Fungi in asthma - investigation of the lung mycobiome and characterisation of allergens

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Abstract

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Asthma is a heterogeneous and complex disease, where sensitisation to, and colonisation with fungi have been associated with decreased lung function. The full spectrum of fungi involved with asthma and their role has not been established due in part to limitations in traditional culture methods. Furthermore, most fungi lack commercially available allergy tests and their allergens have not been characterised. To obtain a better understanding of the fungi involved in asthma, subjects with or without fungal sensitisation provided sputum samples which were subjected to amplicon-based high-throughput sequencing to assess the fungal microbiome (mycobiome). A subset also underwent a bronchoscopy. Fungal sensitised people with asthma showed higher levels of *Candida dubliniensis* in sputum than non-fungal sensitised asthmatics and healthy controls. *Aspergillus tubingensis* and *Cryptococcus pseudolongus* were more prevalent and abundant in bronchoscopy-derived samples, particularly from subjects with asthma, and were significantly associated with decreased sputum neutrophil counts. These fungi could contribute to the asthma phenotype, however, their allergen profile is unknown. This was addressed for *A. tubingensis,* the IgE response of *A. tubingensis* and its close relative *A. niger* were investigated. Only subjects with high *A. fumigatus*-specific IgE levels (> 17 kU/L) showed sensitisation to these fungi. Yet undescribed IgE cross-reactions with *Aspergillus fumigatus* proteins Asp f 3 and 6 could be detected, which may contribute to lung inflammation. Other potential IgE cross-reactive allergens were identified using immunoprecipitation of fungal proteins from crude extract. Both known and yet undescribed allergens from *A. fumigatus* could be detected by these proof-of-principle experiments.

In conclusion, a basic understanding of the lung mycobiome in subjects with asthma and healthy individuals was established. Potentially clinically relevant species were identified whose clinical relevance in asthma is unclear. Immunoprecipitation could be a promising method for specific and reproducible purification and identification of allergens in the future.

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Declaration of joint efforts

My work was central to the development and production of the final work, however a few colleagues were involved in the production of data. Patients were assessed and clinical data collected by Dr Kerry Woolnough, Michelle Craner, Michelle Bourne and Prof Andrew Wardlaw at Glenfield Hospital (Leicester, UK).

The joint efforts for Chapter 3 were: William Monteiro and Hetan Bhatt were responsible for the processing of the sputum and the production of the bronchoalveolar lavage, bronchial wash and sputum cell differentiation data. Dr Abbie Fairs, Dr Catherine Pashley and Dr Kerry Woolnough processed the earlier clinical samples and performed some of the experiments for the mycobiome study. Jack Satchwell provided the spore counts and performed some of the universal quantitative PCR experiments. Dr Catherine Pashley majorly contributed to the bioinformatics analyses. Dr Catherine Pashley and Dr Matthew Richardson were responsible for the statistical analysis of the mycobiome data.

The joint efforts for Chapter 4 were: Prof Ines Swoboda provided plasmid constructs for recombinant allergens. Dr Andrew Bottrill and Prof Ebrahim Razzazi-Fazeli and colleagues Katharina Nöbauer and Kathrin Hummel were responsible for producing the mass peptide fingerprinting data.

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List of abbreviations

ABPA	allergic bronchopulmonary aspergillosis
ABPM	allergic bronchopulmonary mycosis
AFAD	allergic fungal airway disease
ERS/ATS	International European Respiratory Society/American Thoracic Society
ATCC	American Type Culture Collection
BLAST	basic local alignment search tool
BR	broad-range
CF	cystic fibrosis
cfu	colony forming unit
CPA	chronic pulmonary aspergillosis
CRD	component-resolved diagnosis
dNTP	deoxynucleotide
FDA	food and drug administration
FENO	fractional exhaled nitric oxide
FENO ppb	ppb = Fractional exhaled nitric oxide, parts per billion;
FEV_1	forced expiratory volume in one second
FRR	culture collection of CSIRO Sydney Australia
FVC	forced vital capacity
GINA	Global Initiative for Asthma
HiFi	high-fidelity
His-tag	hexahistidine-tag
HS	high-sensitivity
HSP	heat shock protein
HTS	high-throughput sequencing
ICS	inhaled corticosteroids
ICU	intensive care unit
IgE	immunoglobulin E
IgG	immunoglobulin G
ImmunoCAP	immunoassay capture
INSD	International Nucleotide Sequence Database
IPTG	β-D-1 thiogalactopyranoside
ISHAM	International Society of Human and Animal Mycology
ITS	internal transcribed spacer
IUIS	International Union of Immunological Societies
LABA	long-acting inhaled β_2-agonists
LOD	limit of detection
LOQ	limit of quantification
LSU	large subunit
LWB	last wash buffer
MgCl2	magnesium chloride
MnSOD	manganese superoxide dismutase
N_2	liquid nitrogen

NCBI	National Center for Biotechnology Information
NCPF	National Collection of Pathogenic Fungi
NCYC	National Collection of Yeast Cultures
NEB	New England Biolabs
NTC	no template control
OCS	systemic oral corticosteroids
OD	optical density
OTU	operational taxonomic unit
PC_{20}	provocative concentration of methacholine required to cause a 20% fall in FEV_1
PCR	polymerase chain reaction
PDA	potato dextrose agar
PGCF	potato dextrose agar containing gentamicin, chloramphenicol and fluconazole
PMA	propidium monoazide
qPCR	quantitative PCR
rDNA	nuclear ribosomal operon
RFLP	restriction fragment length polymorphism
RT	room temperature
SAFS	severe asthma with fungal sensitisation
SAM	signal accumulation mode
ScedSel+	*Scedosporium* selective agar
SD	standard deviation of the residuals
sIgE	serum IgE
sIgG	serum IgG
S/N	supernatant
SPT	skin prick test
SSU	small subunit
Th2	T-helper cell type 2
UNITE	User-friendly Nordic ITS Ectomycorrhiza Database.
USA	United States of America
WAO	World Allergy Organization
WHO	World Health Organisation
YMA	yeast malt agar

Chapter 1 Introduction

The respiratory tract consists of an upper and a lower part. The airways present a constant access point for microorganisms and foreign particles into the human body. Microorganisms like the fungus *Aspergillus fumigatus* can cause disease such as pulmonary aspergillosis or complicate chronic diseases such as asthma. Allergic asthma is a major health problem worldwide. Studies about the causal factors of asthma mainly focus on patient genetics and environmental contributors such as smoking. Although some fungal species such as *Candia albicans* or *Aspergillus fumigatus* have been implicated in airway diseases, the range of fungi involved and their clinical impact on airway diseases, particularly on asthma, is largely unknown. This may be explained by the low number of microbiome studies investigating which fungi are present in the lung.

1.1 Introduction to fungi

1.1.1 Phylogeny and taxonomy

Fungi are uni- or multi-cellular eukaryotes, which form their own Kingdom with around 100,000 named species [1, 2], although estimations range from 1.5 to 3 million worldwide [3]. The "Assembling the fungal tree of life" project grouped the fungi into 8-10 phyla, of which Ascomycota and Basidiomycota, which together form the Dikarya, represent 98% of described fungal species [4, 5]. Since then, the fungal kingdom was grouped into the phyla Ascomycota, Basidiomycota, Mucormycota, Zoopagomycota, Blastocladiomycota, Chytridiomycota, and Neocallimastigomycota [5, 6]. The largest phylum is the Ascomycota, encompassing > 64,000 described species, followed by the Basidiomycota with > 30,000 species [7]. Ascomycota decay organic matter and are pathogenic to living organisms, whereas Basidiomycota are represented mostly by mushrooms, brackets and jelly fungi [8]. The Mucormycota mostly include fungi involved in plant-associated nutrition, particularly with respect to plant roots, whereas Zoopagomycota are mostly represented by insect parasites [6]. Blastocladiomycota, Chytridiomycota and Neocallimastigomycota include aquatic fungi [9].

Taxonomy is the discipline of identifying, describing and naming fungi, whilst classification deals with the group hierarchy and systematics with their relationship. The

taxonomy and classification are subject to change over the last few decades due to new developments in molecular techniques since the 1990s [10], which provide new insights into the genetic relationship between the fungi and lead to the discovery of a plethora of new species. This includes cryptic species, which represent morphologically indistinct but phylogenetically different fungi. Examples of popular regions to differentiate between different fungal species are the ribosomal operon, β-tubulin and RNA polymerase II (*RPB1* and *RPB2*). Fungal identification may be difficult as fungi can have multiple names based on their different life stages. They can reproduce sexually and/or asexually. Anamorph describes the asexual state of a fungus, whilst the sexual reproductive stage is called teleomorph. The spores of the former are termed conidia (or mitospores), which are formed by mitosis. The spores of the latter are called ascospores or basidiospores depending on which phyla they came from and are generated by meiosis [9, 11]. The identification based on multiple names depending on the life stage is useful for microscopic identification of fungi, but it complicates fungal identification by molecular techniques. Molecular techniques are based on genotypes rather than phenotypes and allow new insights into phylogenetic relationships as well as discovery of uncultivable species. This is why in recent years the "one fungus = one name" policy has been introduced. The oldest and commonly used name is to be maintained. If that is the case for both teleomorph and anamorph names, the teleomorphic name should be chosen except when a formal application in favour of the anamorph name was approved. The naming process also depends on the genome size and its rank in the taxonomic hierarchy [12-15]. Re-naming of fungal species and taxonomic hierarchies is a continuous process. For example, one of the major clinically relevant fungi, *A. fumigatus*, has the teleomorph name *Neosartorya fumigata*, although its anamorph name *A. fumigatus* is more widely used. Its classification as *Aspergillus* is controversial because it is only distantly related to the first *Aspergillus* species described, *A. glaucus* [16, 17]. The International commission of *Penicillium* and *Aspergillus* has therefore allowed a large genus of *Aspergillus* encompassing multiple clades.

1.1.2 The role of fungi

In the environment, fungi are important stabilizers of the ecosystem forming mycorrhizal symbioses or decaying organic matter [9, 18] and many have industrial applications, too. Examples include food production such as cheese, antibiotics like Penicillin and enzymes like xylanase [9, 19, 20]. Their implication in diseases depends on their ability to grow at human body temperature (37°C). The majority of described fungi are mesophilic, which can grow at temperatures ranging from 10 to 40°C, and have an optimum grow temperature of between 18 to 22°C [21] and therefore rarely cause infections in humans. This is also true for thermophilic fungi, which can grow at temperatures ranging from 20°C to 50°C, and have an optimum grow temperature of 40 to 50°C. In contrast, thermotolerant fungi can grow from < 20°C to ~55°C [21, 22], which allows them to survive both in the environment and within or on the body [23]. In humans, these fungi are commensals as part of the microbial communities or cause infections, called mycoses, which are superficial, subcutaneous or systemic [9, 24]. Opportunistic pathogens, which can be commensals, are able to infect immunocompromised individuals. Fungal systemic infections are particularly problematic as they are hard to diagnose and treat, which could explain the high mortality rates of > 50% that exceed those of tuberculosis or malaria [24, 25]. Around 560 fungal species have shown to be clinically relevant, with numbers increasing [15]. These fungi can feed on different nutrient sources and survive the host's countermeasures at least to some degree [9]. In addition, fungi can also be harmful as allergens or by producing mycotoxins [26, 27]. Common antifungal agents are azoles, echinocandins or amphotericin drugs [9] and a major problem for treating fungal diseases is the emergence and spreading of drug resistances [28-30]. *Candida* and *Aspergillus* species are two of the most studied fungal genera involved in fungal diseases.

1.1.2.1 Candida

Candida species, in particular *Candida albicans*, are frequently found at mucosal and skin surfaces in healthy individuals (30-70%) [31, 32]. Although they are commensals, *Candida*-specific antibodies are frequently detected in healthy individuals, which is

suggestive of immune avoidance mechanisms such as the secretion of aspartic proteases to degrade complement proteins, probably as part of their pathogenicity [33]. Other virulence mechanisms include secretion of immunomodulatory substances, morphological changes and biofilm formation [34]. Candidiasis is the 4th most frequent nosocomial infection in the USA [35, 36]. Estimations of systemic candidiasis are as high as 250,000 cases worldwide with mortality rates up to 40% despite treatment [37, 38]. Systemic infections particularly affect immunocompromised individuals like those who underwent invasive clinical procedures, or experienced major trauma. *C. albicans* is the most prominent yeast involved, followed by *C. glabrata* and *C. parapsilosis* [37, 38].

1.1.2.2 Aspergillus

Aspergillus species are usually associated with decaying organic matter [18]. These opportunistic pathogens are a leading cause of nosocomial infections and death in immunocompromised individuals with 700,000 deaths per year worldwide [9]. Invasive aspergillosis usually affects immunocompromised subjects with an estimated incidence of > 200,000 cases per year. The mortality is 50% if diagnosed, or almost 100% if undiagnosed [24]. Examples of lung diseases associated with *Aspergillus* species are chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA). CPA is represented by the formation of hyphal balls in pre-existing lung cavities, which can become invasive and result in necrosis [39]. More than 3 million cases of CPA are estimated, resulting 15% mortality within in the first 6 months after diagnosis, particularly in those with pre-existing lung conditions [24]. The predominant species involved in these diseases are *Aspergillus fumigatus*, followed by *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus lentulus* [9].

Aspergillus species are also potent allergens. Sensitisation and colonisation with these fungi have been associated with ABPA, fungal rhinosinusitis [40] and severe asthma in adults and children [41-43]. The role of *Aspergillus* species and other fungi as allergens is introduced in more detail later (section 1.2.5).

1.2 Role of fungi in asthma

1.2.1 Allergic reactions

Hypersensitivity reactions were first described in the early 1900s, where Clemens von Pirquet coined the term “allergy” from the Greek allos (other or different) and ergia (energy or action) [44]. Four different types of hypersensitivity reactions are described based on the definition of Gell and Coombs, which are not mutually exclusive [45]. They are summarised in Table 1. 1. All of these reactions are based on immune responses that usually target pathogens but instead react towards harmless antigens that otherwise would not elicit any immune response and are therefore called allergens.

The type most involved in allergic rhinitis and allergic asthma is the Type I hypersensitivity reaction. It is based on a dysregulated T-helper type (Th)2 which is usually directed against parasites [46]. The immune reaction encompasses a range of cells and mediators, most prominently Th2 cells, an increased production of immunoglobulin (Ig)E by B cells, eosinophils, and inflammatory mediators secreted by mast cells and basophils [46, 47]. The subsequent allergic reaction includes mucous production from goblet cells, smooth muscle contraction and airway hyperreactivity [45, 46, 48]. The World Health Organisation (WHO) estimates that around 400 million people suffer from allergic rhinitis worldwide in 2013 [49], in Europe alone > 150 million [50]. One in three people is affected in the UK [51]. Allergies are therefore the most common chronic disease with increasing incidence. In Europe, more than half of the population is predicted to be affected by 2025, independent of age, social status or geographical location [50]. Allergic diseases can decrease productivity and quality of life, which decreases the number of working or school days. In Europe, 100 million working days are lost per year, which burdens not only the economy, but also the healthcare industry [50, 52, 53]. The cause of allergic disease is complex. Risk factors include patient genetics, pollution, family size, allergen exposure and insufficient exposure to microorganisms and parasites, which is described as the hygiene hypothesis. Allergies are manifested in a range of conditions such as allergic rhinitis (“hay fever”), rhinosinusitis or asthma, which means that they can trigger a broad range of symptoms from mild symptoms such as a runny nose or sneezing to severe symptoms such as life-threating asthma exacerbations [49]. With respect to fungi, the type 1 hypersensitivity

reactions are the most common dysregulated responses, although others have been observed in ABPA (section 1.2.5.1) [54, 55].

Table 1. 1: Hypersensitivity responses.

Response	Soluble mediators	Characteristics	Examples of diseases
Type I	IgE, histamine, leukotrienes	Th2-mediated, mast cell and basophil activation, eosinophil infiltration, activation of mucosal glands, smooth muscle cell contraction	Allergic asthma
Type II	IgG, IgM, complement	Complement activation by tissue antigens, complex formation with antibodies, chemotaxis and activation of polymorphonuclear leukocytes (neutrophils in particular), lysis of target cells	Autoimmunity
Type III	IgG, IgM, complement	Formation of antibody-antigen immune complexes that are not cleared by granulocytes and cause tissue damage by triggering an inflammatory response	Systemic lupus erythematosus
Type IV	lymphokines	Antigen-driven activation of $CD4^+$ and $CD8^+$ T cells causing a delayed-type inflammatory response, tissue damage, includes contact (epidermal) and tuberculin-type (intradermal) hypersensitivity, granulomatous hypersensitivity (granulomas formed by macrophages and T cells),	Contact dermatitis, tuberculosis, chronic antigen exposure

Adapted from [45, 56]

1.2.2 Asthma

According to the Global Initiative for Asthma (GINA) asthma is described as “a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the patient having a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitations.” [57]. This heterogeneous and complex disease affects > 300 million people worldwide [58] with predictions going as high as 400 million by 2025 [49]. The disease is a burden on the healthcare industry, causing direct medical costs and indirect costs by productivity loss and premature death, which are hard to emphasize. In the UK alone, £500 billion are spent per year [59]. The World Allergy Organization (WAO) estimates that asthma causes 250,000 deaths per year [49]. The causes of asthma are unclear, but genetic and environmental factors as well as the maturation of the immune system are believed to be major risk factors, each of which are heterogeneous underlining the complexity of the disease [11, 57, 60-62].

1.2.2.1 Pathophysiology

The GINA definition is based on the clinical and pathophysiological changes affecting the airways, which may represent repair mechanisms or unrelated processes [63, 64]. These changes include: hyperplasia and hypertrophy of airway smooth muscle (ASM) thickening the airway wall and leading to enhanced ASM contraction [65, 66], bronchoconstriction, bronchospasm and mucus secretion by activation of cholinergic nerves, reflexes of sensory nerves causing cough [67], chest tightness and shortness of breath [68], subepithelial fibrosis [69-71] and mucus hypersecretion as a result of goblet cell hyperplasia within the epithelium and enlarged sub-mucosal glands [72]. These pathophysiological changes lead to airway narrowing and airway hyperresponsiveness, airway oedema, airway thickening and mucus hypersecretion [66, 67], which result in the range of symptoms described in the GINA definition. Upon certain triggers such as allergens [73], the symptoms of asthma transiently worsen (exacerbate). In worst cases, the airflow obstruction can become irreversible (fixed) despite treatment [66, 74], which is one indicator of lung damage next to bronchiectasis and lung fibrosis [23, 71].

1.2.2.2 Diagnosis

Asthma is diagnosed based on the person's history of respiratory symptoms and > 12% variability in expiratory airflow obstruction assessed using spirometric measurements of forced expiratory volume in one second (FEV_1) as a percentage from the predicted value, the forced vital capacity (FVC) and their ratio (FEV_1/FVC) [57]. Direct airway challenges such as inhaled methacholine or histamine are applied when subjects with asthma have no changes in lung function to measure the airway hyperresponsiveness [75], where a positive result means a decrease in FEV_1 usually by 20% [76]. Other markers of asthma are sputum eosinophilia and/or neutrophilia, of which the former can help to predict asthma exacerbations. The concentration of fractional exhaled nitric oxide (FENO) is linked to sputum eosinophilia in patients without corticosteroid treatment, but is not regarded as a reliable monitoring tool yet. The assessment of sensitisation by skin prick test (SPT) or specific IgE can help to identify the underlying trigger of allergic asthma [57, 77, 78].

1.2.2.3 GINA classes

Asthma control encompasses control of asthma symptoms and risk of future adverse events such as exacerbations. Poor control of asthma symptoms is strongly linked to a higher risk of the exacerbations [79-81]. Symptoms are treated predominantly with so-called reliever and/or controller medications. All patients receive the reliever medications (short-acting inhaled β_2-agonists or anticholinergics) for the "rescue" from worsening asthma symptoms and asthma exacerbations, though their use should be limited [82-85]. The controller medications are a regularly treatment to reduce airway inflammation, symptoms, exacerbations and maintain lung function [57]. Typical controller medications are inhaled corticosteroids (ICS) with or without long-acting inhaled β_2-agonists (LABA), leukotriene modifiers, chromones (sodium cromoglycate and nedocromil sodium), systemic oral corticosteroids (OCS) or anti-IgE [57]. The severity of asthma is defined by the amount of treatment required for asthma control, after the patient has been on regular controller treatment over a prolonged period of time [86-88]. The level of treatment is nothing definite as severity may change. Mild, moderate and severe asthma are grouped into the following GINA classes: People

suffering from mild asthma require only low levels of reliever or controller treatment (low dose ICS, leukotriene receptor antagonists or chromones), also known as Step 1 and 2 treatment, to reduce their symptoms. People with moderate asthma require Step 3 treatment such as low dose ICS/LABA [57]. Between 5 to 10% of people with asthma suffer from severe asthma [86, 87], in the UK alone 140 patients/million population [89]. The International European Respiratory Society/American Thoracic Society (ERS/ATS) define severe asthma not only by the need for high corticosteroid doses plus a second controller and/or oral corticosteroids to dampen asthma symptoms such as exacerbations or airflow obstruction (Step 4 or 5 treatment), but also by experiencing symptoms despite optimal treatment [88]. These subjects have more manifested symptoms of asthma [86, 87]. The definition only includes patients with refractory asthma, but not people with uncontrolled asthma deriving from problems with treatment, medication adherence or comorbidities [88]. Severe asthma is linked to severe exacerbations, sinus disease, airway remodelling and fixed airflow obstruction. Consequently these individuals develop one or several symptoms such as varying peak flows, severe chronic airflow limitation, decrease in lung function, varying mucus production and responses to corticosteroid treatment [90].

1.2.2.4 Phenotype and endotype

Recognizable demographic, clinical and/or pathophysiological characteristics have been clustered into several asthma phenotypes such as asthma with obesity, asthma with fixed airflow limitation, late-onset asthma, non-allergic and allergic asthma [57, 91, 92]. Phenotyping asthma helps to understand the complexity of the disease, but fails to describe the underlying causing mechanisms, which is the goal of defining asthma endotypes. This should improve therapeutic management of patients known as precision medicine. They should describe unique pathogenic mechanisms and biomarkers, which can contribute to multiple phenotypes and vice versa. Asthma endotypes are still controversial and therefore not commonly used in practice [63, 74, 93-95]. Specific biomarkers such as blood and sputum eosinophil count, sputum neutrophil count and serum IgE levels are already used to phenotype asthma, though combinations of these are required to increase the specificity of the diagnosis [77, 78].

In the original paper proposing the use of the term endotypes to interrogate the heterogeneity of asthma, fungal allergy was chosen as one of the examples of an asthma endotype [74].

1.2.3 Fungal exposure

In the lungs, foreign particles are removed by mechanical defence mechanisms such as sinus turbulence or the mucociliary escalator and by the innate immune system, above all alveolar macrophages [96, 97]. The air humans inhale continuously contains a plethora of different particles. This includes a varying mixture of different fungi and their hyphal fragments, fungal spores and yeasts [98] which can trigger hypersensitivity Type I responses. It is difficult to determine the amount of fungi that an individual is exposed to because it depends on weather conditions, climate, seasons [98-100], geographic location [101] and particular habitats such as mouldy buildings [102, 103]. It can be measured using air traps [104, 105]. The number of fungal spores per cubic metre of air often exceeds the number of pollen grain concentrations by 100-1000-fold, depending on the time of year [26]. Dikarya comprise the majority of fungi in the air [106]. Conidia comprise 30-60% of total airborne spores [26], in particular of *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium* species [9]. Allergic symptoms can be evoked for example by > 100 *Alternaria* spores/m^3 or 3,000 *Cladosporium* spores/m^3 [107] with the latter often dominating fungal outdoor air biota worldwide [26]. The air indoors contains lower amounts of fungal particles compared to the outdoor air [108, 109]. The spore size is suggestive of how far down the airways spores can go. The size of the spores distributed by wind is usually 1-20 µm [110]. The minority of fungal spores are > 10 µm in size, which means they are usually deposited in the nasopharynx, where they can induce hay fever [26, 111]. This is reflected by high culture rates from nasal mucus [112]. Smaller spores (2-10 µm) like those from *A. fumigatus* (2-4 µm) and fungal fragments from spores or hyphae, whose number can exceed their respective number of spores, are able to reach the lower airways, where they can trigger asthma symptoms in people with asthma [11, 26, 113]. Exposure to fungal allergens is associated with worsening asthma symptoms, severity and asthma-related deaths, which is supported by reports of increased asthma attacks when the fungal exposure increases [42, 114, 115]. The

significance of fungal exposure indoors in asthma disease was demonstrated by a several studies. High levels of fungal exposure, especially to *Aspergillus* and *Penicillium* species, are a risk factor for both childhood and adult-onset asthma, particularly if children have a predisposition to develop asthma [102, 116, 117]. Meta-analysis of studies assessing the relation of asthma and fungal exposure indoors found sufficient evidence that fungal exposure indoors was causative for the development and exacerbation of asthma in children [116]. In adults, the data were only suggestive of an association between asthma and work in mouldy and damp buildings [116], which is often associated with exposure to *A. fumigatus*, *Cladosporium cladosporioides* and *Acremonium kiliense* [118]. The fungal exposure outdoors was associated with hospital admissions of both children and adults [119-121]. Most outdoor-related asthma symptoms in adults are associated with *Alternaria* species, followed by *Cladosporium* and *Didymella* [122-124]. Exposure and sensitisation to fungi, particularly to *Alternaria*, was associated with life-threatening acute asthma attacks requiring ICU admissions [121], asthma-related deaths [125] and risk of adult-onset asthma [126].

1.2.4 Diagnosis of fungal sensitisation

Whether a person is fungal sensitised or not depends on several factors such as genetic background or the state of the immune system. Sensitisation is assessed by anamnesis and allergen-reactivity tests, which can be based on skin reactions or blood IgE levels to allergic compounds. The former includes the SPT, which is the most commonly used allergen-reactivity test, and intradermal test. The intradermal test is more sensitive than the SPT [127], but has a higher false-positive rate [128]. The most commonly used blood test is the immunoassay capture (ImmunoCAP) system (positive result > 0.35 kU/L) [129]. The concordance between the tests is not as high for fungi as for common allergens [130, 131], which is why it was recommended to do both tests [131]. The extracts either contain fungal proteins extracted the whole fungal cell (crude extract) or recombinant allergens representing purified specific allergens of a certain fungal species. Recombinant allergens' names derive from the species of origin such as *A. fumigatus* (Asp f) and an assigned number. These recombinant proteins are listed by the

WHO and International Union of Immunological Societies (IUIS) (WHO/IUIS) [132] (allergen.org, June 2018).

The prevalence of fungal allergies among the general population worldwide is estimated as 3-10% [26]. In contrast, fungal sensitisation is more common in atopic subjects ranging from 12 to 42%, though the highest levels of up to 70% were observed among patients suffering from severe asthma [42, 131, 133]. Fungal sensitisation is very likely to be underdiagnosed and not necessarily species-specific. This means that the exact prevalence among the general population and subjects, in particular polysensitised individuals, suffering from respiratory diseases such as asthma is unknown. This has several reasons:

First, fungal sensitisation is not commonly tested because its clinical relevance is underappreciated and it is often overlooked due to co-sensitisations to more common allergens such as house dust mites or grass pollen. In addition, as the peak of the grass pollen season also coincides with that of fungal allergens, affected individuals are likely to misinterpret the cause of their symptoms as coming from grass pollen rather than fungi [134].

Second, the commercially available protein extracts include a limited range of allergenic fungi. More than 80 fungal genera have been associated with allergic symptoms [26], predominantly from the Ascomycota [135]. Sensitisation to Basidiomycetes other than to *Malasseziales* is rarely assessed despite their high abundance in the environment [26, 106]. From the range of fungal tests available, the Ascomycetes *Alternaria alternata* and *Cladosporium herbarum* are the most frequently included fungi as recommended by the European community health survey [136]. Only a few asthma studies used large test panels [131, 137]. Due to the implications of sensitisation to thermotolerant fungi on asthma, a more comprehensive test panel should be used, including *A. fumigatus, Penicillium chrysogenum, C. albicans, Saccharomyces cerevisiae*, *Malassezia* and *Trichophyton* species [23, 133] (section 1.2.5). Other points to consider are that the prevalence of fungal sensitisation differs depending on age [138, 139] and that sensitisation patterns can vary over time [140-142].

The third problem with the diagnosis of fungal sensitisation are differences between the fungal protein extracts for each test and that fungal tests are not standardised. This

means that the allergen composition and its antigenicity vary between manufacturers as well as different batches from the same manufacturer. For example, Vailes *et al*. [143] detected considerable variations in the quantities of the *A. fumigatus* major allergen Asp f 1 in extracts from 8 manufacturers and repetitive samples from the same company within a year. These variations are based on which fungal strain, culture conditions, protein source (spores, hyphae or secreted proteins) and extraction protocols used to produce the protein extract for the test [26, 144]. None of the protein extracts have been approved by the food and drug administration (FDA) [145].

Forth, IgE cross-reactivity between fungal species, which is common, further complicates the interpretation of the clinical relevance of species-specific sensitisation [146]. Although IgE antibodies react to some allergens that are unique in a certain species, for example *A. fumigatus* (Asp f 1) and *A. alternata* (Alt a 1), they can also bind to fungal allergens that are similar between different fungi or even between fungi and species from other Kingdoms [147, 148]. The basis of antibody cross-reactions is that proteins share similar epitopes, which often derive from protein orthologues [149, 150]. The antibody cross-reactions to different fungal species can occur independent of their relationship [151-153]. Therefore, people suffering from fungal sensitisation can be monosensitised to one fungus or polysensitised to multiple fungi. Monosensitisation is often associated with allergy to *A. alternata*, the latter form to *A. fumigatus*, *C. herbarum*, *P. chrysogenum* and *S. cerevisiae* [154]. It is unclear if the basis for polysensitisation are IgE cross-reactions to allergens of different fungal species or primary sensitisation to multiple fungi during the course of the inflammatory response [146]. A genetic predisposition could play a role for the former [155]. The latter could explain why co-sensitisation to fungi and other airborne allergens is often observed [154].

Fifth, the use of specific recombinant allergens to test for fungal sensitisation is uncommon. Characterising the range of fungal sensitisation in affected individuals would be beneficial as it enables personalized medical diagnostics and treatment. The identification of fungal allergens can be difficult. Whereas dormant conidia cover their antigens with an anergic hydrophobin layer to evade immune recognition [156], germinating conidia and hyphae can express different allergens [135, 157]. For example,

from the 14 *C. herbarum* allergens listed by Simon-Nobbe *et al.* [135], only one is derived from conidia (Cla h hCh-1), whilst the other allergens are hyphal. As all kinds of fungal particles can be inhaled, it seems necessary to include fungi at all their different life stages. Changes in temperature and climate were shown to affect the amount of allergens expressed in fungal spores [158-160]. Allergen expression *in vitro* highly depends on the choice of the strain, culture conditions, and protein extraction methods [26, 144, 161]. The most common technique to identify proteins is mass peptide fingerprinting [162]. Allergens from fungal species outside of the Dikarya are not well characterised. The WHO/IUIS [132] (allergen.org, June 2018) have approved 111 allergens from 29 fungal species from Ascomycota (86 from 22 species), Basidiomycota (23 from 6 species) and Zygomycota (2 from one species). However, the true number of fungal allergens is unknown. Up to 1% of fungal proteins were estimated to be allergens based on homologies to known allergens, predicted allergens and studies using IgE binding to phage libraries [149]. The list of fungal allergens suggested by Simon-Nobbe *et al.* [135] includes 174 allergens identified from 19 genera of Ascomycota and 30 from 4 genera of Basidiomycota. The allergens include secreted, cytoplasmic and structural proteins, such as proteases, stress response proteins or glycosidases [163]. Some approaches for better diagnosis of fungal sensitisation have been undertaken, which include the use of purified native or recombinant allergens by molecular or component-resolved diagnostics (CRD). Studies showed that subjects with ABPA were more likely to be sensitised to Asp f 4 and Asp f 6 from *A. fumigatus* compared to subjects suffering from severe asthma with fungal sensitisation (SAFS) [164]. A recent study showed that Asp f 3 and Asp f 4 are associated with bronchiectasis in fungal sensitised subjects with asthma [165]. Another example is the species-specific recombinant protein Alt a 1 from *A. alternata*, which showed a high efficacy to identify *A. alternata*-sensitised subjects [166], particularly in combination with the IgE cross-reactive proteins Alt a 6 and manganese-dependent superoxide dismutase (MnSOD) [167]. This shows that recombinant allergens are more specific for the diagnosis of species-specific sensitisations and cross-sensitisations. Their use could enable a more personalized medical approach and potential usage for both diagnostics and treatment, but their commercial availability is limited.

1.2.5 Fungal sensitisation and asthma

Fungal sensitisation is frequently found in asthma, particularly in early onset atopic eosinophilic asthma [23], and is considered to be causative of the disease [118, 126, 168]. Fungal sensitisation was described as a distinct endotype of asthma [74] and often occurs in people suffering from severe asthma. The prevalence of fungal sensitisation in children with asthma is about 40%, but as high as 60% if they suffer from severe asthma [169]. It is possible that fungal sensitisation acquired in childhood could persist into adulthood, priming the affected individual for more severe asthma in later life [23, 169]. In adult patients with severe asthma, prevalence ranges between 35 to 70% [42], though the number is up to 76% for those requiring multiple hospital admissions [115]. Often, co-sensitisation to multiple fungi can be found in more than half of severe asthma patients, with the majority being sensitised to thermotolerant fungi, particularly *A. fumigatus* [131, 170]. In people suffering from moderate to severe asthma (without ABPA), sensitisation to *Aspergillus* is associated with impaired lung function represented by reduced FEV_1, increased severity of airway obstruction, eosinophil counts and IgE levels, more cases of bronchiectasis, and higher corticosteroid dose requirement, all of which are indicative of tissue damage and inflammation [41, 43].

Both mesophilic and thermotolerant fungi can be inhaled. The allergic impact of mesophilic fungi is somewhat predictable based on their spore count, but thermotolerant fungi are able to colonise the lungs. This means that allergenic molecules can be released during the growth of the fungi and a more persistent allergenic response can be expected [23]. A cross-sectional study by Agbetile *et al.* [137] demonstrated a 22% drop in lung function in association with fungal sensitisation and fungal-positive sputum culture in moderate-to-severe asthma cases with or without ABPA compared to those subjects who had neither. More than 50% of subjects were culture-positive and in total > 20 taxa were recovered. The predominant fungus was *A. fumigatus* (44%), followed by fungi from the *Aspergillus* section *Nigri* and *Talaromyces piceum* (both 5%) [137]. *A. fumigatus*, which belongs to the *Aspergillus* section *Fumigati*, is the most studied fungus in relation to fungal allergy and asthma. Colonisation by *A. fumigatus* was shown to induce a Th2-driven response, resulting in chronic allergic airway inflammation and bronchial hyper-responsiveness [171]. Fairs *et al*. showed that significantly higher culture rates of *A. fumigatus* were obtained in *A. fumigatus*-IgE-

sensitised moderate-to-severe asthma patients (63%) in comparison to non-sensitised asthmatics (31%) and healthy controls (7%) [41]. These studies are suggestive of hypersensitivity reactions due to a continuous allergic stimulus by fungal colonisation, which may therefore represent a risk factor for asthma development [172]. Fungal colonisation is not exclusive to subjects with respiratory diseases, but can also be found in healthy individuals. People with asthma often experience some degree of lung damage as a result of the chronic inflammation and are frequently treated with antibiotics in addition to their conventional therapy, which damages the existing microbiome. Both factors favour colonisation by other microorganisms such as thermotolerant fungi (as opposed to mesophilic fungi) in lung disease [173], which can worsen the disease to an unknown extent as their allergenic potential is not as predictable as those of aeroallergens.

1.2.5.1 Allergic bronchopulmonary aspergillosis/mycosis

Since the 1950s, clinicians and researchers have tried to define the relationship between asthma and fungal sensitisation, regarding it as a complication of asthma. The term first coined was ABPA [174], which is usually though not exclusively present alongside respiratory diseases like cystic fibrosis (CF) and asthma [128]. Prevalence in each disease ranges between 7 to 9% and 0.7 to 3.5%, respectively [133]. It is usually a progressive inflammatory disease, ranging from mild disease with respiratory exacerbations to fatal lung damage [40] and characterised by Type I, III and IV hypersensitivity responses to fungal colonisation (Table 1. 1) [54]. The term allergic bronchopulmonary mycosis (ABPM) was introduced later which is associated with a range of fungi in addition to species of *Aspergillus* [175] including *C. albicans*, *Bipolaris* species, *Schizophyllum commune*, *Curvularia* species, *Pseudallescheria boydii* species, *A. alternata* and *C. cladosporioides* [55, 175, 176].

According to the International Society of Human and Animal Mycology (ISHAM) [177], the criteria for diagnosis of ABPM are the presence of asthma or CF, increased *A. fumigatus*-specific and total (> 1000 IU/ml) IgE levels, abnormal radiological findings such as bronchiectasis, peripheral and pulmonary eosinophilia with a > 1,000 cells/mL eosinophil count in steroid naïve patients. Cases without bronchiectasis are described

as seropositive ABPM, which has been implicated as “early stage” ABPM [178, 179]. A number of other immunological and pathological markers have been identified, which – including the ones above – do not necessarily occur simultaneously: fleeting lung shadows, progressive tissue damage with lung fibrosis, fixed airflow obstruction [174], recurrent transient chest radiographic lung infiltrates, mucoid impaction, high-attenuation mucus, which is not observed in other asthma endotypes [180, 181], and aspergillomas, which have been diagnosed in 7% of patients with ABPA [128]. Although fungal colonisation is thought to be the cause of ABPA, culture of *A. fumigatus* from sputum is only a minor criteria for ABPA diagnosis [54], which was therefore not assessed by many studies [176]. Although studies have shown that sensitisation to *A. fumigatus*-specific Asp f 2, Asp f 4 and Asp f 6 is more common in patients with ABPA [164, 182], they are not used as a criterion.

1.2.5.2 Severe asthma with fungal sensitisation

Due to the high specificity of the ABPM criteria, clinicians encounter problems diagnosing those patients with fungal sensitisation who lack other pathophysiology parameters defining ABPM [128]. Although fungal sensitisation is common in severe asthma [42, 131], only about 10% of these patients match the criteria for ABPA [47]. This is why Denning *et al*. [42] coined the term “severe asthma associated with fungal sensitisation” (SAFS). The exact prevalence of SAFS is unknown, but as fungal sensitisation is more common in severe asthma with a minimum prevalence of 33%, around 6.5 million patients could suffer from SAFS. The incidence of SAFS is probably lower due to ABPA cases [40]. The diagnosis of SAFS is based on exclusion criteria [42, 183]. The most crucial diagnostic difference to ABPA is that people with SAFS have a total IgE level below 1000 IU/ml [42]. The new SAFS categorisation of people with asthma and fungal sensitisation is questionable concerning some aspects: First, the differentiation between ABPA and SAFS based on the total IgE value would have to imply that the value is an absolute measure, which is not the case as it varies over time. Patients with a mild disease can have high total IgE levels, whilst levels are not necessarily high in patients with severe asthma. A recent study showed that total IgE was unsuitable to determine the risk of lung damage [184]. It also assumes that both

mesophilic and thermotolerant fungi are able to cause SAFS, although mesophilic fungi are unlikely to contribute in persistent lung disease. High IgE levels usually indicate sensitisation to thermotolerant fungi, although they do not necessarily have to derive from pathogens colonising the lung. They could also derive from fungi colonising the skin such as *Candida* or *Malassezia* species, which can cause eczema, a condition frequently accompanying asthma [134]. Some individuals are classified as ABPA cases even when their IgE levels are below the threshold because they fulfil other criteria for ABPA [177]. In addition, the threshold itself is very arbitrary and used to be much lower in earlier reports with 417 IU/ml [23]. The higher value was set in recent years seemingly for greater specificity, but might as well have been wrongly adopted since 417 IU/ml is equivalent to 1000 ng/ml [23, 177]. Third, SAFS encompasses only people with severe asthma, but disregards that fungal sensitisation is also present in mild-to-moderate disease with or without good lung function, or is present in other fungal lung diseases [23].

1.2.5.3 Allergic fungal airway disease

In light of the limitations of ABPA and SAFS, our group has coined the term "allergic fungal airway disease" (AFAD) [23, 184], suggesting a further subgrouping into mild, moderate and severe. ABPA could be diagnosed on its own or described with severe AFAD. The term allows for a broader definition, which encompasses people with asthma who are at risk to develop lung damage with fixed airflow obstruction, bronchiectasis and other radiological abnormalities such as fleeting shadows, mucus plugging and lung fibrosis as a result of their IgE sensitisation to thermotolerant filamentous fungi [23, 134, 184]. Compared to subjects without AFAD, people with AFAD have chronic poor asthma control and their tendency to develop bronchiectasis exacerbations might derive from bacterial bronchitis, which is hard to treat [134]. It was shown that IgE sensitisation to thermotolerant filamentous fungi is a risk factor for the development of lung damage [184]. This association does not hold up for every patient suffering from fungal sensitisation, which implies a high variability among these patients. Reasons for this variability could be the time of diagnosis, variability of the immune control mechanisms, and sensitisation to specific allergens, levels of allergens in the airways, and degree of

IgE cross-reactivity to human proteins [147]. A recent study showed that Asp f 3 and Asp f 4 are associated with bronchiectasis, which means that fungal specific allergens could account for the differences in lung damage among patients with fungal sensitisation [165]. These findings suggest that AFAD might be caused by persistent airway colonisation, but the resulting symptoms and pathophysiology are more likely to derive from the chronic Th2-mediated inflammatory response rather than an active infection.

Despite the associations of asthma, in particular severe asthma, with fungal sensitisation and colonisation, their role in the pathology of asthma is still unclear and understudied, which could impact optimal treatment of the affected individuals. Although antifungal therapy is often used in clinical practice to treat AFAD [23], clinical trials have failed to show a clear benefit [185-190]. It is possible that a better assessment of the fungal sensitisation using recombinant allergens unique to certain fungal species can help diagnosis, treatment and associations with specific asthma outcomes. This may be particularly important with respect to fungi present in the lung of asthmatics that are not currently recognized as potential allergen sources or otherwise due to limitations of traditional detection techniques.

1.3 The lung fungal microbiome

1.3.1 Introduction to the microbiome

The microbiota describes the composition of microorganisms in a given environment, whilst the microbiome describes its genetic composition. Both microbiota and microbiome encompass bacteria, fungi, viruses and other microorganisms. Over recent years the importance of the bacterial microbiome (bacteriome) for health has been more and more recognized, demonstrated by its involvement in immune system development, nutrition and drug metabolism [191-193]. Lifestyle constantly influences the microbiome, which varies highly between individuals [194-197], although all individuals share a core microbiome as demonstrated in a gut study [198].

The respiratory tract used to be regarded as a sterile environment in healthy individuals until sequencing studies showed the presence of a bacteriome [199, 200], which is unequally distributed within the airways [201]. Protective effects of the airway

bacteriome against allergic disease have been observed in mice. Studies in humans showed that some bacterial species in the upper airways of children were associated with an increased risk of asthma [202]. A higher burden and diversity of the airway bacteriome was detected in patients with asthma compared to healthy controls, though the diversity differed depending on the asthma phenotype [199, 203-206]. The full scope of protective and harmful effects of the airway microbiome is still not clear.

The majority of studies on the microbiome investigate the bacteriome and not the fungal component, the mycobiome, a term coined by Ghannoum *et al*. [207, 208], probably because of its lower abundance [209]. It is believed that fungi contribute to the homeostasis of the human microbiome, thereby influencing health and disease [25]. Only a limited number of studies are available looking at a small number of habitations of the mycobiome. The composition of the mycobiome differs depending on the location [210], and varies more over time compared to the bacteriome [211], probably due to environmental factors and the individual's health. Studies on the pulmonary mycobiome in health and disease are not common [209, 212]. A problem encountered with lung samples is that the microbial biomass is usually rather low compared to other body sites like the gut or skin [213] and the mycobiome is part of the rare biosphere, contributing to < 0.1% of the total microbiome [214-216].

1.3.2 High-throughput sequencing of fungal sequences

The mycobiota can be sampled using traditional culture methods or high-throughput sequencing (HTS) determining the mycobiome. HTS is more advantageous for the detection of fungi compared to traditional culture methods as it does not depend on fungal growth conditions. Species could be missed due to different growth rates with faster growing fungi outcompeting slower growing ones [217]. Only 17% of the described fungal species are able to grow *in vitro* using current methods, which includes those that require non-standard medium [20]. Some pulmonary mycobiome studies showed that 36% to > 60% of taxa identified by sequencing were non-culturable fungi [207, 218]. Therefore, HTS allows the identification of a broad range of fungal species from a complex sample [219, 220] and seems to provide a better estimate of the true fungal diversity in a given sample.

The nuclear ribosomal operon (rDNA) (Fig. 1. 1) is the genomic region most routinely used to identify fungi [221]. The region is present with up to 250 copies per cell, making it convenient for PCR amplification [222]. Fungal rDNA has a length of 7 to 50 kb, depending on the species [223]. It encodes the genes of the conserved small subunit (5' end) (SSU), also known as 18S, the internal transcribed spacer (ITS)1 region, conserved 5.8S region, ITS2 region [224], and the large subunit (LSU), also known as 26S or 28S at the 3' end.

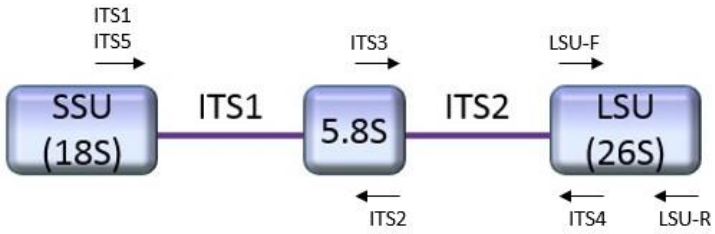


Fig. 1. 1: Structure of fungal nuclear ribosomal operon and primer binding sites.

The D1/D2 variable region of the LSU is suitable for accurate classification [224] of the genus level and above [9]. The 18S rDNA is highly conserved among eurkaryotes and not very divergent, in contrast to the ITS1 and ITS2 regions that are transcribed and spliced away [225]. They are highly variable, but have enough conserved genetic differences to distinguish between closely related species and sometimes also between strains of the same species. They were therefore suggested as formal barcode regions for fungi [9, 222, 226]. The LSU is primarily used for yeast identification, but has also been used to assess fungal diversity albeit to a lesser extent than ITS. Some authors suggest to sequence both regions for a more accurate species-determination [226-228], as ITS is better for discrimination on the species level and LSU for phylogenetic distances as it represents a coding region [229].

1.3.3 The lung mycobiome in health and disease

Only a few studies are available on the lung mycobiome in airway diseases, which usually have low subject numbers, mostly focus on CF and often have no healthy control group. Typical respiratory samples collected were sputum, bronchoalveolar lavage (BAL) and/or mouthwashes.

Studies including healthy controls showed that the lung mycobiome was predominated by environmental fungi such as *Cladosporium*, *Davidiellaceae* [230] and *Malasseziales* [197, 231]. The lung mycobiome was assessed in subjects suffering from the following airway conditions: CF [218, 232-235], asthma [231, 236], lung transplant [230, 237], HIV with or without pulmonary disease [237, 238], pulmonary disease [237], and intensive care unit (ICU) patients with pneumonia [197]. The study on the lung mycobiome in asthma by Fraczek *et al*. [231] was published after completion of the work presented in this thesis [231] and is therefore not presented in this introduction.

Interestingly, *Candida* species predominated the respiratory samples from the majority of these studies except for the asthma study. *Candida* species were previously associated with decreased lung function and increased exacerbation frequency in a culture-based CF study [239], but their clinical relevance is still unclear. Examples of other dominant fungi detected are *Malassezia* [218, 232], and *Aspergillus* [218, 234]. The majority of fungi probably are likely to derive from the environment [218, 240]. Some studies showed that low species richness and diversity of the lung mycobiome were associated with poor clinical status, including lung function [218, 230, 238]. It was further demonstrated that the mycobiome differs between individuals with the same disease [234, 237] and can change over time [218, 233].

The oral mycobiome was the first to be investigated using HTS [207] and may influence the lung mycobiome due to the smooth transition within the respiratory tract as demonstrated for bacteria [241, 242]. Most of the species identified from the oral cavity are likely to derive from the environment by inhalation or food. The composition highly varies within and between subjects [207, 230, 243]. The most predominant fungus found in healthy individuals are species from the genera *Candida*, *C. albicans* in particular [207, 243]. Other prominent species include *Cladosporium*, *Aureobasidium*, the *Saccharomycetales* family, *Cryptococcus*, *Aspergillus* and *Fusarium* and the genus *Malassezia* [207, 230, 243]. Studies comparing the mycobiomes of the oral cavity and the lung showed that microaspiration is possible, but that each sample type also showed a number of distinct species [230, 238]. The influence of the oral mycobiome on the lung mycobiome has yet to be shown for people with asthma.

Most of these mycobiome studies did not address some important aspects: First, distinct mycobiomes between BAL and mouthwash samples were shown in a few studies, but comparisons between sputum and invasive bronchoscopy are rare [238]. Differences between the sample types was indicated by studies on the bacteriota [244-246]. Second, only a small number of studies included contamination controls, most often bronchoscope flushes [197, 230, 237], although other contamination sources during sample processing such as DNA extraction kits exist as demonstrated for bacteria [247] and fungi [248]. Third, none of the studies compared the mycobiome determined from clinical samples to that of matching air samples (aerobiome) to assess the influence of fungal particles that are constantly inhaled (section 1.2.3). Lastly, only one CF mycobiome study [235] discriminated between live and dead fungal cells [249]. This is important as only live cells would be able to grow in the lung and be part of the mycobiota and therefore the mycobiome and can represent permanent allergic triggers.

1.4 Summary

Fungi are ubiquitous in the environment and can cause diseases in humans. The importance of fungal sensitisation in asthma, in particular with respect to *A. fumigatus*, was shown by several studies, however, the full scope of fungal sensitisation, which would be beneficial for a personalised therapy and associations with specific disease outcomes, is unknown. This is due to limitations in the range of fungi used in test panels and ill-defined protein extracts, which emphasizes the need for tests based on recombinant allergens. Their benefit was indicated for the discrimination between SAFS and ABPA, and as possible biomarker for lung damage in AFAD. The identification of individual fungal allergens from fungi colonising the airways would help to understand the role of fungi in asthma. It was demonstrated that both colonisation and sensitisation to the colonising thermotolerant fungi can influence asthma pathophysiology, lung function in particular, compared to non-fungal sensitised people with asthma and healthy individuals. The majority of studies sampling the mycobiota from the respiratory tract are based on culture, which is limited and could miss clinically relevant fungi that subjects could be sensitised to. The mycobiota can also be assessed by investigating the mycobiome using HTS. Current lung mycobiome studies, in particular asthma studies, are rare and limited by small subject numbers. Important questions such as mycobiome differences between distinct lung samples, the influence of fungal particles from the air or risk of contaminations were not addressed by most of them. Investigating the lung mycobiome in asthma could reveal a number of unculturable species whose influence on fungal sensitised people with asthma is yet unknown.

1.5 Hypotheses

- The composition of the mycobiome differs between fungal sensitised and non-fungal sensitised subjects with asthma and healthy controls.
- The composition of the mycobiome depends on the location of sample acquisition from the airway
- High-throughput sequencing provides a better insight into the mycobiota than traditional culture methods.
- Based on the results of the hypotheses above, another hypothesis was that *A. tubingensis* and *A. niger* as representatives of the *Aspergillus* section *Nigri* express unique allergens compared to *A. fumigatus*

1.6 Aims and objectives

The specific aims of this thesis to address the hypotheses were:

- To compare the mycobiome of people with asthma to healthy controls
- To determine whether fungal sensitisation affects the mycobiome in people with asthma
- To determine the fungal load by universal and fungal-specific qPCR
- To assess the influence of the oropharynx during sputum sample acquisition
- To investigate the contribution of dead fungal material to the mycobiome
- To compare mycobiome data derived from sputum samples to invasive bronchoscopy-derived samples
- To compare culture data with the most prominent fungi detected by high-throughput sequencing
- To show the contribution of air spores to the fungi detected in the clinical samples

- To produce protein extracts from the cytosolic fraction of lysed fungal material from fungi of interest detected by high-throughput sequencing using clinical isolates
- To investigate the IgE-mediated allergic response to the protein extracts
- To characterise IgE cross-reactive proteins between related species
- To identify unique allergens and express them as recombinant allergens
- To develop an immunoprecipitation assay to purify allergens from fungal crude protein extract

Chapter 2 Materials and methods

Company names and devices used in this thesis are mentioned only once unless the product of a different manufacturer was used for a specific procedure.

2.1 Patient recruitment

The study was approved by the Leicestershire and Rutland ethics committee (06/Q2502/110 and 14/WM/1099) as well as the National Health Service ethics committee (REACT IRAS 159074:CRN 159074 and ABPA UHL 10111). All subjects gave their written, informed consent under the ethical references. Healthy individuals from staff and associates of the Glenfield Hospital (Leicester, UK) and people with asthma were recruited to the Leicester Respiratory Biomedical Research Unit (BRU) from the outpatient clinics at the hospital and screened by colleagues at Glenfield Hospital (Leicester, UK). They collected clinical samples and carried out the clinical assessments such as sensitivity testing (section 1.2.4), which were kindly provided for this thesis. Subjects were seen at a single stable visit, with a subset returning for a second visit where a bronchoscopy was performed. Sputum samples were processed by members of the sputum lab at the BRU. People diagnosed with asthma were categorised according to the GINA criteria [57]. The inclusion criteria were that subjects were at least 18 years of age. People with asthma were included when they had been diagnosed by a physician and had a clear clinical history of asthma. They had to be at least six weeks post exacerbation, and show historical records of either airflow obstruction with > 12% variability in their FEV_1, or > 12% improvement in FEV_1 15 min after 200 µg inhaled albuterol and/or a provocative concentration of methacholine required to cause a 20% fall in FEV_1 (PC_{20}) of < 8 mg/ml at the time of recruitment [250]. Exclusion criteria were pregnancy or lactating, presence of terminal illness, respiratory diseases other than asthma, or omalizumab treatment. The clinical data collected included gender, age, age of asthma onset, smoking history (never smoked), radiological evidence of bronchiectasis and prescribed inhaled and systemic corticosteroid therapy.

Respiratory measurements performed in both people with asthma and healthy individuals included post-bronchodilator FEV_1 taken 15 min after inhalation with 200 µg albuterol (according to ATS/ERS guidelines [250]) by the best successive readings in 100

ml using a dry bellows spirometer (Vitalograph Ltd, Maids Moreton, UK) [251, 252]. Lung function was assessed by determining FEV_1 (L), FEV_1 (% predicted), FVC (L), FVC (% predicted) prior and after to bronchodilation. The volume change in FEV_1 post bronchodilation was calculated (in mls) as well as the % change in FEV_1 post bronchodilation. Detection of FENO levels was performed at a flow of 50 mL/sec using a Niox chemiluminescence analyser (Aerocrine, Stockholm, Sweden). Bronchiectasis was diagnosed using thoracic scans of the thorax by computed tomography based on the radiologist's clinical report (Picker PQS, Picker International, Cleveland, OH or Siemens sensation 16 scanner, Siemens Healthcare, Knoxville, TN).

2.2 Sensitivity testing

SPTs (Alk-Abello, Denmark) and the ImmunoCAP blood test (UniCAP 250 system, Phadia, UK) were used to determine the subject's sensitivity to several fungi, namely *A. alternata*, *A. fumigatus*, *C. albicans*, *C. herbarum* and *P. chrysogenum*. The blood test also included *Malassezia* species and *Trichophyton rubrum*. Total IgE, *A. fumigatus*-specific serum IgG (sIgG) and *A. fumigatus*-specific serum IgE (sIgE) levels were determined by ImmunoCAP. Sensitisation to common allergens, grass, tree pollen, cat, dog, house dust mite was assessed by SPT. The SPT was positive if the wheel showed ≥ 3 mm diameter compared to the negative control. Sensitisation was recorded if blood IgE values were ≥ 0.35 kU/L. Subjects with asthma were grouped based on their sensitisation to fungi. This thesis presents the data from fungal sensitised people with asthma, non-fungal sensitised people with asthma and healthy controls.

2.3 Sample processing

2.3.1 Fungal culture media

All the work was conducted in a class II safety cabinet cleaned with 10% Microsol (Anachem). All media and their ingredients used for fungal culture are presented in Table 2. 1. All media were autoclaved at 121°C for 15 min and cooled in a water bath to approximately 50°C before pouring plates. The plates were prepared either on the day of autoclaving or from stored media (up to 3 months old). Shelved media were melted

using a microwave and cooled down to approximately 50°C in a water bath. Around 20 ml of medium was poured manually into 90 mm Petri dishes. Antibiotics and antifungal agents were added at optimum concentrations for isolation of fungi before plates were prepared [253]. Stock solutions of the antimicrobial agents were prepared using the recommended solvents and filter-sterilised through 0.2 µm acrodisc filters (Pall cooperation, USA) before they were stored at -20°C. The medium was allowed to solidify in the Petri dishes before the plates were stored at 4°C until use. Plates containing antimicrobial agents were used within 2 months of preparation.

Media based on potato dextrose agar (PDA) were the basic medium used for fungal culture [254]. PDA medium and Sabouraud liquid medium were used to culture fungi for protein extractions (section 4.2.4). Sputum plugs (section 3.2.2.1) and bronchoscopy-derived samples, BAL, bronchial wash and protected bronchial brush (section 3.2.2.3) were cultured on PDA medium with antimicrobial agents chloramphenicol, gentamicin and fluconazole (PGCF). The antifungal fluconazole was added to suppress *Candida* growth and increase the recovery of *A. fumigatus* [255]. Bronchoscopy-derived samples were also split to inoculate yeast malt agar (YMA) and *Scedosporium* selective (ScedSel+) agar to promote growth of yeasts and slow-growing filamentous fungi, respectively [256, 257].

