The theoretical mean conidia detection limit of each assay could be calculated using Equation 2.4:

Equation 2.4

Log_{10} detection limit (DL) = 40 – Mean $C_{T,\ G.\ candidum}$ – a)/b

Where Mean $C_{T,\ G.\ candidum}$ was the average C_T value for *G. candidum* from 15 tubes of undiluted DNA (to give an approximate average for all assays), a and b were the y-intercept and slope respectively calculated in the regression analysis.

The mean C_T reference value used in all analyses was 19.13214, based on duplicate *G. candidum* MSQPCR assay C_T values from triplicate DNA extractions for five target assays. The returned value from Equation 2.4 could then be adjusted to mean conidia detection limit (MCDL) = (10^{DL}). For example, for the *A. fumigatus* target assay, and using the regression line from the ΔC_T plot shown in Figure 2.10B, the MCDL was calculated as follows:

DL = (40-19.13214-26.235)/-4.2228

DL = 1.018871

MCDL = 10^{DL}

MCDL = 10 conidia equivalents

Calculating the amplification efficiency of each target assay

The amplification efficiency (AE) of each target species-specific assay was then analysed using Equation 2.5.

Equation 2.5.

$AE = 10^{-1/b}$ (b = slope parameter from the least-squares regression)

For example, from the *A. fumigatus* target assay, the AE was calculated as follows:

$AE = 10^{-1/b}$

$AE = 10^{-1/-4.2228}$

$AE = 1.73$

2.4.5.4 Calculation of target organisms in air and dust samples

For each target assay, $\Delta C_{T,\ calibrator}$ was generated using Equation 2.6.

Equation 2.6.

$\Delta C_{T,\ calibrator} = \text{Mean } C_{T,\ target\ calibrator} - \text{Mean } C_{T,\ reference}$

Where Mean $C_{T,\ target\ calibrator}$ is the average C_T values for undiluted triplicate DNA from target fungi used in the calibration; and Mean $C_{T,\ reference}$ is the mean *G. candidum* C_T for the undiluted DNA.

Target fungi were quantified in terms of fungal conidia equivalents from air and dust samples using the $\Delta\Delta C_T$ method [203, 228], Equation 2.7.

Equation 2.7.

$\Delta\Delta C_T = \Delta C_{T,\ target} - \Delta C_{T,\ calibrator}$

Where $\Delta C_{T,\ target}$ is the average C_T of spiked *G. candidum* in an unknown sample subtracted from the average C_T of the target assay in the unknown sample and $\Delta C_{T,\ calibrator}$ is the value calculated in Equation 2.6.

Target fungal conidia equivalents in unknown samples were then enumerated using Equation 2.8.

Equation 2.8.

$n\ (AE^{-\Delta\Delta C_T})$

Where n is number of target conidia spiked in the calibrator sample.

In the instance, towards the limit of detection, where one or two of the triplicate C_T values were undetected, the final conidia concentrations were adjusted according to the number of positive detections in the triplicate repeats.

2.5 Statistical analysis

Data was analysed using Microsoft Excel, GraphPad Prism (Versions 4 and 5), SPSS (Version 11.0) and STATA data analysis and statistical software package (Version 10).

Fungal spore concentration data was log-transformed to normalise where appropriate ($Y = Log_{10} (Y+1)$ where Y = fungal spore concentrations per cubic metre of air).

Parametric data was presented as mean (arithmetic) with standard error of the mean (SEM) and analysed using student's t-test, analysis of variance (ANOVA: using Bonferroni post-test for multiple comparisons), repeated measures ANOVA, and linear regression.

Non-parametric data was presented as medians with interquartile range (IQR) and analysed using Kruskal-Wallis (using Dunn's post test for multiple comparisons), Mann-Whitney, Chi-squared, Fisher's exact, Friedman test, Cronbach's alpha internal consistency test, kappa statistics, zero-inflated negative binomial regression and Spearman's rank correlation coefficient.

3 Indoor airborne fungal spore concentrations in non-complaint UK residential properties

A substantial portion of this chapter was published in the Journal of Investigational Allergology and Clinical Immunology, 2010.

Fairs A, Wardlaw AJ, Thompson JR, Pashley CH. 2010. Guidelines on ambient intramural airborne fungal spores. *J Investig Allergol Clin Immunol* 20(6): 490-498

3.1 Introduction

In recent decades, people living in industrialised countries have been spending increasing amounts of time indoors, with up to 95% of an individual's time now being spent inside. This has coincided with increasing prevalence and morbidity of asthma and allergy in industrialised countries [229, 230].

Respiratory exposure to airborne fungal spores is inevitable almost everywhere, due to their ubiquitous nature both indoors and outdoors, which can be problematic for fungal sensitised individuals; triggering symptoms of asthma and allergy and having a detrimental effect on quality of life. The home and work environments are an important source of fungal exposure; however, indoor concentrations of fungi typically reflect outdoor levels unless an indoor source is present [133]. Fungi found indoors include species with known adverse effects on health, including *Cladosporium, Alternaria, Aspergillus* and *Penicillium* species [231].

Despite negative associations of fungal exposure with health, and evidence of home dampness correlated with respiratory symptoms, it is still not possible to provide guidelines on acceptable levels within homes, primarily due to inconsistencies in study design and quantification methods [175, 176]. Threshold levels of indoor fungal exposure which would present an increased risk of clinical implications in healthy individuals or people with underlying disease are unclear, and there is a dearth of knowledge with regard to 'normal' levels of airborne fungal spores in non-complaint homes, resulting in difficulties in data interpretation.

In addition to season and outdoor concentrations of fungi, housing characteristics may influence the types and concentrations of fungi found indoors. Previously reported predictors of indoor fungal spore concentrations include relative humidity, temperature,

presence of cats, old carpets, highly insulated windows, central heating and wooden board flooring [53].

3.2 Materials and methods

3.2.1 Environmental sampling

In order to provide a comprehensive analysis of indoor airborne fungal spore concentrations in non-complaint properties, healthy volunteers were recruited from the University of Leicester, University Hospitals of Leicester and associates of colleagues. Twenty four hour air samples were taken from the living rooms of 124 properties within Leicestershire from August 2006 to January 2008, using the Burkard continuous recording air sampler (described in section 2.1.1.2) to collect samples onto Vaseline-coated slides. Properties sampled were confirmed by participants to be 'non-complaint', defined as having no suspected moisture-related problem within the property. Individual property characteristics were collected by detailed questionnaire for investigation of potential influences on airborne fungal spore concentrations (appendix section 8.5). Outdoor samples of fungi were collected using the Burkard seven day volumetric spore trap located on the roof of a building at the University of Leicester (described in detail in section 2.1.1.1) onto Vaseline-coated slides and analysed by microscopy at corresponding time intervals to indoor samples. Fungal taxa were recorded into the computerised database described in 2.1.1.4. Temperature (°C) and relative humidity (%) data were collected at 10 minute intervals throughout the 24 hour sampling to produce average values for each property (as described in section 2.1.4). Occupants were asked to keep windows closed and refrain from cleaning activities immediately prior to, and

during, the sample period. Open windows and sampler malfunction were among the criteria leading to the exclusion of 24 samples from analysis.

Indoor and outdoor air sample slides were stained with polyvinyl lactophenol cotton blue and a single longitudinal traverse of the air sample trace analysed at x630 magnification, following a standard method [132, 194], described in section 2.4.1.

3.2.2 Between property and within property variations

In order to determine the utility of a single indoor sample for monitoring airborne fungal spore levels, seasonal and daily variation was monitored in a subset of properties. Seasonal variations in indoor air samples from living rooms were measured in 13 properties between February 2007 and February 2009. One 24 hour air sample was taken from the living room of each property during each season, determined by the dates of the vernal and autumnal equinoxes and summer and winter solstices (Table 2.1). Visits were unfortunately only possible on three out of four seasons in two properties.

To measure the variability of air sample data collected on different days within the same property, the living room and a bedroom from three properties were sampled on seven consecutive days during the period of July-October 2008. Properties sampled for validation experiments belonged to volunteer employees and students from the University of Leicester.

3.2.2.1 Statistical analyses

GraphPad Prism software (Versions 4 and 5) was used in initial analyses to generate mean, median, minimum, maximum, upper and lower quartile data for fungal spore and indoor/outdoor (I/O) ratios. In order to account for excess zeros, 1 was added to each data point to generate the I/O ratio (Y+1, where Y = fungal spore concentration/m^3 air).

The correlation between indoor and outdoor concentrations was tested using Spearman's rank correlation coefficient.

STATA data analysis and statistical software package (Version 10) was used to analyse the effect of season and home characteristics on fungal spore concentrations. Indoor and outdoor total fungal spore concentrations and abundant indoor fungi were log-transformed (Y+1) in order to normalise the data for parametric analyses. Initial assessments of seasonal variation were analysed using the chi-square test. Univariate linear regression was used to analyse the effect of home characteristics and seasonal variation on indoor airborne fungal spore concentrations. Since much of the variability of indoor concentrations is explained by outdoors levels, outdoor concentrations for corresponding time periods were incorporated into regression models, enabling season and home characteristics to be investigated for influence on indoor fungal spore concentrations independent to the variability explained by levels outdoors.

Zero-inflated negative binomial regression analyses were performed on non-transformed over-dispersed, positively skewed concentrations of fungal genera with excess zeros within the dataset, with and without adjustment for outdoor fungal spore concentrations. Seasonal variation was investigated by chi-square analyses as per log-transformed data.

Seasonal and within home daily variation was analysed using Dunn-corrected Friedman tests to account for repeated measures and non-parametric data. The internal consistency of repeat samples over seven days was analysed using Cronbach's alpha.

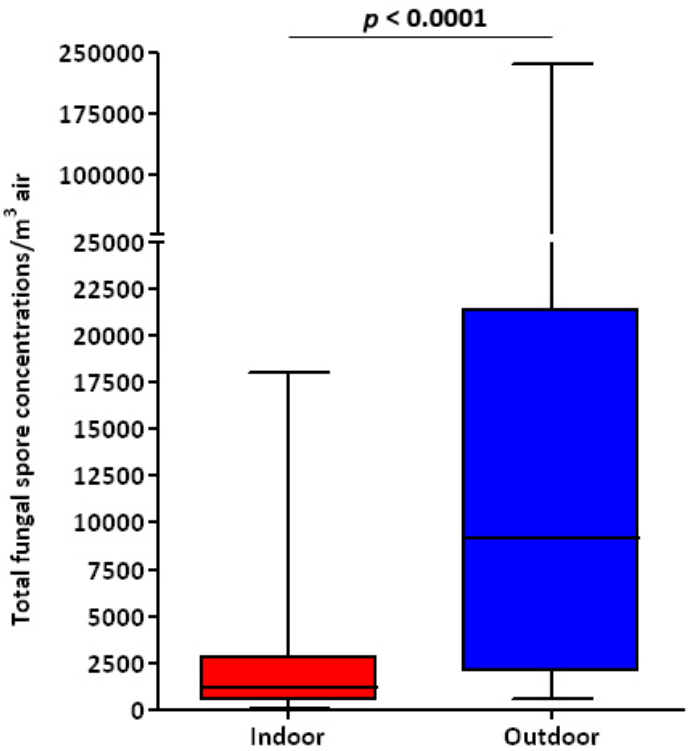
3.3 Results

3.3.1 Distributions of indoor and outdoor airborne fungal spores

The distributions of indoor and outdoor fungal spore concentrations from 24 hour air samples were highly variable and positively skewed (Figure 3.1, Table 3.1). Total airborne fungal spore concentrations were typically much lower indoor, ranging from 25-18,067 (median = 1135), in comparison to outdoor concentrations which ranged from 539-237,144 (median = 9201) spores/ m^3 air (Figure 3.1). Indoor and outdoor total fungal spore concentrations were positively correlated (r_s = 0.5568, $p < 0.0001$).

Figure 3.1. Indoor and outdoor airborne fungal spore distributions

Box and whisker plots showing the distributions of indoor and outdoor fungal spore concentrations from 100 samples. The top and bottom of the box and intermediate line represent the upper and lower quartiles and median, respectively. The whiskers represent the minimum and maximum values of the dataset.



3.3.2 Predominant fungi in indoor air

The indoor and outdoor fungal spore distributions of individual fungal spore types were then investigated to determine predominant fungi found in indoor air. Median indoor concentrations varied considerably between different fungal taxa, ranging from 0-143 spores/m^3 air, and were easily divisible into 'abundant' and 'low abundance' categories based on their presence indoor. Fungi within the 'abundant' group were found in over 50% of samples (Figure 3.2) and had a median indoor concentration greater than zero (Table 3.1A), comprising *Sporobolomyces*, *Tilletiopsis*, hyaline basidiospores, *Asp/Pen*-type conidia, ascospores, *Didymella*, *Cladosporium*, coloured basidiospores and *Leptosphaeria* (Figure 3.3A). Hyaline basidiospores, coloured basidiospores and ascospores are highly generalised categories, where identification by spore morphology is only possible to the level of phylum, enabling only very limited conclusions to be drawn from the data; therefore, basidiospore and ascospore data were excluded from further analyses. No further analyses were conducted on *Leptosphaeria* data since whilst these spores were consistently present indoor, airborne concentrations were very low and never exceeded 167 spores/m^3 air. Fungi from the low abundance group were present in fewer than 50% of samples collected and had a median value of zero. *Alternaria*, an important outdoor aeroallergen with known indoor sources, was only present in 23% of properties and had a median concentration of zero within the 100 properties sampled (Figure 3.2 and Table 3.1B).

Of the abundant indoor fungi, *Cladosporium* spores were most frequently observed (97% of properties), followed by hyaline basidiospores (95%) and *Asp/Pen*-type conidia (95%, Figure 3.2). Indoor concentrations of fungi were lower indoor than outdoor for all taxa, with the exception of *Asp/Pen*-type conidia, which ranged from 0-4117 indoor and 0-2201 outdoor, suggesting indoor sources. *Asp/Pen*-type conidia were thus described

as ‘indoor predominant’ and removed from analyses of total fungal spore concentrations with housing characteristics. All other fungi were described as ‘outdoor predominant’ where the primary source was most likely to be infiltration from outdoor.

Table 3.1. Indoor and outdoor fungal spore distributions for 24 hour sampling periods

Descriptive statistics of indoor and outdoor distributions of fungal spores, showing the minimum (Min) and maximum (Max) values, mean and SEM, median, lower (Q1) and upper (Q3) quartiles (n=100) for abundant indoor fungal taxa, non-identified (other) and total fungal spores (A) and low abundance indoor fungi (B); spores/m^3 air.

A)	Indoor							Outdoor						
Fungal taxa	Min	Max	Mean	SEM	Median	Q1	Q3	Min	Max	Mean	SEM	Median	Q1	Q3
Cladosporium	0	2282	336	47	143	36	443	19	36039	2727	476	1150	186	3319
Hyaline basidiospores	0	5189	339	67	130	29	256	25	27429	2327	336	1479	324	3204
Asp/Pen-type	0	4117	280	57	99	50	298	0	2201	208	30	112	62	217
Ascospores	0	3683	273	54	93	19	245	31	56580	2303	585	933	305	2857
Sporobolomyces-type	0	7552	449	113	71	12	295	6	56826	2886	710	874	164	2562
Tilletiopsis	0	6200	314	83	37	6	144	6	28346	2519	543	474	130	1911
Coloured basidiospores	0	651	81	12	25	6	101	0	8035	741	124	267	68	921
Didymella	0	2474	77	28	6	0	25	0	10732	691	184	25	6	214
Leptosphaeria	0	167	22	4	6	0	25	0	4182	196	43	81	31	268
Other fungal spores	6	1922	327	39	174	98	366	87	34686	2248	379	1240	384	2922
Total fungal spores	25	18067	2540	367	1135	51	272	539	237144	17255	2840	9201	2023	21130

B)	Indoor								Outdoor							
Fungal taxa	%	Min	Max	Mean	SEM	Median	Q1	Q3	%	Min	Max	Mean	SEM	Median	Q1	Q3
Ustilago	27	0	372	13	5	0	0	6	37	0	3869	134	60	0	0	25
Ganoderma	26	0	415	10	5	0	0	6	61	0	246	39	5	16	0	62
Botrytis	33	0	99	6	1	0	0	6	54	0	322	41	7	6	0	43
Rusts/smuts	34	0	37	4	1	0	0	6	85	0	546	45	7	25	6	57
Alternaria	23	0	50	3	1	0	0	0	63	0	632	55	10	9	0	56
Entomophthora	16	0	25	2	1	0	0	0	70	0	1353	41	14	12	0	50
Pithomyces	12	0	19	1	0	0	0	0	47	0	492	15	5	0	0	12
Epicoccum	7	0	31	1	0	0	0	0	34	0	93	8	2	0	0	8
Torula	5	0	12	0	0	0	0	0	25	0	112	4	1	0	0	2
Erysiphe	6	0	6	0	0	0	0	0	61	0	246	12	3	6	0	12
Lewia/Pleospora	5	0	12	0	0	0	0	0	7	0	12	0	0	0	0	0
Polythrincium	5	0	6	0	0	0	0	0	22	0	87	4	1	0	0	0
Stemphylium	2	0	12	0	0	0	0	0	15	0	329	10	5	0	0	0
Drechslera	1	0	6	0	0	0	0	0	9	0	12	1	0	0	0	0

Figure 3.2. Proportion of indoor and outdoor samples positive for individual fungal taxa

Bar chart showing the proportion of indoor (red columns) and outdoor (blue columns) air samples positive for each of 23 fungal taxa recorded, in order of frequency detected indoor (n=100).

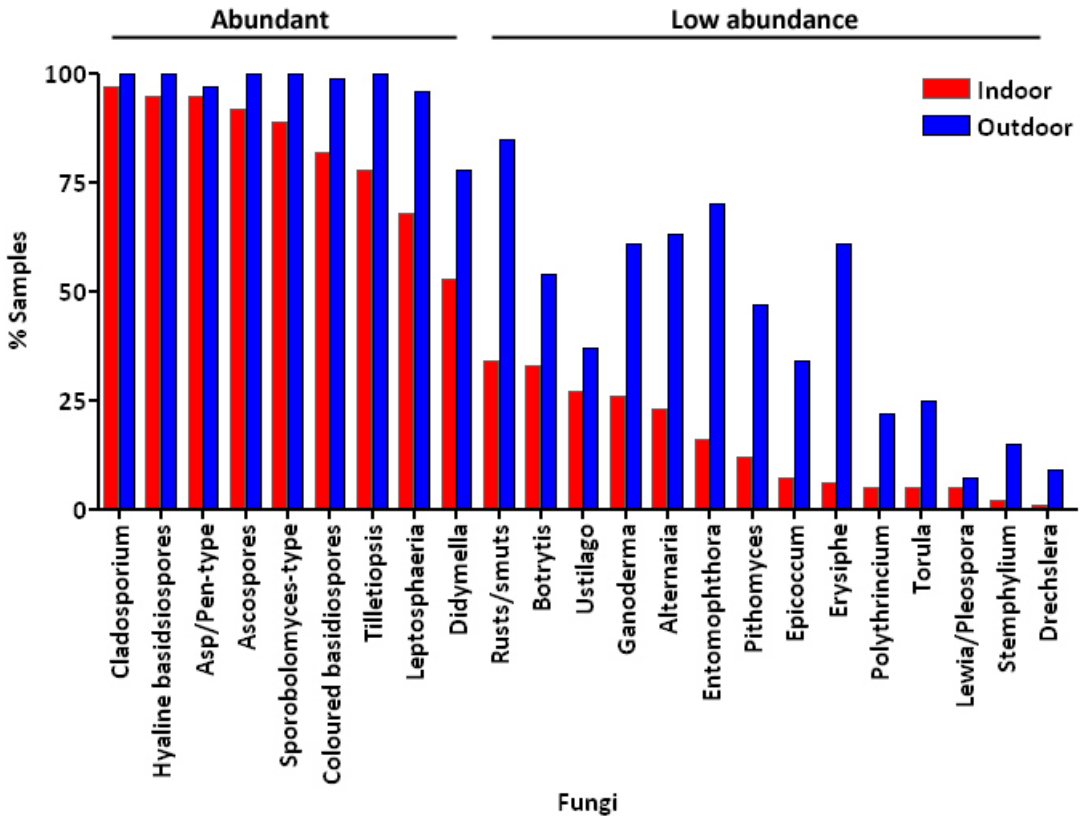
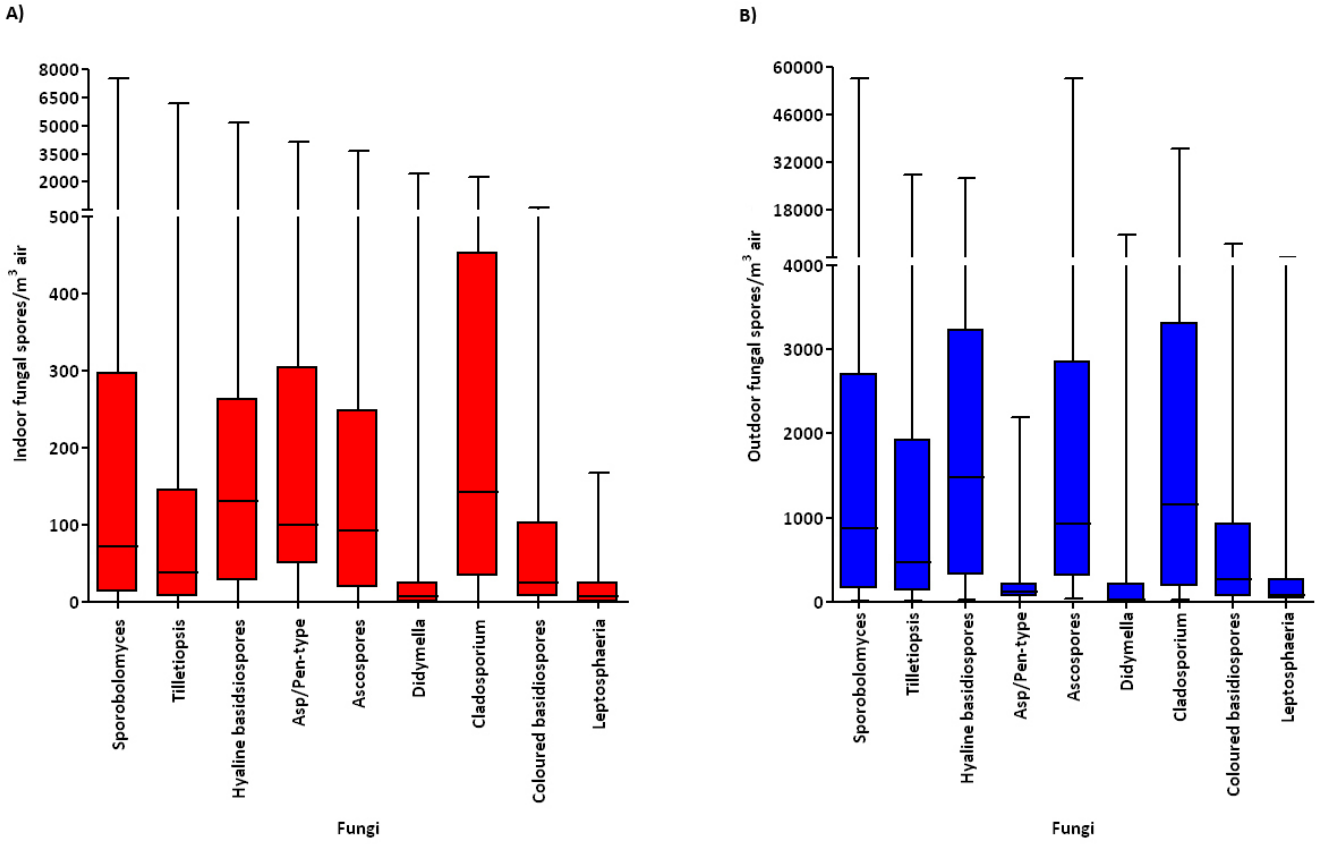


Figure 3.3. Indoor and outdoor airborne spore distributions of abundant indoor fungal genera

Box and whisker plots of indoor (A) and outdoor (B) fungal spore concentrations (n = 100). The top and bottom of the box and intermediate line represent the upper and lower quartiles and median, respectively. The whiskers represent the minimum and maximum values of the dataset.



3.3.3 Indoor-outdoor ratios of abundant indoor fungal taxa

Properties were assigned to seasons according to the dates of the vernal and autumnal equinoxes and summer and winter solstices (Table 2.1), with even spread of samples collected across the four seasons (Table 3.2). Outdoor fungal spore concentrations are highly dependent on season, and very important in analyses of concurrent indoor samples due to infiltration into properties. Therefore, indoor fungal spore concentrations were investigated as an indoor to outdoor (I/O) ratio, and a value of 1 was added to all data for total fungal spores and abundant fungal taxa, to account for zeros within the dataset. All I/O ratios were highly variable and positively skewed (Table 3.3). Median I/O ratios for *Cladosporium* (0.173), *Sporobolomyces* (0.133), *Tilletiopsis* (0.099), and *Didymella* (0.139) showed close consistency with the median I/O ratio for total fungal spores (0.164) and were considered 'outdoor predominant' fungi within the dataset, all with mean I/O ratios less than 1 and median I/O ratios less than 0.2. In contrast, *Asp/Pen*-type conidia showed a higher abundance indoor, with a median I/O ratio of 0.908 (mean 5.907), again suggesting the presence of indoor sources (Table 3.3).

Table 3.2. **Home characteristic variables investigated for influence on fungal levels**

Independent variables (housing characteristics and seasons sampled) incorporated into regression models for influence on indoor airborne fungal spore concentrations following correction of outdoor concentrations (spores/m^3 air). Where total responses were <100, total n is indicated. Relative humidity (%) and temperature (ºC) data were also collected from all properties in ten minute intervals.

Variable	n	Variable	n	Variable	n
Season, n		*Property age, n*		Visible mould, yes	6
Spring	28	0-30	28	Condensation, yes	10
Summer	22	31-60	21	Reported damp, yes	5
Autumn	30	61-90	17	Double glazing, yes	76
Winter	20	>90	31	Wall cavity insulation, yes/n	38/88
Housing type, n		*Floor covering, n*		Cleaning during sample, yes	12
Detached/link-detached	29	Carpet	67	Central heating, yes	88
Semi-detached	37	Hard floor (any)	33	Heating on, yes	46
Terrace/quarter	23	Dog present, yes	11		
Flat	11	Cat present, yes	27		

Table 3.3. Distributions of indoor/outdoor ratios of common indoor fungi

Descriptive statistics of indoor/outdoor (I/O) ratios of total fungal spores and abundant indoor fungal taxa, showing the minimum (Min) and maximum (Max) values, mean and SEM, median, lower (Q1) and upper (Q3) quartiles.

Fungal taxon	Min	Max	Mean	SEM	Median	Q1	Q3
Cladosporium	0.002	8.908	0.339	0.093	0.173	0.078	0.280
Sporobolomyces	0.000	9.716	0.451	0.115	0.133	0.035	0.332
Asp/Pen-type	0.001	100.2	5.907	1.550	0.908	0.332	3.268
Tilletiopsis	0.000	5.537	0.334	0.072	0.099	0.017	0.305
Didymella	0.004	13.40	0.801	0.201	0.139	0.074	1.000
Total fungal spores	0.001	4.082	0.273	0.052	0.164	0.069	0.257

3.3.4 Seasonal variation and relationship between indoor and outdoor air

Chi-square analyses were performed on regression analyses of total fungal spores and abundant indoor fungal taxa to investigate the effect of season on regression models of indoor total fungal spore and abundant fungal spore concentrations and consistency of the I/O ratio (Table 3.4). The consistent presence of *Cladosporium, Sporobolomyces, Asp/Pen*-type and total fungal spore concentrations indoor enabled log transformed (Y+1) linear regression analyses of fungal spore concentrations. However, whilst *Tilletiopsis* and *Didymella* spores are found in outdoor air throughout the year, their presence is more heavily dependent on season. The data was positively skewed and there were excess zeros in the dataset; therefore, *Tilletiopsis* and *Didymella* concentrations were analysed using non-transformed zero-inflated negative binomial regression models.

Chi-square analyses showed significant seasonal variations in indoor fungal spore concentrations of total fungal spores and outdoor predominant fungi; however, *Asp/Pen*-

type conidia did not vary significantly according to season (p = 0.563, Table 3.4). There was no significant seasonal variation of the I/O ratio for total fungal and abundant indoor fungi, with the exception of *Didymella* (p = 0.000; Table 3.4).

Investigation of indoor fungal spore concentrations by individual season using linear regression and zero-inflated negative binomial regression analyses following adjustment for outdoor levels (Table 3.5 and 3.6 respectively) showed significantly higher indoor levels of *Cladosporium* and *Didymella* in the summer (p = 0.020 and p = 0.001 respectively), with lower levels of *Cladosporium* in the autumn (p = 0.017) and *Didymella* in the winter (p = 0.019).

Table 3.4. Seasonal variation in indoor airborne fungal levels and indoor/outdoor ratios

Likelihood ratio (chi square) analysis comparing regression models of indoor fungal spore levels and indoor-outdoor ratio (I/O) of fungal spore concentrations over different seasons. $p < 0.05$ indicates a significant difference between seasons.

	Indoor	I/O ratio
Fungi	p^a	p^a
Cladosporium[b]	< 0.001	0.095
Sporobolomyces[b]	< 0.001	0.148
Asp/Pen-type[b]	0.563	0.351
Tilletiopsis[c]	0.015	0.767
Didymella[c]	< 0.001	< 0.001
Total fungal spores[b]	< 0.001	0.079

[a] Chi-square analysis

[b] Linear regression analysis

[c] Zero-inflated negative binomial regression analysis

3.3.5 Relationship between housing characteristics and indoor airborne fungal spore concentrations

A complete list of home characteristics which were investigated for influence on indoor airborne fungal spore concentrations are presented in Table 3.2.

3.3.5.1 Temperature and humidity

Positive associations of mean temperature and relative humidity (%) with total fungi, *Cladosporium, Tilletiopsis* and *Sporobolomyces* (humidity only) were lost following adjustment for outdoor fungal spore concentrations (Table 3.5 and 3.6). Relative humidity had no significant effect on indoor fungal taxa after the adjustment for outdoor spore concentrations, with the exception of a weak positive association with *Didymella* (p= 0.020, Table 3.6). Decreased humidity was predictive of the absence of *Tilletiopsis* and *Didymella* (p = 0.047 and p = 0.000 respectively, Table 3.6) and mean temperature was negatively associated with the absence of *Didymella* (p = 0.015, Table 3.6).

3.3.5.2 Building age and type of housing

Due to a low number of representatives in each age category, it was necessary to analyse building age by year of construction into 30 year age groups. Following adjustment for outdoor fungal spore concentrations, youngest properties up to 30 years old were shown to have significantly reduced levels of *Didymella* (p = 0.034, Table 3.6). Properties 31-60 years old had significantly higher levels of *Tilletiopsis* (p = 0.024, Table 3.6) and lower levels of *Asp/Pen*-type conidia (p = 0.028, Table 3.5, Figure 3.4), although properties 0-30 years old appeared to have lowest concentrations (Figure 3.4). Oldest properties, over 90 years old had significantly higher levels of *Asp/Pen*-type conidia (p = 0.006, Table 3.5, Figure 3.4).

Significantly reduced total fungal spores (p = 0.008), *Cladosporium* (p = 0.002), *Sporobolomyces* (p = 0.043) and *Didymella* (p = 0.019) were shown in flats (Table 3.5 and 3.6). *Asp/Pen*-type spore concentrations were significantly higher in terraced properties (p = 0.003, Table 3.5, Figure 3.4), with *Tilletiopsis* concentrations highest in semi-detached properties (p = 0.015, Table 3.6).

3.3.5.3 Central heating, presence of pets and other potential factors

Central heating was positively associated with *Cladosporium* concentrations (p = 0.029, Table 3.5); whilst cavity wall insulation was negatively correlated with concentrations of *Asp/Pen*-type conidia (p = 0.002, Table 3.5, Figure 3.4).

The presence of pets within properties was also investigated and the presence of one or more dogs was found to be negatively associated with indoor levels of *Asp/Pen*-type conidia (p = 0.005, Table 3.5, Figure 3.4) and *Didymella* spores (p = 0.037, Table 3.6). The presence of a cat was positively associated with *Didymella* concentrations (p = 0.030, Table 3.6). No significant effect of floor covering, presence of damp, visible mould, condensation or double glazing were shown on levels of any fungal taxa.

