Immunopathogenesis and antifungal therapy for Severe Asthma with Fungal Sensitization and Allergic Bronchopulmonary Aspergillosis

A Thesis Submitted to the University of Manchester for the Degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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School of Medicine
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Thesis Limitation

Discussion of Main Findings

7.3.1 IL-17A has a central role in asthma severity, Independent of cause. Circulating blood neutrophil numbers may reflect neutrophilic airway inflammation, and provide an alternative non-invasive method of assessing airway inflammation.

7.3.2 IgE against staphylococcal aureus (SE-IgE) is associated with reduced lung function with and increased OCS use. Sensitisation against staphylococcal entero-toxins is associated with asthma severity, whereas sensitisation against Aspergillus fumigatus is associated with high frequency of bronchiectasis.

7.3.3 Aspergillus is more abundant in patients with Severe asthma than previously thought. Steroid treatment increases airway fungal load. There is a possible link for airway microbiome with asthma severity.

7.3.4 Voriconazole & posaconazole improves asthma severity, QOL and radiological abnormalities in SAFS & ABPA. Voriconazole has a number of side effects, most of them mild.

7.3.5 Overall efficacy of NAB in this group of patients is poor and associated with bronchospasm. NAB is effective in those that tolerate the initial bronchospasm.
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<th>Description</th>
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<tbody>
<tr>
<td>ACQ</td>
<td>Asthma Control Questionnaire</td>
</tr>
<tr>
<td>ABPA</td>
<td>Allergic bronchopulmonary aspergillosis</td>
</tr>
<tr>
<td>AM</td>
<td>Airway macrophages</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>APC-Cy7</td>
<td>Allophycocyanin-cyanin 7</td>
</tr>
<tr>
<td>APC-H7</td>
<td>Allophycocyanin-H7</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
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<tr>
<td>BALT</td>
<td>Bronchoalveolar lavage T Lymphocytes</td>
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<tr>
<td>BDP</td>
<td>Beclomethasonedipropionate</td>
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<tr>
<td>BTS</td>
<td>British Thoracic Society</td>
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<tr>
<td>BP</td>
<td>band pass filter</td>
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<tr>
<td>CBA</td>
<td>Cytometric Bead Array</td>
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<tr>
<td>CD4</td>
<td>Cluster designation 4</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked Immunosorbent Assay</td>
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<td>ELISPOT</td>
<td>Enzyme linked immunospot</td>
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<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
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<tr>
<td>FEV1</td>
<td>Forced expiratory volume in the first second</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FITC</td>
<td>Flourescein isothiocyanate</td>
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<td>Forkhead Box P3</td>
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<td>Hu</td>
<td>Human</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Laser</td>
<td>Light Amplification by Stimulated Emission of Radiation</td>
</tr>
<tr>
<td>LP</td>
<td>long pass filter</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>m3</td>
<td>IgE antibody against aspergillus fumigatus</td>
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<td>MA</td>
<td>Mild asthma</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>%CD4+ IL-17A</td>
<td>Percentage of CD+ lymphocytes expressing IL-17A+ markers</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PerCP</td>
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<td>PMT</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR (real-time PCR)</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PMA</td>
<td>Phorbol Myristate Acetate</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>SAFS</td>
<td>Severe Asthma with Fungal Sensitization</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
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<tr>
<td>SE-IgE</td>
<td>Specific IgE against staphylococcal enterotoxin</td>
</tr>
<tr>
<td>SEC</td>
<td>Staphylococcal enterotoxin C</td>
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<td>Staphylococcal enterotoxin A</td>
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<td>TSST-1</td>
<td>Toxic shock syndrome toxin 1</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<td>Th</td>
<td>T-helper lymphocyte</td>
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<tr>
<td>Treg</td>
<td>T regulatory cell</td>
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<td>DEFINITIONS</td>
<td></td>
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<tr>
<td>CD3</td>
<td>An antigen cluster or a glycoprotein that is predominantly found on the surface of</td>
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<td></td>
<td>T-Lymphocytes.</td>
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<tr>
<td>CD4</td>
<td>An antigen cluster or a glycoprotein that is predominantly found on the surface of</td>
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<td></td>
<td>Th-Lymphocytes.</td>
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<tr>
<td>DNA sequencing</td>
<td>The process of determining the precise order of nucleotides within a DNA molecule</td>
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<tr>
<td>Fluorochrome</td>
<td>A functional molecule that fluoresces or emits light.</td>
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<tr>
<td>Forkhead Box P3</td>
<td>A protein which is a member of the forkhead/winged-helix family of transcriptional</td>
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<td></td>
<td>regulators.</td>
</tr>
<tr>
<td>Forward scatter</td>
<td>Light that scatters in the same direction as the laser beam.</td>
</tr>
<tr>
<td>Staining or labelling</td>
<td>Adding monoclonal antibodies to a mixture of cells</td>
</tr>
<tr>
<td>Side scatter</td>
<td>Light that scatters roughly perpendicular to the laser beam.</td>
</tr>
<tr>
<td>Permeabilization</td>
<td>The process of making cell membranes permeable using chemical substances to get</td>
</tr>
<tr>
<td></td>
<td>access to the cell's interior without destroying it.</td>
</tr>
<tr>
<td>PCR</td>
<td>A technology in molecular biology used to amplify a single copy or a few copies of</td>
</tr>
<tr>
<td></td>
<td>a piece of DNA across several orders of magnitude, generating thousands to millions</td>
</tr>
<tr>
<td></td>
<td>of copies of a particular DNA sequence.</td>
</tr>
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</table>
ABSTRACT

Immunopathogenesis and antifungal therapy for Severe Asthma with Fungal Sensitization and Allergic Bronchopulmonary Aspergillosis, a Thesis submitted to The University of Manchester for the Degree of Doctor of Philosophy (PhD) in the Faculty of Medical and Human Sciences, Dr Livingstone Chishimba, December 2015.

Introduction: The Pathogenesis and treatment of Allergic bronchopulmonary aspergillosis (ABPA), severe asthma-non fungal sensitised (SANFS) and severe asthma with fungal sensitization (SAFS) is poorly understood. IL-17A, IgE and microbiome may be associated with pathogenesis of asthma, but their role in fungal-associated asthma is uncertain. Further, the efficacy of voriconazole, posaconazole and nebulised amphotericin B (NAB) in ABPA and SAFS has not been fully studied.

Aims and objectives: The aim of this PhD thesis was to evaluate the role of IL-17A, IgE and lung microbiome in patients with SANFS, SAFS and ABPA. We also studied the efficacy and safety of NAB, voriconazole and posaconazole.

Methods: Airway lymphocytes and peripheral blood mononuclear cells (PBMC) from patients with ABPA (n=16), SAFS (n=15), SANFS (n=11), mild asthma (MA) (n=6) and NH (n=11) were characterized by flow cytometric analysis (FACS) to determine the % of CD (+) IL-17A expressing cells. We also evaluated microbiome population using culture and PCR plus sequencing from BAL of these patients. In chapter 3, we analysed total and specific IgE in blood from adult cohorts of SAFS (n=34) and ABPA (n=48) using ImmunoCAP 100. In chapter 5 we studied the efficacy of voriconazole and posaconazole and in chapter 6; we studied the efficacy of NAB.

Results: %CD4+IL-17A expressing cells were significantly higher in patients with severe asthma and correlated positively with serum neutrophil and presence of fungi in the airways. ABPA, SAFS and SANFS were similar but all were significantly higher than MA and NH. There were no differences in IL-17A expression between blood and the lung. Fungi were more frequently associated with severe asthma and low FEV1. Steroid treatment significantly increased airway fungal load. IgE against staphylococcal aureus (SE-IgE) correlated positively with FEV1 and OCS dose. Voriconazole and posaconazole improved asthma severity and radiological abnormalities. NAB was associated bronchospasm, but was extremely effective in the few patients (n=3) that took treatment for >12 months. These responders had unique characteristics.

Conclusions: IL-17A, SE-IgE, and lung microbiome are associated with asthma severity. Steroid use in these patients may increase airway fungal load. Whereas voriconazole and posaconazole are efficacious, the use of NAB is associated with significant bronchospasm. SE-IgE –high asthma patients may be a distinct asthma phenotype. Larger studies are needed.
DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
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I would like to dedicate this Thesis to my wife ‘Matilda’ and my children ‘Chichi’ and ‘Nicholl’ for all their sacrifice during this PhD study and all the achievements without which it could have practically impossible’. I would also like to dedicate this thesis to my parents.
ACKNOWLEDGMENTS

I would like to acknowledge my supervisors; Dr Robert Niven, Dr Lucy Smyth, Prof David Denning and Dr Paul Bowyer for the positive contributions and help in designing the work in particular the project protocol and obtaining the research ethics approval. In particular, I would like to thank Dr Lucy Smyth for all the work involved in designing the laboratory experiments and organizing the training for the lab work. I would also like to thank my advisor, Dr Angela Simpson for the advice during my research years.

My special thanks go to: Simon Stephan - chief lab manager, Leanne Archer - laboratory scientist/technologist, Maryam Safari - laboratory scientist/technologist for their role in helping out with laboratory work (collecting BAL from the bronchoscopy unit and storage). Mrs Ann Bird and Mrs Chris Harris-respiratory medicine secretary for their role in collecting notes and arranging bronchoscopies. Ms Marian Denson - laboratory scientist for her role in helping out with my laboratory training and purchase of lab material plus FACS work for the last 5 patients. Our Collaborators, Prof Claus Buchert team at the University Hospital Ghent, Upper Airways Research Laboratory (URL), De Pintelaan, Ghent, Belgium who performed immunoCAP in their laboratory for total IgE and specific IgE relating to Chapter 3. Mrs Julie Morris and Mr Philip Foden-medical statistics for their role in helping out with statistical and graphical work, Mr Paul Somerset and Mrs Helen Carruthers-Medical illustrations for their role in helping out with images and figures. Finally, I would like to thank my family and everybody else for all the support they have given me during my studies.
The Author

I graduated from the University of Zambia Medical School (Lusaka, Zambia) with an MB ChB. Following that I moved to the UK to pursue my postgraduate training. I completed my Senior House Officer General Medical Training in the East Midland Deanery in November 2007 after obtaining MRCP (UK) and being admitted to the Royal College of Physicians of the UK. In December 2007, I started my Higher Specialist Training in Respiratory and General Internal Medicine in the West Midlands Deanery. In August 2010, I took time out of my training programme and I registered with the University of Manchester to study for a three-year PhD degree (Sept 2010 – Dec 2014). I returned back to the West Midlands to complete specialist clinical training in Respiratory and General Internal Medicine training. I successfully passed the Specialty Certificate Examination in Respiratory Medicine and obtained MRCP (UK) (Respiratory Medicine) in October 2014 following which I got appointed as a Consultant Respiratory Physician at Cardiff University Hospitals (UHW) and the Cardiff University Board (UHB) where I currently run the severe asthma service. Upon obtaining my PhD, I intend to apply for an honouraly senior lecturer’s post with Cardiff University to continue with a combination of clinical, research and teaching work.
AUTHOR’S CONTRIBUTIONS TO THIS THESIS AND OWN LAB WORK

Chapter 1
I derived the concept and conducted literature search. I wrote the whole chapter. Supervisors–mainly my main supervisor helped with revision prior to submission.