Table 2. 1: Composition of media used for fungal culture.

Medium	Basic ingredients	Antibiotics/antifungal agent	End concentration (μg/ml)	Use
PDA	39 g/L PDA			Protein extraction
PGCF	39 g/L PDA	Chloramphenicol (34 mg/ml in ethanol)	16	Clinical samples:
		Gentamicin (50 mg/ml in deionised water)	4	Sputum
		Fluconazole (25 mg/ml dimethyl sulfoxide (DMSO))	5	BAL, wash, brush
YMA	3 g/L Yeast extract	Chloramphenicol (34 mg/ml in ethanol)	16	Clinical samples
	3 g/L Malt extract	Gentamicin (50 mg/ml in deionised water)	4	BAL, wash, brush
	5 g/L Peptone			
	10 g/L Glucose			
	20 g/L Agar			
ScedSel+	6.25 g/L Malt extract	Chloramphenicol (20 mg/ml in ethanol)	100	Clinical samples
	6.25 g/L Maltose	Ciprofloxacin (100 mg/ml in 1M sodium hydroxide)	0.1	BAL, wash, brush
	1.25 g/L Mono-potassium phosphate	Streptomycin sulphate (20 mg/ml in deionised water)	100	
	1 g/L Yeast extract	Dichloran (2 mg/ml in ethanol)	0.002	
	0.625 g/L Magnesium sulphate	Benomyl (1.2 mg/ml in methanol)	6	
	0.625 g/L Soy peptone			
	20 g/L Agar			
Liquid Sabouraud	30 g/L Sabouraud liquid medium			Protein extraction

All basic ingredients were from Oxoid, UK, except for mono-potassium phosphate (Sigma-Aldrich, UK), magnesium sulfate heptahydrate (Acros organics, UK), peptone, glucose and maltose (all Fisher Scientific, UK); all antibidiotics/antifungals were from Sigma-Aldrich, UK

2.3.2 DNA extraction

DNA extractions were performed under aseptic conditions in a class II safety cabinet. One hundred microlitres of PCR-grade water (Sigma-Aldrich, UK) were used as a negative control for the DNA extractions. Contamination control samples (herein called kit controls) were done when the kit was opened or during the use of the kit, and at the last DNA extraction done with the kit. DNA extraction was done using the DNeasy Plant Mini Kit (Qiagen, Netherlands) according to the manufacturer's instructions with some modifications. Multiples of the lysis buffer (400 µl AP1 per sample) and ribonuclease (4 µl RNase per sample) were mixed in a sterile tube by inverting the tube several times. Volumes were adjusted to allow for pipetting errors by adding 1-2 measures to the volumes calculated for the number of samples to be extracted. Cells were disrupted by vigorous bead beating for 2 min using the BioSpec mini-beadbeater 16 at 3450 oscillations per minute (Bartlesville, OK, USA) [98, 228], before samples were incubated in a water bath or heating block at 65°C for 10 min. During the incubation, tubes were inverted 2-3 times. Buffer P3 was added (130 µl per sample) to precipitate proteins and polysaccharides. After vortexing the suspensions, samples were incubated on ice for 5 min. Samples were centrifuged at 14,000 rpm for 5 min and the supernatant was transferred to the QiaShredder Mini spin column. The manufacturer's instructions were followed afterwards. Two hundred microlitres were eluted in two consecutive 100 µl elution steps. The DNA concentration was determined using 10 µl of solution (section 2.3.3). The DNA was stored at -20°C until use.

2.3.3 Determination of DNA concentration

The DNA concentration was either determined using the Qubit® dsDNA high-sensitivity (HS) (range 0-10 ng/µl) or broad-range (BR) (range 2-1000 ng/µl) kit (Invitrogen, UK). For both kits 1 µl of the fluorescent reagent was mixed with 199 µl of the buffer by vortex to produce a working solution. The volumes were multiplied based on the number of samples to be measured plus the two standards and 1-2 extra measures to account for pipetting errors. The work was performed under aseptic conditions in a PCR flow hood (Labcaire, Cantel medical PCR6, UK). For both kits, the concentration within 1 µl or 10 µl of sample was determined. The volumes were mixed with the working solution by vortex

to make up a total volume of 200 µl. Ten microlitres of each DNA standard from the respective kit were added to the working solution by vortex to make up a total volume of 200 µl. The solutions were centrifuged briefly (quick-spinned) before the solutions were incubated for 2 min at RT and the concentrations measured using the Qubit™ fluorometer 1.0 or 3.0 (Invitrogen, UK). The fluorescent dyes within the kit reagent fluoresce when bound to double-stranded DNA. The device measures the fluorescence emitted from the samples and compares the intensity to that emitted from the standards.

2.3.4 Molecular techniques for fungal identification

DNA amplifications were set-up in a PCR flow hood (Labcaire, Cantel medical PCR6, UK) under aseptic conditions unless stated otherwise. PCR reaction products were always handled outside of the PCR flow hood near a Bunsen burner to avoid contamination of samples with high concentrated DNA amplicons. Filtered tips (StarLab) and flat cap PCR tubes (VWR) were used unless stated otherwise. Samples were prepared on a PCR cooler to ensure that the respective polymerase used in each assay remained inactive until running the PCR reaction on the respective cycling machine. Amplification products were stored at 4°C for short-term storage (up to 2 days) and stored at -20°C for long-term storage.

2.3.4.1 Polymerase chain reaction

Identification of yeasts and unknown filamentous fungi recovered from culture of clinical samples (section 3.2.3) was performed targeting suitable regions of the rDNA. Primer combinations used were ITS1/ITS4 or ITS5/ITS4 targeting the entire ITS region, ITS3/ITS4 targeting the ITS2 region [10] and LSU-F/LSU-R to amplify the LSU region [258] (Table 2. 2). Reactions were done in duplicate and contained 5 µl of template DNA or PCR-grade water as a negative control. An optional 0.2 µg/µl BSA (NEB, UK) was added to improve amplification efficiency for any region used. For filamentous fungi DNA was extracted (section 2.3.2) and then amplified whereas for yeasts the PCR was performed directly from the yeast colony without DNA extraction [259]. A small quantity of yeast was taken by touching a pipette tip into a colony and transferred into the tube

containing the PCR reagents. The reactions were conducted in the T100^{TM} Thermal Cycler.

The PCR reaction amplifying the ITS/LSU region is described in Table 2. 3. PCR cycling conditions started with 15 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C and 90 sec at 72°C. The final elongation step was performed for 10 min at 72°C. The presence or absence of the PCR product was determined by gel electrophoresis (section 2.3.4.3).

Table 2. 2: Primer list for amplification of fungal DNA from the nuclear ribosomal operon.

Name	Primer Sequence	Reference
ITS1	5'TCCGTAGGTGAACCTGCGG 3'	[10]
ITS3	5'GCATCGATGAAGAACGCAGC 3'	
ITS4	5'TCCTCCGCTTATTGATATGC 3'	
ITS5	5'GGAAGTAAAAGTCGTAACAAGG3'	
LSU - F	5'GAGTCGAGTTGTTTGGGAATGC 3'	[258]
LSU -R	5'GGTCCGTGTTTCAAGACGG 3'	

Table 2. 3: Reagent mix for fungal ITS/LSU amplification.

	Quantity added per 25 µl PCR-grade water	Final amount/ concentration
PCR buffer with $MgCl_2$ (Qiagen)	2.5 µl	1X
dNTPs (Bioline)	2.5 µl	250 µM (each base)
Forward primer (Invitrogen)	2.5 µl	250 nM
Reverse primer (Invitrogen)	2.5 µl	250 nM
HotStar Taq Polymerase (Qiagen)	0.125 µl	0.625 U
Template DNA/PCR grade water*	5 µl	N/A

N/A = not applicable; * For colony PCR, 5 µl of water was added to the master mix per reaction

2.3.4.2 Clean-up and sequencing

PCR products for fungal identification by Sanger sequencing were purified using a PCR purification kit (Jena Bioscience, Germany) or QIAquick PCR purification kit (Qiagen, Netherlands) according to the manufacturer's instructions. Thirty microlitres were eluted into PCR-grade water. The concentration was determined with the BR assay for Qubit fluorometer using 10 µl of the product (section 2.3.3). Samples were prepared for sequencing by following the instructions of the SmartSeq Kit (Eurofins, Germany). They were then sent to Eurofins (Germany). Sequences were analyzed with FinchTV software (v1.4.0) and BLASTn (NCBI) [260].

2.3.4.3 Amplicon detection by gel electrophoresis

Amplification products were loaded on agarose gels, whose preparation differed depending on the assay. A 1X Tris/Acetate/EDTA (TAE) was prepared from a 10X stock solution containing filtered and autoclaved 39.98 mM Tris (Fisher Scientific), 1.27 mM EDTA (Sigma-Aldrich, UK) with pH 8 adjusted by glacial acetic acid (1.2% v/v) (Fisher Scientific, UK). Two or three percent (w/v) agarose (Sigma-Aldrich or Fisher Scientific, UK) was dissolved using heat and was allowed to cool down to approximately 50°C before pouring the gel. As a rule of thumb, the higher the percentage, the smaller the expected length of the nucleic acid. Amplicons of ~500 bp were loaded onto 2% gels (section 2.3.4.1), whereas RFLP products (section 4.2.3) were loaded onto 3% gels. If PCR reactions had been done in duplicate, products were combined before electrophoresis. To visualize the DNA, 1 µl ethidium bromide (10 mg/ml) (Sigma-Aldrich or BioBasic, UK) per 100 ml molten agarose was dissolved into the molten agar just before pouring the gels. The gels were immersed in 1X electrophoresis buffer before loading. Five microlitres of sample were added to 2 µl of loading dye (Sigma-Aldrich, UK) before it was loaded into the wells of the gel. Seven microlitres of the PCR Sizer 100 bp DNA ladder (Norgen, Canada) were also loaded to estimate the size of the nucleic acid sample. The voltage and time used to run the gels depended on the percentage and size of the gels. Two percent gels were run at 100-120 V for 20-50 min, where small gels (50 ml agarose) had shorter running times compared to larger gels (100 ml agarose) (BioRad Power Pac 300 machine or Fisher Scientific Nanopac 300). After electrophoresis, gels were

visualised and photographed using a suitable UV light imager (UV imager with Image100 software, ChemiDoc Touch machine with ImageLab software v5.2.1 or v6.0.1 (BioRad)).

Chapter 3 The lung mycobiome in people with asthma

3.1 Introduction

Although fungal colonisation has been associated with worsening asthma symptoms [23, 41, 43], a comprehensive understanding of the lung mycobiome and its role in asthma is lacking [212, 261]. The aims of this chapter were:

- To compare the mycobiome of people with asthma to healthy controls
- To determine whether fungal sensitisation effects the mycobiome in people with asthma
- To determine the fungal load by universal and fungal-specific qPCR
- To assess the influence of the oropharynx during sputum sample acquisition
- To investigate the contribution of dead fungal material opposed to living fungal cells on the mycobiome
- To compare mycobiome data derived from non-invasive sputum samples to bronchoscopy-derived samples
- To compare culture data with the most prominent fungi detected by high-throughput sequencing
- To show the contribution of air spores to the fungi detected in the clinical samples

3.2 Materials and methods

3.2.1 Acquisition of clinical samples and outdoor air

3.2.1.1 Sputum

Sputum samples were either spontaneously produced or induced using hypertonic saline [262, 263]. Sputum was expectorated into a sterile container and stored on ice until processing within two hours.

3.2.1.2 Mouthwash

The oral rinse was collected prior to the sputum sample as performed previously [207, 264] to assess the possibility that sputum samples could be contaminated by the oral mycobiota. Mouthwash controls were collected at home for a subset of subjects before they provided a spontaneous sputum sample. Subjects were not allowed to eat, drink or brush their teeth for at least one hour before the mouthwash. They rinsed the mouth with 10 ml 0.9% saline solution for a maximum of 1 min and – if possible – gargled twice for 5 seconds within that time frame. The subjects expectorated the oral rinse into a sterile container.

3.2.1.3 Bronchoscopy

The bronchoscopy procedure was conducted for a subset of patients by Professor Andrew Wardlaw at the Glenfield Hospital in Leicester following the recent British Thoracic Society guidelines [265]. Subjects were treated with nebulised 2.5 ml salbutamol around 20 min before the procedure, received sedatives such as midazolam 0.07 mg/kg when required and up to 8.2 mg/kg of the local antistatic lignocaine. Oxygen was supplied and its blood levels as well as other vital signs such as the heart rate were monitored during the procedure. The subjects were in a supine position while an Olympus fibreoptic bronchoscope (Olympus Company, Tokyo, Japan) was introduced over the nose or mouth cavity and the oropharynx into the trachea to the right middle lobe bronchus to perform a 20 ml bronchial wash using prewarmed normal saline solution, of which usually around 10 ml were retrieved. Afterwards, 150 ml of saline solution was applied to the right lower lobe for bronchoalveolar lavage (BAL) in

60+60+30 ml aliquots, of which usually around 40-50 ml were retrieved. Finally, two brushes were used to collect samples from the epithelium of the right intermediate bronchus. They were collected in 15 ml tubes containing 5 ml Dulbecco's Phosphate-buffered saline (Sigma-Aldrich, UK). One hundred microlitres of the saline used for bronchial wash and BAL samples from four subjects was collected in a sterile tube for DNA extraction (section 2.3.2) as a contamination control. Samples were kept on ice until processing within two hours.

3.2.1.4 Outdoor air samples

Outdoor air samples are routinely collected 24 h a day starting from midnight onwards using a continuous volumetric cyclone sampler with wind orientation, which is placed on the roof of a building on the University of Leicester campus, 12 m above ground level in an urban area 60 m above sea level and approximately 1 km south of the city centre. The location was suitable for aeroallergen analysis for a 41 km area [266]. The trap has an air throughput of 16.5 L/min (Burkard Manufacturing Co.). The device collects the airborne particles into a 1.5 ml microcentrifuge tube, which was stored at -80°C until use.

3.2.2 Clinical samples and outdoor air

3.2.2.1 Sputum

The procedure was conducted by members of the sputum lab at the BRU. Samples were processed in a class II safety cabinet cleaned with 70% IMS. Preparation of sputum plugs was done similar to Fairs *et al*. [41] with some modifications. Briefly, sputum plugs were separated from saliva and mixed with 4x w/v 0.1% DL-dithiothreitol in PBS (both Sigma-Aldrich, UK). The mixture was vortexed and incubated under shaking conditions at 37°C for 15 min. One hundred microlitres of the mixture was used for each, the inoculation of a PGCF plate [41, 254] and up to two sterile 2 ml screw cap tubes containing 600 mg ± 60 mg acid-washed glass beads (212-300 µm, Sigma-Aldrich, UK) for culture and DNA extraction (section 2.3.2), respectively. Any extra sputum material was treated with propidium monoazide (PMA) (Biotium, UK) for a subset of samples to assess the

proportion of dead and live cells within the sample. PMA penetrates the cell wall of dead cells and intercalates with the DNA during light exposure. This prevents DNA amplification [267]. PMA (final concentration 50 µM) was mixed with the homogenized sputum sample, which was incubated for 20 min in the dark, followed by an exposure to light for 15 min using a PMA-Lite™ box ([268, 269], modified). PMA-treated samples and untreated sputum samples were stored at -20°C until the DNA was extracted.

3.2.2.2 Mouthwash

Samples were processed by members of the sputum lab at the BRU. Samples were centrifuged for 10 min at 1700 x g. The supernatant was transferred into a new tube and 250 µl were used to resuspend the pellet. The remaining supernatant was discarded. One hundred microlitres of the solution was applied to a PGCF medium plate, another 100 µl was transferred into a sterile 2 ml screw cap tube with 600 mg ± 60 mg glass beads for DNA extraction (section 2.3.2) and stored at -20°C until use.

3.2.2.3 Bronchoscopy-derived samples

The BAL was filtered through sterile nylon gauze (SEFAR, Switzerland) tissue to filter out clumps of mucus. In some exceptional cases, more than one gauze was necessary to perform the filtration. The brushes were vortexed vigorously. The tubes containing the filtered BAL, bronchial wash and the bronchial brushes, which were left in the tube, were centrifuged at 1500 x g for 10 min. If a pellet was visible, the supernatant was transferred into a fresh tube and the pellet was resuspended in 500 µl of the respective supernatant. If a pellet was not visible, all but < 500 µl of the supernatant was transferred into a new tube and the remaining volume filled up to 500 µl with the respective supernatant. Aliquots were used to inoculate three types of media, namely PGCF (100 µl), YMA and ScedSel+ (both 50 µl) (section 2.3.1). BAL and bronchial wash samples were further aliquoted for cell counting, using 100 µl for cytospins and 10 µl for Trypan blue staining (1:2) (Sigma-Aldrich, UK). For brush samples, 80 µl were used for other purposes. Cell counts were performed by the members of the sputum lab at the BRU. The remaining volume was stored at -20°C until DNA extraction, ranging between 50 and 965 µl.

Samples were centrifuged at 14,000 rpm for 10 min prior to DNA extraction (section 2.3.2). Pellets were resuspended to a maximum volume of 100 µl.

3.2.2.4 Outdoor air samples

Samples were prepared for DNA extraction (section 2.3.2) by adding 600 ± 60 mg sterile glass beads and 100 µl of 0.05% or 0.5% Tween 80 (Sigma-Aldrich or Acros, UK) solutions, which had been filter-sterilised using 0.2 µm acrodiscs (Pall cooperation, USA), to each air sample tube. One hundred microlitres of the filtered Tween 80 solutions were used for DNA extractions as a contamination control.

3.2.3 Fungal culture

3.2.3.1 Clinical samples

All culture plates were sealed with Nescofilm (ThermoFisher) or Parafilm (VWR) to avoid contamination and moved to a dedicated mycology laboratory at the Maurice Shock Building, University of Leicester (Leicester, UK). Clinical samples were cultured at 37°C for 7 days except when ScedSel+ medium was used, which was incubated for 14 days. Plates were monitored regularly and records were kept for the intervals 1-2 days, 3-6 days and day 7 on paper and an excel file. The number of colony forming units (cfu) was recorded for each fungus and photos taken. Plates were kept sealed unless one or several fast-growing filamentous fungi were about to overgrow other clinical isolates. When plates were unsealed, the work was performed in a class II safety cabinet, which had been disinfected with 10% Microsol. Fungi were subcultured onto PDA or PGCF medium as necessary. Filamentous fungi were spread onto the medium using a flame-sterilised wire loop, whereas a small amount of yeasts were inoculated by touching a sterile pipette tip or flame-sterilised wire loop into a colony and transferring a single dot onto the new medium. Representative filamentous fungi and yeast were mixed with glycerol nutrient broth (18.9 g/L nutrient broth (Sigma-Aldrich, UK), 15% glycerol (Fisher Scientific, UK)) and stored at -80°C.

3.2.3.2 Yeast identification

Subcultures for yeast identification were prepared if no distinct colonies or filamentous fungi were growing on the same medium. They were incubated at 37°C for up to 7 days. The number of subcultures was dependent on the number of yeast present (Table 3. 1). Yeast identification was performed using BBL™ CHROMagar™ Candida Medium (Becton Dickinson Ltd.) [270, 271] and for selected yeast by Sanger sequencing as described in section 2.3.4. For CHROMagar, the type strains *Candida albicans* (American Type Culture Collection (ATCC) 18804), *Candida tropicalis* (National Collection of Yeast Cultures (NCYC) 1393), *Candida krusei* (ATCC 6258) and *Candida glabrata* (NCYC 388) were plated as reference colonies. CHROMagar was incubated for 2 days and data recorded in an excel file.

Table 3. 1: Number of yeast cfu and corresponding number of yeast to subculture.

Number of yeast cfu	**Number to subculture**
1	1
2	2
3	3
4-5	3
6-10	4
10-30	5
30-50	6
> 50	≥ 10*

*up to 18 from sputum samples, up to 15 from mouthwash samples

3.2.3.3 Identification of filamentous fungi

Filamentous fungi were subcultured if a pure clinical isolate could not be obtained for identification and storage. They were identified based on macroscopic and microscopic morphology [272] or Sanger sequencing (section 2.3.4). The morphology was recorded photographically. Needle-mounts from both the original culture and subculture were

compared and recorded photographically. Needle-mounts were taken from the rim of a colony, which represents the protruding growth structures, and mounted onto a glass slide with a drop of Lactophenol cotton blue (UKGE Ltd). Slides were examined under a dissecting microscope at 20X magnification. Clumps were separated to improve visibility of growth structures before adding a coverslip. Growth structures were examined at 600X magnification. Microscopic identification was limited to *Aspergillus* section *Fumigati* and section *Nigri* [272]. If an unknown fungus was present, material was taken for DNA extraction and an aliquot stored at -80°C. For DNA extraction (section 2.3.2), approximately 50 mg of fungal material was transferred into a sterile 2 ml screw cap tube filled with 600 mg ± 60 mg acid washed glass beads (212-300 µm) [137] and stored at -20°C. Prior to DNA extraction samples were centrifuged for 1 min at 14,000 rpm to avoid cross-contamination between samples by aerosols, and 100 µl of filter-sterilised 0.5% Tween 80 was added.

3.2.3.4 Harvesting for species-specific quantitative PCR

C. albicans (ATCC 18804) was harvested for species-specific quantitative PCR (qPCR) (section 3.2.5.2) from a PDA plate after 3 days of culture at 37°C when it had grown to confluence. The 0.05% Tween 80 was filter-sterilised using 0.2 µm acrodiscs. The colonies were suspended in 5 ml 0.05% Tween 80 with a sterile cell spreader. The blastospores were counted using a haemocytometer using the average of five small squares (0.004 mm^3) to calculate the number of spores or conidia per millilitre (Equation 3. 1). Samples were diluted for counting as necessary. The final count was then multiplied by the dilution factor used. One hundred microlitres of cell suspension were used for DNA extraction (section 2.3.2).

Equation 3. 1: Calculating the number of fungal conidia per millilitre from a haemocytometer count.

$$\frac{conidia}{ml} = 1000 \times \frac{TC \times DF}{A \times N \times D}$$

TC = Total number of cells counted

DF = dilution factor of the solution

A = area of the counted haemocytometer square (mm^2)

N = number of squares counted

D = Depth of the chamber (0.1 mm)

3.2.4 Library preparation for high-throughput sequencing

DNA from clinical samples and their respective control samples (mouthwash, outdoor air, saline controls, Tween 80 controls and kit controls (sections 3.2.1.3, 3.2.2, 2.3.2) were amplified using the ITS2 region of the rDNA (Fig. 1. 1). Briefly, amplification was performed in two consecutive nested PCR steps, which included a cleaning step after each round of PCR. Afterwards, samples were quantified and analysed using the Bioanalyzer 2100. Multiple samples were pooled, concentrated, cleaned and quantified. The pooled sample was sent to the Centre for Genomic Research, University of Liverpool for HTS using an Illumina MiSeq platform (2 x 250 bp).

3.2.4.1 Nested PCR

Fungal DNA was amplified using labelled TruGrade™ primers (Integrated DNA Technologies, UK) targeting the ITS2 region. The total reaction volume in both rounds of the dual index nested PCR approach was 20 µl containing 1X Kapa HiFi HotStart ReadyMix PCR kit (Kapa Biosystems). A high-fidelity (HiFi) polymerase was chosen to reduce the chance of nucleotide incorporation errors and recombinant amplicons [225, 273]. The 1st round of nested PCR was performed to amplify the ITS2 region (ITS3/ITS4

[10]), whilst the 2nd round served to incorporate sample-specific barcodes and Illumina Nextera Index adapter sequences for the Illumina platform (Table 3. 2). The reactions were conducted in the T100™ Thermal Cycler (BioRad).

For the 1st round of nested PCR, 5 µl of template DNA or controls were added to the reaction mixture. The forward and reverse primer concentration was 125 nM. The negative and positive controls were 5 µl PCR-grade water and 5 µl DNA from *Geotrichum candidum* (UAMH University of Alberta Microfungus Collection and Herbarium (UAMH) 7863, 1 x 10^6 spore equivalents), respectively. *G. candidum* was chosen as a control because it is a food pathogen and rarely associated with lung diseases [274, 275]. The DNA for the positive control was kindly provided by Dr Catherine Pashley (University of Leicester, UK). An optional 0.2 µg/µl of BSA (stock 20 µg/µl, New England Biolabs (NEB), UK) were added to improve the reaction efficiency. Clinical samples were usually amplified in duplicate or triplicate. DNA extracted from air samples were done in single or duplicate. The PCR tubes were placed in a T100™ Thermal Cycler (BioRad) once the block reached 85°C. The PCR cycling conditions in the 1st round of nested PCR were an initial heating step at 95°C for 5 min, followed by 18 cycles of 98°C for 20 s, 62°C for 20 s and 72°C for 30 s, and a final 5 min elongation step at 72°C. Afterwards, the PCR products were cleaned (section 3.2.4.2).

For the 2nd round of nested PCR, 9 µl of the cleaned previous PCR reaction product was added to the reaction mixture. The concentration of each primer was 250 nM. A combination of 8 forward primers and 12 reverse primers was used generating 96 unique barcode combinations (Table 3. 2). PCR cycling conditions were an initial heating step at 95°C for 5 min, followed by 15 cycles of 98°C for 20 s, 65°C for 20 s and 72°C for 30 s, and a final 5 min elongation step at 72°C. The final amplicons were cleaned (section 3.2.4.2). One microlitre of each amplicon sample was quantified using the Qubit® dsDNA HS kit (section 2.3.3). Samples were added to the working solution on the bench to avoid contamination of the PCR flow hood. They were then analysed using the Agilent Bioanalyzer 2100 (section 3.2.4.3).

Table 3. 2: List of primers used for dual index nested PCR.

Round	Forward		Reverse	
1st round	ITS3	5'ACACTCTTTCCCTACACGACGCTCTTCCGATC TNNNNNGCATCGATGAAGAACGCAGC3'	ITS4	5'GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTTCCTCCGCTTATTGATATGC3'
2nd round	DI_N501For	5'AATGATACGGCGACCACCGAGATCTACACT AGATCGCACACTCTTTCCCTACACGACGCTC3'	DI_N701Rev	5'CAAGCAGAAGACGGCATACGAGATTCGCC TTAGTGACTGGAGTTCAGACGTGTGCTC3'
	DI_N502For	5'AATGATACGGCGACCACCGAGATCTACACC TCTCTATACACTCTTTCCCTACACGACGCTC3'	DI_N702Rev	5'CAAGCAGAAGACGGCATACGAGATCTAGT ACGGTGACTGGAGTTCAGACGTGTGCTC3'
	DI_N503For	5'AATGATACGGCGACCACCGAGATCTACACT ATCCTCTACACTCTTTCCCTACACGACGCTC3'	DI_N703REv	5'CAAGCAGAAGACGGCATACGAGATTTCTG CCTGTGACTGGAGTTCAGACGTGTGCTC3'
	DI_N504For	5'AATGATACGGCGACCACCGAGATCTACACA GAGTAGAACACTCTTTCCCTACACGACGCTC3'	DI_N704Rev	5'CAAGCAGAAGACGGCATACGAGATGCTCA GGAGTGACTGGAGTTCAGACGTGTGCTC3'
	DI_N505For	5'AATGATACGGCGACCACCGAGATCTACACG TAAGGAGACACTCTTTCCCTACACGACGCTC3'	DI_N705Rev	5'CAAGCAGAAGACGGCATACGAGATAGGA GTCCGTGACTGGAGTTCAGACGTGTGCTC3'
	DI_N506For	5'AATGATACGGCGACCACCGAGATCTACACA CTGCATAACACTCTTTCCCTACACGACGCTC3'	DI_N706Rev	5'CAAGCAGAAGACGGCATACGAGATCATGC CTAGTGACTGGAGTTCAGACGTGTGCTC3'
	DI_N507For	5'AATGATACGGCGACCACCGAGATCTACACA AGGAGTAACACTCTTTCCCTACACGACGCTC3'	DI_N707Rev	5'CAAGCAGAAGACGGCATACGAGATGTAG AGAGGTGACTGGAGTTCAGACGTGTGCTC3'

	DI_N508For	5'AATGATACGGCGACCACCGAGATCTACACC TAAGCCTACACTCTTTCCCTACACGACGCTC3'	DI_N708Rev	5'CAAGCAGAAGACGGCATACGAGATCCTCT CTGGTGACTGGAGTTCAGACGTGTGCTC3'
			DI_N709Rev	5'CAAGCAGAAGACGGCATACGAGATAGCG TAGCGTGACTGGAGTTCAGACGTGTGCTC3'
			DI_N710Rev	5'CAAGCAGAAGACGGCATACGAGATCAGCC TCGGTGACTGGAGTTCAGACGTGTGCTC3'
			DI_N711Rev	5'CAAGCAGAAGACGGCATACGAGATTGCCT CTTGTGACTGGAGTTCAGACGTGTGCTC3'
			DI_N712Rev	5'CAAGCAGAAGACGGCATACGAGATTCCTC TACGTGACTGGAGTTCAGACGTGTGCTC3'

Dark yellow font = ITS2 sequences amplified [10]; green font = filler sequence for ITS2 primers ITS3/ITS4; black font = matching primer sequences for the 1st and 2nd round of nested PCR;.red font = sample-specific barcodes; blue font = Illumina Nextera Index adapter sequences.

3.2.4.2 Clean-up

The amplicons were cleaned using either Agentcourt® AMPure® XP beads (Beckman Coulter, UK) or AxyPrep Mag PCR clean-up kit (Axygen Biosciences, USA). The clean-up was conducted on the bench near a Bunsen burner using round-bottomed 96-well plates (NUNC™). Beads were added at a ratio of 1:1 to each of the PCR products. The solution was mixed ≥ 10 times by pipetting and incubated for 5 min to allow the DNA to bind to the beads. The samples were incubated on a magnetic stand (either a 96-well ring magnet (Applied Biosystems) or magnetic stand (AxyPrep)) for 2 min for the beads to adhere, and the cleared solution aspirated then discarded. The beads were then washed twice for 30 s using 200 µl 80% ethanol. After the ethanol was aspirated the beads were left to dry for up to 5 min. They were resuspended in 10 µl of PCR grade water. The plate was replaced onto the magnetic stand for 1 min to separate the beads from the fluid, and the cleaned amplicons transferred into new PCR tubes.

3.2.4.3 Bioanalyzer analysis

Samples underwent analysis with the Agilent Bioanalyzer 2100 (Agilent Technologies, Germany) in order to calculate the molarity ratio of the region of interest to other DNA such as primer dimers. One microlitre of cleaned amplicons was transferred onto a DNA HS chip, which had been prepared according to the manufacturer's instructions using the DNA HS kit (all Agilent Technologies, Germany). Equipment and reagents were kindly provided by Dr Nicolas Sylvius and Mrs Reshma Vaghela (NUCLEUS genomics, University of Leicester, Leicester, UK).

The molarity ratio was established by dividing the molarity of the region 50-9000 bp (region 1) from that of region 300-800 bp (region 2) from the sample (example shown in Fig. 3. 1). The ratio was multiplied with the Qubit results of the DNA concentration to yield the concentration from the region of interest (Equation 3. 2). An example is given below for one sample, resulting in a concentration of 0.85 ng/µl of the region of interest (Equation 3. 2):

Equation 3. 2: Calculation of the DNA concentration from region of interest.

$$\frac{Molarity\ region\ 2}{Molarity\ region\ 1} \times Total\ DNA\ concentration = DNA\ concentration\ region\ of\ interest$$

$$\frac{5420\ \frac{pmol}{L}}{14732.4\ \frac{pmol}{L}} \times 2.3\frac{ng}{\mu l} = 0.85\frac{ng}{\mu l}$$

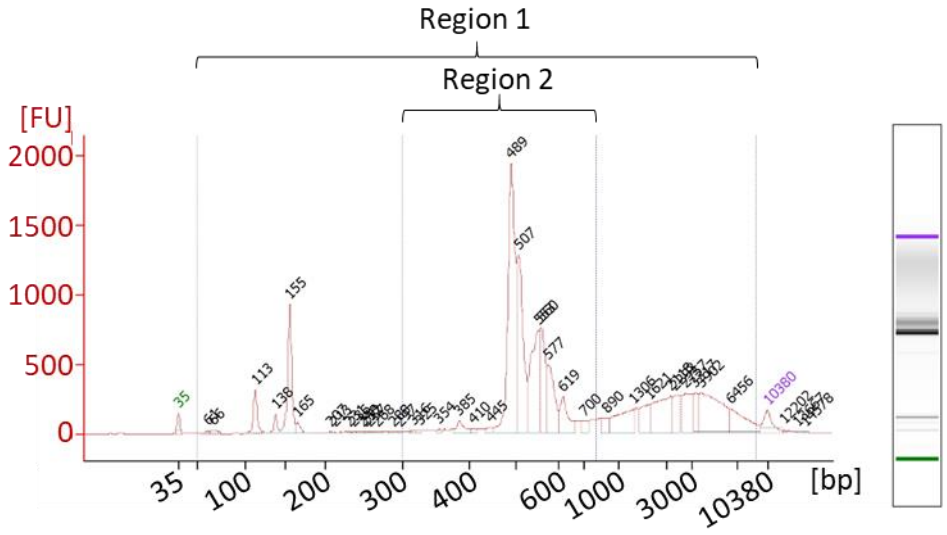


Fig. 3. 1: Electropherogram output of the HS DNA run Agilent Bioanalyzer 2100 using a pooled sample for Illumina MiSeq sequencing. Peaks are generated based on fluorescence (fluorescent units = FU), which is emitted if the fluorescent dye particles are bound to DNA. Region 1 and region 2 depict the regions between 50-9000 bp and 300-800 bp, respectively, which were used to calculate the concentration of the regions of interest. Kit upper and lower marker are indicated in purple and green, respectively. The right-hand image shows the electropherogram converted into a banding pattern.

3.2.4.4 Final processing steps

Amplicons were pooled in batches of up to 96 amplicons based on equal molar concentrations of the region of interest. The pooled DNA was then concentrated using

either a PCR purification kit eluting 30 µl (Jena Bioscience, Germany) or a Speed Vacuum machine. Any residual magnetic beads were removed using the DYNAL MPC®-E-1 (DYNAL, Norway) or the DynaMeg-2 (ThermoScientific, UK) magnetic stand. When the Speed Vacuum machine was used, volumes were adjusted to ≥ 30 µl with PCR-grade water. The concentration was determined with the HS Qubit assay using 1 µl of the pooled sample (section 2.3.3). The molarity ratio was measured with Agilent Bioanalyzer 2100 as described before (example Fig. 3. 1, section 3.2.4.3), before the sample was sent off for HTS.

3.2.5 Quantitative PCR

3.2.5.1 Universal quantitative PCR

The DNA from clinical and outdoor air samples (section 3.2.2) was amplified with primers targeting the SSU region (Chapter 1, 1.3.2) (FungiQuant-F/R [276], Table 3. 3). The primers were selected from a range of other primers [10, 258, 277, 278] as they were the only primers able to amplify the DNA from a range of different fungal species tested (*A. alternata* NCPF 7147, *C. herbarum* NCPF 2564, *C. albicans* ATCC MYA-2876, *A. fumigatus* NCPF 7097, *Penicillium chrysogenum* NCPF 2715, *Penicillium brevicompactum* culture collection of CSIRO Sydney Australia (FRR) 862, *Aspergillus flavus* ATCC 16883, *Aspergillus niger* ATCC 16888, *Fusarium moniliforme* NCPF 2865, *Cryptococcus victoriae* ATCC 208821, *Cryptococcus neoformans* NCYC 3128), showing the best dissociation curves during preliminary experiments (data not shown).

DNA from *C. herbarum* (NCPF 2564) was selected for the standard curve (3.575×10^5 spore equivalents) from a range of three fungi, which included *A. fumigatus* NCPF 7097 (1.045×10^7 spore equivalents) and *C. albicans* ATCC MYA-2876 (3.575×10^5 conidia equivalents) because it showed the best amplification efficiency and dissociation curves during preliminary experiments (data not shown). Spore stocks for all three fungi were kindly provided by Dr Catherine Pashley. One hundred microlitres of spore suspension was used for DNA extraction (section 2.3.2). Standard curves were prepared using serial tenfold dilutions of DNA.

The universal qPCR was performed using FAST PCR plates (Star Labs, UK) and an ABI-7500 Fast machine (ThermoScientific). A total volume of 20 µl per reaction contained 1X Power SYBR Green PCR Master Mix (ThermoScientific, UK), 1.8 µM primers (Sigma-Aldrich, UK) and 2 µl of template DNA or PCR-grade water as no template control (NTC). Cycling conditions were 10 min at 95°C for initial denaturation, followed by 40-50 cycles of 15 s at 95°C and 1 min at 60°C ([276], modified). Standards and samples were done in triplicate.

The data were exported as SDS files, which were analysed using the ThermoScientific Cloud software (July 2017). The baseline was set to automatic and the threshold to 0.1 [276]. The standard curve data generated from two separate runs were used to quantify the unknown products. The amplification efficiency was assessed by the ThermoScientific Cloud software. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as shown in Equation 3. 3 [279], where the standard deviation of the residuals (SD) was determined performing a semilog non-linear regression analysis of the standard curve data using GraphPad Prim (v7.0). Any samples with values below LOD were given the LOD value (0.677) for subsequent analyses.

Equation 3. 3: Calculations to determine the limit of detection or quantification.

$$LOD = 3.3 \times \frac{SD}{slope}$$

$$LOQ = 10 \times \frac{SD}{slope}$$

LOQ = limit of quantification

LOD = limit of detection

SD = standard deviation of the residuals

Slope = slope of the standard curve

3.2.5.2 Species-specific qPCR

The species-specific qPCR was performed using the DNA from a subset of clinical sputum samples with and without PMA treatment (section 3.2.2.1). Specific primers and probes targeting species-specific sequences from *A. fumigatus*, *C. albicans* and *A. niger* were used (Table 3. 3). Blastospores from *C. albicans* were freshly harvested, while spore stocks from *A. fumigatus* and *A. niger* type strain were kindly provided by Dr Catherine Pashley. Standard curves for each species were generated from the extracted DNA of fungi with known spore concentration as determined by haemocytometer count (section 3.2.3.4). DNA extraction was performed as previously described (section 2.3.2), using 100 µl of cell suspension. The products were aliquoted and stored at -20°C until use. The qPCR was performed using FAST PCR plates and an ABI-7500 Fast machine. A total volume of 20 µl containing 1X TaqMan Universal master mix (ThermoScientific), 80 nM probe (Invitrogen), 1 µM of each primer (Sigma), 0.4 mg/ml BSA (NEB) and 5 µl of DNA or PCR-grade water as NTC. Reactions were done in triplicate.

The data were exported as SDS files, which were analysed using the ThermoScientific Cloud software (March-May 2018). The baselines were determined automatically and the threshold manually using the standard curve data from the exponential phase of the logarithmic amplification plot. The data were exported into Excel using thresholds of 0.04-0.17, changing thresholds in 0.01 steps. The threshold was chosen, which had the lowest standard deviation and the highest correlation coefficient compared to the other thresholds. The amplification efficiencies for each primer were assessed by the ThermoScientific Cloud software. The LOD and LOQ were determined as described in Equation 3. 3.

Table 3. 3: Primer and probe list for universal and species-specific qPCR.

Species	Strain	Spore equivalents*	Forward primer	Reverse primer	Probe**
Cladosporium herbarum	NCPF 2564	3.575 x 10^5	FungiQuant-F 5'GGRAAACTCACC AGGTCCAG 3'	FungiQuant-R 5'GSWCTATCCCCAK CACGA 3'	N/A
Aspergillus fumigatus‡	NCPF 7097	1.45 x 10^7	AfumiF1 5'GCCCGCCGTTTC GAC 3'	AfumiR1 5'CCGTTGTTGAAAGT TTTAACTGATTAC 3'	AfumiP1 5'CCCGCCGAAGAC CCCAACATG 3'
Aspergillus niger‡	ATCC 16888	1.035 x 10^6	AnigrF1 5'GCCGGAGACCCC AACAC 3'	AnigrR1 5'TGTTGAAAGTTTTA ACTGATTGCATT 3'	AnigrP1 5'AATCAACTCAGA CTGCACGCTTTCAG ACAG 3'
Candida albicans‡	ATCC 18804	1.485 x 10^8	CalbF1 5'CTTGGTATTTTGC ATGTTGCTCTC 3'	CalbR1 5'GTCAGAGGCTATA ACACACAGCAG 3'	CalbP1 5'TTTACCGGGCCA GCATCGGTTT 3'

N/A = not applicable, NCPF = National Collection of Pathogenic Fungi, ATCC = American Type Culture Collection

*after DNA extraction using 100 µl of spores/conidia, **labelled with 6FAM-TAMRA

‡Primers and probes listed in http://www.epa.gov/microbes/moldtech.htm

3.2.6 Data analysis

3.2.6.1 Data handling and Statistical Analysis

Data were collected and managed using a secure web-based application hosted at the University Hospitals of Leicester NHS Trust [280]. Statistical analysis was performed using GraphPad Prism v7.02. The normality of data was tested using the D'Agostino and Pearson omnibus normality test. Parametric data were analysed by unpaired student-t test and are expressed as means with standard deviation (SD) unless stated otherwise. Between-group comparisons were analysed by Bonferonni-corrected ANOVA. Non-parametric data were analysed using the Mann-Whitney U test for two groups or Kruskal-Wallis test for analysis between more than two groups. They are expressed as medians with interquartile ranges. Categorical variables are presented as proportions (number of subjects and percent of subjects) and analysed by Fisher's exact test for two groups or Chi-square test for more than two groups.

3.2.6.2 High-throughput sequencing

The majority of the HTS data analysis was performed by Dr Catherine Pashley. The statistical analysis for the mycobiome data were performed Dr Catherine Pashley and Dr Matthew Richardson (University of Leicester, Leicester, UK). The recommended quality-filtering guidelines [281] were followed. Primer sequences were removed using Cutadapt 1.9.1 [282], retaining only sequences with a maximum error rate of 0.1. An overlap length of 8 bases between the read and the primer was allowed. A quality cut-off of q30 was applied before paired reads were merged using a python script and stitched together by the FLASH 1.2.11 (Fast Length Adjustment of SHort reads [283]) software. The minimum required overlap length between the two reads was 15 bases, and a maximum overlap length expected in approximately 90% of read pairs of 165 bases. Subsequently, sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) open-source bioinformatics pipeline version 1.9.1 [284]. Chimeric sequences were detected using the usearch61 algorithm [285] against the UCHIME ITS2 dataset released 2016-12-01 [286]. OTUs were assigned using the pipeline *pick_open_reference_otus.py* command and taxonomy was assigned using the QIIME release (2016-11-20) of the UNITE fungal ITS database [287, 288] using dynamic clustering thresholds. Non-fungal hits, singletons and

low abundant sequences were removed (*filter_taxa_from_otu_table.py*). For the latter, a conservative OTU threshold of 0.005% was set (*filter_otus_from_otu_table.py*).

The *core_diversity_analysis.py* command output included the α-diversity (Chao1 index), β-diversity (Bray-Curtis distance), alpha rarefaction plots and the taxonomy summary, rarefying the data to the lowest number of sequences from any sample within the samples being analysed. Comparison of the α-diversity between groups of samples was performed with the script *compare_alpha_diversity.py* using a non-parametric t-test, with *P*-values calculated using 999 Monte Carlo permutations corrected for multiple samples using the Bonferroni correction, and to generate alpha diversity box plots. Three-dimensional principal coordinate analysis plots based on the Bray-Curtis distance values, and the coordinates where the main taxonomic spheres are centred, were generated using the script *make_emperor.py* utilising the EMPeror software [289] bundled with QIIME. The vegan R package [290] was used to determine statistical significance of sample groupings based on the Bray-Curtis distance using the function "adonis" performed through the QIIME script *compare_categories.py* using 999 permutations to calculate statistical significance. Taxa listed in the taxa summary files were ordered and filtered using the *compare_taxa_summaries.py* script. Summary bar charts were then generated by *plot_taxa_summary.py*.

Samples were grouped using the *script filter_samples_from_otu_table.py* and the script *compute_core_microbiome.py* used to define the core mycobiome at various stringencies ranging from 25% to 100% of samples for a defined group. Core mycobiome charts were produced in Prism, and Venn diagrams using Microsoft PowerPoint 2016.

The heat maps illustrating the relative abundances per individual were produced by transforming sequence relative abundance data for the selected taxa using ln(x+1) to allow for the presence of zeros in the dataset, then the plots were produced using R version 3.4.1. Only taxa detected in the core mycobiome of each sample, and with a total OTU observation cut-off of 0.1% across all samples, were included.

The heat maps illustrating the abundance for a single group were produced by averaging the relative abundances for each group and excluding those taxa with average below 1%

in each group or sample type. Only taxa were included above a total OTU observation cut-off of 0.1% and if they were present in at least 25% of each given sample.

Statistical differences between the OTU frequencies across sample groups was assessed using the *group_significance.py* command, where only taxa detected in at least 25% of the samples were included. Significant differences between two groups were determined using the nonparametric t-test calculated using a Monte Carlo simulation with the probability based on 1000 bootstraps, and the Mann-Whitney U test; three or more groups using the Kruskall-Wallis test. *P*-values were corrected by the Benjamini-Hochberg FDR procedure for multiple comparisons [291].

Correlations between continuous variables and fungal taxa were restricted to taxa with a 0.1% total OTU observation cut-off. The tables were then rarefied to the lowest number of reads within a sample using single_rarefaction.py, then Spearman's rho correlation calculated using the script *observation_metadata_correlation.py*. *P*-values were corrected in Prism using the procedure of Benjamini, Krieger and Yekutieli with a desired FDR Q-value of 5% [292].

Statistical analysis of the four lung samples, BAL, protected brush, wash and sputum, were performed using taxa present in at least 25% from any of the four sample types and an overall 1% OTU frequency cut-off for that sample type.

Taxa were excluded that could not be identified to species level as they likely comprised multiple species. The *group_significance.py* script was implemented as described above. Presence versus absence ratios of taxa for samples within a sample type were compared across the four sample types using Chi-square contingency test, with *P*-values corrected using the procedure of Benjamini, Krieger and Yekutieli [292] with a desired FDR Q-value of 5%.

Significant associations between the mycobiota and clinical parameters were run with and without the inclusion of healthy controls for variables such as leucocyte counts, sputum cell differential analyses and smoking status, analyses; only subjects with asthma were included for variables such as lung function, asthma medication usage, bronchiectasis and asthma duration. Analyses were performed in QIIME using the script group_significance.py for categorical variables and the script *observation_metadata_correlation.py* for

continuous variables, as detailed above with P-values corrected in Prism using the procedure of Benjamini, Krieger and Yekutieli [292] with a desired FDR Q-value of 5%.

To determine differences in presence versus absence ratios of taxa between people with asthma and healthy controls, the Chi-square contingency test was performed, with P-values corrected using the procedure of Benjamini, Krieger and Yekutieli [292] with a desired FDR Q-value of 5%.

3.2.6.3 Statistics quantitative PCR data

The qPCR data (section 3.2.5) were analysed with GraphPad Prism v7.0 or v7.04. As the data were not normally distributed, two groups were analysed using the non-parametric Mann-Whitney U test, whilst three groups were analysed using non-parametric ANOVA Kruskal-Wallis test. Corrections for multiple samples were performed with a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli [292] using a desired FDR (q-value) of 5%. Correlations between fungal quantities detected by qPCR and fungal colony-forming units detected by culture or fungal spore counts were performed using two-tailed Spearman correlation. Paired data were analysed using Wilcoxon test.

3.2.6.4 Fungal culture statistics

The statistics for fungal culture rates were performed with GraphPad Prism v7.0 or v7.04. Differences between the numbers of culture-positive samples was analysed with Fisher's exact test for two categories and Chi-Square test for three categories. The concordance between two subsequent culture samples was done using Kappa test for concordance.

3.3 Results

3.3.1 Patients' characteristics

Ninety-seven subjects provided sputum samples for HTS, including 14 healthy individuals and 83 people with asthma, which were categorised into those with or without fungal sensitisation (Table 3. 4). The sex, age and smoking habits between the groups were well matched. No significant differences were detected between the two asthma subgroups regarding spirometry, exhaled nitric oxide levels, blood eosinophilia, sputum neutrophil or eosinophil percent, treatment with inhaled corticosteroids or the number of people with bronchiectasis. A significantly earlier asthma onset (Mann-Whitney U = 441, P = 0.003), longer asthma duration (U = 392.5, $P < 0.001$), defined as time since asthma diagnosis, and trend towards lower lung function (post-bronchodilator FEV_1) (unpaired T-test t = 1.79, P = 0.077) were observed in patients with fungal sensitisation compared to those without fungal sensitisation. Vice versa, more patients in the non-fungal sensitised group were classified and treated according to GINA stage 5 regulations (χ^2 = 6.39, P = 0.041) and uptake of oral steroids (P = 0.025). Compared to people with asthma, healthy subjects did not suffer from bronchiectasis (χ^2 = 6.62, P = 0.036) and showed better lung function ($P \leq 0.001$), lower blood eosinophilia (Kruskal-Wallis kw= 26.64, $P < 0.0001$) and sputum eosinophil percentage (kw = 14.17, $P < 0.0008$). Atopy to common allergens as determined by SPT was similar across the three groups. Total IgE, *A. fumigatus*-specific IgG and *A. fumigatus*-specific IgE were significantly higher in patients with fungal sensitisation compared to the other groups (all $P < 0.0001$) (Table 3. 4). HTS data were obtained from 54 fungal sensitised people with asthma, 26 non-fungal sensitised patients and 13 healthy controls. Seventy-four percent (40/54) of fungal sensitised people with asthma reacted to ≥ 2 fungi of which 88% (35/40) were sensitised to *A. fumigatus*. Concordance of the specific serum IgE test ImmunoCAP and SPT for fungal allergens was moderate to good with *A. fumigatus* and *P. chrysogenum* having the best agreement between the two tests (Table 3. 5).

Table 3. 4: Demographic and clinical characteristics of the study subjects (n = 97). Individuals with asthma are grouped according to their fungal sensitisation. Statistically significant differences ($P < 0.05$) between the asthma groups or between all three groups are indicated in bold.

	Subjects with Asthma (n = 83)			**Control, no asthma**	**3 groups**
	Fungal sensitised (n=54)	**Non-fungal sensitised (n=29)**	***P*-value**	**(n=14)**	***P*-value**
Demographics					
Male [a]	31 (57)	17 (59)	>0.9999	7 (50)	0.856
Age in years [b]	58.89 (23 - 81)	58.86 (32 - 82)	0.993	56 (23 - 72)	0.778
Age of asthma onset, years [c]	24 (3-52)	45 (35-55.5)	**0.003**	-	-
Asthma duration [c]	30 (7.5 - 54.5)	9 (2.25 - 22.25)	**0.0004**	-	-
GINA treatment					
GINA 1-3 [a]	10 (19)	5 (17)	**0.041**		
GINA 4 [a]	32 (59)	10 (34)			
GINA 5 [a]	12 (22)	14 (48)			
Requiring oral steroids [a]	12 (22)	14 (48)	**0.025**	-	-
Dose of ICS- ICS-BM µg/pt/day [d]	1084 (476.8)	1041 (524.1)	0.710	-	-
Spirometry					
FEV_1% of predicted, PB [d, f]	76.3 (26.31)	86.41 (20.73)	0.077	114.4 (14.83) (n=12)	**< 0.0001**
FEV_1/FVC ratio PB [d, f]	65.33 (14.24)	69.82 (10.84)	0.142	79.16 (4.232)	**0.001**
FENO ppb [c, f]	26 (19 - 42.75) (n=44)	34.5 (21 - 61) (n=24)	0.465	21 (14.25 - 27.75) (n=10)	0.154
Smoking and steroid history					
Never-smokers [a]	36 (67)	13 (45)	0.064	9 (64)	0.144
Smoking (pack years) - ex or current [d, f]	8.778 (7.442) (n=18)	12.58 (9.63) (n=16)	0.204	12.91 (18.16) (n=5)	0.493

Leucocyte counts					
Eosinophil count in blood (x10^{-9}/litre) [c]	0.82 (0.44 - 1.388)	0.81 (.29 - 1.575)	0.637	0.135 (0.0875 - 0.225)	**<0.0001**
Sputum Neutrophil percent [d, f]	67.53 (23.67) (n=35)	61.65 (22.56) (n=22)	0.356	78.38 (21.03) (n=9)	0.189
Sputum Eosinophil percent [c, f]	0.75 (2.5-10.5) (n=35)	2 (6.95-20.13) (n=22)	0.204	0 (0-1.25) (n=9)	**0.001**
Immunoglobulins and radiology					
Serum total IgE IU/ml [c]	516 (174 - 1870)	52.9 (32.05 - 115)	**<0.0001**	53.4 (9.9 - 156.5)	**<0.0001**
Atopic [a, f, g]	30 (63) (n=48)	12 (46) (n=26)	0.222	6 (43)	0.255
Aspergillus fumigatus sIgG > 40 mg/L [a]	34 (63)	5 (17)	**<0.0001**	4 (30)	**0.0001**
A. fumigatus sIgE > 0.35 kU/L [a]	40 (74)	0 (0)	**<0.0001**	0 (0)	**<0.0001**
Number with bronchiectasis [a, f]	18 (34) (n=53)	7 (25) (n=28)	0.458	0	**0.036**
Samples analysed by HTS					
Sputum HTS data [a]	53 (98)	26 (90)	0.120	13 (93)	0.233
Bronchcoscopy [e]	12 (100)	9 (89)	0.429	8 (63)	0.056
Bronchial wash [e]	12 (100)	9 (78)	0.171	8 (50)	**0.026**
Bronchoalveolar lavage [e]	9 (89)	9 (89)	>0.9999	8 (63)	0.289
Protected brush [e]	11 (45)	9 (67)	0.406	8 (38)	0.451
Mouthwash [e]	10 (90)	1 (100)	>0.9999	1 (0)	0.063

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

Definitions of abbreviations: FEV1 = Forced expiratory volume during the first second; FVC = Forced vital capacity; PB = post-bronchodilator; FENO ppb = Fractional exhaled nitric oxide, parts per billion; ICS-BM = inhaled corticosteroid, beclomethasone equivalent; IgE = Immunoglobulin E; sIgG = specific Immunoglobulin G; sIgE = specific Immunoglobulin E; HTS = high throughput sequencing;

P values were calculated with an independent t test or ANOVA for parametric values, Fishers exact test or Chi-square test for comparison of proportions and the Mann-Whitney U test or Kruskal-Wallis test for comparison of non-parametric for two groups or three groups respectively.

Table 3. 5: Skin prick test (SPT) and specific serum IgE results for individual fungi and their concordance from people with asthma.

							Cohen's kappa test		
	Total number of patients	**SPT or specific IgE positive***	**SPT and specific IgE both positive***	**SPT positive but specific IgE negative***	**Specific IgE positive but SPT negative***	**Concordance among positive results**	**Kappa (SE)**	**95% CI**	**Strength of agreement**
Aspergillus fumigatus	87	40 (46%)	24 (28%)	5 (6%)	11 (13%)	60%	0.607 (0.087)	0.435-0.778	Good
Penicillium chrysogenum	83	34 (41%)	20 (24%)	7 (8%)	7 (8%)	59%	0.616 (0.093)	0.434-0.797	Good
Candida albicans	75	31 (41%)	15 (20%)	3 (4%)	13 (17%)	48%	0.509 (0.102)	0.308-0.709	Moderate
Alternaria alternata	59	32 (54%)	19 (32%)	3 (5%)	10 (17%)	59%	0.557 (0.105)	0.351-0.764	Moderate
Cladosporium herbarum	60	27 (45%)	15 (25%)	3 (5%)	9 (15%)	56%	0.510 (0.105)	0.304-0.715	Moderate

* n (%), SE = standard error, CI = confidence interval

3.3.2 High-throughput sequencing efficacy for all samples

The ITS2 sequence data is available from the European Nucleotide Archive (www.ebi.ac.uk/ena) under the study accession number PRJEB31078. The minimum number of reads used for all subsequent analyses was ≥ 5085 reads per sample (Table 3. 6). Rarefaction curves represent the quality of sampling effort, which means the number of OTUs with respect to the number of reads. The rarefaction curves reached a plateau, indicating that almost all operational taxonomic units (OTUs) were detected in the respective sample (Fig. 3. 2).

Table 3. 6: Descriptive high-throughput sequencing data. Shown are the number of fungal ITS2 sequence reads that were obtained after quality filtering, and OTUs these represent before and after implementing a conservative OTU threshold of 0.005%. The number of fungal taxa was determined by analysing the sequences to species level of which a certain number of named fungal species were identified, with the remainder being identified to genus or a higher phylogenetic level.