Table 3.5. Associations of home characteristics and season with indoor fungal levels

Significant associations of housing characteristics with airborne fungal spore counts, analysed by linear regression analysis of individual characteristics and bivariate analysis after incorporation of outdoor levels (n<100 when insufficient questionnaire data was received). Significant associations following adjustment for outdoor levels are shown in red. The regression coefficient (coef) represents the difference in log concentrations of indoor fungal spores for one unit change of each independent variable. When the model was adjusted for outdoor levels, these were held constant in the model. When the association between the independent variable and indoor fungal spore concentrations was negative, the coefficient shown is negative. The 95% confidence interval (CI) for the regression model using each predictor variable is also presented.

	Indoor fungal spore concentrations					Indoor levels post-adjustment for outdoors			
Variable	n	Coefficient	*p*	95% CI		Coefficient	*p*	95% CI	
Total fungal spores									
Mean humidity (%)	100	0.03	0.000	0.02	0.04	0.01	0.318	-0.01	0.02
Mean temp (°C)	100	0.05	0.038	0.00	0.10	-0.01	0.810	-0.05	0.04
Heating on	100	-0.53	0.000	-0.75	-0.32	-0.16	0.174	-0.40	0.07
Flat	100	-0.38	0.047	-0.76	-0.01	-0.41	0.008	-0.71	-0.11
Summer	100	0.59	0.000	0.32	0.85	0.24	0.067	-0.02	0.50
Winter	100	-0.53	0.000	-0.81	-0.24	-0.08	0.564	-0.36	0.20

Variable	Indoor fungal spore concentrations				Indoor levels post-adjustment for outdoors			
	n	Coefficient	*p*	95% CI	n	Coefficient	*p*	95% CI
***Asp/Pen*-type**								
31-60 yrs old	97	-0.38	0.027	-0.71	-0.04	-0.38	0.028	-0.72	-0.04
>90yrs old	97	0.42	0.005	0.13	0.71	0.42	0.006	0.13	0.71
Wall cavity insulation	88	-0.45	0.001	-0.72	-0.18	-0.45	0.002	-0.72	-0.18
Presence of a dog	100	-0.62	0.004	-1.05	-0.20	-0.62	0.005	-1.05	-0.20
Terraced	100	0.48	0.003	0.17	0.79	0.48	0.003	0.16	0.79
Cladosporium								
Mean humidity (%)	100	0.03	0.000	0.02	0.05	0.00	0.625	-0.01	0.02
Mean temp (°C)	100	0.11	0.000	0.06	0.17	0.02	0.408	-0.03	-0.07
Central heating	100	0.47	0.041	0.02	0.91	0.35	0.029	0.04	0.66
Heating on	100	-0.78	0.000	-1.03	-0.53	-0.11	0.446	-0.39	0.17
Flat	100	-0.48	0.044	-0.94	-0.01	-0.50	0.002	-0.81	-0.18
Summer	100	0.82	0.000	0.51	1.14	0.32	0.020	0.05	0.59
Autumn	100	-0.35	0.029	-0.67	-0.04	-0.27	0.017	-0.49	-0.05
Winter	100	-0.82	0.000	-1.15	-0.49	-0.05	0.772	-0.37	0.28
***Sporobolomyces*-type**								
Mean humidity (%)	100	0.04	0.002	0.01	0.06	0.01	0.372*	-0.01	0.04
Heating on	100	-0.66	0.001	-1.05	-0.26	-0.32	0.106*	-0.71	0.07
Flat	100	-0.54	0.109	-1.19	0.12	-0.59	0.043	-1.17	-0.02

Table 3.6. Associations between home characteristics and indoor levels of *Tilletiopsis* and *Didymella*

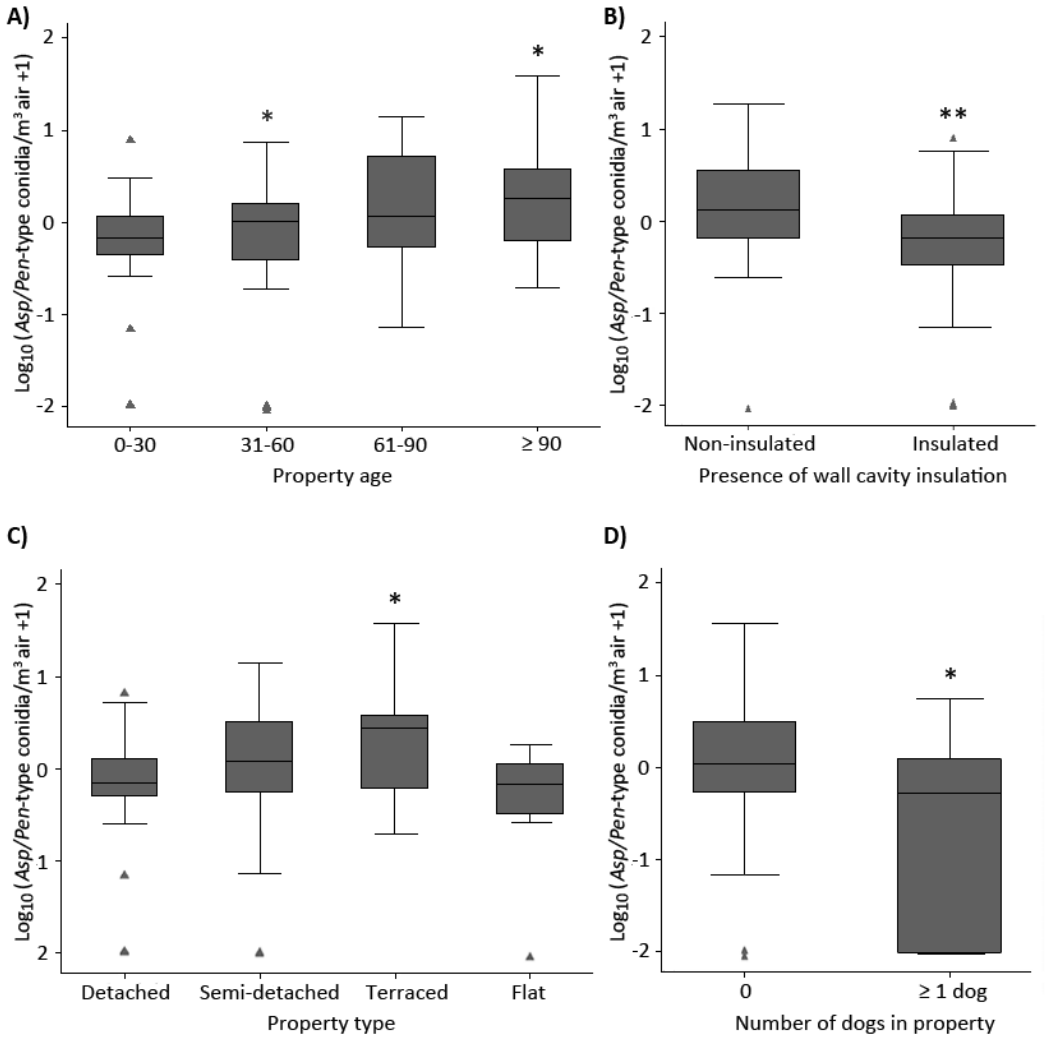
Significant associations of home characteristics and season with indoor concentrations of *Tilletiopsis* and *Didymella* by zero-inflated negative binomial regression analysis. Significant associations of home characteristics with the presence or absence (excess zeros in the indoor dataset) of indoor airborne fungal levels and home characteristics after adjustment for outdoor levels are shown in red. The presence or absence of a given spore type was modelled independently. The regression coefficient (coef) represents the difference in log concentrations of indoor fungal spores for one unit change of each independent variable. When incorporated, outdoor concentrations were held constant in the model. Negative associations have a negative coefficient. The 95% confidence interval (CI) for the regression using each predictor variable is also presented.

	Indoor fungal spore concentrations				Absence indoor				Indoor levels post-adjustment for outdoors				Absence indoors post-adjustment for outdoors			
Characteristic	Coef	*p*	95% CI		Coef	*p*	95% CI		Coef	*p*	95% CI		Coef	*p*	95% CI	
Tilletiopsis																
Mean temperature (°C)	0.15	0.018	0.03	0.28	-0.09	0.592	-0.44	0.250	0.05	0.455	-0.08	0.18	-0.10	0.528	-0.40	0.21
Mean humidity (%)	0.07	0.024	0.01	0.13	-0.08	0.073	-0.17	0.007	0.03	0.272	-0.02	0.08	-0.08	0.047	-0.16	-0.00
Double glazing	0.92	0.044	0.03	1.82	10.25	0.985	-1049	1069	0.34	0.451	-0.54	1.22	0.83	0.596	-2.24	3.91
Visible mould	-2.54	0.013	-4.55	-0.54	1.78	0.317	-1.704	5.266	-1.82	0.060	-3.70	0.07	1.65	0.211	-0.93	4.23
Semi detached	0.77	0.074	-0.07	1.61	0.90	0.565	-2.166	3.966	0.94	0.015	0.18	1.69	0.58	0.513	-1.15	2.30
Autumn	-1.00	0.026	-1.89	-0.12	0.40	0.755	-2.096	2.889	-0.39	0.371	-1.25	0.47	0.52	0.602	-1.42	2.46
Winter	-1.11	0.043	-2.18	-0.04	1.44	0.359	-1.641	4.526	-0.50	0.334	-1.51	0.52	1.24	0.228	-0.78	3.27
31-60 years old	0.78	0.119	-0.20	1.76	-2.11	0.825	-20.818	16.602	0.10	0.024	0.13	1.86	-0.51	0.665	-2.83	1.80
>90 years old	-0.95	0.023	-1.77	-0.13	-16.98	0.997	-10215	10181	-0.76	0.052	-1.52	0.01	-12.19	0.984	-1183	1158

Characteristic	Indoor fungal spore concentrations				Absence indoor				Indoor levels post-adjustment for outdoors				Absence indoors post-adjustment for outdoors			
	Coef	*p*	95% CI		Coef	*p*	95% CI		Coef	*p*	95% CI		Coef	*p*	95% CI	
Didymella																
Mean temperature (°C)	0.35	0.069	-0.03	0.72	-0.45	0.048	-0.90	-0.00	0.02	0.841	-0.18	0.22	-0.32	0.015	-0.58	-0.06
Mean humidity (%)	0.10	0.025	0.01	0.19	-0.13	0.002	-0.22	-0.05	0.05	0.020	0.01	0.09	-0.12	0.000	-0.18	-0.05
Central heating	-2.18	0.029	-4.14	-0.22	-1.52	0.095	-3.31	0.27	0.02	0.977	-1.11	1.14	-0.98	0.150	-2.32	0.35
Presence of a dog	1.67	0.017	0.30	3.04	-11.17	0.989	-1536	1514	-0.82	0.037	-1.60	-0.05	-2.37	0.235	-6.28	1.54
Presence of a cat	-0.10	0.889	-1.46	1.26	1.23	0.583	-3.16	5.61	0.73	0.030	0.07	1.39	0.56	0.238	-0.37	1.49
Visible mould	-3.78	0.003	-6.30	-1.26	-0.67	0.915	-12.832	11.50	-1.39	0.095	-3.02	0.24	0.61	0.507	-1.33	2.69
Condensation	-2.80	0.001	-4.48	-1.11	-3.39	0.932	-81.44	74.65	-0.96	0.063	-1.98	0.05	-0.03	0.974	-1.56	1.51
Flat	-2.91	0.001	-4.59	-1.24	-1.23	0.798	-10.60	8.14	-1.20	0.019	-2.19	-0.20	0.16	0.828	-1.31	1.63
Summer	2.16	0.000	1.29	3.02	-22.04	0.999	-40013	39968	0.91	0.001	0.36	1.46	-3.90	0.041	-7.65	-0.16
Autumn	-2.85	0.000	-3.87	-1.82	-0.31	0.722	-2.04	1.41	-0.67	0.073	-1.40	0.06	0.31	0.516	-0.62	1.24
Winter	-3.56	0.000	-5.17	-1.94	1.24	0.276	-0.94	3.46	-1.25	0.019	-2.30	-0.21	1.42	0.020	0.23	2.61
0-30 years old	1.13	0.110	-0.26	2.51	1.83	0.360	-2.09	5.75	-0.79	0.034	-1.53	-0.06	0.71	0.154	-0.26	1.68

Figure 3.4. Significant associations of airborne *Asp/Pen*-type conidia concentrations with home characteristics

Asp/Pen-type concentrations were highest in properties over 90 years old and significantly lower in properties 31-60 years old (A), lower in properties with wall cavity insulation (B), higher in terraced properties (C) and lower in homes with one or more dogs (D). * $p < 0.05$, ** $p < 0.01$.



3.3.6 'Typical' ranges of indoor airborne fungal spores

Upper quartile limits of seasonal distributions in abundant indoor taxa were used to provide guidelines for 'typical' indoor fungal spore concentrations within non-complaint properties (Table 3.7).

Table 3.7. Guideline upper limits of indoor airborne fungal spores

Guideline upper limits based on upper quartile values from 100 properties for indoor airborne fungal spore concentrations (spores/m^3 air) within normal ranges according to season.

Season	Total fungal spores	*Sporobolomyces*	*Tilletiopsis*	*Asp/Pen*-type	*Didymella*	*Cladosporium*
Spring	2275	110	167	332	6	476
Summer	4520	856	219	116	287	854
Autumn	2443	253	127	321	6	208
Winter	1125	79	60	240	2	51

3.3.7 Analysis of between property and within property variations according to season

Analysis of a subset of 13 properties for between and within property variations showed that total airborne fungal spore concentrations were highly variable according to season, and did not follow a single pattern (Figure 3.5A). Analysis of seasonal variation in 11 properties for which data from all four seasons was available showed that overall, there were significantly lower levels of total airborne fungal spores in the winter in comparison to the spring and autumn ($p < 0.05$) and the summer ($p < 0.001$; Figure 3.5B). *Asp/Pen*-type conidia were also highly variable both within and between properties and did not show any consistent seasonal pattern (Figure 3.6A). When concentrations were low (less than approximately 550 *Asp/Pen*-type conidia/m^3 air), there appeared to be little difference in *Asp/Pen*-type levels over each season; however, greater concentrations were highly variable according to season (Figure 3.6A). Overall, *Asp/Pen*-type concentrations were significantly lower in the 13 properties in the winter in comparison to summer ($p < 0.01$) and autumn ($p < 0.05$; Figure 3.6B).

Figure 3.5 Seasonal variations in total indoor airborne fungal spore concentrations

Total airborne fungal spore concentrations were monitored in a subset of properties. No consistent seasonal pattern was exhibited by raw data from 13 properties (A); however, overall, concentrations were significantly lower in the winter (B). Boxes represent IQR, with median indicated by the intermediate line. Whiskers represent minimum and maximum values.

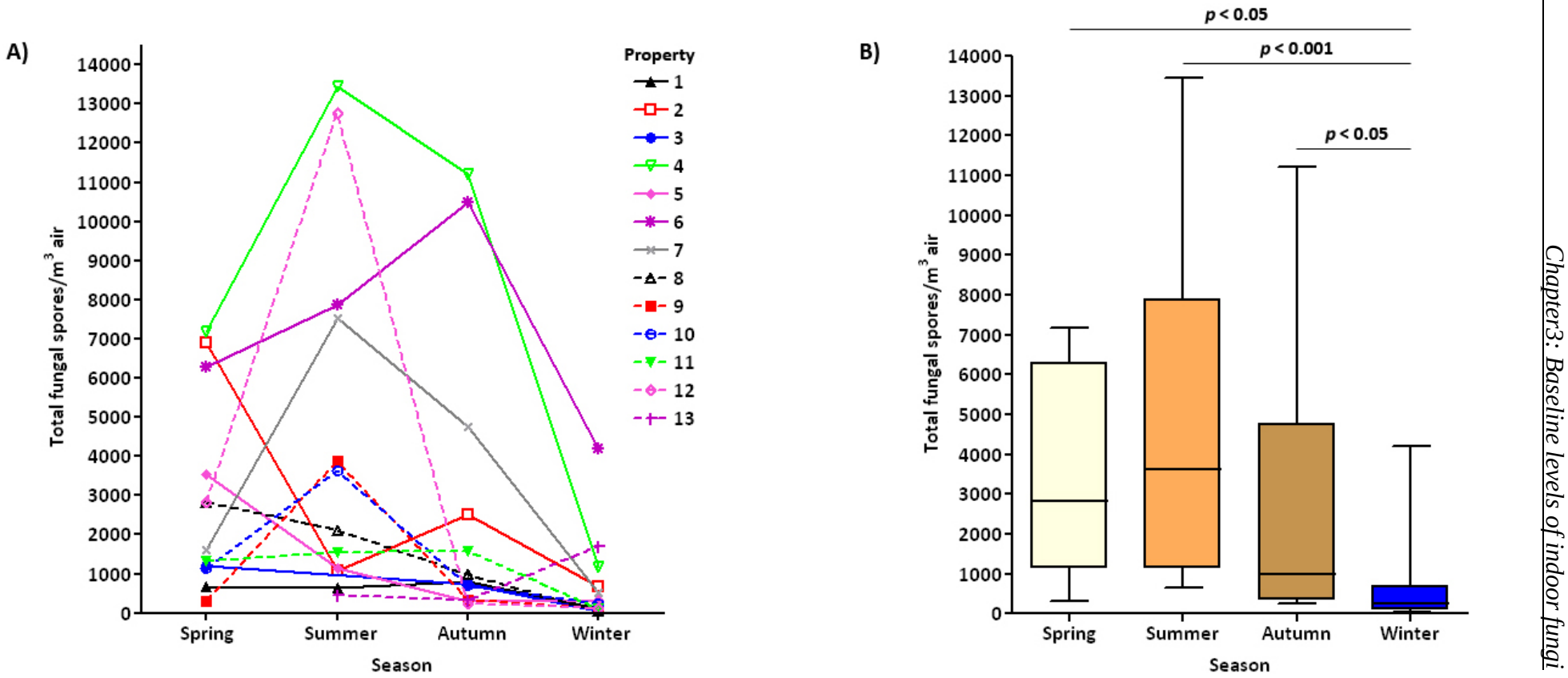
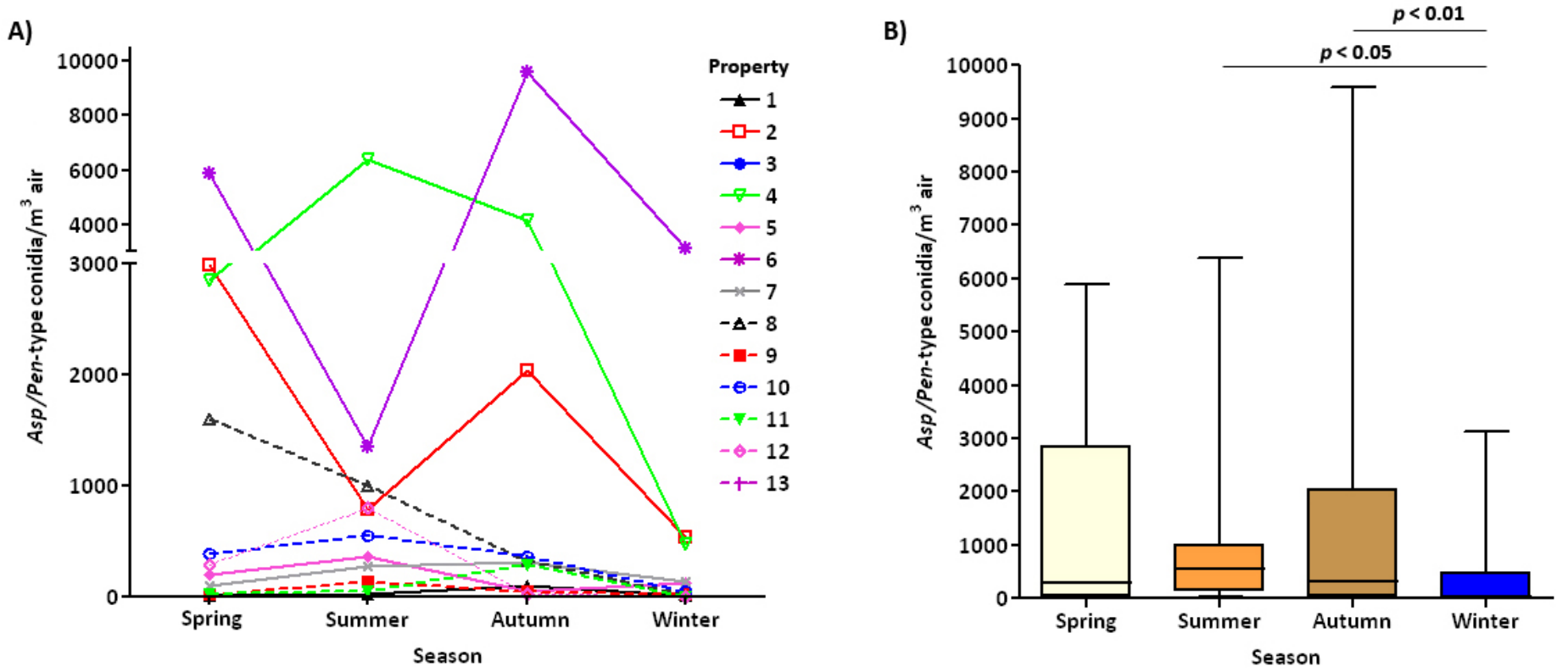


Figure 3.6. Seasonal variation in indoor airborne *Asp/Pen*-type conidia concentrations

Indoor airborne *Asp/Pen*-type conidia concentrations were monitored in a subset of properties. Consistent with total fungal levels, no consistent seasonal pattern was exhibited by raw data from 13 properties (A); however, concentrations were lowest in the winter (B). Boxes represent IQR, with median indicated by the intermediate line. Whiskers represent minimum and maximum values.



The living room and a bedroom from three properties were then sampled over a period of seven days to investigate the daily variation in indoor airborne fungal spore concentrations and differences between rooms in the same property. The three properties sampled were completely different in terms of age, building design and location. The first was a city-centre mid-terrace house over 100 years old; the second was a large, suburban, detached property 72 years old; and the third was a first-floor maisonette approximately 56 years old.

Total airborne fungal spore concentrations were lower in the bedroom than the living room in all three properties; however, this difference was not significant. Property three had significantly lower total airborne fungal spore concentrations in the bedroom in comparison to the other two properties ($p < 0.01$ *vs.* property 1 and $p < 0.05$ *vs.* property 2; Figure 3.7A). Overall, total fungal spore concentrations were significantly higher in the living rooms in comparison to the bedrooms of all three properties ($p < 0.006$). The proportions of different spore types recorded was also different with regard to the room investigated; most noticeable in property two, which had much higher levels of *Sporobolomyces, Tilletiopsis* and *Asp/Pen*-type conidia in the living room in comparison to the bedroom, although levels of basidiospores and *Cladosporium* were similar (Figure 3.8). Overall, however, the proportion of *Cladosporium* and *Asp/Pen*-type conidia were highest in the bedrooms (28.1 vs. 17.4 and 35.0 vs. 13.9% respectively), with *Sporobolomyces* and *Tilletiopsis* higher in the living rooms of the three properties (22.8% vs. 3.5% and 12.9 vs. 3.0% respectively, Figure 3.8). *Asp/Pen*-type conidia were higher in the bedroom of property one in comparison to property two ($p < 0.01$, Figure 3.7B) and in the living room of property one in comparison to property three ($p < 0.01$, Figure 3.7B). The difference in *Asp/Pen*-type conidia concentrations in the bedroom in comparison to the living room was only

significant in property two ($p < 0.001$, Figure 3.7B). Despite variability in daily concentrations of airborne *Asp/Pen*-type conidia, particularly evident in property one where concentrations were highest, analysis of both rooms sampled in all three properties showed no significant variation in levels overall (Figure 3.7B).

The internal consistency of repeated measures from the same room on different days was investigated using the Cronbach's alpha scale, where: $\alpha > 0.9$ internal consistency is considered excellent; $0.9 > \alpha \geq 0.8$ is good; $0.8 > \alpha \geq 0.7$ is acceptable; $0.7 > \alpha \geq 0.6$ is questionable; $0.6 > \alpha \geq 0.5$ is poor; and $\alpha < 0.5$ is unacceptable [232]. Despite the variability in daily concentrations, the internal consistency of repeated measures was found to be acceptable for total fungal spore measurements and *Asp/Pen*-type conidia in the bedrooms ($\alpha = 0.765$, $p = 0.040$ and $\alpha = 0.767$, $p = 0.039$ respectively); good for *Asp/Pen*-type conidia in the living rooms ($\alpha = 0.885$, $p = 0.005$) and excellent for total fungal spore concentrations in the living rooms ($\alpha = 0.912$, $p = 0.002$) [232].

Figure 3.7. Between and within home variation in total fungal spore concentrations and *Asp/Pen*-type conidia

Airborne concentrations of total fungal spores (A) and *Asp/Pen*-type conidia (B) were measured in living rooms (L1-3) and bedrooms (B1-3) of three properties, over seven consecutive days. Significant between-home variation was observed in total fungal spore and *Asp/Pen*-type conidia concentrations. Box plots represent median and IQR values for 24 hour samples taken over seven days, with whiskers representing minimum and maximum values for a 24 hour sampling period. Overall, total fungal spore concentrations were higher in living rooms in comparison to bedrooms. Significant variations in airborne fungal spore concentrations between rooms in the same property were only found in *Asp/Pen*-type concentrations for property two.

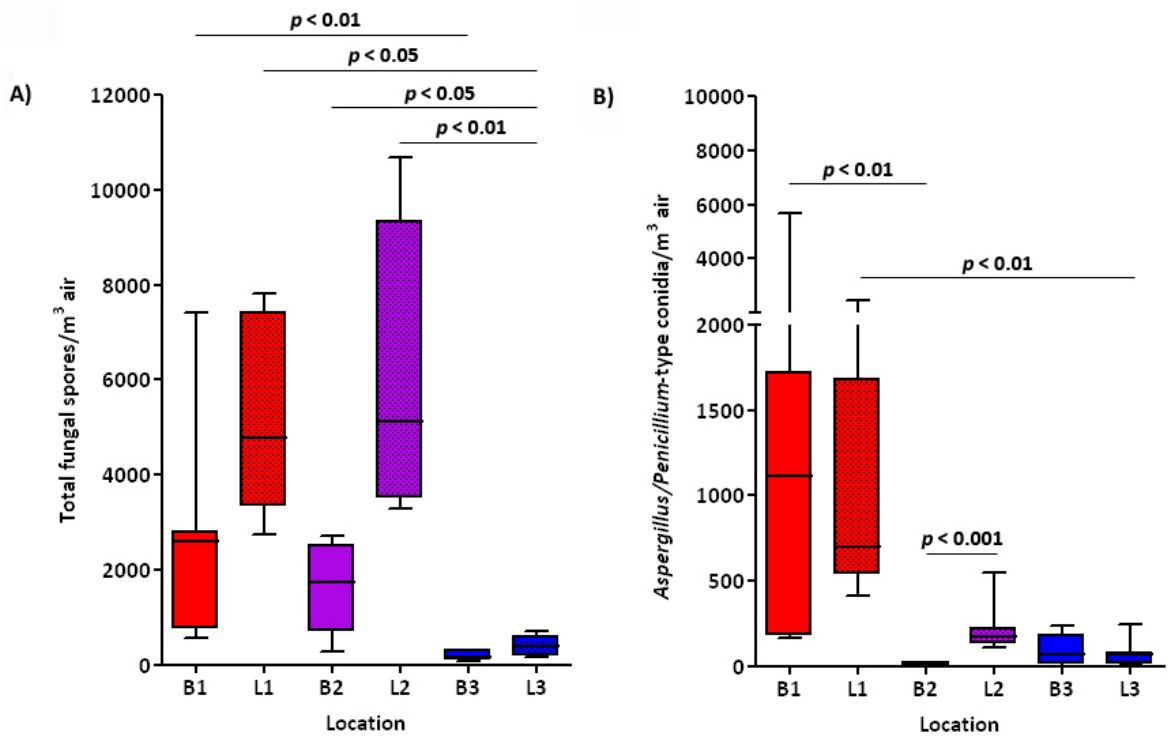


Figure 3.8. Relative proportions of individual fungal taxa in living rooms and bedrooms

Proportions of indoor airborne fungal spores in bedrooms and living rooms from seven day 24 hour air samples from three properties. Fungal taxa listed are ordered clockwise on each respective chart. Other fungi monitored routinely; *Epicoccum, Drechslera, Erysiphe, Stemphylium, Polythrincium, Lewia/Pleospora* and *Pithomyces* were not recorded in any of the three properties.

3.4 Discussion

3.4.1 Indoor fungal spore distributions

Indoor fungal spore concentrations are highly variable and follow non-normal distributions which are positively skewed. Total indoor fungal spore concentrations ranged considerably from 25-18,067 spores/m^3 air, exhibiting higher variability than reported previously [152], with abundant indoor fungi comprising *Cladosporium*, *Asp/Pen*-type conidia, *Sporobolomyces*, *Didymella*, ascospores, hyaline and coloured basidiospores and *Leptosphaeria*. Abundant indoor fungi can be subdivided into outdoor predominant fungi, where indoor levels are predominantly due to infiltration from outside; and indoor predominant fungi, which can colonise indoor substrates and may pose a risk to health or cause building degeneration requiring expensive remediation strategies. Indoor fungal spore concentrations are typically much lower than outdoor unless an indoor source is present [133]. Median I/O ratios of 'outdoor predominant' fungi of abundant indoor genera (*Cladosporium, Sporobolomyces, Didymella* and *Tilletiopsis*) were similar to the median I/O ratio for total fungal spores, suggesting that indoor concentrations represent approximately16% of outdoor levels; although up to 26% of outdoor concentrations may infiltrate indoor, based on the upper quartile limit for total fungal spores.

3.4.2 Seasonal variation

Cladosporium and *Didymella* demonstrated significant seasonal relationships, both being higher in the summer when they reach outdoor peaks (Table 1.1). In contrast to absolute values of total indoor concentrations, the I/O ratio for total fungal spores, *Cladosporium*, *Sporobolomyces* and *Tilletiopsis* demonstrated a lack of seasonal effect.

Seasonal variation in indoor airborne fungal spore concentrations has been well documented; however, whilst absolute values fluctuate according to season, the relative proportion of spores moving indoors from outdoors remains unchanged [175].

A significant effect of season on the I/O ratio for *Didymella* concentrations suggests a disproportionate association between indoor and outdoor airborne concentrations but is likely explained by stark contrasts in daily fluctuations of *Didymella.* Sudden sporadic bursts in release of *Didymella* are triggered by rainfall during the summer months [135], when concentrations can reach peaks exceeding 30,000 spores/m^3 air [135, 136], before rapidly diminishing. Elevated concentrations of *Didymella* during transient periods could enter a property via open windows and remain indoors after outdoor concentrations have subsequently decreased, as previously described [233]. In conjunction with the frequent absence of *Didymella* indoor, this makes interpretation of indoor parameters difficult, since any effect of indoor parameters is likely to be dwarfed by the scale of change in outdoor concentrations during periods of rainfall, and particularly during thunderstorms.

Asp/Pen-type conidia are 'indoor predominant' fungi and species of *Aspergillus* and *Penicillium* are able to colonise a wide variety of indoor materials, including fabric and paper [129, 234], even in non-complaint properties, and may be concealed within wall cavities or building materials [163]. *Asp/Pen*-type conidia are consistently present outdoors in low concentrations, whilst indoor concentrations can easily reach equivalent concentrations or exceed outdoor levels. Within the 100 properties sampled, *Asp/Pen*-type conidia exhibited an independence of season and outdoor concentrations, with no evidence of a seasonal effect on either indoor spore concentrations or the I/O ratio (although the upper limit for indoor airborne *Asp/Pen*-type conidia was lowest in the

summer). However, examining the seasonal variation in a subset of properties sampled in each of the seasons showed significantly lower concentrations in the winter.