Chapter 2
I derived the concept, design of the study, acquisition, analysis and interpretation of the data assisted by my supervisors. I wrote the protocol, patient information sheet (PIS), consent forms, the IRAS ethics application form and obtained ethics approval from the North West Research Ethics and the local NHS R&D committees assisted by my supervisors.

I attended a 3 day FACS course and training in Oxford provided by BD Biosciences. I also received local training for FACS in the laboratory provided by my supervisor (Dr Lucy Smyth) and Marion Denson-laboratory scientist (see acknowledgement section).

I recruited most research subjects and my supervisors helped with some of the patient’s recruitment. I performed all the bronchoscopies for all patients and obtained BAL samples. I performed separation of lymphocytes from BAL and blood, stained the cells, gating, FACS analysis and acquisition of data as detailed in the methods section of this chapter. I wrote the whole chapter. The lymphocyte gating strategy was initially set up by my supervisor (Dr Lucy Smyth) and applied these gating strategy and template for all subjects (hence gating was uniform throughout).

The respiratory research laboratory staff (see acknowledgement) helped with storage of samples and separation of cells from BAL for some of the subjects (approximately 25%). Blood samples were collected by myself and the nursing staff during the bronchoscopy sessions. Marcin Fraczek helped transporting the BAL samples under ice to the respiratory research lab at the education and research centre (ERC) and initial filtration of BAL where appropriate (acknowledged).

The statistics and graphs were done by me verified by medical statistics at the University of Manchester (acknowledged). My supervisors reviewed the chapter and my main supervisor substantial revision prior to submission.
Chapter 3

I derived the concept, design of the study, clinical data acquisition, analysis and interpretation of the data assisted by my supervisors.

Blood samples were packed and transported to our collaborators in Belgium by our ERC laboratory staff supervised by Dr Caroline Moore—laboratory scientist (acknowledged). ImmunoCAP was done by our collaborators in Belgium (Acknowledged).

The statistics and graphs were done by me verified by medical statistics at the University of Manchester (acknowledged). I wrote the whole chapter. My supervisors reviewed the chapter and my main supervisor substantial revision prior to submission—reviewed by my supervisors prior to submission.

Chapter 4

As in chapter 2, I derived the concept and conducted literature search. I wrote the whole chapter. Supervisors—mainly my main supervisor helped with revision prior to submission.

I generated the design of the study—assisted by my supervisors and substantially by Dr Paul Bowyer—Molecular scientist and senior University of Manchester Lecturer. I wrote the protocol, the IRAS ethics application form and obtained ethics approval from the North West Research Ethics and the local NHS R&D committees assisted by my supervisors.

I received local in-house laboratory training for the Health Protection Agency (HPA) standard culture method (BSOP57) protocol (HPA), DNA extraction and PCR techniques provided by Dr Marcin Fraczec at the ERC, University Hospital of South Manchester.

I performed all the bronchoscopies for all patients and obtained BAL samples. I performed all the culture and DNA extraction. Microscopy and identification of microbiome species was done by medical mycology lab staff supervised by Dr Caroline Moore (acknowledged). Dr Marcin Fraczec did the PCR with me. We optimised the PCR reactions together and Dr Paul Bowyer provided the supervision.

Sequencing was done by our collaborators in Liverpool (acknowledged).
I wrote the whole chapter-edited by my supervisors. My supervisors (particularly Prof Denning and Dr Paul Bowyer provided substantial contribution towards the data analysis and interpretation, substantial revision prior to submission (acknowledged).

Chapter 5
I derived the concept and conducted literature search. I wrote the whole chapter. 
Supervisors – supervisors helped with revision prior to submission.
I conducted all data acquisition and wrote the chapter.

My supervisors (Prof Denning and Dr Robert Niven) provided substantial revision prior to submission and publication (acknowledged).

Chapter 6
I derived the concept and conducted literature search.
I drew up the protocol, patient invitation sheets and consent forms. I recruited the patients.

I did all the clinical follow ups, obtained clinical data, analysis and interpretation.

Our research nurse (Georgina Powell) administered the ACQ questionnaires and conducted telephone follow ups to check on safety symptoms.

Our physiotherapist (Phil langridge) administered the investigation drug (see methods, acknowledgement).

My supervisors (Prof Denning and Dr Robert Niven) provided substantial revision prior to submission and publication (acknowledged).

Chapter 7
I wrote the whole chapter- supervisors helped with revision prior to submission.
During this PhD study, I have learnt a number of things including:

- Literature and scientific paper reviews
- Writing research protocols
- Grant application
- Ethics application
- Laboratory techniques including: isolation of lymphocytes from blood and BAL, tissue culture, microbial culture, DNA extraction, PCR, flow cytometry etc.
- Writing and presentation of manuscripts including presentation at local, national and international conferences.
Other research experience and publications

Other than the work presented, I have also been involved in other research projects including The Mepoluzimab (MEPO) trial, an RCT for severe eosinophilic asthma (as an investigator) at the North West Lung Centre, Wythenshawe Hospital, Synairgen Study for asthma and also conducted a retrospective audit for asthma transition to ABPA and Chronic Pulmonary Aspergillosis. I have published 3 papers and 2 of my papers are currently undergoing journal peer review.
CHAPTER 1
General Introduction and Literature Review
1.0 Background
Asthma is a chronic inflammatory airways disease that involves multiple pathophysiological and aetiological mechanisms and affects an estimated 300 million people worldwide. The prevalence of asthma increased significantly in the 20th century and is currently estimated to be around 18% in the UK [1, 2]. The majority of asthma sufferers can be controlled with standard therapy, but about 5-10% have severe asthma that is therapy resistant despite maximal standard therapy. Asthma control can be worsened and complicated by various factors of which fungi is important [3-5]. Allergic bronchopulmonary aspergillosis (ABPA) is an uncommon complication of asthma and cystic fibrosis (CF) occurring in 0.7-4.1% of asthmatics seen in secondary care [6-8]. Severe asthma with fungal sensitization (SAFS) is a new severe asthma phenotype recently described that is closely related to ABPA and refers to patients with severe asthma (SA), sensitized to one or more fungal allergens [3]. Approximately 50-60% of severe asthmatics are sensitised to fungi, hence there are an estimated 7.5-15 million SAFS patients worldwide. The exact immunopathogenesis and treatment of ABPA, SA and SAFS are not fully known.

Patients with ABPA have asthma or CF, total IgE more than 1000 IU/L, evidence of aspergillus sensitization and central bronchiectasis [9]. The precise cause, underlying pathophysiological mechanisms and treatment of these diseases are not fully understood. Therefore, there is a growing need to understand the disease pathogenesis and evaluate potential treatments which are safe and efficacious.

There are probably significant regional differences in prevalence of asthma, with a lower figures likely in the Scandinavia/Baltic States (4.9%), Southern Asia (3.5%), Middle East (5.8%) and higher figures in Western Europe (17.5%) and the USA [10]. Not only do patients with ABPA, severe asthma and SAFS experience progressive, debilitating symptoms, but the costs to the health service are enormous. The number of disability-adjusted life years (DALYs) lost due to asthma worldwide has been estimated to be currently about 15 million per year. Worldwide, asthma accounts for around 1% of all DALYs lost, which reflects the high prevalence and severity of asthma and is the 25th leading cause of DALYs lost among chronic diseases worldwide. In the US, the annual costs per patient at $1907 and the total national medical expenditure at $18 billion. In Europe, estimated total costs of
asthma are approximately € 17.7 billion per annum, with the UK among countries with the most asthma related consultations. The direct costs of asthma comprise the health care expenditure associated with hospitalizations emergency visits, physician visits, diagnostic tests and medical treatment, whereas indirect costs include the impact on employment loss of work productivity and other social costs.

ABPA is characterized by worsening of asthma symptoms not relieved by escalation in treatments, usually with mucus plugging of the airways. Assuming a frequency of ABPA of 2.5% in adult asthmatics, there are an estimated 4,800,000 ABPA patients worldwide [11]. There are probably significant regional differences in prevalence of ABPA, with a lower figure likely in the USA and higher figure likely in India [12-16]. Similar regional differences are likely with SAFS. Diagnosis of ABPA relies on a collection of nonspecific parameters including a history of asthma, peripheral eosinophilia, immediate skin test reactivity to Aspergillus antigen and/or elevated anti-Aspergillus IgE, elevated total serum IgE of greater than 1000 IU/L, and, in older series, central bronchiectasis [9].

The past decade has seen several studies involving molecular, cellular and immune mechanisms of asthma and related allergic diseases using various techniques such as flow cytometry in order to understand the mechanism and pathophysiology of airway inflammation. In the majority of cases, the primary cause of inflammation is sensitisation to environmental allergens such as plant pollen, house dust mites (HDM), dog or cat dander, mixed grass and fungi. In recent years, there has been an increasing interest in immunopathogenesis and treatment of allergic fungal airway diseases associated with asthma. Despite these efforts, the exact mechanisms still remain incompletely understood.

1.1 Definitions, diagnosis and assessment tools

1.1.1 ASTHMA

1.1.1.1 Definition
Asthma is an heterogeneous disease and is defined pathologically as a chronic inflammatory airways disease that involves multiple pathophysiological mechanisms including mediators and cells leading to recurrent attacks of airway narrowing and to
structural alterations of the bronchi. It is defined clinically as variable airflow obstruction [17], characterized by airway hyper responsiveness [17, 18] with resultant characteristic variable symptoms of chest tightness, dyspnoea, and/or cough, recurrent wheeze [19-26]. More recently, the definition of asthma has been expanded in the 2014 GINA clinical definition to incorporate into the above definition features that not only focus on the two key features needed for the diagnosis of asthma (variable respiratory symptoms and variable airflow limitation), but also adds heterogeneity of disease to its definition [27].

1.1.1.2 Diagnosis of asthma

The diagnosis of asthma is clinical and there is no standardised definition. The absence of a gold standard definition means that it is not possible to make clear evidence based recommendations by international task force groups. Nevertheless, the current diagnosis of asthma is based on a combination of characteristic pattern of symptoms and signs, measurement of airflow limitation and the absence of an alternative diagnosis.

In the same manner as the process of diagnosing pulmonary embolism (PE), a clinical diagnosis based on probability (high or low) is currently recommended by the British Thoracic Society (BTS) [28, 29]. This is particular so because most symptoms of asthma do exist in other conditions and as such, the presence of at least two characteristic symptoms such as wheeze, breathlessness, chest tightness and cough especially if worsens at night or early morning or triggered or worsened by exercise, allergen exposure and cold air, NSAIDS, aspirin or beta blockers with or without a history of an atopic disease (hay fever, rhinitis, eczema etc.) increase the clinical probability of asthma. Other factors that increase the clinical probability of asthma include unexplained low forced expiratory volume in the first second (FEV1) or peak expiratory rate (PEF) (historical or serial readings) or unexplained peripheral blood eosinophilia [28]. Confirmation of diagnosis hinges on demonstration of reversible airflow obstruction plus characteristic symptoms.