Sample	Number of samples	Descriptor	Total	Range/sample
Sputum	92	Sequence reads	11,244,211	5085 – 543,347
		OTUs before 0.005%	830	5 - 80
		OTUs after 0.005%	254	4 - 48
		Taxa	206	4 - 44
		Named species	154	3 - 34
Sputum-PMA	10	Sequence reads	1,581,327	46,130 – 446,026
		OTUs before 0.005%	163	7 – 55
		OTUs after 0.005%	73	4 – 43
		Taxa	66	4 - 41
		Named species	44	3 – 28
Bronchoalveolar lavage	21	Sequence reads	2,931,246	32,996 – 250,292
		OTUs before 0.005%	355	8 - 85
		OTUs after 0.005%	125	7 - 32
		Taxa	111	7 - 31
		Named species	83	5 - 27
Protected brush	14	Sequence reads	2,416,809	75,211 – 267,431
		OTUs before 0.005%	464	21 - 54
		OTUs after 0.005%	156	14 - 34
		Taxa	133	14 - 31
		Named species	96	9 - 22
Bronchial wash	23	Sequence reads	2,499,694	10,857 – 277,473
		OTUs before 0.005%	357	8 - 84
		OTUs after 0.005%	132	7 - 49
		Taxa	113	6 - 47
		Named species	78	5 - 34

Mouthwash	10	Sequence reads	948,771	13,707 – 362,663
		OTUs before 0.005%	107	5 – 31
		OTUs after 0.005%	32	3 - 13
		Taxa	31	3 - 13
		Named species	25	3 – 11
Outdoor air	45	Sequence reads	5,196,072	13,220 - 343,054
		OTUs before 0.005%	1713	23 – 365
		OTUs after 0.005%	475	21 – 185
		Taxa	338	20 – 141
		Named species	243	11 - 101

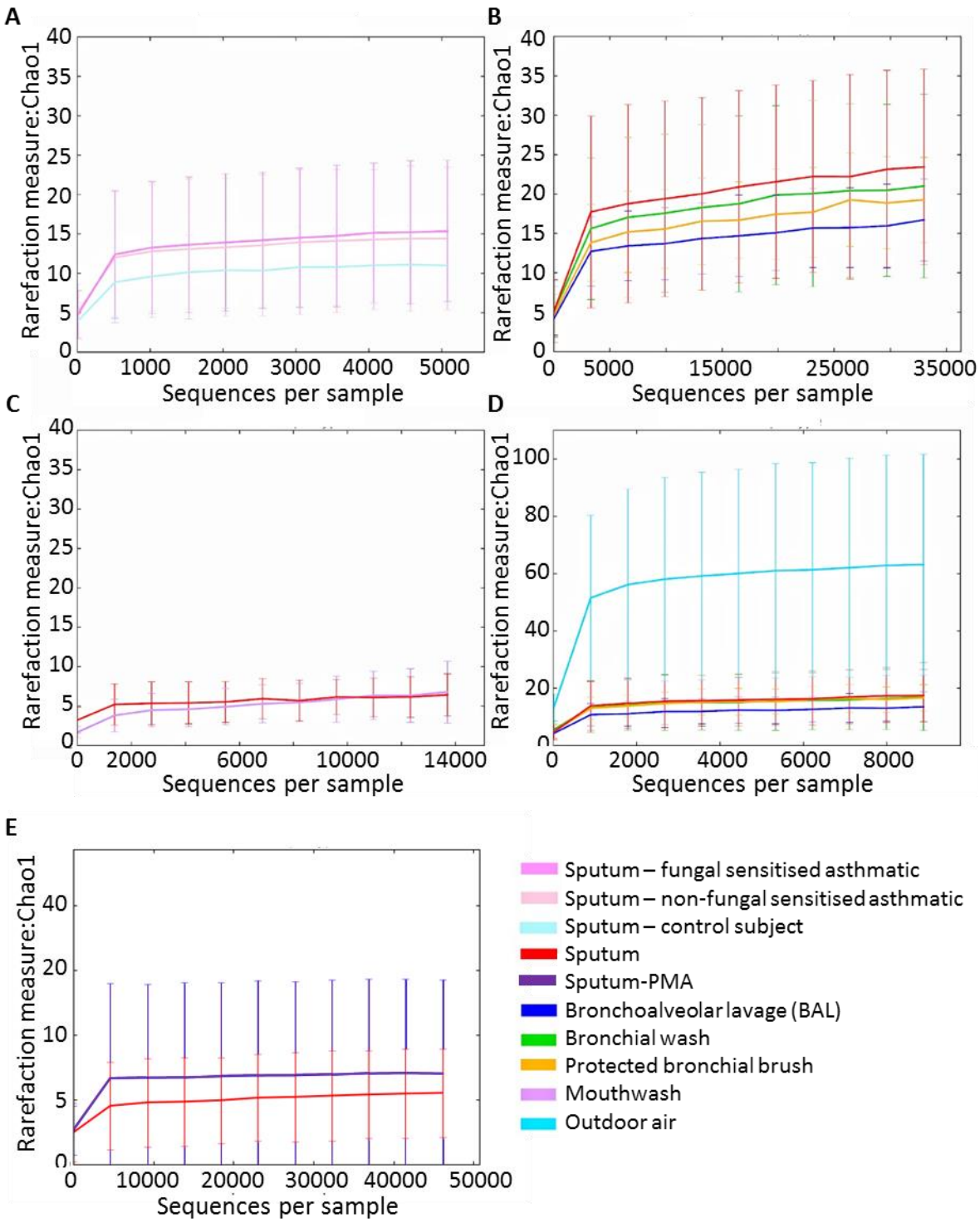

A
B
C
D
E
Rarefaction measure:Chao1
Sequences per sample
Sputum – fungal sensitised asthmatic
Sputum – non-fungal sensitised asthmatic
Sputum – control subject
Sputum
Sputum-PMA
Bronchoalveolar lavage (BAL)
Bronchial wash
Protected bronchial brush
Mouthwash
Outdoor air

Fig. 3. 2: Rarefaction curves (Chao1 alpha diversity) showing the fungal community complexes in the different samples. (A) Sputum samples grouped into healthy controls (n = 13), fungal sensitised asthmatics (n = 53), and non-fungal sensitised asthmatics (n = 26). (B) Samples from patients who underwent a bronchoscopy grouped into bronchoalveolar lavage (BAL) samples (n = 21), bronchial wash samples (n = 23), protected brush samples (n = 14) and sputum samples (n = 25). (C) Samples from patients who had a mouthwash prior to producing sputum (n = 10). (D) Air samples (n = 45) and corresponding clinical samples obtained on the same days as the air samples; BAL (n = 18), bronchial wash (n = 18), protected brush (n = 13) and sputum (n = 31) samples. (E) Sputum samples (n = 5) treated with propidium monoazide (Sputum-PMA) or untreated.

Sputum samples were collected from all individuals, and a subset of 29 underwent bronchoscopy, comprised of 12 fungal sensitised and 9 non-fungal sensitised people with asthma and 8 healthy controls. A fungal amplicon could be amplified from > 90% of sputum samples from all groups (Table 3. 4). In contrast, amplicons from bronchoscopy-derived samples, BAL (21/26 - 81%), bronchial wash (23/29 - 79%) and protected bronchial brushes (14/28 - 50%), were more difficult to obtain. BAL and wash samples were significantly more likely to be amplified compared to brush samples (χ^2 = 7.95, *P* = 0.019). Bronchoscopy-derived samples tended to be less amplifiable when they derived from healthy controls compared to people with asthma (χ^2 = 5.76, *P* = 0.056), though only wash samples reached significance (χ^2 = 7.33, *P* = 0.026) (Table 3. 4). Amplicons were successfully amplified from all sample types for less than half of all subjects who underwent a bronchoscopy (Table 3. 7).

Table 3. 7: Number of bronchoscopy-derived samples successfully amplified from the 29 subjects who underwent bronchoscopy.

Total number of subjects	Fungal sensitised subjects	Non-fungal sensitised subjects	Healthy control subjects	Number of bronchoscopy-derived samples amplified (maximum 3)
14	5	6	3	3
5	3	1	1	2
6	4	1	1	1
4	0	1	3	0

A subset of 12 individuals provided mouthwash samples prior to sputum collection. These were obtained predominantly from fungal sensitised people with asthma. Only one healthy control provided a mouthwash which was not amplifiable (Table 3. 4).

Amplicons were successfully obtained from all 45 air samples. They represented days when clinical samples were provided, including 28 days matching sputum collection from 31 individuals, and 18 days matching bronchoscopy dates of 19 subjects.

Control samples included PCR reagent (PCR grade water subjected to DNA extraction protocol), DNA extraction kit (PCR grade water subjected to DNA extraction protocol), bronchoscopy saline and outdoor air Tween 80 controls (reagents extracted and amplified as per samples) (Table 3. 8).

Table 3. 8: List of different contamination controls from nested PCRs, DNA extraction kits, saline used for washes and BAL, and Tween 80 solvent for air samples.

Contamination controls	**Number of controls**	**Samples controlled**	**Number of matching samples**
PCR negative control	10	PCR	47
Kit contamination control	7	DNA extraction	51
Saline control	4	Bronchoscopy	5*
Tween 80 control	2	Air samples	25

*4 washes, one BAL

Although some fungi were detected in control samples, the fungi detected were either absent from some or all of the samples the control was for. One example for each type of control is shown in Table 3. 9. The number of fungi with more than five sequences detected in the controls ranged from one to four with the lowest number detected in the Tween 80 control (*Aspergillus striatus*) for 13 air samples and the highest number detected in the kit controls (*Bulleromyces albus, Mrakia frigida*, *Aspergillus appendiculatus*, *Aspergillus terreus*). Two fungi with more than five sequences were detected in each a PCR control (*Saccharomycetales* species, *Penicillium spinulosum*) and the saline control (*A. fumigatus*, *C. albicans*). The number of sequences for each fungus did not match with those from the respective samples. No matching sequences were detected for the *Saccharomycetales* species, *Bulleromyces albus* and *Aspergillus appendiculatus*, whilst the matching number of sample sequences highly varied per sample (Table 3. 9). In conclusion, contamination of clinical and air samples by the buffers, solutions or procedures used to prepare the samples could be excluded. The

remaining data can be accessed through the European Nucleotide Archive (www.ebi.ac.uk/ena) under the study accession number PRJEB31078.

Table 3. 9: Examples for each type of contamination control, PCR negative control, kit contamination control, saline control and Tween 80 control. Shown are the respective number of samples matching with each control type. Fungi at the genus or species level with more than 5 sequences are from each control are presented, including their respective number of sequences and the range of the number of sequences from the matching samples.

Type of control	**Matching number of samples**	**Fungi with > 5 sequences**	**Number of sequences in controls**	**Range of number of sequences in matching samples**
PCR negative control	8	*Saccharomycetales* species	9061	0
		Penicillium spinulosum	10	0-6
Kit control*	21	*Bulleromyces albus*	4850	0
		Mrakia frigida	6507	0-1594
		Aspergillus appendiculatus	3799	0
		Aspergillus terreus	1024	0-19,572
Saline control	2	*Aspergillus fumigatus*	1116	0, 9709
		Candida albicans	21	4, 327
Tween 80 control	13	*Aspergillus striatus*	3847	0-4799

*data included from controls analysed from the start and the end of the kit usage.

3.3.3 Fungi in health and disease

Ninety-two sputum samples from 79 people with asthma, including 53 fungal sensitised subjects, and 13 healthy controls yielded fungal ITS2 rDNA sequence data, resulting in 830 fungal OTUs. These included 206 taxa at the species level identified using UNITE fungal ITS database (Table 3. 6). The α-diversity represents the total species richness estimate of a community. The α-diversity as determined by Chao1 index richness

estimator between sputum samples from the three groups were not significantly different (Fig. 3. 3).

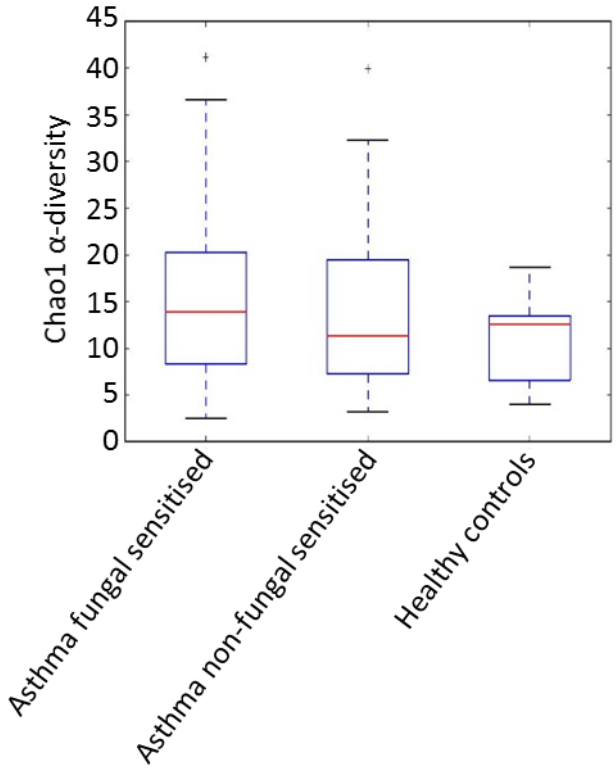


Fig. 3. 3: Box plot of α-diversity index (Chao1) from sputum samples grouped into healthy controls (n = 13), fungal sensitised asthmatics (n = 53), and non-fungal sensitised asthmatics (n = 26). Box plots show mean and standard deviations of the groups' alpha diversities.

The core mycobiome describes the number of taxa shared between given numbers of samples and is herein defined as taxa shared by at least 25% of samples in this study. The most prevalent taxa in all sputum samples, that were identifiable to species level, were *C. albicans* (100%), *A. fumigatus* (84.8%) and *Mycosphaerella tassiana* (82.6%), *A. tubingensis* (44.6%) (Fig. 3. 4 A). The core mycobiome comprised 24 taxa in all 92 sputum samples. The core mycobiome in people with asthma had a higher number of taxa compared to healthy controls, with 27 taxa in non-fungal sensitised people with asthma

and 23 in fungal sensitised people with asthma compared to 14 in healthy subjects (Fig. 3. 4 A). Nine named fungal species, *A. fumigatus*, *C. albicans*, *Malassezia restricta*, *M. tassiana*, *Penicillium digitatum*, *Penicillium roqueforti*, *Saccharomyces paradoxus*, *Sporobolomyces roseus* and *Talaromyces stollii*, were shared between people with asthma (irrespective of fungal sensitisation) and healthy controls, excluding fungi that could not be identified to species level (Fig. 3. 4 B). Six named species, *Alternaria infectoria*, *Aspergillus striatus*, *Aspergillus tubingensis*, *Hyphodontia radula*, *Malassezia globosa* and *Penicillium spinulosum*, were found in both asthma subgroups, and two were shared between healthy controls and each of the asthma subgroups, namely *Aspergillus conicus* and *Phaeoacremonium hungaricum* with the non-fungal sensitised people with asthma, or *Cladosporium sphaerospermum* and *Penicillium decumbens* with fungal sensitised people with asthma. Fungal species unique to a group were found in the asthma subgroups, with five in the non-fungal sensitised and two in the fungal sensitised asthma groups (Fig. 3. 4 B).

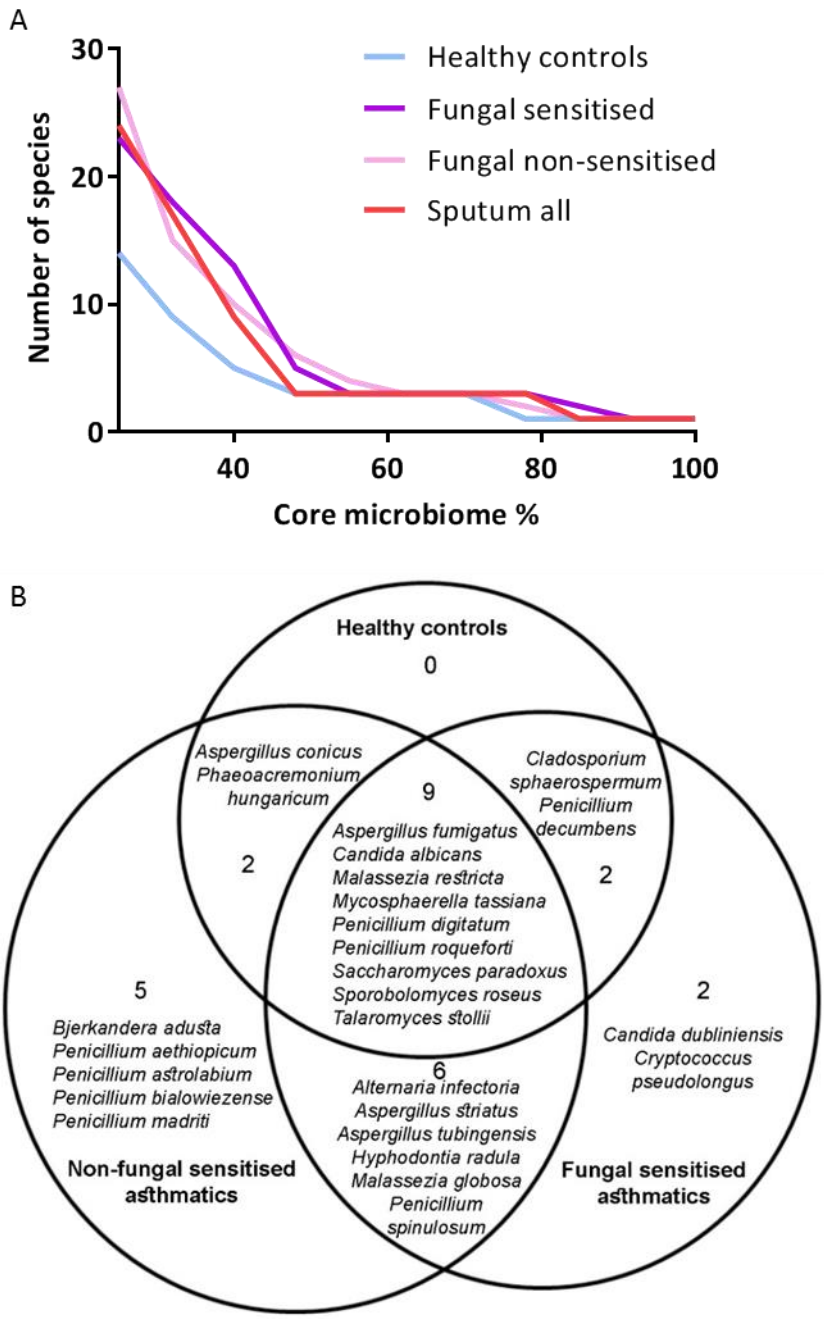
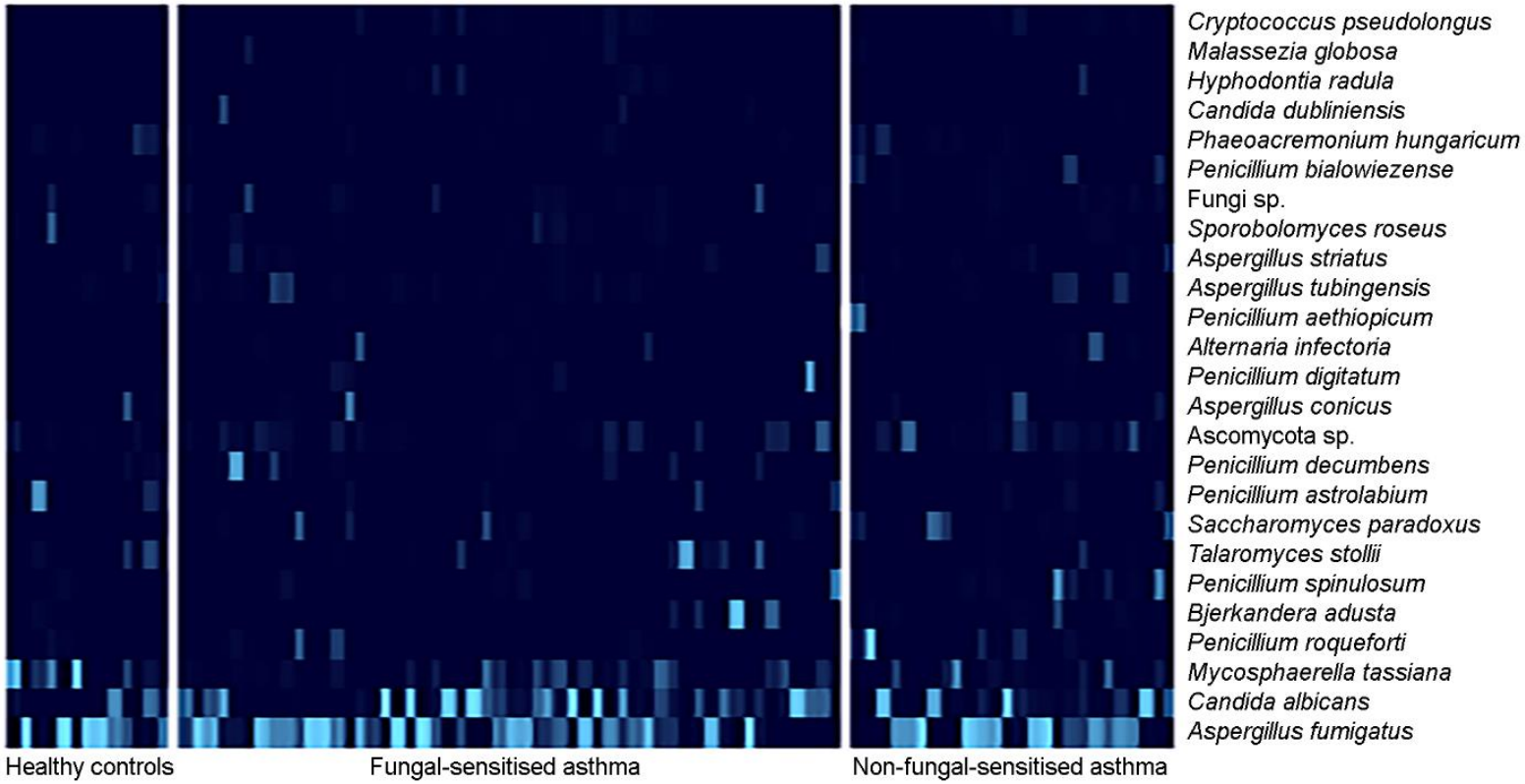


Fig. 3. 4: The core microbiome in sputum samples from either all sputum samples or individual groups of healthy controls (n = 13), fungal sensitised asthmatics (n = 53), and non-fungal sensitised asthmatics (n = 26): (A) The number of taxa shared ≥ 25% of samples; (B) Venn diagram illustrating the species comprising the core microbiome at ≥ 25% prevalence, excluding taxa that could not be identified to species level.

The three most prevalent species also had the highest relative sequence abundances in all sputum samples with means of 19.0% (range 0.0005 to 99.97%) for *C. albicans*, 34.7% (range 0 to 99.70% per individual) for *A. fumigatus* and 6.8% (range 0 to 87.70%) for *M. tassiana*. The number of taxa within healthy controls, fungal-sensitised and non-fungal sensitised people with asthma using 0.1% cut-off for the relative abundances within a group, were 12, 19 and 22, respectively (Fig. 3. 5 A). At 1% average abundance for each sample type, the groups encompassed 10, 9 and 13 taxa, respectively (Fig. 3. 5 B), and the combined sputum samples contained 11 OTU observations.

A


Cryptococcus pseudolongus
Malassezia globosa
Hyphodontia radula
Candida dubliniensis
Phaeoacremonium hungaricum
Penicillium bialowiezense
Fungi sp.
Sporobolomyces roseus
Aspergillus striatus
Aspergillus tubingensis
Penicillium aethiopicum
Alternaria infectoria
Penicillium digitatum
Aspergillus conicus
Ascomycota sp.
Penicillium decumbens
Penicillium astrolabium
Saccharomyces paradoxus
Talaromyces stollii
Penicillium spinulosum
Bjerkandera adusta
Penicillium roqueforti
Mycosphaerella tassiana
Candida albicans
Aspergillus fumigatus
Healthy controls
Fungal-sensitised asthma
Non-fungal-sensitised asthma

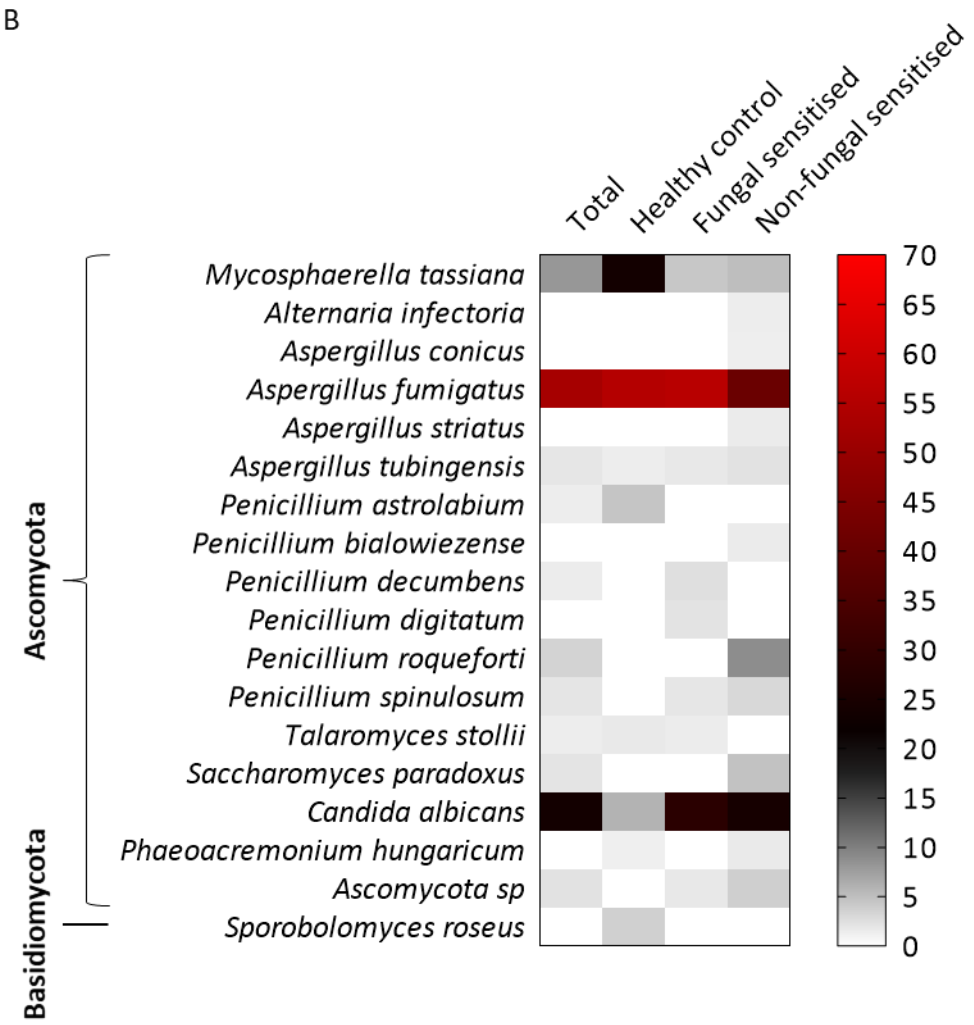


Fig. 3. 5: Heat maps illustrating the distribution of species based on relative abundances using a prevalence of 25% for each sample within the sample groups: All sputum samples (n = 92), healthy controls (n = 13), fungal sensitised (n = 53) and non-fungal sensitised (n = 26) people with asthma. (A) 0.1% OTU frequency cut-off across all samples. The data were transformed using ln(x+1) due to zeros in the dataset. The relative abundance is indicated by colour, with the lighter shades representing the more abundant sequences. (B) At least 1% average abundance within a group and 0.1% OTU frequency cut-off across all samples. The relative abundance is indicated by colour, with darker shades representing the higher frequencies, red frequencies 50% up to 70% abundance.

The heat maps illustrate which species were more abundant in each group, patients with or without fungal sensitisation and healthy subjects (Fig. 3. 5 B) and showed high inter-individual differences (Fig. 3. 5 A). For example, OTU frequencies of *M. tassiana* and *Sporobolomyces roseus* seemed more abundant in healthy subjects compared to people with asthma, though only the former was statistically significant (non-parametric T = -3.66, *P* = 0.002, FDR-*P* = 0.050), the latter only showed a trend (T = -2.41, *P* = 0.081). *C. dubliniensis* was not within the 1% but the 0.1% cut-off from the OTU frequencies. It was significantly more abundant in people with asthma compared to healthy individuals (Mann-Whitney U = 338, *P* = 0.015, FDR-*P* = 0.306), in particular in fungal sensitised subjects ($\chi 2$ = 8.33, *P* = 0.016, FDR-*P* = 0.334), and was only present in the core microbiome of the fungal sensitised group (Fig. 3. 5 B).

The β-diversity based on Bray-Curtis dissimilarity was used for the inter-group comparison by measuring the dissimilarities between individual samples. The principle coordinate plots (PCoA) (Fig. 3. 6) are an ordination technique to visually summarise these β-diversity relationships, where each axis explains a fraction of the variability in the dataset. The β-diversities were not significantly different comparing healthy controls to people with asthma (r^2 = 0.014, *P* = 0.208) or the asthma sensitisation subgroups (r^2 = 0.014, *P* = 0.208). However, significant grouping was detected based on fungal culture (negative culture v yeast only, v *A. fumigatus* culture v other fungal culture; r^2 = 0.088, *P* = 0.002), particularly when the groups only included people with asthma (r^2 = 0.101, *P* = 0.001) (Fig. 3. 6 A). Yeast culture-positive samples were significantly associated with high sequence abundances of *C. albicans* (T = 3.22, *P* = 0.003, FDR-*P* = 0.072; U = 357.5, *P* < 0.0001, FDR-*P* < 0.0001), which cluster in the respective area in the PCoA (Fig. 3. 6 B, C). Yeast culture-positive samples were also significantly associated with high abundances from *Talaromyces stollii* (U = 659, *P* = 0.007, FDR-*P* = 0.088), and lower abundances of *M. globosa* (T = -1.66, *P* = 0.017, FDR-*P* = 0.204). These relationships (*C. albicans* (T = 2.94, *P* = 0.006, FDR-*P* = 0.144; U = 284, *P* < 0.0001, FDR-*P* < 0.001), *Talaromyces stollii* (U = 458, *P* = 0.009, FDR-*P* = 0.109), and *M. globosa* (T = -1.76, *P* = 0.021, FDR-*P* = 0.252)) remained significant when healthy control patients were removed from the analysis (Fig. 3. 6 C). The same clustering pattern for yeast culture-positive samples was observed for *A. fumigatus* and sequences with high *A. fumigatus*

abundances. Sputum samples, which were culture-positive with *A. fumigatus*, clustered around the sphere that represented the co-ordinates for *A. fumigatus* (Fig. 3. 6 B, C). There was a significant relationship between abundance of *A. fumigatus* sequences and *A. fumigatus* positive culture (T = 2.41, *P* = 0.019, FDR-*P* = 0.228; U = 243, *P* = 0.007, FDR-*P* = 0.081). These *A. fumigatus* culture-positive samples were also significantly associated with abundances from *A. tubingensis* (T = 3.62, *P* = 0.001, FDR-*P* = 0.024), *M. globosa* (T = 2.84, *P* = 0.029, FDR-*P* = 0.232; U = 267.5, *P* = 0.003, FDR-*P* = 0.075), *C. dubliniensis* (T = 1.95, *P* = 0.044, FDR-*P* = 0.264), and *Penicillium decumbens* (U = 319.5, *P* = 0.018, FDR-*P* = 0.144). Within the asthma group, these significant relationships were maintained for *A. fumigatus* (T = 2.78, *P* = 0.018, FDR-*P* = 0.216; U = 192, *P* = 0.005, FDR-*P* = 0.072), *A. tubingensis* (T = 3.50, *P* = 0.005, FDR-*P* = 0.120) and *M. globosa* (T = 2.57, *P* = 0.030, FDR-*P* = 0.240; U = 235.5, *P* = 0.009, FDR-*P* = 0.072). After adjusting the *P*-values to allow for multiple testing the relationship between yeast culture-positive samples and the respective *C. albicans* sequence abundances remained significant (U = 357.5, *P* < 0.0001, FDR-*P* < 0.0001), as well as the association between *A. fumigatus* culture-positive samples and *A. tubingensis* sequence abundances (T = 3.62, *P* = 0.001, FDR-*P* = 0.024).

Table 3. 10: Fungi detected in sputum that had significantly different OTU abundances from subjects who were culture positive for yeast compared to those who were culture negative.

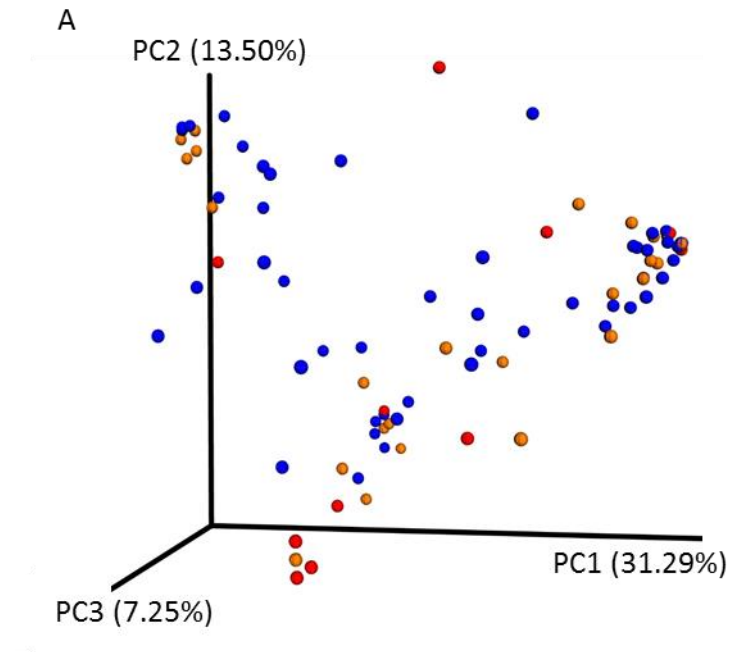
Fungus	Test statistic	Mean OTU frequency		*P*-value	Corrected *P*-value (FDR-P)
		Culture positive	Culture negative		
All subjects (n = 91)		n = 59	n = 32		
Candida albicans	T = 3.21	31601.36	2228.906	**0.003**	0.072
	U = 357.5	31601.36	2228.906	**< 0.0001**	**< 0.0001**
Talaromyces stollii	U = 659	1100.542	1880.094	**0.007**	0.088
Malassezia globosa	T = -1.66	27.74576	318.625	**0.017**	0.204
Asthmatics (n = 78)		n = 52	n = 26		
Candida albicans	T = 2.94	34045.63	2716	**0.006**	0.144
	U = 284	34045.63	2716	**< 0.0001**	**< 0.001**
Talaromyces stollii	U = 458	906.0962	1960.423	**0.009**	0.109
Malassezia globosa	T = -1.76	30.26923	392.1538	**0.021**	0.252

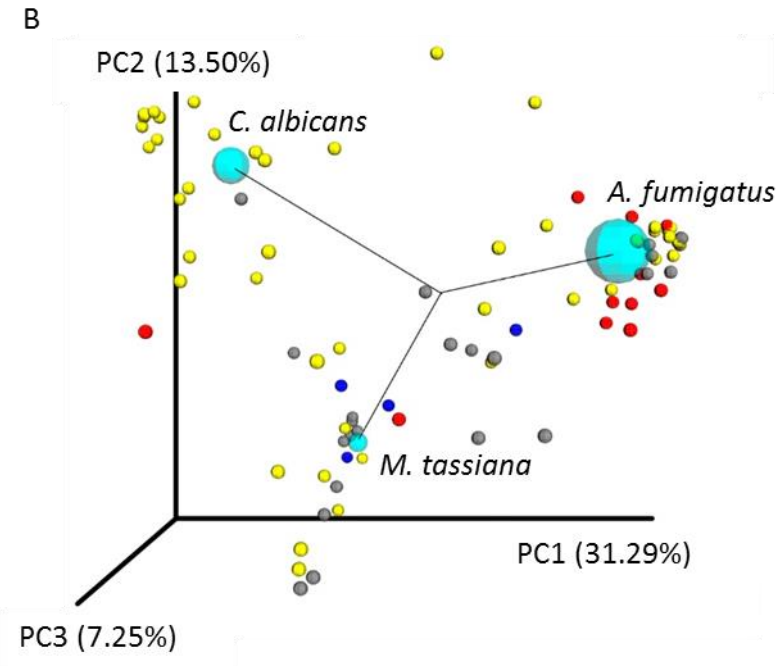
P values were calculated using two approaches; the Mann-Whitney U test and the non-parametric t-test (with *P*-values calculated using 999 Monte Carlo permutations) corrected for multiple samples by the Benjamini-Hochberg FDR procedure for multiple comparisons [291]. Significant *P*-value's ($P < 0.05$) are given in bold.

Table 3. 11: Fungi detected in sputum that had significantly different OTU abundances from subjects who were culture positive for *A. fumigatus* compared to those who were culture negative.

Fungus	Test statistic	Mean OTU frequency		*P*-values	Adjusted *P*-value (FDR-P)
		Culture positive	Culture negative		
All subjects (n = 91)		n = 12	n = 79		
Aspergillus fumigatus	T = 2.41	105,063.8	43,349.63	**0.019**	0.228
	U = 243	105,063.8	43,349.63	**0.007**	0.081
Aspergillus tubingensis	T = 3.62	7549.333	991.5063	**0.001**	**0.024**
Malassezia globosa	T = 2.84	722.5833	40.02532	**0.029**	0.232
	U = 267.5	722.5833	40.02532	**0.003**	0.075
Candida dubliniensis	T = 1.95	1180.5	217.3038	**0.044**	0.264
Penicillium decumbens	U = 319.5	4295	973.3291	**0.018**	0.144
Asthmatics (n = 78)		**n = 12**	**n = 66**		
Aspergillus fumigatus	T = 2.78	105063.8	38534.44	**0.018**	0.216
	U = 192	105063.8	38534.44	**0.005**	0.072
Aspergillus tubingensis	T = 3.50	7549.333	857.3636	**0.005**	0.120
Malassezia globosa	T = 2.57	722.5833	46.95455	**0.030**	0.240
	U = 235.5	722.5833	46.95455	**0.009**	0.072

Only taxa detected in at least 25% of the samples were included. Taxa that could not be identified to species level or those with an OTU frequency below 1% are not shown. *P* values were calculated using two approaches; the Mann-Whitney U test and the non-parametric t-test (with *P*-values calculated using 999 Monte Carlo permutations) corrected for multiple samples by the Benjamini-Hochberg FDR procedure for multiple comparisons [291]. Significant *P*-value's ($P < 0.05$) are given in bold.


A
PC2 (13.50%)
PC1 (31.29%)
PC3 (7.25%)


B
PC2 (13.50%)
C. albicans
A. fumigatus
M. tassiana
PC1 (31.29%)
PC3 (7.25%)

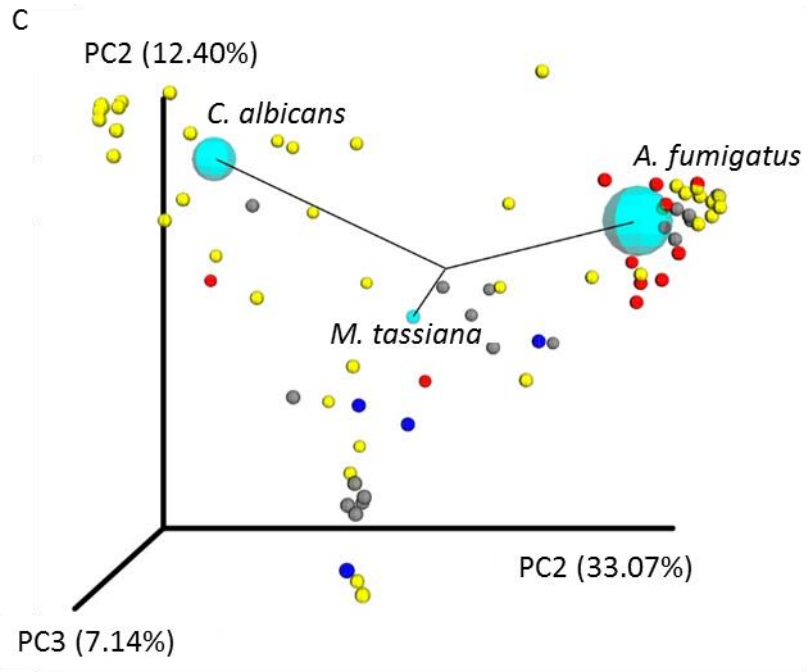


Fig. 3. 6: Principal Coordinate Analysis plots based on the Bray-Curtis distance values. (A) All culture-positive samples from people with asthma who are fungal sensitised (blue) or non-fungal sensitised (orange) and healthy individuals (red). Graphs (B) and (C) indicate culture results from (B) all sputum samples and (C) samples from subjects with asthma. Samples are coloured according to whether they were culture positive for *Aspergillus fumigatus* (red) or another filamentous fungus (blue), with or without the presence of yeast, culture positive for yeast only (yellow) or were culture negative (grey). Aqua spheres indicate the coordinates where the main fungal species, according to both sequence abundance and prevalence, are centred. The size of the sphere is correlated with the relative abundance of the labelled organism. The closer the sample spheres are to the taxa sphere, the greater the relative proportions that taxa comprises within that sample.

3.3.3.1 Fungal load in people with asthma and healthy controls

The total fungal load was determined by universal qPCR for all samples using a standard curve prepared from a serial dilution of a known *C. herbarum* spore concentration. The results are described as *C. herbarum* spore equivalents (*Ch*SE). The fungal load could be determined for the majority of samples. Technical replicates > 0.5 C_T apart were not included. Weakly positive NTCs ($C_T \geq 37$) were detected in some of the universal qPCR runs, therefore the detection threshold was set at 37 and any samples with a C_T value of 37 or higher were treated as negative. The baseline was set to automatic and the threshold to 0.1 ΔRn [276], based on which the ThermoScientific Cloud software calculated the Ct-value for each reaction. The amplification efficiency of the universal qPCR was 90.5%, using the automatic baseline determined by the software. Based on the weakly positive NTCs, the limit of quantification was 6.18 *Ch*SE. Within the three groups depicted in Table 3. 4, 50/53 samples from fungal sensitised people with asthma, 25/26 from non-fungal sensitised patients, and 13/13 from healthy controls could be amplified. No significant differences were detected between the fungal sensitised subjects with asthma (median 136.3 *Ch*SE, IQR 30.04-333.4), non-fungal sensitised subjects with asthma (median 104.9 *Ch*SE, IQR 42.6-434.5) and healthy controls (median 166 *Ch*SE, IQR 111.3-2355) (kw = 3.88, *P* = 0.1469) (Fig. 3. 7), although there was a trend towards higher fungal load in the sputum of healthy controls (U = 318, *P* = 0.0523).

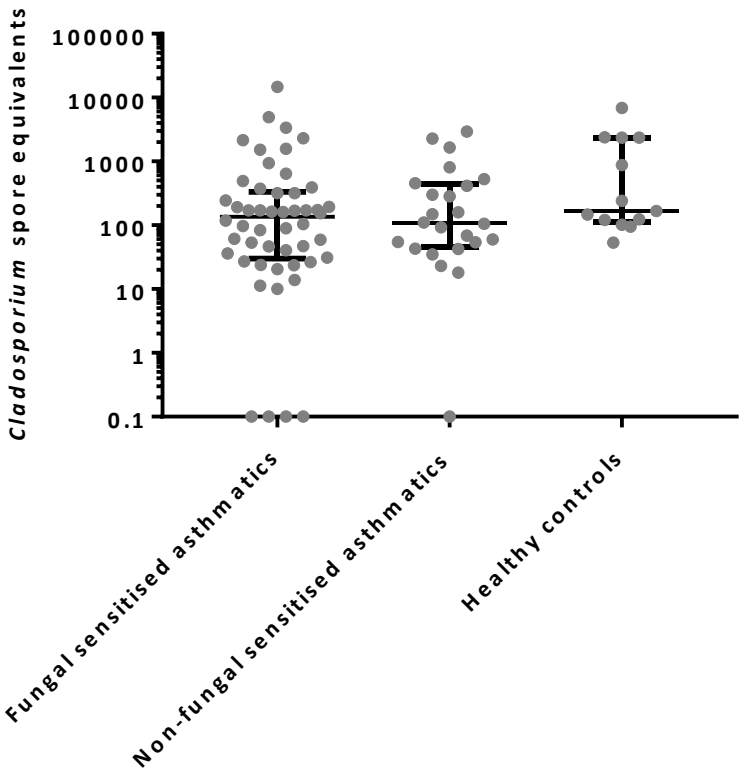


Fig. 3. 7: Amount of *Cladosporium herbarum* spore equivalents as determined by universal qPCR in fungal sensitised and non-fungal sensitised people with asthma, as well as healthy controls. Statistical analysis was performed with Kruskal-Wallis test (GraphPad Prism 7.00). Samples that did not amplify are indicated as 0.1 as otherwise they would not appear due to the logarithmic scale.

There was a positive correlation between the total number of cfus of all fungi cultured and the fungal load determined by universal qPCR (n = 87, r = 0.2151, *P* = 0.047, Fig. 3. 8 A). In contrast, there was no significant correlation between fungal spores detected by microscopy in the air on a given day and the fungal load of the air sample by qPCR (n = 30, r = -0.1711, *P* = 0.366, Fig. 3. 8 B).

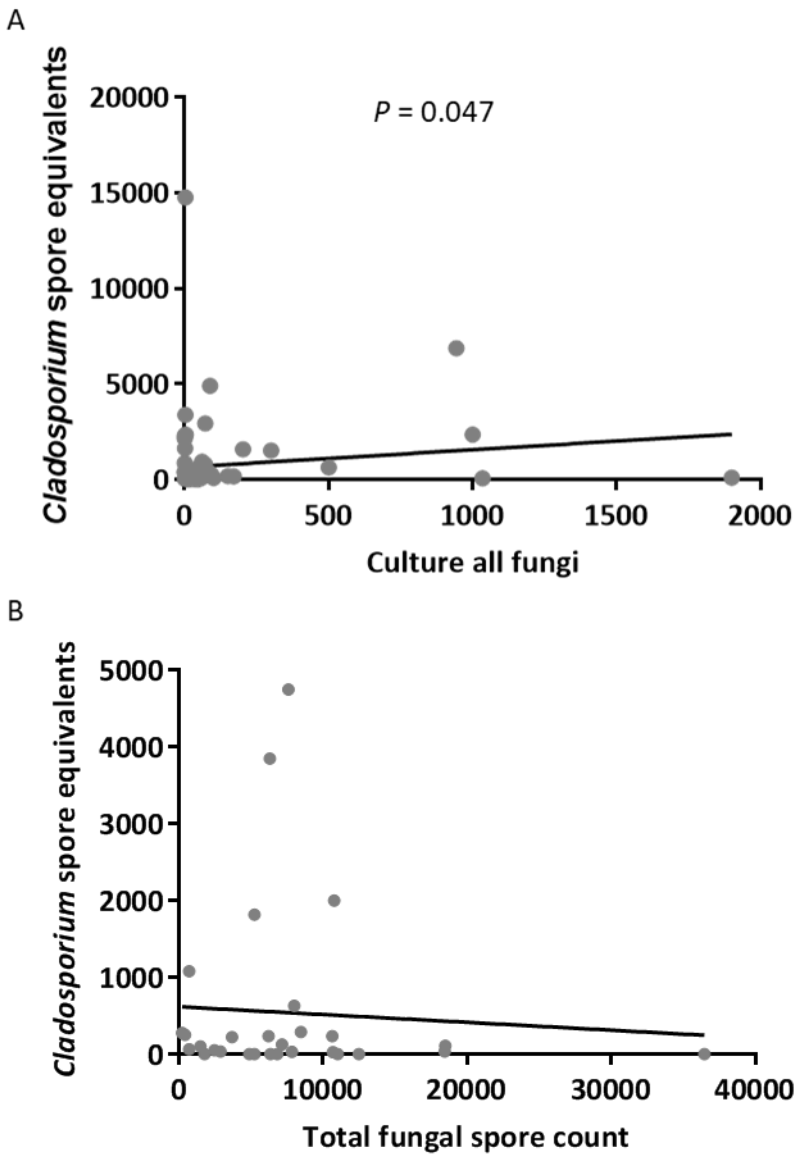


Fig. 3. 8: Correlation between *C. herbarum* spore equivalents as measured by universal qPCR and (A) total number of cfus of all fungi cultured or (B) total fungal spores counted from slides on the corresponding day. Statistics were performed using the two-tailed non-parametric Spearman correlation (GraphPad Prism 7.04). Significant *P*-values are shown in the graph.

The HTS adjusted abundance was determined by multiplying the species' relative abundances with the universal qPCR mean quantities obtained [281]. The adjusted abundance of fungal species was compared for species detected in the core mycobiome of each study group with a 0.1% OTU frequency cut-off (n = 25). A significantly higher amount of *C. dubliniensis* was detected in people with asthma (n = 74) compared to

healthy individuals (n = 13) (U = 318.5, *P* = 0.019, q-value 0.475). The load of this fungus was significantly higher in fungal-sensitised subjects (n = 50) compared to those without fungal sensitisation (n = 24) or healthy controls (n = 13) (kw = 6.927, *P* = 0.031, q value = 0.520). The amount of the plant pathogen *Bjerkandera adusta* tended to be higher in fungal sensitised asthmatics compared to the other groups (kw = 5.908, *P* = 0.052, q-value = 0.520). These significant differences did not remain significant after allowing for multiple testing.

Species-specific qPCR was performed on a subset of DNA samples to determine the amount of *C. albicans*, *A. fumigatus* and *A. niger*. *A. niger* was investigated due to its ability to ability to amplify *A. tubingensis*, which was associated with bronchoscopy-derived samples and clinical parameters described in later sections (3.3.4, 3.3.7). Not all samples could be amplified or had reliable data with reproducible technical replicates (> 0.5 C_T apart). The thresholds for the species-specific qPCR were determined manually (Table 3. 12) using the automatic baseline from the software. The primer amplification efficiencies for the three species were 72.46% for *A. fumigatus* (n = 25), 87.16% for *C. albicans* (n = 24) and 77.35% for *A. niger* (n = 17). The fungal loads did not statistically differ across all three study groups for any of these fungi (Table 3. 12). A statistical comparison for *A. niger* could not be performed across all groups due to the low number of healthy controls (n = 1) but was not significant between the two asthma groups (Table 3. 12).

Table 3. 12: Species-specific qPCR values (median and interquartile range in brackets) across asthma patients with or without fungal sensitisation and healthy controls.

	A. fumigatus	***C. albicans***	***A. niger***
Threshold (ΔRn)	0.1	0.105	0.13
Amplification efficiency (%)	72.46	87.16	77.35
LOD	0.32	0.22	0.19
LOQ	0.97	0.67	0.59
Total number of samples (n)	25	24	17
Fungal sensitised (n = 16)[a]	255.1 (16.36-2036) (n = 16)	3003 (0-9026) (n = 15)	0 (0-196.7) (n = 9)
Non-fungal sensitised (n = 6) [a]	1633 (0-3860) (n = 6)	2005 (664.7-6775) (n = 5)	0 (0-45.66) (n = 6)
Test statistics	39	35	22
P value	0.5244	0.8647	0.4857
Healthy controls (n = 3) [a]	218 (0-436.9) (n = 2)	0 (0-2410) (n = 3)	145.6 (n = 1)
Test statistics	1.026	2.248	N/A
P value	0.5986	0.3250	N/A

LOD = Limit of detection, LOQ = limit of quantification, [a]Data missing for some patients, number data available for indicated in brackets

P-values were calculated with the Mann-Whitney U test or Kruskal-Wallis test for comparison of non-parametric for two groups or three groups, respectively, using GraphPad Prism v7.0.

The robustness of the species-specific qPCR was determined by correlating the measured quantities and number of fungi recovered by culture. This could not be done for the filamentous fungi as their recovery by culture was too low (section 3.3.5) and the qPCR only included eight samples with matching culture data for *A. fumigatus*. The correlation between *C. albicans* conidia equivalents and yeast culture data (n = 23) was significant (r = 0.9126, $P < 0.0001$) with or without the inclusion of the outlier (1900 cfu ≈ 9237.87 conidia equivalents, r = 0.9103, $P < 0.0001$) (Fig. 3. 9 A). The correlation remained significant when considering only the *C. albicans* yeast culture positive samples (n = 13) as determined using CHROMagar and Sanger sequencing (section 3.3.5) (Fig. 3. 9 B). This suggested robust data by the *C. albicans*-specific qPCR.

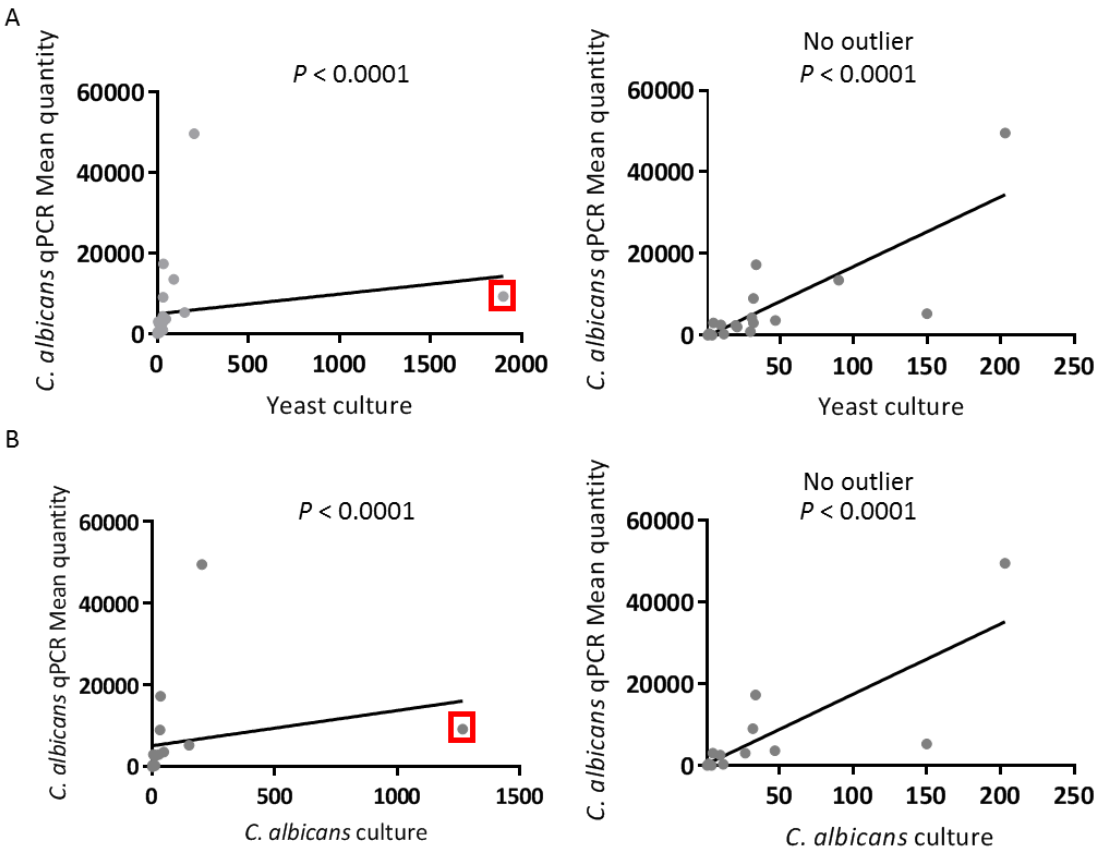


Fig. 3. 9: Spearman correlations (two-tailed) between *C. albicans*-specific qPCR mean quantities and culture data for (A) yeast in total (n = 23) and (B) *C. albicans* culture-positive samples as determined by CHROMagar and Sanger sequencing (n = 13). Graphs are shown with and without the outlier at 9237.87 conidia equivalents (indicated by a red box), which had (A) 1900 colony-forming units (cfu) (r = 0.9103, $P < 0.0001$), of which (B) 1267 were *C. albicans* cfu (r = 0.9127, $P < 0.0001$). *P* values for the respective assay are provided in the graphs. Statistics were performed using the two-tailed non-parametric Spearman correlation (GraphPad Prism 7.04).

A subsequent analysis of the correlation between the species-specific qPCR and the HTS adjusted abundance values showed, that the correlation was positive overall for *A. fumigatus* (r = 0.929, 95% CI 0.837-0.970, $P < 0.0001$, Fig. 3. 10 A), *C. albicans* (r = 0.8217, 95% CI 0.6112-0.9236, $P < 0.0001$, Fig. 3. 10 B) and *A. niger* (r = 0.5309, 95% CI 0.03177-0.8181, $P = 0.034$, Fig. 3. 10 C). A direct comparison of people with asthma with or without fungal sensitisation to healthy controls was not possible due to the low sample number from the latter.

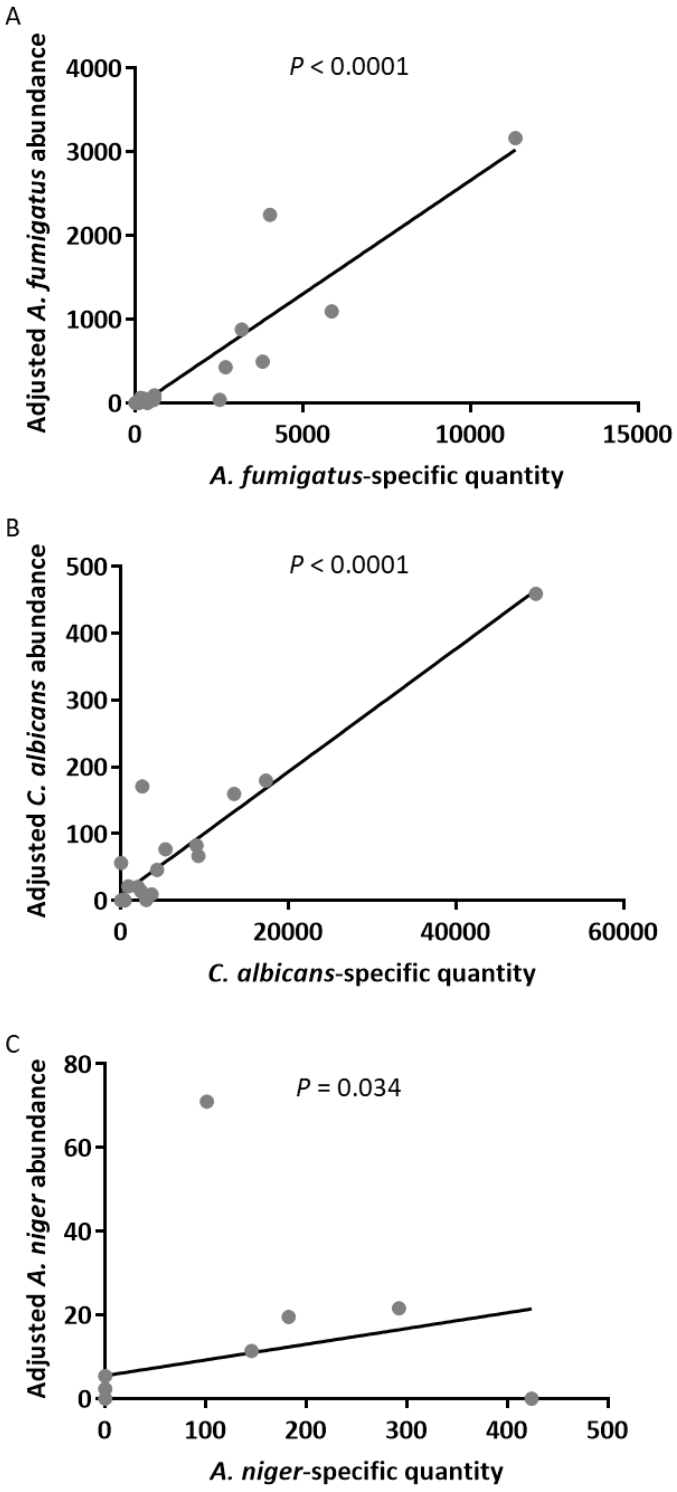


Fig. 3. 10: Spearman correlations (two-tailed) between HTS adjusted abundances and species-specific qPCR quantities for (A) *A. fumigatus*, (B) *C. albicans* and (C) *A. niger*. Significant *P*-values for the respective assay are provided in the graphs. Statistics were performed using the two-tailed non-parametric Spearman correlation (GraphPad Prism 7.04).