3.4.3 Home characteristics

3.4.3.1 Relative humidity

Increased indoor relative humidity has been associated with elevated levels of fungal spores and respiratory symptoms [161, 178, 196]. However, due to our focus on non-complaint properties, none of the properties sampled in our study reached levels of 80%, which would be expected to encourage fungal contamination [235]. The positive associations of relative humidity with *Cladosporium*, *Sporobolomyces* and *Tilletiopsis* concentrations were lost after adjusting for outdoor levels, suggesting that, in some studies not allowing for outdoor fungal spore concentrations, previous associations with humidity may have been misleading, with outdoor factors playing a key role on indoor levels. This supports a previous study (where 80% of properties had mean humidity under 60%), which showed that adjustment for outdoor concentrations removed associations with relative humidity [161].

The weak association of *Didymella* with relative humidity is interesting, but not surprising, since *Didymella* is a wet weather spore, released during periods of increased humidity, reaching high outdoor concentrations. A further observation was the negative relationship between relative humidity and an absence indoor of *Didymella* and *Tilletiopsis*. Although more consistently present in outdoor air than *Didymella, Tilletiopsis* is also a wet weather spore released following periods of precipitation. Thus low outdoor concentrations during dry conditions would lead to low infiltration indoors.

3.4.3.2 Temperature

Increased temperature has been associated with elevated indoor fungal spore levels [178]; however, this was not shown after adjustment for outdoor levels. As with humidity, previous associations with mean temperature may be explained by increasing outdoor concentrations and infiltration indoors during the warmer summer months. After adjustment for outdoor concentrations, the only association of indoor airborne fungal levels was a negative association with the absence of *Didymella*, a pathogen of vegetation which is diminished during the winter months when temperatures are reduced.

3.4.3.3 Type of housing

Indoor fungal spore concentrations of outdoor predominant fungi were lower in flats than houses, as described previously [196], which may be explained by reduced infiltration or carriage of spores indoor. *Tilletiopsis* concentrations were higher in semi-detached properties and those 31-60 years old, with *Didymella* spore concentrations lower in new properties 0-30 years old. There are no obvious reasons for these results; *Tilletiopsis* concentrations are consistent outdoors throughout the year and rarely observed in elevated concentrations indoor, but can occur. Therefore, it is possible that building materials or design of properties 31-60 years old encourage growth of *Tilletiopsis*.

Higher concentrations of *Asp/Pen*-type conidia were found in old, terraced properties, indicating that differences in building design and materials are important determinants of indoor levels. Properties over 90 years old had significantly elevated levels of *Asp/Pen*-type conidia, whereas properties 31-60 years old had significantly reduced levels. This is an interesting observation since properties 31-60 years old were built in

the period after the end of the Second World War and prior to the energy crisis of the 1970s, which led to changes in building construction and design. Properties under 30 years old, which were built after this period, showed no significant reduction in *Asp/Pen*-type conidia (although a trend towards lowest concentrations in these properties was observed). Whilst other abundant indoor fungi were shown to be lower in flats, *Asp/Pen*-type conidia were not reduced in these properties. This reiterates the likelihood that outdoor fungal spore concentrations are reduced in flats due to reduced carriage and infiltration indoors, whereas *Asp/Pen*-type conidia predominantly have indoor sources, and thus are unaffected by reduced infiltration.

3.4.3.4 Floor covering

There was no association between airborne fungal spore concentrations and type of floor covering, confirming previous studies showing elevated levels of fungi in dust, but not air samples, taken from rooms with carpeted floors in comparison to hard floors [196, 199], although one study showed significantly higher levels of *P. chrysogenum* aerosolised from carpet in comparison to vinyl flooring [236]. This highlights the problem with comparative analysis of dust and air samples since elevated levels of dust-borne mould concentrations do not automatically indicate increased airborne levels [196]. Carpets are more likely to harbour larger quantities of dust and may therefore contain higher concentrations of fungal spores than dust from hard floors; however, this may be a poor indication of exposure to airborne spores.

3.4.3.5 Central heating, double glazing and insulation

Increased dustborne levels of (1→3) β -D glucans (a major cell wall component of fungi and yeasts), as a measure of fungal contamination, have been shown in houses with central heating (and built after 1970), with no effect of double glazing [237].

Cladosporium species are important aeroallergens with growth potentiated on water-damaged substrates or areas of increased moisture, such as condensation on windows [161]. The presence of central heating was positively associated with *Cladosporium* levels and it is possible that the location of radiators in centrally heated houses, typically beneath windows, would encourage release of *Cladosporium* spores from window frames; however, no associations were shown between levels of *Cladosporium* spores and reports of condensation or use of heating during sampling.

Previous research has shown that installation of double glazing and central heating in flats is associated with increased relative humidity, a decrease in thermotolerant species of *Aspergillus* and an increase in *A. fumigatus,* which has a high water affinity (0.98-0.99) [238]. Indoor fungal spore concentrations observed in this study were not affected by the presence or absence of double glazing; however, the presence of cavity wall insulation was negatively correlated with *Asp/Pen*-type spore concentrations, supporting a previous study which showed lower levels of *Penicillium* in homes with wall, floor and ceiling insulation in comparison to ceiling insulation only [161]. Cavity wall insulation would be expected to add to effects of central heating and double glazing. Therefore, a negative association of *Asp/Pen*-type conidia with the presence of cavity wall insulation may be explained by species of *Aspergillus* or *Penicillium* within these properties having lower water affinities; however, species-specific analysis of indoor air samples would be required to investigate this further. The lack of a relationship between levels of *Asp/Pen*-type conidia and relative humidity reported here is probably explained by the focus on non-complaint properties; none of which reached levels which would be expected to encourage fungal contamination [235].

3.4.3.6 Presence of pets

There was no effect of the presence of cats or dogs on total indoor fungal spore concentrations or individual outdoor predominant fungal taxa, with the exception of *Didymella* concentrations, which were negatively associated with dogs and positively associated with cats. There are no obvious explanations for these results, other than the influence of other factors such as building design. The lack of an association between pets and indoor fungal spore concentrations contradicts previous reports where the presence of cats has been associated with elevated levels of fungal spores within properties [178, 199], in addition to positive associations between cat allergen (*Fel d 1*) and β (1→3) glucans, which are pro-inflammatory glucose polymers found in the cell walls of fungi, yeasts, and some bacteria and plants [239]. The presence of dogs within properties has not consistently been correlated with increased concentrations of fungal spores [178], although elevated levels of *Alternaria* from homes where a dog is present have been shown using dust samples [196]. Indoor airborne concentrations of *Alternaria* were not high enough in this study to be able to analyse any potential associations.

Significantly lower levels of *Asp/Pen*-type conidia were found in properties where a dog was present; however, this is likely explained by housing age and type, since there were no dogs within old terraced properties sampled.

3.4.4 Calculating ranges of abundant indoor fungi

Typical ranges generated from upper quartiles of the abundant indoor fungal taxa data provide a guide which can be used in conjunction with outdoor data to identify potential indoor fungal contaminants by air sample collection and microscopic identification and quantification. Seasonal variation is apparent in all outdoor predominant abundant

indoor fungal taxa, with highest concentrations indoor typically during the summer. In contrast, indoor *Asp/Pen*-type spore concentrations exhibited an independence of season. Since these fungi are indoor predominant, elevated concentrations indoor are possible due to colonisation of indoor substrates. *Asp/Pen*-type conidia were shown to reach levels up to 4117 spores/m^3 air within properties, which far exceeds typical ranges. Properties investigated which exhibit distorted distributions of indoor fungal spores relative to outdoor concentrations and exceed 'typical' ranges, may warrant further investigation.

3.4.5 Variation in indoor fungal spore concentrations in repeat samples within and between properties

The baseline study suggested an effect of season on indoor airborne fungal spore concentrations; however, this was based on 100 properties sampled on a single occasion at varying times of year. To investigate this further, 13 properties were sampled on a single occasion in each of the four seasons. Again, significant seasonal variation was exhibited in the data with both *Asp/Pen*-type and total fungal spore concentrations lowest in winter. Different rooms in homes would be expected to have different fungal concentrations due to differences in room use and potential growth substrates present. In order to investigate whether a single 24 hour sample provides adequate information regarding typical airborne fungal spore concentrations within a property, the living room and bedroom of three properties was sampled for seven consecutive days. Living rooms and bedrooms are the rooms in homes where people are most likely to spend the majority of their time. Living rooms were focused on for sampling in the main study because sampling of bedrooms was considered too disruptive due to the noise made by the samplers. Whilst shown to be variable over seven days, repeat sampling of living rooms over a seven day period showed good and excellent consistency of absolute

values measured by intra-class correlation (Cronbach's alpha) in approximation of *Asp/Pen*-type and total airborne fungal spores respectively, suggesting that a single 24 hour sample may be sufficient to produce a reliable indication of fungal spore concentrations over a seven day period. Variability in fungal spore concentrations within seven days of the same property could be caused by many factors, including differences in room occupancy and domestic activities [239, 240]. Total airborne fungal spore concentrations (but not *Asp/Pen*-type conidia) were consistently higher in living rooms in comparison to bedrooms. The proportions of different fungal spore types varied between living rooms and bedrooms, with *Sporobolomyces* and *Tilletiopsis* (both yeasts) comprising a higher proportion of fungal taxa present in the living rooms in comparison to bedrooms and a number of other fungi including *Cladosporium* and *Asp/Pen*-types spores (both filamentous fungi) sharing a larger proportion of taxa present in the bedrooms of properties in comparison to the living rooms. This is consistent with a previous culture-based study of air samples which identified *Cladosporium* and *Penicillium* species to comprise the majority of fungi found in bedrooms [199]. This is an important observation, since different rooms may contain different sources of fungal exposure. The presence of houseplants or bird cages would be expected to have an effect on fungal concentrations within a given room, and bathrooms for example, can be very humid environments, particularly due to the use of showers, encouraging the growth of fungi such as *Cladosporium* and *Asp/Pen*-type [127, 241]. Accumulation of moisture and inadequate airing or extraction of humidity in kitchens would similarly be expected to affect fungal concentrations, and contamination of foodstuffs would also provide an obvious source. Therefore, sampling one site within a property may not give a complete picture of fungal exposure within the home.

Consistent with the main study, property three, a maisonette flat, had lower levels of airborne fungal spores that the terraced and detached properties, with the terraced property again having the highest levels of airborne *Asp/Pen*-type conidia. The detached property, whilst having high levels of total fungal spores, had lower levels of *Asp/Pen*-type conidia.

3.4.6 Utility of continuous sampling

Continuous air sampling and analysis by microscopy was chosen as the most suitable method for investigation, as a validated and widely used technique for indoor and outdoor airborne fungal spore quantification, enabling detailed analysis of longer duration samples (which can aid capture of transient fluctuations in fungal spores) without the biases of viability and growth media associated with culture-based methods. Fungal spore concentrations are often underestimated by viable culture since only a fraction of fungi can be cultured and of these only viable spores will grow. Continuous air sampling enables a more accurate representation of total fungal spore levels; however, caveats of this approach include the inability to distinguish species to the level of species, and in some cases, to the level of genus. There may also have been some issues with the efficiency of collection of smaller spores. The cut off size (d_{50}) of an orifice is the diameter of spores at which collection will be 50% and is higher (3.7-5.2 μm) using the standard orifice (14 mm x 2 mm), to an alternative orifice (14 mm x 0.5 mm) which has been developed with a d_{50} of 2.17 μm. The orifice of samplers used in this study was the standard size, which has been shown to have a greater collection efficiency of larger spores such as *Alternaria,* but significantly lower collection efficiency of concentrations of *Asp/Pen*-type conidia, which range from 2-10 μm.[242] Therefore, the smaller spores of *Aspergillus* and *Penicillium* may have been underestimated in this study.

Analysis of continuous samples by microscopy by trained individuals can provide a fast, reliable indication of indoor airborne fungal spore levels. The continuous sampling method is much cheaper and less time-consuming than more modern methods such as liquid impaction and filtration. Even when culture-based techniques are used for identification, considerable time and expense may be incurred for analysis, increasing the time delay in providing a result. Therefore, culture-based techniques are not suitable for routine analysis of outdoor daily airborne fungal spore concentrations which are reported routinely as an allergy service in the East Midlands. Furthermore, species-specific identification using molecular-based methods is not straightforward due to the current lack of a fungal DNA barcode (although ITS and LSU have been proposed as potential targets) and can become very expensive for routine analysis.

Occupant activity is likely to have a large effect on indoor fungal spore concentrations; during the study, repeat analysis of one of the properties sampled, which was excluded due to associated moisture-related problems, showed clear contamination with *Asp/Pen*-type conidia, which increased ten-fold during cleaning and removal activities associated with relocation from the property. Disturbance of fungi from dust reservoirs in flooring or neglected areas could cause increases in airborne concentrations which would be missed in a short term viable sample but would be captured by continuous sampling. However, it is very difficult to accurately monitor disturbance and activity within a property. Despite room occupancy being requested on the corresponding questionnaire for the study, differences in reporting meant that no meaningful data could be collected and analysed. The development of personal samplers [184, 185, 192, 193] may enable more accurate analysis of variability in fungal spore concentrations during activity and give a more accurate indication of actual exposure.

3.4.7 Summary

Based on data from Leicestershire (UK), indoor airborne fungal spore concentrations typically represent 16% of outdoor levels in non-complaint properties; although indoor levels may reach 26% of outdoor concentrations based on the upper quartile limit for total fungal spores. Ideally, corresponding outdoor data should be taken into consideration when analysing indoor samples. Where outdoor comparative data is not available, the limits suggested in this study may be a useful guide for interpretation of results for UK-based studies using continuous sampling methods. It should be stressed that these limits are intended as a guide, and concentrations exceeding these ranges are by no means definitive of an indoor source. Infiltration from outdoors during transient periods, increased occupancy or indoor activities such as cleaning, restoration, refurbishment and remediation works may all influence indoor airborne fungal spore concentrations, and should be taken into account during interpretation of air sample data.

Further studies will be important to determine long-term effects of changes in building materials and design on ambient levels of fungal spores, particularly species of *Aspergillus* and *Penicillium*. Species-specific analysis of *Asp/Pen*-type concentrations in old, terraced properties in particular is necessary to investigate the clinical relevance of fungi present within these properties, to determine whether predominating species are classified as potentially harmless or have known adverse health effects.

4 *A. fumigatus* sensitisation and sputum culture in asthma

Fairs, A*, Agbetile, J*, Hargadon, B *et al.* 2010. IgE sensitisation to *Aspergillus fumigatus* is associated with reduced lung function in asthma. *Am J Respir Crit Care Med* 182(11): 1362-1368. Official Journal of the American Thoracic Society.

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4.1 Introduction

A. fumigatus is the most pathogenic and widely studied *Aspergillus* species and is a common environmental mould, found indoors and outdoors, particularly in soil, decaying vegetation and water damaged building materials [62]. Sources include areas with high levels of humidity and condensation, such as kitchens, bathrooms and cellars, with documented indoor substrates including insulation material, potted plants and used pillows [128, 243, 244]. *A. fumigatus* is also occasionally isolated from water-damaged floor materials and concrete [165]. Due to its fundamental role in the degradation of organic matter, outdoor concentrations of *A. fumigatus* can reach very high levels, particularly around compost sites [245, 246]. Environmental exposure to *A. fumigatus* can trigger symptoms of asthma and allergy in sensitised individuals; however, the small spore size and thermotolerant properties of the fungus can also lead to airways colonisation in people with asthma and CF, where areas of damage and retained mucus provide a favourable environment in which the fungus can thrive. In immunocompromised individuals, *A. fumigatus* can disseminate from the airway to other organs of the body, causing invasive disease with mortality rates ranging from 30-95% [247, 248].

ABPA is a florid hypersensitivity reaction to airways colonisation by *Aspergillus* species, predominantly *A. fumigatus*, involving IgE and IgG antibodies [83], affecting around 40,000 people in the UK alone [73]; where recurrent periods of airway inflammation, bronchial obstruction and mucoid impaction [49] lead to the development of bronchiectasis and fixed airflow obstruction. Therefore early diagnosis of the disease is preferable in order to limit bronchial injury; however, many asthma patients who are

sensitised to *A. fumigatus* do not fulfil all of the criteria for a diagnosis of ABPA, causing difficulties for clinicians in prescribing the best course of treatment.

Sputum culture of *A. fumigatus* is only utilised as a secondary diagnostic tool for ABPA due to low detection rates; only 36% of respiratory samples from patients with confirmed or probable aspergillosis were positive for *A. fumigatus* in one study [98]. In routine laboratories in the UK, the methods of sputum culture utilised follow guidelines outlined by the Health Protection Agency (HPA) [73], which heavily dilutes sputum samples prior to culture due to parallel investigations for bacterial growth.

A. fumigatus sensitisation and ABPA has been associated with a progressive reduction in lung function in CF [49, 249, 250]; however, the relationship of sensitisation, colonisation and lung function is poorly understood in the absence of CF or ABPA.

Preliminary studies of treatment of ABPA patients with itraconazole, an antifungal agent, as a monotherapy or combined therapy with corticosteroids have shown promising results, with reduced total IgE, *A. fumigatus*-IgE, airway inflammation and increased FEV_1[112]. However, in the absence of a definitive diagnostic tool for demonstrating airways colonisation, determining the efficacy of these treatments in eliminating fungi from the airways is difficult. Moreover, the efficacy of these treatments in *A. fumigatus*-associated asthma without a diagnosis of ABPA is unclear. A trial of itraconazole in patients with SAFS showed a significant improvement in asthma-related quality of life; however, this disease entity does not take into consideration airways colonisation [114].

4.2 Materials and methods

4.2.1 Recruitment and clinical characterisation

Patients were recruited from the difficult asthma clinic at Glenfield Hospital, Leicester and underwent allergy testing, standard spirometry and clinical characterisation as described in section 2.2.1. Asthma patients were divided into three groups based on *A. fumigatus* sensitisation: 1) *A. fumigatus*-IgE sensitised (positive SPT to *A. fumigatus* with wheal ≥3mm above negative control and/or *A. fumigatus*-IgE > 0.35 kU/L; 2) IgG-only sensitised with *A. fumigatus*-IgG >40 mg/L; and 3) non-sensitised. A fourth group comprised healthy controls.

Sputum was obtained either spontaneously or by induction and processed for differential inflammatory cells counts and fungal culture (section 2.3.2). *A. fumigatus* was identified based on macroscopic and microscopic morphology (Figure 2.7).

4.2.2 Statistical analysis

All data were entered into a secure Microsoft Access database and analysed using GraphPad Prism (Versions 4 and 5) and SPSS for windows (Version 11.0). Samples were analysed blind to clinical characteristics of patients. Parametric data was expressed as means ± SEM and analysed by Bonferroni-corrected ANOVA. Non-parametric data was expressed as medians with IQR and analysed by Mann-Whitney, chi squared and Dunn-corrected Kruskal-Wallis tests. Repeatability of sputum culture was assessed by kappa statistics.

4.3 Results

4.3.1 Study cohort demographics

The study comprised 79 asthma patients, with and without sensitisation to *A. fumigatus* (Table 4.1). Gender and age were well matched between the asthma groups; however, the healthy controls were significantly younger ($p < 0.001$; Table 4.1). Smoking history was well matched across the asthma groups and healthy controls, all of which having a median pack year history of zero (Table 4.1). The majority (94.4%) of participants were no longer smokers (four current smokers in the *A. fumigatus*-IgE sensitised group). Within the asthma groups, there was no significant difference in age of asthma onset; however, IgE sensitised asthma patients had significantly longer duration of asthma in comparison to non-sensitised asthmatics ($p <0.05$, Table 4.1).

In terms of asthma treatment, there was no significant difference in the prescription of oral or inhaled corticosteroids between the three asthma groups. Of those treated with oral steroids, the median dose prescribed was 10 mg in all three groups (Table 4.1). Forty three (54.4%) of the asthma patients were classed as having refractory asthma which is a term used to describe patients with severe, poorly-controlled asthma, requiring high dose inhaled steroids, with frequent (two or more) severe exacerbations of their symptoms per year despite compliance with asthma medication and optimal management of co-morbidities [251]. The Global Initiative for Asthma (GINA) guidelines are used for the treatment of asthma, ranging from mild and intermittent to severe and persistent symptoms (www.ginasthma.org). Most newly diagnosed patients start treatment at step two, with stepwise increases in treatment to high dose inhaled steroids and oral steroids at step four. Inadequate control of symptoms at step four is considered ‘difficult-to-treat’ asthma [252, 253]. The majority (88.6%) of the patients in

this study were classed as severe asthma and on GINA step four or five treatment (Table 4.1).

Table 4.1. Study cohort demographics and clinical characteristics

	Asthma and *A. fumigatus* sensitisation					
	IgE (+/- IgG)	*Af*–IgG only	Non sensitised	*p*	Healthy	*p*
n	40	13	26		14	
Gender, male n (%)	19 (47.5)	5 (38.5)	11 (42.3)	0.82	9 (64.3)	0.51
Age, years[a]	58.3 (2.0)	57.7 (4.9)	53.4 (2.6)	0.36	33.4 (2.5)	< 0.001
Smoking, pack years[b]	0 (0-14.5)	0 (0-10)	0 (0-4)	0.69	0 (0-3.8)	0.41
Duration of asthma[b]	27 (14.5-45.0)[c]	18 (7.5-33.5)	9 (5.0-28.5)	0.03		
Age of asthma onset[b]	24 (2-46)	30 (14-59)	39 (23-49.5)	0.21		
GINA treatment step, n (%)				0.33		
1-3	4 (10.0)	2 (15.4)	1 (3.85)			
4	25 (62.5)	6 (46.2)	12 (46.2)			
5	11 (27.5)	5 (38.5)	13 (50.0)			
Prescribed oral prednisolone (mg), n (%)	12 (30)	6 (46)	11 (42)	0.44		
Prednisolone dose (of those prescribed, mg)[b]	10 (10-10)	10 (3.8-15.0)	10 (7.5-15)	0.93		
Prescribed inhaled steroids (µg/day)[a]	965.1 (61.8)	978.3 (130.7)	991.7 (100.5)	0.97		

[a] Mean with SEM in parentheses.

[b] Median with IQR in parentheses.

[c] $p < 0.05$ *vs.* non-sensitised asthma by Dunn's-corrected Kruskal-Wallis test

4.3.2 *A. fumigatus* sputum culture and sensitisation

Significantly higher rates of *A. fumigatus* sputum culture were found in *A. fumigatus*-IgE-sensitised asthmatics (62.5%) in comparison to non-sensitised asthmatics (30.8%) ($p < 0.05$; Figure 4.1, Table 4.2). Whilst *A. fumigatus* sputum culture rates were higher in *A. fumigatus*-IgG only sensitised and non-sensitised asthma patients than healthy controls, this was not significant ($p = 0.08$ and $p = 0.52$ respectively). There was also no significant difference in sputum culture rates between the *A. fumigatus*-IgG only sensitised group and either the *A. fumigatus* IgE sensitised or non-sensitised asthma groups.

Figure 4.1. Isolation of *A. fumigatus* from sputum in asthma and healthy controls

Bar chart representing the proportion of asthma patients and healthy controls with a positive sputum culture for *A. fumigatus*. Asthma patients were grouped according to sensitisation to *A. fumigatus* (IgE sensitised (+/- IgG), IgG-only sensitised, and non-sensitised). Higher rates of culture were shown in *A. fumigatus* IgE sensitised asthmatics in comparison to non-sensitised asthma patients and healthy controls.

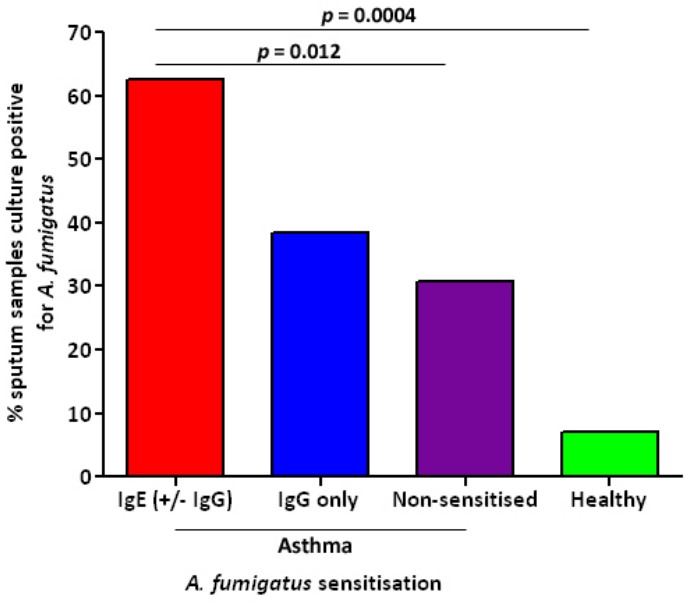


Table 4.2. Sputum culture and sensitisation data for asthma patients and healthy controls

Asthma patients were separated into groups based on *A. fumigatus* sensitisation: *A. fumigatus* IgE sensitised (+/- IgG), *A. fumigatus* IgG- only sensitised and non-sensitised.

	Asthma and *A. fumigatus* sensitisation			
	IgE (+/- IgG)	IgG only	Non-sensitised	Healthy
Sputum culture of *A. fumigatus*, n (%)	25 (62.5)[b]	5 (38.5)	8 (30.8)	1 (7.1)[c]
Total IgE (kU/ml)[a]	791 (361.5-2285)[d]	74.5 (36.9-141)	150 (81.2-326)	36.9 (7-53.0)[e]
A. fumigatus–IgE >0.35 (kU/L), n (%)	39 (97.5)	0 (0)	0 (0)	0 (0)
A. fumigatus-IgE (kU/L) [a]	11.6 (1.0-44.1)	0.03 (0-0.07)	0.05 (0.03-0.08)	0.05 (0.04-0.07)
A. fumigatus-IgG >40 mg/L, n (%)	20 (50)	13 (100)	0 (0)	2 (14)
A. fumigatus-IgG >40 mg/L[a]	44.4 (14-63.6)	62.1 (45.3-72.6)	13.1 (6.2-22.9)	20.6 (5.7-33.4)
Atopy (%)	55	38	54	21
A. fumigatus SPT, n (%)	26 (68)	0 (0)	0 (0)	0 (0)
Sensitisation to other fungi,%	24.4	0	0	7.1
Blood eosinophils	0.1 (0.1-0.4)	1.0 (0.7-1.7)	0.8 (0.6-1.2)	1.0 (0.5-1.5)
CF genotype mutations not detected, n	37	13	23	
Heterozygous, n	3	0	1	

[a] Median with IQR in parentheses.

[b] $p = 0.02$ *vs.* non-sensitised asthmatics by Fisher's exact test.

[c] $p = 0.001$ by chi squared analysis.

[d] $p < 0.001$ *vs.* IgG only sensitised and non-sensitised asthmatics by Dunn's corrected Kruskal-Wallis test.

[e] $p < 0.001$ *vs. A. fumigatus* IgE-sensitised and $p < 0.05$ *vs.* non-sensitised asthmatics by Dunn's corrected Kruskal-Wallis test

4.3.3 Repeatability of sputum culture for *A. fumigatus*

Repeatability of sputum culture was analysed in 17 asthma patients (8 negative on the first visit) where sputum was obtained on two occasions within six months when the patient was clinically stable (i.e. periods when asthma symptoms were adequately controlled. Exacerbation visits were not included in analyses). Seven out of nine samples originally positive remained positive, whilst seven of eight initially negative samples remained negative, showing substantial agreement by kappa statistics (k = 0.649, standard error = 0.184) [254, 255].

4.3.4 Lung function, *A. fumigatus* sensitisation and sputum culture

Reduced post-bronchodilator FEV_1 (% predicted) was observed in *A. fumigatus*-IgE-sensitised patients in comparison to non-sensitised asthmatics, independent of *A. fumigatus* sputum culture ($p < 0.05$; Figure 4.2, Table 4.3). *A. fumigatus*-IgE-sensitised patients also showed significantly lower airway reversibility ($p < 0.05$) and significantly higher rates of bronchiectasis ($p = 0.011$) in comparison to non-sensitised asthmatics (Table 4.3). *A. fumigatus*-IgG-only sensitised asthma patients had better lung function than IgE sensitised asthma patients, and worse lung function than non-sensitised asthmatics, although this was not statistically significant. Overall, there was a trend towards lower lung function in sputum culture positive asthma patients in comparison to culture negative patients; however, this was non-significant.

Healthy subjects had significantly higher FEV_1 in comparison to all three asthma groups ($p < 0.001$ *vs. A. fumigatus* IgE-sensitised, $p < 0.01$ *vs.* IgG only sensitised and $p < 0.05$ *vs.* non-sensitised asthmatics, Table 4.3).

Figure 4.2. Lung function of people with asthma and healthy controls as measured by post-bronchodilator FEV_1

Reduced lung function, measured by post-bronchodilator FEV_1, was shown in *A. fumigatus* IgE (+/- IgG) sensitised asthma patients in comparison to non-sensitised asthmatics. Closed symbols denote *A. fumigatus* sputum culture positive subjects and open symbols denote sputum culture negative subjects. Squares represent the four asthma patients who fulfilled all of the diagnostic criteria for ABPA. Closed circles represent mean and SEM.

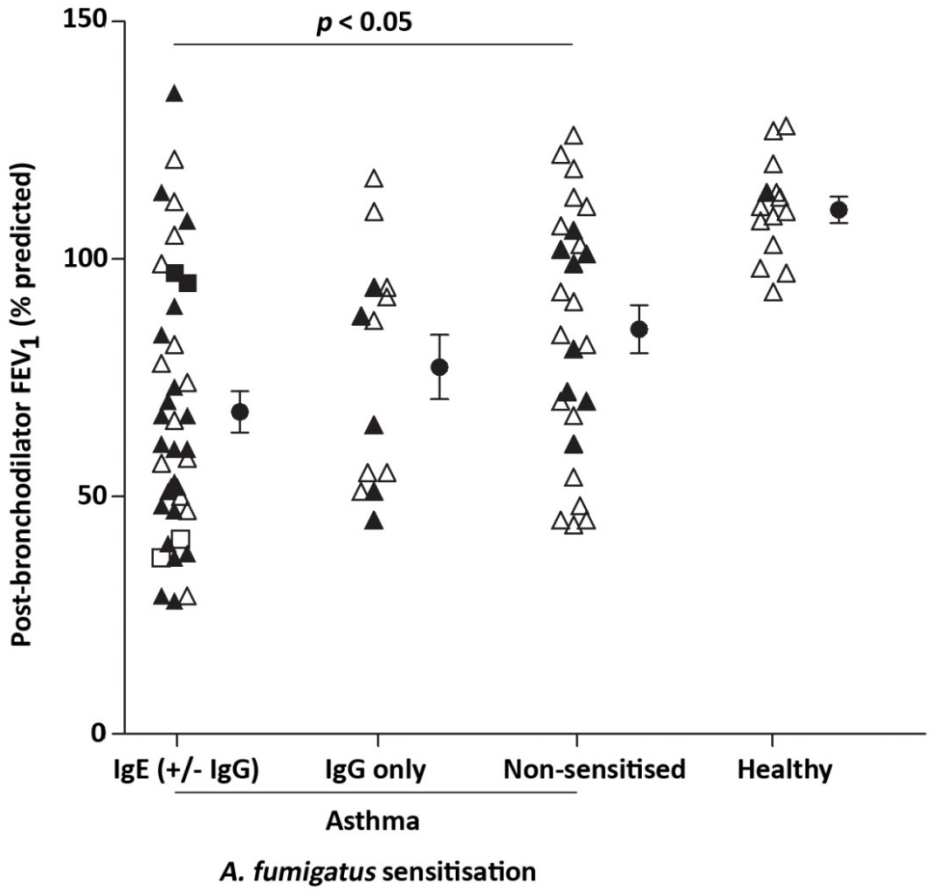


Table 4.3. Lung function and airway damage

	Asthma and *A. fumigatus* sensitisation			
	IgE (+/- IgG)	IgG only	Non-sensitised	Healthy
Post-BD FEV_1[a]	68.1 (4.6)[c]	77.2 (6.8)	87.5 (5.2)	110.5 (3.0)[d]
Post-BD FEV_1/FVC[b]	63.25 (49-73.5)[e]	68 (46.5-73.5)	73 (62.9-82.9)	83.6 (79.0-87.8)[f]
Post-BD FEV_1 with positive *A. fumigatus* culture[a]	66.6 (5.5)	68.6 (9.8)	86.50 (6.2)	114 (0)
Bronchiectasis, n (%)	27 (69.2)[g]	5 (38.5)	9 (36.0)	0 (0)

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

[a] Mean with SEM in parentheses.

[b] Median with IQR in parentheses.