Spirometry is preferable to PEF because it allows clearer identification of airflow obstruction, and is less effort dependent [28, 29]. However, a normal spirometry or PEF obtained when the patient is not symptomatic does not exclude the diagnosis of asthma. Hence repeated measurements of lung function are often more
informative than a single assessment. In some doubtful cases, a trial of high does OCS for a period of 2-3 weeks is advocated.

All patients with suspected asthma should undergo reversibility testing. A Diagnosis of asthma is confirmed by post-bronchodilator (PBD) increase in FEV1 \( \geq 12\% \) and or \( \geq 200 \text{ mL} \) compared with baseline [29] (except in some cases of chronic asthma who might have fixed airflow obstruction [30, 31], in the absence of features suggestive of COPD). It is now recommended that patients with symptoms suggestive of asthma, but with no evidence of PBD reversibility features of asthma (provided no fixed airflow obstruction) should undergo bronchial hyperresponsiveness (BHR) testing using methacholine challenge test (MCT) or histamine [32]. In a patient with typical asthma symptoms, a diagnosis of asthma is considered when there is a significant bronchial responsiveness as demonstrated by a maximum provocative dose (PD) of methacholine or histamine producing a 20% fall in FEV1 (PC20 or PD20 FEV1) of \( \leq 8 \text{mg/ml} \) on a 5-breath dosimeter protocol [33, 34].

1.1.1.3 Bronchial challenge tests

In clinical setting, bronchial challenge testing is used to measure airway hyperresponsiveness (AHR). AHR may be used in confirming the diagnosis of asthma, occupational asthma, or reactive airways dysfunction syndrome (RADS). The two most commonly used agents are methacholine (MCT) and histamine (HCT), although mannitol is also used in some centres [35]. AHR may vary over time, often increasing during exacerbations and decreasing during treatment with anti-inflammatory medications. Thus it is important to consider these factors when interpreting these tests, especially in borderline test results [36, 37].

MCT is most often considered when asthma is a serious possibility and traditional methods, most notably post-bronchodilator (PBD) reversibility, have not confirmed or excluded the diagnosis [28]. Diagnosis is based on the maximum concentration of methacholine or histamine (provocation concentration/dose) able to drop FEV1 by 20% or more from baseline (PC20 or PD20), usually <8mg/ml [33, 34]. A positive test is often defined as a \textbf{PC20} < 8 mg/ml. A negative MCT result is commonly defined as non-response to the highest concentration (PC 20> 8-25 mg/ml).
A diagnosis of asthma is made when a patient has typical clinical features and positive MCT/HCT. However, it is important to exclude false positive and false negative MCTs.

**False positive MCT**

While sensitivity of the MCT in diagnosing clinically significant asthma is excellent, its specificity is poor. Since there are many conditions which have been associated with AHR, a positive test must be interpreted cautiously [38]. A false-positive MCT (*PC20 is < 8 mg/ml but the patient does not have asthma*) must be born in mind and should always be considered when interpreting a positive MCT [38, 39]. Factors which may be associated with a false positive MCT include **test methodology**, normal variation of AHR in the general population, and numerous medical conditions such as allergic rhinitis, smokers and COPD where MCT has relatively high false-positive rates and, therefore, poor positive predictive power. About 30% of patients without asthma but with **allergic rhinitis** have a PC20 in the borderline BHR range [38].

**False negative MCT**

False negative MCT results are less frequently encountered than false-positive results.

Common causes of false negative MCT are patients taking intensive anti-inflammatory agents prior to the MC, although this may not be relevant in symptomatic patients. A small fraction of workers with occupational asthma due to a single antigen or chemical sensitizer may respond only when challenged with the specific agent. More recently, there have been concerns that MCT may be insufficient to exclude AHR in a symptomatic military population [40]. Therefore, these factors should be considered before accepting a negative test as ruling out asthma [10].
1.1.2 ASTHMA CONTROL

The assessment of asthma control is important for the evaluation of treatment response in clinical practice and interventional studies. Asthma control refers to a spectrum of characteristics which describes the extent to which clinical manifestations of asthma have been removed or reduced by treatment. In clinical practice, it refers to the achievement of an acceptable clinical state, often based on goals of asthma management. These characteristics include symptoms, exacerbations, health care utilisation, exercise tolerance and severity of airflow obstruction [27, 41-43]. However, there is currently no single outcome measure that can adequately assess asthma control.

There are four main international criteria used for asthma control i.e.

i. Global Initiative for Asthma (GINA) [47-50].
ii. The Goal (Gaining Optimal Asthma Control).
iii. National Asthma Education and Prevention Program (NAEPP) criteria.
iv. The new ERS/ATS 2013 task force criteria [44].

1.1.2.1 GINA criteria for asthma control

The GINA criteria for assessing control relies on frequency of symptoms and their effect on day to day activity, level and frequency of medication use and lung function. In these criteria, levels of asthma control have been proposed that are grouped into three main categories (controlled, partially controlled or uncontrolled) (table 1.1) [47-50]. The GINA criteria are the most frequently and widely used and are usually updated yearly. The latest is the 2015 classification which is essentially same as the GINA 2012, except that lung function now appears only in the assessment of risk factors (suppl). Two domains (symptom control + risk factors for adverse outcomes) have been included in the new GINA 2015 asthma control criteria [27, 43, 45, 46]. These risk factors include risk factors for exacerbations (ever intubated for asthma, uncontrolled asthma symptoms, low FEV1), risk factors for fixed airflow limitation (No ICS treatment, smoking, occupational exposure, mucus hypersecretion, blood eosinophilia) and risk factors for medication side-effects (frequent OCS, high dose/potent ICS, P450 inhibitors) [27].
Table 1.1: Levels of asthma control according to GINA (2006)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Well controlled</th>
<th>Partly controlled</th>
<th>Uncontrolled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daytime symptoms</td>
<td>None (2 or less/week)</td>
<td>&gt;twice/week</td>
<td>3 or more features of partly controlled asthma present per week</td>
</tr>
<tr>
<td>Limitations of activities</td>
<td>None</td>
<td>Any</td>
<td></td>
</tr>
<tr>
<td>Nocturnal symptoms/awakening</td>
<td>None</td>
<td>Any</td>
<td></td>
</tr>
<tr>
<td>Need for rescue/reliever medication for symptoms &gt;twice/week</td>
<td>None (2 or less/week)</td>
<td>&gt;twice/week</td>
<td></td>
</tr>
<tr>
<td>Lung function (FEV1 or PEFR)</td>
<td>Normal</td>
<td>&lt;80% predicted or personal best on any day</td>
<td></td>
</tr>
<tr>
<td>Exacerbations</td>
<td>None</td>
<td>One or more per year</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1: Levels of asthma control according to GINA (2006). GINA=Global Initiative for Asthma. Need for rescue/reliever medication excludes reliever taken before exercise, because many people take this routinely. (Table adopted and modified from the 2006 GINA report)

1.1.2.2 Gaining Optimal Asthma Control (GOAL) criteria

The Goal criteria for asthma control are based on the use of the ACT and ACQ scores. Thus according to these criteria;

- **Poorly controlled asthma** is ACQ consistently > 1.5 or ACT <20.
- **Well-controlled asthma** is ACQ <0.75.
1.1.2.3 The 2013 ERS/ATS criteria for poor control

According to the 2013 ERS/ATS criteria, uncontrolled asthma is defined as at least one of the following [44, 47, 48]:

- **Poor symptom control**: ACQ consistently >1.5, ACT<20 (or “not well controlled” by GINA guidelines-above)
- **Frequent severe exacerbations**: two or more bursts of systemic CS (≥3 days each) in the previous year.
- **Serious exacerbations**: at least one hospitalisation, ICU stay or mechanical ventilation in the previous year
- **Airflow limitation**: FEV1 <80% predicted together with reduced FEV1/FVC (defined as less than the lower limit of normal) [49].

There are some similarities between the latest ERS criteria and the 2015 GINA criteria in that both highlight and include the importance of risk factors for exacerbations in defining poor disease control [27, 44]. Accordingly, the new BTS 2015 draft guideline (unpublished) suggests the need for assessment of patient risk from death, decline or severe exacerbations. Such approach might improve the quality of care and patient outcomes.

1.1.3 ASTHMA CONTROL CLINICAL ASSESSMENT TOOLS

Various clinical tools have been used to assess asthma control including the Annual recording of the Royal College of Physicians three questions (RCP3Q) [50], the validated Asthma Control Questionnaire (ACQ) and the Asthma Control Test (ACT) [67] [51] administered by a self-completed questionnaire. The superiority of either of each assessment tool is debatable.
1.1.3.1 Annual recording of the Royal College of Physicians three questions (RCP3Q)

The RCP3Q morbidity score which is rewarded within the UK ‘pay-for-performance’ Quality and Outcomes Framework uses three scores and is usually used in General Practice [50]. RCP3Q scoring is based on a self scoring questionnaire. Patients are asked to state ‘yes or no’ to each of the 3 questions which asks whether patients have had the following in the last 4 weeks:

i. **Sleep** disturbance due to asthma (including cough).

ii. Day time **asthma symptoms** or

iii. Disturbance of **daily activity**.

Each of yes scores 1 and no scores 0, maximum 3.

In the RCP3Q,

- **Good asthma control** is an RCP3Q score of zero,
- **Poor control** is a score of 2 or 3.

1.1.3.2 Asthma control test (ACT)

The ACT is a simple, 5-question (5-dormain) tool that is self-administered by the patient in which the patient scores herself/himself on each of the 5 questions (each scored on a scale of 0-5) [67] (appendix A1.1):

i. How much of the time did their asthma affected their ability to perform **activity** at work, school or at home in the preceding 4 weeks,

ii. frequency of **breathlessness**,

iii. frequency of **nocturnal awakening** as a result of asthma,

iv. frequency of **reliever medications** and

v. how the patient would **rate their asthma control** over the preceding 4 weeks.

The results can help to determine the level of asthma control [42]. Patients are encouraged to record and score their ACT at home and the tool can be used by attending practitioners to assess the levels of disease control and alter treatment accordingly. It is also available online for patients to use. Each question has maximum 5 points and the maximum score is 25. The higher the score, the better the asthma control. On the basis of the ACT, **poorly controlled asthma** is an ACT <20 (Appendix A1.1) [51] 58.
1.1.3.3 Asthma control questionnaire (ACQ)

The ACQ is a 7-point asthma control assessment tool and is the most widely used assessment tool in clinical practice. ACQ measures the adequacy of asthma treatment as identified by international guidelines and is a well validated tool [52]. It consists of seven items (the five highest scoring symptoms, rescue bronchodilator use and FEV$_1$% of predicted, each scoring from 1-6, giving a total score of 42 normal) [52]. Patients are asked to recall and score their experiences during the previous 7 days and respond to each question using a 7-point scale [52]:

i. The severity of five highest scoring symptoms during the previous 7 days;
   a. Frequency of **night awakening** (0=Never, 6=Unable to sleep because of asthma).
   b. Severity of **asthma symptoms when they woke up** in the morning during the previous 7 days (0=No symptoms, 6=Very severe symptoms).
   c. Severity of **limitation of activity** because of asthma during the previous 7 days (0=Not limited at all, 6=a very great deal).
   d. Frequency of **shortness of breath** experienced because of asthma during the previous 7 days (0 none, 6=very great deals).
   e. Frequency of **wheezing** during the past week (0=not at all, 6=all the time).

ii. Number of puffs of **short-acting bronchodilator** used each day during the previous 7 days (0=none, 6=more than 16 puffs most days).

iii. The value of FEV1 completed by the clinic staff (0>95% predicted, 6<50% predicted).