In summary, the fungal load was not significantly different between groups using either universal or species-specific qPCR. The HTS adjusted abundance data correlated with the species-specific qPCR assay.

3.3.3.2 Mouthwash control

HTS data were obtained from 10/12 subjects who provided mouthwash samples prior to sputum collection. One hundred and seven fungal OTUs representing 31 taxa at species level were identified (Table 3. 6). Sensitisation groups were not statistically assessed due to the limited number of samples. Since the mouthwashes were collected as control samples for the corresponding sputa, a comparison of the α-diversity and the core mycobiome showed that the species richness estimate was not significantly different (Fig. 3. 11).

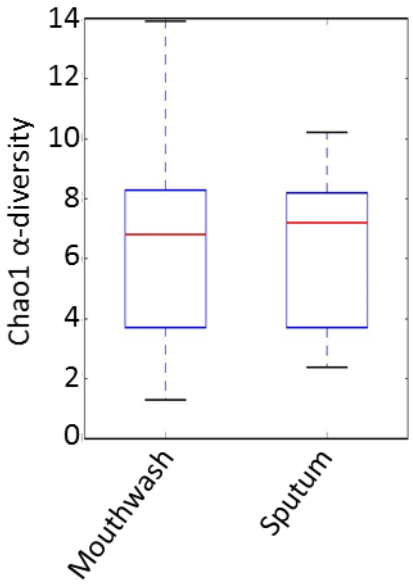


Fig. 3. 11: Box plot of α-diversity index (Chao1) from mouthwash and matching sputum samples (both n = 10). Box plots show mean and standard deviations of the groups' alpha diversities.

The two most prevalent fungi in matched sputum and mouthwash samples (n = 10) were *C. albicans* (100%) and *M. tassiana* (60 and 70%, respectively). This was followed by *A. fumigatus* (60%) and *M. globosa* (40%) in mouthwash samples compared with

unidentifiable Ascomycota species (60%) and *A. fumigatus* (50%) in sputum samples. The core mycobiome of mouthwash samples encompassed 12 species and sputum samples contained 9. In the core mycobiome, seven taxa identifiable to species level were unique to mouthwash, four unique to sputum and three species shared between them (Fig. 3. 12).

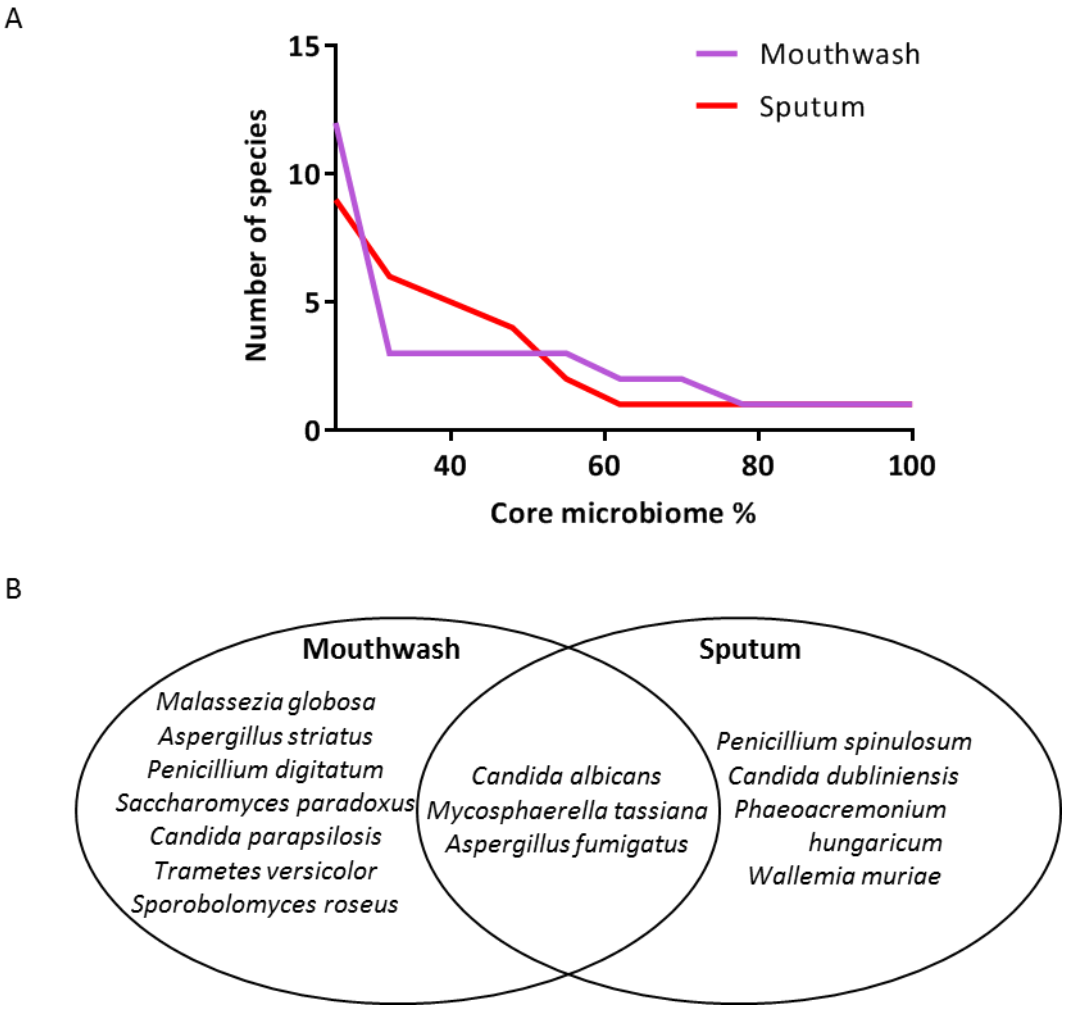


Fig. 3. 12: The core microbiome in mouthwash and matching sputum samples (n = 10). (A) The number of taxa shared between 25% and 100% of samples (n = 10). (B) Venn diagram illustrating the core microbiome, excluding taxa that could not be identified to species level.

The highest OTU frequencies were not represented by the most prevalent species with the exception of *C. albicans*, which was more abundant in mouthwashes (71.96%) compared to sputa (46.06%). In mouthwash, the next most abundant identifiable fungi

were *Candida smithsonii* (6.5%), *C. tropicalis* (5.5%) and *Saccharomyces paradoxus* (3.3%). *C. smithsonii* and *C. tropicalis* were not present in the core mycobiome of any sample type including mouthwash. No significant differences between the OTU frequencies of species from the core mycobiomes of mouthwashes and matching sputum samples were detected. A heat map comparison of relative abundances from individual samples is illustrated in Fig. 3. 13. Statistical analyses on species prevalence was not performed due to the small sample size.

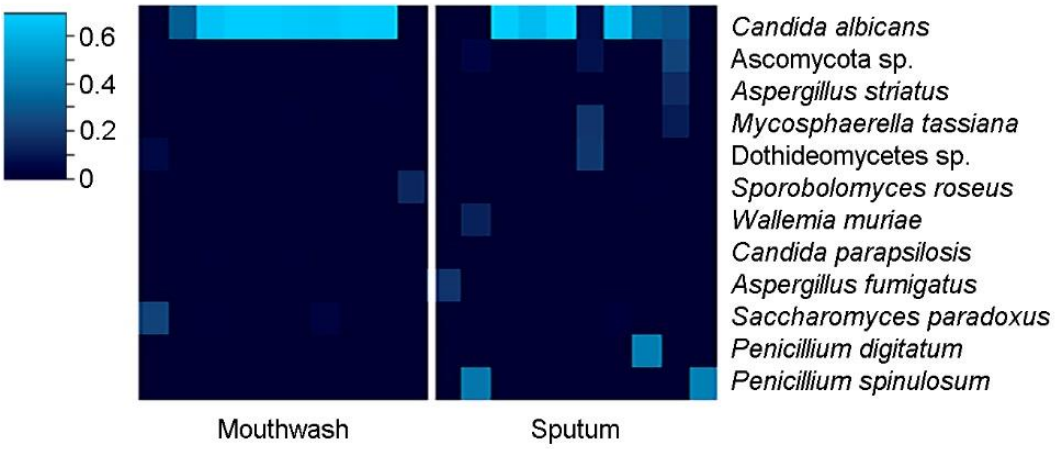


Fig. 3. 13: Heat map illustrating the relative abundances of fungal ITS2 sequences between mouthwash and matching sputum samples (n = 10). The data were transformed using ln(x+1) due to zeros in the dataset. Only taxa detected in the core mycobiome of each mouthwash or sputum and with a total OTU observation cut-off of 0.1% across all samples were included. The relative abundance is indicated by colour, with the lighter shades representing the more abundant sequences.

3.3.3.3 Proportion of alive and dead fungi in sputum

A subset of sputum samples from fungal sensitised subjects with asthma were split for treatment with PMA (n = 12). HTS data were only obtained from 5 samples. The median α-diversity value was lower in PMA treated samples, however, the differences were not significant (*P* = 0.771) (Fig. 3. 14).

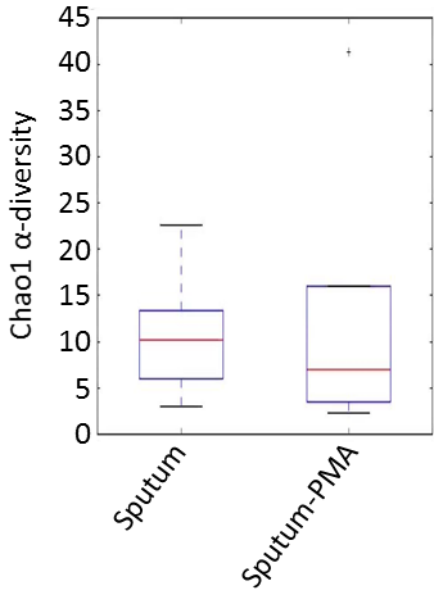


Fig. 3. 14: Box plot of α-diversity index (Chao1) from sputum samples which were either treated with propidium monoazide (PMA) or untreated (n = 5). Box plots show mean and standard deviations of the groups' alpha diversities.

The most prevalent fungus was *C. albicans* in 100% of sputum samples with or without PMA-treatment, followed by *M. tassiana* (80% sputum, 60% sputum-PMA) and *A. fumigatus* (each 60%). More species were detected in PMA-treated samples in 2/5 sample pair comparisons using a 1% OTU frequency cut-off (Fig. 3. 15). Due to the small sample size, statistical analyses were not performed.

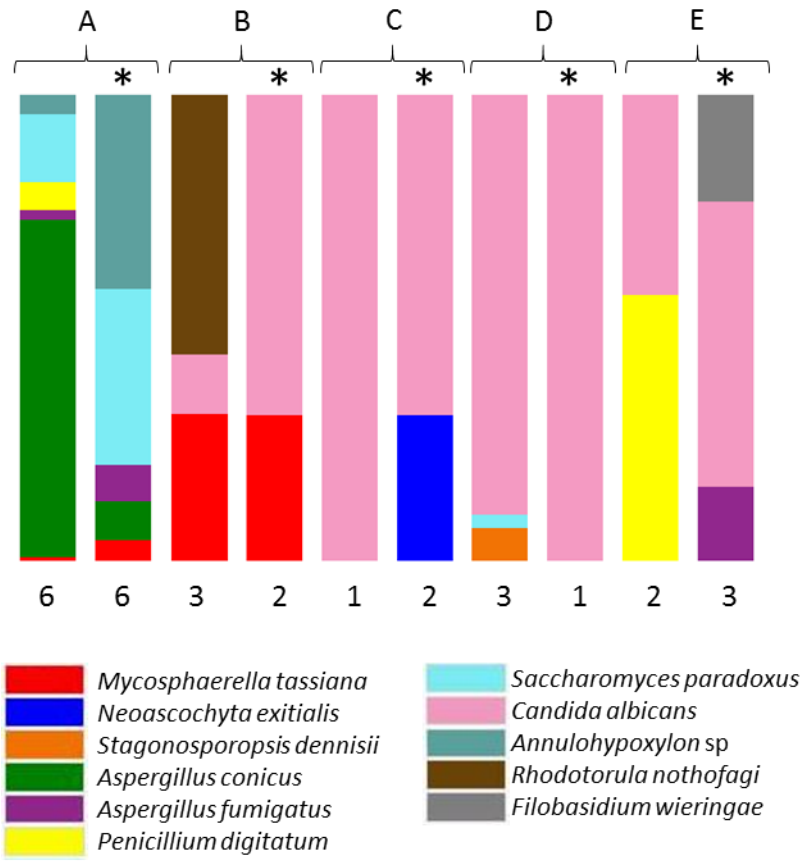


Fig. 3. 15: Overview of species detected in PMA-treated and untreated sputum samples from five fungal sensitised subjects with asthma (A-E) using a 1% OTU frequency cut-off across all samples. PMA-treated samples are marked with an asterix. The number of species detected in each sample is indicated below the bar charts.

A species-specific qPCR was performed on 11 matching pairs of PMA-treated and PMA-untreated sputum samples, of which four pairs had matching HTS data. Amplification efficiencies for *A. fumigatus* (threshold 0.11 ΔRn) and *C. albicans* (threshold 0.14 ΔRn) were 74.08% and 97.06%, respectively. The LOD and LOQ values for *A. fumigatus* were 0.24 and 0.74, respectively, for *C. albicans* 0.20 and 0.60, respectively. The species-specific qPCR for *A. fumigatus* was negative for all samples. To note, four sputum samples that had been included in the previous *A. fumigatus*-specific qPCR remained negative, indicating the reproducibility of the method (section 3.3.3.1). In contrast, *C. albicans* was amplifiable from six pairings, including the four that had matching HTS data (Fig. 3. 16). The *C. albicans*-specific qPCR showed increased quantities of this fungus for two samples, and decreased quantities for the other two samples comparing the PMA-

treated samples to the matched untreated samples. This reflected the observations of the relative abundances for this fungus for three samples. The relative abundances for two samples were increased and decreased for another sample when they were treated with PMA.

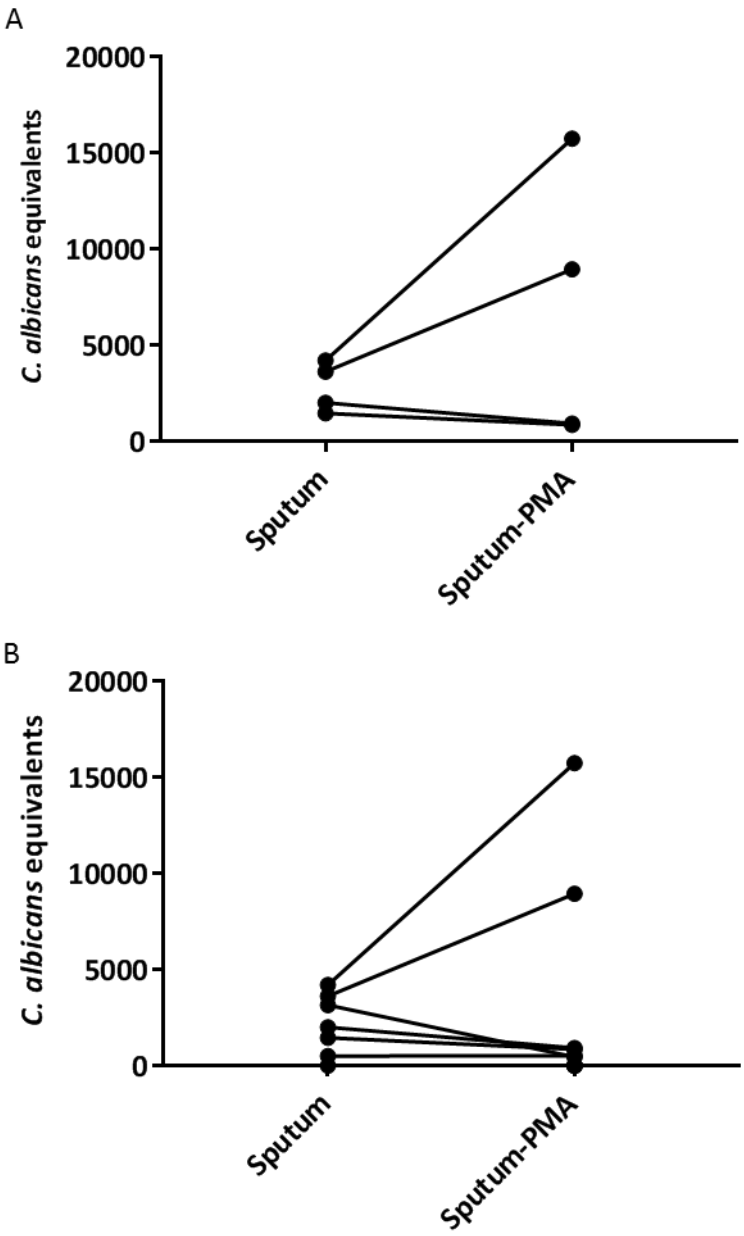


Fig. 3. 16: *C. albicans*-specific qPCR amplification of sputum samples with and without PMA treatment. Readings are *C. albicans* conidia equivalents. (A) HTS matching samples only, (B) all amplified pairs (n = 11).

3.3.4 Mycobiome differs depending on the sample type used

Twenty-nine subjects underwent bronchoscopy and HTS data were obtained from 25 sputa matching at least one of the bronchoscopy-derived samples, BAL (n = 21), washes (n = 23) and brushes (n = 14). The comparison between healthy controls and people with asthma showed that *A. tubingensis* was significantly more prevalent in BAL samples (75% v 20%, *P* = 0.048) or all bronchoscopy-derived samples (72% v 33%, *P* = 0.020) from people with asthma compared to healthy controls. The OTU frequencies of *A. tubingensis* (U = 361, *P* = 0.018, adjusted *P*-value = 0.192), *C. pseudolongus* (U = 369, *P* = 0.020, adjusted *P*-value = 0.192) and *P. decumbens* (U = 409, *P* = 0.042, adjusted *P*-value = 0.190) were significantly higher in people with asthma compared to healthy controls. Conversely, the OTU frequency of *M. tassiana* (T = -3.2, *P* = 0.010, adjusted *P*-value = 0.190) was significantly higher in healthy controls (Table 3. 13). *C. pseudolongus* remained significantly higher in people with asthma when matching sputum samples were excluded from the analysis (U = 175, *P* = 0.041, adjusted *P*-value = 0.333, Table 3. 13). None of the four fungi remained significantly different after allowing for multiple testing (adjusted *P*-values). This is why the main focus of this section is on the comparison between the different sample types in order to assess whether the invasive bronchoscopy procedure shows any differences to the corresponding sputum sampling. Seven hundred and fifty-five OTUs representing 173 taxa at species level were detected from all the different sample types, where the taxa detected within the sample types ranged from 111 to 133 (Table 3. 6). The Chao1 indices (α-diversities) for species richness estimation between the different sample types were not significantly different (Fig. 3. 17).

Table 3. 13: Fungal species that differed between healthy controls and people with asthma in OTU frequency. Only taxa detected in at least 25% of the samples were considered. Taxa that could not be identified to species level, and likely comprised multiple species, were excluded.

Sample(s)	Fungus	Test-Statistic	Asthma mean	Healthy mean	*P*-value	Adjusted *P*-value
Bronchoscopy & sputum	*Aspergillus tubingensis*	U = 361	10,796	8,659	**0.018**	0.192
	Cryptococcus pseudolongus	U = 369	1,361	98	**0.020**	0.192
	Penicillium decumbens	U = 409	1,426	240	**0.042**	0.264
	Mycosphaerella tassiana	T = -3.2	2,664	14,056	**0.010**	0.190
Bronchoscopy	*Cryptococcus pseudolongus*	U = 175	1,679	138	**0.041**	0.333

P values were calculated using two approaches; the Mann-Whitney U test and the non-parametric t-test (with *P*-values calculated using 999 Monte Carlo permutations) corrected for multiple samples by the Benjamini-Hochberg FDR procedure for multiple comparisons [291]. Significant *P*-value's ($P < 0.05$) are given in bold.

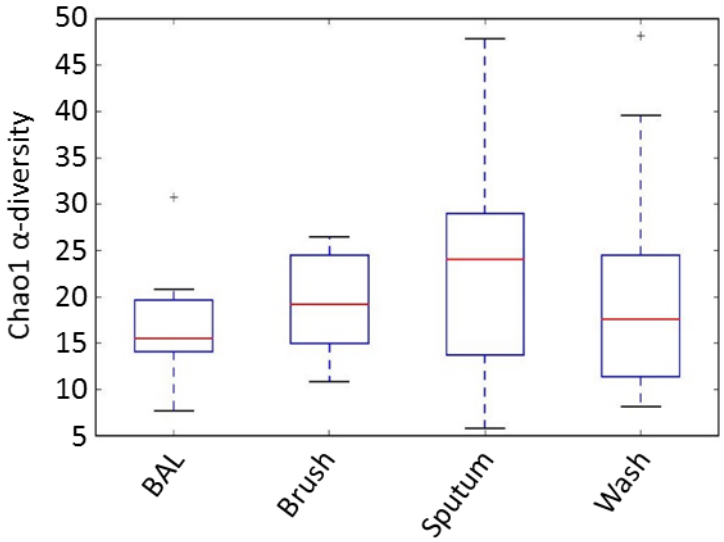


Fig. 3. 17: Box plot of α-diversity index (Chao1) of samples from subjects who underwent a bronchoscopy grouped into bronchoalveolar lavage (BAL, n = 21), protected brush (n = 14), and bronchial wash (n = 23) and matching sputum (n = 25). Box plots show mean and standard deviations of the groups' alpha diversities.

The core mycobiome was represented by 19, 26, 21 and 23 species in BAL, brush, sputum and wash samples at 25% prevalence, respectively (Fig. 3. 18 A). The majority of species (n = 12) was shared between all sample types, and only one to three species were unique in each sample type such as *Trametes versicolor* in brush samples (Fig. 3. 18 B).

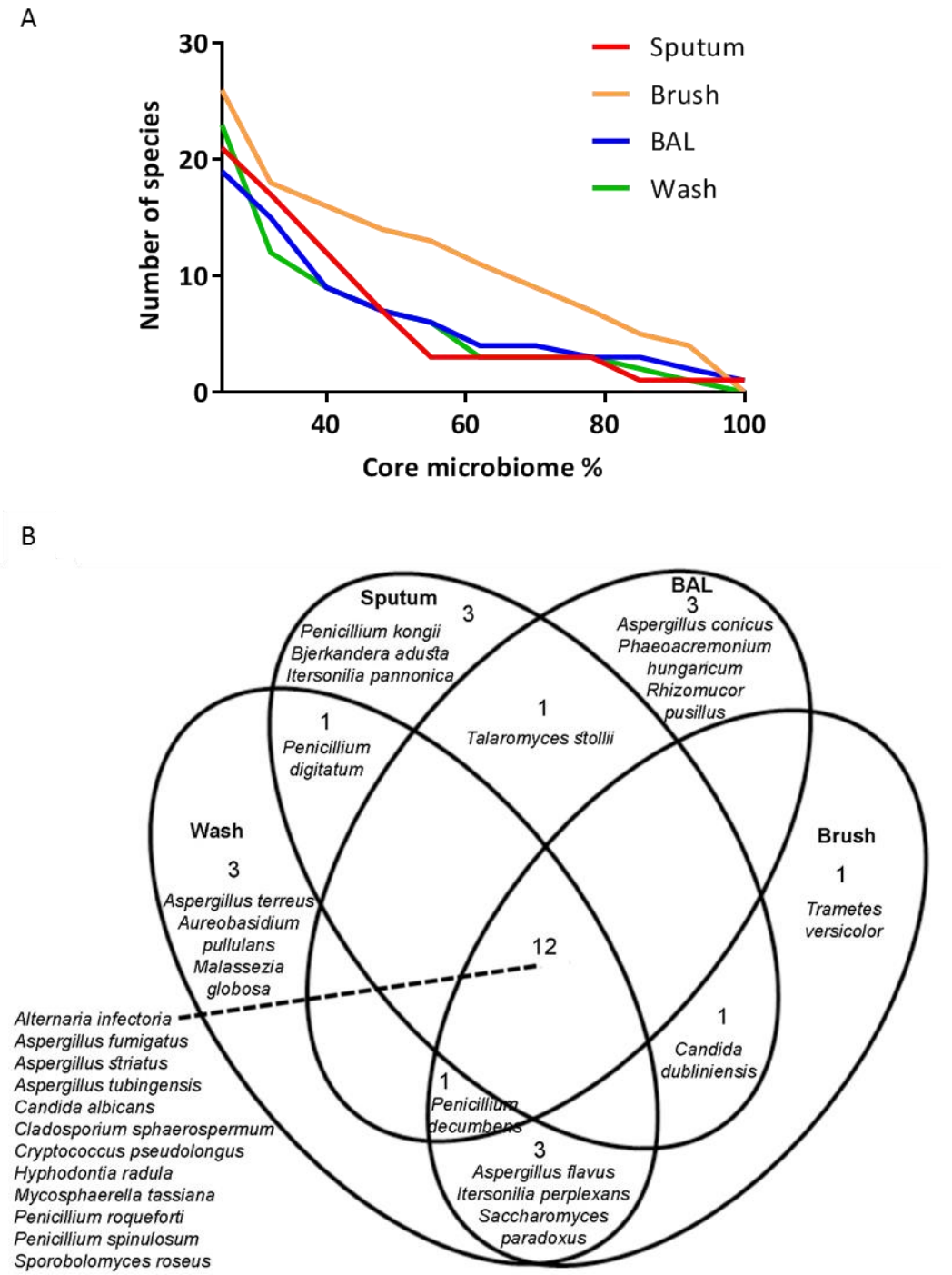


Fig. 3. 18: The core mycobiome in bronchoalveolar lavage (BAL) samples (n = 21), bronchial wash samples (n = 23), protected brush samples (n = 14) and matching sputum samples (n = 25): (A) The number of taxa shared between 25% and 100% of samples; (B) Venn diagram illustrating the core microbiome, excluding fungi not able to be identified to species level.

Similar to sputum samples, only a few species were shared between the majority of samples with the most prevalent taxa being *C. albicans*, *A. fumigatus* and *M. tassiana* (> 82%) in all sample types, except for *C. albicans* from brushes (78%). Brush samples had a significantly higher prevalence of *Cryptococcus pseudolongus* (92.9%, χ^2 = 13.45, *P* = 0.004, FDR-*P* = 0.035), *A. tubingensis* (92.9%, χ^2 = 12.65, *P* = 0.006, FDR-*P* = 0.035) and *S. roseus* (71.4%, χ^2 = 8.80, *P* = 0.032, FDR-*P* = 0.135) compared to the other sample types, however, *S. roseus* did not remain significant after adjusting for multiple samples (Table 3. 14).

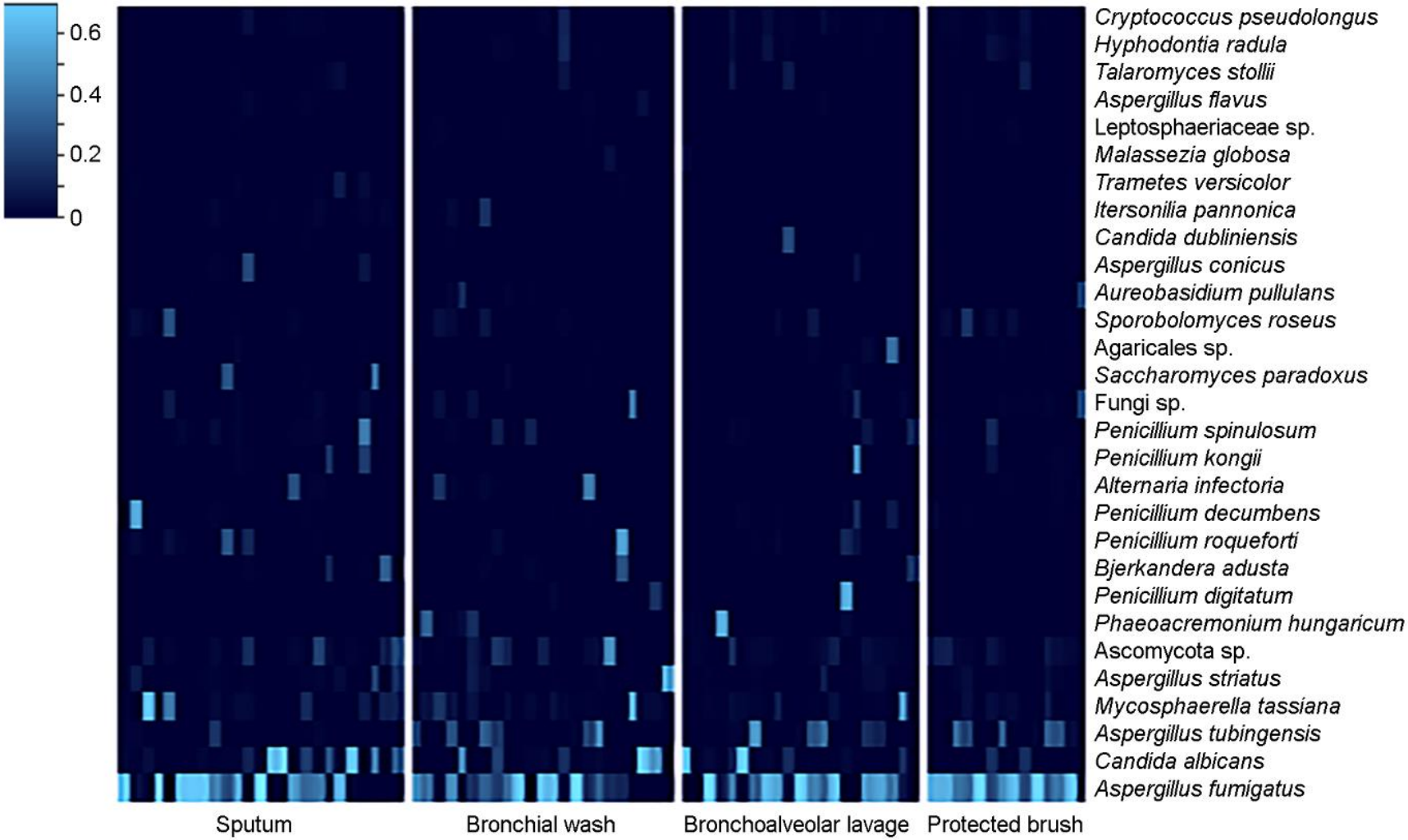
Table 3. 14: Fungal species that differed in their presence or absence between the samples obtained from different lung compartments. Only taxa detected in at least 25% of the samples from any of the four sample types, and with a total OTU observation cut-off of 1% for that sample type, were considered. Taxa that could not be identified to species level, and likely comprised multiple species, were excluded.

	Test-Statistic	**BAL mean**	**Brush mean**	**Wash mean**	**Sputum mean**	***P*-value**	**Adjusted *P*-value**
Candida albicans	7.64	95.2	78.6	95.7	100.0	0.054	0.170
Aspergillus fumigatus	1.22	90.5	92.9	82.6	84.0	0.749	0.767
Aspergillus tubingensis	12.65	61.9	92.9	47.8	36.0	**0.006**	**0.035**
Cryptococcus pseudolongus	13.45	47.6	92.9	34.8	40.0	**0.004**	**0.035**
Sporobolomyces roseus	8.80	28.6	71.4	26.1	40.0	**0.032**	0.135

P values were calculated with a Chi-square contingency test for presence versus absence ratios, with *P*-values corrected using the procedure of Benjamini, Krieger and Yekutieli with a desired false-discovery rate Q-value of 5% (adjusted *P*-value) [292]. Significant *P*-value's (*P* < 0.05) are given in bold.

The most abundant fungus in all sample types was *A. fumigatus* with mean of 39.13% (range 0-96.8%) in BAL, 32.00% in washes (range 0-97.11%), 60.13% in brushes (range 0-99.32%) and 39.3% in matching sputum samples (range 0-99.74%). This was followed by *C. albicans* in BAL (mean = 10.63%, range = 0-87.18%), washes (mean = 10.70%, range = 0-92.35%) and sputa (mean = 16.75%, range = 0-99.98%), or *A. tubingensis* in brushes (mean = 11.25%, range = 0-36.35%), which was the third most abundant species in BAL (mean = 7.49%, range = 0-55.38%) and washes (mean = 7.77%, range = 0-50.19%). *C. albicans* was around 5-8 times less abundant in brushes compared to the other sample types. The number of taxa within the core mycobiomes of matching sputum samples, BAL, wash, brush and with a 0.1% OTU frequency cut-off was 19, 16, 20 and 20 taxa, respectively (Fig. 3. 19 A), with a 1% average abundance cut-off 14, 12, 10 and 11 taxa, respectively (Fig. 3. 19 B). The OTU frequencies of *A. fumigatus* (kw = 9.54, *P* = 0.023, FDR-*P* = 0.098), *A. tubingensis* ($\chi 2$ = 10.74, *P* = 0.013, FDR-*P* = 0.098), *C. pseudolongus* ($\chi 2$ = 9.49, *P* = 0.023, FDR-*P* = 0.098) and *S. roseus* ($\chi 2$ = 9.09, *P* = 0.028, FDR-*P* = 0.098) were significantly higher in brush samples. The OTU frequency of *S. roseus* in sputum samples was similar to the one found in brush samples (Fig. 3. 19 A, Table 3. 15), whilst that of *C. albicans* ($\chi 2$ = 6.96, *P* = 0.073) tended to be higher in brush and BAL.

A


0.6
0.4
0.2
0
Cryptococcus pseudolongus
Hyphodontia radula
Talaromyces stollii
Aspergillus flavus
Leptosphaeriaceae sp.
Malassezia globosa
Trametes versicolor
Itersonilia pannonica
Candida dubliniensis
Aspergillus conicus
Aureobasidium pullulans
Sporobolomyces roseus
Agaricales sp.
Saccharomyces paradoxus
Fungi sp.
Penicillium spinulosum
Penicillium kongii
Alternaria infectoria
Penicillium decumbens
Penicillium roqueforti
Bjerkandera adusta
Penicillium digitatum
Phaeoacremonium hungaricum
Ascomycota sp.
Aspergillus striatus
Mycosphaerella tassiana
Aspergillus tubingensis
Candida albicans
Aspergillus fumigatus
Sputum
Bronchial wash
Bronchoalveolar lavage
Protected brush

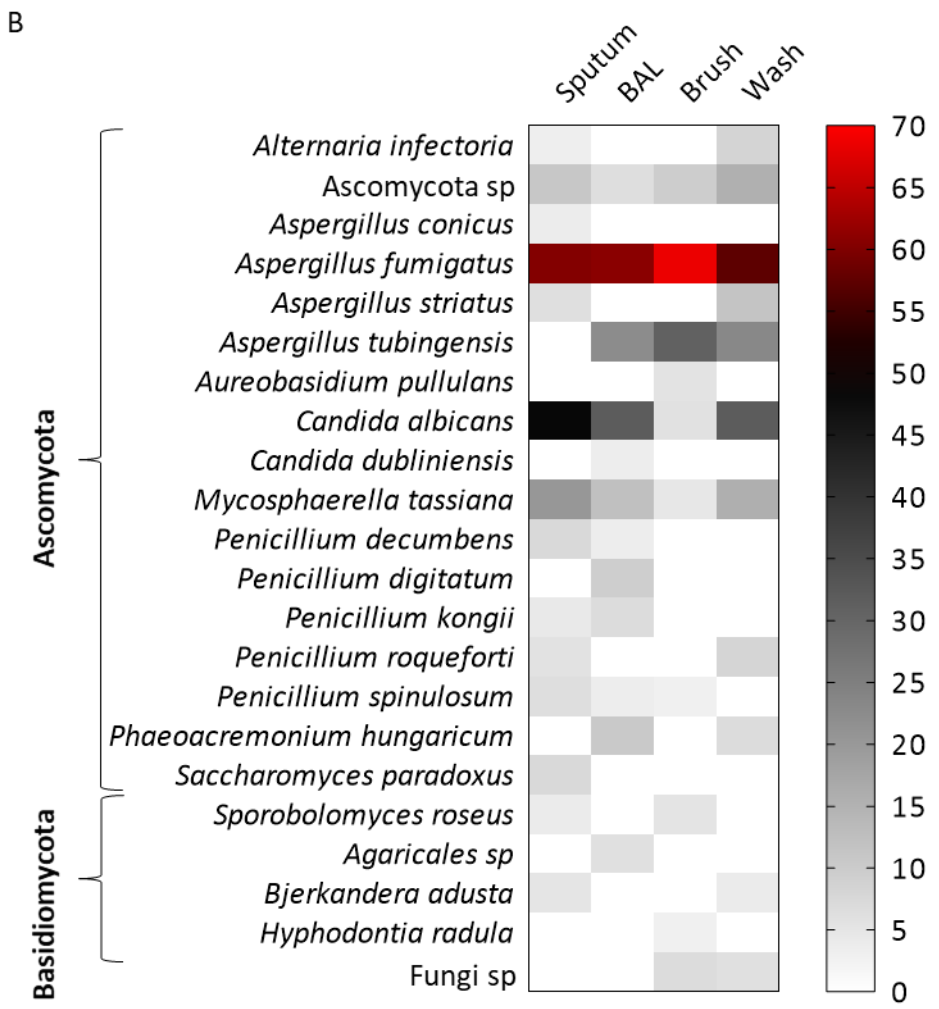


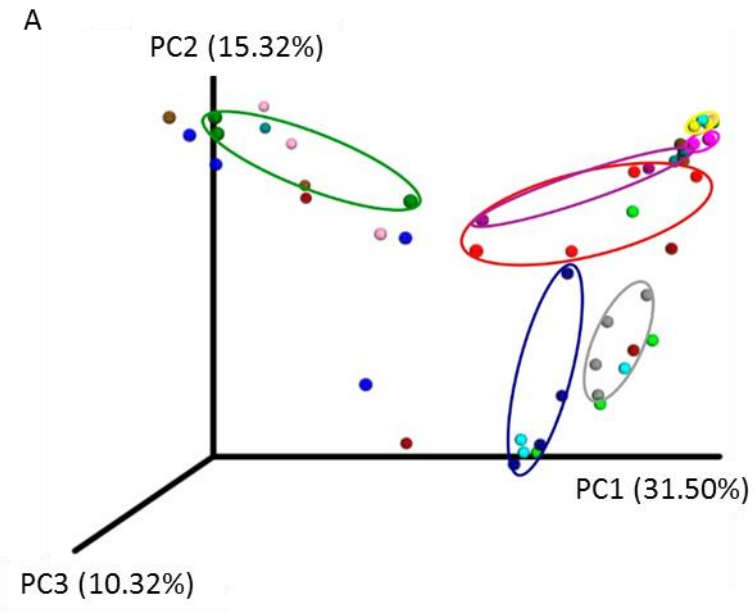
Fig. 3. 19: Heat maps illustrating the distribution of species based on relative abundances using the core mycobiome for each bronchoalveolar lavage (BAL) (n = 21), bronchial wash (n = 23), protected brush (n = 14) and matching sputum samples (n = 25). (A) A 0.1% OTU frequency cut-off was used across all samples. The data were transformed using ln(x+1) due to zeros in the dataset. The relative abundance is indicated by colour, with the lighter shades representing the more abundant sequences. (B) At least 1% average abundance within a sample type and 0.1% OTU frequency cut-off across all samples. The relative abundance is indicated by colour, with darker shades representing the higher frequencies, red frequencies 55% up to 70% abundance.

Table 3. 15: Fungal species whose OTU frequency significantly differed between the samples obtained from different lung compartments. Only taxa detected in at least 25% of the samples from any of the four sample types, and with a total OTU observation cut-off of 1% for that sample type, were considered. Taxa that could not be identified to species level, and likely comprised multiple species, were excluded.

	Test-Statistic	**BAL mean**	**Brush mean**	**Wash mean**	**Sputum mean**	***P*-value**	**Adjusted *P*-value**
Aspergillus fumigatus	9.54	57,596	102,527	40,020	55,482	**0.023**	0.098
Aspergillus tubingensis	10.74	10,821	20,351	13,892	1,123	**0.013**	0.098
Cryptococcus pseudolongus	9.49	1,329	1,450	1,334	502	**0.023**	0.098
Sporobolomyces roseus	9.09	672	3,058	822	2,544	**0.028**	0.098

P values were calculated with the Kruskal-Wallis test for comparing OTU frequencies correcting *P*-values by the Benjamini-Hochberg FDR procedure for multiple comparisons with a desired false-discovery rate Q-value of 5% [291]. Significant *P*-value's ($P < 0.05$) are given in bold.

A comparison between the β-diversity distance measures based on Bray-Curtis showed no significant difference between the different sample types (BAL v wash v brush v sputum; $r^2 = 0.048$, $P = 0.126$). The majority of the β-diversity was influenced by the patient identity, explaining approximately 56% of the variation in distances ($r^2 = 0.555$, $P = 0.001$). The matching sample types for half of the subjects were clustered closer together (Fig. 3. 20 A). Similar to the sputum data, β-diversity distance measures were slightly different when patients were grouped according to their culture data (negative culture v yeast only v *A. fumigatus* culture v other fungal culture; $r^2 = 0.072$, $P = 0.013$). *A. fumigatus* culture-positive samples clustered around samples with high *A. fumigatus* sequence abundance, which was also observed for yeast culture-positive samples and samples with high *C. albicans* sequence abundance (Fig. 3. 20 B).


A
PC2 (15.32%)
PC1 (31.50%)
PC3 (10.32%)

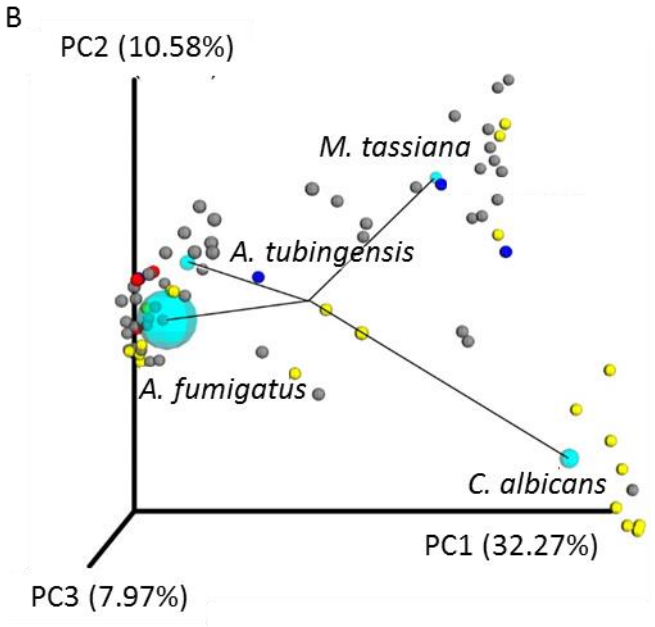

B
PC2 (10.58%)
M. tassiana
A. tubingensis
A. fumigatus
C. albicans
PC1 (32.27%)
PC3 (7.97%)

Fig. 3. 20: Principal Coordinate Analysis plots based on the Bray-Curtis distance values using samples from patients who underwent bronchoscopies. (A) Samples from the 14 individuals who underwent bronchoscopies and have sequence data for sputum, bronchial wash, bronchoalveolar lavage and protected brushes. Samples are coloured individually for each subject. Ellipses indicate 7 patients whose samples showed the least intraindividual differences between the sample types represented by the distances on the plot. (B) Samples are coloured according to whether they were culture positive for *A. fumigatus* (red) or another filamentous fungus (blue), with or without the presence of yeast, culture positive for yeast only (yellow) or were culture negative (grey). Aqua spheres indicate the coordinates where the main fungal species, according to both sequence abundance and prevalence, are centred. The size of the sphere is correlated with the relative abundance of the labelled organism. The closer the sample spheres are to the taxa sphere, the greater the relative proportions that taxa comprises within that sample.

3.3.5 Identification of fungi by culture

Fungal recovery from sputum samples was low, with 67%, 15% and 5% of people with asthma being culture-positive for yeast, *A. fumigatus* and non-*A. fumigatus* filamentous fungi respectively. No significant differences were detected for yeasts, *A. fumigatus* or other filamentous fungi when comparing fungal sensitised and non-fungal sensitised people with asthma to each other or to healthy individuals, although filamentous fungi were only recovered from people with asthma (Table 3. 16). Yeasts were identified for a subset of sputum samples using CHROMagar and Sanger sequencing, with a minimum of 10 and maximum of 18 colonies identified per culture plate. They were predominated by *C. albicans* (79.7%), followed by *Saccharomyces cerevisiae* (9.7%) and *C. glabrata* (8.0%) (Table 3. 17). The CHROMagar results supported the previous observation that yeast culture-positive samples were significantly associated with high abundances from *C. albicans* (sections 3.3.3, 3.3.4).

Table 3. 16: Summary of culture data from sputum samples cultured on PGCF medium, according to subject groups. When sputum has been obtained on multiple occasions, the most recent sample was used for the analysis.

	HTS data n	**Culture data n**	**No growth n (%)**	**Culture-positive n (%)**	**Yeast n (%)**	***A. fumigatus* n (%)**	**Other filamentous n (%)**
Total	92	91	26 (29)	65 (71)	59 (65)	12 (13)	4 (4)
Fungal-sensitised asthmatics	53	52	12 (25)	40 (77)	33 (63)	9 (17)	3 (6)
Non-fungal sensitised asthmatics	26	26	7 (27)	19 (73)	19 (73)	3 (12)	1 (4)
Fisher's exact test	-	-	-	0.7822	0.4534	0.7410	0.6538
Healthy controls	13	13	6 (46)	7 (54)	7 (54)	0 (0)	0 (0)
Chi-Square test	-	-	-	0.2484	0.4709	0.2456	> 0.9999

n = number of

Table 3. 17: CHROMagar and Sanger sequencing results for yeast identification from culture of sputum and mouthwash samples recovered from PGCF medium. Indicated are the number of patients, the total number of yeast cfus counted and the number of yeast colonies taken for identification. Species are ranked from the highest to the lowest proportions.

	Number of			**Proportion calculated for total number of yeasts counted**							
Sample type	**patients**	**Total yeast cfu**	**Clinical isolates identified**	**CA**	**CT**	**SC**	**CG**	**UI**	**KL**	**CGu**	**CK**
Sputum	35	6834	226	79.7%	0.4%	9.7%	8.0%	1.4%	0.6%	0%	0.1%
Mouthwash	9	4967	90	79.6%	20.1%	0%	0%	0%	0%	0.3%	0%

CA = *C. albicans*, CT = *C. tropicalis*, SC = *S. cerevisiae*, CG = *C. glabrata*, UI = unidentified, KL = *Kluyveromyces lactis*, CGu = *C. guilliermondi*, CK = *C. krusei*

Twenty-two patients provided more than one sputum sample, represented by one healthy control and 21 people with asthma, of whom 16 were fungal-sensitised. The average intervals between samples were 14.3 months (range 6-19 months) for all samples, 14.4 (range 8-18) for samples from fungal sensitised people with asthma, 14.4 (range 6-19) for samples from non-fungal sensitised people with asthma and 12 months for the heathy control. No significant differences between the sample intervals were detected for the repetitive samples from patients with or without fungal sensitisation (U = 34, *P* = 0.641). Changes between repetitive cultures were detected from nine subjects, all of which were fungal sensitised. In contrast to their previous culture results, six samples were culture-negative and three culture-positive. Reproducibility was classed as moderate overall (Kappa = 0.554, 95% CI 0.300-0.808) and fair within the fungal sensitised people with asthma group (Kappa = 0.389, 95% CI 0.064-0.715) (Table 3. 18).

Table 3. 18: Reproducibility of culture data, showing the number of culture-positive (culture(+)) and culture-negative (culture(-)) samples from the oldest and latest samples of 22 subjects. Samples that were culture-positive the first and the second time showed growth of the same fungal species.

		Oldest		
		Culture (+)	Culture (-)	**Total**
Latest	Culture (+)	13	3	**16**
	Culture (-)	6	19	**25**
	Total	**19**	**21**	

A comparison of culture data between sample types showed the following: More fungi were cultured from mouthwash compared to sputum samples (100% versus 71%, respectively), in particular yeasts (100% versus 65%, respectively). Similar to previous observations for sputum samples, the high prevalence and abundance of *C. albicans* was also reflected in the culture data where it represented 79.6% of clinical isolates, followed by 20.1% of *C. tropicalis* (Table 3. 17). The latter was based on the sample from one patient, whose relative sequence abundance was also the highest for this fungus

(55.1%), followed by *C. albicans* (37.1%). Only two samples showed growth of filamentous fungi. Significantly less fungi were cultured from the bronchoscopy-derived samples BAL, wash and brush with ≤ 33% compared to sputum ($\chi^2 = 30.25$, $P < 0.0001$) or mouthwash samples ($\chi^2 = 20.83$, $P = 0.0001$) using PGCF medium. More fungi were cultured on PGCF medium compared to the other two types of media used, YMA and ScedSel+, except for wash samples, though this difference was not statistically significant (Table 3. 19).

Table 3. 19: Overview of fungal recovery overall, for *A. fumigatus*, any filamentous fungi and yeasts, from samples obtained from the bronchoscopies cultured on three media, PGCF, ScedSel+ and YMA. The recovery of specific fungal groups are indicated as total number and percentage of positive cultures in brackets.

	Medium	**Total**	**No growth**	**Culture-positive (%)**	***A. fumigatus* n (%)**	**Any filamentous n (%)**	**Yeast n (%)**
BAL	**PGCF**	21	15	29	0 (0)	1 (17)	5 (83)
	ScedSel+	21	15	29	0 (0)	0 (0)	6 (100)
	YMA	18	15	17	1 (33)	1 (33)	2 (67)
Wash	**PGCF**	21	16	24	0 (0)	1 (20)	4 (80)
	ScedSel+	20	16	20	0 (0)	0 (0)	4 (100)
	YMA	18	12	33	1 (17)	3 (50)	4 (67)
Brush	**PGCF**	14	11	21	2 (67)	2 (67)	1 (33)
	ScedSel+	14	13	7	0 (0)	0 (0)	1 (100)
	YMA	11	9	18	2 (100)	2 (100)	0 (0)

3.3.6 Fungal aerobiome only showed a low contribution to the mycobiome in clinical samples

Outdoor air samples were collected on dates of sputum collection or bronchoscopy in order to estimate the contribution of inhaled particles to the mycobiome detected. The air trap was representative of a wide geographical region [266]. HTS data were obtained from 45 dates and 1713 fungal OTUs represented by 338 taxa at species level were detected (Table 3. 6). Twenty-eight days matched 31 sputum samples, 18 days matched days when subjects underwent bronchoscopy. In comparison to the corresponding clinical samples, the α-diversity measures showed that the species richness was significantly higher for air samples ($P \leq 0.02$, Fig. 3. 21), which was previously indicated by the higher number of OTUs with respect to the number of reads shown by the rarefaction analysis (Fig. 3. 2).

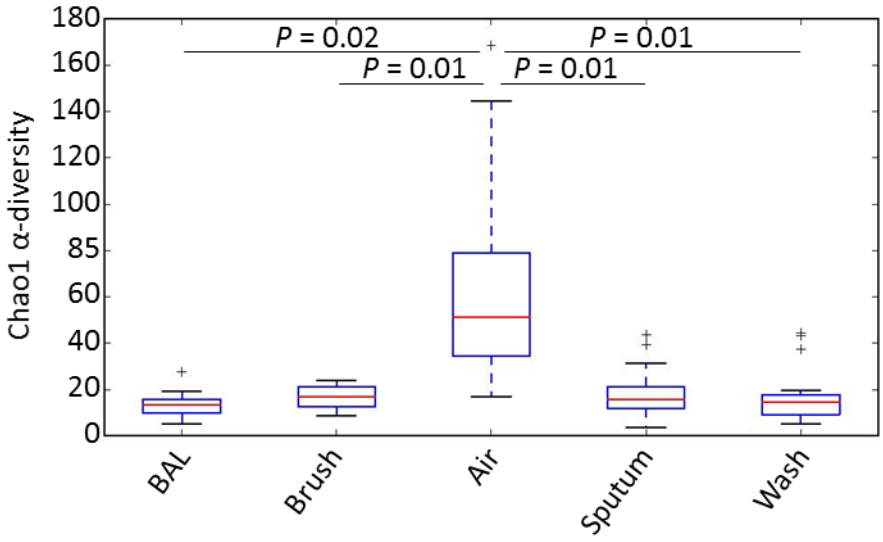


Fig. 3. 21: Box plot of α-diversity index (Chao1) from samples obtained on days when outdoor air was analysed, BAL (n = 18), brush (n = 13), air (n = 45), sputum (n = 31) and wash (n = 18). Box plots show mean and standard deviations of the groups' alpha diversities.

The number of species shared by 50% of air samples was higher compared to those shared by 25% of any other sample type (Fig. 3. 22). Eighty-seven taxa, encompassing 47 identifiable species were shared between 25% of air samples (Fig. 3. 22). Thirty-two of these species were unique to the air. In contrast, clinical samples had 20 unique

species. Air samples shared 15 taxa with at least one of the clinical groups. The most prevalent fungi in air samples were *M. tassiana* (100%), *Cladosporium sphaerospermum* (93.33%) and the *Agaricales* genus (84.44%).

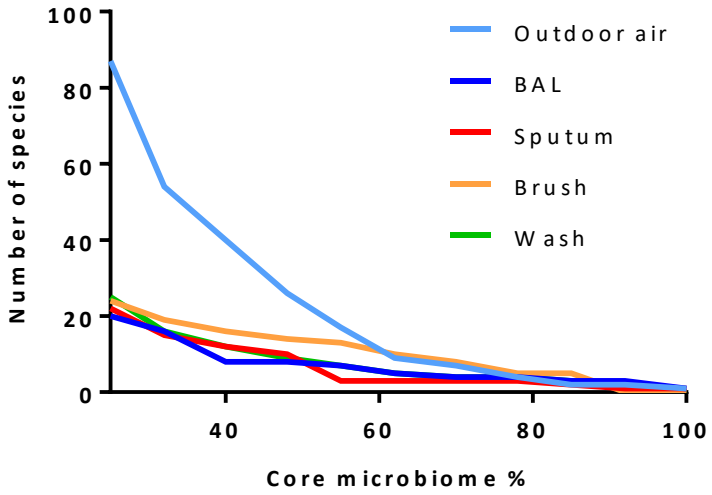
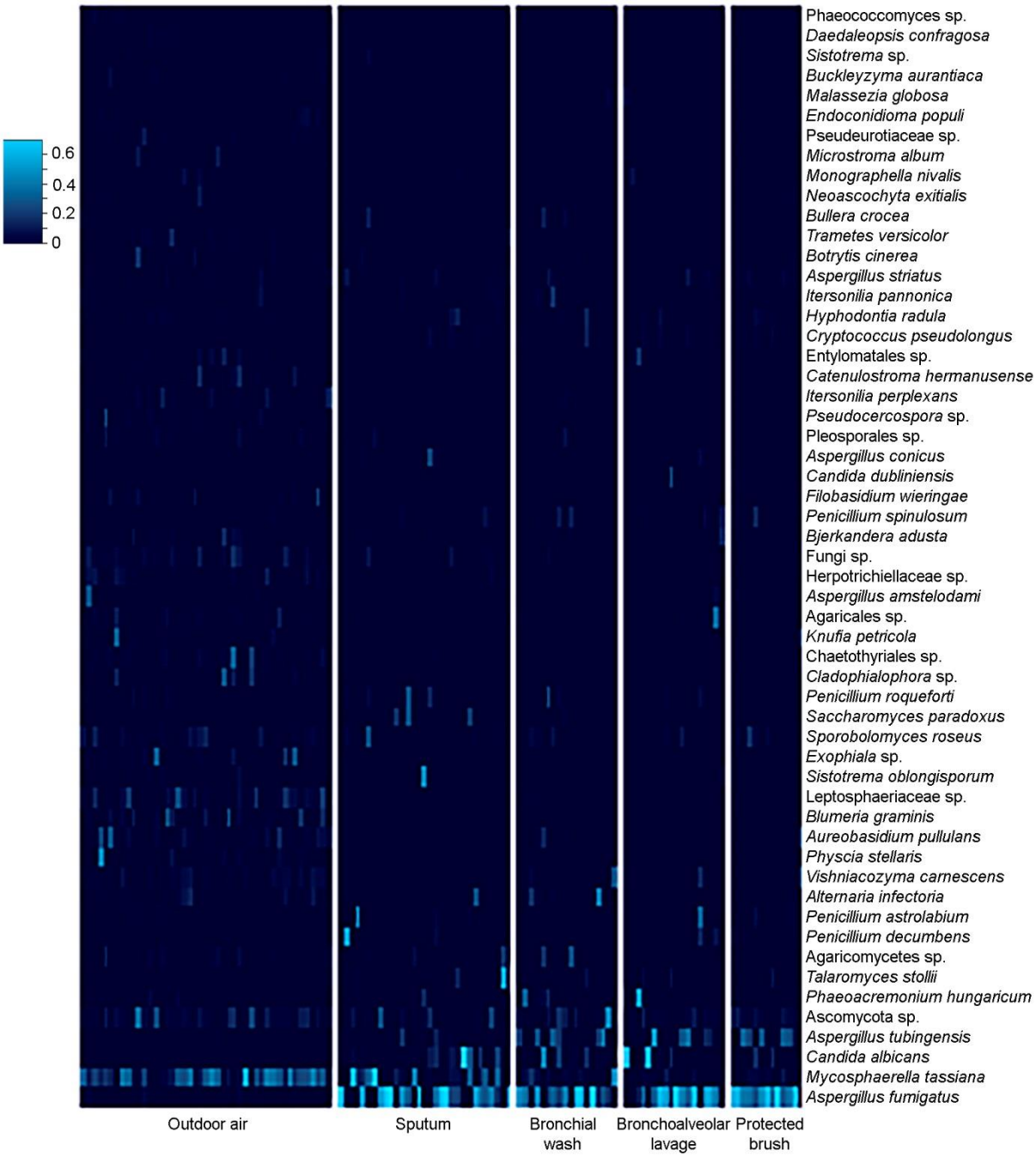


Fig. 3. 22: The core microbiome in air samples (n = 45) and matching BAL (n = 18), brush (n = 13), sputum (n = 31) and wash (n = 18) samples, shown as the number of taxa shared between 25% and 100% of samples.