[c] $p < 0.05$ *vs.* non-sensitised asthmatics by Bonferroni-corrected ANOVA.

[d] $p < 0.001$ *vs. A. fumigatus* IgE sensitised, $p < 0.01$ *vs. A. fumigatus* IgG only sensitised, $p < 0.05$ *vs.* non-sensitised by ANOVA.

[e] $p < 0.05$ *vs.* non-sensitised asthmatics by Dunn's-corrected Kruskal-Wallis test.

[f] $p < 0.001$ *vs. A. fumigatus* IgE sensitised, $p < 0.01$ *vs. A. fumigatus* IgG only sensitised and $p < 0.05$ *vs.* non-sensitisedby Kruskal-Wallis test.

[g] $p = 0.011$ *vs.* non- sensitised asthmatics by Fisher's exact test

4.3.5 Airway inflammation and clinical characteristics

There was no difference in differential cell counts of neutrophils between *A. fumigatus* sputum culture positive and negative asthmatics (Figure 4.3A); however, proportions of neutrophils were significantly higher in *A. fumigatus*-IgE sensitised asthma patients in comparison to non-sensitised asthmatics ($p < 0.01$; Figure 4.3B). Sputum eosinophils were significantly lower in healthy controls in comparison to sputum culture negative ($p < 0.001$) and sputum culture positive asthmatics ($p < 0.05$); and were also lower in comparison to the asthma groups grouped by *A. fumigatus* sensitisation ($p < 0.001$ *vs.* non-sensitised asthmatics and $p < 0.05$ *vs. A. fumigatus* IgE (+/- IgG) sensitised and *A. fumigatus* IgG-only sensitised asthmatics, Figure 4.4B). There was no difference in proportions of eosinophils between the asthma sensitisation groups, concordant with peripheral blood eosinophils, which also did not differ (Table 4.2).

97.5% of *A. fumigatus*-IgE-sensitised asthmatics had elevated *A. fumigatus*-IgE >0.35kU/L, with 65% having a positive SPT to *A. fumigatus* (Table 4.2). *A. fumigatus*-IgE-sensitised asthmatics demonstrated significantly higher total serum IgE (IU/ml) than non-sensitised asthmatics and *A. fumigatus*-IgG-only-sensitised asthmatics ($p < 0.001$). 48% of *A. fumigatus*-IgE-sensitised patients also had *A. fumigatus*-IgG sensitisation, and 24.4% of *A. fumigatus*-IgE-sensitised patients had a positive SPT to other fungi. Mutations in the CF gene (ΔF508) were found in four patients, all of whom were heterozygous (Table 4.2). Only four patients with elevated *A. fumigatus*-IgE fulfilled all the major criteria for ABPA (Figure 4.1).

Figure 4.3. Airway neutrophils in asthma patients and healthy controls

Airway neutrophils were quantified by differential cell counts of sputum cytospins. There was no difference in the proportion of sputum neutrophils in *A. fumigatus* sputum culture positive and negative asthma patients or healthy controls (A); however, *A. fumigatus* IgE sensitised asthma patients had significantly a higher percentage of sputum neutrophils than non-sensitised asthmatics (B). Lines represent mean with SEM (A) and median with IQR values (B) due to parametric and non-parametric fluctuations in the distributions of the data respectively.

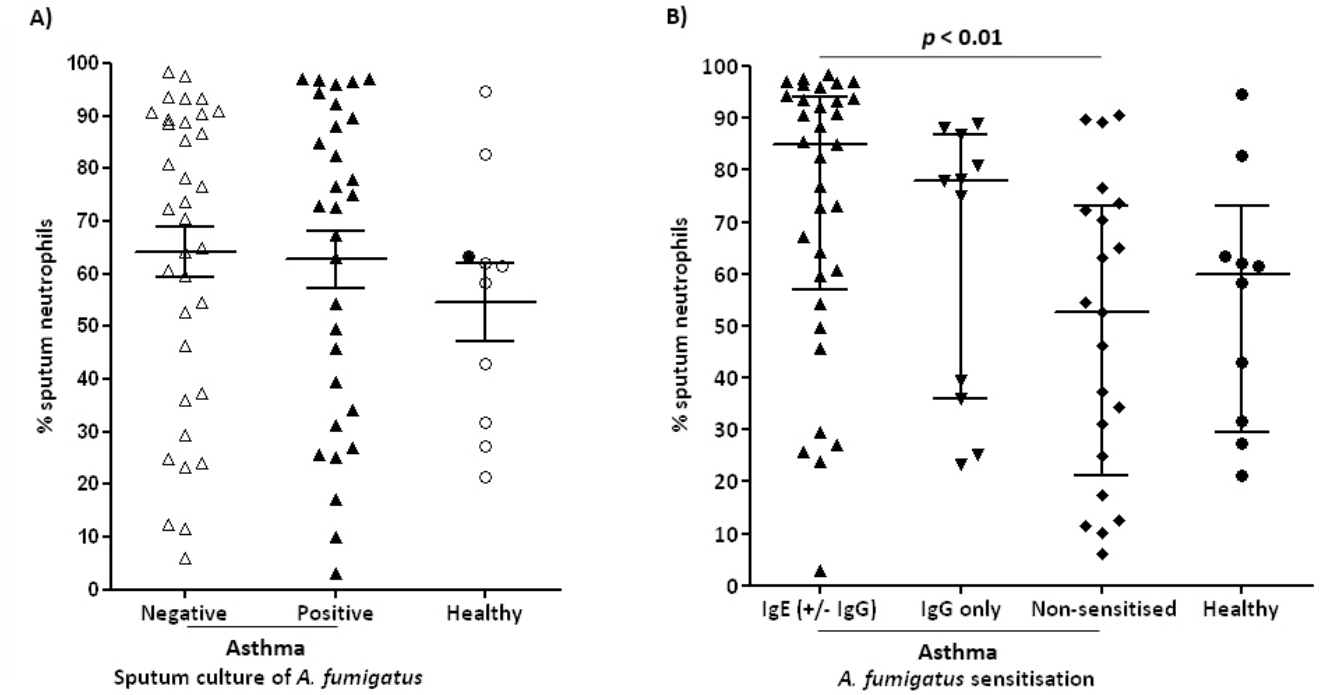
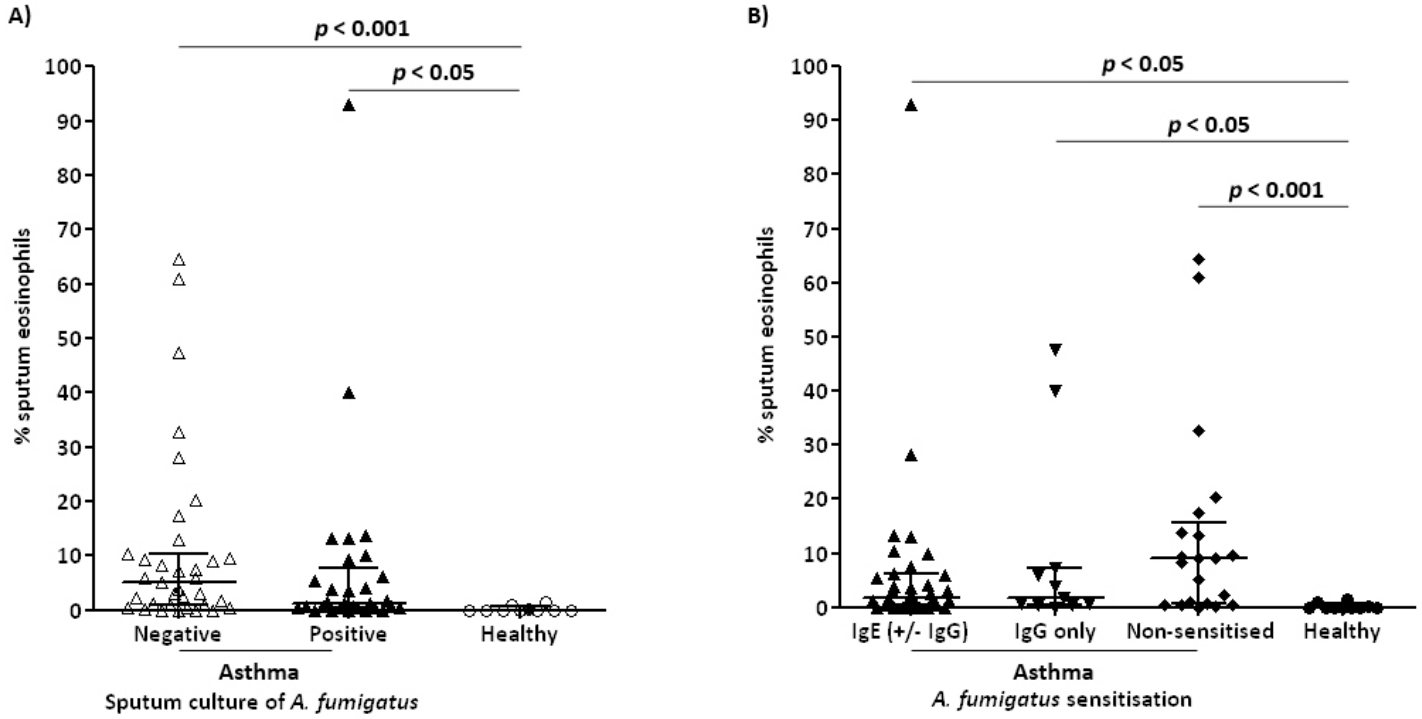


Figure 4.4. Airway eosinophils in asthma patients and healthy controls

Airway eosinophils were quantified by differential cell counts of sputum cytospins. There was no difference in the proportion of sputum eosinophils with regard to *A. fumigatus* from sputum culture (A) or sensitisation (B) in patients with asthma; however, the proportion of sputum eosinophils was significantly lower in healthy subjects in comparison to all asthma groups. Lines represent median with IQR.



4.4 Discussion

This was the first study to show an association between impaired lung function and *A. fumigatus*-IgE sensitisation alone in asthma [255]. This finding has since been confirmed in another study cohort of 133 severe asthma patients, where *A. fumigatus* sensitisation was also associated with a two-fold increased risk of bronchiectasis [256]. Asthma severity has been associated with fungal sensitisation in a number of studies [3, 11, 257, 258] and *A. fumigatus* sensitisation has previously been associated with a progressive decline in lung function over time in CF-ABPA patients, likely due in part to coinciding *Pseudomonas aeruginosa* bacterial infection [49, 249, 250]. A more severe reduction in lung function has been shown in children with CF and *A. fumigatus* sensitisation in combination with elevated total IgE [249]. The data reported here supports these earlier studies, showing specifically that *A. fumigatus*-IgE sensitisation, regardless of other ABPA diagnostic criteria, is an important indicator of reduced lung function. All data was collected whilst patients were stable and optimally treated, therefore the post-bronchodilator FEV_1 was taken to reflect fixed airflow obstruction in patients with asthma.

It is not possible to determine whether the relationship observed in this study between *A. fumigatus* IgE sensitisation and impaired lung function is causal; however, it is possible that sensitisation could transpire through airways colonisation, which may be more likely in areas of retained mucus in damaged airways. This explanation would be consistent with the finding that *A. fumigatus* IgE sensitised asthmatics had significantly longer asthma duration, an observation which has been previously reported [258].

4.4.1 Diagnosis of ABPA

Whilst ABPA represents the most florid hypersensitivity response to *A. fumigatus* airways colonisation, this study shows that *A. fumigatus* IgE sensitisation is clinically important with regard to reduced lung function and bronchiectasis within the wider spectrum of *A. fumigatus*-associated disease. Many patients with *A. fumigatus* sensitisation do not fulfil all of the criteria for ABPA, reducing the clarity of investigations into immunological and inflammatory responses to airways colonisation. Of the 53 *A. fumigatus* sensitised patients recruited into the study, only four satisfied all of the diagnostic criteria for classical ABPA, the majority of IgE-sensitised patients either not having elevated total IgE (>1000 ng/ml) or having no evidence of central bronchiectasis.

A. fumigatus-IgE and IgG have been suggested to be the most useful immunological diagnostic tools for ABPA [259]. Airway colonisation and sensitisation rates to *A. fumigatus* were examined in patients with elevated IgG to the fungus in the absence of IgE sensitisation. The results from this study showed that patients with IgG-only sensitisation to *A. fumigatus* had similar clinical profiles and rates of positive sputum culture to non-sensitised patients; however, the number of patients with *A. fumigatus* IgG only sensitisation were small (13 patients), and larger studies may be required to explore this further.

4.4.2 Sputum culture for *A. fumigatus* as a diagnostic tool

Detection of *A. fumigatus* in sputum is only utilised as a minor criterion in diagnosing ABPA due to low isolation rates, even in proven aspergillosis [98]. This study has shown that a focused approach toward culture of *A. fumigatus* in sputum enables detection of the fungus in sputum samples from almost two thirds of *A. fumigatus*-IgE

sensitised moderate-severe asthma patients, and as such should be considered in parallel with immunological measures of *A. fumigatus* allergy and lung function. Differences in culture rates shown in this study in comparison to routine laboratories and previous studies is almost certainly explained by the quantity of material inoculated onto culture plates. Within the UK, the standard protocol followed in clinical laboratories follows the BSOP 57 issued by the HPA, [73], which is designed for the identification of both bacteria and fungi from a single sample. According to this protocol, 0.1% DTT or equivalent is added to a mixture of saliva and sputum plug at equal volume, agitated for 10 seconds, incubated for 15 minutes at 35-37°C, agitated gently for 15 seconds and diluted 1:500 with sterile water. One microlitre of this diluted solution is then inoculated onto Sabaraud agar plates, followed by incubation for 2 days at 37°C. One microlitre of the sputum/DTT solution is added directly to plates in investigations of immunocompromised or CF patients for two to five days [73]. Some clinical laboratories use a modification of this protocol for mycological investigations of sputum, where 10 µl of undiluted homogenised sputum/DTT mix is inoculated onto plates and cultured at 37°C for up to five days. The method utilised in this study uses a much greater quantity of inoculating material than the BSOP 57 protocol, and the sputum plug is also separated from saliva prior to inoculation in our approach, therefore any oropharyngeal contaminants present in saliva should be removed or drastically reduced [97, 260]. This technique has been shown to have significantly higher rates of detection of *A. fumigatus* in sputum than the standard methods for mycological investigations [216]. Undiluted sputum has been previously been used to investigate the prevalence of *A. fumigatus* in patients with CF [261].

An alternative to traditional culture methods is the galactomannan assay, detecting galactomannan from fungal membranes using an ELISA-based method which has been

developed for diagnosis of invasive aspergillosis. This assay can be useful but is expensive and may have problems with sample to sample variability and specificity, such as false positives from non-*Aspergillus* fungi and also false negative results [100, 101, 262, 263].

Detection of fungi in sputum has been suggested to be indicative of either airway colonisation (whether it be transient or chronic) or environmental contamination [64]. There is the possibility that sputum culture of filamentous fungi could reflect oropharyngeal or environmental contaminantion during sputum induction and processing, as opposed to a measure of airways colonisation; however, this is unlikely for two reasons. Firstly, the sputum is separated from saliva prior to plating out plugs, therefore, any oropharyngeal contaminants present in saliva should be removed or drastically reduced prior to plating [97, 260]. Secondly, the low rate of positive cultures in the healthy controls in comparison to asthma patients, which were processed in an identical manner, blinded to clinical characteristics of subjects, in addition to negative control plates left open during spot checks of sputum processing and aseptic techniques employed. Therefore, environmental contamination was unlikely but cannot be ruled out during the sputum induction process when the sample was expectorated into a sterile container in a hospital room. The repeatability of the sputum culture method based on 17 patients from whom stable visit sputum samples were collected on two occasions within 6 months, a time interval which is relevant to re-testing after an intervention such as anti-fungal treatment, showed substantial agreement, suggesting that the method is reasonably robust considering the inherent variability in the amount and quality of sputum obtained by induction.

The healthy controls who participated in the study were significantly younger than patients in the asthma groups; therefore a possible effect of age cannot be ruled out and must be investigated in future studies.

4.4.3 Airways colonisation by *A. fumigatus*

The results of this study suggest that *A. fumigatus* colonisation can occur in 63% of *A. fumigatus*-IgE-sensitised patients, significantly higher than found in non-sensitised patients. *A. fumigatus*-IgE-sensitised asthmatics with a negative sputum culture for *A. fumigatus* may represent a subpopulation of patients with a continued IgE response to a previous transient *A. fumigatus* infection; or *A. fumigatus* may have been present but was not detected in the study. This could only be addressed through future longitudinal studies of sputum culture and allergy.

Differences in airway function which cause susceptibility of patients to ABPA remain unknown [62]; however, the potential involvement of immunogenetic factors has been suggested [78, 90, 95] such as the heterozygous 24 base pair duplication in the CHIT-1 gene, found in a small study of children with SAFS, which may contribute to reduced host defense to inhaled fungal pathogens [92-94]. Increased risk factors for ABPA may also include over-activity or increased number of CD4+ T cells, HLA-DR2 and HLA-DR5 restriction and elevated sensitivity to stimulation by Il-4 [50, 90].

4.4.4 Inflammatory mediators

Sputum eosinophilia is a response normally regarded as a hypersensitivity reaction to environmental antigens, whereas a neutrophilic profile usually suggests an infection [264]. ABPA is characterised by eosinophilia, which is used as a diagnostic criterion for the disease, however, ABPA has also been correlated with increased sputum neutrophils, airway obstruction and increased levels of Il-8 [265]. Elevated levels of

neutrophils shown by the data from *A. fumigatus*-IgE-sensitised patients in comparison to non-sensitised asthmatics suggests infection, further substantiated by significantly higher *A. fumigatus* culture rates in *A. fumigatus*-IgE-sensitised patients. Neutrophils and macrophages provide the first line of host defence against *Aspergillus* infection [266]. *In vitro* experiments have shown that bronchoalveolar macrophages phagocytose and kill *Aspergillus* conidia, and neutrophils damage *Aspergillus* hyphae on germination of conidia [267, 268].

4.4.5 Bronchiectasis

Fixed airflow obstruction may affect around 23% of patients with moderate to severe asthma [269]. Airway remodelling and evidence of bronchiectasis have been associated with neutrophilic and eosinophilic inflammation [270, 271]. The number of patients with bronchiectasis was significantly higher in *A. fumigatus*-IgE-sensitised asthmatics, indicative of increased asthma duration and severity. Approximately twice as many *A. fumigatus*-IgE sensitised asthma patients had evidence of bronchiectasis in comparison to non-sensitised asthmatics (69.2% *vs.* 36.0%). These data have been supported by a recent study, where 53.7% of *A. fumigatus*-IgE sensitised asthmatics and 26.7% of non-sensitised asthmatics had evidence of bronchiectasis. In the same study, evidence of airflow limitation was also reported, with comparable data to this study [256]. Future studies will be required to determine the causality of fungal colonisation, sensitisation and development of fixed airflow obstruction in asthma.

4.5 Summary

We found 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, IgE sensitisation was demonstrated in patients with longer asthma duration, elevated neutrophilic airway inflammation and fixed airflow obstruction. Future studies are necessary to assess the benefit of antifungal agents in these patients, particularly with regard to sputum culture indicating airways colonisation.

5 Characterisation and clinical implications of filamentous fungi in the airways of asthma

A substantial portion of this chapter has been published in Clinical and Experimental Allergy.

Agbetile J*, Fairs A*, Desai D, Hargadon B, Bourne M, Mutalithas K, *et al*. 2012. Isolation of filamentous fungi from sputum in asthma is associated with reduced post-bronchodilator FEV1. *Clin Exp Allergy* 42(5): 782-791.

* Joint first authors

5.1 Introduction

A. fumigatus is the most common and widely studied cause of airways colonisation and ABPA; however, a number of other fungi, primarily species of *Aspergillus,* whilst less frequently isolated, have also been implicated in causing similar or identical disease [14]. Of *Aspergillus* species alone, 20 species have been implicated in human disease [69]. *A. flavus, A. niger, A. terrerus, A. versicolor* and *A. nidulans* have been isolated from sputum in asthma or CF [64, 67, 83, 261, 272, 273] and *Aspergillus lentulus* and *Neosartorya pseudofischeri,* both from *Aspergillus* section *Fumigati* have also been reported [274]. In addition to *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* [52, 83, 275]. However, the clinical significance of other fungi found in sputum, indicating airways colonisation, is unknown and rarely discussed with the exception of limited case studies. *Penicillium* species are only rarely identified to the level of species in reports of airways colonisation or infection; therefore, the prevalence of *Penicillium* species in sputum of patients with asthma is poorly understood.

The prevalence of sensitisation to one or more fungi from a panel of five common fungal aeroallergens: *A. alternata, C. herbarum, A. fumigatus, P. chrysogenum* and the yeast *C. albicans,* has been shown to be significantly higher (76% sensitised to any fungi) in severe asthma requiring multiple hospital admissions in comparison to mild-moderate asthma with no hospital admissions (16% sensitised to any fungi), with patients having one hospital admission having comparable rates of sensitisation to the no admissions group [3]. Sensitisation rates to individual fungi in the multiple

admissions group were variable, with sensitisation to *Cladosporium* most common (41%) and *Alternaria* least common (26%) [3]. This phenotype of asthma is classified as SAFS [12], and a trial of itraconazole in these patients has been shown to improve asthma-related quality of life in 60% of patients, with reductions in total serum IgE and better morning peak flow [114].

Antifungal treatment options for people with invasive aspergillosis, airways colonisation or SAFS are limited and can have severe adverse side effects. Furthermore, suboptimal treatment can lead to the development of resistance in clinical isolates and interest is increasing in identification of fungi from clinical samples in order to prescribe optimum treatment. The development of resistance in some species; azole resistance in *A. fumigatus*, [115, 119, 276, 277] and amphotericin B resistance in *Aspergillus* species including *A. nidulans, niger* and *A. terreus* [278, 279], highlights the need for species-specific identification of pathogenic fungi from clinical isolates.

During the study investigating the prevalence of *A. fumigatus* in the sputum of moderate to severe asthmatics (Chapter 4), it was noted that a surprisingly high proportion of other fungi were also being cultured from sputum, either as a monoculture or with co-culture of *A. fumigatus.* Whilst the fungal biota detected in sputum of patients with CF has been thoroughly examined, no study has focused on the fungal biota found in sputum of asthmatics.

5.2 Materials and methods

Sputum was obtained from 126 patients with asthma and 18 healthy controls (including 79 patients and 9 healthy controls from Chapter 4) from the difficult asthma clinic at Glenfield Hospital, Leicester (as outlined in section 2.2.1). Clinical assessment and sputum collection were carried out as described in sections 2.2.1.1-2.2.1.5. Neat sputum

plugs were plated onto PGCF plates (prepared as per section 2.3.1) and incubated at 37ºC, with sputum samples also processed for differential cell counts (described in section 2.3.2).

Fungal isolates from sputum were identified as *A. fumigatus, Aspergillus* section *Nigri* or broadly classified into other fungal groups such as *Aspergillus* or *Penicillium* species based on macroscopic and microscopic features, followed by DNA extraction from pure subcultures and PCR amplification using ITS and LSU primers. DNA was then cleaned up and sequenced at PNACL, University of Leicester (procedures for identifying filamentous fungal isolates are detailed in sections 2.3.3.1, 2.3.5 and 2.4.4).

The effects of therapeutic doses of dexamethasone on the phagocytic ability of macrophages were examined *in vitro,* using lung filtrate from three patients undergoing upper lobe lobectomies (sections 2.3.4 and 2.4.3).

All data were analysed using GraphPad Prism (Versions 4 and 5). Parametric data was expressed as means with SEM and analysed by Bonferroni-corrected ANOVA. Non-parametric data was expressed as medians with IQR and analysed using Mann-Whitney, Fisher's exact, chi squared and Dunn's-corrected Kruskal-Wallis tests. Macrophage data was analysed by repeated-measures Bonferroni-corrected ANOVA.

5.3 Results

5.3.1 Identification of filamentous fungi

The majority of fungi could be identified by PCR amplification of DNA using primers targeting the ITS1 region of the fungal nuclear ribosomal operon. However, primers targeting LSU were required for a subset of fungi; notably species of *Penicillium,* although *P. piceum,* the predominant *Penicillium* species isolated, was consistently and

reliably identified using ITS primers. *Aspergillus* section *Nigri* isolates were not consistently amplified using ITS primers; however, all but one isolate were amplified after repeated attempts. Due to lack of resolution within the ITS1 region of *Aspergillus* sections *Flavi, Nidulantes* and *Nigri,* these fungi could only be identified to section. All named matches from GenBank had ≥ 99% sequence coverage and ≥ 95% sequence identity; the lowest was for one isolate of *Thermoascus crustaceus* at 95% and one isolate of *Gymnascella citrina* at 97%. All other named matches reported had ≥ 98% sequence identity.

5.3.2 Fungi isolated from sputum

One or more filamentous fungi were isolated from the sputum of around half (54%) of asthma patients, the vast majority of fungal species isolated being from the *Aspergillus* and *Penicillium* genera (Figure 5.1, Table 5.1). Eight species of *Aspergillus* were detected in sputum from asthmatics. The majority of isolates were of *A. fumigatus* (55 isolates), of which 37 were in monoculture. Of the other *Aspergillus* species, the most common isolate was *Aspergillus* section *Nigri* (seven isolates), of which four were isolated in co-culture with *A. fumigatus*; two were monocultures. *Aspergillus* species from the sections *Nidulantes* and *Flavi* were isolated from two patients; all other species were isolated from a single patient. Twelve species of *Penicillium* were detected in sputum from patients with asthma, most commonly *P. piceum,* which was isolated from seven patients, one as a monoculture, three in co-culture with *A. fumigatus* (+/- other filamentous fungi) and three in co-culture with another filamentous fungus. *Penicillium* subgenus *Penicillium* fungi were isolated from three asthma patients (*P. chrysogenum* and *Penicillium gladioli* are taxonomically grouped within this subgenus); one was isolated in monoculture and two in co-culture with *A. fumigatus.* All other *Penicillium* species were isolated from a single patient only. *Paecilomyces variotii* and two isolates

sequenced as *T. crustaceus*, (the teleomorph of *Paecilomyces crustaceus*) were isolated from three patients; all in co-culture with *A. fumigatus.* Zygomycetes were detected in the sputum of two asthma patients, both in co-culture of *A. fumigatus*. One monoculture of a Sordariomycete and a species of Agaricomycete in co-culture with *A. fumigatus* were isolated from sputum of patients with asthma, but could not be discriminated even to genus. Three healthy controls were sputum culture positive for filamentous fungi; one for *A. fumigatus* in monoculture, *Chaetomium bostrychodes* (a Sordariomycete) in monoculture from another subject and co-culture of *Coprinus xanthothrix* and two species of *Penicillium* from the third subject.

Figure 5.1. Proportion of fungal isolates attributed to individual fungal taxa from sputum of people with asthma

A total of 98 fungal isolates from sputum were predominated by *A. fumigatus*, other *Aspergillus* and *Penicillium* species.

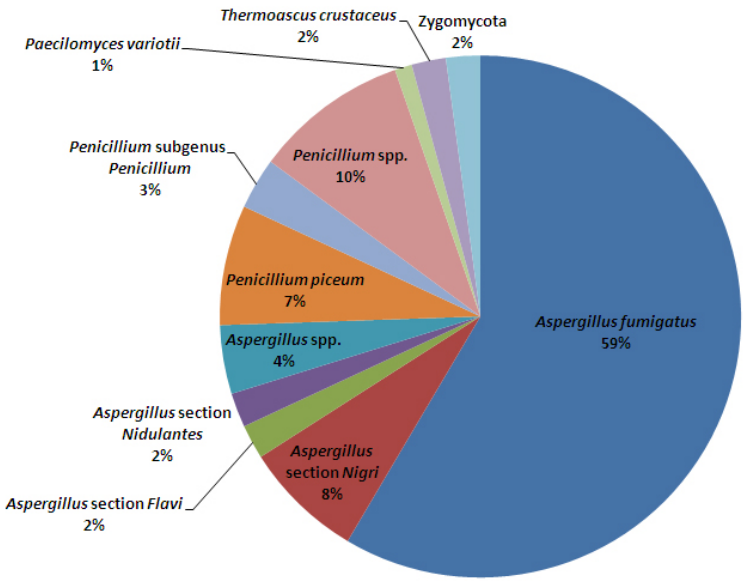


Table 5.1. Incidence and molecular identification of filamentous fungi cultured from sputum of asthma patients and healthy controls

Fungal isolates were cultured in isolation (mono), in co-culture with *A. fumigatus* (co-*Af*), or in co-culture with one or more non-*A. fumigatus* filamentous fungi in the absence of *A. fumigatus* (co-other).

Class	**Genus**	**Species/taxonomic identification**	**Asthma (n=126)**			**Healthy (n=18)**		
			Mono	**co-*Af***	**co-other**	**Mono**	**co-*Af***	**co-other**
Eurotiomycetes	*Aspergillus*	*A. fumigatus*	37		18	1		
		A. fischeri var. *glaber*		1				
		section *Nigri* (species undetermined)	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				

Class	Genus	Species/taxonomic identification	Asthma (n =126)			Healthy (n = 18)		
			Mono	co-*Af*	co-other	Mono	co-*Af*	co-other
	Paecilomyces	*P. variotii*		1				
		Thermoascus crustaceus (*Paecilomyces* teleomorph)		2				
	Gymnascella	*G. citrina*			1			
Zygomycota (class undetermined)	*Rhizomucor*	*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		

5.3.3 Clinical characteristics of study cohort

Demographic data for the study are shown in Table 5.2. In contrast to Chapter 4, the focus of this study was specifically investigating fungal culture from sputum; therefore, for analysis, asthma patients were divided into those with a positive sputum culture for fungi and those without. Whilst the age of asthma patients were closely matched between those culturing filamentous fungi from their sputum and those culture negative (median age 58 and 55 respectively), the healthy controls were significantly younger (median age 40; Table 5.2, p <0.001). Amongst the asthma patients, there was no difference in smoking history with regard to sputum culture of fungi, nor any difference in total serum IgE, atopy to common aeroallergens, age of asthma onset or duration of asthma. The healthy controls had significantly lower levels of total serum IgE (p <0.001), reduced atopy (p <0.01, Table 5.2) and lower rates of fungal sensitisation (p <0.001, Table 5.3).

Asthma patients with a positive sputum culture for any filamentous fungi had significantly higher rates of fungal sensitisation overall (p = 0.05) and higher rates of sensitisation to *A. fumigatus* and *P. chrysogenum* (p = 0.02 and p = 0.04 respectively, Table 5.3) but not to the other fungal aeroallergens tested (*A. alternata, C. herbarum* and *B. cinerea*) which were not isolated from sputum. Sputum culture positive asthma patients had been prescribed significantly higher levels of inhaled corticosteroids (p = 0.04, Table 5.2), and there was a trend towards higher levels of bronchiectasis in the culture positive group (p = 0.06, Table 5.2). Post-bronchodilator FEV_1 (% predicted) was significantly lower in asthma patients sputum culture positive for any filamentous fungi (p < 0.01, Table 5.2, Figure 5.2); however, there was no significant difference in sputum inflammatory cells between the culture positive and negative groups (Table 5.3).

Table 5.2. Demographic and lung function data of study participants

	Asthma (n=126)				Comparison of three groups
	No fungi cultured (n=58)	Any fungi (n=68)	*p*	Healthy (n=18)[a]	*p*
Age, years (range)	55 (21-84)	58 (24-83)	0.23	40 (21-67)	<0.001
Smoking history (pack years)[b]	0 (0-4)	0 (0-10)	0.51	0 (0-3)	0.44
Gender (male)	41%	53%	0.20	50%	0.42
Serum total IgE kU/L[b]	159 (43-494)	207 (89-718)	0.08	31 (9-50)	<0.001
Atopic[c]	55%	61%	0.57	17%	0.01
Age of asthma onset, years[b]	34 (9.5-47.25)	25 (5.25-46)	0.65	-	-
Duration of asthma, years[b]	22 (10.75-42.5)	23 (7-41.75)	0.89	-	-
FEV_1% of predicted, post BD	82.8 (24.8)[f]	70.8 (25.4)[e]	<0.01	111.6 (11.0)[e]	<0.001[f]
Volume change post BD, (ml)[b]	100 (50-250)	50 (0-150)	0.01	-	-
GINA treatment					
GINA 5	38%	44%		-	-
GINA 4	55%	51%	0.58		
Inhaled corticosteroid dose (µg)[bd]	1600 (800-2000)	2000 (1600-2000)	0.04	-	-
Bronchiectasis, n (%)	17 (35)	32 (51)	0.06	-	-

Abbreviations: BD = bronchodilator, CI = confidence interval

[a] Three subjects had positive fungal cultures.