The items are equally weighted and the ACQ score is the mean of the 7 items (0-6). The total score is the average of all scores. In contrast to the ACT, the lower the score, the better the asthma control and therefore **0 (well controlled) and 6 (extremely poorly controlled)**.

Three shortened versions of the ACQ (symptoms alone (ACQ-5) symptoms plus FEV$_1$ (ACQ-6) and symptoms plus short-acting β$_2$-agonist (ACQ-6) have been developed, but data from randomised trial suggests no difference between different versions of the ACQ suggesting that any of these versions (short or long version)
can be used in large clinical trials without loss of validity or change in interpretation [53].

Whether one assessment tool is superior to others is not clear, but recent data indicate that the ACT may be superior to the ACQ [54]. In a recent systematic review and meta-analysis that compared the diagnostic performances of the ACT (n=11,000) and the ACQ (n=12,483) in twenty-one studies, ACT was preferable to ACQ. However, neither the ACT nor the ACQ was useful for the assessment of uncontrolled asthma [60].

One problem of the original asthma control assessment tools is that they focussed greatly on current symptom level for an individual patient, but while a patient current symptom status is a good measure, patients who report few symptoms may still be at risk of asthma exacerbations [50, 52]. For this reason, there has been recent improvements to the original assessment tools in the recent GINA reports and the ATS/ERS which have now included risk of exacerbations in the assessment tool [44]. In this thesis, the earlier ACT and ACQ scores have been used to assess asthma control and the AQLQ-J has been used for QOL assessments.

1.2 SEVERE ASTHMA

The vast majority of asthma patients can be controlled with standard therapy. However, a substantial group of patients remain uncontrolled despite adequate standard therapy and are termed ‘severe’, ‘refractory’ or ‘difficult to control’ asthmatics [44, 55]. These terms are usually used interchangeably although difficulty to control asthma implies asthma whose asthma is difficult to control because of uncontrolled co-morbidity. Severity is not static – it may change over months or years, or as different treatments become available or optimised.

Severe asthma is a debilitating disease and is associated with high morbidity and mortality. It is estimated that about 1-3% of the total world population (children and adults) and about 5-10% (15-30 million) of those with asthma have severe asthma that is difficult to treat despite adequate standard asthma therapy [56-58] [59-61]. This form of asthma represents a high unmet medical need and continues to be a
challenge despite several international initiatives to better understand the disease process. The pathogenesis and treatment of severe asthma is partially understood.

1.2.1 Definitions of severe asthma

The precise definition of severe asthma is important for clinical and research in order to produce reliable results by allocating research subjects to the correct study groups. However, its definition has undergone several changes over the years and there is no universally accepted definition of severe asthma although progress has been made recently to standardise the definition [62-65]. The absence of clear, objective definitions allows for subjective judgments; individual physicians and patients focus on different assessment components.

Historical perspectives

Historically, the definition of severe asthma has undergone various modifications over the years. Various clinical definitions have been proposed through national and international guidelines, working groups, task forces and workshops which incorporate symptoms, lung function, exacerbations, and, in many cases, specific use of high-dose inhaled corticosteroids (ICS) [66, 67][31].

In 1999, an ERS taskforce adopted the term ‘difficult asthma’ [67]. In 2000, the ATS–sponsored workshop defined refractory asthma [68]. This definition included one of two major criteria i.e. continuous high-dose ICS or OCS for ≥50% of the previous year, plus two of seven additional minor criteria [68]. Both the 1999 ERS and the 2000 ATS criteria focussed on the level of medication needed to achieve the best possible control [42, 69], but this is subjective as the choice of medication depends on individual physicians and is subject to inter-observer bias and interpretation.

1.2.1.1 BTS severe asthma criteria

According to the BTS criteria, severe asthma is defined as treatment steps 4 i.e. poorly controlled asthma on high dose ICS (≥800 mcg BDP daily) plus long acting B2-agonist (LABA) or continuous use of OCS or frequent use of OCS to provide disease control (step 5) [70]. Therefore, an element of treatment of responsiveness is incorporated in the definition.
However, the use of medication as a marker of severity remains somewhat arbitrary, as and both assessment strategy and frequency may vary within and between observers.

1.2.1.2 World Health Organization (WHO)-2009 severe asthma definition
In April 5-6, 2009, the WHO’s definition of severe asthma includes 3 groups [71]:

i. Untreated severe asthma.

ii. Difficult-to-treat severe asthma (as a result of non-adherence, persistent triggers, or co morbidities), and

iii. Treatment-resistant severe asthma.

1.2.1.3 ATS/ERS task force 2013 definition of severe asthma
More recently, the ATS/ERS task force 2013 defined severe asthma as asthma that requires treatment with high dose ICS (≥2000 µg (DPI or CFC MDI) ≥1000 µg (HFA MDI) plus a second controller and/or OCS to prevent it from becoming "uncontrolled" or that remains "uncontrolled" despite this therapy [62-65]. This definition requires the diagnosis of asthma confirmed, triggers, compliance and co morbidities addressed before the patient can be labelled to be severe asthmatic. Such co morbidities include COPD [72], rhino sinus disease [72], CCF, laryngeal dyskinesia, laryngopharyngeal reflux (LPR), vocal cord dysfunction (VCD), mechanical obstruction, bronchiectasis, obliterative bronchiolitis and obesity. Thus, the ideal diagnosis of severe asthma requires a correct diagnosis of asthma itself as demonstrated by typical asthma symptoms [19-26], together with objective evidence of variable airflow limitation and/or [17, 18] plus addressing the co morbidities. Patients with difficult to control or refractory asthma need to have their asthma evaluated and managed by an asthma specialist for more than 3 months and severe asthma according to the ATS/ERS definition only applies to patients with refractory asthma and those in whom treatment of co morbidities remains incomplete. In this thesis, we used the BTS definition as the 2013 ERS/ATS definition was not in place at the design and commencement of the study.
1.2.2 Asthma control versus asthma severity

Concepts of asthma severity and control are important in evaluating patients and their response to treatment, as well as for research, but the terminology applied is not standardized, and terms are often used interchangeably. ‘Asthma control’ refers to a spectrum of characteristics which describes the extent to which clinical manifestations of asthma have been removed or reduced by treatment. In clinical practice, it refers to the achievement of an acceptable clinical state, often based on goals of asthma management and is assessed by tools such as the ACT and ACQ (hence well controlled, partly controlled and poorly or uncontrolled). ‘Severe asthma’ refers to asthma that remains poorly controlled, despite maximum asthma treatment after addressing modifiable factors (co-morbidities) and inhaler techniques. The current ERS/ATS 2013 definition of severe asthma is an ACT<20 or ACQ>0.75 during high-dose ICS therapy with an additional controller or during OCS therapy for more than 6 months per year [55-58].

The approach now is to sub classify severe asthma patients into phenotypes and endotypes, severe controlled asthma/uncontrolled [73].

1.2.3 PHENOTYPES (OBSERVABLE CHARACTERISTICS) OF SEVERE ASTHMA

Asthma, in particular, severe asthma is increasing being recognised as a syndrome with a heterogeneous presentation, consisting of multiple different pathogenetic subgroups with different cellular and molecular characteristics. Thus, the earlier classification of asthma as “allergic or “intrinsic “originally proposed by Francis M. Rackemann in 1918 [74-77] is slowly being replaced by phenotyping of asthma for targeted treatment of particular subtypes [73, 78, 79]. A phenotype is an observable characteristic of a patient [77]. The concept of asthma severity has also changed [44]. Thus, the GINA 2014 now recognizes the heterogeneity of the disease, its phenotypes [27, 46] and endotypes [80, 81].

An endotype is defined as the integration of a specific identifiable underlying pathobiologic process, the inhibition of which contributes critically to elemental clinical characteristics [80, 81].
1.2.3.1 Clinical Phenotypes

Different clinical phenotypes have distinct and unique features of the disease characteristics of symptoms, health status, response to treatment, nature of airway obstruction [82], type of airway inflammation, atopy, frequency of exacerbations [82], reversibly of lung function, age of onset [83, 84], corticosteroid dependence or resistance [85-87], obesity, triggers such as aspirin sensitivity, BMI [88].

The advent of non-invasive diagnostic approaches to assess airway inflammation using sputum induction and the measurement of exhaled nitric oxide (FENO) techniques have revolutionised our approach to diagnosing and subgrouping asthmatics into a subgroup of severe asthmatics with predominant airway eosinophilia that is steroid responsive and neutrophilic phenotype that is poorly responsive to ICS [89, 90]. In addition, the emergence of targeted molecular-based therapies, together with the use of unbiased statistical clustering approaches have rapidly advanced our concept of phenotyping [89, 90]. Thus, we can now identify severe asthma patients belonging to distinct subgroups with distinct features of severe asthma (phenotypes):

a) Early onset-usually allergic versus late onset (usually obese) severe asthmatics
b) Eosinophilic, neutrophilic, and paucigranulocytic asthma.
c) Aspirin Exacerbated Respiratory Disease (AERD).
d) Corticosteroid dependant or corticosteroid resistant.
e) Severe asthma with fixed airflow obstruction.
f) Allergic severe asthma.
g) Severe asthma with fungal sensitisation (SAFS) [3].

With phenotyping, we can now stratify treatment accordingly. For example, high dose steroids for eosinophilic phenotype, anti IL-5 monoclonal antibody (mepolizumab) or its receptor (benralizumab) for eosinophilic phenotype [91-100], anti-IgE (omalizumab) therapy for severe persistent allergic asthma (for IgE-mediated asthma) [101-103], anti-fungals for SAFS [104] and bronchothermoplasty for patients with remodelled airways [105-107], tiotropium and/or azithromycin (for non-eosinophilic asthma) and irreversible airflow obstruction [108-113].
1.2.3.2 Molecular Phenotypes

The observation that only a portion of clinical asthma was associated with an underlying Th2-like immune-inflammatory process (present) in about 50% of adult asthma. [78], has led us to subtype asthma patients into a a group with Th2-driven inflammation and that with non-Th2 type inflammation.

a) Th2-like molecular phenotypes

This group usually has a Th2 driven type of inflammation and usually orchestrated by Th2 cytokines IL-4, IL-5 and IL-13. They include some of those patients traditionally known as allergic asthma, some patients with exercise-induced asthma (EIB) and eosinophilic asthma (table 1.2).

Patients with a Th2-like molecular phenotype are usually corticosteroid (CS) sensitive although the range of this sensitivity is variable across the group, hence supporting the overall heterogeneity even within this same molecular phenotype. Biomarkers, including blood eosinophils, periostin and exhaled nitric oxide (FENO) can be used to identify this Th2-like phenotype. In fact, using these Th2-like biomarkers improves the ability to identify responders to Th2 targeted therapies and improve outcomes [100, 114, 115].