M. tassiana was not only the most prevalent, but also the most abundant (mean 22.4%, range 0.02 to 72.0%) species. Its abundance was significantly higher in the air compared to the clinical samples ($\chi 2 = 35.50$, $P < 0.0001$, FDR-$P < 0.0001$). The core mycobiome using a 0.1% OTU frequency cut-off across all sample types air and matching sputum, BAL, brush and wash encompassed 20, 22, 20, 24 and 25 taxa, respectively (Fig. 3. 23 A) and using at least 1% average abundance in each sample type 20, 12, 12, 11 and 12 taxa, respectively (Fig. 3. 23 B). In total, significant differences in the OTU frequencies between air and clinical samples were detected for 18 taxa, including 13 known species, which remained significant after correcting for multiple samples. *A. fumigatus* ($\chi 2 = 53.50$, $P < 0.0001$, FDR-$P < 0.0001$), *C. albicans* ($\chi 2 = 45.20$, $P < 0.0001$, FDR-$P < 0.0001$), *A. tubingensis* ($\chi 2 = 41.0$, $P < 0.0001$, FDR-$P < 0.0001$), and *C. pseudolongus* ($\chi 2 = 45.0$, $P < 0.0001$, FDR-$P < 0.0001$) were significantly more present in clinical samples than air (Fig. 3. 23). The abundance of the species in air as well as matching clinical samples were highly variable between days and between subjects (Fig. 3. 23 A).

A


0.6
0.4
0.2
0
Phaeococcomyces sp.
Daedaleopsis confragosa
Sistotrema sp.
Buckleyzyma aurantiaca
Malassezia globosa
Endoconidioma populi
Pseudeurotiaceae sp.
Microstroma album
Monographella nivalis
Neoascochyta exitialis
Bullera crocea
Trametes versicolor
Botrytis cinerea
Aspergillus striatus
Itersonilia pannonica
Hyphodontia radula
Cryptococcus pseudolongus
Entylomatales sp.
Catenulostroma hermanusense
Itersonilia perplexans
Pseudocercospora sp.
Pleosporales sp.
Aspergillus conicus
Candida dubliniensis
Filobasidium wieringae
Penicillium spinulosum
Bjerkandera adusta
Fungi sp.
Herpotrichiellaceae sp.
Aspergillus amstelodami
Agaricales sp.
Knufia petricola
Chaetothyriales sp.
Cladophialophora sp.
Penicillium roqueforti
Saccharomyces paradoxus
Sporobolomyces roseus
Exophiala sp.
Sistotrema oblongisporum
Leptosphaeriaceae sp.
Blumeria graminis
Aureobasidium pullulans
Physcia stellaris
Vishniacozyma carnescens
Alternaria infectoria
Penicillium astrolabium
Penicillium decumbens
Agaricomycetes sp.
Talaromyces stollii
Phaeoacremonium hungaricum
Ascomycota sp.
Aspergillus tubingensis
Candida albicans
Mycosphaerella tassiana
Aspergillus fumigatus
Outdoor air
Sputum
Bronchial wash
Bronchoalveolar lavage
Protected brush

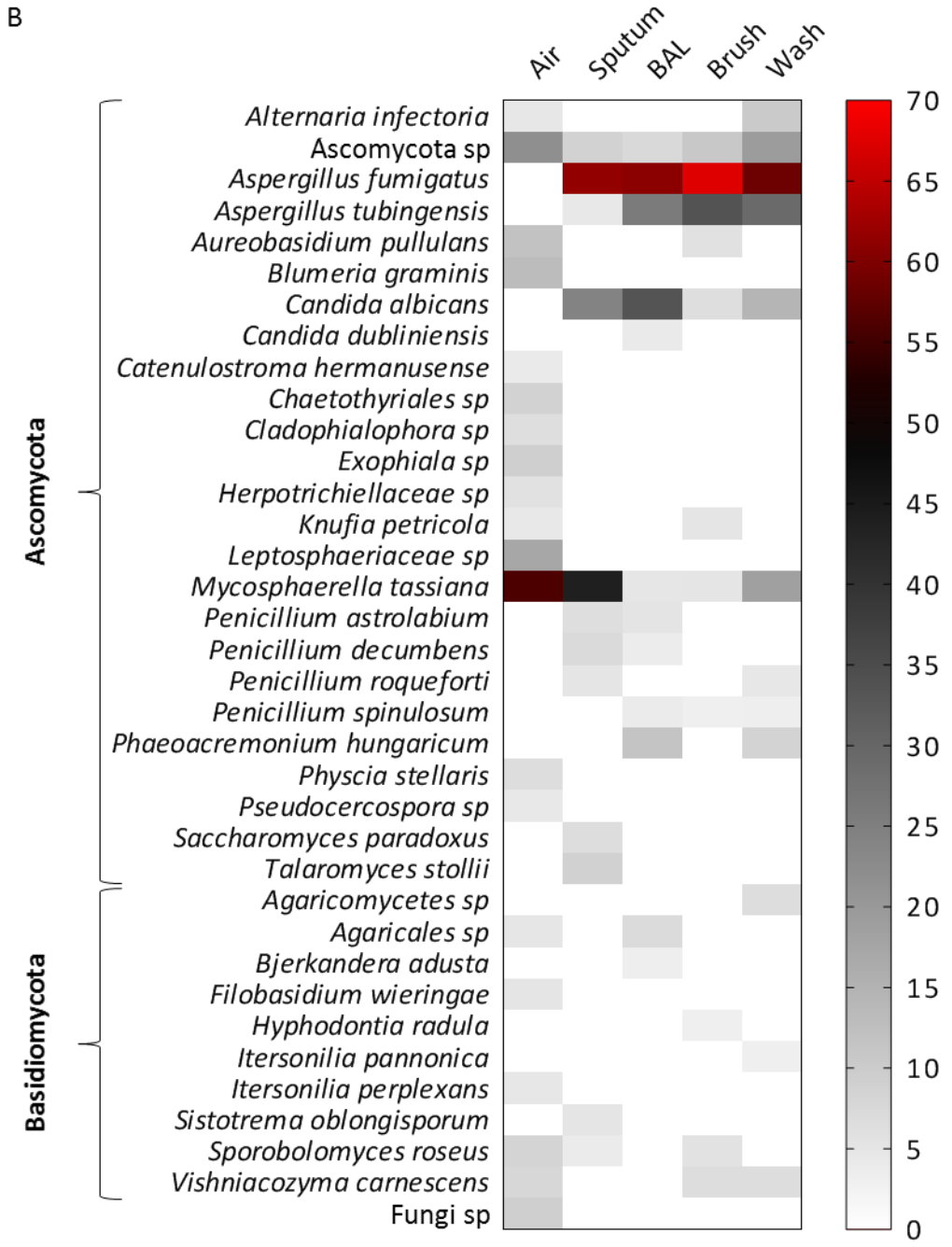

B
Air
Sputum
BAL
Brush
Wash
Ascomycota
Basidiomycota
Alternaria infectoria
Ascomycota sp
Aspergillus fumigatus
Aspergillus tubingensis
Aureobasidium pullulans
Blumeria graminis
Candida albicans
Candida dubliniensis
Catenulostroma hermanusense
Chaetothyriales sp
Cladophialophora sp
Exophiala sp
Herpotrichiellaceae sp
Knufia petricola
Leptosphaeriaceae sp
Mycosphaerella tassiana
Penicillium astrolabium
Penicillium decumbens
Penicillium roqueforti
Penicillium spinulosum
Phaeoacremonium hungaricum
Physcia stellaris
Pseudocercospora sp
Saccharomyces paradoxus
Talaromyces stollii
Agaricomycetes sp
Agaricales sp
Bjerkandera adusta
Filobasidium wieringae
Hyphodontia radula
Itersonilia pannonica
Itersonilia perplexans
Sistotrema oblongisporum
Sporobolomyces roseus
Vishniacozyma carnescens
Fungi sp
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0

Fig. 3. 23: Heat maps illustrating the distribution of species based on relative abundances using a prevalence of 25% for each sample within the sample groups comprised of bronchoalveolar lavage (BAL) (n = 18), bronchial wash (n = 18), protected brush samples (n = 13), sputum samples (n = 31) and air samples (n = 45). (A) A 0.1% OTU frequency cut-off was used across all samples. The data were transformed using ln(x+1) due to zeros in the dataset. The relative abundance is indicated by colour, with the lighter shades representing the more abundant sequences. (B) At least 1% average abundance within a sample type and 0.1% OTU frequency cut-off across all samples. The relative abundance is indicated by colour, with darker shades representing the higher frequencies, red frequencies 55% up to 70% abundance.

3.3.7 Clinical significance of the most predominant fungi detected

It was hypothesised that for a fungal species to be influencing clinical outcomes in asthma it should be i) more prevalent in asthma than healthy subjects, and ii) be present in the lower airways. Only fungi found in the core mycobiome of people with asthma, but not in the core mycobiome of healthy controls (Fig. 3. 4 B) that were also found in the core mycobiome from bronchoscopy samples (Fig. 3. 18 B) were considered. *Alternaria infectoria*, *Aspergillus striatus*, *A. tubingensis*, *C. pseudolongus*, *Hyphodontia radula* and *Penicillum spinulosum* (detected in BAL, brush and wash), *C. dubliniensis* (brushes) and *M. globosa* (wash) matched these criteria. Only *C. dubliniensis* was significantly more prevalent in asthma than healthy controls (34% versus 0%, $P = 0.009$), particularly in fungal-sensitised people with asthma (40%) compared to non-fungal sensitised subjects with asthma (23%). Significant relationships between six of the eight fungi mentioned above and clinical features were detected (Table 3. 20).

Table 3. 20: Significant relationships between members of the mycobiota and clinical characteristics. Some variables were assessed across all subjects before being restricted to people with asthma, whilst asthma specific variables were only assessed against people with asthma.

Category	n	Fungus	Test-statistic	*P*-value	Adjusted *P*-value
All subjects					
FEV1	92	*Malassezia globosa*	$r^2 = -0.213$	**0.041**	0.282
Smoking status	92	*Malassezia globosa*	$\chi^2 = 8.593$	**0.014**	0.114
Sputum neutrophil (%)	62	*Aspergillus tubingensis*	$r^2 = -0.369$	**0.003**	**0.019**
		Cryptococcus pseudolongus	$r^2 = -0.339$	**0.007**	**0.021**
		Penicillium spinulosum	$r^2 = -0.279$	**0.028**	0.058
Total IgE value	90	*Penicillium spinulosum*	$r^2 = -0.256$	**0.015**	0.124
		Hyphodontia radula	$r^2 = 0.212$	**0.045**	0.188

Asthma only					
Asthma duration	78	*Aspergillus tubingensis*	r^2 = -0.301	**0.007**	0.060
Bronchiectasis	77	*Malassezia globosa*	U = 494	**0.045**	0.187
FeNO ppb	65	*Penicillium spinulosum*	r^2 = -0.292	**0.018**	0.151
GINA class	79	*Malassezia globosa*	χ^2 = 6.606	**0.037**	0.309
Smoking status	79	*Malassezia globosa*	χ^2 = 9.444	**0.009**	0.075
Sputum neutrophil (%)	54	*Aspergillus tubingensis*	r^2 = -0.363	**0.007**	**0.019**
		Cryptococcus pseudolongus	r^2 = -0.347	**0.010**	**0.021**
		Aspergillus striatus	r^2 = -0.281	**0.039**	0.161
Total IgE value	79	*Penicillium spinulosum*	r^2 = -0.243	**0.030**	0.255

Definitions of abbreviations: n = number of subjects; FEV_1 = Forced expiratory volume during the first second; FeNO ppb = Fractional exhaled nitric oxide, parts per billion; IgE = Immunoglobulin E.

Spearman's Rho (r_s), *P*-values and corrected *P*-value (corrected by the Benjamini-Hochberg FDR procedure for multiple comparisons [291]) were calculated for all taxa with a total OTU observation cutoff of 1%. Taxa that could not be identified to species level and species with a prevalence of less than 25% of sputum samples are excluded. Only species with significant *P*-value's ($P < 0.05$, shown in bold) or *P*-value approaching significance ($P < 0.08$) are shown.

P values were calculated with the Mann-Whitney U test or Kruskal-Wallis test for comparison of categorical variables, with *P*-values corrected by the Benjamini-Hochberg FDR procedure for multiple comparisons [291]. Spearman's rho correlation was used for continuous variables, with *P*-values corrected using the procedure of Benjamini, Krieger and Yekutieli with a desired false-discovery rate Q-value of 5% [292]. Significant *P*-value's ($P < 0.05$) are given in bold.

No significant associations were determined between any of the eight fungi and blood eosinophil count, sputum eosinophil percentage, oral steroid usage or inhaled steroid dose. The taxa *A. tubingensis*, *C. pseudolongus*, *P. spinulosum* and *A. striatus* were negatively associated with the sputum neutrophil percentage: This relationship was only significant for *P. spinulosum* when healthy controls were considered in addition to people with asthma (r2 = -0.279, *P* = 0.028) and for *A. striatus* when healthy controls were excluded (r2 = -0.281, *P* = 0.039). Only *A. tubingensis* (with healthy controls r2 = -0.369, *P* = 0.003, adjusted *P* = 0.019; without controls r2 = -0.363, *P* = 0.007, adjusted *P*

= 0.019) and *C. pseudolongus* (with healthy controls r2 = -0.339, *P* = 0.007, adjusted *P* = 0.021; without controls r2 = -0.347, *P* = 0.010, adjusted *P* = 0.021) maintained the significant relationship after allowing for multiple testing. *A. tubingensis* was also negatively associated with asthma duration (r^2 = -0.301, *P* = 0.007, adjusted *P* = 0.060).

3.4 Discussion

In this study the lung mycobiome in people with asthma and in healthy controls was investigated using HTS. The role of fungi in asthma was described in previous studies. Fungal exposure is considered as a risk factor for asthma development in both childhood and adulthood. High indoor exposure was even considered as a cause for childhood asthma development recently [116]. Sensitisation to fungi is more prevalent in people with asthma, in particular with severe asthma [42, 115, 169]. The relationship between asthma and fungal sensitisation was defined as a distinct endotype of asthma [74], which was associated with impaired lung function and risk to develop lung damage [23, 40, 134, 184]. The main method of fungal identification is to culture respiratory samples [41, 137], which is suggestive of colonisation of the lower airways causing a continuous exposure to fungal allergens [172]. The main problem with this approach is that not all fungi can be identified by culture as they could be overgrown by other fungi [217] or do not readily grow *in vitro* [20]. This is why the present study used HTS to obtain a more comprehensive overview of the fungal species present in the lungs from people with asthma. To our knowledge, this study is the largest study exploring the mycobiome in asthma to date. For this study people with asthma were recruited and assigned to different groups based on their sensitisation to fungi, and healthy controls. The demographics for sex, age and smoking habits between the groups were well matched. Sputum samples and bronchoscopy derived samples, which were obtained from a subset of subjects, contained both shared and unique fungal species. The presence and number of fungal taxa was dependent on the health status of the individual and the sample examined. Although a high inter-sample diversity was detected for the less prevalent or abundant species, some of the more predominant fungi highlighted differences of the mycobiomes between the three groups or the different sample types collected. A comparison to other asthma studies investigating the lung mycobiome is limited as only two other studies have been published [231, 236], of which one discriminated between subjects with asthma based on their fungal sensitisation [231]. The other study from van Woerden *et al.* [236] is not included in the discussion for this thesis. Their way of sample processing risked contamination by environmental fungi and may have missed important fungi as only a fraction of the pooled samples was sequenced.

3.4.1 Fungi in health and disease

The hypothesis was that the airway mycobiome composition would be different between asthma and healthy subjects and between asthmatics with and without fungal sensitisation. People with asthma had an eosinophilic phenotype as indicated by their significantly decreased lung function, and higher blood and sputum eosinophil levels compared to healthy controls [57]. The study recruited mostly people with asthma who were on the highest asthma treatment levels GINA4 and GINA5. Fungal sensitisation was assessed using SPT and ImmunoCAP. The IgE test was more sensitive than the SPT test, which agrees with O'Driscoll *et al.* [131] and was given priority in case of discordance between the two tests. The concordance among positive results for all five fungi tested were higher compared to previous studies [127, 131, 293], but lower than those known for common allergens [130]. These findings might be the result of differences between fungal IgE test and SPT extracts used for diagnosis, the fact that extracts are not standardized, unreproducible extract compositions, and differences between companies [26, 144, 294]. Although people with asthma sensitised to fungi were specifically recruited for this study, the proportion of subjects sensitised to more than one fungus (74%) was similar to previous studies with a randomized selection of asthmatics [115, 131, 170].

Subjects showed strong inter-individual differences (Fig. 3. 5), which has also been observed in other mycobiome studies [234, 237]. The mycobiomes were predominated by a few species, *C. albicans*, *M. tassiana* and *A. fumigatus*. *M. tassiana* was significantly more abundant in healthy controls compared to subjects with asthma. This species, formerly known as *Davidiella tassiana*, is the sexual form of *C. herbarum*. As sequencing of the ITS region is not discriminative enough for the *herbarum* complex [295], it can be assumed that *M. tassiana* represents a number of *Cladosporium* species. These fungi are mesophilic and commonly found in the air [98], which **suggests that the mycobiome in healthy individuals is significantly influenced by inhaled fungal spores**. This supports observations by a previous mycobiome study [230]. The commensal and opportunistic pathogen *C. albicans* was not associated with health status or any clinical parameter. This was supported by the results of the *C. albicans*-specific qPCR between subjects with asthma with or without fungal sensitisation. Similar findings were reported by Fraczek

et al. [231], although this fungus was detected at lower levels compared to the present study. Interestingly, another *Candida* species, *C. dubliniensis*, was significantly more abundant and had a higher adjusted abundance in people with asthma, in particularly fungal sensitised subjects, compared to the other groups. It was initially identified in the mouth of HIV+ subjects and to a much lower extent in healthy subjects [296]. Since then, it was only detected from mucosal surfaces [233, 296-299]. It is the second most common *Candida* species after *C. albicans* using culture or mycobiome analysis in CF patients [233, 299]. To our knowledge, this study is the first to demonstrate its presence in asthmatics. This species is less virulent than its relative *C. albicans*, but expresses higher levels of aspartyl protease, which plays a role during active infection, has a stronger adhesion to the mucosa and more readily develops resistance to fluconazole [298, 300-303]. **It can be assumed that *C. dubliniensis* is colonising the lung of people with asthma. This finding was also consistent with the hypothesis, showing that there are marked differences in the mycobiome among asthma patients.** The role of *C. dubliniensis* in asthma pathogenesis still has to be clarified. It is not known whether subjects with asthma have allergic responses to this yeast, but IgE cross-reactions to its relative *C. albicans* are likely where IgE sensitisation is relatively common.

The results for *A. fumigatus* were unexpected as this fungus was frequently cultured from *A. fumigatus*-sensitised people with asthma in previous studies [41, 137] and is associated with lung damage in sensitised asthmatics [23, 184]. Although filamentous fungi were exclusively recovered from people with asthma by culture, the culture data were limited as discussed later (section 3.4.6). In contrast to this study, Fraczek *et al.* [231] showed a significantly higher load of the *A. fumigatus* complex in asthma and allergic fungal disease, particularly in relation to corticosteroid therapy, supporting previous culture-based studies [41, 137]. It can only be speculated why this association was not shown in this study. This may be important as an increased fungal load has been associated with higher corticosteroid doses [231], which can impair fungal phagocytosis by immune cells located in the airways [304]. It is likely that a higher number of participants would have allowed for more statistical analysis accounting for asthma treatment.

3.4.2 Determination of the fungal load

One of the aims in this study was to determine the fungal load because the sequence abundances resulting from HTS are only a semi-quantitative measure of the fungi present in a sample at best [305]. Both universal and species-specific quantification were done. While species-specific qPCR is considered to be more efficient and sensitive than a universal quantification, the latter can be used to determine absolute quantities (HTS relative abundance adjusted using universal qPCR) [306]. The limitations of taxon-specific primers are that they exist only for a limited number of fungi and that DNA samples would be frequently used, encouraging damage by multiple freeze-thaw cycles [307]. Using universal primers for the qPCR coupled with HTS enables quantification for a broader range of fungi equal to the range obtained by the HTS. The FungiQuant primers used in this study have previously been used with both TaqMan and SYBR green based assays, promising successful amplification of a range of fungi, including rare species, with a low chance of human contamination and a low false negative rate [232, 276]. They amplify the ribosomal small subunit (SSU) gene, which could have resulted in amplification bias as the SSU can have variable lengths [222, 308]. The universal qPCR had a high amplification efficiency (90.5%). **The overall fungal load as determined by qPCR were not significantly different between the three groups. The results showed an unexpectedly higher fungal load in healthy controls compared to subjects with asthma.** A significant correlation was observed between HTS adjusted abundances calculated from the universal qPCR quantities and quantities determined by species-specific qPCR, which was also observed previously [232]. The quantities determined by universal qPCR also significantly correlated with the total number of cfus counted from sputum culture. **The fungal quantities in air samples did not correlate with spore counts from the respective days.** This was unexpected as our group previously demonstrated a high correlation between species-specific qPCR from air traps and equivalent spore counts for that day [309], and also between spore counts and relative abundances generated using a clone-sequencing approach [98]. This shows that i) differences between the spore traps used for counting and DNA extraction do not account for differences between the quantified DNA and counted spores observed in the present study; and ii) a correlation between the quantities determined by universal

qPCR and number of counted spores would have been expected. This should be addressed in future studies using these primers.

The species-specific qPCR had limited sample numbers. It did not show any differences between the two asthma subgroups. **The *C. albicans*-specific qPCR significantly correlated with the number of total yeasts and *C. albicans* in particular cultured from sputum samples.** A comparison between *A. fumigatus*-specific qPCR and culture was not possible due to the limited culture data (section 3.4.6). In addition, the amplification efficiency for *A. fumigatus* or *A. niger* were not as high as previously reported [310]. Similar efficiency values were obtained for *A. fumigatus* in our group, but not for *A. niger* using the same strains [311]. However, the values were still within the range of those reported for *Aspergillus* species [310]. The amplification efficiency of *C. albicans* was similar to those obtained in the literature [312].

The aim to determine the fungal load was fulfilled, though it remains to be elucidated why the quantities from air samples did not correlate with the matching spore counts. The data obtained from both universal and species-specific qPCR showed that the absolute quantities and species-specific quantities correlated well, despite the limitations of each method. **The data implied that the fungal loads did not differ depending on the health status.**

3.4.3 Mycobiome differs depending on the sample type

It was hypothesised, that the mycobiome composition would depend on the location of sample acquisition from the airway. A subset of patients underwent bronchoscopy for this purpose. To our knowledge, bronchial wash and protected brush samples have not been used in other mycobiome studies. **Interestingly, *C. albicans* and *A. fumigatus* remained the most prevalent and abundant species irrespective of the sample type or health status.** As *C. albicans* is a known commensal on skin and mucosal epithelium [31, 32], it can be assumed that it is a commensal in the lower airways, which has not been suggested previously. The core mycobiome identified in the bronchoscopy samples shared at least twelve species with matching sputum samples (Fig. 3. 18). Some species were unique to bronchoscopy samples, including the skin pathogen *M. globosa* or

Aspergillus flavus. These findings support previous observations, that the risk of contamination by the introduction of the bronchoscope from the oropharynx is rather low [230, 238, 313]. The shared species included *A. tubingensis* and *C. pseudolongus*, which were significantly more prevalent and abundant in bronchoscopy-derived samples, in particular protected brush samples obtained directly from the bronchial epithelium (Fig. 3. 19, Table 3. 14, Table 3. 15). In addition, *A. tubingensis* and *C. pseudolongus* were more abundant in people with asthma using all four sample types. *C. pseudolongus* was also more abundant when only the bronchoscopy samples were considered (Table 3. 13). These species were associated with decreased sputum neutrophil counts (Table 3. 20). Neutrophils are crucial for the primary immune response to fungi [23, 314]. Studies in mice and *in vitro* studies with human cells showed that neutrophils play a significant role in clearing resting or germinating conidia and hyphae [315-319]. It was also suggested that they may contribute to a break in tolerance to harmless antigens through the digestion of resting conidia and subsequent induction of an inflammatory response [320]. No virulence factors for *C. pseudolongus* have been reported. This fungus has previously been detected as part of the anal mycobiome [321]. *C. pseudolongus* belongs to the *Cryptococcus humicola* complex [322], which can be a rare cause of non-*neoformans* cryptococcal infection [323, 324]. Another relative, *Cryptococcus neoformans*, is involved in pulmonary infections and has immune defence mechanisms such as a dormancy state and ability to induce a Th2-type response [324]}. These properties might be present in *C. pseudolongus*. *Cryptococcus* species were also detected in BAL samples of subjects with other respiratory conditions [237], but their clinical implication is unknown. Both *A. fumigatus* and *A. niger*, which belongs to the same section as *A. tubingensis*, were shown to secrete the polysaccharide galactosaminogalactan [325], which among other things, can trigger neutrophil apoptosis [326, 327] and promotes fungal resistance to neutrophil killing by NADPH-oxidase dependent extracellular traps [325]. It is therefore possible that *A. tubingensis* has this ability. In addition, *A. tubingensis* might express toxins previously detected in *A. fumigatus* that were shown to impact neutrophil functions [328]. *A. tubingensis* was negatively associated with asthma duration (Table 3. 20). **Fungal sensitised people with asthma showed a significantly longer duration of asthma and earlier asthma onset compared to non-fungal sensitised patients** (Table 3. 4). This supports the observation,

that fungal sensitisation is particularly found in early-onset atopic eosinophilic asthma [23]. *A. tubingensis* and *A. niger* are more frequently detected in respiratory samples from subjects with chronic respiratory diseases [137, 175, 329], where they might be involved in exacerbations [329]. In addition, the incidence of antifungal triazole drug resistance is higher in *A. tubingensis* compared to *A. fumigatus* [329].

These findings confirmed that unique species can be identified in bronchoscopy-derived samples and showed the importance of bronchoscopy samples as opposed to sputum samples with respect to *A. tubingensis* and *C. pseudolongus*. Although they were shared by bronchoscopy and sputum samples, they predominated in the bronchoscopy-derived samples, in particular brush samples, suggesting that some potentially clinically relevant species might be missed using sputum alone and therefore consistent with the hypothesis. **It would be recommended that subjects provide sputum and undergo bronchoscopy because species from either sample collection method were identified, which might have a potentially crucial role in asthma yet to be determined.**

3.4.4 Influence of contamination from reagents, the aerobiome and oral mycobiome

3.4.4.1 Contaminants

It is important to establish if the sequencing results were affected by contamination from laboratory reagents. The sample preparation for HTS is prone to contamination due to multiple processing steps [225] and reagents like those used for DNA extraction [248]. All of these factors could be mistaken as low abundant species and influence relative abundances. In the present study all rare OTUs were therefore excluded as part of the quality control filtering before analysis began. Saline used during bronchoscopy and reagents such as Tween 80, buffers from the DNA extraction kit and nested PCR sample preparation were included in the analysis. Contamination based on the buffers and solutions could be excluded due to the fact that not all samples were affected where the control showed a highly abundant taxa and the inconsistent number of sequences in the supposedly affected samples (examples presented in Table 3. 9). Although HTS is a very sensitive technique, it is not common that other studies account for contaminations acquired during sample processing [231, 237], risking

misinterpretations of their results. **This study showed that the risk of contamination was negligible with the sample and data processing methods used.**

3.4.4.2 Air

Another aim of this study was to assess the aerobiome, defined as the mycobiome detected in air samples, to assess the influence of inhaled fungi to the mycobiome found in the clinical samples. This depicts a major advantage compared to other airway mycobiome studies, which have not investigated this question. The airways are constantly exposed to the air and the fungal particles in it [26, 98], which could comprise a major part of the mycobiome, influencing relative abundances and leading to misinterpretations. The air samples in this study matched days when sputum or bronchoscopy-derived samples were provided. Similar to sputum samples, air samples showed inter-sample variation (Fig. 3. 23), which can be expected as the fungal spore concentration in the air is affected by daily and seasonal fluctuations [9, 330]. The aerobiome data showed that all clinically relevant species, *A. fumigatus*, *C. albicans*, *A. tubingensis* and *C. pseudolongus*, were significantly less abundant in air compared to clinical samples. Conversely, the sputa of healthy subjects were predominated by mesophilic fungi, likely to be represented by several *Cladosporium* species, which also dominated the air samples. The aim of this control was fulfilled, indicating that these **clinically relevant species likely represent colonising fungi rather than inhaled spores or conidia**.

3.4.4.3 Oral mycobiome

As sputum samples are coughed up from the lower airways [262], a contamination by the oropharyngeal mycobiome is possible. The risk was reduced in this study by using sputum plugs, which are dense accumulations of cells from the bronchial mucosa and contain less saliva-derived cells such as squamous epithelial cells and microbes from the oropharynx [244, 254, 262, 263]. The aim was to compare the mycobiomes from mouthwash samples to matching sputum samples, however, there was only data for ten samples. Differences between mouthwash and either sputum or BAL samples has been

demonstrated in a few studies and shown both shared and unique species between these samples [230, 237, 238]. A similar pattern was observed in this study, where species such as *C. tropicalis* and *S. paradoxus* were only found in mouthwash samples, or *P. spinulosum* was unique to the core mycobiome of sputum samples. Both sample types contained high abundances of *Candida* species; their predominance in the oral cavity has frequently been reported in oral mycobiome studies [207, 230, 243, 331]. It cannot be excluded that the amount of ***C. albicans*** in sputum samples was influenced by its high relative abundances in the oropharynx, however, it is also possible that this fungus migrated from the upper to the lower airways and could survive in both habitats. As this fungus **predominated in all the respiratory samples collected in this study, it is likely that it represents a commensal in both the upper and the lower airways independent of the health status of the subject.** Further conclusions from the mouthwash samples cannot be drawn due to the limited number of samples. These samples were collected from fungal sensitised people with asthma and might not have been as representative of that group because the species richness estimate in the sputum samples was much lower from this subset of samples compared to all subjects from this group (Fig. 3. 3, Fig. 3. 11). This means that the comparison between this control and sputum samples could not be covered sufficiently and future studies should account for possible contaminations by the oropharynx in more depth.

3.4.5 Proportion of live and dead fungi in sputum

It is possible that many of the sequences detected derived from dead fungi [113, 332]. Only live cells are detected by culture, but for molecular analyses substances like PMA and ethidium monoazide (EMA) are needed [333]. Both substances can permeate through compromised cell membranes of dead cells and intercalate into the DNA during photolysis, which prevents its amplification [267]. PMA has been successfully applied in bacterial [334, 335] and fungal [235] microbiome studies, however, in comparison to bacterial and fungal culture-based studies, or the bacterial microbiome studies mention above, Nguyen *et al*. [235] used a much stronger concentration of PMA (50 mM compared to < 100 µM) [249, 269, 336-338]. This could be problematic as it was previously shown that high PMA concentrations affect live cells [337]. This highlights the

major limitation of fungal PMA-based studies. No recommendations exist with respect to PMA concentration and incubation conditions, which should be addressed by future studies. To date, most authors optimised these factors for specific species using culture or food samples. The conditions vary highly depending on the study [268, 269, 336, 337, 339, 340]. In this study, a small number of sputum samples was halved after homogenisation, where one half was treated with PMA before DNA extraction following recommendations for a range of fungi [268, 269]. The aim of this experiment was to show the proportion of dead cells in the mycobiome. The species richness estimates were not significantly different between PMA-treated and untreated samples. Changes in relative abundances for a number of species in each sample could be observed (Fig. 3. 15), however, the sample number was quite small so that no reliable statistical analysis was possible. The total number of taxa changed in 4/5 samples. It was increased in two samples treated with PMA compared to the matching untreated samples. This can be expected as the proportion of live cells increases once the dead cells were treated with PMA. Despite the use of a much higher PMA concentration, similar observations were made by Nguyen *et al.* [235]. It is possible that taxa showing zero relative abundance in the non-treated sample might represent a rare fraction of the mycobiome, which could be determined by species-specific qPCR. The quantity for *A. fumigatus* could not be measured in this study, which may be explained by the low sequence abundances in the respective samples. Interestingly, the species-specific qPCR showed an increase of *C. albicans* quantities for two PMA-treated samples compared to the matched untreated samples. This was unexpected as the quantities of certain species should either remain the same or decrease if PMA would be applied, however, insufficient homogenisation and splitting of the sputum samples before PMA-treatment could be a reason for that. **It was demonstrated that PMA treatment had an effect on the mycobiome composition and quantities in sputum samples**, fulfilling the aim of this experiment, however, future studies should include higher sample numbers to assess statistical differences.

3.4.6 Sequencing compared to culture results

It was hypothesised that HTS would provide a more comprehensive understanding of the fungi in the lung compared to culture methods. Culture is the traditional approach to identify fungi from respiratory samples, however, the recommended processing of these samples by the UK Health Protection Agency standard procedure BSOP57 [341] frequently underestimates the number and type of fungi present [254, 342]. It was recommended to use undiluted samples for culture [254, 342, 343] and to inoculate sputum plugs separated from saliva samples to avoid contamination by salivary squamous cells and microbes in the oropharynx [254, 262, 263]. The method has been successfully applied in other respiratory studies [41, 137], but had to be adapted for the needs of the present study by diluting samples for both culture and DNA extraction from sputum and bronchoscopy-derived samples. This would explain why the culture rates obtained were lower compared to these studies. Culture was less sensitive than the HTS approach, which identified overwhelmingly more species compared to those recovered from medium. The higher sensitivity of sequencing methods was previously demonstrated, particularly with respect to non-culturable fungi [207, 218, 344], which were also detected in the present study (e.g. *Malasseziales* which requires specialised media to culture). As mentioned earlier (section 3.4.1), filamentous fungi were exclusively cultured from people with asthma, but not healthy controls. Culture rates in total were lower for healthy controls (54%) compared to people with asthma (76%). Other studies have also shown significantly lower culture rates from healthy controls [41, 137]. A possible reason might be that sputum plugs, which contain a high burden of cellular material, are not necessarily present in the respiratory secretions from healthy individuals, whose samples often show low viscosity [72]. It is also possible that these subjects might have a higher proportion of so-called viable but non-culturable fungi [345] or that it has to do with their better health status compared to subjects with asthma. An indication that culture status can be transient over time was provided by repetitive sputum samples and has been found in other respiratory mycobiome studies [218, 233]. **Despite the low culture rates, culture data were representative of the sequence abundance data with respect to certain fungi.** The yeast culture-positive results were significantly related to the sequence abundance of *C. albicans* in the sputum mycobiome data. A similar relationship was also present yet not significant for

A. fumigatus. Interestingly, *A. fumigatus* culture-positive samples were significantly associated with the sequence abundance of *A. tubingensis* even after correction for multiple samples.

Bronchoscopy-derived samples were diluted more than sputum samples so that samples could be plated onto three different types of media. This was reflected by significantly lower culture rates obtained from bronchoscopy-derived samples cultured on PGCF medium compared to sputum or mouthwash samples. The aim of this method was to promote growth of fungi in general using PGCF, yeasts in particular using YMA or slow-growing fungi that are rarely detected on other media using ScedSel+ [256, 257]. To our knowledge, no comparable studies exist with respect to the media used in this study. The aim was not fulfilled as no significant advantage of YMA or ScedSel+ media was detected compared to PGCF. **It would be recommended to use only PGCF as the medium of choice for future comparison studies as a good fungal recovery from this medium was demonstrated in a few studies** [41, 137, 254]. In addition, it could be advantageous to use a higher centrifugation speed to separate the bronchoscopy-derived samples [342, 346]. Bronchoscopy samples showed a similar relationship between *C. albicans* and *A. fumigatus* culture-positive samples and their respective sequences abundances as sputum samples.

Another aim was to identify yeasts from a subset of samples. Yeasts represented the majority of fungi recovered by culture. They were identified from a subset of samples using CHROMagar [270, 271] and colony PCR sequencing [259]. From the seven species identified from sputum samples, *C. albicans* was the most predominant fungus (80%), which also dominated the few available bronchoscopy-derived samples (100%) and mouthwash samples (~80%). Although the identification by CHROMagar is subjective, it is a good and cost-effective standard method to discriminate between most of the dominant yeast species, including *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. dubliniensis* [270, 271, 297]. Ambiguous isolates were identified by colony PCR sequencing, which is a quick way to identify yeasts from fungal culture by PCR and sequencing without performing a DNA-extraction step. It is possible that some species were missed, particularly from plates with > 100 colonies. The risk was reduced by the random selection of colonies, in which discrepancies between colony morphologies

were taken into account as these might have represented distinctive species. The aim of this experiment was fulfilled as up to six other species could be identified.

The hypothesis could be confirmed as HTS yielded a plethora of results, which could not be detected by culture, although it has to be acknowledged that the recovery by culture was limited, most likely due to the dilution of the sample for the purpose of this study. Despite this limitation, a significant relationship between yeast culture data and *C. albicans* sequence abundances could be detected in sputum samples. Although yeast identification was only done for approximately 60% of the yeast culture-positive samples, the predominance of *C. albicans* in these samples supported this significant relationship.

3.4.7 Limitations of molecular techniques

As mentioned earlier, a higher number of participants would have allowed for more statistical analysis accounting for asthma medication, but also for other factors such as age. In particular the mouthwash controls and the viability assay lacked the numbers to perform reliable statistical analysis. There were also too few bronchoscopy samples to enable reliable analyses to address clinical questions.

3.4.7.1 DNA extraction

An efficient DNA extraction is crucial for all molecular based fungal detection and quantification assays. The lysis of fungal cells is challenging as they have a robust cell wall, which can have different levels of rigidity depending on the fungus. Lysis of some species such as *Aspergillus* is particularly challenging as their rodlet layer, which covers the cell wall [156], was shown to increase cell wall elasticity that could protect the spores during bead-beating [347]. The major problem is that no guidelines about the extraction of fungal DNA from sputum or bronchoscopy samples exist, although some recommendations have been proposed for BAL samples [346]. Sputum samples from people with asthma are usually more viscous compared to those from healthy individuals [72], which could impair the DNA extraction. In addition, DNA extraction efficacy depends on the fungus and the kit used [348, 349]. This limits the comparison

between the results from mycobiome studies using respiratory samples as the extractions methods vary highly, including the choice of DNA extraction kit, which can affect the number and number of taxa extracted [230, 232, 234].

In this study, fungal cell walls were lysed using mechanical disruption by beat-beating before the DNA was extracted using a plant extraction kit to account for the rigid cell wall of filamentous fungi. It was previously shown that bead-beating is the most efficient method to extract fungal DNA from cultured fungi [350-352]. It is also a cheap and quick method that reduces the risk of cross-sample contamination, and is more efficient than enzymatic lysis [346]. One minute oscillation was shown to be very efficient for DNA extraction from fungi, however, the recovery of DNA from larger conidia was significantly higher than from smaller conidia [352]. Our group optimised this approach using two minutes of oscillations, which was previously applied for fungi from sputum cultures [137]. Some other mycobiome studies also performed this type of mechanical disruption to lyse fungal cells prior to DNA extraction with a kit [230, 232, 234] and bead-beating is also recommended to process BAL samples [346].

It cannot be excluded that some fungal taxa might have been missed by the DNA extraction method used in this study, particularly as differences in sputum viscosity were not considered. However, bead-beating and plant DNA extraction kits were shown to be most effective to extract fungal DNA in previous studies [98, 353]. It was also demonstrated that the method was robust as no cross-contaminations were detected in the sample controls (section 3.4.4.1).

3.4.7.2 High-throughput sequencing

The Illumina platform is frequently used in biome studies and has some limitations compared to other platforms such as the Roche/454-pyrosequencer or Ion Torrent. The read length is limited due to dephasing [354-356] and substitution errors can occur [357, 358]. Conversely, the paired-end Illumina MiSeq platform has the lowest error rates [356], highest resolution [359] and a deeper sequencing depth for complex fungal communities [360] in comparison to the other common platforms. Index hopping, which is characterised by incorrect barcode identifiers being incorporated into growing

clusters during bridge amplification [361] could have occurred, but this would only affect the rarer OTUs which have been excluded in the analyses.

The ITS2 region of the fungal nuclear ribosomal operon for fungal identification was used in this study. This region is rarely used in respiratory mycobiome studies in contrast to ITS1 [230-232]. Although unique taxa can be detected using either region, the identification of fungi was shown to be comparable, in particular for the most abundant species [219, 353, 362]. ITS2 is believed to be superior to recover the molecular diversity compared to ITS1 [219]. A problem of the ITS region is that certain taxa such as the aforementioned *Cladosporium herbarum* complex [295], *Alternaria* or *Fusarium* [363], cannot be identified reliably down to species level. In addition, PCR-based approaches for studies of the micro/mycobiome are susceptible to amplification bias resulting from differences in the length of the region of interest, and primer mismatch bias [222, 362, 364]. For example, PCR is biased towards shorter amplicons [365, 366], which potentially favours the amplification of ascomycetes as their ITS regions are shorter than from basidiomycetes, especially with respect to ITS2 [229]. Despite these differences, the ITS region is recommended for mycobiome studies [9, 222, 226]. The risks were reduced here through the choice of primers and for index hopping by using a conservative quality-filtering approach [281] and focusing on the more abundant (> 0.1% sequence abundance) and prevalent (shared between at least 25% of samples within a group) taxa.

It also has to be acknowledged that fungal identification is dependent on the sequence database used. The mycobiome results of this study were based on the fungal taxonomic database UNITE [367, 368] for fungal identification. It would also be possible to use GenBank, which is a partner of the International Nucleotide Sequence Database Collaboration (INSD) [369, 370], but it was demonstrated that > 27% of ITS sequences from the INSD were insufficiently identified and a majority of annotations were out-of-date and lacked description [371]. UNITE mirrors the INSD, but retains only high quality sequences and offers third-party annotation capacities [368].

To account for variations in qPCR batches and sequencing, it might be advisable to use a specific positive control with a known DNA concentration such as a plasmid, which has a similar size to the expected amplicons.

3.4.8 Conclusions

The main conclusions of this study are: Both unique and shared taxa were detected based on health status or location of sample acquisition in the airways, which were mostly influenced by less prominent species. Although the qPCR data showed some limitations, no changes of the overall fungal loads between subjects with asthma and healthy individuals could be detected. The presence of dead fungal biomass was indicated by using PMA in a small number of samples, which should be further investigated in future mycobiome studies. Contamination by reagents or during sample processing could be excluded. Samples were predominated by *C. albicans*, *A. fumigatus* and *M. tassiana*. *C. albicans* can be considered as a potential commensal in both the upper and the lower airways of both people with asthma and healthy individuals. It is often part of the normal microflora on both skin and mucosal surfaces and predominated all respiratory samples collected. Its predominance in sequencing data was represented by culture samples despite the limitations for these samples in this study. With respect to culture, it was shown that the media used should be reduced to a minimum to avoid high dilution of samples, which could miss species. *A. fumigatus* was predominantly cultured from sputum samples of asthmatics, but its role could not be clearly defined. Prevalence and abundance of *A. fumigatus* was independent of health status and sample types similar to *C. albicans*, except for the oral mycobiome. However, unlike *C. albicans* it is frequently associated with a more severe course of asthma, particularly in subjects sensitised to this fungus. Therefore it won't be described as a commensal in this thesis. A longitudinal study with more participants would be suitable to address whether the lung mycobiome composition changes, particularly with respect to *A. fumigatus*, and if this has an impact on structural changes observed in subjects with asthma.

Another *Candida* species, *C. dubliniensis* was only identified in fungal sensitised subjects with asthma, suggesting a yet unknown role in this asthma phenotype. Other species with a potential impact on asthma were *A. tubingensis* and *C. pseudolongus*, which were predominantly found in brush samples particularly in subjects with asthma. The sequence abundance of the former was significantly associated with *A. fumigatus* culture-positive sputum samples. The results also highlight the importance of

bronchoscopy to assess the mycobiome of the lower airways. It was indicated that these fungi might affect the anti-fungal immune response as they were significantly negatively associated with sputum neutrophil numbers. Air control samples showed that these fungi are probably more a part of the mycobiota rather than inhaled.

The results of this study therefore fulfilled most of the aims and confirmed the hypotheses that the mycobiome would differ depending on health status or based on the sample type used. The role of *C. dubliniensis*, *A. tubingensis* and *C. pseudolongus* in asthma is largely unknown, which should be investigated in future studies. In that context, *A. tubingensis* and *A. niger* as potential sources of allergens compared to *A. fumigatus* are elucidated in the next chapter.

Chapter 4 Characterisation of *Aspergillus* allergens

4.1 Introduction

The mycobiome data presented in Chapter 3 revealed *A. tubingensis* as a fungus of interest. It was significantly more abundant in the lower airways of people with asthma compared to healthy controls and its sequence abundance associated with *A. fumigatus* culture-positive samples (Chapter 3, 3.3.4). It was significantly more prevalent and abundant in brush samples (Chapter 3, 3.3.4) and significantly associated with decreased sputum neutrophil counts (Chapter 3, 3.3.7). These data indicated that *A. tubingensis*, which belongs to the *Aspergillus* section *Nigri*, could play a role in lung inflammation. Species from the *Aspergillus* section *Nigri* are the second most common filamentous fungus recovered from sputum samples of asthma and COPD patients after *A. fumigatus* [137, 372]. They are associated with ABPM [175] and are close relatives of *A. fumigatus* [373, 374]. Sensitisation to *A. fumigatus* is associated with ABPA [175, 177], SAFS [42] and AFAD [23, 184] in asthma. It is presumed that *A. fumigatus* could represent a persistent allergen source in the lower airways as part of the mycobiota (Chapter 1, 1.2.5). Based on the data from the previous chapter, this theory might also apply to *A. tubingensis*. *A. tubingensis* is a close relative of *A. niger*, which belongs to the same section. These fungi are morphologically indistinguishable. The ITS2 region, which is recommended for identifying fungi [221] and was used to generate the results presented in Chapter 3, is not variable enough to discriminate between the species of the *Aspergillus* section *Nigri*. They can be identified using RFLP of the entire ITS region [375, 376], or sequencing of the β-tubulin region, which is the recommended region for identification [377]. It may be important to discriminate between these species as some characteristics are more unique to one compared to the other. For example, the resistance to antifungal azole drugs is more frequently found in *A. tubingensis* compared to *A. niger* [378, 379], which might be relevant for the outcomes in asthma disease. Although commercial fungal extracts are available for *A. niger*, their use is associated with the same problems as for the other fungal extracts (Chapter 1, 1.2.4) such as missing standardisation, reproducibility, [26, 144, 145] and IgE cross-reactivity [146]. All of these factors prevent a specific diagnosis, which could help to determine the role of this fungus or its relative *A. tubingensis* in asthma pathology. This was demonstrated for some *A. fumigatus* recombinant proteins [164, 165] (Chapter 1, 1.2.4), however, their

commercial availability is limited, in particular for other species. The WHO/IUIS lists 23 fully characterised allergens for *A. fumigatus*, but only three for *A. niger* and none for *A. tubingensis* [132] (http://www.allergen.org, Feb 2018). The three *A. niger* allergens were discovered in the setting of occupational asthma. They are enzymes which are used widely in food processing and animal feedstuffs [19, 380]. Potential IgE cross-reactions of *A. niger* to other fungal species have rarely been investigated and unique allergens of *A. niger* or *A. tubingensis* are unknown.

The hypothesis of this chapter was that *A. tubingensis* and *A. niger* as representatives of the *Aspergillus* section *Nigri* express unique allergens compared to *A. fumigatus*.

The specific aims of this chapter were:

- To determine the identity of clinical isolates from *Aspergillus* section *Nigri* and produce protein extracts of representative species
- To test the IgE-reactivity of subjects with asthma with varying levels of *A. fumigatus*-specific IgE to protein extracts from fungi within the *Aspergillus* section *Nigri*
- To test IgE cross-reactivity between fungal proteins of *A. fumigatus* and those from the *Aspergillus* section *Nigri*
- To identify novel allergens and express them as recombinant allergens

4.2 Materials and methods

4.2.1 Serum samples

Serum samples from 19 people with asthma were used whose IgE reactivity to fungi was determined as described in section 2.2. Respiratory samples from all subjects had undergone HTS. Subjects were assigned to three groups based on low, high and negative *A. fumigatus*-specific IgE levels, which are referred to as group 1, group 2 and group 3, respectively (Table 4. 1).

Table 4. 1: IgG and IgE reactivity of selected people with asthma to *A. fumigatus*.

	Group 1	Group 2	Group 3
Inclusion level of *A. fumigatus*-specific IgE (kU/L)	Low	High	Negative
	> 1, < 5	≥ 17	< 0.35
Number of subjects	3	4	7
Total IgE (kU/L) *A. fumigatus*	1.64 - 3.85	17.5 - 66	0.0 - 0.13

4.2.2 Fungal culture

Characterised clinical isolates of *Aspergillus* section *Nigri* and *A. fumigatus* as well as *A. fumigatus* type strain National Collection of Pathogenic Fungi (NCPF 7097) were cultured between 3-14 days at 37°C. Hyphae and spores were harvested. Both liquid broth and agar were inoculated with a -80°C glycerol stock (section 3.2.3.1) using an inoculation loop. Biomass of *A. fumigatus* was derived from a 200 ml liquid culture using Sabouraud liquid medium, whereas material from *Aspergillus* section *Nigri* was derived from a PDA for the spores and liquid Sabouraud agar, shaking at 200 rpm, for the hyphae (Table 2. 1, section 2.3.1).

For a second batch of protein extract from the clinical isolate of *A. fumigatus* for immunoprecipitation (section 4.2.8.3), 200 ml of Sabouraud dextrose liquid agar was inoculated with 10 µl of a -80°C glycerol stock (section 3.2.3.1) from the fungus and incubated at 200 rpm, 37°C overnight, before the solution was transferred to 2 L of

Sabouraud dextrose liquid agar, which was incubated for 14 days at 37°C like the previous batch.

Harvesting was performed by filtration through a sterile Whatman filter No. 1 membrane (pore size 11 µm) (Whatman® GE Healthcare Life Sciences, UK) and washed with deionized water sterilised by autoclaving at 121°C for 15 min. Samples were stored at -80°C until use in a sterile 50 ml centrifuge tube.

4.2.3 Fungal identification

Both the ITS and the β-tubulin regions were used for species identification of *Aspergillus* section *Nigri* clinical isolates [381]. The clinical isolates from sputum samples of people with asthma were kindly provided by Dr Catherine Pashley (Maurice Shock Building, University of Leicester, UK). Reactions were done in duplicate and contained 5 µl of template DNA or PCR-grade water as a negative control. An optional 0.2 µg/µl BSA (NEB, UK) were added to improve amplification efficiency. The ITS region was amplified using primers ITS5/ITS4 and PCR conditions described in Table 2. 2 and Table 2. 3, section 2.3.4.1. The presence of an amplicon was confirmed using gel electrophoresis (section 2.3.4.3) and subsequently underwent restriction digest using restriction enzymes *Hha*I, *Nla*III and *Rsa*I (NEB) for 2 h at 37°C (Table 4. 2, Appendix, 6.1) [375, 376]. Reactions were done separately for each restriction enzyme in total reaction volumes of 10 µl. The reaction was stopped by addition of 2 µl loading buffer (Sigma-Aldrich, UK) and incubation at 4°C for 10 min. Digestion products were analysed by gel electrophoresis on a 3% agarose gel (section 2.3.4.3).

Table 4. 2: Reagents and concentrations for the restriction digest of amplified ITS region using *Hha*I, *Nla*III and *Rsa*I restriction enzymes. All reagents were from NEB.

Reagents	Quantity added per 10 µl deionised water	End concentration
10X CutSmart buffer	1 µl	10X
*Rsa*I	0.2 µl	20 U
*Nla*III	0.2 µl	10 U
*Hha*I	0.2 µl	40 U

The β-tubulin region was amplified using the reaction composition described in Table 4. 3 using primers Bt2a (5' GGTAACCAAATCGGTGCTGCTTTC 3') and Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC 3') [381]. PCR cycling conditions were 15 min at 94°C, followed by 32 cycles of 60 s at 94°C, 60 s at 58°C, 90 s at 72°C, before the final elongation step at 72°C for 10 min. The presence or absence of the PCR product was confirmed by gel electrophoresis (section 2.3.4.3).

Table 4. 3: Reagent mix for fungal β-tubulin amplification.

	Quantity added per 25 µl PCR-grade water	Final amount/ concentration
PCR buffer with $MgCl_2$ (Qiagen)	2.5 µl	1X
dNTPs (Bioline)	2.5 µl	250 µM (each base)
Forward primer (Invitrogen)	0.5 µl	200 nM
Reverse primer (Invitrogen)	0.5 µl	200 nM
HotStar Taq Polymerase (Qiagen)	0.2 µl	1 U
Template DNA/PCR grade water	5 µl	N/A

N/A = not applicable, U = unit

4.2.4 Protein extraction

After the identification of *Aspergillus* section *Nigri* clinical isolates using RFLP and β-tubulin sequencing (section 4.2.3) strains were cultured (section 2.3.1). A clinical strain

and type strain NCPF 7097 of *A. fumigatus* were used as a control. Protein extraction was done either by cooling with liquid nitrogen (N_2) and grinding with pestle and mortar, or bead beating for 2 x 30 sec using a Mini-Beadbeater-16 at 4°C [161, 382, 383]. Tubes for bead beating contained fungal material, 600 ± 60 mg acid-washed glass beads (212-300 µm) and lysis buffer. Five millilitre lysis buffer (0.5X or 1X cOmplete, EDTA-free protease inhibitor cocktail (Roche, Germany), 150 mM NaCl, 2 mM EDTA (Sigma-Aldrich, UK), 50 mM sodium bicarbonate (Acros, UK), 2 mM PMSF (Sigma-Aldrich, UK)) was applied per gram of material [161, 383]. Two rounds of centrifugation were used to get rid of the solid fraction of the suspension, first at 4500 x g for 20-30 min, then 10 min at 10,000 x g at 4°C. If less then 5 ml suspension was present, suspensions were only centrifuged for 10 min at 10,000 x g at 4°C. . Supernatants were aliquoted and stored at -20°C or -80°C until use. Protein concentrations were determined using the Bradford assay (Protein Assay Dye Reagent Concentrate, BioRad) at A_{595} according to the manufacturer's instructions for tube assays. The absorbance (A_{595}) was measured with a suitable spectrophotometer (Jenway 6305 or Hitachi U-2000).

4.2.5 SDS-PAGE

Two SDS-PAGE assays were used as work was performed in two different laboratories (Table 4. 4). For method A, SDS-PAGE was performed using 4-20% Mini-PROTEAN® TGX stain-free precast gels. In some cases pre-cast 12% Mini-PROTEAN® TGX stain-free precast gels were used as indicated in the figure legends. For method B self-cast 12% SDS-PAGE gels with 5% stacking gels were used. The 1X electrophoresis buffer was prepared from 10X stock (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3), which was either commercial (BioRad, UK) or self-made (ingredients from Roth, Germany). Four micrograms of crude protein extracts or 2 µg, 5 µg and 10 µg of recombinant proteins were used for electrophoresis. Recombinant proteins rAsp f 3 (2.52 µg/µl), rAsp f 4 (2.06 µg/µl) and rAsp f 6 (3.68 µg/µl) were kindly provided by Professor Ines Swoboda (FH Campus Wien, Vienna) (MADx Macro Array Diagnostics, Appendix, 6.2). Alternatively, rAsp f 3 and rAsp f 6 produced using a large-scale protein expression protocol were used (section 4.2.7.3). Proteins were mixed with 4X Laemmli buffer containing β-mercaptoethanol (1X end concentration) [384] and water to equalise the volumes

loaded, before the suspensions were heated for 10 min at 95°C using a thermal cycler or a heating block (Eppendorf, Germany). The Laemmli buffer [384] was either commercial (method A, BioRad) or kindly provided by Prof Ines Swoboda, FH Campus Wien, Vienna, Austria (method B). In order to verify binding of the secondary antibody and exclude a cross-reaction between the anti-human IgE to IgE and IgG, 100 or 50 ng of human IgE (Abcam) or IgG (BioRad) were used. Human IgE antibodies were not heated or mixed with β-mercaptoethanol as not all anti-human IgE secondary antibodies used for immunoblotting were able to detect denatured IgE (Appendix, 6.3, Fig. A. 1 to Fig. A. 5).

Table 4. 4: Steps of two SDS-PAGE methods.