[b] Median (IQR)

[c] Assessed by SPT ≥3 mm or specific IgE to common aeroallergens

[d] Beclomethasone dipropionate equivalent

[e] Post-test comparison p <0.05

[f] Post-test comparison p < 0.01

Table 5.3. Fungal sensitisation and airway inflammation

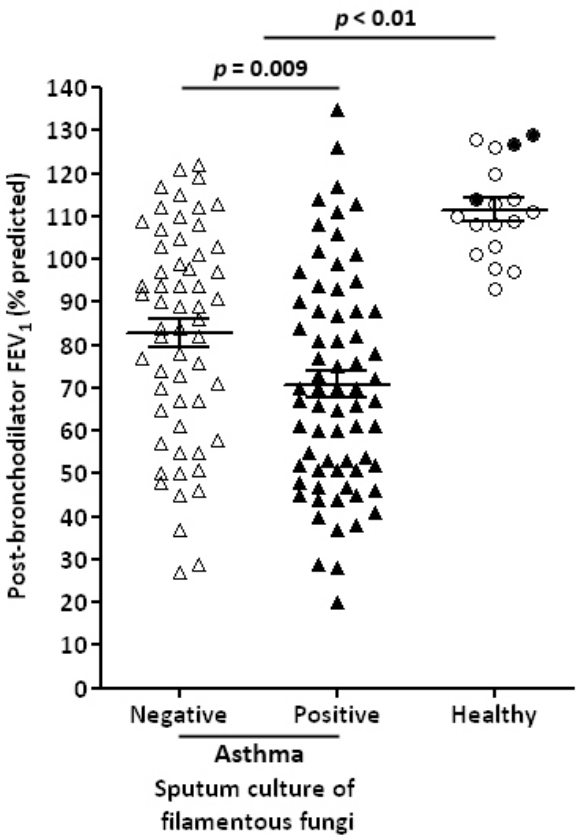
	Asthma patients (n=126)			Healthy (n=18)[b]	Comparison of three groups
	No fungi cultured (n=58)	Any fungi (n=68)	*p*		*p*
Fungal sensitisation (any)	38%	56%	0.05	6%	<0.01
• *A. fumigatus* (positive/n)	17/58	35/68	0.02	0/18	
• *P. chrysogenum*	5/36	17/48	0.04	0/12	
• *Botrytis cinerea*	3/31	8/41	0.30	0/12	
• *Alternaria alternata*	6/39	11/58	0.80	1/8	
• *Cladosporium herbarum*	7/38	13/57	0.80	0/8	
Total cell count x 10^3/mg sputum[a]	3.7 (1.6-7.8)	3.7 (1.3-6.9)	0.96	3.8 (2.6-6.6)	0.90
Sputum neutrophil (%)[a]	69.8 (50.4 -89.1)	67.3 (38.4-85.3)	0.43	60.0 (30.6-68.3)	0.32
Sputum macrophage (%)[a]	12.4 (4.7-28.0)	18.0 (5.7-34.8)	0.35	31.3 (9.5-60.1)	0.16
Sputum eosinophil (%)[a]	3.4 (0.4-10.2)	2.3 (0.5-8.1)	0.58	0.0 (0.0-0.4)	0.001

[a] Median with IQR in parentheses
[b] Three were sputum culture positive for filamentous fungi

The healthy controls had significantly better lung function (p <0.01, Table 5.2 and Figure 5.2), lower rates of fungal sensitisation ($p < 0.01$) and lower differential counts (%) of sputum eosinophils ($p < 0.001$) in comparison to the two asthma groups; however, there was no difference in the number of sputum neutrophils (Table 5.3).

Figure 5.2. Lung function of people with asthma and healthy controls in comparison to sputum culture of any filamentous fungi

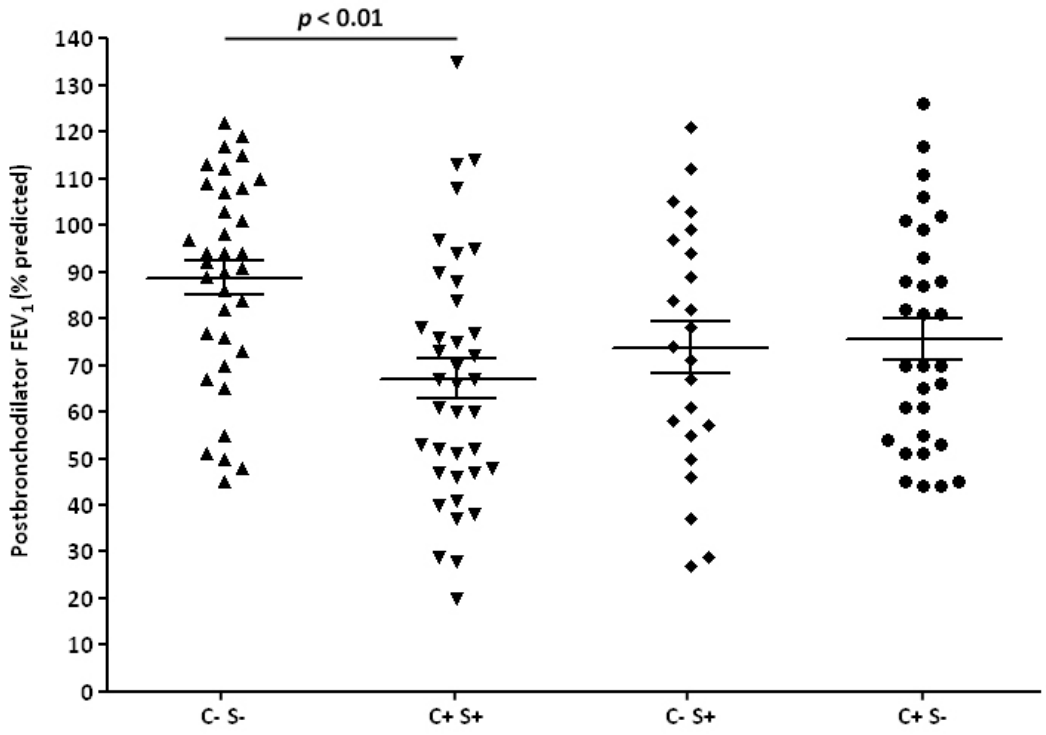
Asthma patients with a positive sputum culture for any filamentous fungi had significantly reduced lung function in comparison to sputum culture negative patients. Healthy controls had significantly better lung function than asthma patients. Filled symbols denote sputum culture positive subjects, open symbols represent sputum culture negative.



Division of asthma patients into groups based on sputum culture positivity and fungal sensitisation showed that patients with both a positive sputum culture and fungal sensitisation had significantly reduced post bronchodilator FEV_1 (% predicted) in comparison to non-fungal sensitised and sputum culture negative patients, with a mean drop in lung function of 21.6%. The presence of either fungal sensitisation or sputum culture was associated with an intermediate reduction in FEV_1 but this was not significant (Figure 5.3).

Figure 5.3. Lung function with and without fungal culture from sputum and fungal sensitisation in asthma

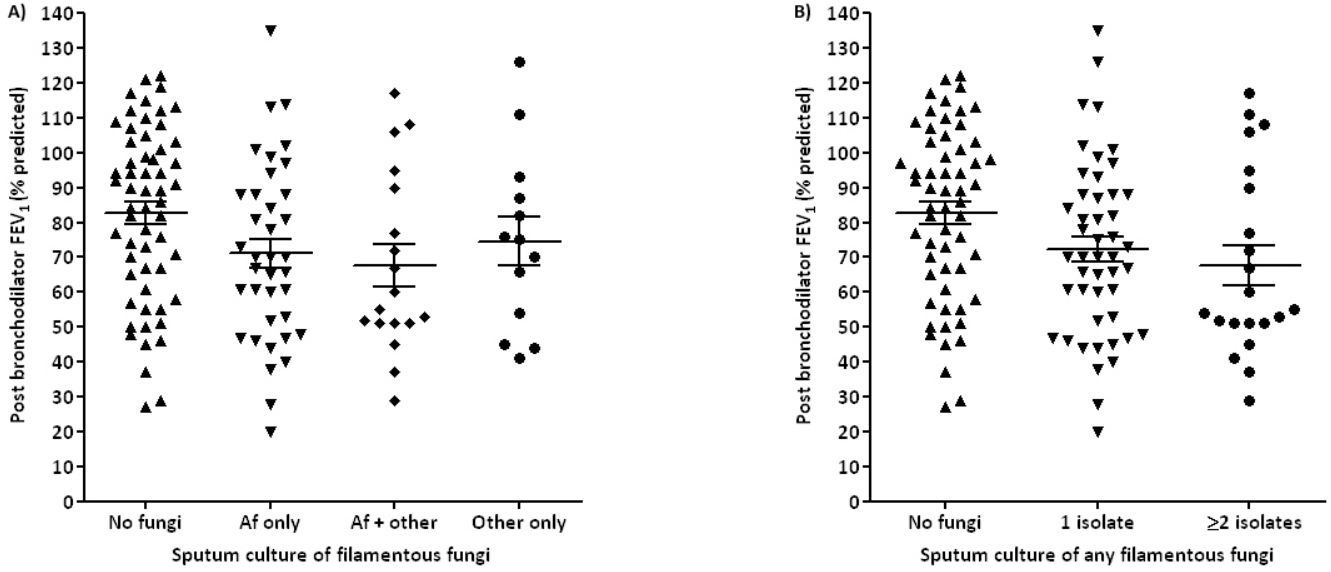
Scatter plot showing reduced post bronchodilator FEV_1 in sputum culture positive and fungal sensitised asthmatics in comparison to non-sensitised and sputum culture negative asthma patients. An intermediate reduction in post bronchodilator FEV_1 was shown by patients either sputum culture positive or fungal sensitised only. C= sputum culture, S= fungal sensitisation positive (+) and negative (-). Lines represent mean and SEM.



To investigate the clinical significance of non-*A. fumigatus* fungi, asthma patients were separated into groups based on isolation from sputum of *A. fumigatus* only, *A. fumigatus* in the presence of one or more other fungi, non-*A. fumigatus* isolates only and culture negative patients. Whilst not significant, a trend towards reduced post bronchodilator $FEV_1$was shown in patients with a positive sputum culture for non-*A. fumigatus* fungi in comparison to sputum culture negative patients. Lowest lung function was shown in patients with a positive sputum culture for *A. fumigatus* in co-culture with one or more other fungi, although this was not significant (Figure 5.4A). Reduced lung function was shown in patients with a positive sputum culture for one fungal isolate in comparison to sputum culture negative patients, with a trend towards even worse lung function in patients sputum culture for two or more types of fungi (Figure 5.4B). The means in these three groups were significantly different ($p = 0.03$); however, there was no significant difference between the groups following Bonferroni adjustment for multiple comparisons.

Figure 5.4. Associations between lung function and type of fungi isolated from sputum in asthma

Further investigation of associations of different fungi with post-bronchodilator FEV_1 (% predicted) shows consistent trends towards lower lung function in asthma patients with a positive sputum culture for any filamentous fungi, with greater reductions shown where more than one fungus was isolated from sputum. Trends towards reduced lung function were shown in asthma patients with a positive sputum culture for *A. fumigatus* (Af) only, *A. fumigatus* in addition to one or more other fungi (Af + other) or one or more non-*A. fumigatus* fungi (Other only) in comparison to sputum culture negative patients (A). Trends in reduced post-bronchodilator FEV_1 were greater in patients with a positive sputum culture for more than one fungus (≥ 2 isolates) in comparison to a single isolate (B). Lines represent mean and SEM.

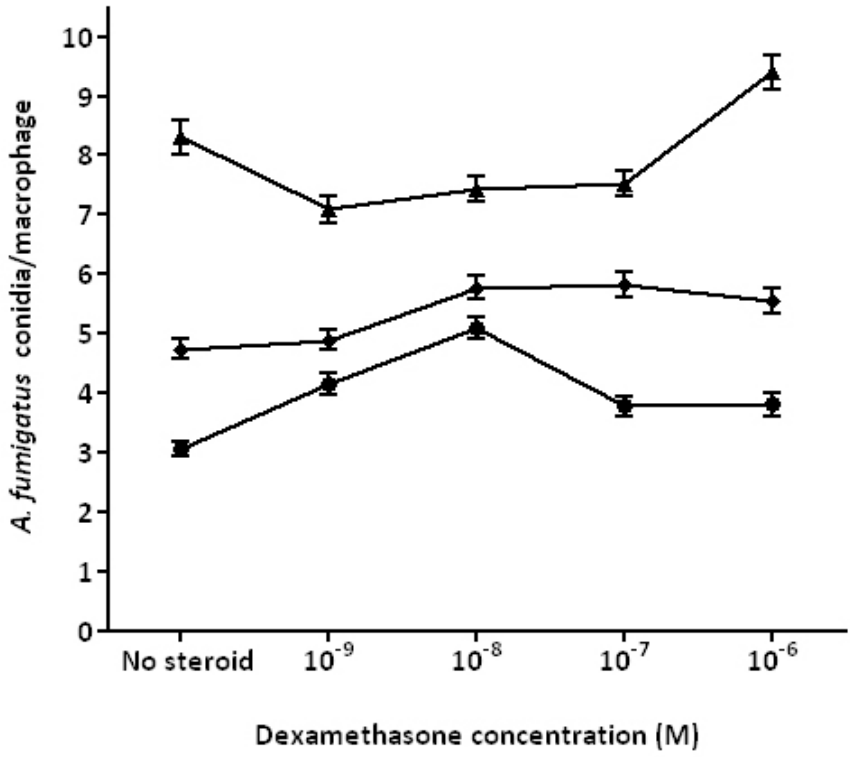


5.3.4 The influence of corticosteroids on *A. fumigatus* phagocytosis by bronchoalveolar macrophages

Due to the significant difference in inhaled corticosteroid dose between the sputum culture positive and culture negative asthmatics, the potential effect of increased steroid on the ability of bronchoalveolar macrophages to clear *A. fumigatus* conidia was investigated *in vitro*. There was no difference in the number of conidia phagocytosed by bronchoalveolar macrophages following 24 hours pre-incubation with 10^{-6}-10^{-9} M dexamethasone (p = 0.538, Figure 5.5). Mean viability of macrophages was 94%, assessed by the trypan blue exclusion method.

Figure 5.5. The influence of therapeutic concentrations of glucocorticoids on the phagocytic ability of macrophages

Phagocytic index of bronchoalveolar macrophages engulfing *A. fumigatus* conidia four hours post-phagocytosis, following 24 hr pre-incubation with therapeutic concentrations of dexamethasone (10^{-9} – 10^{-6} M) or HBSS negative control (no steroid). Symbols represent mean values for 600 macrophages (200 per coverslip, in triplicate), with lines representing SEM. Different symbols denote different subjects.



5.4 Discussion

This is the first study to fully describe the fungal biota, as assessed by culture, associated with the airways of patients with moderate to severe asthma, showing significantly reduced lung function in patients with a positive sputum culture for filamentous fungi. Whilst the predominant fungus isolated was *A. fumigatus,* a number of other species were also cultured, predominantly from the *Aspergillus* and *Penicillium* genera, either as a monoculture or in co-culture with *A. fumigatus.* Whilst a trend towards reduced lung function was shown in asthma patients without fungal sensitisation but with a positive sputum culture, or vice versa, fungal sensitisation in combination with a positive sputum culture had a stronger association with reduced lung function, which was statistically significant. A trend towards step-wise reduction in lung function has been shown from asthma patients with a negative sputum culture, to those from whom one fungus was isolated, with lowest lung function in patients with a positive sputum culture for multiple fungi.

5.4.1 Molecular identification of clinical isolates

Molecular identification of fungal species cultured from sputum was possible to the level of species, or closely related species, in the majority of isolates targeting the ITS1 region of the fungal nuclear ribosomal operon. When DNA from fungal isolates could not be amplified using primers targeting the ITS1 region, primers targeting the LSU D1/D2 domain were used. Neither ITS or LSU primers were able to identify all fungi to the level of species. The ITS region, as reported previously, enabled greater discrimination between clinical isolates [109]; however, closely-related fungi in the *Aspergillus* sections *Nigri, Nidulantes* and *Flavi* could not always be distinguished using ITS1 primers. This supports the results of a previous study, where species-specific

identification of clinically important *Aspergillus* species, including *A. niger, A. nidulans* and *A. flavus* was not always possible due to the molecular similarity of closely related species [109]. A recent study also showed that accurate identification of environmental fungi is not possible using GenBank, where only 13% of fungi could be identified to the level of species using ITS sequences only. Comparative analysis of ITS and β-tubulin sequences yielded species-specific identification of 38% of isolates. Further issues with species-specific identification of fungal isolates were highlighted by disparities in results between identification in GenBank when compared to other databases of type strains [166]. In the absence of a definitive tool for species-specific identification of clinically important *Aspergillus* species, future studies may require more comprehensive sequence data for ITS regions 1 and 2 for closely related species to be uploaded to GenBank for combined analysis of clinical isolates, which may enable greater differentiation between species. Misidentified species sequences must also be identified and removed or reclassified [109]. Targeting the β-tubulin and calmodulin genes has been shown to be beneficial in discriminating between species of *Emericella* and *Aspergillus* sections *Fumigati* and *Usti;* and targeting the β-tubulin has also been shown to be useful for species discrimination of *Aspergillus* section *Terrei* [280]. Therefore, better discrimination of *Aspergillus* clinical isolates may be possible targeting these protein-coding genes.

P. piceum, the *Penicillium* species most frequently isolated from sputum in asthma, was reliably amplified using ITS primers. Other *Penicillium* species were often not amplified by ITS primers, requiring identification using primers targeting the LSU. However LSU amplification of fungal isolates and sequence comparison in GenBank could often not distinguish between closely related species, such as relatives of *P. chrysogenum* in *Penicillium* subgenus *Penicillium*. Previous studies have shown the

potential for primers targeting *cox1* in the identification of *Penicillium* species, enabling species-specific identification of two thirds of *Penicillium* subgenus *Penicillium* species. However, analyses of other *Penicillium* complexes were not as successful and primers targeting the β tubulin gene enabled identification of some species which could not be distinguished by amplification of *cox1* [281].

The importance of identifying clinical isolates to the level of species within a particular section or subgenus is important due to differences in antifungal susceptibilities *in vitro*, which may have clinical relevance in antifungal treatment. For example, clinical isolates of *A. lentulus (Aspergillus* section *Fumigati)* and *A. calidoustus (Aspergillus* section *Usti)* have been shown *in vitro* to have lower susceptibilities to antifungal treatment than other members of their respective sections, whereas *Aspergillus tubingensis* from *Aspergillus* section *Nigri* was found to be more susceptible [282]. There is no current target which can be used to universally amplify and identify important clinical species. In combination with the progress being made towards the development of fungal barcodes, routine analysis of common clinical isolates to pre-determine optimal molecular targets would benefit future studies of airways colonisation in asthma.

5.4.2 Sputum culture as a diagnostic tool for airway colonisation

A positive sputum culture is taken to indicate airways colonisation; however, whether the relationship between airways colonisation and reduced lung function is causal remains to be determined. The majority of respiratory units do not routinely utilise sputum culture for diagnoses; however, some specialist centres screen patients vulnerable to invasive fungal infection (typically immunocompromised patients), followed by antifungal susceptibility testing of isolates [64].

ABPA and related diseases are most commonly caused by *A. fumigatus.* Other *Aspergillus* species are occasionally reported but in the majority of cases are considered

to represent either environmental contamination or colonisation without representing clinical disease [64]. In this study, one or more filamentous fungi were cultured from the sputum of 54% of asthmatics, in comparison to 17% of healthy controls. This may represent either transient or chronic colonisation of the airways and warrants further investigation into the clinical and immunological implications of colonisation. Since airway colonisation can occur without penetration of surrounding tissues or detrimental effects on health in the absence of allergy to the colonising fungus [17], it is likely that incidences of fungal respiratory infection pass without being detected and that transient infections would rapidly resolve in healthy individuals [77]. Longitudinal studies would be required in order to determine: i) which fungi are capable of long-term colonisation of the airways, ii) which fungi are harmless commensals or environmental contaminants (more likely in healthy individuals due to the small quantity of sputum typically produced, causing the possibility of greater saliva contamination), and iii) the persistence of airway colonisation in asthmatics in comparison to healthy controls.

A problem with using culture as a means of characterisation of the fungal biota from the airway is that the majority (estimated to be over 95%) of fungi cannot be maintained by current culture methods [283]; therefore investigations utilising culture methods bias analyses towards identification of particular fungi, and comprehensive analyses of the fungal biota are only possible using molecular methods. A potential issue when using sputum as an indicator of airways colonisation which must be considered is that, due to the ubiquitous nature of fungi, there is the possibility that detection of fungi could occasionally represent culture of an inhaled spore which did not represent clinical disease. Fungi have been found in the nasal mucus of over 90% of healthy volunteers and people with chronic rhinosinusitis, with the most prevalent fungi isolated being common environmental contaminants: *Aspergillus* (most commonly *A. fumigatus*),

Penicillium, Alternaria and *Cladosporium* [284]. Similarly, fungi are commonly present within the oral cavity [285] and, whilst saliva was separated from sputum prior to plating for culture reducing the likelihood of oropharangeal contaminants [97, 260], this cannot be completely ruled out.

5.4.3 Non-*A. fumigatus* fungi isolated from sputum

Whilst *A. fumigatus* was the most common fungus isolated from sputum of asthma patients, many other filamentous fungi capable of growing at body temperature were also frequently isolated; notably *Aspergillus* section *Nigri* and *P. piceum,* each of which represented 7% of fungal isolates. *Aspergillus* and *Penicillium* species comprised the majority of fungi isolated from sputum of ashmatics, spores of which are of respirable size and able to penetrate the lower airway. Unfortunately, due to the relatively low incidence of individual fungal types cultured from sputum, it was not possible to investigate associations of individual fungi with lung function, and this would need to be addressed in future studies with larger study cohorts. However, it is interesting to note that whilst numbers were too small to show a significant difference, asthma patients with a sputum culture for non-*A. fumigatus* fungi showed a trend towards reduced lung function in comparison to sputum culture negative asthmatics.

There are currently no guidelines on the reporting of mixed fungal species in culture from respiratory samples; however, a recent study [64] demonstrated repeat cultures of mixed *Aspergillus* and *Penicillium* species in a subset of patients, and suggested that, whilst *A. fumigatus* isolates are probably the most important due to the known allergenicity and pathogenicity of the fungus, it is possible that other fungi contribute to airways disease and should not be disregarded clinically. Furthermore, where multiple morphologies of *A. fumigatus* are isolated, antifungal susceptibility testing should be performed on each isolate [64]. The prevalence of non-*A. fumigatus* species is likely to

be underestimated by culture-based methods due to the prolific growth of *A. fumigatus*; which may outgrow co-cultured fungi, preventing detection. Future studies utilising PCR of sputum may prove valuable in detecting fungi; however, culture-based analyses are very important to first characterise the fungal biota of sputum in order that species-specific primers can be designed.

A. flavus has been reported as the second most pathogenic *Aspergillus* species [69] and *Aspergillus* section *Flavi* fungi were found in two patients in this study. However, higher detection rates suggest that fungi from *Aspergillus* section *Nigri*, which have been previously been described in cases of ABPA, could be more clinically important in moderate to severe asthma. The relatively high detection rate of *P. piceum* is also very interesting, since it has not been previously reported in asthma but is a member of the *P. marneffei* complex, an emerging human pathogen [71, 286]. Species of *Paecilomyces*, *Rhizomucor*, *Coprinus* and *Chaetomium*, were also detected in sputum which have previously been described in case reports of infection [287], mucormycosis [288], pulmonary infection [289], and invasive mycotic infections [290] respectively, suggesting that these fungi should not be disregarded clinically.

Despite efforts being made to recruit older healthy controls following analyses in Chapter 4, the healthy controls were still significantly younger than both groups of asthmatics; therefore, further studies would be required in order to investigate the possibility of an effect of age on fungal airways colonisation. However, as described in Chapter 4, sputum samples from asthma patients and healthy controls were processed blind to the clinical characteristics of each subject. Therefore, environmental contamination during processing as an explanation for elevated levels of fungal culture in the asthmatics can be rejected since the processing of all samples was identical.

5.4.4 Clinical relevance of isolation of fungi from sputum

Significantly reduced lung function in patients who are sputum culture positive for any filamentous fungi supports the hypothesis that airway colonisation by fungi could lead to the development of fixed airflow obstruction in asthma. However, it is also possible that the presence of some filamentous fungi within the lungs represents transient fungal infection or environmental exposure in the absence of clinical implications. A longitudinal study of mild asthmatics with and without fungal sensitisation is the only means of fully addressing the question as to whether the relationship between fungal colonisation and reduced lung function is causal; or that fungal colonisation is more likely in already damaged lungs. Longitudinal studies are also necessary to follow the clinical implications of long-term colonisation by non-*A. fumigatus* fungi.

Sputum culture positive asthmatics also had significantly higher rates of fungal sensitisation which has been shown previously in severe asthma [3] and higher rates of sensitisation to *A. fumigatus* and *P. chrysogenum* in comparison to sputum culture negative asthmatics. Since the majority of fungi cultured from sputum were from *Aspergillus* and *Pencillium* genera, this suggests an association between sputum culture and sensitisation and that sputum culture for any filamentous fungi, not just *A. fumigatus*, should be considered in the medical evaluation of asthma patients.

Furthermore, there was no difference in sensitisation to common aeroallergens not routinely associated with colonisation, such as *Alternaria, Cladosporium* and *Botrytis,* in sputum culture positive and negative asthmatics. This may suggest a different pathophysiology between patients sensitised to potential pathogenic or colonising species and non-colonising aeroallergens. It has previously been suggested that fungal sensitisation may be attributed to either environmental exposure or internal exposure to colonising fungi [12]; however, it is not known whether patients in whose sputum more

than one fungus is cultured indicates impaired clearance or elevated exposure. Overall, the rates of sensitisation to *A. fumigatus* found in this study cohort are comparable with the data from a study of asthma patients requiring multiple hospital admissions [3], where 37% patients were shown to be sensitised to *A. fumigatus* (in comparison to 7 and 6% of asthma patients requiring no admissions or one admission for exacerbations of their symptoms respectively) [3]. The proportion of patients with bronchiectasis in the culture negative group was consistent with another recently published study in severe asthma [256]; however, there was a trend towards higher rates of bronchiectasis in the culture positive patients.

5.4.5 The implications of glucocorticoid treatment on clearance of *A. fumigatus* conidia by alveolar macrophages

The majority of patients recruited into the study were on high dose inhaled steroids and sputum culture positive asthmatics were prescribed significantly higher doses compared with sputum culture negative patients. Glucocorticoids have been reported to elicit immunosuppressive effects including impaired activity of lymphocytes, neutrophils and macrophages, leading to increased susceptibility to fungal infection [291]. Chronic treatment with high dose steroids has been associated with the development of fungal pneumonia in patients with pre-existing respiratory disease [292] and invasive aspergillosis in transplant patients [293].

In immunocompetent individuals, inhaled fungal conidia can be cleared by bronchoalveolar macrophages. Any conidia which escape phagocytosis germinate into hyphae and are then targeted by neutrophils [266, 268, 294]. Treatment with corticosteroids have been shown in bronchoalveolar macrophages from both mice [295] and humans [221] to cause impaired clearance and killing of *A. fumigatus* conidia, and have also been associated with enhanced growth of *Aspergillus* species [248]. Whilst no

significant effect of therapeutic levels of glucocorticoids was found on the phagocytic index of macrophages, consistent with earlier studies [221], it has been reported that whilst the phagocytic index of macrophages remains unchanged, the ability of macrophages to actually kill spores is impeded by glucocorticoid treatments [221]. *A. fumigatus* conidia were not pre-opsonised prior to phagocytosis and the effect of this may warrant further investigation. Future studies investigating the prescribed dose and metabolism of corticosteroids, in relation to clearance of inhaled conidia from the lung and rates of fungal culture from sputum in asthma, will be required to fully understand the role of steroids in rates of airway colonisation.

5.5 Summary

In summary, this study has shown a direct link specifically between isolation of fungi from sputum and reduced lung function, an effect greatest in combination with fungal sensitisation. It is not possible to determine the allergenicity of, and patient sensitivity to, many of the fungi isolated from sputum due to limitations in SPT reagents available; however generic fungal sensitisation and specific sensitisation to *A. fumigatus* and *P. chrysogenum* was significantly higher in asthmatics with a positive sputum culture. The clinical implications of airways colonisation by non-*A. fumigatus* species of fungi is unclear, and steroid treatment may predispose asthmatics to airways colonisation; however the association with reduced lung function supports the hypothesis that filamentous fungi may, at least in part, cause the progressive lung decline observed in severe asthma. As previously suggested, consensus guidelines on the reporting of mixed fungal species from clinical samples are required, based on conclusive clinical data and longitudinal studies of the clinical relevance of non-*A. fumigatus* species cultured [64].

6 Home exposure to fungi, airway colonisation and sensitisation in asthma

6.1 Introduction

The ubiquitous nature of fungi means that exposure is inevitable, and in patients with lung disease such as asthma and CF, germination of inhaled fungi (predominantly *A. fumigatus*) within the bronchial tree can lead to airways colonisation. Sputum samples are frequently used in microbiological investigations of respiratory infections. A focused approach towards detection of fungi in sputum significantly increases culture rates in comparison to standard procedures used in routine national health service clinical laboratories [216], with 63% of *A. fumigatus*-IgE sensitised asthma patients being sputum culture positive for *A. fumigatus*, indicating airways colonisation, in comparison to 31% of non-sensitised asthmatics and 7% of healthy controls (Chapter 4). We have shown that people with asthma who were IgE sensitised to *A. fumigatus* had reduced lung function in comparison to non-sensitised asthmatics (Chapter 4). Furthermore, isolation of any filamentous fungi from sputum (predominantly *Aspergillus* and *Penicillium* species) is also associated with reduced lung function in asthma (Chapter 5).

It is recommended that optimal management of asthma should include identification and minimisation of environmental exposures to irritants and allergens, including fungi [296]. Therefore the identification and eradication of sources of fungal contamination may be of benefit to fungal-sensitised asthmatics. *Aspergillus* and *Penicillium* species are common indoor contaminants, even in non-complaint properties, although levels vary considerably [178, 297] (Chapter 3). Analysis of fungal contamination of

properties typically utilises fungal culture from air or dust samples, or standard microscopy of air samples (as used in Chapter 3). Quantification by culture-based methods is susceptible to growth media bias, short sampling times and culture only of viable spores. Traditional microscopy methods enable longer duration of sampling and time-dependent analyses, without the bias of viability, but prevent discrimination between *Aspergillus* and *Penicillium* conidia, which are grouped together as '*Asp/Pen*-type'. An alternative method is MSQPCR which enables species-specific quantification of 130 fungi, including *Aspergillus* and *Penicillium* species, with no limits on sample duration, or bias of viability. MSQPCR can be applied to both air and dust samples, enabling detection of higher levels and greater diversity of fungal contamination than traditional culture-based methods [298]. This technique has been used to generate environmental relative mouldiness index values (ERMI, the ratio of fungi indicative of water-damage or visible mould contamination to common indoor fungi within properties) demonstrating an association between high ERMI values from home dust samples and risk of asthma in children aged seven [299]. Using more traditional approaches, viable levels of *Aspergillus* and *Cladosporium* in house dust have been associated with allergic sensitisation in children [173], and dustborne occupational exposure to viable fungi has been associated with new onset asthma, post-building occupancy in adults [300]. Remediation of moisture damage in properties has been associated with an improvement of asthma symptoms in children [170] and a meta-analysis of the literature has shown that mould and home dampness is associated with a 30-50% increase in asthma and a variety of respiratory symptoms [301]. A recent epidemiological review concluded that, whilst indoor mould and home dampness have been consistently associated with asthma development and exacerbations, allergy and respiratory symptoms, it is still not possible to describe dose-response relationships

between microbial exposure and respiratory disease, to attribute causality, or to define safe levels of mould and damp-related exposures. Future studies recommended include efforts to determine the causality of exposure to mould or damp and respiratory infections or asthma [302].