However, responses still vary, even within the Th2-predominant patients. Thus, it is likely that some Th2-like molecular phenotypes (eventually endotypes) will respond better to IL-4/IL-13 targeted therapy while another group will respond better to an IL-5 directed therapy [100, 114, 115]. Studies that link these molecular targeted therapies to improvements in specific characteristics, pathobiology and biomarkers will ultimately identify more asthma endotypes.
b) Non-Th2 associated asthma
This is a broad asthma phenotype that exhibits little or no evidence of Th2 airway inflammation. It is defined generally by the absence or low biomarkers that are normally associated with Th2-like asthma (table 1.2). Examples are obesity associated asthma, neutrophilic asthma, paucigranulocytic asthma and smoking associated asthma, all of whom are generally poorly CS responsive. It is likely that the non-Th2 like asthma have different exacerbation profile and disease progression from Th-2 like molecular phenotype. The IL-17 driven asthma appears to be in this group (table 1.2) and studies of this type of asthma can help identify molecular targets for targeted therapy.
Table 1.2: Th2-like (eosinophilic) and non-Th2 molecular phenotype of severe asthma

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Th2-like (eosinophilic) molecular phenotype</th>
<th>Non-Th2 asthma (non-eosinophilic) molecular phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>FENO</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Periotin</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Blood or sputum eosinophils</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>IL-17A</td>
<td>Little role</td>
<td>Likely significant role</td>
</tr>
<tr>
<td>IL-4, IL-5, IL-13</td>
<td>Major role</td>
<td>Little role</td>
</tr>
<tr>
<td>Steroid responsiveness</td>
<td>ICS, OCS responsive</td>
<td>Less responsive</td>
</tr>
<tr>
<td>Clinical asthma examples</td>
<td>• High eosinophilic asthma</td>
<td>• obesity associated asthma</td>
</tr>
<tr>
<td></td>
<td>• Some allergic asthma (but not all)</td>
<td>• neutrophilic asthma</td>
</tr>
<tr>
<td></td>
<td>• Some with exercise-induced asthma (EIB).</td>
<td>• paucigranulocytic asthma</td>
</tr>
<tr>
<td></td>
<td>• Adult onset,</td>
<td>• smoking associated asthma</td>
</tr>
</tbody>
</table>

Table 1.2: Molecular phenotypes of severe asthma. FENO=exhaled nitric oxide; Th2=lymphocyte type 2.

Limitations of phenotyping based airway inflammation

Although patients with asthma can be defined on the basis of presence and type of inflammation, the relationship is not a straight forward one. This is due to many factors;
Firstly, numerous pathogenic mechanisms appear to be involved in the pathogenesis of asthma other than inflammation alone. Secondly, eosinophilic and neutrophilic asthma are not mutually exclusive subtypes of asthma and both eosinophilia and neutrophilia can co-exist within the same patient. In addition, the
predominant airway cells vary with time within the same patient. For example, neutrophils are prominent in airway secretions during acute severe asthma exacerbations, where it is possible that they have roles in both the initiation and resolution of attacks. Thirdly, around 50% of patients with severe asthma have very little identifiable inflammation.

1.2.3.3 Aspirin sensitive asthma
Aspirin sensitive asthma (so called ‘aspirin exacerbated respiratory disease (AERD) is a distinct clinical syndrome seen in 5-10% of patients with asthma, usually adult onset. It is characterized by history of acute dyspnoea usually accompanied by nasal symptoms (rhinorrhea and/or nasal congestion) within two hours after ingestion of acetylsalicylic acid (ASA). These patients usually suffer from chronic, usually severe, rhino sinusitis, recurrent nasal polyps and hypersensitivity to a cyclooxygenase-1 (COX-1) inhibitor, aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs) [116, 117]. AERD is associated with increased risk for severe asthma, frequent exacerbations, although they are a heterogeneous group with respect to asthma severity. Up to 70% AERD patients are atopic. Diagnosis of AERD is with aspirin challenge. It is usually treated by aspirin desensitization followed by continued daily aspirin [116-121].

1.2.3.4 Allergic severe asthma
Patients with this type of asthma have a high frequency of atopic manifestations such as rhinitis, eczema, hay fever [122-127]. This group is usually early onset type and constitute around 40–50% of all severe asthmatics [128]. Severe asthmatics with this phenotype are likely to have a high number of allergic skin test positive reactions, a high levels of total and allergen specific blood immunoglobulin (Ig)E, greater environmental exposures and a family history of asthma [128]. The presence of a high IgE levels and multiple allergic sensitisation makes this group an ideal target for anti-IgE (omalizumab) therapy [101-103, 129].
1.3 Social economic burden of severe asthma

The disability associated with asthma varies with the level of asthma control, with uncontrolled asthma having a considerably more disabling than moderate angina [130]. Not only are patients with severe asthma disabled with their disease and have poor quality of life due to numerous comorbidities often as a result of treatment or the disease itself, the health cost burden is enormous [1, 131]. In the UK, latest data from the BTS Difficult Asthma Registry (n=596) that estimated direct healthcare treatment costs from the National Health Service (NHS) show that the annual mean treatment costs attributable to severe refractory asthma patients alone is around £2912–£4217 per person per year, with the largest proportion due to medication [132].

Patients with severe asthma also present a special challenge because of multiple diagnostic tests required and insufficient evidence regarding personalized treatments. The high prevalence and increasing of co-morbid disorders such as obesity, cardiovascular disease, upper airways disease, psychosocial factors such as depression may lead to substantial direct and indirect costs of disease. This situation is further compounded by high frequency non-adherence and persistent triggers [133, 134]. As such, continued research into the mechanisms driving the underlying pathophysiology to improve outcomes is vital.

1.4 FUNGAL ALLERGY AND ASTHMA

The link between asthma severity and fungal allergy is strong [3-5]. Multiple fungi are involved including *Aspergillus, Alternaria, Penicillium, Candida, Trichophyton, Mucor, and penicillium*. Sensitisation to mould allergens is related to asthma severity and increased sudden respiratory death [135], risk of severe asthma exacerbations and multiple hospital & ICU admissions [136]. In a cross-sectional study of 1,132 adults with asthma, sensitisation to Alternaria (odds ratio (OR) 2.03) or Cladosporium (OR 3.2) was a significant risk factor for severe asthma in several European countries, Australia, New Zealand and Portland (OR, USA) [137]. In an Indian ICU setting, Ritesh Argawal and colleagues evaluated the occurrence of Aspergillus hypersensitisation (AH) and ABPA in patients with acute severe asthma (ASA) and found that the occurrence of AH was significantly higher in patients with
ASA admitted to a respiratory ICU compared to outpatients (51\% versus 39\%), (p=0.01). Similarly, a significantly higher prevalence of ABPA in ASA in ICU compared to outpatients (39\% versus 21\%, \(p=0.001\)) was reported [138]. These findings support the rationale for the use of fungal skin testing to exclude associated fungal allergy in patients admitted with acute asthma at discharge. Such screening may help identify more patients sensitised to fungi who may benefit from targeted antifungal therapy with a potential to improve asthma control, reduce morbidity, mortality and the cost of care.

1.4.1 SEVERE ASTHMA WITH FUNGAL SENSITISATION (SAFS)

In recent years, a new asthma variant called ‘Severe Asthma with Fungal Sensitisation (SAFS)’ has been described by Denning et al following clinical observations, supported by more than a century of published links between asthma severity and fungal exposure and/or sensitization [3]. These patients have severe asthma, are sensitized to one or more fungi but have normal or only slightly elevated total IgE concentrations [5]. The clinical and diagnostic manifestations of SAFS are thought to arise from an allergic response to multiple antigens expressed by \(A.\ fumigatus\) and possibly other fungi, colonizing the bronchial mucus [139]. The exact immunopathogenesis of SAFS has not been established.

The prevalence of SAFS is not completely understood partly, because of lack of agreement regarding the criteria required to define severe asthma and variation in serological tests. There are probably geographic variations with higher numbers in Latin America and the Caribbeans. Recent studies using specific populations at risk derived from literature search and the fungal infection frequencies suggest prevalences of 529/100,000 in Dominican Republic [140], 198/100,000 in Spain [141], 124/100,000 in Nigeria [142] and 140/100,000 population in Ireland [143].

1.4.2 ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS (ABPA)

ABPA is the most frequently recognized manifestation of allergic aspergillosis and is an uncommon complication of asthma and cystic fibrosis occurring in 0.7-4.1\% of asthmatics seen in secondary care [6-8]. ABPA was first described by Hinson et al in 1952 among patients at London Chest Hospital in the United Kingdom [144]. In
the USA, the first case was reported in 1968 [145]. Today, there are an estimated 4,800,000 ABPA patients worldwide [11]. There are probably significant regional differences in prevalence of ABPA, with a lower figure likely in the USA and higher figure likely in India [12-16]. It’s likely that an increase in awareness combined with increased use of screening using serological tests will increase the prevalence of ABPA. The prognosis and natural history is variable, but usually chronic diseases associated with frequent exacerbations, chest infections, and finally; respiratory failure and death. The clinical and pathological course of ABPA [16] is variable but exposure to fungus may lead to either recurrent exacerbations or chronic persistent symptoms.

ABPA is characterized by Th2 allergic inflammatory response to A. fumigatus allergens in the bronchial airway of atopic asthma and cystic fibrosis (CF) patients when A. fumigatus spores are inhaled and germinate in bronchial mucus releasing allergens including proteases [146-149].

1.4.2.1. Diagnosis and definition of ABPA

The diagnostic criteria for ABPA include; i) asthma (of any severity), ii) total serum IgE ≥1000 KIU/mL, iii) immediate cutaneous reaction to A. fumigatus of > 3mm compared to control or or iv) elevated A. fumigatus-specific serum IgE levels, v) precipitating antibodies to A. fumigatus in the serum, vi) a history of pulmonary infiltrates (transient or fixed), vii) in older series, central bronchiectasis [9], viii) a history of expectoration of brown plugs or flecks and ix) isolation of A. fumigatus from sputum [150, 151], of which ii) and iv) are essential.

The diagnostic criteria for ABPA have been a topic of debate and more recently, new classifications and diagnostic criteria have been proposed. These criteria includes the proposed radiological classification which has subsequently classified ABPA into 4 classes i.e. serological ABPA (ABPA-S), ABPA with bronchiectasis (ABPA-B), ABPA with high attenuation mucus (ABPA –HAM) and ABPA with chronic pleural pulmonary fibrosis (ABPA-CPF) [152]. For the purpose of this thesis, the older series definition was used [9], [150, 151].
Clinical course of ABPA

The clinical and pathological course of ABPA is variable including recurrent exacerbations or chronic persistent symptoms [153]. The disease may remit temporarily but is more commonly a progressive, unremitting disorder and untreated patients have a chronic course characterized by recurrent pulmonary consolidation and in many cases, progression to bronchiectasis or pulmonary fibrosis [153], bronchiolitis obliterans [154], granulomatous bronchiolitis [154], lung destruction [155], chronic cavitary aspergillosis (CPA) [156] and in rare cases, pleural effusion [157]. Other complications of ABPA are lobar shrinkage, persistent cavitations (>3 months), and pleural fibrosis, which are features of chronic pulmonary aspergillosis (CPA).