	Method A	**Method B**
Gel type	4-20% Mini-PROTEAN® TGX stain-free precast gels*	12% SDS-PAGE gels with 5% stacking gels**
4X Laemmli sample buffer (1X end concentration)	277.8 mM Tris-HCl, pH 6.8, sample buffer 44.4% (v/v) glycerol 4.4% LDS 0.02% bromophenol blue* plus 1:10 β-mercaptoethanol (VWR)	62.4 mM TrisBase, 80 mM SDS, 10% glycerol (both Roth, Germany), 5% β-mercaptoethanol (Calbiochem, Germany), 0.025% bromophenol blue (Sigma-Aldrich), 2 µl glycerol added per sample
Ladder	5 µl of Precision Plus Protein™ WesternC™ blotting standard*	5 µl PageRuler™ Prestained (ThermoScientific)
SDS-PAGE	200-250 V for ~30-40 min	First, 90 V for ~15 min, then 200 V for 35-45 min
Staining	Stain-free	Coomassie***
Imaging	ChemiDoc Touch* stain-free imaging, 5 min imaging for faint bands Image Lab software v5.2.1 or v6.0.1*	FluorChem E (ProteinSimple, Austria) Automatic setting AlphaViewSA software (Version 3.4.0.0)

*all BioRad, UK, **Self-cast (Appendix 6.4), ***Procedure for Coomassie staining Appendix 6.5

For method B, a Canon scanner (600 dpi) was used for imaging in some cases, which is indicated in the respective figure legend (Chapter 4, 4.3.3.2, Fig. 4. 10). They were further processed for peptide mass fingerprinting as necessary (section 4.2.9). Some

stained gels were transferred into deionised water for short-term storage or dried for 2 h using a gel dryer model 583 (BioRad), where gels were sandwiched between a filter paper moistened with deionised water and cling film.

4.2.6 Immunoblotting

Immunoblotting experiments were performed with protocols adapted from the Clarity Western Introductory Manual (BioRad) [385]. Although the major steps of the procedure were basically the same (Fig. 4. 1), the protocols differed depending on the transfer technologies available as well as the buffers and staining techniques used. The different methods (A-C) are depicted in Table 4. 5. All steps were performed at room temperature unless stated otherwise. All blots were incubated with the protein side up under continuous agitation (20-30 rpm).

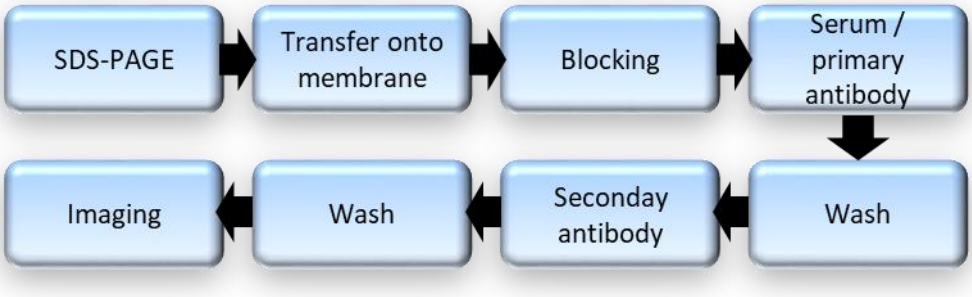


Fig. 4. 1: Main steps of the immunoblotting procedure.

Proteins from SDS-PAGE were transferred onto 0.2 µm nitrocellulose membranes using either semi-dry transfer (TransTurbo^{TM} blot, BioRad) (method A or B) or wet transfer (method C) (Table 4. 5). Membranes were blocked for 1 h using the ingredients described in Table 4. 5. In certain instances, blots prepared by method A were blocked with 5% milk (in 0.05% Tween 20 in PBS), incubated with secondary antibody in 0.05% Tween 20 in PBS, and washed with 0.05% Tween 20. They were incubated using ECL Clarity substrate (BioRad) before imaging as indicated in the figure legend.

Blots were incubated with human serum overnight at 4°C. For some experiments, serum from different subjects was pooled. Serum or serum pools were usually diluted 1:10. If

a negative control blot was prepared, it was not incubated with human serum or primary antibody, but in blocking buffer. After washing, membranes were incubated with the secondary antibody for 1 h at room temperature (Table 4. 5, Table 4. 6). Both chemilumescent and fluorescent assays were performed using the respective antibodies (Table 4. 6). The anti-human IgE antibodies were selected from a range of anti-human IgE antibodies from different companies (Appendix 6.3, Table A. 1) because they had the highest sensitivity (Appendix 6.3, Fig. A. 2 to Fig. A. 4). The secondary antibodies did not react to human IgG (Appendix 6.3, Fig. A. 2, Fig. A. 3, Fig. A. 5). The sensitivity was assessed using dilution series of pure human IgE (Abcam, UK) and different dilutions of secondary antibody. The specificity was tested using a 100 ng pure human IgG (BioRad, UK) control.

The membranes for the fluorescent assays were incubated in the dark from that time onwards. After imaging, these blots were dried on sterile filter paper in the dark and stored in aluminium foil for several months. For chemiluminescent assays, the excess liquid from the membranes was soaked up by tissue paper using the edge of the membrane. The membrane was wrapped in cling film before imaging.

Table 4. 5: Three immunoblotting methods, including two methods for chemiluminescent and one for fluorescent blotting.

	Method A	**Method B***	**Method C**
Protein transfer technique	Semi-dry Trans-Blot® Turbo™ System		Wet transfer
Transfer ingredients	Turbo™ Mini Nitrocellulose Transfer Packs (BioRad)		Amersham™ Protran™ nitrocellulose blotting membrane (GE Healthcare Life Sciences, Germany)
Transfer time	3 min at 2.5 A (“1 Mini-TGX” = 5-150 kDa) 7 min at 1.3 A (“Mixed MW” = 5-150 kDa) 10 min at 1.3 A (“High MW” > 150 kDa)**		100 V for 1 h in 1x transfer buffer***
Control imaging	stain-free imaging on the ChemiDoc Touch		Ponceau red**** for a few seconds and 5-10 min destaining with deionised water
Blocking	1 h in 0.5% Tween 20 in PBS‡	1 h in Odyssey’s proprietary blocking buffer (PBS-based) (Li-COR)	1 h in 0.5% Tween 20 (Sigma) in PBS‡
Incubation buffer for the human serum	1 h in 0.5% Tween 20 in PBS	Odyssey’s proprietary blocking buffer with 0.05% Tween 20 in PBS	0.5% Tween 20 in PBS

Washing	6 x 5 min using 0.5% Tween 20 in PBS	5 x 5 min using 0.05% Tween 20 in PBS	6 x 5 min using 0.5% Tween 20 in PBS
Secondary antibody incubation conditions (1 h)	0.5% Tween 20 in PBS; the ladder was incubated separately in a 1:10,000 dilution Precision Protein™ StrepTactin-HRP conjugate (BioRad)	0.05% Tween 20 in PBS in the dark	0.5% Tween 20 in PBS
Washing	6 x 5 min using 0.5% Tween 20 in PBS	4 x 5 min using 0.05% Tween 20 in PBS in the dark	6 x 5 min using 0.5% Tween 20 (Sigma) in PBS
Substrate	SuperSignal® West Pico chemiluminescent substrate (ThermoScientific) for 5 min in the dark	Not applicable	SuperSignal® West Pico chemiluminescent substrate (ThermoScientific) for 5 min in the dark
Imaging instrument	ChemiDoc Touch	Odyssey CLx	FluorChem E (ProteinSimple)
Imaging software	Image Lab v5.2.1 (BioRad)	ImageStudio v2.1	AlphaViewSA software (Version 3.4.0.0)

*[386], **[387], *** prepared from 10X stock solution (2.4 M glycine (Roth or Sigma), 315.8 mM TrisBase (both Roth or Fisher Scientific)), plus 20% methanol (both Roth or Fisher Scientific), ****30 mM Ponceau S (Santa Cruz, Austria), 1.8 M trichloroacetic acid (Roth, Germany) and 1.4 M 5-Sulfosalicylic acid (Merck, Germany), kindly provided by Prof Ines Swoboda (FH Campus Wien, Austria), ǂFor method A, PBS was prepared from tablets (Gel lifesciences); for method C, PBS was prepared from a 10X stock solution (0.1 M $Na_2HPO_4 \cdot 2\ H_2O$, 22 mM KH_2PO_4 and 1.4 M NaCl, pH 7.4 (all Roth, Germany)).

Table 4. 6: Secondary antibodies with fluorescent (DyLight and IRDye®) or chemiluminescent (HRP) conjugates used for immunoblotting experiments.

Name	Supplier	Clonality	Conjugate	Dilution
Mouse anti-human IgE HRP	SouthernBiotech	Monoclonal*	HRP	1:5000
Goat anti-human IgE	Novus Biologicals	Polyclonal	DyLight 680	1:15,000
Goat anti-mouse IgG HRP antibodies	Jackson Labs	Polyclonal	HRP	1:10,000
Donkey anti-mouse IgG	Li-COR	Polyclonal	IRDye® 800CW	1:15,000

HRP = horseradish peroxidase, *clone B3102E8

Inhibition blots were prepared by running the protein samples in duplicate on an SDS-PAGE gel. After transfer onto a nitrocellulose membrane, the blot was halved. Each half was incubated with human serum (1:10 in 0.5% Tween 20 in PBS). One part of the human serum was pre-incubated with the fungal protein extract or with a mixture of recombinant proteins, whose amount was 10X the amount of recombinant protein or crude protein extract per millilitre loaded on a SDS-PAGE gel. The other part was pre-incubated with wash buffer only. The pre-incubation was carried out on a spinning wheel at 40 rpm, overnight at 4°C. Detection of bound IgE antibodies was performed using method C (Table 4. 5).

The different gels and blotting methods were compared and showed compatible results as presented in Appendix, 6.6. The comparison between the gels was only possible for pre-cast 12% and 4-20% gels as self-cast gels were not produced on site. Despite this limitation, the experiments showed that the blots were highly consistent and therefore results using either method were comparable.

4.2.7 Production of recombinant allergens from *A. fumigatus*

4.2.7.1 Bacterial media

Liquid or solid Lurea Broth (LB) medium was used for growing *Escherichia coli* BL21. Both media were composed of 10 g/L peptone and NaCl, 5 g/L yeast extract (all Roth, Germany) (pH 7.2-7.5). The solid medium used for plates also contained 15 g/L agar-agar (Roth, Germany). Media were autoclaved for sterilisation at 121°C for 15 min. The medium was cooled down to approximately 55°C before pouring agar plates (90 mm Petri dish). For selection, a 50 mg/ml ampicillin 3-hydrate (PanReac AppliChem) stock solution was added to the broth or solid medium before pouring to a final concentration of 100 µg/ml. Selective medium is referred to as LB-Amp medium.

4.2.7.2 Transformation and culture

Competent *E. coli* BL21 cells and plasmid constructs encoding rAsp f 3 and rAsp f 6 with C-terminal hexahistidine-tags (His-tags) (both Eurofins genomics, Germany) were kindly provided by Professor Ines Swoboda. The vector construct (Appendix 6.7) contained an ampicillin resistance gene allowing the selection of transformed bacteria from LB-Amp medium. The bacteria were taken from -80°C and thawed on ice. They were transformed for protein expression using 1 µl of plasmid constructs per 100 µl of cells. After mixing by gentle pipetting, the cells were incubated on ice for 30 min. Cells underwent heat shock for 1 min at 42°C before a 2 min incubation on ice. Four hundred microlitres of liquid LB medium (section 4.2.7.1) was added and cells were incubated for 30 min at 37°C, shaking at 600 rpm. A LB-Amp medium plate (section 4.2.7.1) was inoculated using 10 µl of the transformed cell suspension. The remaining suspension was centrifuged at 6800 x g for 2 min. The pellet was resuspended in 100 µl of LB medium, which was plated out. *Escherichia coli* BL-21 cells were incubated on LB-Amp medium at 37°C for up to three days. Afterwards, plates were stored at 4°C for up to a week. One colony was used to inoculate a 20 ml of liquid LB-Amp medium (section 4.2.7.1) referred to as pre-culture, which was incubated at 37°C at 200 rpm overnight.

4.2.7.3 Large-scale protein expression

Four millilitre of the pre-culture was used to inoculate 400 ml LB-Amp medium, which was incubated at 37°C, 200 rpm, until an OD_{600} of ~0.5 was reached as determined by spectrophotometry (Genesys 6, Thermo Spectronic). Protein expression was then induced by 0.5 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) (ThermoScientific). Bacteria were harvested after 2 h of IPTG-induction by centrifugation at 4500 x g for 10 min, at 4°C. Proteins were extracted using three cycles of alternating freeze-thawing using N_2 and a hand-warm water bath, respectively, for 10 min each. Cells were resuspended in 40 ml of native lysis buffer (10 mM Tris, 300 mM NaCl, 0.1% Triton X-100 (all Roth), 20 mM imidazole (Calbiochem), pH 8.0, modified from [388]) by vortex and homogenized for 3 x 30 sec (Glas-Col GKH GT motor control and Glas/Col® Homogenizer) with 30 s intervals on ice in between. The homogenate was centrifuged at 4500 x g for 10 min at 4°C and the supernatant transferred into a separate tube. As the proteins were His-tagged (Appendix 6.2), they could be purified from the supernatant by adding 4 ml of Protino® Ni-NTA agarose (Macherey-Nagel) for 1 h at 4°C, rotating at 30 rpm. The suspension was transferred into a 6 ml Chromabond column (Macherey-Nagel) and washed with 40 ml of the native buffer. His-tagged proteins were eluted using elution buffer in 1 ml fractions (10 mM Tris, 300 mM NaCl, 250 mM imidazole, pH 8.0, modified from [388]). After a control SDS-PAGE (section 4.2.5, method B) using 20 µl of each eluate (data not shown), protein fractions were combined and dialysed against 0.01 M sodium phosphate buffer (pH 6.9), prepared from a 10X stock solution, overnight at 4°C due to the high concentration of imidazole in the elution buffer. For this procedure, proteins were transferred into a Slide-A-Lyzer cassette (MWCO 3.5) (Thermo Scientific). The stock buffer was prepared by mixing 42.3 ml of 1 M sodium dihydrogen phosphate with at least 20.6 ml of 1 M disodium phosphate (both Roth) until pH 6.9 was obtained. The buffer was exchanged at least twice within 24 h and was continuously stirred at low speed using a magnetic stirrer. The protein concentration was determined using a Micro BCA Protein assay kit (Pierce, ThermoScientific) at A_{562} (Hitachi U-2000 spectrophotometer) according to the manufacturer's instructions. The purity of the proteins was analysed by SDS-PAGE (section 4.2.5, method B) using 2 µg, 5 µg and 10 µg of recombinant protein per well, followed by Coomassie staining (Appendix 6.5). Impurities were further assessed by

immunoblotting with 1:1000 diluted Penta-His™ antibodies (Qiagen), which were detected by goat anti-mouse IgG HRP antibodies (1:10,000) (Jackson labs) (section 4.2.6, method C, Table 4. 5, Table 4. 6). Proteins were aliquoted and stored at -20°C.

4.2.8 Immunoprecipitation

Only protein low binding tubes (Eppendorf) were used for these experiments. Beads were separated from a solution either with a DynaMag™-2 (ThermoScientific) or DYNAL MPC®-E-1 (DYNAL, Norway) magnets. For these experiments, serum from patient R083 was selected due to the large amounts of serum available with a reasonable content of total IgE (1746 kU/L = 4190.4 ng/ml), where roughly a forth comprises *A. fumigatus*-specific IgE (66 kU/L = 158.4 ng/ml). Experiments were performed using the Dynabeads Co-Immunoprecipitation kit (ThermoScientific, UK) according to the manufacturer's instructions with a few modifications as described below. The main steps of the immunoprecipitation are illustrated in Fig. 4. 2. First, anti-human IgE antibodies were coupled to magnetic Dynabeads, which where used to bind human IgE antibodies from human serum in the next step. These were used to purify proteins from fungal crude extract.

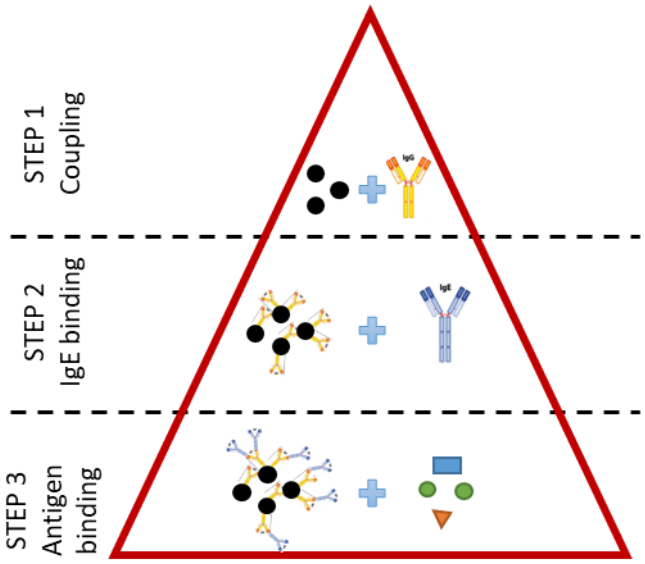


Fig. 4. 2: Schematic overview of the steps for immunoprecipitation of fungal proteins. Step 1 shows coupling of mouse anti-human IgE antibodies (SouthernBiotech, yellow) to magnetic Dynabeads (black dots) (ThermoScientific). The coupled antibodies are used to capture human IgE (blue) from serum during step 2. In step 3, the coupled beads with bound human IgE from serum are used to capture fungal proteins (blue, green, orange geometric structures).

4.2.8.1 Antibody coupling

Unlabelled mouse anti-human IgE antibodies (SouthernBiotech, clone B3102E8) were covalently bound to Dynabeads. The same clone was used as the one used for immunoblotting (Table 4. 6). Beads were washed with C1 solution, before adding the C1 and C2 solution together with the antibodies to the beads (7 µg of antibodies per milligram beads). The coupling reaction was conducted overnight at 37°C rotating in a hybridisation chamber (approximately 21 h). Beads were washed once with each HB and LB buffer, both containing 0.05% Tween 20 (VWR), and three times with SB buffer (SB 1-3). The last wash lasted 15 min on a roller at room temperature, before beads were resuspended in 6 ml SB buffer containing 0.02% sodium azide (G Biosciences) as a preservative and stored at 4°C until use. Washes were stored as well to estimate loss of antibodies after the coupling reaction. The coupling efficiency of the beads was assessed by immunoblotting (sections 4.2.5 method B, 4.2.6 method B), loading the washes,

beads and anti-human IgE antibodies equivalent of 100% and 65% of antibodies that potentially bound to the beads. If none of the antibodies would have been coupled, an amount of 2.36 µg of mouse anti-human IgE would be expected. One hundred nanograms of pure human IgE to exclude non-specific binding of the donkey anti-mouse IgG fluorescent antibodies to human IgE (Table 4. 6, Appendix 6.8, Fig. A. 9). Although the washes indicated that some beads had been washed off, it should be noted that some beads were present within the washes. The IgG light chain (23 kDa) in the beads' lane indicated that most fragments dissociated from the beads by the sample buffer was represented by the light chain (23 kDa) and that at least 50% of anti-human IgE was bound. This is equivalent of 3.5 µg antibody per milligram of beads. The secondary antibody did not show any cross-reactivity with the human IgE control.

4.2.8.2 Immunoprecipitation of human IgE

One milligram of coupled beads was washed first with 900 µl sterile PBS containing 0.1% BSA (Sigma-Aldrich, UK) for 5 min on a rotator and then 3 x with 900 µl of PBS to remove the sodium azide. Afterwards, beads were incubated with serum or pure human IgE antibodies (Abcam) as a control whose amount was equivalent to the serum total IgE. For IgE harvesting from serum, the serum was diluted 1:10 in PBS; for fungal protein harvesting 1:8. The mixture was incubated at 4°C for 30 min on a roller. The supernatant was removed and stored at -20°C. Beads were washed three times in 200 µl PBS, though for immunoprecipitation of fungal proteins, only the first wash was performed using PBS, whereas the subsequent two washes were done with extraction buffer (section 4.2.4). Proteins were eluted in 60 µl of elution buffer using 5 min incubation on a rotator after a last 5 min wash with 200 µl of LWB buffer containing 0.02% Tween 20 on a rotator. For IgE harvesting from serum, IgE binding was confirmed using immunoblotting (sections 4.2.5 method B, 4.2.6 method B). Loading was limited to the size of the wells (30 µl), allowing 22.5 µl of sample plus 4X buffer to be loaded. The expected maximum amount of human IgE in the serum or eluate was 70 ng. The maximum amount of IgE that could be expected in the washes was 9.5 ng. Equivalent amounts of pure human IgE were loaded. IgE antibodies from both human serum and the control were bound by the coupled beads (Appendix 6.8, Fig. A. 10). In contrast to the IgE control assay, a small

fraction of IgE was not harvested from the serum as indicated by a red fluorescent signal in the serum supernatant. Cross-reactivity between mouse and human IgG detection by the secondary antibody was observed in the lanes containing human serum and serum supernatant (green) (Appendix 6.8, Fig. A. 10 A), which is a known complication according to the manufacturer, Li-COR [389].

4.2.8.3 Immunoprecipitation of fungal proteins

Fungal proteins were immmunoprecipitated using a new batch of *A. fumigatus* extract (section 4.2.2). Samples were spiked with 10 µg/ml of recombinant Asp f 3 (section 4.2.7.3). Coupled beads were processed as described in section 4.2.8.2 to bind human IgE from serum. Afterwards, beads were washed initially with PBS, followed by two washes with extraction buffer (section 4.2.4). The beads were then incubated with 40 µg/ml of protein extract within 1 ml of extraction buffer overnight at 4°C on a roller. The supernatant was removed and beads were washed three times with extraction buffer, followed by the LWB and elution using 60 µl of elution buffer. Samples were analysed using immunoblotting according to method A (section 4.2.6, Table 4. 5). Samples for mass spectrometry were prepared according to the manufacturer's instructions. Four milligrams of beads were prepared as described above using 750 µl of serum (1:8 dilution in PBS) and 80 µg/ml of protein extract from *A. fumigatus*. After an incubation overnight, washes were performed with twice with 900 µl of extraction buffer and once with 900 µl of PBS. The sample was eluted using the HPH EB buffer containing 0.5 M ammonium hydroxide (Sigma-Aldrich, UK) and 0.5 mM EDTA. To perform a control blot as described in method A (section 4.2.6, Table 4. 5), 26.7% of the sample was eluted with the commercial elution buffer. Samples were sent to the PNACL facility for vacuum speed centrifugation and peptide mass fingerprinting (section 4.2.9).

4.2.9 Mass spectrometry

Bands of IgE-reactive proteins were excised using a scalpel, transferred into protein low-binding tubes and sent for LC-MS/MS analysis either to the VetCore – Facility for Research (Vienna, Austria) or the Protein Nucleic Acid Chemistry Laboratory (PNACL) (University of Leicester, UK). Proteins were digested with trypsin. The former facility

used the TripleTOF mass analyser, while the latter PNACL facility used an Orbitrap Velos mass analyser. The PNACL facility was also used to analyse samples from the immunoprecipitation (section 4.2.8.3).

4.2.10 Mass spectrometry data

4.2.10.1 Gel-based protein identification

Data from the TripleTOF mass analyser were analysed using the ProteinPilot™ (SCIEX) software. Database search was accomplished by searching against proteins from different strains of *Aspergillus* in the UniProt database by setting the thorough identification mode. Using a "cRAP" database (https://www.thegpm.org/cRAP/) common contaminants were identified. Sequences from other kingdoms than fungi were excluded by comparison to a separate data file containing common contaminants [390-392]. It was allowed for 1% false-discovery rate (FDR) for each, peptides, proteins and spectra.

Data from the OrbiTrap mass analyser were processed using Proteome Discoverer (version 1.4, Thermo Scientific), searching each file in turn using Mascot (version 2.2.04, Matrix Science Ltd.) [393] against the human reference proteome downloaded from UniProtKB [394] (Proteome ID: UP000005640). The peptide tolerance was set to 5 ppm and the MS/MS tolerance was set to 0.6 Da. Fixed modifications were set as carbamidomethyl (C) with variable modifications of oxidation (M). Trypsin was selected as the enzyme and up to 3 missed cleavages were allowed. A decoy database search was performed.

The output from Proteome Discoverer was further processed using Scaffold Q+S [395] (version 4.0.5, Proteome Software). Upon import, the data was searched using X!Tandem [396] (The Global Proteome Machine Organization). PeptideProphet [397] and ProteinProphet [398] (Institute for Systems Biology) probability thresholds of 95% were calculated from the decoy searches and Scaffold was used to calculate an improved 95% peptide and protein probability threshold based on the data from the two different search algorithms.

4.2.10.2 Immunoprecipitation-based protein identification

The .raw data file obtained from each LC-MS/MS acquisition was processed using Proteome Discoverer (version 1.4.0.288, Thermo Scientific), searching each file in turn using Mascot2 (version 2.2.04, Matrix Science Ltd.) against the UniProtKB-Swissprot3 database. The peptide tolerance was set to 10 ppm and the MS/MS tolerance was set to 0.02 Da. Fixed modifications were set as carbamidomethyl (C) and variable modifications set as oxidation (M). A decoy database search was performed. The output from Proteome Discoverer was further processed as described in section 4.2.10.1.

4.2.11 Protein sequence comparisons

Sequences of proteins identified by mass spectrometry were compared using Allergen Online (FASTA Version 35.04) [399] or the Structural Database of Allergenic Proteins (SDAP) (FASTA 3.45) [400, 401]. The searches included both FASTA search and 80mer sequence alignments (Codex Alinorm 03/34, 2003) [402]. Protein names were also searched using the IUIS/WHO database [132] (allergen.org). The EMBOSS Needleman-Wunsch algorithm (ebi.ac.uk/Tools/psa/) [403-405] was used to align the sequences of all detected proteins, which were obtained from the UniProt database [394].Proteins not present in either of these databases were searched in Allergome (allergorme.org) [406-408] to account for allergens that are not officially listed (this means unofficially listed). Any proteins not found in any of these databases are herein described as “unlisted”.

4.3 Results

4.3.1 Preparation of protein extracts

4.3.1.1 Selection of *A. tubingensis* and *A. niger* clinical isolates

Clinical isolates representing species from the *Aspergillus* section *Nigri* were kindly provided by Dr Catherine Pashley because culture rates were low as presented in Chapter 3 (3.3.5). From a range of fungi tested, this section focuses on those six isolates, which were used for subsequent experiments. A few clinical isolates of each *A. niger* and *A. tubingensis* were chosen to account for possible variances of protein expression in each fungus. Both RFLP and sequencing of the β-tubulin region were performed to identify clinical isolates of the *Aspergillus* section *Nigri*. Whilst the RFLP was applied to three samples, the β-tubulin sequencing was performed for all six isolates. Restriction digest of the ITS region using the restriction enzymes *Hha*I and *Nla*III, resulted in the same banding pattern across all the samples tested (Fig. 4. 3). Four bands were detected for *Hha*I at sizes around 80, 150, 180 and 210 bp, whereas three were detected for *Nla*III around 100, 120 and 350 bp. This is indicative of *A. niger* or *A. tubingensis* as opposed to other *Aspergillus* section *Nigri* species such as *Aspergillus aculeatus* [375, 376]. Distinctive banding patterns were observed using the enyzme *Rsa*I. Amplicons were either digested (~500 and 100 bp bands) or not digested (~600 bp), depending on whether *A. niger* or *A. tubingensis* were present [375, 376].

Sanger sequencing of the β-tubulin region and subsequent BLASTn [260] results showed that half of clinical isolates chosen for subsequent experiments were *A. tubingensis* and the other half *A. niger* (Appendix 6.9, Table A. 3). The species identified by RFLP were confirmed by β-tubulin sequencing, showing that the methods were compatible.

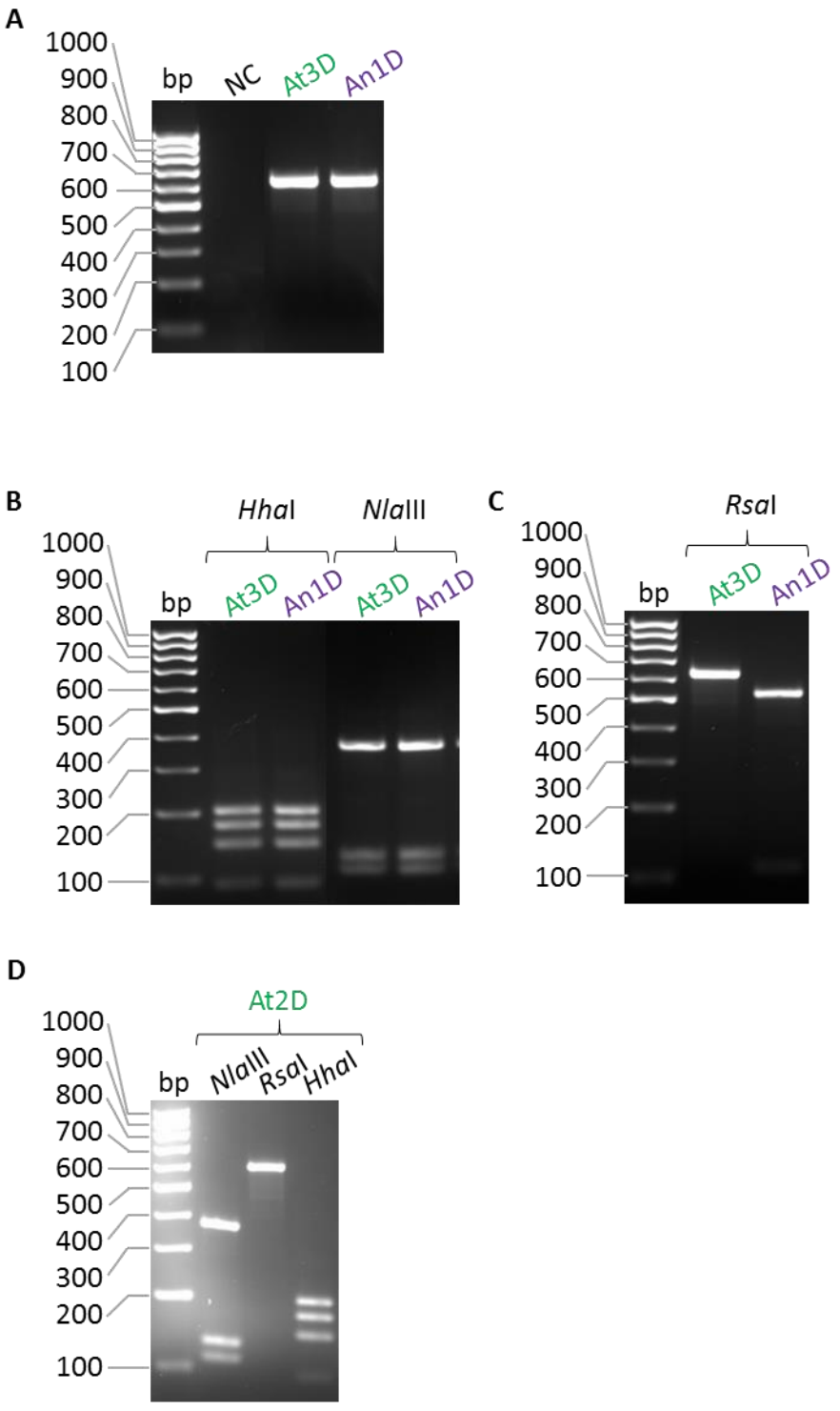


Fig. 4. 3: RFLP from *Aspergillus* section *Nigri*. (A) Amplification of the ITS region using ITS1/ITS4 primers [10] (2% agarose gel containing ethidium bromide). (B-D) Restriction digest using restriction enzymes NlaIII, HhaI and RsaI for three *Aspergillus* section *Nigri* species, which were identified as *A. tubingensis* (At2D, At3D) and *A. niger* (An1D) (3% agarose gel containing ethidium bromide). "D" is the abbreviation for DNA. Bands were visualised using ChemiDoc Touch (automatic exposure).

4.3.1.2 Comparison between *Aspergillus* protein extracts

Protein extracts were prepared from hyphae and spores of *A. fumigatus*, *A. tubingensis* and *A. niger* (Table 4. 7) to account for potential differences in protein/allergen expression between these two fungal growth structures. The protein concentrations ranged between 0.21 and 1.26 µg/µl.

Table 4. 7: Protein extracts obtained from *Aspergillus* clinical isolates and a type strain of *A. fumigatus* (NCPF 7097) (section 4.2.4).

Fungus	Abbreviation for each isolate/type strain	Culture time (days)	Protein concentration (µg/µl)
A. fumigatus NCPF 7097	Af1	8	0.40
A. fumigatus	Af2	14	0.21
	Af2*	14	0.34
A. niger	An1	3	0.38
	An2	4	0.26
	An3	4	0.43
A. tubingensis	At1	3	0.22
	At2	4	1.26
	At3	4	0.55

*second batch of the same clinical isolate

SDS-PAGE was performed using the same amount of protein extract for each gel and western blot. An example is shown in Fig. 4. 4. Several protein bands for each extract could be detected from a Coomassie brilliant blue stained gel, where the darker bands indicate higher amounts of protein compared to weaker bands. Most of the *A. tubingensis* and *A. niger* extracts showed a similar banding pattern, where the most intense bands could be found at low molecular weights ≤ 15 kDa, and at ~23 kDa and 26 kDa. Interestingly, At2 (Table 4. 7) showed a slightly different pattern with several bands between 40 kDa and 130 kDa, which were more intense than in the other *Aspergillus* section *Nigri* clinical isolates. The banding patterns of the *A. fumigatus* species were different to the *Aspergillus* section *Nigri* with the most predominant bands observed around 15 kDa and 35 kDa.

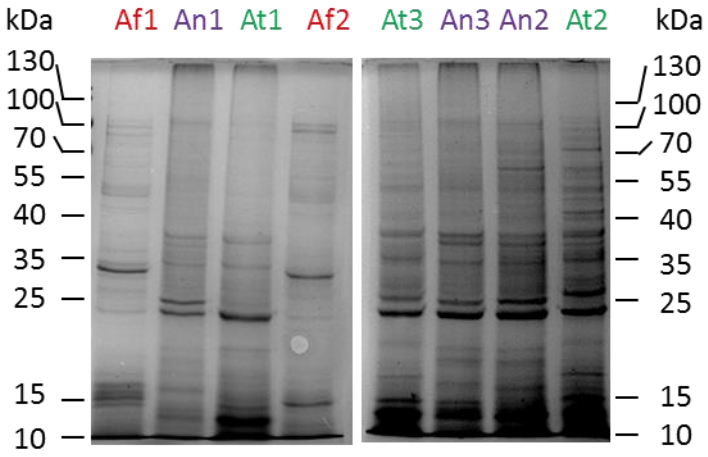


Fig. 4. 4: Example of an SDS-PAGE gel loaded with fungal extracts from different *Aspergillus* species, namely *A. fumigatus* (red) Af1 and Af2, *A. niger* (purple) An1-3, *A. tubingensis* (green) At1-3. The gel was prepared and imaged following method B (section 4.2.5, Table 4. 4) using Coomassie brilliant blue staining.

4.3.2 IgE reactivity of people with asthma to *Aspergillus* species

Immunoblot experiments were performed subjects with different levels of IgE reactivity to *A. fumigatus* (group 1-3, Table 4. 1, Fig. 4. 5 A-C, Appendix 6.10, Fig. A. 11 to Fig. A. 14). No reaction was observed from non-fungal sensitised people with asthma (group 3) (Fig. 4. 5 C). Only subjects with a low or high *A. fumigatus*-specific IgE reactivity (group 1 or 2, respectively) reacted to several proteins within the *A. fumigatus* extracts (Appendix 6.10, Fig. A. 11 to Fig. A. 13), yet only patients with high IgE levels to *A. fumigatus* (> 25 kU/L) (group 2) reacted with allergens from the *Aspergillus* section *Nigri* (Fig. 4. 5 B, Appendix 6.10, Fig. A. 12, Fig. A. 13). More than 10 bands were detected for *A. fumigatus*, where the most distinctive bands were observed at around 13, 15, 18, 23, 27, 30, 35, 40, 55, 60 and 100 kDa. Although the banding patterns between Af1 and Af2 was quite similar, subjects from group 2 reacted more prominently to the clinical isolate Af2, which was therefore used for subsequent experiments (Appendix 6.10, Fig. A. 12). In comparison, only six IgE-reactive protein bands were detected for the *Aspergillus* section *Nigri* at 15, 18, 23, 35, 50 and 100 kDa (Fig. 4. 5 B). As the 50 kDa band was only identified from *A. tubigensis* At2, this extract was used for subsequent experiments (Appendix 6.10, Fig. A. 12).

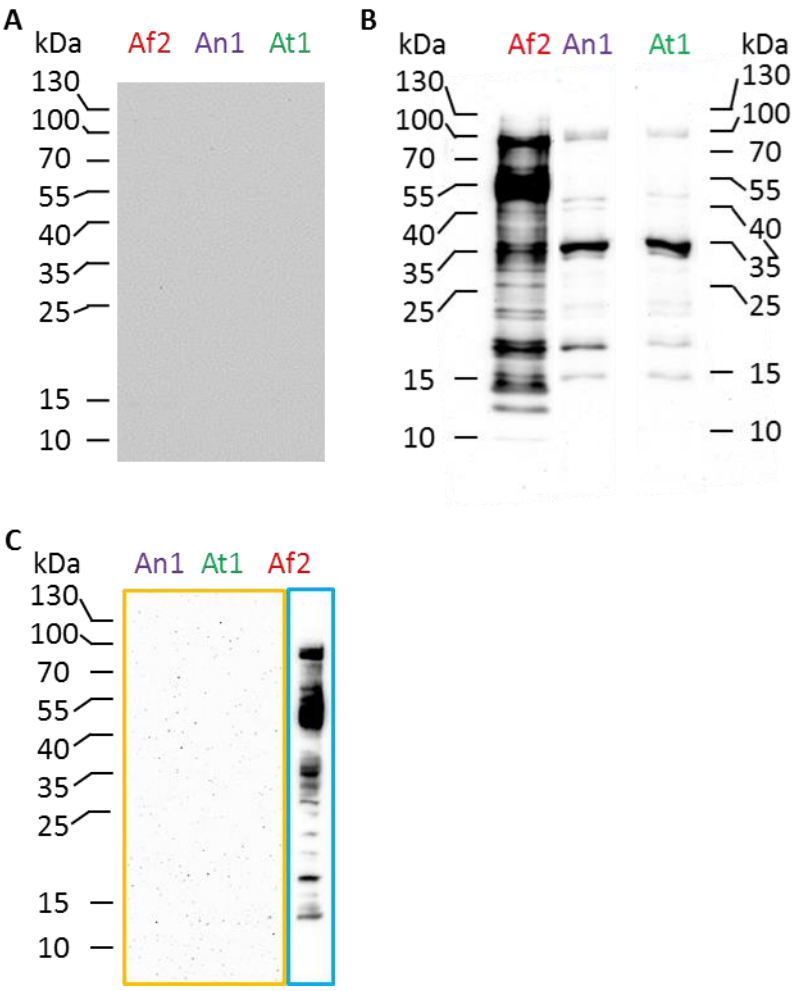


Fig. 4. 5: Immunoblots incubated with serum of asthmatics with varying IgE levels to *A. fumigatus*. Examples of protein extracts from *A. fumigatus* Af2 (red), *A. niger* An1 (purple) and *A. tubingensis* At1 (green) were loaded into the wells of an SDS-PAGE gel according to method B (section 4.2.5, Table 4. 4). After blotting, membranes were incubated with serum pools of asthmatics with *A. fumigatus*-specific IgEs (A) group 1 (> 1, < 5 kU/L), (B) group 2 (> 25 kU/L) and (C) group 3 (< 0.35 kU/L). A positive control was done for (C) (blue box), using serum of an *A. fumigatus*-sensitised patient R083 (66 kU/L specific IgE) for half of the Af2 lane. Blots were processed according to method C (section 4.2.6, Table 4. 5). Imaging times were (A) 25 min, (B) 25 min and (C) 30 min (orange box), except for the positive control (blue box), where the imaging time was 2 min.

4.3.3 IgE cross-reactivity between *Aspergillus* section *Nigri* and *A. fumigatus*

IgE cross-reactivity between allergens from the *Aspergillus* section *Nigri* and *A. fumigatus* was investigated using IgE inhibition immunoblot. When the serum of subject R083 (66 kU/L *A. fumigatus*-specific IgE) was pre-incubated with Af2, the IgE-reaction to the *Aspergillus* section *Nigri* species and the control *A. fumigatus* extracts was completely inhibited (Fig. 4. 6). This subjects' serum was chosen because it strongly reacted with Af2, An1 and At2 extracts and was available in high quantities (Appendix 6.11, Fig. A. 15).

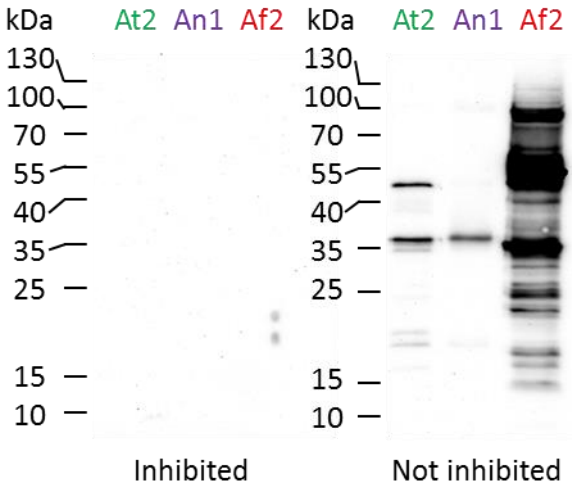


Fig. 4. 6: Inhibition blot and regular blot using serum from R083. Protein extracts from *A. fumigatus* (Af2), *A. niger* An1 and *A. tubingensis* At2 were loaded into the wells of an SDS-PAGE gel according to method B (section 4.2.5, Table 4. 4). Serum was either pre-incubated with buffer or 40 µg/ml of Af2 overnight. Blots were either incubated with the serum pre-incubated with Af2 (inhibited) or with buffer (not inhibited). The blots were processed according to method C (section 4.2.6, Table 4. 5) and imaged for 30 min.

As crude extracts might contain molecules that could interfere with IgE-binding to specific allergens, IgE cross-reactions to the recombinant *A. fumigatus* allergens rAsp f 3, rAsp f 4 and rAsp f 6 and the *Aspergillus* section *Nigri* species were determined. Sera from patients R083 and R084 were used for this experiment because high amounts of these sera were available, and they showed distinctive IgE-mediated reactions to the recombinant proteins as determined by ImmunoCAP (Chapter 2, 2.2, Table 4. 8), which

was confirmed by immunoblotting (Appendix 6.11, Fig. A. 16). The pre-incubation with the recombinant proteins inhibited IgE-binding to protein bands at 15, 18 and 23 kDa. The latter two are the respective molecular weights of rAsp f 3 (19 kDa) and rAsp f 6 (23 kDa). Inhibition by rAsp f 4 (30 kDa) was not detected (allergen.org, June 2018) [132]. This suggested that *A. tubingensis* and *A. niger* protein extracts contained orthologous proteins of the peroxisomal protein (Asp f 3) and MnSOD (Asp f 6), respectively (Fig. 4. 7).

Table 4. 8: ImmunoCAP values of patients R083 and R084, showing their IgE-reactivity to Asp f 3, Asp f 4 and Asp f 6.

	IgE (kU/L)	
	R083	**R084**
Asp f 3	73.4	12.9
Asp f 4	30.9	1.57
Asp f 6	8.66	0.00

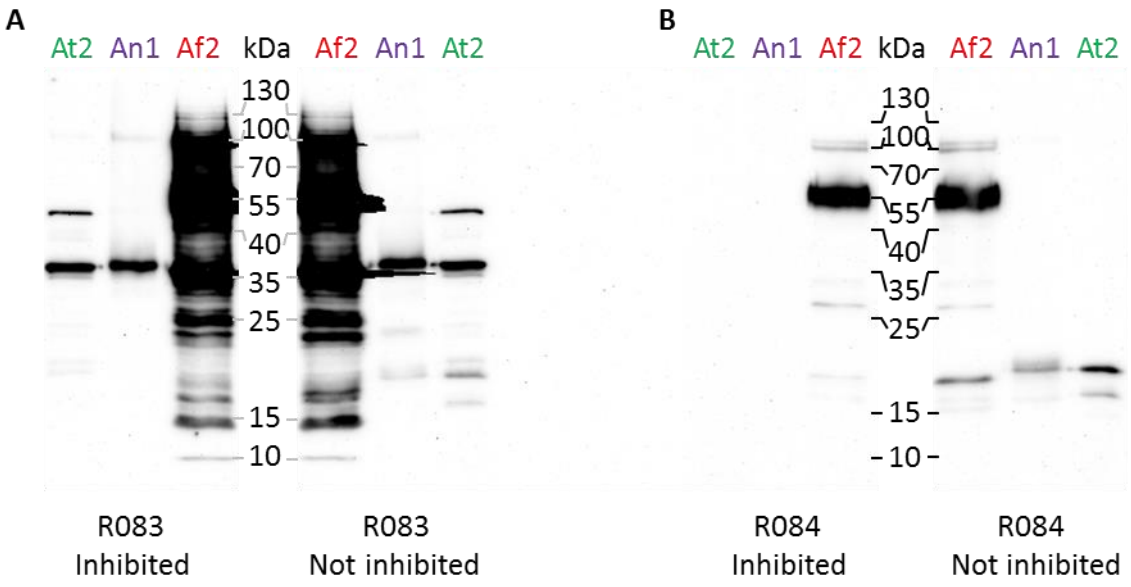


Fig. 4. 7: Inhibition blots using recombinant proteins of *A. fumigatus*. Protein extracts from *A. fumigatus* (Af2), *A. niger* An1 and *A. tubingensis* At2 were loaded into the wells of an SDS-PAGE gel according to method B (section 4.2.5, Table 4. 4). Serum of patient (A) R083 or (B) R084 was either pre-incubated with buffer or 10 µg/ml of each recombinant protein, rAsp f 3, rAsp f 4 and rAsp f 6 as a mixture overnight. Blots were either incubated with the serum pre-incubated with recombinant proteins (inhibited) or with buffer (not inhibited). The blots were processed according to method C (section 4.2.6, Table 4. 5) and imaged for 30 min.

4.3.3.1 Production of recombinant proteins

To produce large amounts of recombinant Asp f 3 and Asp f 6 for further experiments, bacteria (*E. coli* BL21) were transformed with the respective expression vectors containing the genes coding for Asp f 3 and Asp f 6 with C-terminal His-tags. After purification by nickel-NTA affinity chromatography, the concentrations of rAsp f 3 and rAsp f 6 were 3.77 µg/µl and 1.62 µg/µl, respectively. SDS-PAGE analysis showed that besides the protein bands at the respective molecular weights of Asp f 3 and Asp f 6, some weak additional protein bands with lower and higher molecular weights were present (Fig. 4. 8). The lower molecular weight bands were more prominent in each case, in particular at 10 kDa for rAsp f 3 or 10 kDa, 13 kDa and 18 kDa for rAsp f 6. Faint higher

molecular weight proteins were detected at about 38 kDa and 70 kDa, respectively, when high amounts of the recombinant proteins were loaded on the gels.

To determine whether the additional protein bands detected represent degradation or dimer or oligomer products of these His-tagged recombinant proteins, immunoblotting was performed using an anti-His antibody (section 4.2.6, Table 4. 5, Table 4. 6). The blots showed that the lower molecular weight protein bands also contained His-tags, indicating the presence of degradation products (Fig. 4. 9). Interestingly, a band at ~38 kDa was also detected by the anti-His immunoblotting for rAsp f 3, suggesting the presence of a dimeric form of the protein. The contaminants only represented a small proportion of the extract and were detectable when using high protein concentrations (5 µg and 10 µg).

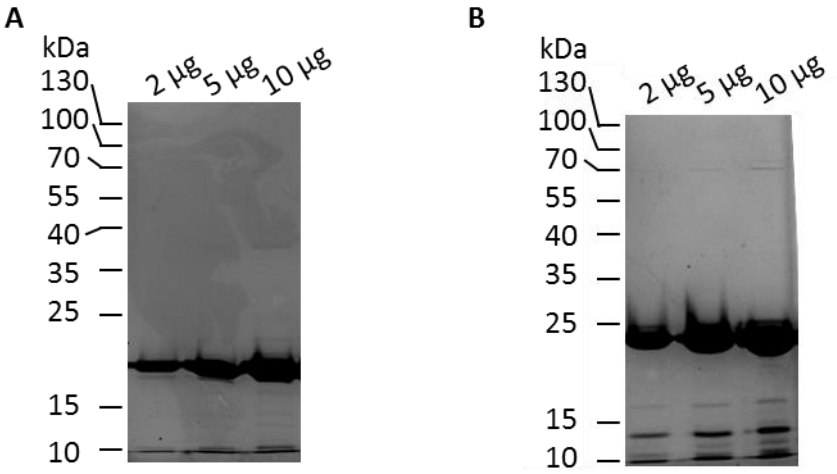


Fig. 4. 8: SDS-PAGE analysis of recombinant proteins rAsp f 3 and rAsp f 6 after purification. The gel was loaded with 2 µg, 5 µg and 10 µg of (A) rAsp f 3 or (B) rAsp f 6 according to method B (section 4.2.5, Table 4. 4) using Coomassie brilliant blue staining.

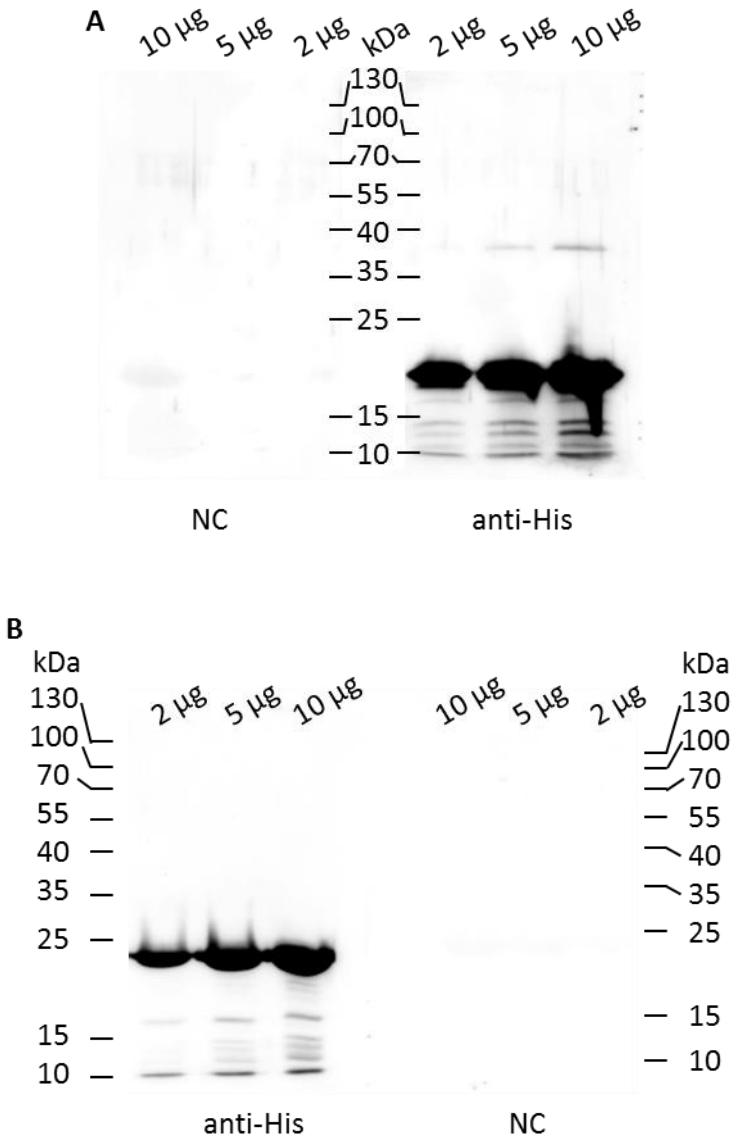


Fig. 4. 9: Anti-His-tag immunoblot. The gel was loaded with 2 µg, 5 µg and 10 µg of (A) rAsp f 3 or (B) rAsp f 6 according to method B (section 4.2.5, Table 4. 4). After blotting, the anti-His blot was incubated with an anti-His primary antibody before incubation with the secondary antibody. The negative control (NC) blot was incubated with the secondary antibody only. The blots were processed according to method C (section 4.2.6, Table 4. 5) and imaged for 2 min.

4.3.3.2 Identification of allergens by peptide mass fingerprinting using protein bands from SDS-PAGE gels

Based on the IgE cross-reactivity between the *Aspergillus* section *Nigri* fungi and *A. fumigatus*, the aim to identify unique proteins from *A. tubingensis* and/or *A. niger* was omitted and IgE cross-reactive proteins were further investigated. To identify the 35 kDa allergen and the other IgE-reactive proteins of *Aspergillus* section *Nigri* and *A. fumigatus,* protein bands were cut out from Coomassie-stained gel and sent for LC-MS/MS mass spectrometry analysis either to the VetCore facility (Vienna, Austria) or the PNACL facility (Leicester, UK) (section 4.2.9, Fig. 4. 10).

An overview of the total number of proteins detected in all bands of a respective species is shown in Table 4. 9. With the exception of *A. fumigatus*, more proteins were detected by the VetCore facility compared to the PNACL facility. It was apparent that more unknown proteins were detected in the *Aspergillus* section *Nigri* compared to *A. fumigatus*. The number of proteins detected for each fungus by both mass peptide fingerprinting facilities varied between 24 for *A. fumigatus*, 82 for *A. niger* and 85 for *A. tubingensis*. As the mass spectrometry results derived from gel bands yielded a huge number of potential allergen candidates, immunoprecipitation was performed to reduce this number (section 4.3.3.3).

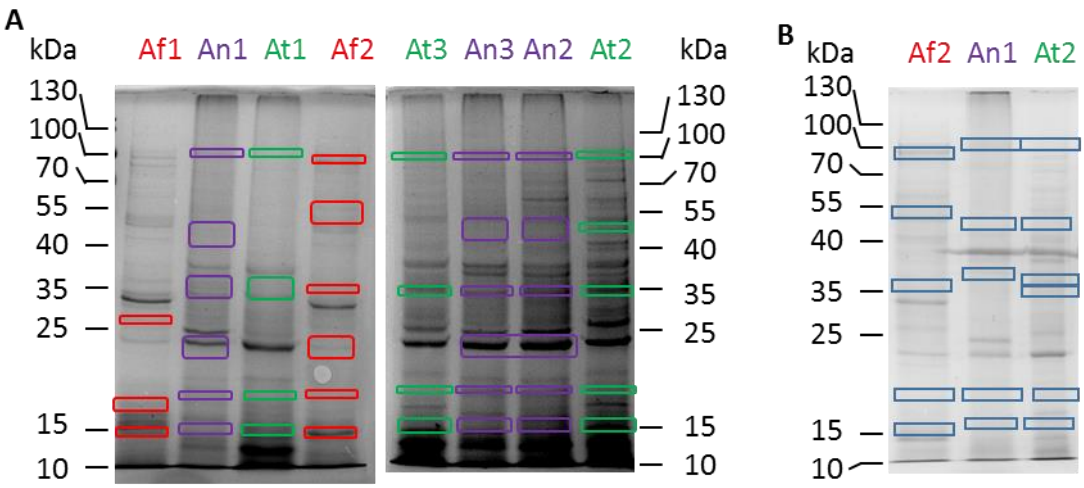


Fig. 4. 10: Bands of Coomassie-stained 12% SDS-PAGE gels sent to mass peptide fingerprinting at the (A) VetCore facility (Vienna, Austria) and (B) PNACL facility (University of Leicester, UK). Bands were excised at positions where IgE reactivity of detected (Fig. 4. 5): using *A. fumigatus* (red) Af1, Af2, *A. niger* (purple) An1-3, *A. tubingensis* (green) At1-3 protein extracts. (A) Bands from *A. tubingensis* and *A. niger* were combined before sample processing and mass peptide fingerprinting. (B) Imaged was obtained using a scanner.

Table 4. 9: Total number of proteins identified from all gel bands from *A. fumigatus*, *A. niger* and *A. tubingensis* by the VetCore (Vienna, Austria) and PNACL (Leicester, UK) facilities.

		A. fumigatus	*A. niger*	*A. tubingensis*
VetCore	Raw data total	134 (1 U)	1658 (833 U)	1811 (1273 U)
	After duplicate removal	54 (1 U)	Name: 923 (552 U)* Code: 940 (443 U)	Name: 997 (763 U)* Code: 965 (731 U)
PNACL	Raw data total	187 (6 U)	125	114 (66)
	After duplicate removal	155 (6 U)	92	84 (50 U)
Shared proteins	By name	24	N/A	50 (16 D)*
	By code	24	82 (34 U)	84 (50 U)

U = number of uncharacterised/unknown/unnamed proteins within total number of proteins, D = number of duplicates by name or code, * no duplicate removal of uncharacterised/unknown/unnamed proteins, N/A = not applicable as only putative name/gene region or function indicated

4.3.3.3 Immunoprecipitation as alternative to allergen characterisation

The proteins identified from the gel bands did not necessarily represent all the allergens that the subject/s reacted to. The aim of the immunoprecipitation experiments was to obtain only specific allergens from fungal crude extract using IgE antibodies from the serum of subject R083 who had a strong *A. fumigatus*-specific IgE-reactivity (Appendix 6.11, Fig. A. 15). After successful coupling of anti-human IgE antibodies to magnetic Dynabeads and purification of human IgE antibodies from serum (sections 4.2.8.1, 4.2.8.2, Fig. 4. 2), beads were then used to purify fungal proteins from a new crude extract of the clinical isolate of *A. fumigatus* (Af2*). A new extract was prepared to obtain sufficient amounts of protein to perform these experiments.

Several bands were detected in the immunoprecipitation eluate from *A. fumigatus* Af2* with molecular weights of approximately 170, 100, 70, 40, 35, 30, and 25 kDa (Fig. 4. 11). Immunoprecipitation products that had been spiked with rAsp f 3 (section 4.3.3.1) showed a similar banding pattern compared to the unspiked eluate, showing a more prominent staining at 37 kDa. The 18 kDa band representing rAsp f 3 was detected in

the supernatant and protein control, but a clear band within the immunoprecipitation eluate was not detected. Several very prominent bands were detected within the protein supernatant, which were not present within the eluates, indicating that not all allergens in the extract were harvested. The bands' intensities were comparable to the ones of the protein control, which was an amount of protein equivalent to the amount within the supernatant given that no proteins were precipitated. Bands detected in the first washes after the incubation with the protein extract showed bands at molecular weights of 35, 40 and 70 kDa. The wash from the rAsp f 3 spiked assay showed an IgE-reactive band at 18 kDa. As these bands were also detected in the respective eluates, it is possible that some of the bound proteins were washed away during the washing steps. Pure human IgE, which was used as a secondary antibody control, was detected as well. Therefore, it can be assumed that the band at around 70 kDa within the immunoprecipitation eluates represented denatured human IgE co-eluted from the magnetic beads.