To our knowledge, there have been no studies to date relating home exposure to fungi with airway colonisation or sensitisation to *A. fumigatus*, with the exception of a small study of eight patients with ABPA and atopic controls, where no differences in exposure were found and host susceptibility to ABPA was considered more important [139]. Therefore, we investigated home exposure to fungi as a predictor for airways colonisation and fungal sensitisation through analysis of airborne and dustborne concentrations of a subset of fungi detected in sputum, focusing primarily on *A. fumigatus*.

6.2 Materials and methods

6.2.1 Environmental sampling

Home sampling was conducted for 58 asthma patients and nine healthy controls. Sixty three of the 67 volunteers were recruited from the *Aspergillus*-associated asthma study cohort (Chapter 4; Figure 2.6). Twenty four hour air samples were collected from the living rooms of all properties using the Burkard continuous recording sampler as described in section 2.1.1.2. Concurrent air samples were also collected from 62 properties using the Burkard multi-vial cyclone sampler. Collection of air samples was not possible from five properties due to sampler malfunction or inconvenience to participants. Dust samples were collected from 66 properties and prepared for DNA extraction, as described in section 2.1.2.

6.2.2 Preparation of MSQPCR calibration curves

The fungi chosen as targets for investigation in this study were *A. fumigatus, A. flavus, A. niger* and *P. chrysogenum* due to isolation from sputum from more than one patient in Chapters 4 and 5 and availability of primers from the US EPA. Concentrations of target fungi in air and dust samples were analysed by MSQPCR as described previously [203, 228], using validated assays [205]. These assays are known to also amplify close relatives of *A. fumigatus, A. niger* and *A. flavus* (amplifying *Neosartorya fischeri, Aspergillus awamori* and *foetidus,* and *Aspergillus oryzae* respectively) [205]. For ease of reporting, the assays are referred to by the target fungal conidia used in initial calibrations: *A. fumigatus, A. niger, A. flavus* and *P. chrysogenum* (Table 2.3). It was also not always possible to identify clinical isolates to the level of species in Chapter 5; therefore, clinical isolates are referred to as *A. fumigatus, Aspergillus* section *Nigri, Aspergillus* section *Flavi,* and *Penicillium* subgenus *Penicillium.*

Conidia were harvested and adjusted to concentrations of 1-2 x 10^{7-8} conidia/ml (Table 2.3). DNA was extracted from target conidia in triplicate (section 2.3.5), spiked with 2 x 10^6 *G. candidum* conidia, serially diluted and analysed by MSQPCR to determine ΔC_T calibration curves (Figure 6.1). When reagents were replaced, assays were recalibrated; resulting in *A. fumigatus* and *P. chrysogenum* being calibrated twice. Baseline and threshold fluorescence was manually adjusted to maximise assay efficiency and theoretical mean conidia detection limits (MCDL), as described in section 2.4.5.3.

Fungal DNA was extracted from air and dust samples from study participant homes spiked with 2 x 10^6 *G. candidum* conidia and analysed by qPCR using the $\Delta\Delta C_T$ method (sections 2.4.5.4) [203, 228].

6.2.3 Quantification of *Asp/Pen*-type conidia by microscopy

Airborne concentrations of *Asp/Pen*-type conidia were quantified by microscopy at a magnification of x630 by counting a single longitudinal traverse of one field width down the centre of each slide stained with polyvinyl lactophenol cotton blue, in a standard method described previously [132]. Each longitudinal traverse was counted in 4 mm intervals to provide fungal concentrations divided into two hourly periods, which could be used to examine daily maximum concentrations. *Asp/Pen*-type conidia concentrations were adjusted to conidia/m^3 air (as described in section 2.4.1) or per two hour period to investigate daily maximum concentrations.

6.2.4 Categorisation of asthma patients according to sensitisation

Asthma patients were divided into groups according to *A. fumigatus* sensitisation as previously described in Chapter 4: 1) *A. fumigatus*-IgE sensitised as defined by a positive skin prick test to *A. fumigatus* with wheal ≥3 mm and/or *A. fumigatus*-IgE >0.35 kU/L; 2) IgG-only sensitised with *A. fumigatus*-IgG >40 mg/L; and 3) non-sensitised.

6.2.5 Statistical analysis

All data was analysed using GraphPad Prism (Versions 4 and 5). Concentrations of fungal conidia were shown to be non-normal using the D'Agostino and Pearson omnibus normality test and also had zeros in the dataset. Raw data was log-transformed to normalise ($Log_{10}(Y+1$; where Y = fungal spore concentration)) and parametric data expressed as mean with SEM, and analysed by ANOVA and two-tailed, unpaired t-tests. Non-parametric data were expressed as median with IQR and analysed by

Kruskal-Wallis, Mann-Whitney and Fisher's exact tests, Cronbach's alpha and Spearman's rank correlation coefficient.

6.3 Results

6.3.1 Study cohort

Around half of patients recruited into the study were male, with a mean age of 56.8 years. The majority of patients were on GINA step 4 or 5 treatment and prescribed high dose inhaled steroids (Table 6.1). 56.4% of patients had evidence of bronchiectasis and 57.9% of patients were atopic to common aeroallergens (not including the fungal panel). 29 patients had evidence of IgE sensitisation to *A. fumigatus* (wheal ≥ 3mm above negative control and/or *A. fumigatus*-IgE >0.35 kU/L), 11 patients had only evidence of IgG sensitisation (*A. fumigatus*-IgG >40 mg/L) and 18 patients were non-sensitised. Asthma patients and healthy controls were well matched according to gender and smoking history, but healthy controls were significantly younger (p <0.0001) and less atopic to common aeroallergens (p < 0.01; Table 6.1).

44.8% of patients had a positive sputum culture for *A. fumigatus;* 58.6% for any filamentous fungi (Table 6.1), of which 13.8% were isolated in the absence of *A. fumigatus* co-culture. Fungi identified as *Aspergillus* section *Flavi* were isolated from sputum samples from two asthma patients, one in co-culture with *A. fumigatus* and one in co-culture with *A. fumigatus, Aspergillus* section *Nigri* and *P. variotii. P. chrysogenum* or close relative, defined as *Penicillium* subgenus *Penicillium* fungi were also isolated from the sputum of two patients, one as a mono-culture and one in co-culture with *A. fumigatus. Aspergillus* section *Nigri* fungi were isolated from sputum of five patients; one in co-culture with *A. fumigatus, P. variotii* and *Aspergillus* section

Flavi as described above, two as a mono-culture and two in co-culture with *A. fumigatus.*

Table 6.1. Study cohort demographic data and clinical characteristics

Characteristic	Asthma (n = 58)	Healthy (n = 9)	*p*
Gender male, n (%)	30 (51.7)	5 (55.6)	1.00
Age, mean years (range)[a]	56.8 (24-84)	35.3(24-53)	<0.0001
Smoking, pack years[a]	0 (0-5.75)	0 (0-5.63)	0.37
Post bronchodilator FEV_1[a]	75 (51.75 – 103.0)	111 (106-117)	<0.001
Age of asthma onset, years[a]	35 (3.5 – 51.5)		
Duration of asthma, years[b]	24.0 (2.5)		
GINA treatment n, (%)			
GINA 1-3	7 (12.1)		
GINA 4	28 (48.3)		
GINA 5	23 (39.7)		
Inhaled corticosteroid dose (μg)[b]	1571 (108.9)		
Bronchiectasis, n (%)[c]	31 (56.4)		
Atopic, n (%)[d]	33 (57.9)	11.1	0.01
Total IgE (IU/ml)[a]	218.5 (81.2-751.5)	17.6 (6.0-53.0)	<0.0001
A. fumigatus IgE (kU/L)[a]	0.14 (0.05-11.6)	0.04 (0.04-0.06)	0.03
A. fumigatus IgG (mg/L)[a]	27.55 (10.5-61.8)	18.5 (5.0-30.4)	0.15
Sputum culture of filamentous fungi, n (%)	34 (58.6)	1 (11.1)	0.01
Sputum culture of *A. fumigatus*, n (%)	26 (44.8)	1 (11.1)	0.02

[a] Median with IQR in parentheses

[b] Mean with SEM in parentheses

[c] Of those scanned

[d] To common aeroallergens, with wheal ≥3mm (not including fungal panel)

6.3.2 MSQPCR calibration curves for fungal quantification

All non-template controls for each MSQPCR assay were negative for all data presented. Calibration curves were created for all target fungi (Figure 6.1) and the universal PenAsp assay (Figure 6.2). Manually adjusted baseline levels and thresholds were comparable for all fungi with the exception of the PenAsp assay, which required a lower threshold to enable detection ($\Delta Rn = 0.03$, Table 6.2). Amplification efficiencies for ΔC_T assays (after adjustment for extraction efficiency through subtraction of *G. candidum*) for *A. flavus, A. niger* and *P. chrysogenum* were comparable (1.90, 1.92 and 1.90 respectively); however, the amplification efficiency for the *A. fumigatus* and PenAsp assays were lower (1.73 and 1.78 respectively; Table 6.2). Similarly, theoretical mean conidia detection limits were lowest for *A. flavus, A. niger* and *P. chrysogenum* (<1, 3 and 5 respectively) and higher for *A. fumigatus* and PenAsp (both with a theoretical detection limit of 10 conidia).

For investigations using PenAsp primers, initial calibration was conducted using DNA extracted from a spore mix of the four target fungi *(A. fumigatus, A. niger*, *P. chrysogenum* and *A. flavus)* plus *P. variotii*. The amplification of fungal DNA was poor using this technique and enumeration of fungal levels predicted concentrations much lower than the sum of the target assays. MSPCR analysis of neat *A. fumigatus, A. niger, A. flavus, P. chrysogenum* and *P. variotii* showed that *A. niger* and *P. variotii* DNA were either not at all, or very weakly amplified. *A. fumigatus, A. flavus* and *P. chrysogenum* were amplified at consistent but differing efficiencies. Therefore, a calibration curve based on a single species would provide a much better correlation coefficient; however due to the heterogeneity of air and dust samples and the wide variety of *Aspergillus* and *Penicillium* species likely to be present other than the target species which were the focus of this study, a calibration curve was created for the

PenAsp assay based on the combined efficiencies of *A. flavus*, *A. fumigatus* and *P. chrysogenum* for approximations of *Aspergillus* and *Penicillium* fungi. The average of the three target fungi conidia concentrations used in initial DNA extractions were plotted against ΔC_T (Figure 6.2).

Figure 6.1. MSQPCR ΔC_T calibration curves for target fungi assays (*A. fumigatus*, *A. flavus*, *A. niger* and *P. chrysogenum*).

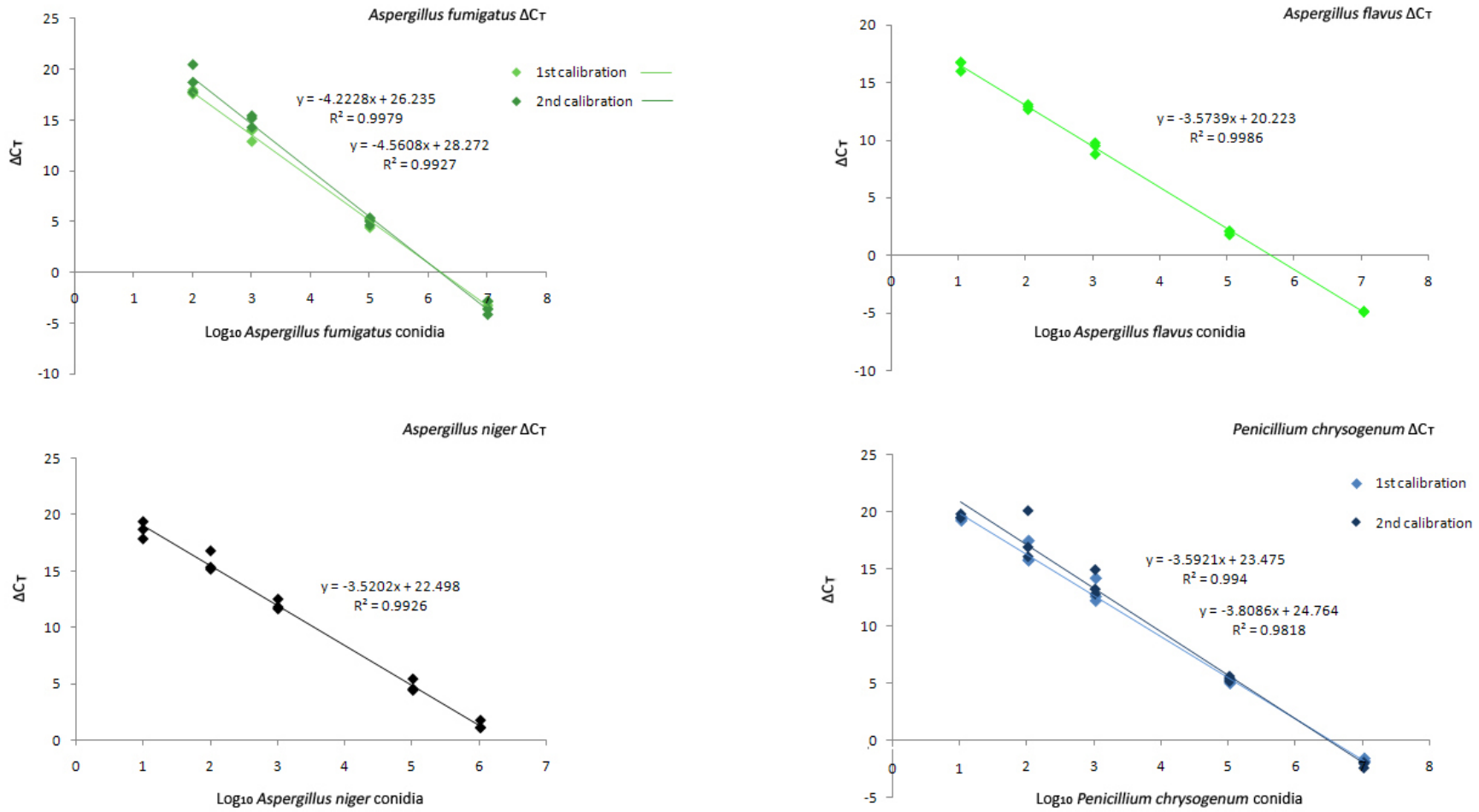


Figure 6.2. Calibration curve for the PenAsp assay based on DNA from *A. fumigatus, A. flavus* and *P. chrysogenum*

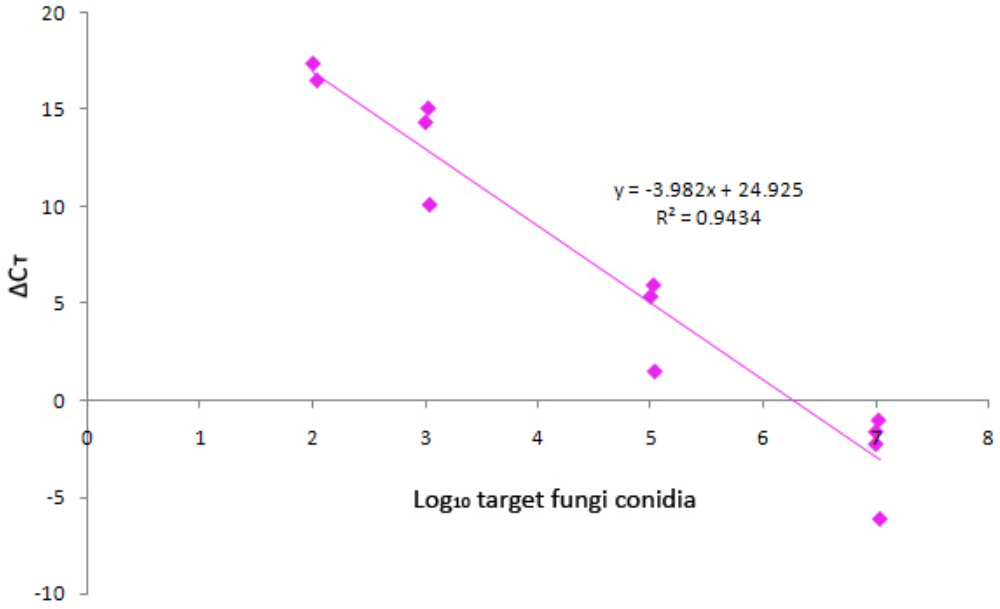


Table 6.2. Baseline and threshold values, amplification efficiencies and detection limits of assays

Summary of baseline ranges and threshold values selected for optimum efficiency of calibration curves, in addition to theoretical detection limits for target fungi and the generic PenAsp assay.

Primer	Range (C_T)	Threshold (ΔRn)	ΔC_T AE	MCDL
A. fumigatus	3-12	0.08	1.73 (1.66)	10 (42)
A. niger	3-13	0.08	1.92	3
A. flavus	3-12	0.07	1.90	<1
P. chrysogenum	3-12	0.06	1.90 (1.83)	5 (10)
PenAsp	3-11	0.03	1.78	10

Abbreviations: ΔRn: reporter dye (FAM)/passive reference dye; ΔC_T AE = amplification efficiency of delta C_T assay; MCDL = mean conidia detection limit.

6.3.3 Airborne concentrations of *A. fumigatus* by MSQPCR and *Asp/Pen*-type conidia by microscopy

Airborne concentrations of *A. fumigatus* ranged from 0-138 conidia/m^3 air. Only one healthy control was sputum culture positive for *A. fumigatus* and this subject was exposed to 129 conidia/m^3 air; the highest recorded for healthy controls (although the dustborne concentrations did not correlate). The highest airborne concentrations were recorded in the home of this healthy subject and two asthma patients, all of whom were sputum culture positive for *A. fumigatus.* There was a trend for higher levels of airborne *Asp/Pen*-type conidia, as determined by microscopy, in homes of asthma patients with a sputum culture of *A. fumigatus* in comparison to culture negative patients although this did not quite reach significance (p = 0.0575; Figure 6.3A). There was also a trend towards higher daily maximum *Asp/Pen*-type conidia concentrations, for a two hourly period, in asthma patients with a positive sputum culture of *A. fumigatus* in comparison to those without (median (IQR) = 46.5 (12.4-186.0) *vs.* 24.8 (12.4-71.3) conidia/m^3 air respectively, p = 0.1). Normalising the data and adjusting for zeros within the dataset showed the trend approaching significance (p = 0.06, Figure 6.3B) However, the concentrations of airborne *Asp/Pen*-type conidia in homes of asthmatics fell within the normal ranges of *Asp/Pen*-type conidia from non-complaint properties recorded in Chapter 3 (Figure 6.3A). Specifically targeting airborne levels of *A. fumigatus* by MSQPCR demonstrated significantly higher airborne levels in culture positive patients (p = 0.040; Figure 6.3C). No association was found, however, between airborne levels of *Asp/Pen*-type or *A. fumigatus* conidia and sensitisation to *A. fumigatus* (Figures 6.4A and B respectively).

Airborne concentrations of *A. fumigatus* conidia equivalents detected by MSQPCR were positively correlated with concentrations of *Asp/Pen*-type conidia (per m^3 air)

determined by microscopic analysis (Figure 6.5). The relationship was strengthened by two highly correlated data points. Removal of these data points and reanalysis of the correlation weakened the association and removed significance of the correlation ($r = 0.239$, $p > 0.05$).

Figure 6.3. Airborne levels of *A. fumigatus* and *Asp/Pen*-type conidia in comparison to isolation of *A. fumigatus* from sputum

Airborne levels of *Asp/Pen*-type conidia detected by microscopy in homes of *A. fumigatus* sputum culture positive and negative asthma patients and healthy controls, in comparison to levels of *Asp/Pen*-type conidia from 100 non-complaint (Baseline) properties in Chapter 3 (A); daily maximum *Asp/Pen*-type concentrations for a two hour period determined by microscopy (B); and *A. fumigatus* conidia equivalents detected by MSQPCR (C) in homes of *A. fumigatus* sputum culture positive and negative asthma patients and healthy controls. With the exception of the baseline data, *A. fumigatus* sputum culture positive and negative subjects are represented by closed and open symbols respectively. Lines represent mean and SEM.

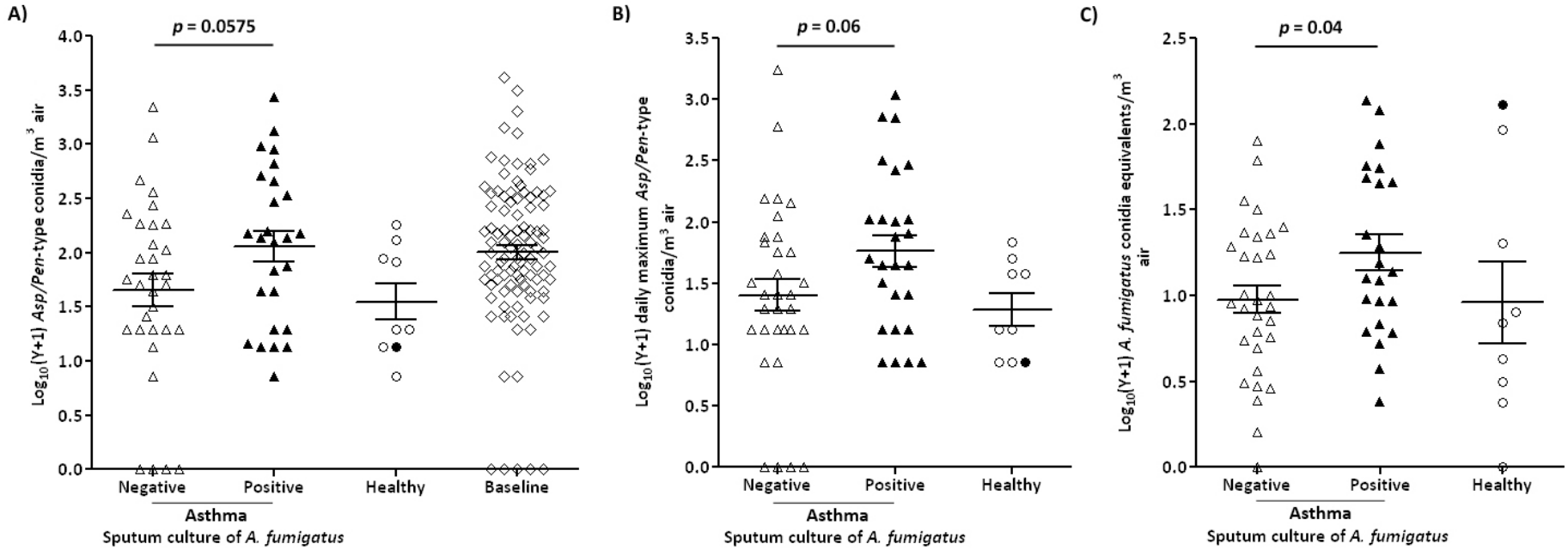


Figure 6.4. Airborne *Asp/Pen*-type and *A. fumigatus* conidia in relation to measures of *A. fumigatus* sensitisation

Airborne *Asp/Pen*-type conidia (A) and *A. fumigatus* conidia equivalents (B) per m^3 air in homes of *A. fumigatus* IgE (+/- IgG) sensitised, IgG only sensitised and non-sensitised asthma patients, in comparison to healthy controls. Lines represent mean and SEM.

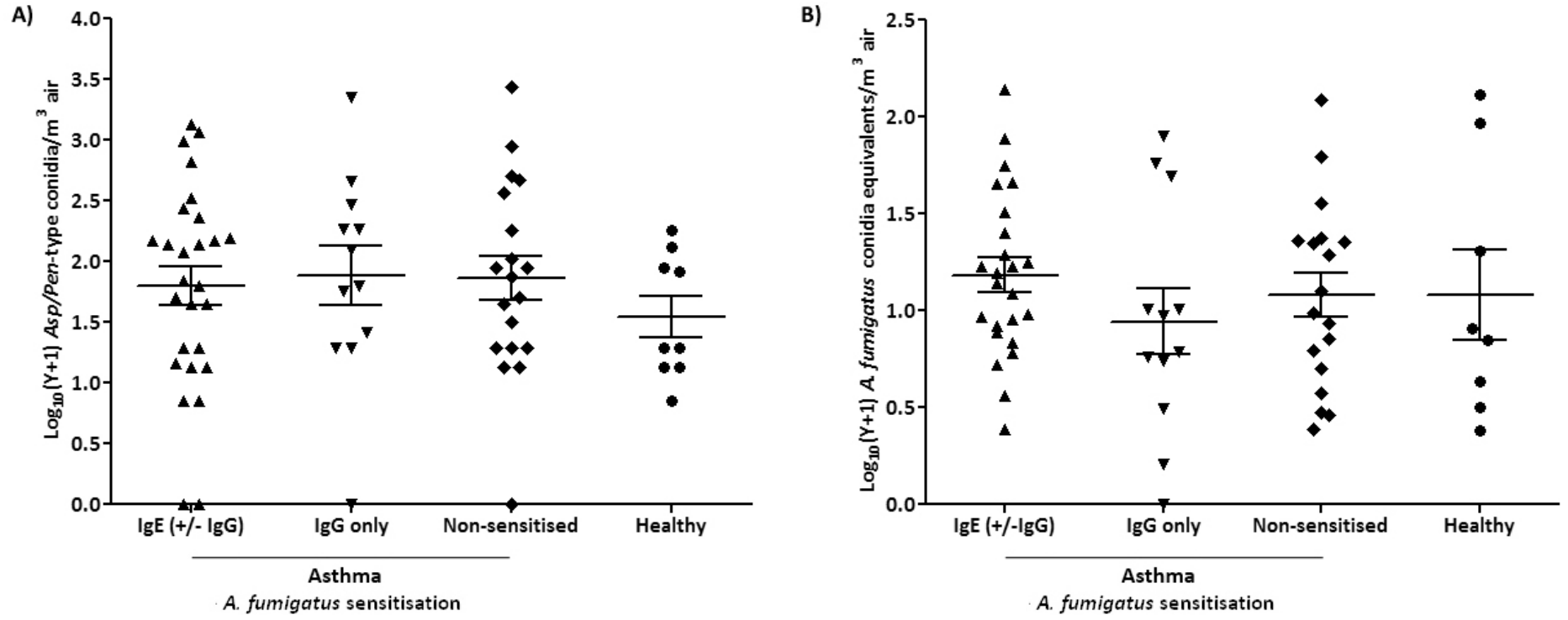
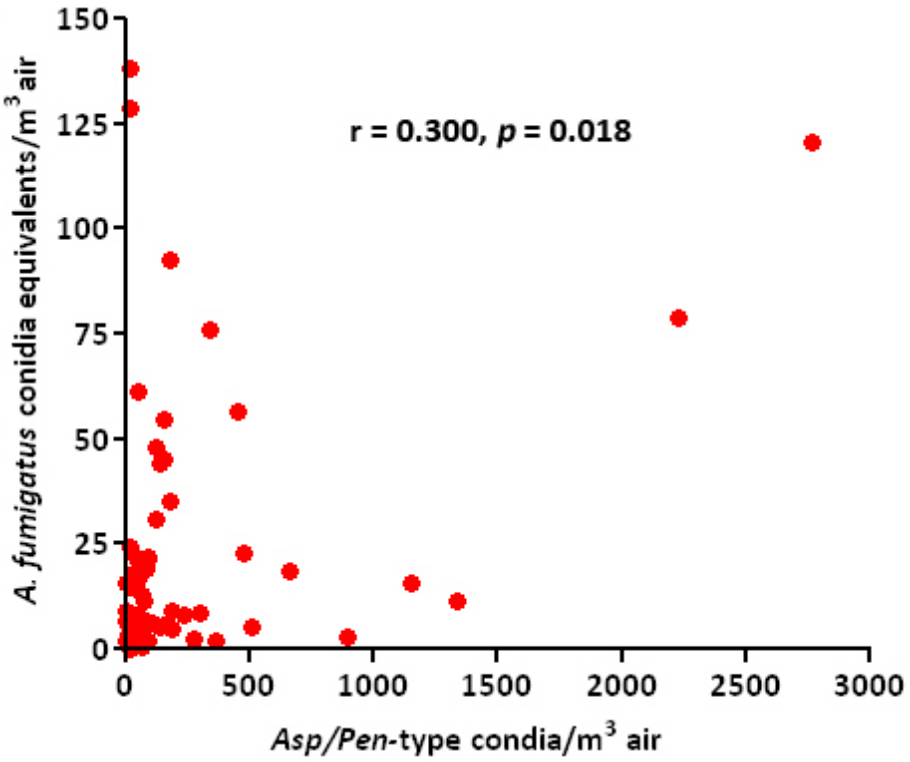


Figure 6.5. Correlation of *A. fumigatus* concentrations with *Asp/Pen*-type conidia

Correlation of airborne concentrations of *A. fumigatus* conidia equivalents by MSQPCR with airborne *Asp/Pen*-type conidia concentrations (conidia/ m^3 air) determined by microscopy (Spearman's rank correlation coefficient r = 0.300, p = 0.018).



6.3.4 Dustborne concentrations of *A. fumigatus* by MSQPCR

Dustborne concentrations of *A. fumigatus* ranged from 0-3180 conidia equivalents per 5 mg dust. No association was found between dustborne levels of *A. fumigatus* and either isolation from sputum (Figure 6.6A), or sensitisation to *A. fumigatus* (Figure 6.6B). Quantities of dust obtained from properties were highly variable between properties and ranged from 0.02-4.96 grams (median = 0.346 g, IQR: 0.139-0.679 g) and significantly higher quantities of dust were obtained from properties which were carpeted in comparison to laminate flooring ($p < 0.05$, Figure 6.7). However, there was no

difference between quantity of dust collected and either *A. fumigatus* sputum culture or sensitisation and no difference between groups following extrapolation of data to account for absolute quantities of *A. fumigatus* conidia equivalents collected from each property using the quantity of dust obtained.

Figure 6.6. Dustborne concentrations of *A. fumigatus*

Dustborne levels of *A. fumigatus* (conidia equivalents/5 mg of dust) in: homes of *A. fumigatus* sputum culture positive and negative asthmatics and healthy controls (the single culture positive healthy control is represented by a closed circle; lines represent median with IQR) (A); and homes of *A. fumigatus* IgE sensitised (IgE +/- IgG), IgG only sensitised and non-sensitised people with asthma and healthy controls. Lines represent mean with SEM (B).