Some patients develop aspergilloma, which is definitive evidence of CPA. The frequency of these non-bronchiectasis features in ABPA has been reviewed and overall is present in approximately 10% of cases [158]. With one exception, all studies were earlier than 1985, and the epidemiology of asthma, some aspects of clinical management and referral patterns have changed since then [159-164]. The more recent study was a small radiology series from India [165].

1.4.2.2 Aspergillus sensitisation versus ABPA

The term aspergillus sensitisation (AS) refers to the presence of serological or skin prick positivity without radiological or clinical features of ABPA. The last two decades have seen a considerable debate about the relative merits of the radiological, microbiological and immunological tests utilized, and how to best differentiate Aspergillus sensitization, Aspergillus colonization and ABPA as clinical entities [166-168].

The prevalence of AS varies depending on the type of test used and is higher with skin test compared to specific IgE testing [169]. A major problem is the lack of standardized allergens used in the determination of specific IgE. In addition, the precise cut-off value for total IgE and the method by which to demonstrate Aspergillus sensitization (cutaneous hyper-reactivity or specific IgE) has been questioned. Although a serum total IgE level of >1000 is currently used as a cut off, most patients attending secondary care have had long standing poorly controlled asthma receiving steroids and other immune modulators which alter the IgE levels
at presentation and therefore the use of this IgE cut off usually leads to under diagnosis of ABPA.
1.4.2.3 Asthmatic ABPA versus CF ABPA

The diagnosis of ABPA in CF presents a major challenge compared to that of asthma patients. This is due to a number of reasons. First, *Aspergillus* species are commonly isolated from airway samples of CF patients and markers of immunological sensitisation to Aspergillus are frequently encountered. Therefore the distinction between pathogenic and non-pathogenic aspergillus is problematic. Secondly, many of the criteria used to diagnose ABPA in asthma are the same as the usual symptoms of CF. Thus, the diagnosis of ABPA in CF is more complicated and disagreement exists in the literature regarding the diagnostic criteria. The difficulty lies in the fact that the usual criteria for ABPA and the common signs and symptoms of CF overlap.

The most recent Cystic Fibrosis Foundation Consensus Conference proposed the following diagnostic criteria: (1) acute or subacute pulmonary deterioration not attributable to another aetiology, (2) total serum IgE > 1000 IU/mL, (3) immediate cutaneous reactivity to Aspergillus or in vitro specific IgE antibodies to Aspergillus, and (4) one of the following: Aspergillus serum precipitins, elevated specific IgG anti-Aspergillus antibodies, or new or recent chest radiographic or chest CT abnormalities that have not cleared with antibiotics and chest physiotherapy [170].

A recent systematic review and meta-analysis suggested that the prevalence of ABPA in CF is around 8.9% and is higher in adults compared to children (10.1% vs. 8.9%, p<0.0001) [169]. The main limitation is the wide variation in the diagnostic criteria used, with around half of the new studies published since 2004 having used criteria other than the CF foundation criteria for diagnosing ABPA [171]. The prevalence of ABPA in asthma is around 12.9% and is also limited by a wide variation in diagnostic criteria used [171].
IMMUNOPATHOGENESIS

1.5. PATHOGENESIS OF ASTHMA
The pathogenesis and pathology of asthma is complex, but can be viewed in two ways;

1) A chronic inflammatory process that is characterised by infiltration of inflammatory cells and various mediators and
2) A remodelling process that is characterised by airway smooth muscle and mucosal glandular hypertrophy.

1.5.1 Airway inflammation
The inflammatory process involves multiple cell types, and several distinct cellular and molecular pathways. These pathways include adaptive and innate immunity and involve Th2 cells, IgE, activation of mast cells, basophils, eosinophils, neutrophils, airway epithelial cells, and natural killer T (NKT) cells [172]. Recently, oxidative stress has been implicated in the pathogenesis of asthma [173, 174]. The inflammatory response is characterized by infiltration of the airways by eosinophils, mast cells and lymphocytes; disruption of the epithelium; thickening of the reticular basement membrane; and increases in smooth muscle mass. Through mechanisms that are not yet entirely clear, airway inflammation leads to a persistent augmentation of bronchoconstriction in response to allergens, adenosine monophosphate, and to nonspecific stimuli such as inhaled methacholine. An infiltration of the bronchial mucosa with activated T lymphocytes (T cells), eosinophils, and to a lesser extent, polymorphonuclear leukocytes is found in the airways of asthmatics [175, 176]. These cells, along with resident airway mast cells, secrete a variety of soluble mediators that directly damage the mucosal surface and perpetuate tissue damage by amplifying the original inflammatory response. As a result, the asthmatic airway undergoes remodelling which is characterised by epithelial denudation, sub-epithelial fibrosis, mucus gland hypertrophy, mucus hypersecretion, and smooth muscle hypertrophy.

Although these mechanisms have been implicated, it is not clear which cell type or mediator plays a more dominant role. Further, the pathological findings described
above are qualitatively found in mild as well as severe asthmatics, and attempts to
draw quantitative correlations between severity and intensity of inflammation have
yielded discordant results. In addition, although anti-inflammatory therapy with
corticosteroids decreases the intensity of the inflammation in association with
improved clinical variables, the association is circumstantial and it is unclear which
marker of inflammation correlates best with the severity of acute episodes or the
severity of the disease in patients who are stable.

1.5.2 Th1/Th2 Paradigm and the Th2 –Driven Airway Inflammation of Asthma
Pathogenesis

For years, studies of immunopathogenesis of asthma focused on the two types of
cells of the immune system (Th1 and Th2) [177-182]. In the Th1/Th2 paradigm of
asthma pathogenesis, an allergen enters the airway, professional antigen
presenting cells (APC) such as dendritic cells (DCs), macrophages, and B cells,
take up the allergen and present it to naïve CD4+ T helper cells receptors via major
histocompatibility complex (MHC) proteins which are expressed on APCs (fig 1.1). T
cell receptors can only recognize antigen fragments in complex with MHC proteins.
Naïve CD4+ T helper cells differentiate into Th1 or Th2. The differentiation of naïve
CD4+ T helper cells into Th1 and Th2 is driven by IL-12 and IL-4, respectively,
through intracellular signalling pathways that activate specific transcription factors
such as GATA-3 and induce changes in the chromatin structure of cytokine genes
[183, 184].

Th2 cells produce cytokines such as IL-4 and IL-5 that attract and activate
eosinophils and drive differentiation of B-cells to IgE-secreting plasma cells. The
pre-formed IgE antibodies affix to tissue mast cells and circulating basophils to
trigger release of PGs, histamine etc upon re-exposure to similar allergens (fig 1.1).
Th1 are involved in cellular and humoral immunity, whereas Th2 are involved in the
inflammatory process and allergy. Activation of Th2 cells results in the release of a
plethora of inflammatory mediators (fig 1.1) by regulating B cell- and eosinophil-
mediated responses that induce changes in airway wall geometry and produce the
symptoms of asthma and allergy [177, 185, 186] [184, 187-189] (fig 1.1).
Th1 cells produce IFN-γ and IL-2 which is involved in humoral [189, 190] and cell mediated immunity, hence the Th1/Th2 paradigm of asthma pathogenesis (fig 1.1).
Fig 1.1: The Th1/Th2 paradigm of asthma pathogenesis.

LTs=leukotrienes, PGs=prostaglandins, PAF=platelet activating factor, TGF-β=transforming growth factor beta, AHR=airway hyperresponsiveness, IFN-γ=interferon gamma, ECP= Serum eosinophil cationic protein.
1.5.3 Role of Th2 cytokines in Th2-driven airway inflammation

In this paradigm, IL-4, IL-5 and IL-13 have been found to play an instrumental role in asthma pathogenesis in that IL-4 and IL-13; i) activate the vascular endothelium promoting the attachment and infiltration of T cells and eosinophils in lung tissues [191, 192], ii) promote the synthesis of mediators of the acute allergic reaction through recruitment of IgE antibody producing B cells, mast cells and eosinophils [193]. In addition, IL-4 stimulates mucus cell metaplasia and the production of mucins, thus contributing to airflow obstruction (fig 1.1).

Th2 cytokines IL-4, IL-5, IL-9 and IL-13 mediate atopic manifestations (IL-4 and 5 pathway), airway remodelling, chronic eosinophilic asthma, eosinophilic airway inflammation (IL-5 pathway) and airway hyperresponsiveness (IL-13 pathway) (fig 1.1).

IL-5 is responsible for eosinophil recruitment which is believed to play important roles in the pathogenesis of asthma through the release of inflammatory mediators [194]. Indeed, in refractory eosinophilic asthma, anti-IL-5 monoclonal antibodies (mAb) reduces exacerbations and steroid dose, thus suggesting a pivotal role of eosinophils and IL-5 in severe eosinophilic asthma pathogenesis [195].
Limitation of the T1/Th2 paradigm in asthma pathogenesis

For years, studies of immunopathogenesis of asthma focused on the two types of cells of the immune system (Th1 and Th2). According to the Th1/Th2 paradigm, allergy and asthma result from an imbalance in favour of a Th2 response, and is negatively regulated by Th1 cells. However, while numerous studies support this view [177-182], emerging data are accumulating that do not fit with the model in its original form. Several weaknesses appear in this Th2/Th1 model:

i. We know from gene expression study of mild asthma that only approximately 50% of mild asthmatics manifested a Th2 gene expression profile [78]. These results therefore suggest that not all asthma can be explained by the Th2 paradigm only.

ii. These models do not adequately explain non-Th2-driven asthma and do not explain non-allergic asthma [196-198].

iii. T-helper type 2-driven inflammation does not explain the newly described neutrophilic phenotypes, remodelling or steroid resistant asthma [128, 199-201].

Thus there is need for continued efforts to understand the immunopathogenesis of asthma with the potential to develop targets for therapy. Tregs and Th17 cells are now accepted as representing other CD4+ subsets, which have led to the resolution of some inconsistencies in the Th1/Th2 paradigm.

1.5.4. Current model of CD4+ subsets and immunopathogenesis of asthma (Th2/Th17/Tregs)

Recent advances have provided more insights into the mechanisms by which T cells alter airway inflammation. The original Th1/Th2 paradigm has been expanded to include IL-17-producing Th17 cell lineages, expressing IL-17 family (IL-17A, IL-17E, IL-17F) of cytokines and IL-23-mediated functions on T cells [202-204]. These cells and cytokines play a role in immune response to fungi, allergic asthma [205], extracellular pathogen handling and autoimmune inflammatory disorders. In
addition, IL-9-producing 'Th9' helper T cells [206] and regulatory T (Treg) cells have immunosuppressive functions (fig 1.2).