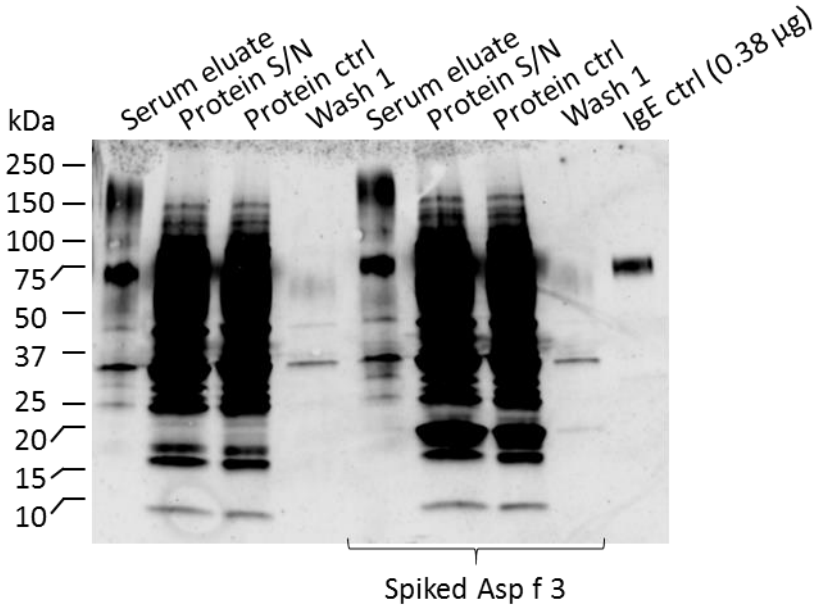


Fig. 4. 11: Immunoblot showing immunoprecipitation results for Af2*. Gels were loaded according to method A (section 4.2.5, Table 4. 4) using the elution from Dynabeads with bound serum IgE and fungal proteins (serum eluate); the protein supernatant (S/N) left after incubating the IgG-IgE magnetic beads with 40 µg/ml of protein extract Af2*; protein control (ctrl), representing an equivalent amount of protein present in the protein S/N if no protein would have been bound; first wash (wash 1) after retrieving the protein S/N from the beads; human IgE control (ctrl) (0.38 µg) as equivalent amount to be expected in the eluent. Protein extract was untreated or spiked with 10 µg/ml of recombinant Asp f 3. The chemiluminescent immunoblot was performed according to method A (section 4.2.6, Table 4. 5). The blot was imaged for 2 min using the signal accumulation mode (SAM).

Proteins from *A. fumigatus* were immunoprecipitated again to be sent for peptide mass fingerprinting. The same procedure was used as before, except around 73% was eluted using a volatile substance suitable for mass peptide fingerprinting. The smaller elution volume was used for a control blot to check whether results were reproducible. Given the smaller sample volume (20% less than used previously), bands were fainter but showed the same most prominent bands (Appendix 6.11, Fig. A. 17). Similar to the previous blot, the results indicated that material might have been washed away during the washing steps and most of the proteins did not seem to be immunoprecipitated.

The other, larger eluate was sent for mass peptide fingerprinting, which identified 38 proteins (IP-based proteins) (Table 4. 10). Eighteen of the proteins identified matched the 24 proteins that were identified from gel bands by both mass spectrometry facilities referred to as gel-based proteins (Table 4. 9, Table 4. 10). Interestingly, an extra number of 15 proteins of the IP-based proteins matched only gel-based proteins identified by the VetCore facility (Table 4. 10), and only one by the PNACL facility. Five protein codes were only found in the immunoprecipitate, including the isochorismatase family hydrolase (putative), methyltransferase GliN, Actin Act1, the 60S ribosomal proteins L22 and L36. The first protein name was also listed in the VetCore results, but the protein codes did not match. Gel-based proteins that were only identified by both mass spectrometry facilities included the 14-3-3 family protein ArtA (putative), malate dehydrogenase, O-methyltransferase GliM-like (putative), and the 60S ribosomal proteins L5 (putative), L20 and L27 (Table 4. 10).

For all IP-based proteins detected, the exclusive unique peptide counts ranged from 3 to 28 and the coverage ranged from 3.77% to 74%. A higher peptide count did not indicate a higher coverage. Peptides with the highest exclusive unique peptide counts (> 10) were alanine aminotransferase, cobalamin-independent methionine synthase, coproporphyrinogen III oxidase, fucose-specific lectin FleA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), malate dehydrogenase (MDH), phosphoglycerate kinase and translation elongation factor eEF-1 subunit (eTEF 1β). The coverage for these proteins ranged from 24.2% to 74%. Molecular weights ranged from 34.7 to 86.9 kDa (all detected proteins from 13.4 to 131.3 kDa) and most matched the bands detected by immunoblotting. Some IP-based proteins did not have the exact molecular weight as detected by immunoblotting. For example, from the six proteins detected between 70 and 131.3 kDa, the heat shock protein (HSP) 90 and the ATP citrate lyase have known molecular weights of around 80 kDa, which were not apparent in the immunoblot (Fig. 4. 11). Both their peptide counts and their sequence coverage values were low with 3% and around 6%, respectively. Interestingly, the HSP90 was also detected by the gel-based protein identification approach. The output by both facilities showed a low sequence coverage (< 16%), but the number of unique peptides was higher with 12 confident peptides detected by the Vetcore mass peptide fingerprinting. The low coverage and amount of unique peptides could indicate that only fragments of HSP90

and ATP citrate lyase were detected. This was supported by the observation that several proteins were identified from multiple gel bands cut at different molecular weights (Fig. 4. 10) (data not shown). For example, HSP90 was identified in bands excised at 13, 18, 23, 35 and 55 kDa. The highest unused ProtScore obtained from the VetCore facility presented in Table 4. 10 derived from the 35 kDa band. It was also detected in the 35 kDa band used for mass peptide fingerprinting at the PNACL facility.

Table 4. 10: List of proteins with their respective UniProt accession number and molecular weight identified from immunoprecipitation using R083 and Af2* protein extract (80 µg) by peptide mass fingerprinting at the PNACL facility (Leicester, UK) (IP PNACL). In comparison, the proteins from Af2 gel bands identified by both PNACL and VetCore (Vienna, Austria) are presented (gel-based). The proteins from the gel-based identification matched the codes or names from the proteins identified from IP PNACL. Also shown are matching gel-based identifications from both facilities. "x" marks identical protein names from gel-based *A. tubingensis* or *A. niger* identification by both facilities used.

			IP PNACL		**Gel-based PNACL**		**Gel-based VetCore‡**			**Gel-based**	
Protein name	**Protein accession numbers**	**Molecular weight (Da)**	**Exclusive unique peptide count***	**Percentage sequence coverage**	**Exclusive unique peptide count***	**Percentage sequence coverage**	**Unused ProtScore****	**Confident Peptides*****	**PercentageSeq Cov - peptides >95% conf**	***A. niger* match**	***A. tubingensis* match**
14-3-3 family protein	Q4WND4	30,104.20	3	16.60%	2	33.20%	15.23	12	31.7%	x	x
6-phosphogluconate dehydrogenase, decarboxylating	Q4WN06	55,801.60	6	17.70%	10	36.40%	14.00	9	18.9%	x	x
Adenosylhomocysteinase	Q4WT91	48,490.40	3	11.90%	4	14.60%	5.02	3	7.8%	x	
Alanine aminotransferase, putative	Q4WN34	55,135.60	12	31.50%	9	27.90%	8.00	4	10.6%		
Allergen, putative	Q4WE17	21,951.00	4	23.80%	7	55.00%	10.00	6	27.2%		x

Cobalamin-independent methionine synthase MetH/D	Q4WNY2	86,895.70	12	24.20%	19	39.80%	18.00	11	18.0%	x	x
Eukaryotic translation elongation factor 1 subunit Eef1-beta, putative	Q4WSY6	25,245.30	5	41.40%	8	51.50%	4.00	2	16.7%	x	x
Fructose-bisphosphate aldolase, class II	Q4WY39	39,791.60	8	24.20%	2	9.44%	2.00	1	3.1%		x
Glyceraldehyde-3-phosphate dehydrogenase	Q4WE70	36,314.70	13	45.90%	6	24.90%	4.66	4	12.4%	x	
Heat shock protein 90	P40292	80,643.60	3	5.67%	1	8.36%	19.87	12	15.6%	x	x
Malate dehydrogenase, NAD-dependent	Q4WDM0	34,810.60	12	52.40%	6	27.00%	2.00	1	6.4%	x	
Mannitol-1-phosphate 5-dehydrogenase	Q4X1A4	43,004.40	7	22.20%	9	30.40%	10.00	5	13.7%		
Molecular chaperone Hsp70	Q4WJ30	69,662.10	7	16.60%	10	30.10%	21.77	11	21.6%	x	x
Phosphoglycerate kinase	Q4WT69	44,763.10	12	31.20%	3	7.91%	4.01	2	4.3%		
Putative peroxiredoxin pmp20	O43099	18,453.50	7	43.50%	3	29.80%	14.05	9	45.8%	x	x
Transketolase	Q4WSA0	74,829.10	9	19.00%	8	15.90%	9.89	5	8.5%		
Translation elongation factor eEF-1 subunit gamma, putative	Q4WDF5	54,184.20	17	35.10%	15	42.30%	28.00	23	29.7%		x

UDP-glucose 4-epimerase	Q4WV46	40,609.70	3	13.20%	3	9.70%	1.24	1	2.4%		
14-3-3 family protein ArtA, putative	Q4WI29	29,102.40			11	69.70%	36.00	22	68.6%	x	x
60S ribosomal protein L20	Q4WJW9	17,404.20			3	40.50%	10.94	6	45.3%		x
60S ribosomal protein L27	Q4WJD7	21,450.80			3	22.60%	8.08	6	23.7%		
60S ribosomal protein L5, putative	Q4WSG1	35,484.60			3	16.90%	2.00	1	2.9%		
Malate dehydrogenase	Q4WGP3	35,898.60			13	58.50%	40.00	34	71.8%	x	x
O-methyltransferase GliM-like, putative	Q4WYG1	48,678.80			11	40.00%	7.47	8	11.1%		
Uncharacterized protein	Q4WQH8	25,033.80	3	20.10%			5.35	4	19.2%		
Aspartate transaminase, putative	Q4X1K5	51,184.00	6	18.60%			9.54	6	11.9%		
ATP citrate lyase, subunit 1, putative	Q4WM99	78,831.50	3	6.49%			2.00	1	1.9%		
Coproporphyrinogen III oxidase, putative	Q4WJ26	50,180.00	14	33.20%			1.42	1	2.3%	x	
Elongation factor 1-alpha	Q4WJD2	53,842.80	9	26.10%			4.82	3	5.5%		x
Fucose-specific lectin FleA	Q4WW81	34,661.50	28	74.00%			18.00	18	37.1%		
Glucose-6-phosphate isomerase	Q4X1J1	61,316.80	8	18.80%			16.65	9	20.8%		x
Histone H2A	Q4WWC6	14,092.90	3	29.30%			4.00	2	13.5%		

Histone H2B	Q4WWC5	14,955.90	3	25.00%			3.17	2	14.3%		
Isochorismatase family hydrolase, putative	Q4WLV1	20,904.80	3	26.80%			2.00	2	7.0%		
Methyltransferase GliN	Q4WMJ1	32,189.30	4	15.60%							
NAD-dependent formate dehydrogenase AciA/Fdh	Q4WDJ0	45,746.00	3	11.00%			9.44	6	14.1%		
Phosphoglucomutase	Q4WY53	60,503.40	5	12.80%			20.50	12	20.9%		
Phosphoribosyl-AMP cyclohydrolase, putative	Q4WRZ4	92,882.10	4	7.11%			2.00	1	1.1%		
Pyruvate carboxylase	Q4WP18	131,275.6	3	3.77%			4.19	2	3.4%		x
Actin Act1	Q4WDH2	43,893.80	5	22.40%							
60S ribosomal protein L22, putative	Q4WYA0	13,422.40	3	35.00%							
60S ribosomal protein L36	Q4WNZ0	19,006.20	4	20.50%							
60S ribosomal protein L4, putative	Q4WEH4	40,504.20	3	11.30%			12.00	6	20.9%		
O-methyltransferase, putative	Q4WED7	47,751.10	3	13.70%	7	26.90%					

‡Presented are the highest Unused ProtScore, confident peptides and sequence coverage for a protein irrespective of the molecular weight that the respective band was excised.

*Exclusive unique peptide count reflects the number of different amino acid sequences that are associated only with the respective protein.

**Unused ProtScore reflects the amount of total, unique peptide evidence that supports the presence of a given protein

*** Confident peptides: Peptide confidence is based on the number of matches between the data and the theoretical fragment ions (FDR).

Protein Thresholds for PNACL data 5% FDR and 3 peptides minimum, 1% FDR for VetCore data.

Potential IgE cross-reactive proteins were assessed using sequence comparisons of the proteins identified from immunoprecipitation to known allergens using the UniProt codes. Table 4. 11 illustrates search hits for the immunprecipitated proteins in the WHO/IUIS list (June 2018) [132], Allergen Online [399], Structural Database of Allergenic Proteins (SDAP) [400, 401] and to some extent Allergome [406-408]. Proteins presented in the WHO/IUIS, Allergen Online or SDAP databases are referred to as listed proteins. These different approaches were used to account for differences between algorithms and listed proteins in the databases. The searches identified the percent similarity and identity to known allergens. The main purpose of these searches was to identify proteins that have more cross-reactive potential than others. This may indicate that orthologues of these allergens could be present in the *Aspergillus* section *Nigri*, of which only three allergens are listed by the WHO/IUIS (June 2018) [132].

In comparison with the WHO/IUIS list of *A. fumigatus* allergens [132], the only known allergens detected from the immunoprecipitate were the peroxiredoxin Asp f 3 (O43099, Q4WE17) and the HSP90 Asp f 12 (P40292). Some of the other identified proteins are known allergens in other fungal species or even Kingdoms, which could allow for antibody cross-reactions (Table 4. 11). It remains to be elucidated whether the 13 proteins identified from *A. niger* and 16 from *A. tubingensis* that matched the IP-based or gel-based proteins identified from *A. fumigatus* could be potential candidates of IgE cross-reactive allergens (Table 4. 10). Several IP-based proteins identified from *A. fumigatus* showed cross-reactive potential. Most of these proteins had high sequence identity to one or two known allergens from species of the kingdom of fungi and/or plants. The NAD-dependent malate dehydrogenase was > 50% identical to an unlisted malate dehydrogenase from *A. fumigatus* and the listed Mala f 4 from *M. furfur*. A high sequence identity (~68%) from *A. fumigatus* GAPDH to Tri a 34 from wheat (*Triticum aestivum*) was detected using all databases. Similarly, protein sequences of the eukaryotic translation elongation factor 1 subunit Eef1-beta (eTEF 1 β) showed 78.1% identity between *A. fumigatus* and *Penicillium citrinum*. Some of the *A. fumigatus* proteins identified from the immunoprecipitate including the fructose-bisphosphate aldolase and formate dehydrogenase matched allergens that have not been officially approved sharing > 50% of their sequences with *Candida* species.

The highest cross-reactive potential was detected for the peroxysomal protein Asp f 3 and HSP70 identified from the *A. fumigatus* immunoprecipitate. As expected Asp f 3 (O43099) was 100% identical to the peroxisomal protein (O43099) detected in the immunoprecipitate. A putative allergen (Q4WE17) also showed a high sequence identity to this known *A. fumigatus* allergen. Both the peroxisomal protein and the HSP70 had high sequence similarities to *Penicillium citrinum*, which belongs to the same class as *A. fumigatus* [7]. The respective proteins' sequences were ~39% and ~29% identical to sequences to *Basidiomycetes* (*Malassezia* species). The low sequence identity between the *A. fumigatus* HSP70 and Alt a 3 (14.4%), which was mostly attributable to the different sequence lengths. The overlapping regions of the two proteins were highly similar as shown by the Allergen Online FASTA and 80mer results, which are focussed on the amino acid overlap (data not shown). Although Mala s 10 (HSP88) showed low sequence identity with the *A. fumigatus* HSP70, the 80mer analysis of Allergen Online indicated a sequential and therefore structural overlap. High sequence identities of ≥ 60% were also detected between *A. fumigatus* HSP70 or Asp f 3 and other kingdoms such as plants (hazelnut (*Corylus avellana*)) and animals (mites (*Dermatophagoides farina*, *Tyrophagus putrescentiae*), insects *Aedes aegypi*). However, these matches only derived from either of the databases Allergen Online and SDAP.

Table 4. 11: Immunoprecipitation results were compared and searched within the IUIS/WHO list [132] (allergen.org), Allergen Online (FASTA Version 35.04) [399, 409] or the Structural Database of Allergenic Proteins (SDAP) (FASTA 3.45) [400, 401]. Searches within the latter two not only included FASTA search, but also 80mer alignments (Codex Alinorm 03/34, 2003) [402], where Allergen Online uses FASTA3. This table only lists those results with > 35% sequence ID and/or those with positive 80mer (> 35% ID within 80 amino acids), except for Mala s 10 and Cand b 2 due to reported cross-reactions in the literature [135, 151]. Additionally, the protein sequences were compared with EMBOSS Needleman-Wunsch algorithm (ebi.ac.uk/Tools/psa/) [403-405].

***A. fumigatus* immunprecipitated proteins**		**Allergens identified in searches**				**Needleman-Wunsch**		**Allergen Online FASTA within overlap**			**SDAP FASTA**		
Name	**UniProt**	**Species**	**Name**	**Code**	**aa**	**ID %**	**Sim %**	**ID %**	**Sim %**	**80mer**	**ID %**	**aa match**	**80mer**
HSP70 (638 aa)	Q4WJ30	*C. herbarum*	Cla HSP70‡	P40918	643	82.9	90.5	84.1	93.6	Y	84.48	539	Y
		P. citrinum	Pen c 19	Q92260	503	72.3	75.1	90.7	96.1	Y	72.26	461	Y
		A. alternata	Alt a 3	P78983	152	14.4	17.5	66.9	0.875	Y	14.58	93	N
		Corylus avellana	Cor a 10	CAC14168	668	58.3	73.9	-	-	-	61.91	395	N
		Dermatophagoides farinae	Der f 28.0101	AGC56218.1/ AIO08848.1	654	66.7	72.3	74.6	89.9	Y	-	-	-
		M. sympodialis	Mala s 10*	Q8TGH3/CAD20981	773	26.4	41.9	30.3	62.8	Y	31.50	201	N
			N/A	CCU97864.1	773	26.5	42	30.4	63.0	Y	-	-	-
		Aedes aegypti	Aed a 8.0101	ABF18258.1	655	60.9	74.8	64.8	86.1	Y	-	-	-

		Tyrophagus putrescentiae	Tyr p 28.0101	AOD75395.1	659	75.9	85.2	79.6	92.1	Y	-	-	-
Malate dehydrogenase (330 aa)	Q4WDM0	*M. furfur*	Mala f 4	Q9Y750/AAD25927.1	342	50.7	63.3	55.7	78.6	Y	55.15	182	Y
		A. fumigatus	Asp f MDH‡	Q4WGP3	340	54.3	68.8	-	-	-	-	-	-
MetE (774 aa)	Q4WNY2	*Salsola kali*	Sal k 3	C1KEU0 / ACO34814	757	47.0	63.2	48.0	73.8	Y	47.80	370	Y
eTEF 1β (227 aa)	Q4WSY6	*P. citrinum*	Pen c 24	Q69BZ7/AAR17475	228	78.1	88.6	78.1	91.7	Y	78.41	178	Y
GAPDH (338 aa)	Q4WE70	*Triticum aestivum*	Tri a 34	C7C4X1/CAZ76054	337	67.4	80.3	68.2	88.7	Y	67.75	229	Y
		Aedes aegypti	N/A‡	J9HYM2	332	66.6	78.4	-	-	-	-	-	-
Allergen, putative (202 aa)	Q4WE17	*A. fumigatus*	Asp f 3	AAB95638.1	168	55.7	66.0	68.5	89.1	Y	55.94	113	Y
		P. citrinum	Pen c 3	AAD42074.1	167	54.2	66.0	65.9	87.4	Y	54.46	110	Y
		M. sympodialis	Mala s 5	CAA09883	172	34.1	46.4	41.1	70.9	Y	35.64	72	N
		M. furfur	Mala f 3	P56578/ BAA32436.1	166	32.5	48.2	40.5	72.0	Y	33.66	68	N
			Mala f 2	P56577/BAA32435.1	177	31.5	44.3	38.5	69.5	Y	33.17	67	N
Pmp20 (Asp f 3) (168 aa)	O43099	*A. fumigatus*	Asp f 3	O43099/ AAB95638.1	168	100	100	100.0	100.0	Y	100.00	168	Y
		P. citrinum	Pen c 3	AAD42074	167	81.0	87.5	82.4	92.1	Y	80.95	136	N
		M. furfur	Mala f 3	P56578/BAA32436.1	166	37.3	57.6	38.1	73.2	Y	38.00	64	N
			Mala f 2	P56577/BAA32435.1	177	38.7	58.6	41.7	72.0	Y	41.67	70	N
		M. sympodialis	Mala s 5	CAA09883	172	42.1	57.4	43.6	70.9	Y	44.05	74	N

		C. bondii	Cand b 2	AAA34358/AAA34357	167	33.5	54.5	-	-	-	36.31	61	N
		Blattella germanica	Bla g 1	AAD13530.2	412	9.5	17.8	35.6	62.2	N	-	-	-
Fructose-bisphosphate aldolase (360 aa)	Q4WY39	*C. albicans*	Cand a FPA**‡	C4YHS0/ Q9URB4	359	70.5	79.9	-	-	-	-	-	-
		Rhizopus arrhizus	N/A**‡	I1CF64	358	62.5	78.1	-	-	-	-	-	-
Formate dehydrogenase (418 aa)	Q4WDJ0	*C. bondii*	Cand b FDH‡	O13437	364	55.8	69.9	-	-	-	-	-	-

aa = amino acids, eTEF = eukaryotic translation elongation factor subunit Eef1-beta, HSP = heat shock protein, MetE = Cobalamin-independent methionine synthase, GAPDH = Glyceraldehyde-3-phosphate dehydrogenase,

*putative heat shock protein, which had been listed as HSP70 [410], but was characterised as HSP88 later on [411], **predicted proteins, ‡Not listed by the WHO/IUIS (allergen.org, June 2018) [132]

4.4 Discussion

The results presented in Chapter 3 showed a potential role of *A. tubingensis* in asthma, which has yet to be determined. The fungus particularly detected in lower respiratory tract samples, particularly from people with asthma (Chapter 3, 3.3.4). It belongs to the *Aspergillus* section *Nigri* along with its close relative *A. niger*, which has been associated with ABPM [175]. Fungi of this section were cultured from sputum samples of people with moderate-to-severe asthma or COPD in previous studies [137, 372]. They could therefore represent a permanent allergen source. Sensitisation is usually assessed by fungal extracts, but they lack standardization, which means variations from batch-to-batch and between suppliers occur [26, 144]. Diagnosis of fungal sensitisation is further complicated by IgE cross-reactions to different allergens, which makes it difficult to interpret the clinical relevance of specific fungi for example in asthma. Therefore, standardised recombinant allergens have distinct advantages allowing for more specific diagnosis. CRD is now widespread in areas of food and aeroallergens but is still uncommon in fungal allergy. Only three allergens are known for *A. niger* and none for *A. tubingensis* [132] (http://www.allergen.org, Feb 2018). An aim of this study was to characterise unique allergens from these fungi using clinical isolates from people with asthma. The hypothesis was that these two fungi as representatives of the *Aspergillus* section *Nigri* express unique allergens compared to *A. fumigatus*. *A. fumigatus* was used as a reference due to its relationship with the *Aspergillus* section *Nigri* [373, 374].

4.4.1 *Aspergillus* species selection and *Aspergillus* sensitisation of people with asthma

4.4.1.1 Characterisation and protein extraction of *Aspergillus* species

The first aim was to characterise clinical isolates of *Aspergillus* section *Nigri* fungi and extract proteins. *A. tubingensis* and *A. niger* are cryptic species, meaning that the two species are morphologically indistinguishable. Species identification can be done based on RFLP from the ITS region. The restriction enzymes *Hha*I and *Nla*III enable a discrimination between *A. niger* and *A. tubingensis* versus *A. tubingensis*-like and *A. carbonarius coccodes* species, whereas *A. tubingensis* and *A. niger* can be distinguished using *Rsa*I [375, 376]. It is more common to sequence the β-tubulin or calmodulin region

[373, 412, 413]. This study was limited with respect to the clinical isolates from black fungi of the *Aspergillus* section *Nigri* recovered from the subjects recruited (Chapter 3, 3.3.5) and therefore other clinical isolates derived from sputum culture of people with asthma had to be used. To account for discrepancies between different isolates, three isolates of each *A. tubingensis* and *A. niger* were selected. Although RFLP analysis was only applied for three of the six samples used in this study, the results were 100% concordant with the β-tubulin sequencing results. To our knowledge, a direct comparison of the RFLP and β-tubulin sequencing identification methods has not been done previously. The results showed that both methods are equally suitable to discriminate these species and sequencing of the β-tubulin region was used for the remaining samples. Protein extracts of these clinical isolates, the *A. fumigatus* type strain NCPF 7097 and an *A. fumigatus* clinical isolate were successfully prepared, therefore fulfilling this aim of the present study.

4.4.1.2 IgE-reactivity of people with asthma to *Aspergillus* species

The next aim was to test how people with asthma that had varying levels of *A. fumigatus*-specific IgE would react to the *A. fumigatus* species and the *Aspergillus* section *Nigri* fungi tested. *A. fumigatus* was used as a control. IgE immunoblots using protein extracts showed that **only patients with high levels of *A. fumigatus*-specific IgE reacted to protein extracts from clinical isolates of the *Aspergillus* section *Nigri***. The results indicated that IgE cross-reactions to the different *Aspergillus* species was possible. The number of bands detected for these isolates was lower compared to those observed for the *A. fumigatus* species (Fig. 4. 4), which could be due to differences in culture times, the slightly different culture conditions used and the choice of the strains (Table 4. 7), all of which can affect protein expression [26, 144, 161]. Whilst it cannot be excluded that the culture and extraction conditions could have affected the results, allergens from *A. niger* and *A. tubingensis* were detected nonetheless represented by six bands in the immunoblot, which were further assessed. The aim of this experiment was therefore fulfilled.

4.4.2 Characterisation of *Aspergillus* allergens

4.4.2.1 IgE cross-reactions between allergens from different *Aspergillus* species

Investigating IgE cross-reactions to the different allergens detected from the different *Aspergillus* species is a way to identify homologous allergens. Since *A. fumigatus* and *Aspergillus* section *Nigri* are closely related species [373, 374], IgE cross-reactions are likely to occur, but only one cross-reactive protein, the vacuolar serine protease (Asp f 18 and Asp n 18), was previously reported [414, 415]. IgE cross-reactions between these fungi were indicated by the fact that only subjects with high *A. fumigatus*-specific IgE levels reacted to *A. niger* and *A. tubingensis*. This was supported by IgE inhibition immunoblots as IgE-reactions to the *Aspergillus* section *Nigri* were inhibited when sera were pre-incubated with *A. fumigatus* extracts. **Specific homolgous allergens could be identified using inhibition blots with recombinant rAsp f 3, a peroxysomal protein [151], and rAsp f 6, a MnSOD [416].** IgE reactions to the 15, 18 and 23 kDa from *Aspergillus* section *Nigri* species were inhibited. To our knowledge, these IgE cross-reactions have not been reported in the literature to date. IgE cross-reactions to Asp f 3 are particularly interesting as they were previously associated with bronchiectasis in people with AFAD [165]. The allergens Asp f 3 and Asp f 6 were subsequently produced on a large scale. Although impurities were detected by SDS-PAGE, anti-His immunoblotting showed that most of these proteins were degradation products (Fig. 4. 8, Fig. 4. 9). Impurities at high molecular weights were only detected at higher concentrations. Due to their low amounts, it is unlikely that the impurities would have affected any subsequent immuno-based experiments. Interestingly, the anti-His tag blot for rAsp f 3 was suggestive of a dimeric form of the protein, which had been reported for the orthologue Cand b 2 from *Candida bondii* [151] and might also occur in case of Asp f 3 from *A. fumigatus*. **These experiments indicated IgE cross-reactions to all three *Aspergillus* species examined.** Inhibition of IgE binding to *Aspergillus* section *Nigri* allergens by pre-incubation with the whole *A. fumigatus* extract suggested that all *Aspergillus* section *Nigri* allergens are IgE cross-reactive to allergens present in *A. fumigatus*. Specific *A. fumigatus* allergens were identified, Asp f 3 and Asp f 6, which cross-reacted with the *Aspergillus* section *Nigri* species used in this study. These results suggest that the *Aspergillus* section *Nigri* fungi could be allergen sources in the lungs of

people with asthma and may promote the Type I inflammatory response, perhaps in addition to other allergen sources such as *A. fumigatus*.

The clinical relevance of IgE cross-reactions is manifold. Antibody cross-reactions supposedly complicate diagnosis as to identify a specific fungus related to a clinical outcome [146], however, they were shown to improve diagnosis of fungus-specific sensitisation in combination with unique allergens [167]. IgE cross-reactive allergens could contribute to the observation that patients sensitised to fungi are often (> 50%) polysensitised to multiple fungi like the patient used for the inhibition blot and immunoprecipitation experiments. In this study, 74% patients were co-sensitised to > 1 fungus (Chapter 3, 3.3.1). Monosensitisation to *Alternaria* or *Candida* is more common in contrast to *A. fumigatus*, *C. herbarum*, *P. notatum*, and *S. cerevisiae* (< 1%), where polysensitisation occurs more often [115, 131, 154]. Another interesting point of IgE cross-reactions are those between fungi and self-antigens as previously demonstrated for MnSOD [416, 417]. These self-antigens are believed to be clinically relevant by causing type I hypersensitivity responses even in absence of the environmental allergen and contributing to the chronic symptoms observed in long-lasting diseases such as ABPA. These proteins are stress-induced cytoplasmic proteins, which are usually not secreted and therefore do not interact with antibodies, but they could be released from tissue damaged by continuous inflammation as present in asthma [147, 148, 416]. The identification of common allergens and their epitopes could be helpful in designing anti-fungal therapy with for example immunotherapy or biomolecules. This might be able to neutralize autoimmunity and result in symptom relief in chronic diseases. They could also help to assess the likelihood of sensitisation to multiple fungi and other kingdoms, as well as autoimmunity and its contribution to the disease. In addition, by ruling out IgE cross-reactive proteins, unique allergens can be more easily identified, which enable a specific diagnosis of fungal sensitisation. Despite the advantages of identifying both unique and cross-reactive allergens, only about twenty proteins are known to cross-react, most of which are located in the cytoplasm, whereas species-specific allergens tend to be secreted [135, 148, 418, 419]. Therefore, the original aim to identify unique allergens from *A. tubingensis* or *A. niger* was omitted and IgE cross-reactive allergens further elucidated using mass peptide fingerprinting.

4.4.2.1.1 Gel-based protein identification

Mass peptide fingerprinting is the most common way to characterise proteins as it allows protein identification from complex samples and is sensitive enough to detect low abundant proteins [162, 420-422]. The gel-based protein identification by peptide mass fingerprinting yielded a plethora of different potential allergens present (Table 4. 9). These data are hard to interpret: First, many proteins are listed with unknown biochemical function, making it difficult to compare the mass spectrometry data from the different facilities and finding mRNA sequences for amplification. Most unknown proteins were detected from the *Aspergillus* section *Nigri* fungi, which can be explained by the fact that the identification of the fungal proteome is somewhat biased towards certain species. For example, in 2011, < 10% of the predicted *A. fumigatus* proteome was known [423], whereas around > 60% has been characterised from *Saccharomyces cerevisiae* [424, 425]. The current number of identified proteins from the *A. fumigatus* reference proteome listed by UniProt [394] is 9648 proteins (8% reviewed, UP000002530, Apr 2018), 6035 for *C. albicans* (16.6% reviewed, UP000000559, Apr 2018), 6049 for *S. cerevisiae* (100% reviewed, UP000002311, Apr 2018). In comparison, 14,068 proteins *A. niger* (2.5% reviewed, UP000006706, Aug 2018) and 12,319 *A. tubingensis* (all unreviewed, UP000184304, Aug 2018) are listed. These numbers illustrate that a lot more work has to be done to characterise the proteome of filamentous fungi, which are lagging behind the *Saccharomycetes*.

Second, proteins have to be considered with bigger or smaller molecular weights than the respective bands detected based on factors such as presence of signal peptide and propeptide sequences, glycosylation, domains cut off or partial degradation. An example of the latter was HSP90 from *A. fumigatus* (Asp f 12), which was found at different molecular weights, most prominently from the 35 kDa band. This suggests that not all of the protein bands detected in the immunoblotting experiments represented unique allergens and could have contributed to the strong reaction observed for *A. fumigatus* (Fig. 4. 5, Fig. 4. 10).

Third, the identification of the proteins depended on the way the gel had been cut, the mass analyser and the algorithm to detect the proteins. Each mass analyser used in this study had its own specific resolution and sensitivity (Appendix 6.12). In addition, both

different protein identification software packages were used (section 4.2.10). It was therefore encouraging that both facilities were able to identify 24 (*A. fumigatus*), 82 (*A. niger*) and 84 (*A. tubingensis*) identical protein codes. The main problem with these results was the high number of potential candidates, particularly the unknown proteins from the *Aspergillus* section *Nigri* fungi, which would have taken a long time to be processed by cloning and to be tested in immunoblotting experiments.

4.4.2.1.2 Immunoprecipitation-based protein identification

To narrow down the number of allergen candidates, an immunoprecipitation method was developed to harvest specific proteins (section 4.2.8). Protein identification from Af2* was prioritised as more known allergens have been listed from *A. fumigatus* WHO/IUIS compared to the *Aspergillus* section *Nigri* fungi [132]. To our knowledge, approaches to use a subject's antibodies to purify allergens have been rarely performed [426, 427]. It was demonstrated that this method is very specific [426, 427], which represents a main advantage of this method, avoiding the repetitive gel-based results observed for example for HSP90. Second, the method promises high reproducibility [428], which might not be obtained from the 2-dimensional gels commonly used for protein identification [429]. Reproducibility was demonstrated in the present study using immunoblot detection (Fig. 4. 11, Appendix 6.11, Fig. A. 17). Third, denaturing conditions were not present during the immunoprecipitation. The native state of the protein was preserved as much as possible, for example by using aliquoted extracts and lysis buffer with protease inhibitors for the incubation with the extracts and washes (section 4.2.8). Allergen identification usually relies on denaturing conditions during SDS-PAGE, as performed for example for rAsp f 6 [416]. The majority of proteins refold after transfer to the membrane during immunoblotting due to the removal of SDS [430], but the protein conformation could have been affected by improper refolding and some proteins may have remained denatured, for example HSP90 (Table 4. 10). This affects antibody affinity, which is a lot lower to peptide fragments compared to the intact respective protein because the peptide is only a fraction of the epitope, and is more flexible, which does not represent its normal conformation and results in the loss of

entropy upon binding. Most antibodies recognize non-sequential (conformational) epitopes [150, 430, 431].

The immunoprecipitation method had limitations. The majority of the IgE-reactive proteins were not captured by the coupled beads with bound human IgE antibodies from serum. It is possible that the amount of proteins in the supernatant exceeded the levels of allergen-specific IgE. In addition, it seemed as if some of the material was washed out (Appendix 6.8, Fig. A. 9). Despite these limitations, the preliminary immunoprecipitation experiments showed that IgE-reactive proteins could be successfully harvested. The prominent band at around 70 kDa could be indicative of denatured IgE antibodies, which were co-eluted from the beads [426, 427]. The band at around 170 kDa might represent native IgE antibodies or an unidentifiable smear.

Thirty-eight proteins were identified from the immunoprecipitation experiment. Interestingly, both IP-based and gel-based approaches identified Asp f 3 and Asp f 12, representing a peroxysomal membrane protein at 18 kDa and HSP90 at 80 kDa, respectively, although the sequence coverage was low for the latter. The detection of Asp f 3 supports the use of immunoprecipitation as a purification technique for IgE-reactive proteins as the subject was known to react to Asp f 3. The results are also conflicting as the subject showed IgE reactivity to Asp f 6, a MnSOD and the unknown protein Asp f 4 (Table 4. 8) – neither of which showed up in the IP results (Table IV.4). There are several potential explanations for this: First, the composition of the lysis buffer used for the overnight incubation could have affected IgE-binding although the salt concentrations were within the range recommended by the kit [432, 433]. Second, it is possible that only low amounts of these proteins were in the protein extract used, although low protein concentrations were accounted for by increasing the amount of proteins 20X to that used for immunoblotting. A low amount of Asp f 4 in the extract was indicated by inhibition blotting as it could not be detected using the same serum (Fig. 4. 6). Third, not enough IgE antibodies specific to Asp f 4 and Asp f 6 could have been captured by the coupled beads as previously indicated for the total IgE (Appendix 6.8, Fig. A. 10 A). IgE represents only a low proportion of the total immunoglobulins with 1-400 ng/ml in a normal non-atopic human compared to 8-16 mg/ml of IgG, which means that it is difficult to extract high amounts of this immunoglobulin. It is possible

that some components within the serum could have lowered the immunoprecipitation efficiency as the pure IgE control was completely harvested from the solution (Appendix 6.8, Fig. A. 10 B). A pre-purification of IgE antibodies using other precipitation methods such as protein L or ammonium sulfate precipitation [434] were not used in this study because of the potential loss of IgE antibodies by this extra step. Instead, immunoprecipitation could be improved by prolonged incubation times and a higher serum volume as previously suggested [426], which should be explored by future studies.

These results indicated that the immunoprecipitation approach to purify fungal proteins was suitable to identify many gel-based proteins previously identified by two mass spectrometry facilities despite the aforementioned limitations (Table 4. 9, Table 4. 10). Almost half of the IP-based proteins (18/38) were also detected by the gel-based approach using two mass spectrometry facilities. The number was even higher when only the mass peptide fingerprinting results of the VetCore facility were considered (33/38). Interestingly, proteins were identified by immunoprecipitation whose molecular weights did not match to the ones detected by immunoblotting. This could be due to partial protein degradation, absence of signal and propeptide sequences, and migration properties through the SDS-PAGE gel. This is dependent for example on glycosylation, phosphorylation or acidity of the protein [435-437]. Therefore, proteins without the exact molecular weight could be at the same band as a protein with the proper molecular weight. **Unique allergen candidates, which were not detected by the gel-based identification by either facility were the methyltransferase GliN, Actin Act1, the 60S ribosomal proteins L22 and L36.** They have not been reported as allergens before and homologous known allergens were not detected by the allergen database searches (Table 4. 11). Based on the detection of rAsp f 3, it can be assumed that immunoprecipitation only harvested those proteins to which more specific IgE antibodies were present in serum as rAsp f 3 had the highest IgE levels from the three recombinant allergens tested (Table 4. 8). **This means that the immunoprecipitation may just pull out major allergens that the particular individual reacts to.** These proteins are therefore potential new unique allergens, which should be further investigated. Several proteins were detected that showed cross-reactive potential and could be

responsible for the cross-reactivity observed between *A. fumigatus* crude extract and the *Aspergillus* section *Nigri* fungi (Fig. 4. 6).

4.4.2.1.3 Candidates for IgE cross-reactions

IgE cross-reactions between fungal allergens are frequently observed, half of them even to non-fungal species [135, 145, 146, 148]. These include conserved orthologous proteins, which can be described as pan-fungal allergens. Homologous sequences have to be interpreted with caution as they do not necessarily represent epitope similarity [150]. Antibodies usually recognise 6-10 amino acids, which do not necessarily have to lie next to each other [150]. In general, the higher their sequence similarity, the more likely are cross-reactions as they mostly rely on shared protein structures. This means that proteins with > 70% sequence identity are very likely to be cross-reactive, whereas sequences < 50% sequence identity are less likely to cross-react [430]. It was determined, that two protein sequences should be ≥ 35% identical or similar over a segment of 80 amino acids to identify the allergenicity of novel proteins [150, 402, 406]. Identification of IgE cross-reactive allergens can be performed *in vitro*, for example by inhibition blots, or *in silico*, using on homology searches with sequenced allergens. *In vitro*, IgE cross-reactivity to *A. fumigatus* and *Aspergillus* section *Nigri* fungi *A. tubingensis* and *A. niger* could be demonstrated in the present study (section 4.3.3), in particular cross-reactions to rAsp f 3 and rAsp f 6, which to our knowledge have not been reported before (section 4.4.2.1). This was supported for Asp f 3 by the IP-based peptide mass fingerprinting results identifying the same protein (O43099), which was therefore a 100% sequence match in the database searches (Table 4. 11). Interestingly, a putative allergen (Q4WE17) identified from the immunoprecipitation eluate also matched this known *A. fumigatus* allergen (> 50% sequence identity), indicating a close yet unidentified relative of the peroxisomal protein. Asp f 3 is an example of a pan-fungal allergen sharing IgE-binding epitopes with the peroxisomal membrane proteins A and B from *C. boidinii* [151], *Malassezia furfur* Mala f 2 and Mala f 3, and *Penicillium citrinum* Pen c 3 [148, 438]. Homologous proteins to both Asp f 3 and the putative allergen were detected in both *A. niger* and *A. tubingensis* (Table 4. 10).

Asp f 6 was not identified in the immunoprecipitation product, although the patient is known to react to the recombinant allergen as assessed by ImmunoCAP. This allergen is known to show IgE cross-reactivity to *Malassezia sympodialis* Mala s 11 [148] and to a certain degree with human MnSOD. The database searches did also not yield any results for Asp f 12 (HSP90), but the protein was detected in both IP-based and gel-based identification assays and in all three *Aspergillus* species investigated. The chaperone HSP90 controls the function of at least 10% of the proteome [439, 440] and is involved in anti-fungal drug resistances in yeasts [441-444] and *A. fumigatus* [445, 446]. It could be a pan-fungal allergen as homologous proteins are present in *C. albicans*, *S. cerevisiae* and humans [135, 447]. These two proteins, in particular Asp f 6, could induce autoreactivity in humans, which could contribute to the severity of asthma [416, 417].

The other allergens detected from immunoprecipitation eluate included proteins that were not listed as *A. fumigatus* allergens by the WHO/IUIS [132], but are listed as unofficial allergens (allergome.org, June 2018) [406-408]. The malate dehydrogenase, an enzyme involved in the citric acid cycle, is listed as an allergen from *M. furfur* by the WHO/IUIS [132] and involved in atopic dermatitis infection mediated by this fungus [448]. It was > 50% identical with the one identified from *A. fumigatus*. A malate dehydrogenase from *A. fumigatus* is listed as an unofficial allergen, which shares 54.3% of its sequence with the one detected in the present study. This protein, which is involved in conidia formation in *A. fumigatus* and host interaction [449], could therefore be regarded as an allergen. IgE-reactivity to malate dehydrogenase was previously demonstrated in subjects with ABPA [450, 451], which suggests that it could play a role in fungal sensitised subjects with asthma. This protein is also a strong candidate for the 35 kDa allergen detected from *A. tubingensis* and *A. niger* as orthologues were detected in the gel-based identification (Table 4. 10). Other unofficial allergens listed from *A. fumigatus* that were detected using the IP-based identification included glucose-6-phosphate isomerase (Q4X1J1), formate dehydrogenase (Q4WDJ0) (allergome.org, June 2018) [406-408]. This showed that the IP-based protein identification was able to harvest known allergens of *A. fumigatus*, which supports the use of this method to purify allergens from fungal protein extracts.

Some proteins are not known listed or unlisted allergens of *A. fumigatus*. This included the HSP70 protein, which showed high cross-reactive potential as high sequence similarities to other fungi and even species from other kingdoms were detected (Table 4. 11), suggesting that this chaperone is highly conserved. Most of its sequence was shared with *P. citrinum* HSP70 and an unlisted allergen of *C. herbarum*. IgE Cross-reactions to the fungal protein and other Kingdoms were not assessed. Inter-fungal IgE cross-reactions for HPS70 proteins have been reported for Alt a 3 [452] and *C. herbarum* [161]. The protein was also detected from the *Aspergillus* section *Nigri* (Table 4. 10). The house-keeping gene GAPDH is a known wheat allergen involved in baker's asthma [453] and insect allergy [454], which showed high sequence identity with the GAPDH protein detected in this study (Table 4. 11). High sequence identities between *A. fumigatus* proteins fructose-bisphosphate aldolase, formate dehydrogenase or eTEF 1β and allergens of *Rhizopus oryzae* [455] and *C. albicans* (Cand a FPA) [456], *C. bondii* (Cand b FDH) [457], or *P. citrinum* [458] were detected, respectively. The eTEF 1β was also detected in the *Aspergillus* section *Nigri* fungi. IgE cross-reactions between the *Aspergillus* section *Nigri* and the other fungi to *A. fumigatus* for the given proteins have yet to be demonstrated.

The *in silico* approach provided a good indication of the allergenicity from the newly identified allergen candidates based on sequence similarities to known allergens. The high degree of identical amino acid sequences between *A. fumigatus* and *Penicillum* species observed here was not as surprising since these species are closely related [459] and IgE cross-reactions between allergens of these species have been reported [415, 460]. The computational tools have their limitations due to the heterogeneity of the data sources and structures [406]. Therefore, a genuine IgE response of the patients using recombinant allergens should be obtained and the degree of IgE cross-reactivity determined with for example inhibition ELISA or blot.

4.4.3 Limitations

Other limitations besides the ones that were already discussed, were the limited number of subjects and serum used. It cannot be excluded that more IgE-reactive protein bands could have been detected from the *Aspergillus* section *Nigri* fungi if the

IgE-reactivity of more subjects would have been tested. However, the main purpose of the immunoblotting experiments was to identify IgE reactions and characterise allergens, which can be achieved using a small number of subjects that react to the main allergens of the respective species tested. Similarly, the protein identification from immunoprecipitation products was a proof-of-principle experiment, where serum from only one subject was sufficient to harvest known and unknown allergen candidates as determined by peptide mass fingerprinting.

The IgE antibodies used for this experiment derived from a subject with the highest *A. fumigatus*-specific IgE levels (66 kU/L). The protein extract from the clinical isolate of *A. fumigatus* was freshly prepared (Af2*) as Af2 was almost used up. The extracts were very similar, yet some discrepancies were detected (Appendix 6.6, Fig. A. 6, Fig. A. 7). Possible reasons included: First, the culture conditions were slightly different as it involved a pre-culture and a larger volume of liquid medium to obtain enough material for all subsequent experiments, however, as the growth temperature and culture time was the same, this should have had only a minor effect. It is more likely that the fungal growth in liquid medium was inhomogeneous as observed in previous gene expression studies [461, 462]. Similar to gene expression [463], the use of solid medium could result in a more reproducible protein expression, which should be investigated in future studies. Second, the amount of proteases could have been different, which can degrade potentially important allergens [144, 172, 294, 464]. They could have influenced the protein composition of Af2 over time or during the preparation of the new batch Af2*. It should also be noted that no guidelines for fungal protein extraction exist, so various methods can be found in the literature. The extraction method used in this study used grinding with pestle, mortar and N_2, which was shown to be efficient in previous studies [383, 465, 466]. The lysis buffer contained protease inhibitors to minimize protein degradation by proteases present in the suspension and followed the recipe by our collaborators at the FH Campus Wien (Vienna, Austria) to keep the differences between the laboratories to a minimum.

Another limitation could be the use of different gel types and protein transfer mechanisms as immunoblotting and immunoprecipitation experiments were performed in two different laboratories, however, the methods were very compatible. The

difference between self- and pre-cast protein gels was not tested in parallel as despite commercial self-cast stain-free kits available, the self-cast gels prepared for the majority of the experiments did not exploit the stain-free technique and were not prepared using kits but self-made buffers whenever possible. Although the bands were sharper in the gel and on the blot using gradient gels, a comparison between pre-cast 12% and 4-20% gels showed similar banding patterns for the respective molecular weights (Appendix 6.6, Fig. A. 6). Comparisons between wet tank and semi-dry transfer also showed similar results. Although the signal strength of the former was better for proteins at lower molecular weights, the transfer times of the semi-dry transfer can be varied as necessary (Appendix 6.6, Fig. A. 7). Both are known to yield compatible and reliable results, and are therefore commonly used [467, 468]. Differences were detected between fluorescent and chemiluminescent blotting, which had been prepared in parallel, showing that the former is more sensitive than the latter (Appendix 6.6, Fig. A. 8). This observation was contradicted by the results of another chemiluminescent blot showing an identical pattern compared to the previous fluorescent blot (Appendix 6.6, Fig. A. 7), indicating some experimental error in the direct comparison between these two blotting techniques. It should be noted that the same secondary anti-human IgE antibody was used for the chemiluminescent blotting experiments showing reproducible results despite the different techniques used (Appendix 6.6, Fig. A. 7 B, Fig. 4. 6).

Protein extracts could be affected by protein degradation and multiple freeze-thawing, which was avoided by freezing aliquots at -20°C and -80°C. With the exception of proteins at the highest and lowest molecular weights, blots performed with the same extract half a year apart and showed identical banding patterns. This means that the extracts could be used over a long period of time in the present study (Appendix 6.6, Fig. A. 7 B, Fig. 4. 6).

4.4.4 Conclusions

The main findings and conclusions from this study were: Clinical isolates of the *Aspergillus* section *Nigri* could be successfully characterised as *A. niger* or *A. tubingensis*, showing that either identification method, RFLP or sequencing of the β-tubulin was suitable for this aim. Protein extracts were successfully produced for these fungi and the

A. fumigatus species, which were used as a control. Only subjects sensitised to *A. fumigatus* showed IgE-reactivity towards the *Aspergillus* section *Nigri* fungi. This indicated potential IgE cross-reactions between these species, which was tested using inhibition blots. The respective aim was fulfilled as IgE cross-reactivity between these species was indicated, leading to the identification of unknown IgE cross-reactions between Asp f 3 and Asp f 6 to orthologous proteins of *A. tubingensis* and *A. niger*. Although not unique, the aim to produce recombinant proteins was at least partially fulfilled as Asp f 3 and Asp f 6 were successfully produced in *E. coli*. The experiments to date did not indicate that unique allergens were present in either *A. niger* or *A. tubingensis* compared to *A. fumigatus*, which means that the initial hypothesis was rejected. A further investigation of IgE cross-reactive proteins using a gel-based identification by mass peptide fingerprinting yielded a large number of potential unique allergens for the *Aspergillus* section *Nigri* fungi. To narrow down the number of potential candidates, a proof-of-principle immunoprecipitation experiment was developed, which showed highly similar results to the gel-based identification approach with a large overlap in the proteins identified through either methodology. Although not all proteins were detected to which the subject was known to react to, the results indicated that only those allergens could have been detected to which the subject expressed high amounts of IgE antibodies such as Asp f 3. The results also showed several listed and unlisted allergens of *A. fumigatus*, which supported the use of the immunoprecipitation. Some proteins were not known to be allergenic and could represent new potential allergens such as HSP70. A technique which enables specific and reproducible detection of allergens that a subject is sensitised to was introduced. Not all allergens could be identified that the subject was known to be sensitised to, however, both known and unknown allergens of *A. fumigatus* could be detected. The allergen candidates require further testing using patient serum, and may be added to short list of known *A. fumigatus* allergens [135, 145]. The technique may be a better alternative protein identification method compared to the more common gel-based approaches and that could be used for a number of different fungal species.

Chapter 5 General discussion and future directions

5.1 Summary

The primary aim of these studies was to identify clinically relevant fungi in people with asthma under the overarching hypothesis that the mycobiota plays a more significant role in asthma than is currently known. This was assessed by analysis of the mycobiome, which lead to a subsequent investigation into the IgE-reactivity of *Aspergillus tubingensis* and its relatives. These studies presented significant new insights into this understudied research area using and developing innovative techniques.

Since the discovery of a bacteriome in the airways [199, 200], interest has grown in studying the lung mycobiome. Compared to traditional culture techniques, utilising HTS to analyse the mycobiota allows for the identification of multiple species in a sample without any of the culture-related limitations [20, 218, 219]. The cross-sectional study presented in Chapter 3 is the largest study performed in subjects with asthma to date. It is also the most comprehensive due to the inclusion of multiple clinical and control samples, not included in the other asthma studies. Sputum samples and samples obtained by bronchoscopy, specifically BAL, wash and protected brush, were obtained from people with asthma who were grouped based on fungal sensitisation, and from healthy individuals as controls. A possible contamination of the samples was excluded based on the results of the controls from sample processing steps such as kit controls and inclusion of only high abundant sequences. Although culture data were limited based on the needs of this study, it was able to represent the most dominant species *C. albicans* in sputum, mouthwash controls and bronchoscopy-derived samples. The mycobiome was dominated by a few species, *C. albicans*, *A. fumigatus* and *M. tassiana* in all subjects. *M. tassiana* was also highly abundant in control air samples suggesting that these fungi are predominantly inhaled. Other potentially clinically important fungi were significantly less abundant in the air compared to clinical samples, which means that they were unlikely to be inhaled. Enough evidence was present to define *C. albicans* as a commensal of the upper and lower airways as it was highly prevalent and abundant in all subjects and all sample types collected including mouthwash control samples, although the latter were limited by low sample numbers. Interestingly, *A. fumigatus* was present in all sample types collected except mouthwash samples and could not be

associated with fungal sensitisation or lung damage, which was likely the result of a type II error. Future studies should therefore include higher subject numbers. In contrast to *A. fumigatus*, *C. dubliniensis* was associated with fungal sensitisation in asthma, whose role in clinical airway and other diseases is understudied. This is the first time that this fungus has been associated with asthma as to date, it has only been associated with one other lung disease, CF [233, 299].

Although sample numbers were limited, the analysis of the mycobiome from bronchoscopy samples revealed two other species that could play an important role, *A. tubingensis* and *C. pseudolongus*, particularly from subjects in asthma. Their role in the disease's pathology is unclear. This also highlighted the importance of investigating both sputum and bronchoscopy samples in mycobiome studies. Both of these fungi were associated with decreased sputum neutrophil counts. The role of neutrophils is primarily the antimicrobial response [315, 318], but a role in allergic inflammation has also been suggested [320]. The decreased neutrophil counts could be caused by certain virulence factors similar to those reported for related species [324, 325, 327], which have not been reported yet for *A. tubingensis* and *C. pseudolongus*. In contrast to bacterial studies, the influence of the mycobiota on the immune system is understudied. Future studies investigating the mycobiota in subjects with asthma should therefore further elucidate the interaction between the immune response and the fungi identified, in particular with respect to neutrophils. In addition, a longitudinal study could help to assess mycobiome changes and associated signs of progressive lung damage, which could not be observed in this study. Another factor that makes *C. dubliniensis* and *A. tubingensis* interesting candidates for future drug studies is their potential to develop resistance to azole drugs [303, 378, 379], which could be a reason why antifungal drug trials in fungal sensitised people with asthma showed only limited benefits [185-187, 190].

The role of *A. tubingensis*, which belongs the *Aspergillus* section *Nigri*, as an allergen source was investigated. To enable specific diagnosis and personalised medical treatment in the future, allergens from fungi of potential clinical interest need to be identified as commercial extracts based on total fungal proteins lack standardisation and consistency [26, 144, 161]. As presented in Chapter 4, no unique allergens could be characterised from the *Aspergillus* section *Nigri* fungi, due to the IgE cross-reactions

observed between the crude extracts of *A. fumigatus* and the *Aspergillus* section *Nigri* fungi. Interestingly, experiments showed a yet unknown IgE cross-reactivity between the *A. fumigatus* recombinant allergens rAsp f 3 and rAsp f 6 based on the immunoblotting results. Asp f 3 was previously associated with signs of lung damage [165], which may also be the case for the homologous allergens found from *A. niger* and *A. tubingensis*. This would support the hypothesis that *A. tubingensis* is a persistent allergen source which contributes to asthma pathology. The other IgE cross-reactive proteins were further investigated as they may contribute to the inflammatory response observed in asthma as well. A higher cross-reactive potential of allergens may also help to identify unique allergens from other fungal species. To optimise allergen identification, the study introduced an immunoprecipitation technique to purify specific allergens using serum samples. This method showed reproducible results for *A. fumigatus* and identified proteins that were not picked up by the gel-based approach such as Actin Act1. Whereas some of the detected allergens were known such as the officially listed Asp f 3 (WHO/IUIS, [132]) or the unofficial malate dehydrogenase [450, 451], some unknown allergen candidates of *A. fumigatus* were identified, which may be used for CRD or could be associated with disease outcomes in asthma by future studies.

5.2 Techniques

The limitations of the techniques used were discussed in detail in Chapters 3 and 4. Some of the key points that should be addressed in future studies were:

- The discrimination between live and dead cells should include a higher sample number and guidelines for the use of PMA for fungi should be established.
- Although it is believed that the extraction methods used in this thesis were sufficient for their respective purpose, official guidelines should be established for fungal DNA extraction from clinical samples and protein extractions.
- The IP-based identification of allergens was efficient to purify proteins. Some but not all allergens that the subject was sensitised to could be harvested, which means that the current method missed IgE-reactive proteins. This could be

related to the amounts of serum used which affects the levels of allergen-specific antibodies, or to other factors, and therefore requires further optimisation.