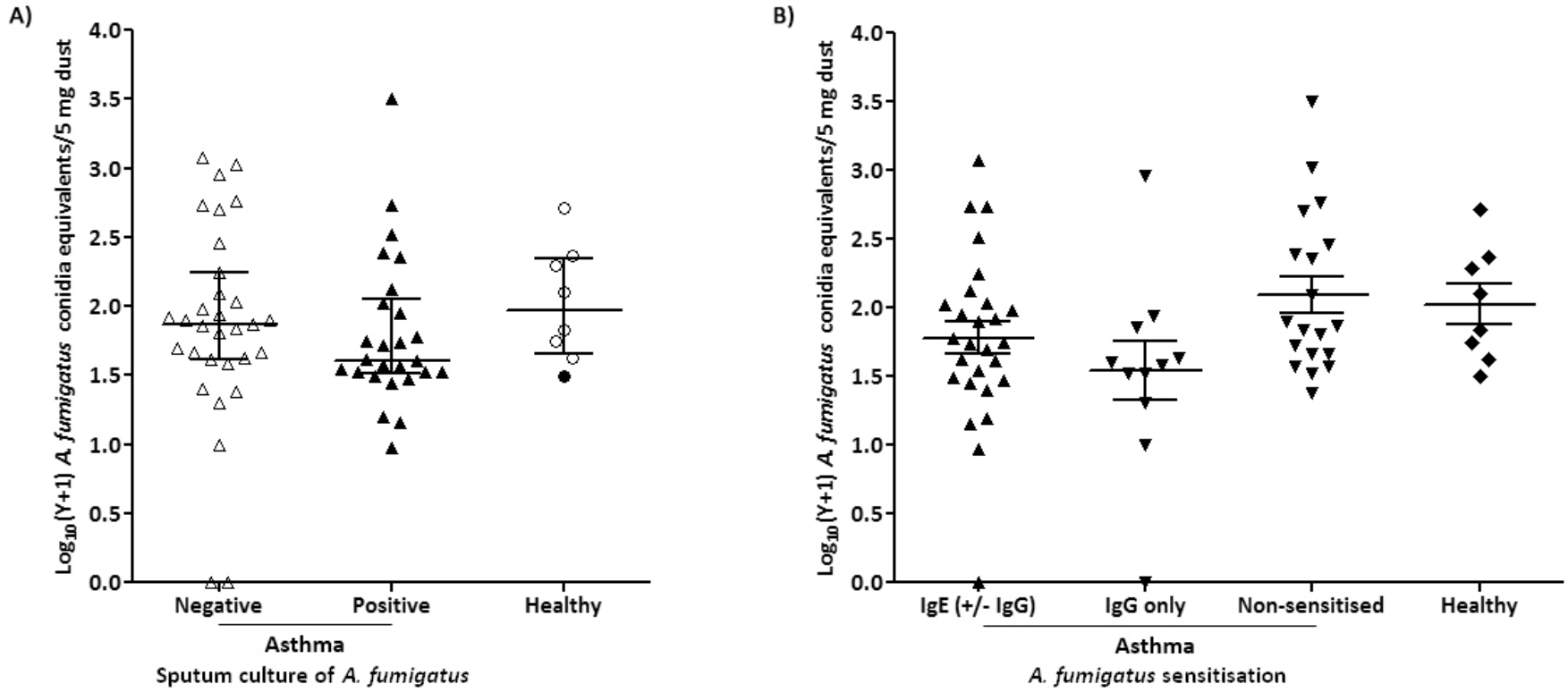
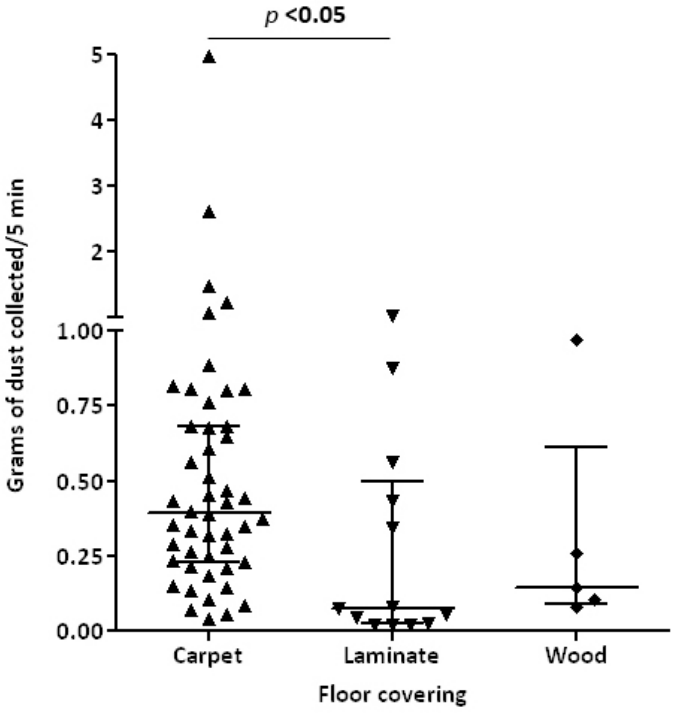


Figure 6.7. Quantity of dust collected from different types of flooring

Quantity of dust collected according to type of flooring in room sampled from participant homes. Lines represent median with IQR.

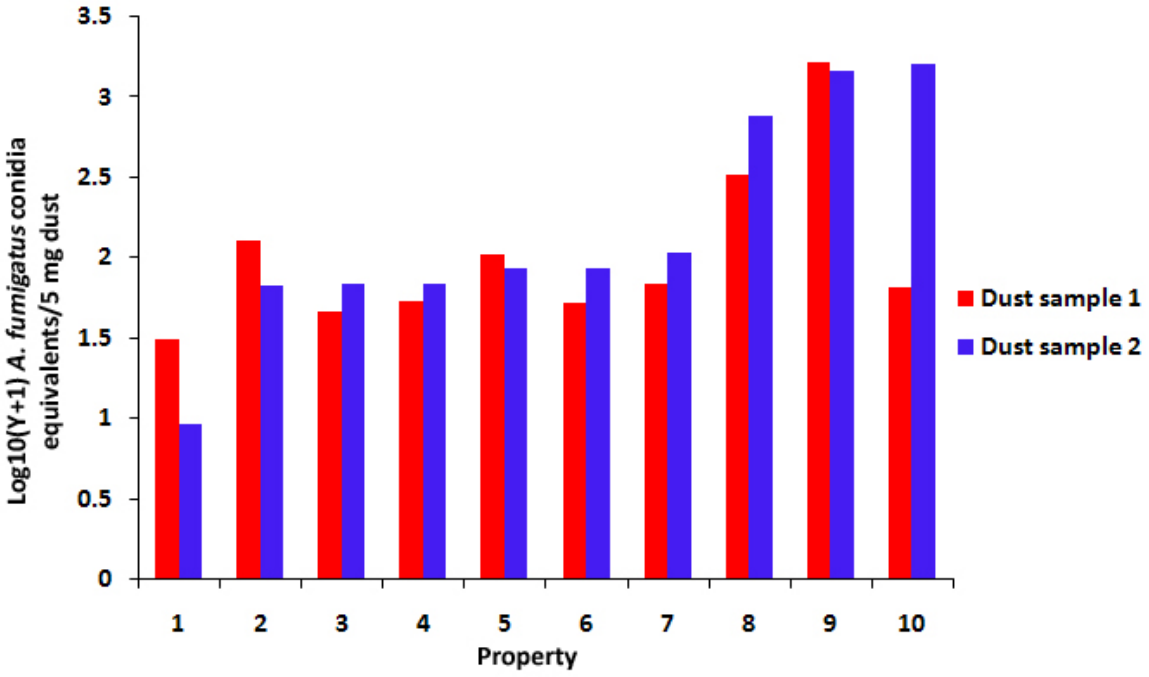


6.3.5 Variability between dust samples

In order to assess the variability in dustborne *A. fumigatus* conidia concentrations between duplicate 5 mg dust samples, *A. fumigatus* DNA extraction and MSQPCR was performed on two dust samples from each of ten properties. An acceptable level of consistency in *A. fumigatus* dustborne conidia equivalents was shown between duplicate 5 mg samples using Cronbach's alpha reliability coefficient ($\alpha = 0.795$, $p = 0.014$). Eight of the ten duplicate samples showed very good consistency, with differences noted in two of the properties (one and 10, Figure 6.8) [232].

Figure 6.8. Variation between duplicate dust samples

Dustborne concentrations of *A. fumigatus* conidia equivalents were measured from duplicate dust samples from ten properties, showing acceptable consistency (Cronbach's $\alpha = 0.795$, $p = 0.014$).



6.3.6 Airborne and dustborne levels of target fungi

Figure 6.9 shows the non-transformed data from MSQPCR analysis of *P. chrysogenum*, *A. flavus* and *A. niger* conidia equivalents respectively, from air and dust samples in homes of asthma patients and healthy controls. Airborne *P. chrysogenum* was converted to conidia equivalents /m^3 air (Figure 6.9A); however, due to very low levels recorded of *A. flavus* and *A. niger* (Table 6.4), concentrations of these fungi from air samples are presented as airborne concentrations per 24 hour period (Figure 6.9C and 6.9E respectively).

No healthy controls were sputum culture positive for any of the target fungi investigated other than *A. fumigatus* (for which one was positive). Five asthma patients were sputum culture positive for *Aspergillus* section *Nigri* fungi; two for *Penicillium* subgenus

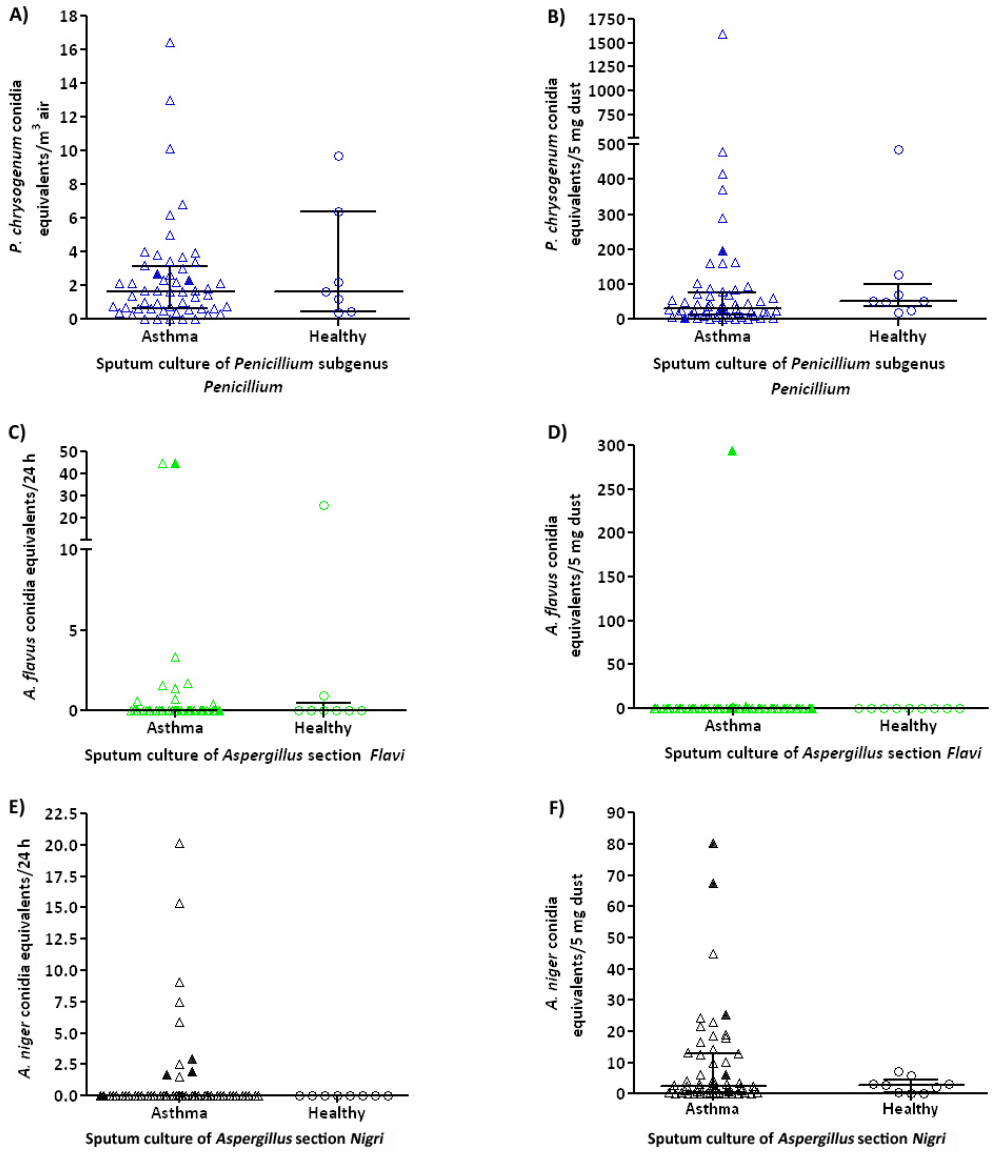
Penicillium (which contains the species *P. chrysogenum)* and two were sputum culture positive for *Aspergillus* section *Flavi*. Airborne concentrations of *P. chrysogenum* ranged from 0-16 conidia/m^3 air for the 67 homes analysed. Airborne levels in the homes of the two *Penicillium* subgenus *Penicillium* sputum culture positive asthma patients had detectable levels within this range (2 and 3 conidia/m^3 air respectively); one of which also had moderate levels of dustborne concentrations (197 conidia/5 mg) (Figures 6.9B).

One of two patients sputum culture positive for *Aspergillus* section *Flavi* had the second highest levels of airborne *A. flavus* conidia equivalents (45 conidia/day) and the highest recorded dustborne concentrations in their home (294 conidia/5 mg dust, Figure 6.10A), whereas the second culture positive patient had undetectable levels in their home. Two of the five *Aspergillus* section *Nigri* sputum culture positive asthmatics were exposed to the highest levels detected of dustborne *A. niger* conidia equivalents (80 and 68 conidia/5 mg dust), whilst having very low but detectable airborne concentrations (3 and 2 conidia/day respectively). Airborne *A. niger* conidia equivalent concentrations were <1 for a 24 hour sample period in two of the five sputum culture positive asthma patients (Figure 6.10B).

There was a significant negative correlation between airborne and dustborne concentrations of *A. fumigatus* ($r = -0.287$, $p = 0.025$); however, there were no other significant associations between airborne and dustborne concentrations of target and PenAsp fungi.

Figure 6.9. *P. chrysogenum, A. flavus* and *A. niger* in air and dust samples

P. chrysogenum (B) conidia equivalents per m^3 air (A) and per 5 mg dust (B); *A. flavus* conidia equivalents in 24 hr air samples (C) and 5 mg dust (D); and *A. niger* conidia equivalents in 24 hour air samples (E) and 5 mg dust samples (F) in sputum culture positive and negative asthma patients and healthy controls. Closed symbols denote sputum culture positive subjects. Lines represent median with IQR.



6.3.7 Influence of home characteristics and seasonal variation

The majority of study participants (73.1%) had carpeted living rooms, central heating (89.6%), double glazing (94.0%) and wall cavity insulation (63.8% of those able to answer). Home samples from asthma patients were collected across seasons; however, sampling from homes of healthy volunteers was predominantly restricted to the winter (Table 6.3). 19% of asthma patients reported the presence of visible mould and condensation; however this was predominantly identified in the kitchen or bathroom of properties, with only 6.9% of properties reporting mould or condensation in the living room. Five properties had history of flooding or damp remediation known to occupants, again predominantly affecting rooms other than the living room. 32.8% of asthma patients had one or more pets, 27.6% had a cat or dog, in comparison to 55.6% of healthy subjects who had pets. There was no significant difference in either airborne or dustborne concentrations of *A. fumigatus* in relation to any of the home characteristics examined, although associations were difficult due to limited numbers in each category. A significant effect of season was shown on airborne concentrations, but not dustborne concentrations of *A. fumigatus*, with higher levels in winter in comparison to the spring and summer ($p < 0.05$, Figure 6.10).

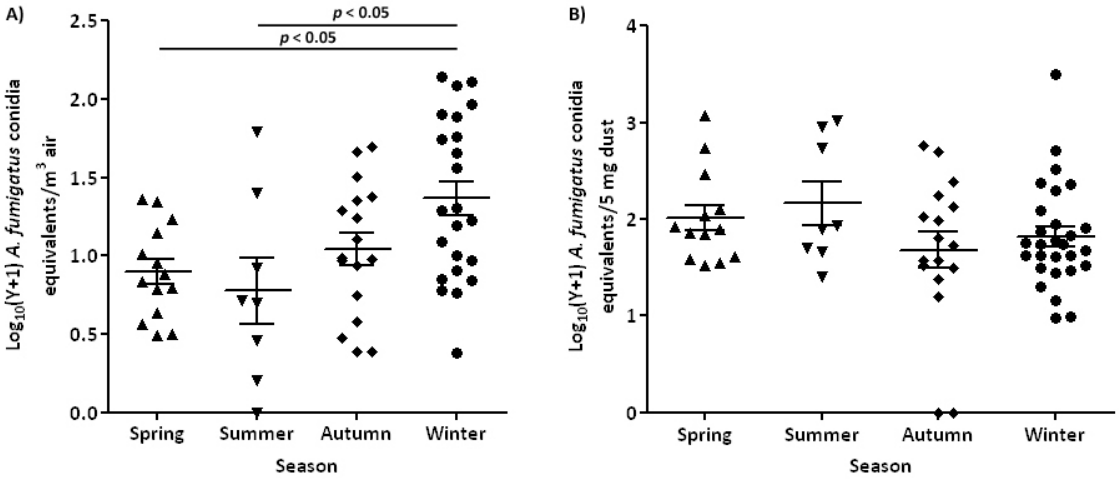
Table 6.3. Home characteristics and seasons sampled

Variable	Asthma	Healthy
Season		
Spring	13 (22.4)	2 (22.2)
Summer	9 (15.5)	0 (0)
Autumn	16 (27.6)	0 (0)
Winter	20 (34.5)	7 (77.8)
Central heating, n (%)	52 (89.7)	8 (88.9)
Flooring, n (%)		
Carpet	44 (75.9)	5 (55.6)
Laminate	10 (17.2)	3 (3.3)
Hard wood	4 (6.9)	1 (1.1)
Type of housing, n (%)[a]		
Detached	16 (27.6)	5 (55.6)
Semi	22 (37.9)	3 (33.3)
Terrace	10 (17.2)	1 (11.1)
Flat	3 (5.2)	0 (0)
Bungalow	6 (10.3)	0 (0)
Other	1 (1.7)	
Wall cavity insulation, yes/n	32/51	5/7
Double glazing, n (%)	54 (93.1)	9 (100)
Age of housing		
0-30	15 (26.8)	3 (33.3)
31-60	24 (42.9)	4 (44.4)
61-90	8 (14.3)	0
>90	9 (16.1)	2 (22.2)
Pets, n (%)		
Cat(s)	8 (13.8)	4 (44.4)
Dog(s)	12 (20.7)	3 (33.3)
Cat(s) and dog(s)[b]	3 (5.2)	2 (22.2)
Birds	2 (3.4)	0
Other	3 (5.2)[c]	1 (11.1)
Visible mould	11 (19.0)[d]	1 (11.1)
Condensation	11 (19.0)[d]	2 (22.2)
Water intrusion	8 (13.8)[e]	0
History of damp problem	5 (8.6)	0

[a] Only one property had a cellar
[b] Included separately and together
[c] No cat or dog
[d] 4 in living room
[e] 1 in living room

Figure 6.10. Seasonal variation in airborne and dustborne *A. fumigatus* concentrations

Airborne (A) and dustborne (B) concentrations of *A. fumigatus* according to season (n = 62 and n = 66 respectively)



6.3.8 Quantification of *Asp/Pen*-type fungi by MSQPCR

There was a trend towards higher airborne concentrations of PenAsp species in homes of asthma patients sputum culture positive for *Aspergillus* or *Penicillium* species in comparison to sputum culture negative asthmatics ($p = 0.09$). Airborne *Aspergillus/Penicillium* concentrations quantified by microscopy or MSQPCR were positively correlated ($r = 0.268$, $p = 0.035$, Figure 6.11); however whilst quantities appeared comparable prior to adjustment for litres of air sampled, when converted to conidia/m^3 air, concentrations determined by microscopy were much higher. The weak positive correlation shown between *Asp/Pen*-type conidia concentrations determined by MSQPCR and microscopy were influenced by two obvious datapoints, which when removed, reduced the correlation coefficient and removed the significance of the association ($r = 0.200$, $p > 0.05$). Average concentrations of dustborne conidia determined using PenAsp assay were much higher than the sum of the species-specific assays used, as would be expected, since few *Aspergillus/Penicillium* species were

targeted in this study; however, detection in air was much lower using the generic PenAsp assay (Figures 6.12A and B). There were no significant correlations between concentrations of PenAsp conidia equivalents determined by MSQPCR and detection of fungi in sputum.

Descriptive statistics of concentrations of airborne and dustborne concentrations of fungi targeted are summarised in Table 6.4. The standard deviations of triplicate reaction ranged from 0-1.48 for target fungi assays (average of 0.41-0.67), but were more variable for the PenAsp assay (Table 6.5).

Figure 6.11. Correlation between *Aspergillus/Penicillium* fungi quantified by microscopy and MSQPCR

Aspergillus/Penicillium-type conidia calculated by MSQPCR were positively correlated with concentrations determined by microscopy (conidia/m^3 air)

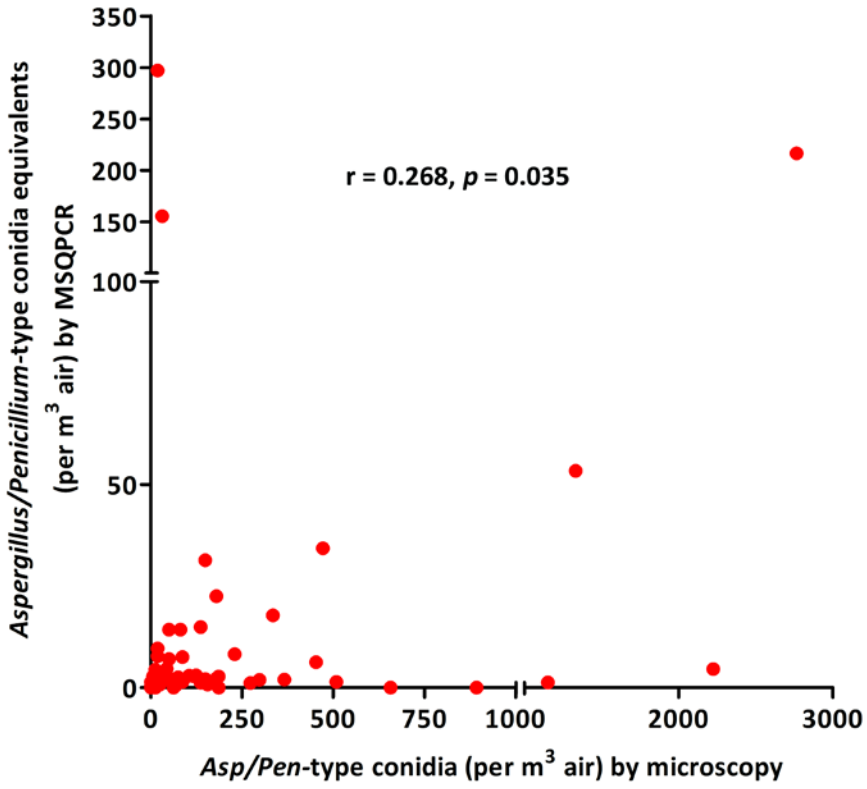


Figure 6.12. Quantification of *Aspergillus/Penicillium*-type fungi using species-specific assays in comparison to the PenAsp assay

Total concentrations of airborne (A) and dustborne conidia (B) from the sum of the species-specific assays and generic PenAsp assay. Charts represent median values with IQR.

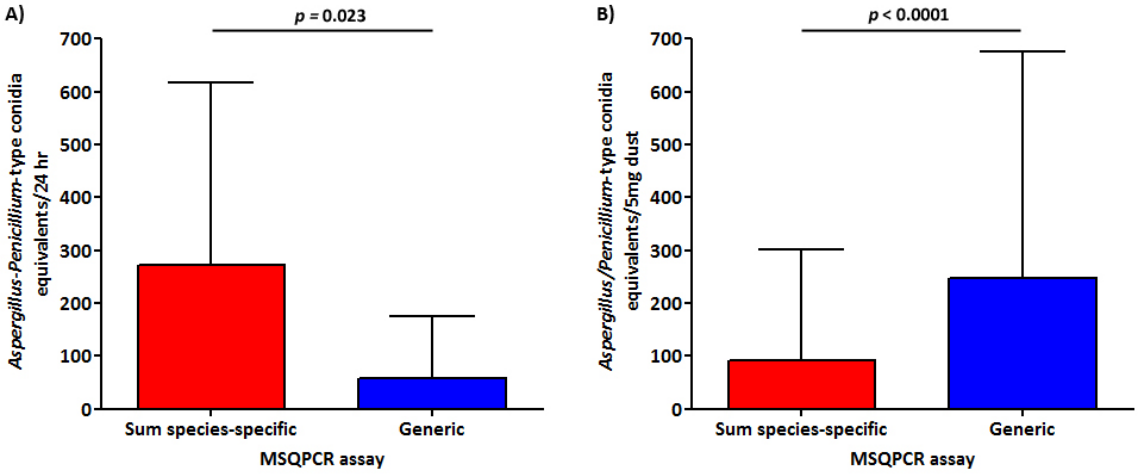


Table 6.4. Indoor airborne and dustborne distributions of target fungi

Descriptive statistics showing the indoor airborne distributions of *A. flavus, A. niger, P. chrysogenum, A. fumigatus* and PenAsp fungi by MSQPCR in 24 hour air samples (n = 62); *Asp/Pen* type conidia by microscopy (conidia/m^3 air, n = 67); dustborne distributions of target fungi by MSQPCR (per 5 mg dust, n = 66); and number of asthma patients sputum culture positive for target fungi (Culture +ve).

Target fungi/assay	*A. flavus*	*A. niger*	*P. chrysogenum*	*A. fumigatus*	PenAsp	*Asp/Pen*-type
Culture +ve, n (%)	2 (3.4)	5 (8.6)	2 (3.4)	26 (44.8)[a]	34 (58.6)	34 (58.6)
Air						
Minimum	0	0	0	0	0	0
Maximum	45	20	391	3288	7060	2765
Mean	2	1	58	552	380	240
Median	0	0	39	211	52	68
GM (log GM)	1.2 (0.081)	1.3 (0.108)	28.5 (1.455)	241.4 (2.383)	50.1 (1.700)	61.2 (1.787)
% Properties	16	16	92	98	87	94
Dust						
Minimum	0	0	0	0	0	Not measured
Maximum	294	80	1593	3180	17556	Not measured
Mean	5	8	95	202	738	Not measured
Median	0	2	37	61	229	Not measured
GM (log GM)	0.8 (-0.075)	2.8 (0.449)	31.6 (1.500)	71.4 (1.85)	181.9 (2.260)	Not measured
% Properties	11	73	97	97	98	Not measured

Abbreviations: GM, geometric mean

[a] Plus one healthy control

Table 6.5. Variability of MSQPCR reactions

Variation of individual MSQPCR assays showing mean (range) values of the standard deviation of triplicate reactions.

	A. flavus	*A. niger*	*P. chrysogenum*	*A. fumigatus*	*PenAsp*	*G. candidum*
Dust	0.50 (0.11-1.45)	0.52 (0.03-1.35)	0.55 (0.03-1.38)	0.60 (0.06-1.48)	0.84 (0.00-2.14)	0.14 (0.00-0.58)
Air	0.41 (0.08-0.71)	0.64 (0.25-1.17)	0.57 (0.00-1.36)	0.67 (0.04-1.48)	0.54 (0.08-2.65)	0.11 (0.02-0.49)

6.4 Case study

An interesting case study which transpired through collection of data for this study was a non-*A. fumigatus*-sensitised asthma patient, who was sputum culture positive for three of the four target fungi (*A. fumigatus, Aspergillus* section *Flavi* and *A. niger*) and *P. variotii*. Airborne and dustborne *A. flavus* concentrations found in the patient's property represented the maximum levels found in any of the study participant homes; *A. niger* dust concentrations were second highest recorded (airborne concentrations, whilst very low, were one of the few with detectable levels); highest dustborne concentrations of *A. fumigatus,* and third highest airborne concentrations; second highest airborne *Asp/Pen* concentrations by MSQPCR (dustborne levels did not correlate) and highest *Asp/Pen*-type concentrations by microscopy. Amplifiable levels of *P. variotii* were also discovered but were not quantified for this study.

6.5 Discussion

6.5.1 Sputum culture and airway colonisation

This is the first study to show a direct link between indoor exposure to fungi and a positive sputum culture, indicating airways colonisation in patients with asthma. *Aspergillus* and *Penicillium* conidia can be found in high concentrations indoors, even within non-complaint homes without obvious damp (Chapter 3, [178, 297]). Fungal sensitisation may be attributed to either environmental exposure or internal exposure to colonising fungi [12]; however, it is unclear whether isolation of multiple fungi from sputum indicates environmental contamination, impaired clearance or elevated exposure. The data from this study indicates a trend towards increased exposure to

Asp/Pen-type fungi and significantly higher levels of exposure to airborne *A. fumigatus* in homes of asthma patients who are sputum culture positive for *A. fumigatus.* However, no direct association between home exposure and *A. fumigatus* sensitisation was shown. As discussed in previous chapters, very low detection rates of fungi in sputum of healthy controls compared with sputum from people with asthma suggests that fungal isolates were not representative of environmental contamination or inhaled conidia in the oropharageal cavity at the time of sputum collection; although high dose inhaled steroids may predispose asthmatics to airways colonisation by fungi.

Due to the limited numbers of patients who were culture positive for other fungi (non-*A. fumigatus*) targeted in this study, it is difficult to attribute clinical relevance to the data. However, it interesting that those patients culturing specific fungi from their sputum often had detectable home exposures to the fungi cultured. A larger scale study would be required to address longitudinal aspects of colonisation of fungi other than *A. fumigatus* and to better evaluate the association between culture of fungi other than *A. fumigatus* and exposure. Furthermore, genotyping of environmental and clinical isolates, to confirm matching strains in the home and lung, would be necessary to confirm a direct link.

6.5.2 MSQPCR to detect indoor fungi levels

The comparative C_T method for identification of fungi enables approximation of fungal conidia in environmental samples utilising *G. candidum* as an external reference organism which controls for variability in DNA extraction efficiencies [203, 207, 228]. The MSQPCR technique has been shown to detect both higher levels and a greater diversity of fungal contamination than traditional culture-based methods [298]. All of the primers utilised in this study have been validated for identification of *Aspergillus, Penicillium* and *Paecilomyces* species found indoor [205]. The amplification

efficiencies for some of the target fungi presented in this study were comparable to data previously reported [205] (*A. niger* 1.92 *vs.* 1.98; *A. flavus* 1.90 *vs.* 1.89 *P. chrysogenum* 1.90 *vs.*1.93 respectively); however the efficiencies of the *A. fumigatus* and generic PenAsp assay were much lower than previously reported (*A. fumigatus* 1.73 *vs.* 1.91 and PenAsp 1.76 *vs.* 1.98 respectively). Furthermore, recalculation of primer efficiencies in assays which required new reagents (*P. chrysogenum* and *A. fumigatus*) had lower amplification efficiencies in the second calibration (1.83 and 1.66 respectively), which could be explained by possible degradation of target DNA. The efficiencies of these assays were, however, within the ranges reported previously for MSQPCR of *Aspergillus* and *Penicillium* species, where the lowest assay amplification efficiency was 1.64, and the highest, an ideal amplification efficiency of two [205].

The standard deviation of triplicate repeats in each target fungi assay ranged from 0-1.48 C_T, which is within the acceptable inherent variability of the technique (1.5 C_T, Dr Stephen Vesper, personal communication). The PenAsp assay was more variable due to problems with amplification of DNA, particularly towards the limit of detection. The variability of the *G. candidum* assay was greatly reduced due to the large quantity of DNA co-extracted with each sample.

The ERMI was developed in the US to help distinguish fungi associated with water-damage from common indoor fungi from non-complaint homes, using 36 widely distributed fungi from over 130 published MSQPCR assays [298, 303]. 'Group 1' fungi, indicative of water-damage or visible mould include *A. fumigatus*, *A. niger* and *A. flavus*, three of the target fungi investigated in this study. ERMI group 2 fungi, common indoor fungi in reference homes, include *P. chrysogenum*; however, two recent European studies have shown *P. chrysogenum* to be a common contaminant of water-damaged buildings [165, 166]. This study showed that *P. chrysogenum* was very

common in UK homes, where the majority sampled were considered non-complaint; only 18% of occupants reported the presence of mould or condensation and only 12% reported any evidence of damp within the property.

There have been few previous studies investigating home exposure to fungi in the UK. One study utilised culture-based methods from air samples and found that *Penicillium* species were most commonly detected, followed by *Cladosporium* species, and found significantly lower levels of total viable fungal concentrations in semi-detached homes in comparison to other building types [304]. A more recent study [210] investigated the concentrations of fungi in house dust by MSQPCR in a random selection of 45 non-contaminated (reference) homes in Ohio (USA) in comparison to 11 UK homes. The study found that the majority of the 81 fungal species or assay types investigated were not significantly different; however, 13 species, predominantly *Penicillium* (but not including *P. chrysogenum*) were significantly different in the USA compared with the UK including concentrations of *A. niger*, which were significantly lower in the UK [210]. The data reported here supports this finding, with *A. niger* conidia very rarely detected and in much lower quantities than *A. fumigatus, P. chrysogenum* or generic *Asp/Pen*-type conidia. Quantities of *A. niger* and *P. chrysogenum* conidia equivalents per 5 mg dust were comparable to those reported in British homes previously (log geometric mean = 1.500 *vs.* 1.467 (reported previously) for *P. chrysogenum* and 0.449 *vs.* 0.223 for *A. niger*); however, concentrations of *A. fumigatus* were much higher in this study in comparison to the previous study (log geometric mean = 1.854 *vs.* -0.149) and *A. flavus* concentrations were lower (-0.075 *vs.* 0.240) [210].