As allergens enter the airway, professional antigen presenting cells (APC) take up each allergen and present it to naïve CD4+ T helper cells (fig 1.1). Contrary to the original Th1/Th2 paradigm that proposed differentiation into only two T lymphocyte subsets (Th1/Th2), it has recently been appreciated from cytometric analysis that activation of naïve CD4+ T helper cells differentiate into at least 5 T-cell subsets (Th1, Th2, Th9, Th17, Tregs) each with distinct functions and cytokine profiles that are specialized in terms of the effector molecules that they express on their membranes (figs 1.2). Therefore, pathophysiologically, the mechanism can be summarised by the balance of 2 pro-inflammatory pathways (Th2/Th17) and one or more anti-inflammatory pathways (Treg) (fig 1.2, 1.3).
Fig 1.2: Differentiation of naïve T-cell into CD4+ cell subsets (Th1/Th2/Tregs and Th17)

An allergen (e.g., fungal allergen) enters the airway, professional antigen presenting cells (APC) take up the allergen and present it to naïve CD4+ T cell receptors (TCR) via major histocompatibility complex (MHC) proteins which are expressed on APCs. The receptor of T cells can only recognize antigen fragments in complex with MHC proteins. Naïve CD4+ T helper cells differentiate into Th1 or Th2 (cell with brown nucleus), Th17 (cell with blue nucleus) and Treg cells. Differentiation into the Th1 and Th2 cells (cell with brown nucleus) is mediated by IL-12 and IL-4 respectively. STAT5= Signal Transducer and Activator of Transcription 5; STAT3= Signal Transducer and Activator of Transcription 3; TGFβ-1=transforming growth factor 1; TGF-β= transforming growth factor β; FoxP3= forkhead box P3 (is a member of the FOX protein family. FoxP3 functions transcription factor in the development and function of regulatory T cells (Tregs); RORγ= Retinoic acid receptor-related orphan receptor; IRF4=IFN regulatory factor 4
Fig 1.3: Current model of CD4+ subsets and immunopathogenesis of asthma

Upon antigenic stimulation, naive CD4+ T cells differentiate into at least 4 subsets (Th1/Th2/Th17/Tregs) (fig1.3A) which are characterized by different cytokines they produce Th2 cytokines that mediate atopic manifestations airway remodelling, chronic eosinophilic asthma, eosinophilic airway inflammation and AHR.

Th17 cytokines (IL-17) mediate neutrophilic airway inflammation, LTs=leukotrienes, PGs=prostaglandins, PAF=platelet activating factor, TGF-β=transforming growth factor beta, AHR=airway hyperresponsiveness, IFN-Ɣ=interferon gamma, ECP=Serum eosinophil cationic protein, DTH=delayed-type hypersensitivity, CH=contact hypersensitivity, CIA=collagen-induced arthritis.
The Th2-driven airway inflammation (Th2-molecular phenotype)
In the Th2-pathway (fig 1.2, 1.3, table 1.3), Th2 cells produce:
   o IL-4, which induces B-cell activation and IgE antibody production.
     IL4 is also required for optimal Th2 differentiation [207].
   o IL-5, which induces eosinophil recruitment [207]
   o IL-13, which promotes mucus production and airway hyperreactivity (AHR).

The Th17-driven airway inflammation (Th-17 molecular phenotype)
In the Th17 pathway, Th17 cells produce IL-17 and IL-22 (fig 1.3, table 1.3). These cytokines induce epithelial cells to produce the proteins MUC5B and MUC5AC and granulopoietic factors such as IL-8 and G-CSF, which cause neutrophil recruitment and expansion and mucus hypersecretion, ultimately leading to airway narrowing with resultant symptoms (fig 1.3).

The Treg/IL-10 inflammatory suppressor mechanism
IL-10-producing Treg cells and Foxp3 (+)Tregs have an immunosuppressive effect on Th2 cell activation that occurs during asthmatic immune processes, especially in decreasing production of IgE antibody [208]. It is possible that the numbers or function of these cells may be deficient in patients with allergic fungal asthma.
Studies of therapeutic targets of this anti-inflammatory pathway to enhance Treg function in the airway may prove clinically relevant in the treatment of inflammatory airways disease including asthma, ABPA and SAFS.
Table 1.3: Immunological and biological function of the Th2, Th17, Treg type cytokines in the pathogenesis of asthma

<table>
<thead>
<tr>
<th>Th Cell</th>
<th>Cytokine</th>
<th>Function</th>
<th>Effect on inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th2</td>
<td>IL-4</td>
<td>B-cell activation and IgE, Optimal Th2 differentiation.</td>
<td>Pro-inflammatory</td>
</tr>
<tr>
<td></td>
<td>IL-5</td>
<td>Eosinophil recruitment</td>
<td>Pro-inflammatory</td>
</tr>
<tr>
<td></td>
<td>IL-13</td>
<td>Mucus production and AHR</td>
<td></td>
</tr>
<tr>
<td>Th17</td>
<td>IL-17, IL-22.</td>
<td>Induce epithelial cells to produce MUC5B &amp; MUC5AC; - Induces mucus production and IL-8 GM-CSF - Neutrophil recruitment and expansion. Targets structural cells; - epithelia, fibroblasts and smooth muscle cells, inducing the production of chemokines that promote neutrophilic inflammation</td>
<td>Pro-inflammatory</td>
</tr>
<tr>
<td>Treg cells</td>
<td>IL-10</td>
<td>Immunosuppressive effect on Th2 cell activation, ↓ IgE</td>
<td>Anti-inflammatory</td>
</tr>
</tbody>
</table>

Table 1.3: Immunological and biological function of the Th2, Th17, Treg type cytokines in the pathogenesis of asthma. *GM-CSF = Granulocyte-macrophage colony-stimulating factor (GM-CSF)*

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1.6. IMMUNOPATHOGENESIS OF ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS

1.6.1 Th2 cells driven airway inflammation in ABPA

ABPA is characterized by CD4+ Th2 allergic inflammatory response to A. fumigatus allergens in the bronchial airway of atopic asthma and CF patients when A. fumigatus (Af) spores are inhaled and germinate in bronchial mucus releasing allergens including proteases (fig 1.5) [146-149]. In support of the CD+Th2-driven inflammatory process is the finding that Af -specific T-cell lines isolated from peripheral blood [146] and BAL fluid [147] of ABPA patients express CD4+ Th2-like cytokine profile [146-149].

The underlying susceptibility to develop ABPA following an asthmatic allergic response to A. fumigatus (Af) allergens, however, is not fully understood although genetic predisposition including polymorphisms in toll-like receptor (TLR) genes [209], HLA-DR2 and DR5 restriction [210], IL-4 alpha chain receptor (IL-4Ralpha) polymorphisms [211] may play a role.

Aspergillus proteases damage the respiratory epithelial barrier, exposing the bronchoalveolar lymphoid tissue (BALT) to high concentrations of Aspergillus antigens that alter the immune response. Aspergillus proteases also promote epithelial activation and a potent chemokine response that induces neutrophilic airway inflammation [212].

A. fumigatus mycelia release allergens that are processed by antigen-presenting cells (APC) bearing HLA-DR2 or DR5 and presented to T-cells within the bronchoalveolar lymphoid tissue (BALT). This immune response is similar to asthma in many ways. The T-cell response to Aspergillus allergens becomes skewed toward a Th2 CD4+ T-cell response with IL-4, IL-5 and IL-13 cytokine synthesis and secretion (fig 1.4). Aspergillus allergens stimulate an IL-5-mediated Th2 pathway responsible for the eosinophilic inflammatory response. IL-4 and IL-13 drive differentiation of B-cells to IgE-secreting plasma cells [146]. IgE antibodies affix to tissue mast cells and circulating basophils to trigger immediate hypersensitivity reactions upon re-exposure to A. fumigatus allergens (fig 1.4).

The strong Th2-type immunological response induced by Af antigens lead to markedly elevated Aspergillus-specific (Asf IgE) and total serum IgE levels and this may explain the presence of IgE and eosinophilia used in the diagnosis of ABPA.
Aspergillus fumigatus germinating spores and hyphae activate dendritic and respiratory epithelial cells via innate recognition receptors, such as Toll-like receptor (TLR)2/4 to produce Th2 immune-deviating cytokines; chemokines and costimulatory molecules that include TSLP, IL-25, IL-33, OX40 ligand (OX40L) and CCL17, which in turn orchestrate differentiation, chemotaxis and activation of CD4+ Th2 cells. CCL17 also attracts regulatory T-cells (Treg) capable of suppressing protective Th1 responses and suppressing macrophage activation, thereby impairing fungal killing. Th2 cytokines attract and activate eosinophils and drive differentiation of B-cells to IgE-secreting plasma cells. IgE antibodies affix to tissue mast cells and circulating basophils to trigger immediate hypersensitivity reactions upon re-exposure to A. fumigatus allergens.
AM: alveolar macrophage; DC: dendritic cell; TNF: tumour necrosis factor. CCR4=chemokine receptor type 4, TLR2/4=toll-like receptor type 2/4, TSLP=thymic stromal lymphopoietin, AM=airway microphages, CCL17=thymus-activated and -regulated chemokine (Reproduced and modified with permission from R.Moss and ‘Inflamm Allergy Drug Targets 2006; 5: 219–228).
1.6.2 Pathological changes in asthmatic ABPA airways

Pathologically, airway remodelling is said to occur in both severe asthma [213-216] and ABPA [217]. It is proposed that fungal proteases induce strong repair process, similar to that described in asthmatic patients, followed by release of epithelial derived growth factors resulting in severe changes in airway structure (remodelling). Furthermore, Th2-type cytokines and proteases from inflammatory cells and mast cells, are also said to promote the release of growth factors from airway epithelial cells driving a strong and irreversible remodelling reactions in airways of patients with ABPA, resulting in bronchiectatic lesions and fibrosis [218].

1.6.4 Limitation of the Th2 paradigm in ABPA pathogenesis

Although the original Th2 paradigm attempted to explain the immunopathogenesis of ABPA, not all aspergillus fumigatus (Af) peptides lead to Th2 secretion. Indeed, molecular studies using cloned Af peptides have shown that different Af peptides differentially express Th1 and Th2 cytokines suggesting that a number of epitopes of diverse activities are present in individual molecules and may be involved in the pathogenesis of ABPA through differential cytokine secretions [219, 220].

The original Th1/Th2 paradigm in ABPA is overly simplistic in that human T cells do not always adhere to this classifications and many of the allergic features of clinical ABPA have been reproduced in murine models but very few in human models as data derived from murine models of Aspergillus-induced allergic airway disease or asthma may be different in human tissues and airways. Secondly, most of what we know now is derived from experiments performed on peripheral blood that may not reflect uncommitted B cells or T-cells in the lungs. It is likely that prospective human studies that utilise airway samples may yield variable information that can increase our understanding of the precise immune mechanisms of airway inflammation in these diseases.
1.7 LABORATORY CHARACTERIZATION of T-CELL SUBSETS

T-cells can be characterised by actual quantification of the cells themselves or by quantifying related cytokines. A vast array of different cell surface molecules is involved in mediating immune responses. During cell maturation and proliferation, precursor T cells transiently or stably express specific molecules including T cell lineage markers such as T-cell receptor (TCR), CD3, CD4, CD8 and CD25. By analysing expressed patterns of various markers, including cell surface receptors and intracellular signalling molecules, different cell populations can be isolated and quantified from different tissue samples.

The following platforms can be used to study T-cell subpopulations:

a) **Cell Surface Markers** to identify cells from heterogenous samples
b) **Intracellular Cytokine Staining (ICS)** to measure cytokines within individual cells;
c) **Phosflow™ technology** to measure the phosphorylation of key proteins;
d) **Cytometric Bead Array (CBA)** to measure secreted cytokines within a sample.