5.3 Overall conclusions

The present work established a comprehensive cross-sectional understanding of the lung mycobiome in subjects with asthma and healthy individuals, which was previously lacking, and was able to identify fungal species that may play an as yet undetermined role in asthma. With respect to the original aims and hypotheses, this thesis demonstrated:

- The potentially clinically relevant fungus *C. dubliniensis* in subjects with asthma, in particular those who were fungal sensitised, compared to healthy individuals.
- No significant differences in the fungal load between the different study groups using either universal or species-specific qPCR.
- A high prevalence of *C. albicans* in the oropharynx, sputum and bronchoscopy samples, which is suggestive of a role as a commensal in the upper and lower airways.
- No contamination of sputum and bronchoscopy samples by the mycobiome of the oropharynx, though sample numbers should be increased.
- Differences in sputum samples that were treated or not treated with PMA to assess viability, although the aim was only particially fulfilled due to the low number of samples.
- Potentially clinically relevant species *A. tubingensis* and *C. pseudolongus* in bronchoscopy derived samples compared to sputum samples. They were associated with decreased neutrophil counts. This showed that both sputum and bronchoscopy samples should be obtained.
- Limitation of the culture technique probably due to dilution of samples, although culture data were representative of the sequence abundance data with respect

to certain fungi. A lower dilution of samples and use of a minimal number of media would be advisable.

- A low contribution of the aerobiome to the lung mycobiome, showing low abundances of the clinically relevant fungi described above.
- Successful production of protein extracts from a selection of clinical isolates of *A. niger*, *A. tubingensis* and *A. fumigatus* as well as a type strain of *A. fumigatus* (NCPF 7097).
- IgE-reactivity to fungi from the *Aspergillus* section *Nigri* only by those subjects with asthma who had a high IgE-reactivity to *A. fumigatus*.
- IgE cross-reactivity between allergens of *A. fumigatus* and the *Aspergillus* section *Nigri* fungi tested, in particular Asp f 3 and Asp f 6.
- No unique allergens were identified from *A. niger* or *A. tubingensis* and therefore only IgE cross-reactive proteins were examined. They may contribute to allergic inflammation in the lungs of people with asthma and could be associated with asthma outcomes.
- Successful application of an immunoprecipitation method using protein extract from *A. fumigatus*, although the method should be optimised in the future to purify less abundant specific IgE antibodies.

Future asthma studies should further elucidate the role of the mycobiome particularly with respect to its influence on the immune system and on their long-term effects. These studies should include specific allergens to determine sensitisation to enable a specific diagnosis of fungal sensitisation, which could help to understand their clinical relevance.

Chapter 6 Appendix

6.1 Restriction sites

Restriction enzyme	Restriction sites
*Hha*I (NEB)	5′ GCG|C 3′ 3′ C|GCG 5′
*Nla*III (NEB)	5′ CATG| 3′ 3′ |GTAC 5′
*Rsa*I (NEB)	5′ GT|AC 3′ 3′ CA|TG 5′

6.2 Protein sequence recombinant proteins

Recombinant protein	Protein sequence
rAsp f 3	MSGLKAGDSFPSDVVFSYIPWSEDKGEITACGIPINYNASKEWADKKVILFAL PGAFTPVCSARHVPEYIEKLPEIRAKGVDVVAVLAYNDAYVMSAWGKANQV TGDDILFLSDPDARFSKSIGWADEEGRTKRYALVIDHGKITYAALEPAKNHLE FSSAETVLKHLHHHHHH
rAsp f 4	MRLHGHERRHLHHAGEKREVGDTVYATINGVLVSWINEWSGEAKTSDAPV SQATPVSNAVAAAAAASTPEPSSSHSDSSSSSGVSADWTNTPAEGEYCTDG FGGRTEPSGSGIFYKGNVGKPWGSNIIEVSPENAKKYKHVAQFVGSDTDPW TVVFWNKIGPDGGLTGWYGNSALTLHLEAGETKYVAFDENSQGAWGAAK GDELPKDQFGGYSCTWGEFDFDSKINQGWSGWDVSAIQAENAHHEVQG MKICNHAGELCSIISHGLSKVIDAYTADLAGVDGIGGKVVPGPTRLVVNLDYK EHHHHHH
rAsp f 6	MSQQYTLPPLPYPYDALQPYISQQIMELHHKKHHQTYVNGLNAALEAQKKA AEANDVPKLVSVQQAIKFNGGGHINHSLFWKNLAPEKSGGGKIDQAPVLKA AIEQRWGSFDKFKDAFNTTLLGIQGSGWGWLVTDGPKGKLDITTTHDQDP VTGAAPVFGVDMWEHAYYLQYLNDKASYAKGIWNVINWAEAENRYIAGD KGGHPFMKLHHHHHH

6.3 Secondary antibodies

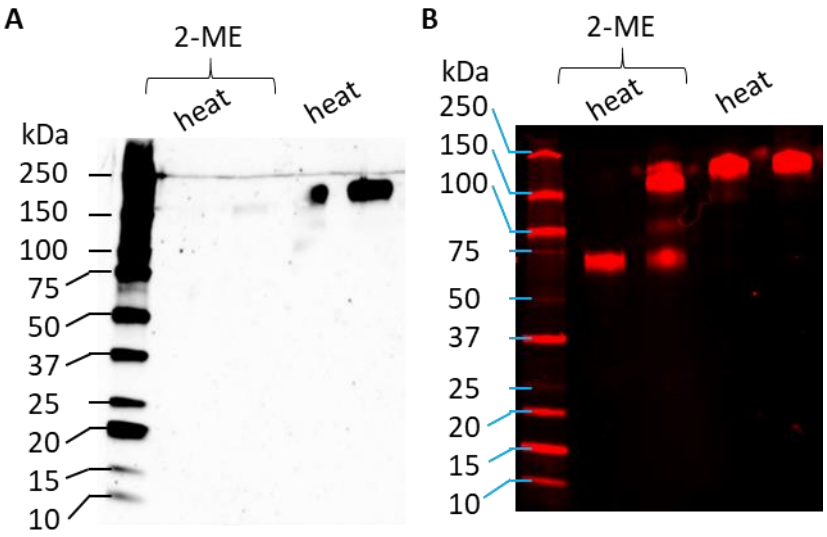


Fig. A. 1: Reactivity of secondary anti-human IgE antibodies to denatured human IgE. Loading was performed according to method A using 100 ng of human IgE with or without heat and/or 2-ME treatment (Chapter 4, 4.2.5, Table 4. 4). (A) The blotting procedure was performed according to method A (Chapter 4, 4.2.6, Table 4. 5) using anti-human IgE monoclonal secondary antibody SouthernBiotech (Chapter 4, Table 4. 6) and an imaging time of 5 min. (B) The blotting procedure was performed according to method B (Chapter 4, 4.2.6, Table 4. 5) using polyclonal NovusBio DyLight 650 (Chapter 4, Table 4. 6).

Table A. 1: List of anti-human IgE secondary antibodies tested

Name	Clonality	Company	Clone name	Conjugate	Dilution
Mouse anti-human IgE	Monoclonal	SouthernBiotech	B3102E8	HRP	1:5000
		BioRad	E411 (5H2)	HRP	1:2000
IgE Antibody		Novus Biologicals	E411	DyLight 680	1:1000
Goat anti-human IgE	Polyclonal	Novus Biologicals	N/A	DyLight 680	1:15,000

N/A not applicable, HRP = horseradish peroxidase

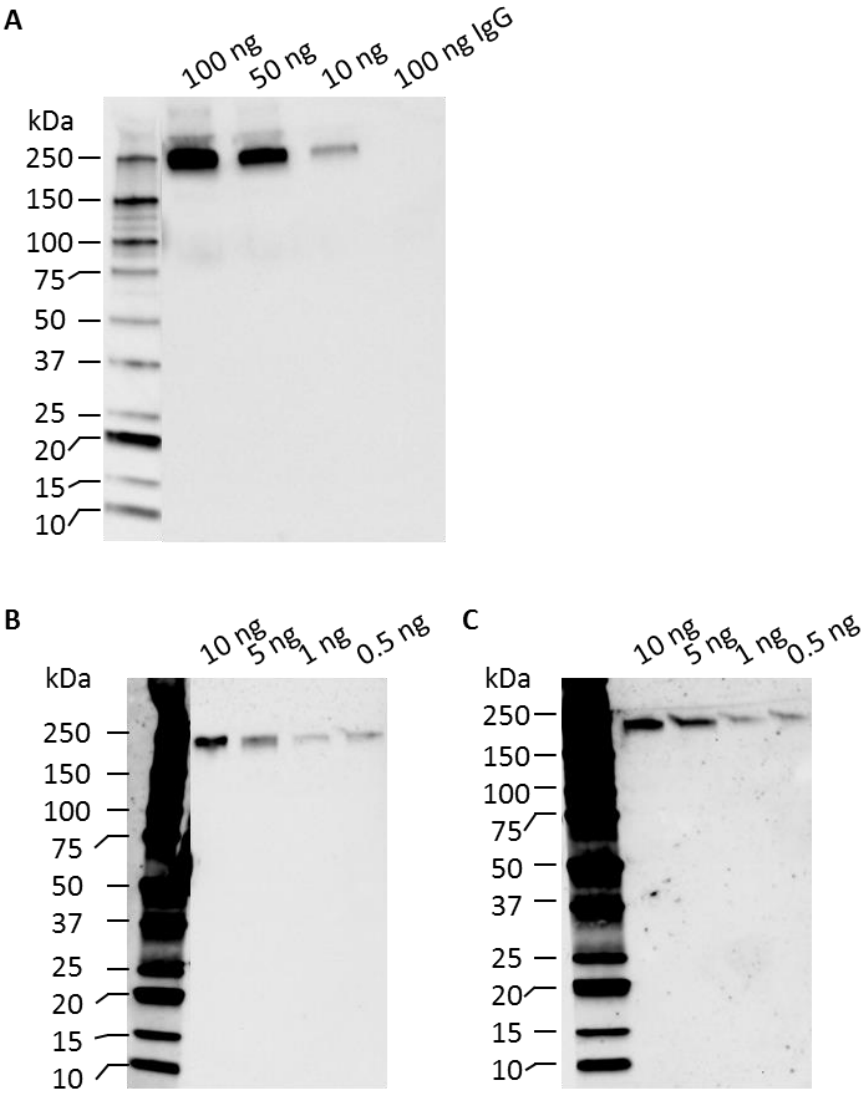


Fig. A. 2: Sensitivity test monoclonal mouse anti-human IgE (SouthernBiotech) (Chapter 4, Table 4. 6). Blots were loaded according to method A (Chapter 4, 4.2.5, Table 4. 4) using (A) 100, 50 and 10 ng or (B, C) 10, 5, 1 and 0.5 ng of not denatured (no addition of 2-ME in loading buffer, no heat) pure human IgE (Abcam). Blotting was performed according to method A (Chapter 4, 4.2.6, Table 4. 5). Bots were incubated with (A) Clarity ECL substrate (BioRad), or (B, C) Pico PLUS substrate (ThermoScientific) for 5 min before imaging. (C) Blot also contained 1:10,000 dilution of the Precision Plus Streptactin-HRP conjugate used to visualize the ladder, which was incubated separately in (A, B). (A) Cross-reaction control were 100 ng human IgG (BioRad). Blot (A) was blocked with 5% non-fat dry milk and washed with 0.05% Tween 20 in PBS. The exposure time was either (A) ~25 min SAM exposure or (B, C) automatic 1 min SAM exposure (both imaged simultaneously).

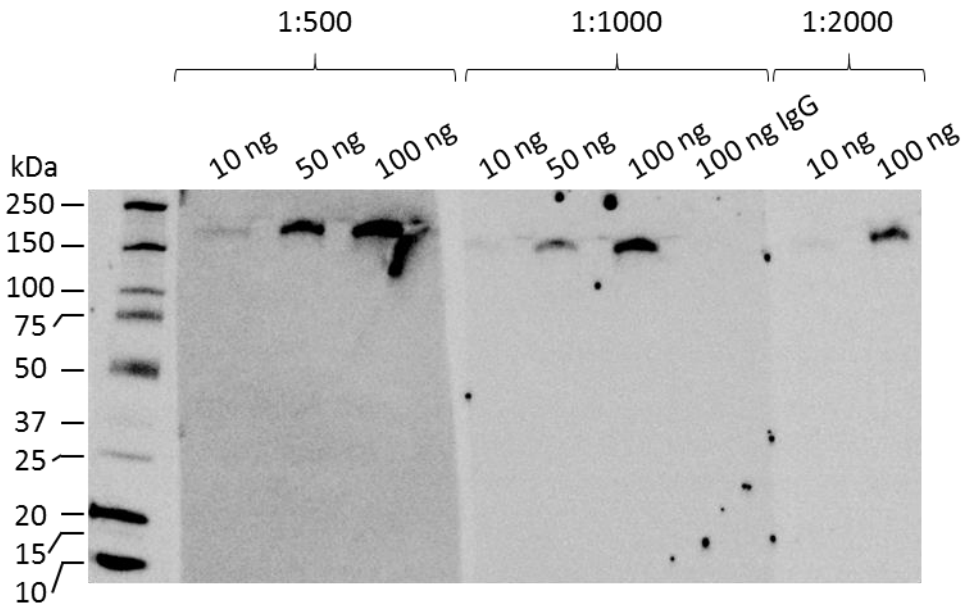


Fig. A. 3: Sensitivity testing for monoclonal mouse anti-human IgE antibody (BioRad). Blots were loaded according to method A (Chapter 4, 4.2.5, Table 4. 4). Blotting was performed according to method A (Chapter 4, 4.2.6, Table 4. 5). Monoclonal mouse anti-human IgE antibody (BioRad) was diluted 1:500, 1:1000, and 1:5000. Ten, fifty and one hundred nanograms of pure human IgE (not denatured, meaning no 2-ME in loading buffer, no heat) were loaded. Cross-reaction control were 100 ng human IgG (BioRad). The blot was blocked with 5% non-fat dry milk and washed with 0.05% Tween 20 in PBS. Ladder was incubated separately with Precision Plus Streptactin-HRP conjugate (BioRad) and imaged at the same time. Substrate was Clarity Western ECL substrate (BioRad) and imaging was performed on the ChemiDoc Touch (automatic exposure).

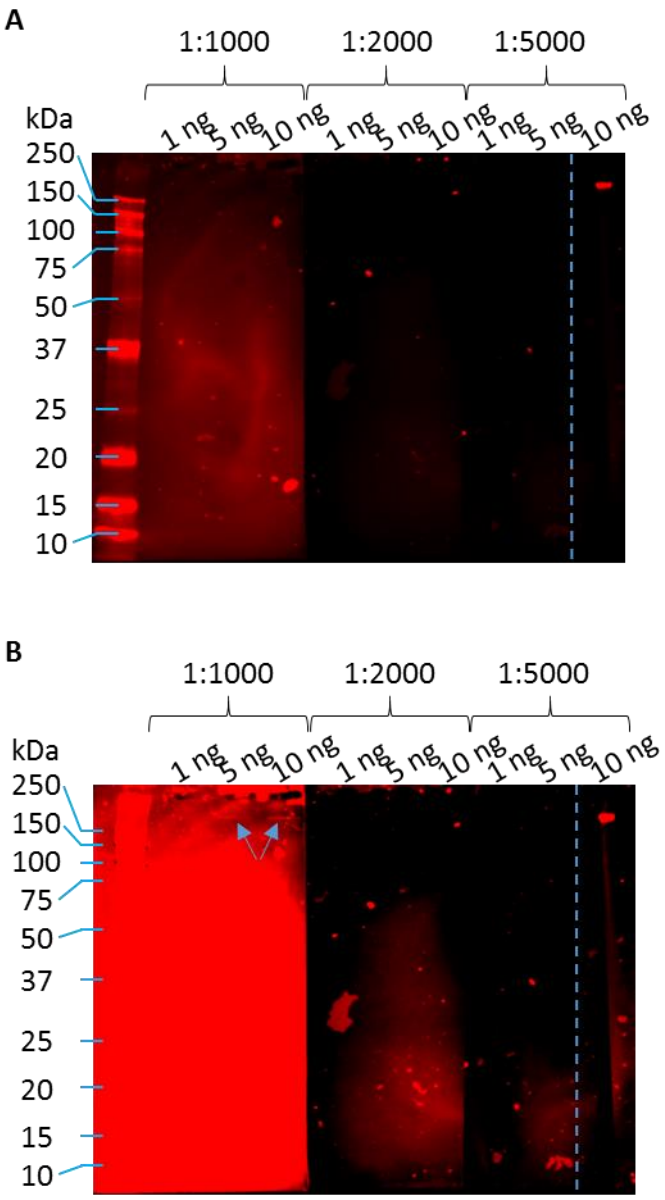


Fig. A. 4: Sensitivity testing for monoclonal anti-human IgE (NovusBio, DyLight 650). Blots were loaded according to method A (Chapter 4, 4.2.5, Table 4. 4) using 1, 5 and 10 ng of pure human IgE (undenatured), which were incubated with 1:1000, 1:2000 and 1:5000 dilutions of secondary antibody. Blotting was performed according to method B (Chapter 4, 4.2.6, Table 4. 5). The last lane (indicated by dotted line) was divided in two in order to apply polyclonal anti-human IgE (NovusBio, DyLight 650) as a positive control. The same blot is shown in (A) and (B) with the only difference being the upper threshold of pixel values that will shade to the same colour on the viewed image. Blue arrows indicate bands detected within the 1:1000 dilution (B), which were not visible with a lower upper threshold of pixel values (A).

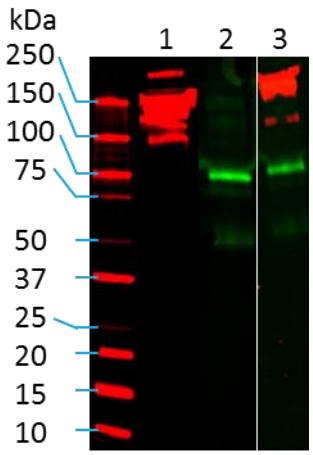


Fig. A. 5: Specificity of polyclonal secondary antibodies. Blots were loaded according to method A (Chapter 4, 4.2.5, Table 4. 4). Blotting was performed according to method B (Chapter 4, 4.2.6, Table 4. 5). Polyclonal anti-human IgE (DyLight 650, NovusBio) (lanes 1 and 3) and IgG (DyLight CW800, Li-COR) (lanes 2 and 3) fluorescent secondary antibodies were applied for the detection of human IgE (Abcam) and IgG (BioRad), of which 100 ng were loaded in lanes 1 and 2, respectively. In lane 3, 50 ng of each antibody were loaded and both secondary antibodies were applied. DyLight 650 fluorescence is shown in red, CW800 in green.

6.4 Preparation of self-cast SDS-PAGE gels (method B)

The 12% polyacrylamide resolving gels with 5% polyacrylamide stacking gels were prepared using glass plates with 1.0 mm spacers, gel cassettes and casting stands (all BioRad) with rubbers to prevent leaking. The inserted combs are for 10 wells fitting a maximum volume of 30 µl. The 12% resolving gel was composed of 12% acrylamide (Ripophorese Gel 40), 0.004% APS (10% stock), 0.0001% TEMED, 405 mM TrisBase and 0.108% SDS (all Roth). The solution was mixed by gently inverting the tube before it was poured between the glass plates until about 2-3 cm below the rim. A layer of isopropanol (Roth) was added on top to get rid of air bubbles and allow for an evenly horizontal gel line. Polymerisation was complete after around 30-40 min. The isopropanol was drained of and the rim of the gel dried using tissue paper. The 5% stacking gel was prepared encompassing 5.2% acrylamide, 0.1% APS, 0.0001% TEMED, 70 mM TrisBase and 0.056% SDS. At least twice the volume of solution was prepared than required to account for

extra volume needed. The solution was mixed by gently inverting the tube before it was applied on top of the resolving gel. The comb was inserted sideways directly afterwards to avoid air bubbles. Polymerisation was complete after 30-40 min. After the gels within the glass plates were released from the gel cassettes and casting stands, they were wrapped in tissue paper moistened with deionised water and cling film. Gels were stored at 4°C and used within 1-2 weeks.

6.5 Coomassie staining

For protein identification by peptide mass fingerprinting, gels were stained with Coomassie brilliant blue by fixing for 30 min, staining for 30 min and destaining overnight (Table A. 2). The gel was shaken continuously at ~30 rpm.

Table A. 2: Buffer composition for Coomassie brilliant blue staining.

Buffer	Ingredients
Fixation buffer	50% methanol 10% acetic acid 100 mM ammonium acetate
Staining buffer	1.25 g Coomassie brilliant blue R250 45% methanol 10% acetic acid
Destaining buffer	10% methanol 7% acetic acid

All ingredients from Roth, Germany

6.6 Compatibility of immunoblotting methods

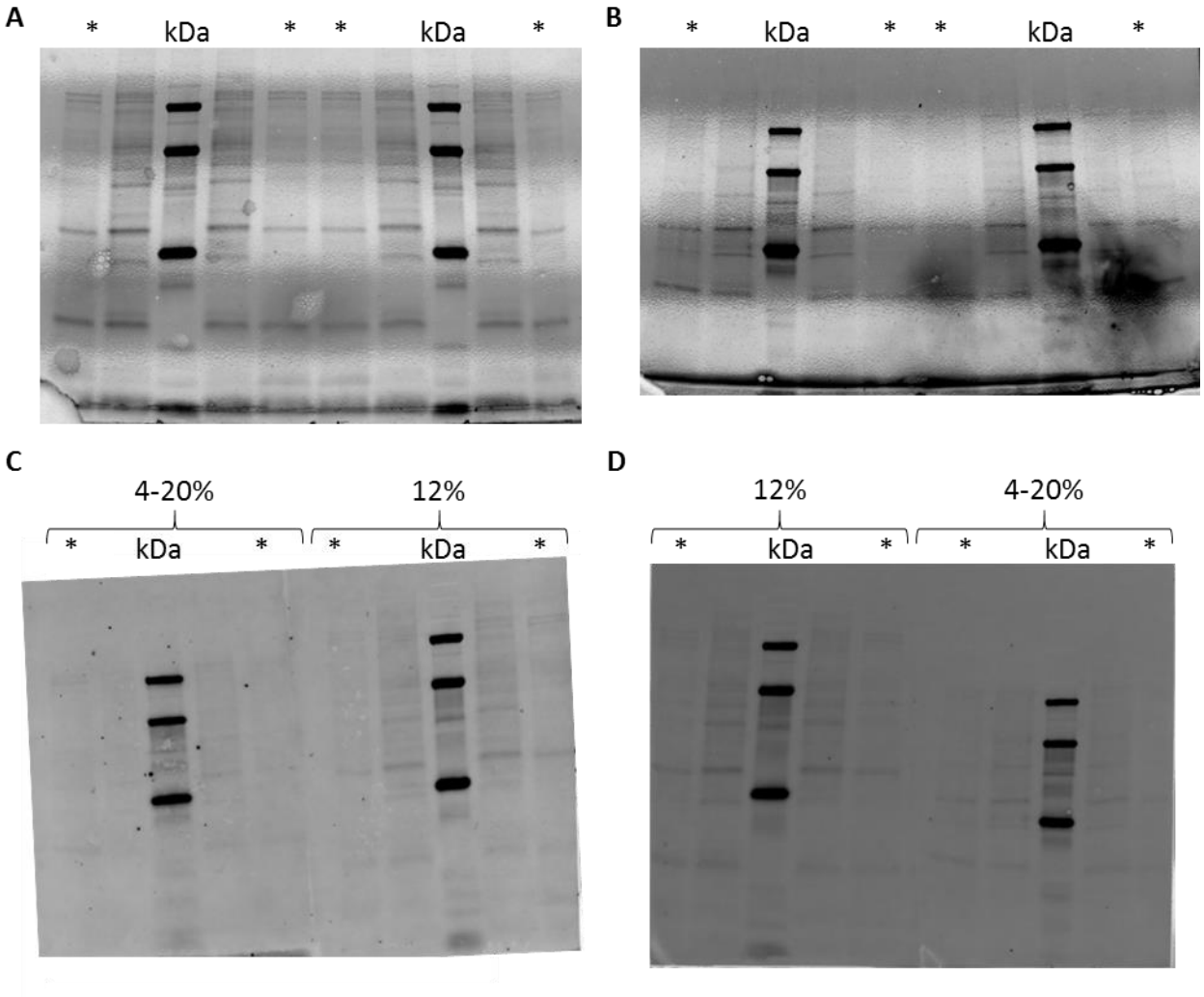


Fig. A. 6: Blots were loaded with Af2 and Af2* crude extract from *A. fumigatus* according to method A (Chapter 4, 4.2.5, Table 4. 4) using (A) 12% and (B) 4-20% gradient gels. For imaging, these stain-free gels were activated for 45 s and exposed for 1 s (faint band setting) on the ChemiDoc Touch. Background signals were observed for both gels due to problems with the imager. Blotting was performed using (C) wet tank transfer or (D) semi-dry transfer (Chapter 4, 4.2.6, Table 4. 5). Once transfer was complete, blots were imaged again with 1 s exposure time. Thick bands in the marker (kDa) represent the 75, 50, and 25 kDa bands top to bottom. (*) indicates the latest protein extract (Chapter 4, Table 4. 7).

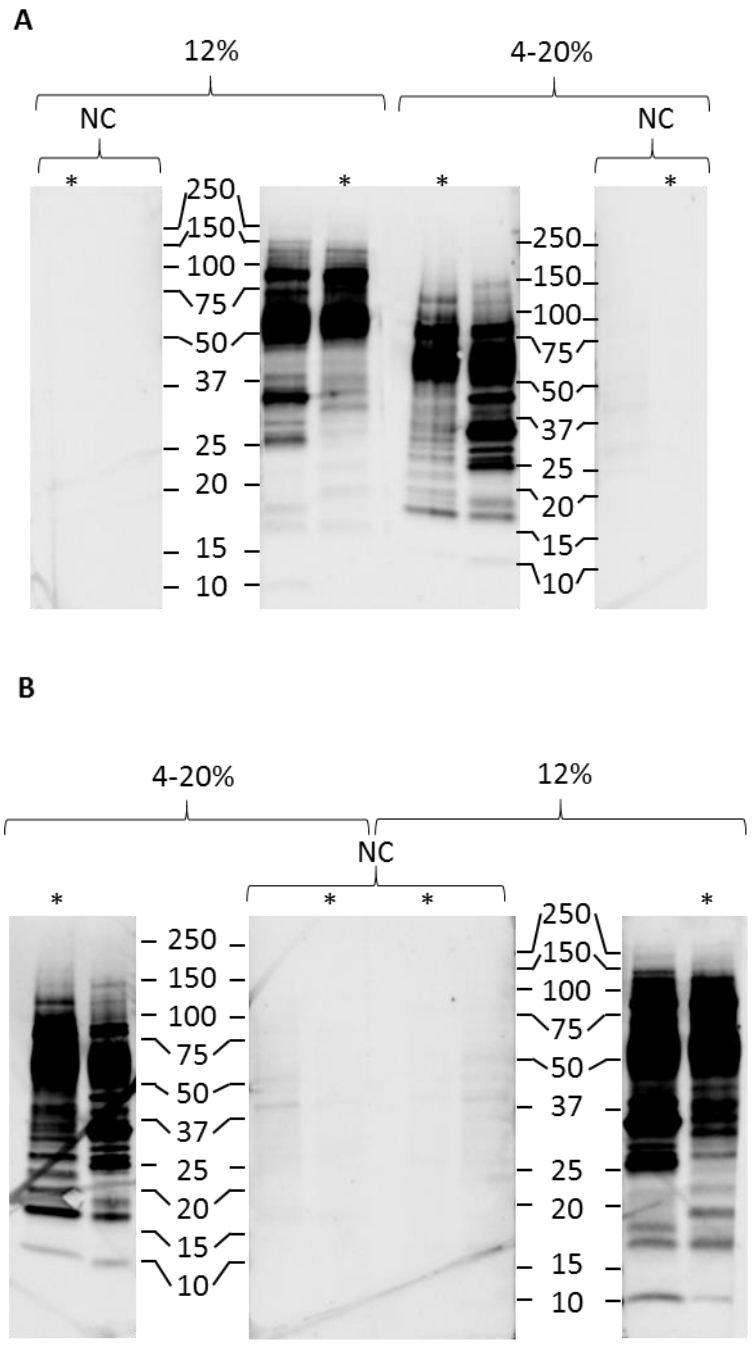


Fig. A. 7: (A) Semi-dry and (B) wet tank transfer (Chapter 4, 4.2.6, Table 4. 5) of *A. fumigatus* proteins using serum from subject R083. Negative controls (NC) were blots incubated with secondary antibody only. Samples Af2 and Af2* (indicated with a *) were used with 4 µg each on either a 4-20% gradient or 12% TGX stain-free SDS-PAGE gels (BioRad) (Chapter 4, 4.2.5, Table 4. 4). Imaging was done using the ChemiDoc Touch for 10 s using the stain-free imaging application with automatic pre-exposure time.

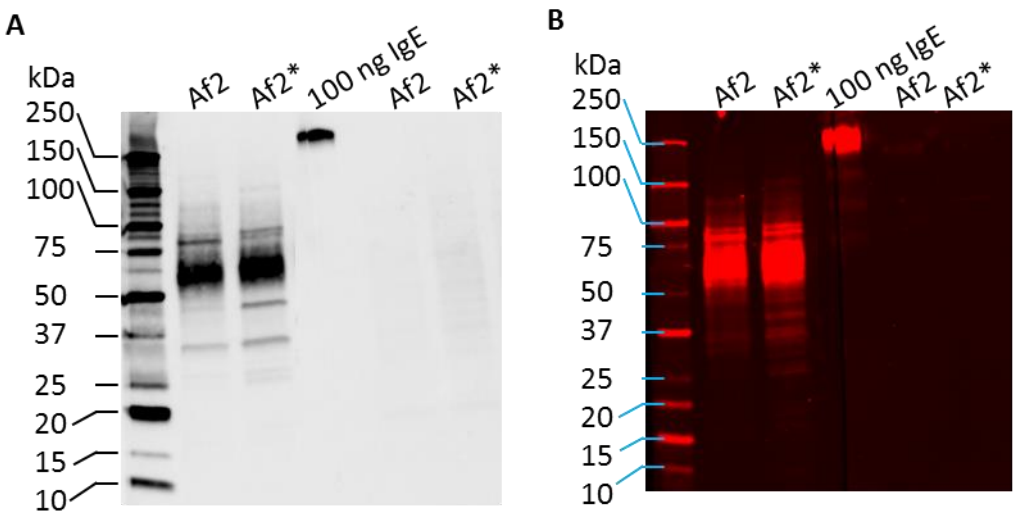
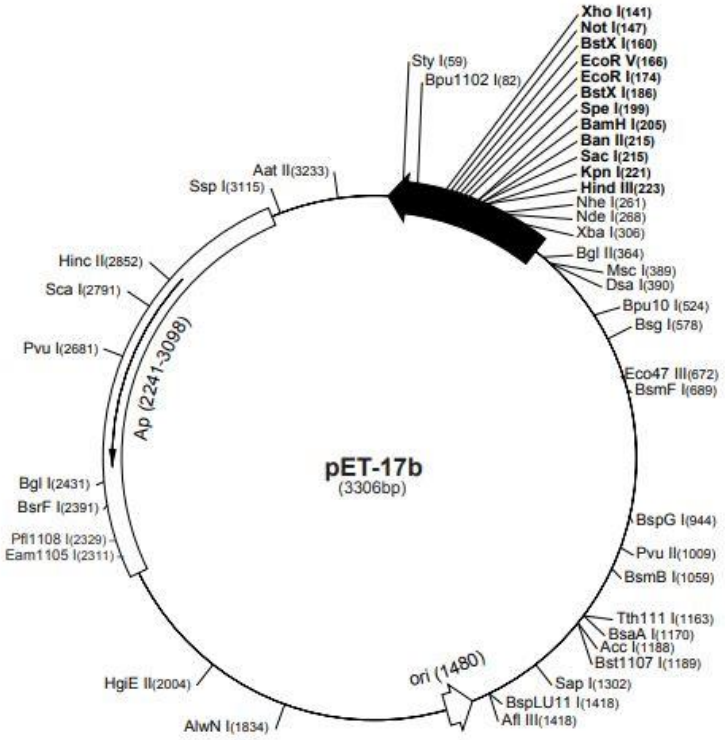


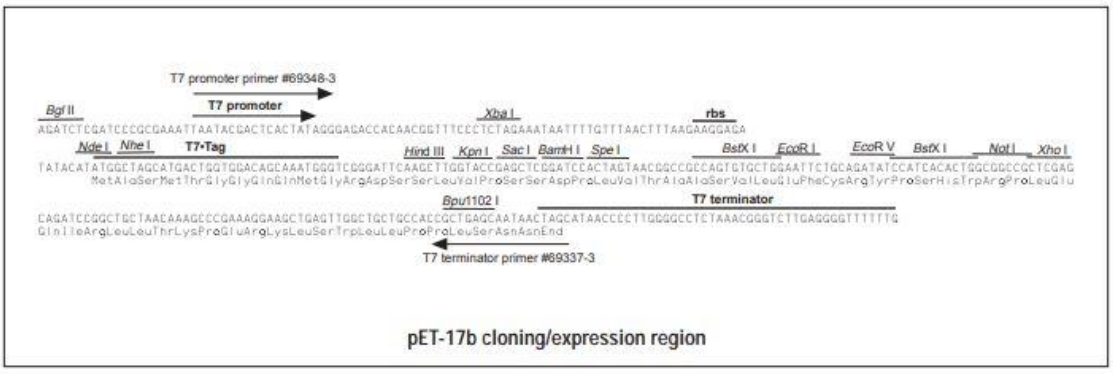
Fig. A. 8: Comparative blots using 4 µg old (Af2) and new (Af2*) extracts from *A. fumigatus* loaded as described by method A (Chapter 4, 4.2.5, Table 4. 4). (A) chemiluminescent and (B) fluorescent blots using monoclonal SouthernBiotech and polyclonal NovusBio DyLight 650 anti-IgE antibodies, respectively (Chapter 4, Table 4. 6). Blots were halved through the positive control (100 ng human IgE) and the right-handed parts were incubated with the respective secondary antibodies only as negative controls. The left-handed blot parts were incubated with serum of patient R083 overnight. (A) Processed according to method A (Chapter 4, 4.2.6, Table 4. 5) and imaged for 1 min (SAM), (B) processed according to method B (Chapter 4, 4.2.6, Table 4. 5).

6.7 Vector pET-17b

Information obtained from manufacturer (Novagen) [469]:

pET-17b sequence landmarks	
T7 promoter	333-349
T7 transcription start	332
T7•Tag coding sequence	237-269
Multiple cloning sites (*Hin*d III - *Xho* I)	141-228
T7 terminator	28-74
pBR322 origin	1480
bla coding sequence	2241-3098





pET-17b cloning/expression region

6.8 Immunoprecipitation preparation results

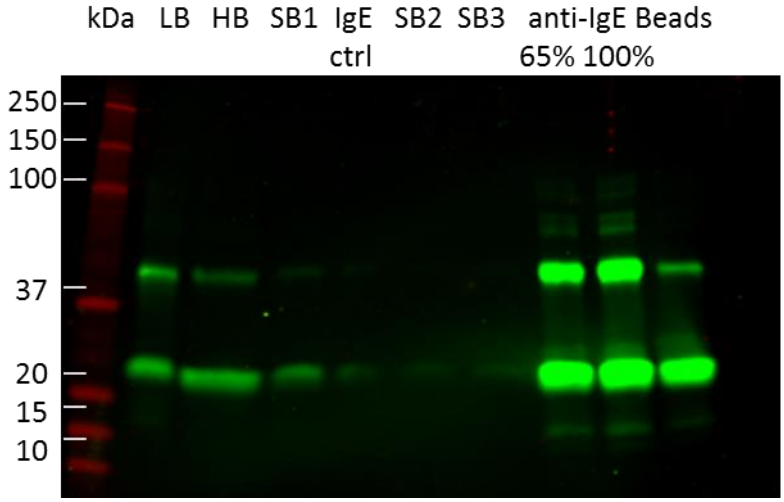


Fig. A. 9: Coupling efficiency of anti-human IgE and Dynabeads. Gels were loaded according to method A (Chapter 4, 4.2.5, Table 4. 4). The maximum volume was loaded for the washes. The volumes of the beads and 100% mouse anti-human IgE control (anti-IgE) were adjusted to the amounts of IgE expected in the washes (LB, HB, SB1-3) if no antibodies would have bound. The 65% anti-IgE control represented a theoretical amount of anti-human IgE that could have bound to the beads. Controls also included 100 ng of pure human IgE to test the specificity of the donkey anti-mouse IgG fluorescent antibodies (Chapter 4, Table 4. 6). Remark: Some material spilled over from SB1 to the IgE control during loading. Blotting to nitrocellulose and incubation of the blot was performed according to method B (Chapter 4, 4.2.6, Table 4. 5).

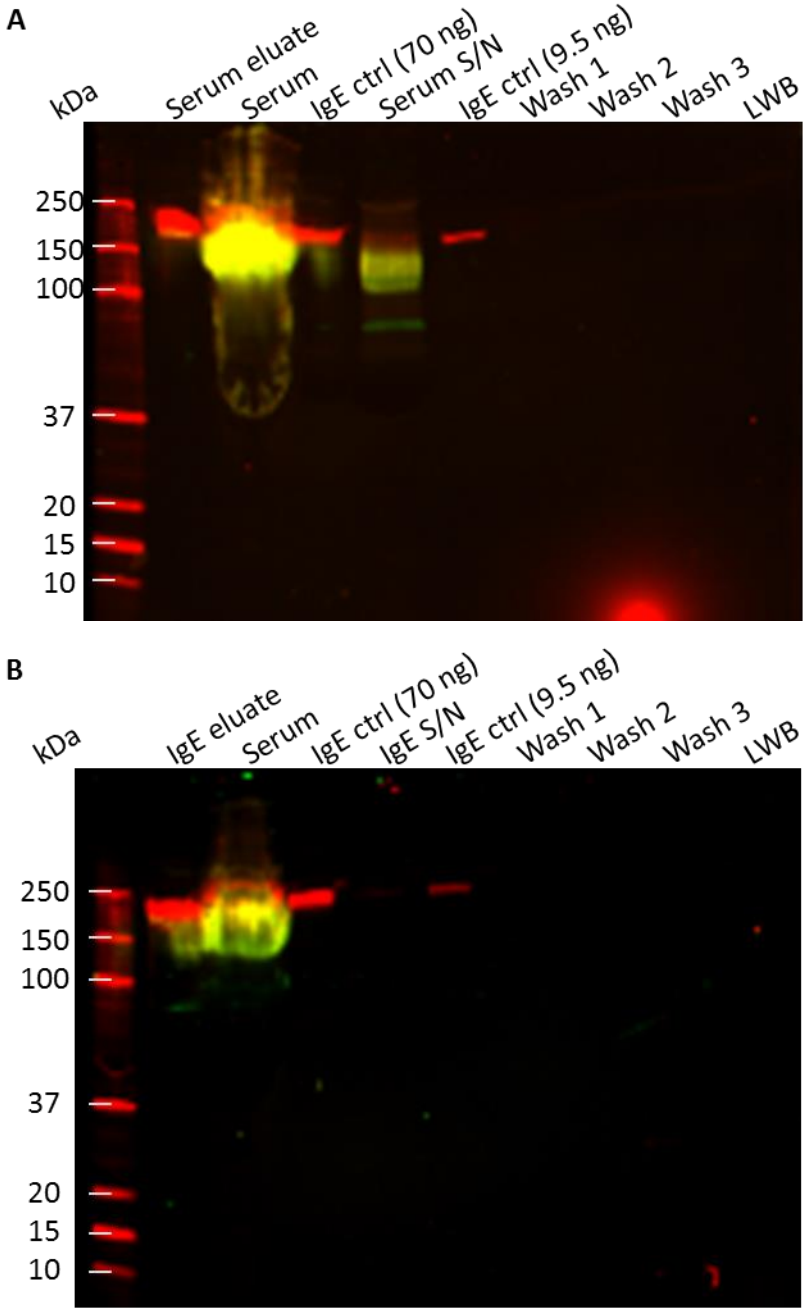


Fig. A. 10: IgE purification from serum or human IgE control using coupled Dynabeads. Gels were loaded according to method A (Chapter 4, 4.2.5, Table 4. 4) using (A) serum eluate and (B) human IgE eluate from immunoprecipitation of human serum and pure human IgE, respectively; serum and an IgE control (ctrl) (70 ng) with an equivalent amount of IgE as the eluate; washes 1-3 and LWB as well as another IgE ctrl (9.5 ng) representing the equivalent amount of IgE in the washes, if no IgE would have been bound by the beads. Fluorescent immunoblots were performed according to method B (Chapter 4, 4.2.6, Table 4. 5) using polyclonal goat anti-human IgE and polyclonal donkey anti-mouse IgG (Chapter 4, Table 4. 6).

6.9 Beta-tubulin sequences

Table A. 3: Beta-tubulin sequences from *A. niger* samples (An1-3) and *A. tubingensis* samples (At1-3) obtained by Sanger sequencing.

Sample	DNA sequence (5' → 3')
An1	GGAACATCGTCTCTTAGGCTATCTCAGCTTGAGTTCAGATGTTGTCCATTA GGTACATGCTATCGGTCTAAGAACACGTCTAACAATTCAACAGGCAGACC ATCTCTGGCGAGCACGGCCTTGACGGCTCCGGTGTGTAAGTGCAACTTTTT CACACCTCTCAATTGGTCAACAATGGGCAAAGGGTTGGGTCTTCTGACAC GCAGGATAGTTACAATGGCACCTCCGACCTCCAGCTGGAGCGCATGAACG TCTACTTCAACGAGGTGAGATCCATCGGACCTTGGCTTTTACACGACAATA TCATCAATGTCCTAATCACTTAAGCAGGCTAGCGGTAACAAGTATGTTCCT CGTGCCGTCCTCGTCGACCTCGAGCCCGGTACCATGGACGCCGTCCGTGCC GGTCCTTTCGGCCAGCTCTTCCGCCCCGACAACTTCGTCTTCGGCCAGTCC GGTGCTGGTAACAACTGGGCCAAGGGTCACTACACTGA
An2	CGTCTCTTAGGCTATCTCAGCTTGATTTCAGATGTTGTCCATTAGGTACATG CTATCGGTCTAAGAACACGTCTAACAATTCAACAGGCAGACCATCTCTGGC GAGCACGGCCTTGACGGCTCCGGTGTGTAAGTGCAACTTTTTCACACCTCT CAATTGGTCAACAATGGGCAAAGGGTTGGGTCTTCTGACACGCAGGATAG TTACAATGGCACCTCCGACCTCCAGCTGGAGCGCATGAACGTCTACTTCAA CGAGGTGAGATCCATCGGACCTTTGCTTTTACACGACAATATCATCAATGT CCTAATCACTTCAGCAGGCTAGCGGTAACAAGTATGTTCCTCGTGCCGTCC TCGTCGACCTCGAGCCCGGTACCATGGACGCCGTCCGTGCCGGTCCTTTCG GCCAGCTCTTCCGCCCCGACAACTTCGTCTTCGGCCAGTCCGGTGCTGGTA ACAACTGGGCCAAGGGTC
An3	GCTTGAGTTCAGATGTTGTCCATTAGGTACATGCTATCGGTCTAAGAACAC GTCTAACAATTCAACAGGCAGACCATCTCTGGCGAGCACGGCCTTGACGG CTCCGGTGTGTAAGTGCAACTTTTTCACACCTCTCAATTGGTCAACAATGG GCAAAGGGTTGGGTCTTCTGACACGCAGGATAGTTACAATGGCACCTCCG ACCTCCAGCTGGAGCGCATGAACGTCTACTTCAACGAGGTGAGATCCATC GGACCTTTGCTTTTACACGACAATATCATCAATGTCCTAATCACTTCAGCAG GCTAGCGGTAACAAGTATGTTCCTCGTGCCGTCCTCGTCGACCTCGAGCCC GGTACCATGGACGCCGTCCGTGCCGGTCCTTTCGGCCAGCTCTTCCGCCCC GACAACTTCGTCTTCGGCCAGTCCGGTGCTGGTAACAACTGGGCCAAGG
At1	GCTATCTTAGCTTGAGTTCAGATGTTATCCATCGGGTATATAGCTATCGGG TTAAGAACACGTCTAACAACTCAACAGGCAGACCATCTCTGGCGAGCACG GCCTTGACGGCTCCGGTGTGTAAGTACAACTTTTTCACACCTCTCAATTGGT CAACAATGTGGAAAGGATTGGGTTTCCTGACGCGCAGGATAGTTACAATG GCACCTCCGACCTCCAGCTGGAGCGCATGAACGTCTACTTCAACGAGGTTA GATCACACCGTCCCTGAGTTTTTTCACGACAATATCATCAATGTCCTGACCA CTTCAGCAGGCTAGCGGTAACAAGTATGTCCCCCGTGCCGTCCTCGTCGAT

	CTCGAGCCCGGTACCATGGACGCCGTCCGTGCCGGTCCCTTCGGCCAGCTC TTCCGCCCCGACAACTTCGTCTTCGGCCAGTCCGGTGCTGGTAACAACTGG GCCAAGGGTC
At2	GCTATCTTAGCTTGAGTTCAGATGTTATCCATCGGGGATATAGCTACGGTT AAGAACACGTCTAACAACTCAACAGGCAGACCATCTCTGGCGAGCACGGC CTTGACGGCTCCGGTGTGTAAGTACAACTTTTTCACACCTCTCAATTGGTCA ACAATGTGGAAAGGATTGGGTTTCCTGACGCGCAGGATAGTTACAATGGC ACCTCCGACCTCCAGCTGGAGCGCATGAACGTCTACTTCAACGAGGTTAG ATCACACCGTCCCTGAGTTTTTTCACGACAATATCATCAATGTCCTGACCAC TTCAGCAGGCTAGCGGTAACAAGTATGTCCCCCGTGCCGTCCTCGTCGATC TCGAGCCCGGTACCATGGACGCCGTCCGTGCCGGTCCCTTCGGCCAGCTCT TCCGCCCCGACAACTTCGTCTTCGGCCAGTCCGGTGCTGGTAACAACTGGG CCAAGGGTC
At3	TCATCTCTCAAGCTATCTTAGCTTGAGTTCAGATGTTATCCATCGGGGATAT AGCTACGGTTAAGAACACGTCTAACAACTCAACAGGCAGACCATCTCTGG CGAGCACGGCCTTGACGGCTCCGGTGTGTAAGTACAACTTTTTCACACCTC TCAATTGGTCAACAATGTGGAAAGG

6.10 IgE reactivity of subjects with different *A. fumigatus*-specific IgE levels to different protein extracts

Fig. A. 11: Immunoblot incubated with serum of asthmatics with low IgE levels to *A. fumigatus*. Protein extracts from *A. fumigatus* in red (Af1, Af2), *A. niger* (purple) An1-3, *A. tubingensis* (green) At1-3 were loaded into the wells of an SDS-PAGE gel according to method B (Chapter 4, 4.2.5, Table 4. 4). After blotting, membranes were incubated with serum pools of asthmatics with *A. fumigatus*-specific IgEs ranging from > 1 to < 5 kU/L). The blot was processed according to method C (Chapter 4, 4.2.6, Table 4. 5) and imaged for 25 min.

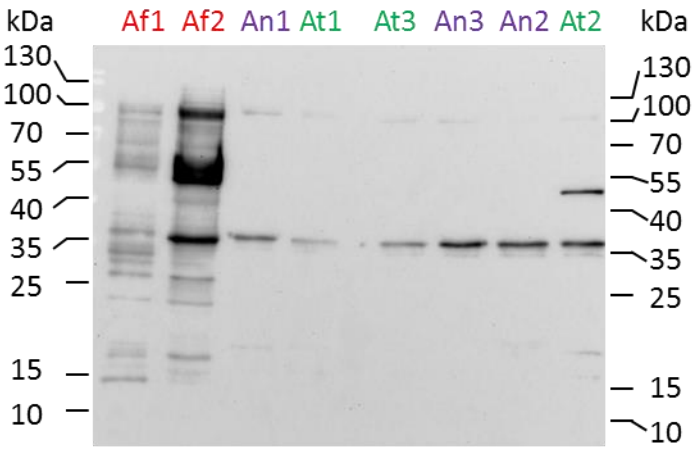


Fig. A. 12: Immunoblot incubated with serum of asthmatics with high IgE levels to *A. fumigatus*. Protein extracts from *A. fumigatus* in red (Af1, Af2), *A. niger* (purple) An1-3, *A. tubingensis* (green) At1-3 were loaded into the wells of an SDS-PAGE gel according to method B (Chapter 4, 4.2.5, Table 4. 4). After blotting, membranes were incubated with serum pools of asthmatics with *A. fumigatus*-specific IgEs > 25 kU/L. The blot was processed according to method C (Chapter 4, 4.2.6, Table 4. 5) and imaged for 15 min.

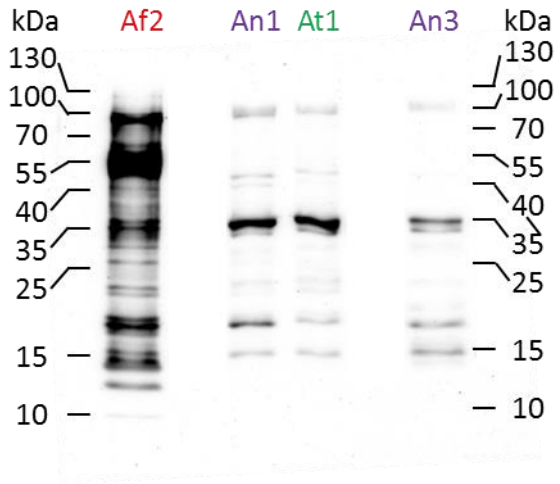


Fig. A. 13: Immunoblot incubated with serum of asthmatics with high IgE levels to *A. fumigatus*. Protein extracts from *A. fumigatus* in red (Af2), *A. niger* (purple) An1 and An3, *A. tubingensis* (green) At1 were loaded into the wells of an SDS-PAGE gel according to method B (Chapter 4, 4.2.5, Table 4. 4). After blotting, membranes were incubated with serum pools of asthmatics with *A. fumigatus*-specific IgEs > 25 kU/L. The blot was processed according to method C (Chapter 4, 4.2.6, Table 4. 5) and imaged for 25 min.

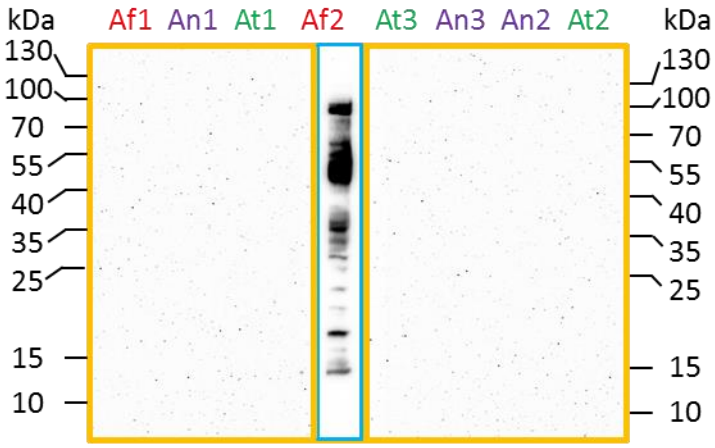


Fig. A. 14: Immunoblot incubated with serum of asthmatics with no sensitisation to *A. fumigatus*. Protein extracts from *A. fumigatus* in red (Af1, Af2), *A. niger* (purple) An1-3, *A. tubingensis* (green) At1-3 were loaded into the wells of an SDS-PAGE gel according to method B (Chapter 4, 4.2.5, Table 4. 4). After blotting, membranes were incubated with serum pools of asthmatics with no sensitisation to *A. fumigatus*-specific IgE (< 0.35 kU/L). A positive control was done using serum of an *A. fumigatus*-sensitised patient R083 (66 kU/L specific IgE) for half of the Af2 lane (blue box). The blot was processed according to method C (Chapter 4, 4.2.6, Table 4. 5) and imaged for 30 min (orange box), except for the positive control (blue box), which was imaged for 2 min.

6.11 IgE reactivity of subjects R083 and R084 to protein extracts from *Aspergillus* species

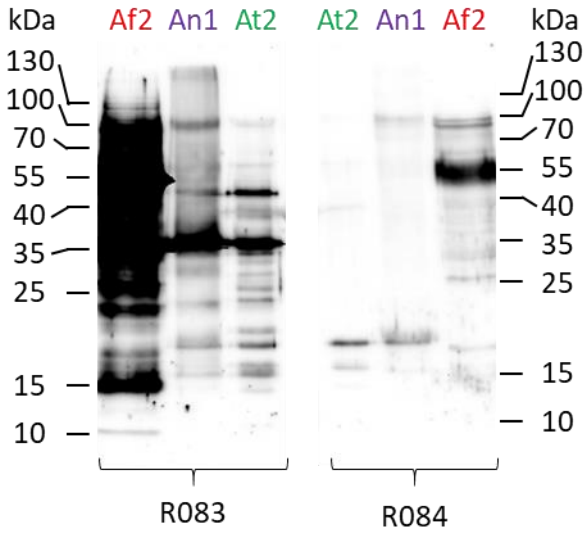


Fig. A. 15: Immunoblot showing the IgE reactivity of subjects R083 (66 kU/L specific IgE) and R084 (17.5 kU/L specific IgE) to *Aspergillus* extracts from *A. fumigatus* (Af2), *A. niger* (An1) and *A. tubingensis* (At2). Proteins were loaded into the wells of an SDS-PAGE gel according to method B (Chapter 4, 4.2.5, Table 4. 4). The blot was processed according to method C (Chapter 4, 4.2.6, Table 4. 5) and imaged for 5 min.

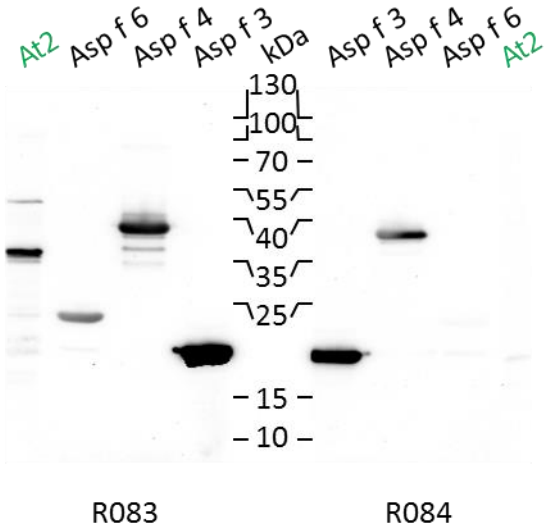


Fig. A. 16: Immunoblotting using sera from patients R083 and R084 against recombinant proteins rAsp f 3, rAsp f 4 and rAsp f 6, and *A. tubingensis* extract At2 as a control. Two micrograms of each recombinant allergen and 4 µg of At2 were loaded into the wells of an SDS-PAGE gel according to method B (Chapter 4, 4.2.5, Table 4. 4). The blot was processed according to method C (Chapter 4, 4.2.6, Table 4. 5) and imaged for 10 min.

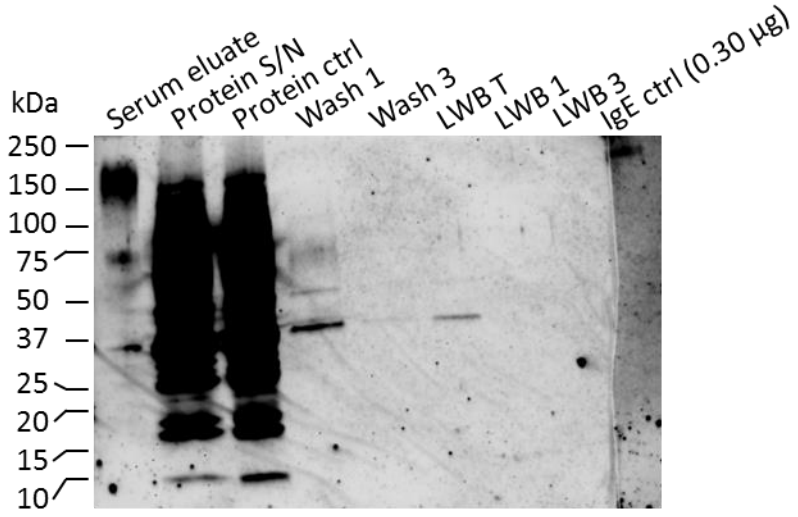


Fig. A. 17: Immunoblot showing repetitive immunoprecipitation results for Af2*. Gels were loaded according to method A (Chapter 4, 4.2.5, Table 4. 4) using the elution from Dynabeads with bound serum IgE and fungal proteins (serum eluate); the protein supernatant (S/N) left after incubating the IgG-IgE magnetic beads with 80 µg/ml of protein extract Af2*; protein control (ctrl), representing an equivalent amount of protein present in the protein S/N if no protein would have been bound; first wash (wash 1) after retrieving the protein S/N from the beads; human IgE control (ctrl) (0.38 µg) as equivalent amount to be expected in the eluate. The chemiluminescent immunoblot was performed according to method A (Chapter 4, 4.2.6, Table 4. 5) and imaging was done for 15 min signal accumulation mode (SAM), 20 min post-incubation with the substrate.

6.12 Mass spectrometers

Table A. 4: Comparison between LTQ Orbitrap Velos[1] (Thermo Scientific) and TripleTOF 5600[2] (SCIEX) mass analysers.

	LTQ Orbitrap Velos[1]	**TripleTOF 5600[2]**
Mode of analysis	quadrupole mass analyser and time-of-flight	quadrupole mass analyser and Orbitrap
Resolution	Max 100,000 $_{FWHM}$	≥ 35,000$_{FWHM}$
Mass range of *m/z*	50-2000, 200-4000	5-40,000
Mass accuracy	< 1 ppm with internal calibration	< 3 ppm*
Dynamic range	> 5000	≥ 10^4

m/z = mass-to-charge ratio, FWHM = Full width at half maximum, [1][470], [2][421], *high MS/MS spectral acquisition rates (20Hz)

Both machines were designed to identify a larger amount of low abundant proteins from a complex sample [421, 422].

Chapter 7 References

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