6.5.3 Links between asthma and indoor exposure to fungi

This study has shown significantly higher levels of airborne *A. fumigatus* conidia equivalents in homes of asthma patients with a positive sputum culture for the fungus.

However, *A. fumigatus* concentrations in homes of asthma patients were no higher than levels in the homes of healthy controls and *Asp/Pen*-type conidia did not exceed typical ranges reported for non-complaint properties in Chapter 3. Many indoor studies focus on indoor exposure and development of asthma and allergy in children, probably due to the reduction in confounding variables in collection of measurements, such as occupation, longevity of disease and smoking status. A small study investigating fungal concentrations in 13 homes of people with and without ABPA concluded that host susceptibility was more important than environmental exposure; however, after sensitisation, environmental exposure to *Aspergillus* was thought to exacerbate symptoms, particularly over the winter months [139]. Culture of *Cladosporium* and *Alternaria* from nasal lavage has been shown to reflect environmental exposure [305]. No associations between the presence of fungi in dust and either sputum culture or sensitisation were found in this study. Due to the ease of sampling and potential for a large quantity of sample material, dust samples are commonly utilised for investigations of environmental exposures and respiratory symptoms. Exposure to hydrophilic fungi has been associated with post-building occupancy onset asthma in adults working in a water-damaged office building [300] and dustborne fungal concentrations have also been associated with childhood asthma [298, 299]. Specifically, airborne concentrations of *Penicillium* and dustborne concentrations of *Alternaria, Cladosporium* and zygomycetes (collected by culture) have been associated with lower respiratory tract illness in children within the first year of life [306]. Remediation of mould and moisture sources from water-damaged homes was associated with reduced numbers of exacerbations and symptom days (following adjustment for asthma severity and season) [170]; however, children living on farms can be exposed to a larger diversity of fungi (based on culture studies) which has been associated with a reduced risk of asthma

[307]. Therefore, more research is required in order to ascertain the potential risk *versus* beneficial effects of mould exposure.

6.5.4 Alternative sites of exposure

The link between sputum culture and exposure shown in this study is a very interesting observation, particularly taking into consideration the numerous alternative sites of exposure, such as the work or garden environments, and the potential confounding factors. For example, whilst data is available on the duration of asthma, for patients sensitised to *Asp/Pen*-type fungi, there is no way of knowing when initial exposure, infection or airways colonisation may have been, or how long the patients have been sensitised. Regarding the home environment, changes in home design may have had a large effect on airborne and dustborne fungal conidia concentrations, such as change of flooring in the room sampled. Reported indoor sources of *A. fumigatus* include houseplants insulation material, soil of potted plants and used pillows [128, 243, 244], with the fungus also occasionally isolated from water-damaged floor materials and concrete [165]. Questioning of study participants during home investigations suggested that anecdotally, a number of patients (on advice from clinicians) had made alterations to their properties, such as removal of pets or carpets to reduce allergen exposure; one patient had made drastic attempts to reduce allergen exposure by removing all vegetation within the vicinity of the property. Personal communications with the patients also suggested other potential sources of fungal exposure, such as the workplace (in packaging or workshop environments handling damp wood-based materials), during water intrusion problems within properties, holiday activities, and one patient reported a build up of bird excrement behind a fireplace which had been rectified prior to sampling for this study. Many asthma patients recruited into the study were either of, or approaching, retirement age and some were too ill to work; however, many

were avid gardeners. The presence of high quantities of *A. fumigatus* in compost and bird excrement means that avid gardeners or bird keepers could be exposed to very high levels of the fungus [33, 62].

It is unclear whether airways colonisation transpires through either long-term exposure to moderate levels of fungi, or through a single exposure to very high concentrations. It is probable that colonised patients may result from a combination of the two types of exposure; however, it is not possible to investigate these theories since relating exposure to clinical outcomes is currently only possible via *post hoc* analysis. Large scale studies examining detailed histories of potential sources of exposure from fungal sensitised and/or colonised asthmatics may shed some light on this issue, with future studies of personal exposure and models of airways colonisation required to find a link between exposure limits and sustained colonisation.

6.5.5 MSQPCR in comparison to traditional microscopy of air samples

The positive correlation between *Asp/Pen*-type spore concentrations analysed by microscopy and *A. fumigatus* conidia equivalents analysed by MSQPCR is encouraging; however, some data did not correlate, and removing two obviously better correlated data points resulted in loss of significance of the association. High *Asp/Pen*-type concentrations predominated by species not measured in this study, suboptimal DNA extraction or inhibition of amplification could explain high *Asp/Pen*-type and low *A. fumigatus* concentrations; vice versa contradictions in the data may be explained by differences in orifice size and presentation for the two samplers affecting efficiency of collection, since the cyclone sampler has been shown to be superior in collection efficiency of spores smaller than 2.5µm in size in comparison to the standard spore traps [186, 242].

The data from the universal PenAsp primers were disappointing, especially with regard to the airborne analysis. Despite a weak positive correlation between *Aspergillus* and *Penicillium*-type concentrations analysed by microscopy and MSQPCR, concentrations determined by MSQPCR were much lower when adjusted to conidia equivalents per cubic metre of air. Furthermore, removal of the best correlated data points resulted in loss of significance of the association. The universal PenAsp primers have been designed to amplify multiple species and amplification efficiencies for individual species will vary (as shown in the preliminary data for this study). Comparison of the sum of species-specific target assays with the universal assay in air and dust samples suggests that either something in the air samples interfered with fungal DNA amplification for the universal amplification of air samples, or that fungal species present (with different amplification efficiencies) were different between the air and dust samples.

6.5.6 Correlations of dust *versus* air

There was a significant negative correlation between *A. fumigatus* levels in air and dust; however, no other correlations were found between concentrations of the other three target fungi in air and dust samples. It is generally accepted that air sampling gives a measure of current exposure, whereas dust samples can reflect long-term exposure [156]. Hyphal fragments could be present within dust samples but do not readily become airborne [222]; therefore, the presence of hyphae within dust samples could potentially distort associations between airborne and dustborne levels of fungal DNA. Many studies focus on dust samples as a means of quantifying fungal exposure; due to ease of sampling and large quantites of material on which multiple investigations can be made. However, this study has shown that dust sampling alone can miss potentially important observations, and that both air and dust samples should be analysed together

in investigations of fungal exposure or suspected contamination. Airborne exposure to allergens is particularly relevant in airways disease, since it is inhaled fungi which have the potential to germinate within the bronchial tree and to colonise the human airway.

6.5.7 Study limitations

A limitation to this study is that the environmental sampling and sputum sampling was not conducted at the same time (an average of five months apart, due to logistics of organising visits). We have shown previously that indoor levels of *Asp/Pen*-type conidia do not exhibit seasonal variation and exhibit an independence of outdoor concentrations (Chapter 3) and there is substantial agreement between repeat sputum samples up to six months apart [255]. However, airborne *A. fumigatus* concentrations were shown to be significantly higher in the winter; therefore a seasonal effect cannot be ruled out. This would support previous studies which suggest exacerbation of symptoms of ABPA on exposure to higher *A. fumigatus* concentrations in the winter [139], which could be caused by condensation and increased humidity [161]; however, in contrast, the data from Chapter 3 showed that within the same property, concentrations of *Asp/Pen*-type conidia were lowest in the winter.

Concurrent outdoor concentrations were not taken into consideration in this study; however, species-specific analysis of concurrent indoor and outdoor concentrations of fungi by MSQPCR has shown that the majority of fungal species do not correlate, with the exception of *Aspergillus penicillioides, C. herbarum* and *Cladosporium cladosporioides,* which have been shown previously to dominate their respective taxa in indoor samples. This suggests that incorporating outdoor fungal concentrations in species-specific analyses may generate misleading results [308].

6.6 Summary

MSQPCR analysis of indoor airborne and dustborne fungal concentrations in the main living area of homes showed that patients with a positive sputum culture of *A. fumigatus* were exposed to significantly higher concentrations of airborne (but not dustborne) *A. fumigatus*; a trend that was mirrored by quantification of airborne *Asp/Pen* concentrations by microscopy. This is the first time a link has been shown between home exposure to fungi and airway colonisation in patients with asthma. Future studies will be required to further investigate this association and longevity of exposure and colonisation. Genotyping studies may be required to provide conclusive evidence of a direct link.

7 General discussion

7.1 Summary of findings

The primary aim of these studies was to better understand the role of fungi in asthma; through investigations of exposure, airways colonisation and analyses of clinical characteristics of patients, with the general hypothesis that fungal allergy is more important in asthma than is currently recognised. A number of novel and striking findings have been uncovered through these studies; showing specifically that *A. fumigatus* IgE sensitisation, regardless of other diagnostic criteria used for ABPA, is significantly associated with reduced lung function in moderate to severe asthma. Isolation of filamentous fungi from the sputum of patients with asthma was shown to be common, with *Aspergillus* and *Penicillium* species predominating, which are also common indoor contaminants. Significantly higher rates of sensitisation to *A. fumigatus* and *P. chrysogenum* in patients with a positive sputum culture for filamentous fungi (in which *Aspergillus* and *Penicillium* species predominated) suggests that in addition to ABPA and SAFS, a clinically important asthma phenotype which should be considered is airways disease associated with colonising fungi. This is further substantiated by the data in Chapter 5, which showed that there was no difference in sensitisation rates to common environmental fungi (*A. alternata, C. herbarum* and *B. cinerea*) in asthma patients sputum culture positive or negative for filamentous fungi. ABPA is defined by a stringent set of diagnostic criteria [62], which represent the most severe form of a spectrum of disease. Early recognition of *A. fumigatus*-associated asthma may enable limitation of lung function decline. SAFS is a clinically important asthma phenotype describing fungal sensitisation in severe asthma [3, 12]; however, this disease entity

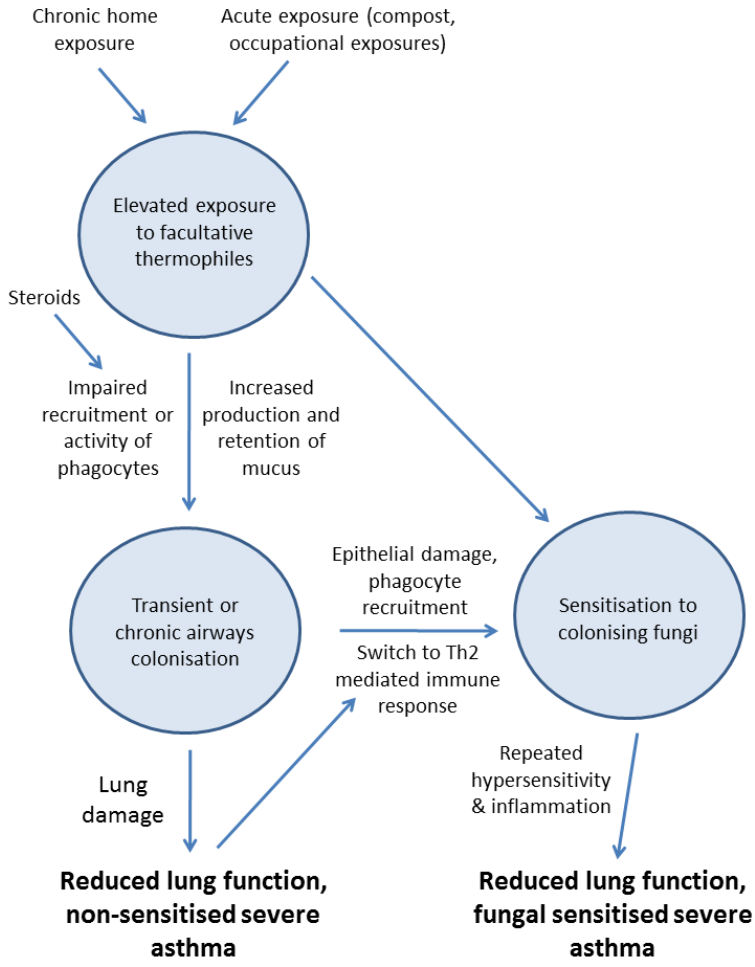
describes severe asthma with sensitisation to any fungi, which includes *A. alternata* and *C. herbarum* for example, which do not colonise the airway.

Initial studies of baseline concentrations of fungi showed that airborne concentrations of *Asp/Pen*-type conidia were highest in non-insulated, terraced properties over 90 years old. Subsequent home exposure studies utilising MSQPCR showed that detection of *A. fumigatus* in sputum was associated with higher levels of airborne exposure in the home environment, for the first time demonstrating a possible link between exposure to fungi and airways colonisation in asthma. Fungal isolates from sputum other than *A. fumigatus* are commonly regarded as environmental contaminants and disregarded clinically; however, associations of a positive sputum culture for any filamentous fungi with reduced lung function suggests that non-*A. fumigatus* fungal isolates should not be ignored.

These data support the hypothesis that elevated exposure, through chronic exposure in the home (or work) environment as one source, or acute exposure through interactions with compost or heavily contaminated substrates leads to airways colonisation in asthma, through impaired clearance in areas of retained mucus, which may be transient or chronic. This hypothesis is supported by research demonstrating improvement of respiratory symptoms following remediation of moisture sources and fungal contamination in patient homes [170, 171]. Immunogenetics and high dose inhaled steroids prescribed in moderate-severe asthma may predispose some asthmatics to fungal colonisation of the airways, although these studies showed that the phagocytic ability of macrophages is not affected by steroid treatment. Progressive lung function decline may then be caused by destruction of host tissues through repeated or chronic colonisation and inflammation. Fungal sensitisation could transpire through either exposure to allergens from colonising fungi, or due to elevated environmental exposure.

Repeated hypersensitivity reactions to colonising fungi may then lead to fixed air flow obstruction through hyphal damage and inflammatory cell recruitment (Figure 7.1).

Figure 7.1. Proposed model of fungal exposure, lung damage and development of sensitisation



7.2 Techniques developed and limitations

A number of techniques have been developed as a result of this thesis for use in environmental and respiratory investigations, which are likely to be continued in future studies. The indoor air data collected from non-complaint properties provides a comprehensive dataset of indoor and outdoor fungal spore concentrations, which can be

used as a guide of typical concentrations during different seasons in the absence of concurrent outdoor data in the UK. Furthermore, the data acquisition database developed can be used in future aerobiological studies for recording and exporting indoor and outdoor fungal spore data to greatly improve efficiency of counting. Whilst continuous air sampling onto slides analysed by microscopy can enable detection of fluctuations in levels of fungi, a caveat of this approach is the inability to quantify fungi to the species level; a property which is essential for attributing clinical exposure effects. The use of MSQPCR methods for the identification of pathogenic fungi in environmental samples shows promise; however, the utility of the generic PenAsp primers warrants further investigation. The methods described here for the extraction of DNA from air samples may be used in future studies as an alternative to washing collected material from tubes; however, further experiments using multiple samplers are necessary to determine the efficiency of this sampling and extraction technique in comparison to alternative methods such as collection onto filters.

Significantly higher levels of airborne, but not dustborne, *A. fumigatus* were shown in homes of asthma patients with a positive sputum culture for the fungus, and were mirrored in analyses of airborne *Asp/Pen*-type conidia concentrations by microscopy. The majority of studies investigating exposure to filamentous fungi in the home or work environment focus on dust as a means of monitoring exposure and this study has shown that airborne and dustborne concentrations do not correlate, and focusing on one route of exposure could potentially miss important clinical associations. One of the major limitations of the study relating home exposure to fungi with airways colonisation was that the measurements were not taken at the same time and respiratory and environmental sampling were not restricted to a particular time of year, which would need to be rectified and repeated measures taken in future studies.

Defection from the use of diluted sputum to culture of neat sputum plug for aiding diagnoses of respiratory fungal infections or colonisation is an important step which can be used in future studies to detect periods of transient and chronic colonisation of the airways. A drawback of our culture technique is that airways colonisation cannot be quantified, and this problem may be rectified in future studies with developments in PCR. Nonetheless, culture of fungi from sputum provides an easy, cost-efficient and effective indication of airways colonisation. This tool would be invaluable in future studies aimed at determining the causality of asthma severity and airways colonisation, and also in studies investigating the development of fungal sensitisation. Sputum is diluted in routine laboratories due to the focus of culture on detection of bacteria in samples; however, this technique is often insufficient for detection of fungi and a comparison of our technique with the standard method followed in routine laboratories has recently been published, showing superiority in detection [216]. A caveat of our sputum culture technique for detection of fungi from the lower airways is that fungi from the oral cavity could inadvertently be cultured. For example, *Aspergillus* species were detected in the oral cavity of around one third of healthy individuals in one study [285]. Removal of saliva reduces the likelihood of contamination from the oral cavity [97, 260] but cannot be completely ruled out in sputum culture results. Furthermore, non-homogenised sputum was used for fungal culture in these studies. The homogenisation of sputum through combined use of DTT and sonication has been shown to increase the sensitivity of detection of *A. fumigatus* by culture in comparison to homogenisation with DTT alone [309]; therefore, this may warrant further study. It is important to note that fungal culture itself biases identification of certain fungi, since the majority of fungi cannont be maintained by current culture methods [283].

7.3 Future studies

A limitation to the studies in asthma reported here is that the majority of patients had a moderate to severe form of the disease. Future studies need to focus on determining the causality of the association between airways colonisation, fungal sensitisation and reduced lung function in patients with asthma and would benefit from investigations of mild asthma, with and without fungal sensitisation. The hypothesis is that chronic colonisation of the airways by fungi leads to the development of fungal sensitivity and decline in lung function. Asthma patients such as those identified in these studies that were either sputum culture positive and non-sensitised, or those sensitised but sputum culture negative, should be followed in longitudinal studies to determine the consequences of colonisation and to better understand the development of fungal sensitisation. Furthermore, a better understanding is required of the underlying immunopathology and potential risk factors for the development of airways colonisation and *A. fumigatus*-associated asthma. It is possible that steroid treatment predisposes asthmatics to airways colonisation and further investigations of steroid dosage, metabolism and airways colonisation are necessary to determine potential doses which pose a risk factor for colonisation.

It is still not possible to provide guidelines on acceptable fungal spore concentrations in the home or work environment with regard to the development of clinical symptoms of exposure. Future studies including markers of exposure, sensitisation and airways colonisation should be used in combination with studies attempting to determine the causality of fungal sensitisation and lung function decline. Whilst this study has provided a link between home exposure and airways colonisation, genotyping of clinical and environmental isolates is necessary to attribute causality.

8 Appendix

8.1 Equipment, software and reagents

Supplier	Equipment/reagent
Alk-Abello, Hørsholm, Denmark	Skin test solutions
Anachem Ltd., Luton, UK	MicroSol 3+
Applied Biosystems, Warrington, UK	Applied Biosystems Fast 7500 Real-Time PCR instrument
	TaqMan®Universal PCR mastermix
Audon Electronics, Chilwell, UK	LogBox-RHT-dataloggers
Biostain Ready Reagents Ltd, Manchester, UK	Rapi-Diff
Burkard Manufacturing Company Ltd, Rickmansworth, UK	Continuous recording air sampler
	Multi-vial cyclone sampler
	Seven day recording volumetric spore trap
Clintech, Guildford, UK	Lactophenol
Fisher Scientific UK Ltd., Loughborough, UK	Acetic acid
	255 µm aperture sieves
	DPX
	Nescofilm®
	Non-essential amino acids
	Tris base
Fisons Pharmaceuticals, Loughborough, UK	Wright nebuliser
Flaemnuova, Brescia, Italy	EasyNEB II Ultrasonic nebuliser
Geneflow, Fradley, UK	PCR sizer 100 bp DNA ladder

Geospiza, Inc, Washington, USA	FinchTV (Version 1.4.0)
Gibco, Invitrogen, Paisley, UK	Hepes buffer
GraphPad Software Inc, California, USA	GraphPad Prism, Versions 4 and 5
Indoor Biotechnologies, Warminster, UK	Mitest sampler and filter insert
Invitrogen, Paisley, UK	Dulbecco's modified eagle's medium (DMEM)
	Foetal bovine serum
	Forward and reverse primers
	Qubit fluorometer
	Quant-iT dsDNA broad range (BR) assay kit
	Quant-iT dsDNA high sensitivity (HS) assay kit
Microsoft, Redmond, USA	Microsoft Access and Excel
Organon, Cambridge, UK	Dexamethasone (4 mg/ml)
Oxoid, Hampshire, UK	Potato dextrose agar (PDA)
Pall Corporation, Hampshire, UK	0.2 µm acrodisc filters
Phadia, Thermo Fisher Scientific, Loughborough, UK	ImmunoCAP 250 system
Qiagen, Crawley, UK	DNeasy plant mini kit
	10x polymerase buffer
	Hotstar Taq DNA polymerase
	QIA quick PCR clean up kit

Sigma-Aldrich Company Ltd,	212-300 µm acid-washed glass beads
Gillingham, UK	Azure-B-thiocyanate
	Chloramphenicol
	Dimethyl sulfoxide (DMSO)
	Dithiothreitol (DTT)
	Dulbecco's phosphate buffered saline
	Eosin Y
	Ethidium bromide (10 mg/ml)
	Hank's balanced salt solution (HBSS)
	Molecular biology grade (0.1 µm filtered) water
	Ethylenediaminetetraacetic acid (EDTA)
	Fluconazole
	Fluorescent probes
	Gel loading solution
	Gentamicin
	Lactophenol blue solution
	Penicillin G
	Streptomycin sulphate
	Trypan blue, 0.4%
	$Tween_{80}$
SPSS, Inc., Chicago, USA	SPSS, Version 11
StataCorp LP, Texas, USA	STATA data analysis and statistical software package, Version 10
Stratech Scientific Ltd, Suffolk, UK	Biospec mini-beatbeater-1 and 16
	Biospec mini-beatbeater-16
Vitalograph, Buckingham, UK	Dry bellows spirometer

8.2 Ethics approval

The studies were approved by the Leicestershire and Rutland Ethics committee (research ethics committee reference numbers 06/Q2502/110 and 07/MRE08/42). University Hospitals of Leicester indemnity insurance for studies reported expires June 2012.

8.3 Participant information sheets

8.3.1 Studies on *Aspergillus* lung disease participant information sheet

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 CF.

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 CF. 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 CF 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*. 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 30 ml). 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.

8.3.2 Lung studies subject information leaflet

Study Title: Use of Tissue from Lung Resections to Investigate the Molecular and Functional Mechanisms of Human Lung Disease

Principle Investigator: Professor Andrew Wardlaw

Study Funded by: AstraZeneca

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.

Introduction

Lung disease causes pain, discomfort and can prevent sufferers from carrying out everyday activities. Whilst available treatments including steroids and other drugs may relieve symptoms, none provide a cure. Operations may help some people. More research is needed to find new treatments that can cure lung disease.

What is the purpose of the study?

In order to find the causes of lung disease such as COPD and lung cancer and to find new ways of treating these diseases we have to do more research. It is ideal to do the research on tissue from human lungs because we are investigating a human disease. The lung research teams at Glenfield Hospital, Leicester Birmingham Heartlands Hospital, Walsgrave Hospital, Coventry and AstraZeneca (a pharmaceutical company)

have joined together in order to collaborate on studies into lung disease using human lung tissue. A numbers of diseases will be studied and the lung tissue will be used in a number of different laboratory studies. AstraZeneca will use the lung tissue in the understanding of lung disease and development of new treatments for lung disease. These experiments will be done on lung tissue that has been removed from patients as part of their medical treatment, which would otherwise be destroyed. In some cases we may also wish to take a blood sample to compare the findings in the lung tissue and blood.

Why have I been chosen?

You have been chosen because your doctor has said that you may need to have some of your lung removed to treat your disease.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do 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?

You are about to undergo an operation for your current condition. We would like to retain some of the spare lung tissue that will be removed as part of your operation, which would otherwise be destroyed. If you are willing to take part in this research, we will pass the surplus lung tissue to the collaborating hospitals and AstraZeneca.. The

surgeon will not remove any extra lung tissue for this research. We will also record some information about your recent medical history, medicines taken and reason for the operation from your medical records. In terms of your operation, stay in hospital and subsequent follow up there will be no difference to what will happen to you whether you take part in the study or not except that in a few cases we may wish to take an extra blood sample of approximately 15mL (about three tablespoons full) before your operation.

What happens if I don't want to take part?

Nothing, you simply don't sign this form. This will not affect your medical care or your legal rights in any way.

What rights do I have to the results of the research?

You are being asked to donate your tissue as a gift to the researchers in the hospitals involved and AstraZeneca. Any information derived directly or indirectly from this research by the collaborating hospitals or by AstraZeneca, as well as any patents, diagnostic tests, drugs, or biological products developed directly or indirectly as a result, are the sole property of the company (or their successors, licensees, and assigns) and may be used for commercial purposes. You have no right to this property or to any share of the profits that may be earned directly or indirectly as a result of this research. However, in signing this form and donating a blood sample, you do not give up any rights that you would otherwise have as a participant in research.

What do I have to do?

There is nothing extra to do as a result of being part of this study.

What are the possible disadvantages and risks of taking part?

There are no disadvantages or risks to taking part in the study over and above the normal risks associated with this surgery, which you require as part of your care. If you are asked to donate an additional blood sample there may be some discomfort of the needle being inserted into a vein in your arm and the possibility of bruising developing afterwards around the area that the needle was inserted. This should disappear in a few days.

What are the possible benefits of taking part in the study?

There are no direct benefits. Taking part in this study means that you may possibly help suffers of lung disease in the future, as information about the changes that occur in the lung may be used to develop new treatments.

What if something goes wrong?

We do not think there is any significant risk of any harm occurring as a result of participating in this study. However if you are harmed 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 a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms would be available to you.

Will my taking part in this study be kept confidential?

All information resulting from you taking part in the study will be stored both in paper and computerised form, and will be treated confidentially. You will be identified in the computer by a number and only your doctor will be able to identify the number as belonging to you. The study records and your medical records will not be made available in any form to anyone other than authorised representatives of all three health authorities (Leicester, Birmingham, and Coventry & Warwickshire) and AstraZeneca. In all instances, your confidentiality will be maintained, in accordance with the Data Protection Act or as local laws permit.

AstraZeneca and Regulatory authorities may wish to check that this research has been done properly, they may have access to your files and know your identity, but they are under a duty of confidentiality not to disclose details to others.

What will happen to the samples that I have donated?

The samples will be processed by the research team and used in a range of experiments into the causes of lung disease. Samples may be transported to AstraZeneca or other hospitals in the collaborating group to do further experiments including tests to develop new drugs. Those samples that are not fully used up in experiments may be stored by the research team or by AstraZeneca, for use in future experiments, for up to 20 years.

Who is organising and funding the research?

The research is a collaboration between the lung research teams at the hospitals in Leicester, Coventry, Birmingham and AstraZeneca. The study is organised and operated by the individual hospitals involved and the overall collaboration has been funded by

AstraZeneca. The income obtained from AstraZeneca will only be used to support the work carried out as part of this project.

Can I withdraw my consent?

You may withdraw your consent to the use of your data and samples at any time. If you withdraw your permission consent before your donated tissue and data are used, we will not use the data and the samples will be destroyed. If you withdraw your consent after your tissue sample has been sent for analysis we will ensure that your sample(s) are destroyed. However, if analysis has already been performed neither AstraZeneca nor ourselves are obliged to destroy results of this research.

Who has reviewed the study?

The study has been reviewed by the research teams within the consortium and by the members of the Department of Respiratory Medicine, Allergy and Thoracic Surgery. Individual research projects where we use the lung tissue have been reviewed by a variety of charities and funding organisations

If you have any further questions about this study please do discuss them with:

Professor Wardlaw or Mr Waller (0116 2563841)

8.4 Consent forms

8.4.1 *Aspergillus* lung disease

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

11.11.2010: Version 2

8.4.2 Lung studies

Study Title: Use of Tissue from Lung Resections to Investigate the Molecular and Functional Mechanisms of Human Lung Disease

	Initials
1. I confirm that I have read and understood the patient information form on the above project and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being done and any foreseeable risks involved.	☐
2. I agree to donate as a gift a sample of tissue for research in the above project. I understand how the sample will be collected and that giving the sample is voluntary. I am free to withdraw my approval for use of the sample at any time without giving any reason and without my medical treatment or legal rights being affected.	☐
3. I give permission for my medical records to be looked at and information taken from them to be treated in strict confidence by responsible people from Glenfield Leicester**,** Heart of England Birmingham and University Hospitals Coventry & Warwick and AstraZeneca.	☐
4. I understand that my doctor will be informed if any of the results of the tests done as part of the research are important for my health.	☐

5. I understand that I will not benefit financially if this research leads to a new treatment or medical test. ☐

6. I do know where to contact Professor Wardlaw, if I need further information. ☐

7. Do you agree to take part in this study? YES ☐ NO ☐

Signed: ……. Date: ……..

Name (Block capitals) ……..

I, (Name of investigator, block letters) ……

have explained the nature and purpose of the study to …….

and believe that he/she understands what the study involves.

Signed: …….. Date: ……..

8.5 Indoor air sample questionnaire

Slide reference N^{o}	
Trap A / B / C	
Name	
Address	
Speed setting (should be 3):	
Height of trap from floor (approx 1m):	
Time and date on:	
Time and date off:	
Room trap located (should be lounge/living area)	
Floor covering in room where trap is located	Carpet / Tiles / Laminate / Floorboards / Rugs (delete as appropriate) Other:
Number of houseplants in room (type if known)	
Hours when room with trap is occupied (Please specify times anybody is in the room and when the room is unoccupied)	
Building type (e.g. terraced, detached, semi-detached)	
Age of building (If not known, please attempt a guess)	
Number of floors in the property	
Level of floor where trap is located (i.e. ground / first floor)	
Number of rooms in the property	
Presence of a cellar	Yes / No (delete as appropriate)

Presence of wall cavity insulation	Yes / No (delete as appropriate)
Number of adults in the property	
Number of children in the property	
Number and type of pets	
Rooms accessed by pets (include times)	
Windows shut (windows should preferably be shut to prevent outdoor air affecting results)	Yes / No (delete as appropriate)
Type of heating	Gas central heating /Electric storage heating / Open fire (delete as appropriate) Other:
Heating on/off during air sampling	On / Off (If yes, answer below) Hours during which heating was on:
Presence of visible mould (if present, include room(s) located)	
Presence of damp (if present, include room(s) located)	
Condensation (if present, include room(s) located)	
Any previous history of damp problems	Yes / No (If yes, answer below) Room/location and date/year of problem:
Presence and type of double glazing in room where trap is located	Yes / No (If yes, answer below) Secondary / Sealed units (delete as appropriate)
Garden	Yes / No (If yes, answer below) Approx size – Striking features – (trees present, presence of running water, stream, pond

	etc.)
Building surroundings (built up / rural – identify any nearby crops / orchards / compost bins etc)	
Did any cleaning activity take place during sampling? (preferably no cleaning should take place during the sample as this could interfere with results)	Yes / No (if yes, answer below) Type of cleaning: o Dusting o Vacuuming o Both Time of cleaning:
Number of years since onset of symptoms (if applicable)	
Number of years occupied property at above address	

8.6 Preparation of stains

Kimura stain

The Kimura stain was prepared by research personnel at Glenfield Hospital as previously described [310], thoroughly mixed and filtered.

Main ingredient	Ingredient components	Final volume
Toluidine blue solution 0.05%	0.05 g Toluidine blue in 50 ml NaCl 1.8% (1.8 g in 100 ml ultra pure water)	11ml
Light green solution 0.03%	0.03 g in 100 ml ultra pure water.	0.8ml
Saponin saturated in 50% ethanol	4 teaspoons saponin in 10 ml 50% ethanol	0.5ml
Phosphate buffer 6.4 pH 1/15M	100 ml 1/15 M Na_2HPO_4 and 100 ml 1/15M KH_2PO_4, adusted to pH 6.4	5ml

Romanowsky stain

The Romanowsky stain was prepared by William Monteiro at Glenfield Hospital and comprised 1.5g Azure-B-thiocyanate dissolved by heating to 37°C in 200 ml DMSO and 0.5g Eosin Y dissolved in 300 ml ethanol. The Azure-B and Eosin solutions were slowly mixed and filtered, constituting the neat stain. A dilute version of the stain was produced by adding 2.0 ml neat Romanosky stain and 1.5 ml DMSO to 27ml 10 mM Hepes buffer at pH 7.2. Sputum cytospins were stained for 5 minutes with neat Romanowsky stain, rinsed with distilled water and air dried, followed by a further 25 minutes staining with the dilute stain.

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