The Th17 cell type can be characterized using specific cell surface markers and identified by FACS or by characterizing or quantification of IL-17 coated and stained with IL-17 Ab and determined using flow cytometry. Other methods include: immunohistochemistry, combined immunofluorescence and immunohistochemistry especially from lung biopsy specimen [221] or IL-17 measured by ELISA [222].

1.8 ANTIFUNGAL THERAPY IN ABPA AND SAFS

ABPA and SAFS are chronic and devastating diseases associated with poor quality of life, high healthcare costs and morbidity. Without treatment, these diseases are usually progressive in the majority of cases. However, some patients remain stable for many years without treatment. Thus, mechanisms for predicting the prognosis of an individual patient are not clear. A major area of debate is the timing of antifungals in an individual patient.

Traditionally, treatment for allergic fungal diseases involves the use of systemic oral corticosteroids (OCS) to suppress eosinophilic airway inflammation [223-228]. Despite good efficacy, the chronic use of OCS has a number of well-known adverse effects. OCS do not appear to prevent the development of ABPA [225]. In SAFS, even high dose, prolonged OCS courses may be only moderately successful. High dose inhaled corticosteroids also appear to be only partially effective in controlling disease and preventing exacerbations.
1.8.1 Itraconazole

Since the introduction of itraconazole, the first oral antifungal agent with activity against *Aspergillus* spp., in 1991, some efficacy in ABPA has been documented with good improvement in clinical, eosinophilia and immunological markers. In addition, itraconazole acts as a corticosteroid sparing agent [66, 229-232].

Clinical data suggest that the early institution of treatment with these agents is likely to prevent progression of ABPA to end-stage fibrosis [66, 233]. Indeed evidence from two randomized controlled trials (RCTs) suggests that treatment with oral itraconazole can offer a therapeutic benefit to approximately 60% of patients although any effect on lung function has not been formally demonstrated [234-236]. A marked beneficial effect on quality of life has been demonstrated among patients with SAFS treated with itraconazole [236, 237]. The number needed to treat (NNT) is ~3.5 for both ABPA and SAFS.

However, a major problem with the use of itraconazole is the high failure rate of about 40% and frequent adverse events (AEs) [238].

Itraconazole is a triazole antifungal agent, usually given orally and has a broad spectrum of activity against a wide range of fungi and works by inhibiting the fungal mediated synthesis of ergosterol. Oral preparations are available as blue/pink Capsules (*Sporanox®*-Pulse) and as oral liquid formulation. Itraconazole capsules require an acid environment in the stomach for optimal absorption.

1.8.1.1 Itraconazole metabolism and drug interactions

The main problem with itraconazole stems from its metabolism in relation to the CYP3A4 (the human Cytochrome P450 (CYP) [239]). CYP3A enzymes are expressed in the liver, kidney, and intestine are responsible for the metabolism of >50% of drugs. Itraconazole is usually metabolized in the liver and has a high ability to inhibit or induce CYP3A4 and as such induces or inhibits the metabolism of drugs usually metabolized by CYP450 including steroids and is a major factor in drug-drug interactions (DDI) that exists with itraconazole [240]. Clinically important mechanism-based CYP3A4 inhibitors include antibacterials (e.g. clarithromycin, erythromycin, sulphonamides and isoniazid), sex steroids and their receptor modulators and protein pump inhibitors (PPIs) like omeprazole [216]. Thus, concomitant use of itraconazole with these drugs should be used with caution due to the likelihood of increased side effects as a result of drug interactions with itraconazole. Most importantly, itraconazole should be given with caution when considering co-administration with drugs with narrow therapeutic index such as warfarin and theophylline [241]. Fortunately, there are
about twenty genetic variants in the CYP3A4 gene [242]. The presence of these genetic variants partly contribute to inter-individual differences in the extent of CYP3A4-mediated drug-drug interactions and the difference in clinical side effects and drug interactions exhibited by different individuals.

1.8.1.2 Itraconazole and steroid metabolism

Itraconazole is often used in combination with corticosteroids (OCS or ICS) in patients with asthma or CF ABPA. Reversible adrenal suppression with inhaled corticosteroids and itraconazole in 50% of patients is a real concern [235], given the rapid development of Cushing’s syndrome and/or complete adrenal suppression, which may be permanent in some patients [243].

Budesonide is a potent glucocorticoid that is metabolized in the liver by the cytochrome P450, especially the CYP3A isoforms (CYP3A4) to inactive metabolites. Itraconazole is a potent cytochrome P450 inhibitor and can inhibit the metabolism of oral or inhaled corticosteroids [244, 245], since steroids are partly metabolized by CYP3A4, causing an elevation in systemic steroid concentration. Excess cortisol may lead to Cushing’s syndrome and adrenal insufficiency [218, 219] [246]. Therefore, combination of these drugs even with moderate doses of budesonide should be closely monitored.

In a two-period cross-over study, Lebrun-Vignes and colleagues (2001) studied the interaction between itraconazole and OCS. Ie (1) itraconazole (400 mg of orally for 4 days followed by 200 mg daily for 3 days) and prednisone (single oral 60 mg dose), (2) itraconazole plus methylprednisolone (48 mg single oral dose) in 14 healthy male subjects. Itraconazole increased methylprednisolone concentrations markedly (P<0.001), with enhanced suppression of endogenous cortisol secretion [220][245].

Thus, despite its efficacy, drug interruption and consequent side effects is a real concern and should be monitored closely when co-administration of itraconazole and a number of other drugs including steroids. Furthermore, there is not yet any reliable evidence that this treatment ameliorates any progressive lung damage [234].

Recent developments in the field of allergic fungal disease have led to a renewed interest in finding more potent therapy. Some emerging data from studies of ABPA in cystic fibrosis indicate that voriconazole may be a useful adjunctive therapy for ABPA in CF [247, 248]. However, a recently published double-blind, placebo-controlled, randomized study (EVITA3) in patients with asthma with A fumigatus sensitization by Agbetile and colleagues did not
demonstrate improvement in QOL or reduction in severe exacerbations after 3 months of
treatment with voriconazole [249]. It is not clear whether the lack of efficacy in their patients
was due to inadequate duration of therapy. Nevertheless, we have learned from the results
of previous study involving itraconazole that treatment with azole antifungals probably
require a minimum of 6 months to get clinical benefit [250]. The optimal duration of therapy
with voriconazole is unclear and it is unknown whether this treatment can ameliorate
progressive lung damage, often seen in patients with ABPA. little is is known regarding the
use of voriconazole or posaconazole in ABPA or SAFS.

1.8.2 Nebulised amphotericin B

Some published case reports and series indicate that nebulised amphotericin (liposomal)
(NAB) may have a role in the treatment of CF ABPA and in corticosteroid dependant ABPA
[251, 252]. A major benefit has been demonstrated among 3 CF ABPA patients with
improvement in hypoxemia, FEV1, reduction of blood eosinophilia, total and specific serum
IgE antibodies to *A. fumigatus* and a disappearance of precipitins to *A. fumigatus* [253].

However, these are few case series and mostly in haematological and heart and lung
transplant. It is not clear whether the same effect can be observed in patients with SAFS and
ABPA. The safety profile for both short and long term use is not clear, and there are
unanswered questions as to whether NAB can prevent long term complications.

Little is known about the response rates or appropriateness of alternative antifungal
treatment for those with ABPA or SAFS.
1.9 **Summary**

- The role IL-17A in fungal-associated asthma is uncertain.
- There are no prospective studies that have compared IL-17A expression in airways and blood.
- The relationship between IL-17A and airway microbiome population in humans has not been studied fully.
- The role of IgE in asthmatic patients with ABPA and SAFS is not completely understood. Little is known regarding the association between IgE against staphylococcal enterotoxin and disease activity in SAFS or ABPA.
- The range of microbial population and the effect on asthma severity in patients with fungal associated asthma is not fully understood.
- Little is known about the response rates or appropriateness of alternative antifungal treatment for those with ABPA or SAFS.
- Very few case series and mostly in haematological and heart and lung transplant have reported the use of NAB, but the role of NAB in patients with SAFS and asthmatic ABPA have not been fully studied.
- The safety profile for both short and long of NAB is not clear.
1.10 **HYPOTHESES**

We hypothesised that:

1. Percentage of CD4+ cells expressing IL-17A (% CD4+IL-17A+) would be different between ABPA, SAFS, SANFS, MA and NH.

2. IgE expression would be higher in ABPA than SAFS. We also hypothesised that levels of specific IgE against staphylococcal aureus enterotoxins (SE-IgE) would be higher in patients with severe asthma compared to milder asthma.

3. The airway microbiome population would be higher in patients with fungal-associated asthma and that the microbiome density would be associated with asthma severity. We also hypothesised that steroid use would increase the fungal population and diversity in ABPA, SAFS and SANFS.

4. Voriconazole and Posaconazole would be effective in ABPA and SAFS.

5. Nebulised Amphotericin B (NAB) would be effective in ABPA and SAFS.

1.11 **AIMS AND OBJECTIVES**

The overall aim in this PhD thesis was to evaluate the immunopathogenesis and antifungal therapy in patients with ABPA, severe asthma-non-fungal sensitised (SANFS) and SAFS. Therefore this thesis objectives are:

- To investigate the %CD4 lymphocytes expressing IL-17A (%CD4+IL-17A) in patients with fungal associated asthma.
- To investigate the relationship between total IgE, specific IgE to Aspergillus and *Staphylococcus aureus* and features of asthma in ABPA and SAFS.
- To investigate the bacterial and fungal microbiome in ABPA, SAFS and severe asthma (SANFS) and explore the association of this microbiome population with features of asthma and steroid use.
- To investigate the efficacy and safety of voriconazole and posaconazole in ABPA and SAFS.
- To investigate the efficacy and safety of nebulised amphotericin in ABPA and SAFS.
1.12 LIST OF PUBLICATIONS MAKING UP THE THESIS

The list of publications making up the thesis is as outlined below. Each chapter has a separate abstract, introduction, hypothesis, methods, results, discussion and references.

1. Chapter 1 deals with the main introduction.
2. In chapter 2, we have studied the role of IL-17A in ABPA, severe asthma and SAFS.
3. In chapter 3, we have studied IgE expression in ABPA and SAFS.
4. In chapter 4, we have studied role of lung microbiome in ABPA, SANFS and SAFS.
5. In chapter 5, we have studied the role of Voriconazole and posaconazole in ABPA and SAFS.
6. In chapter 6, we have studied the role of nebulised amphotericin B (NAB) in ABPA and SAFS.
7. Chapter 7 is a final discussion of all studies. In this chapter, summaries of each thesis study (chapter) are given. In addition, the limitations of the main findings, directions for future work have been discussed.
8. Chapter 8: Conclusions.
CHAPTER 2

The role of IL-17A in severe asthma, severe asthma with fungal sensitisation and allergic Bronchopulmonary aspergillosis
2.1 ABSTRACT

The University of Manchester
Dr Livingstone Chishimba
Submitted for the Degree of Doctor of Philosophy
December 2015

Background and rationale: IL-17A is implicated in the pathogenesis of severe, often steroid resistant asthma, but its role in fungal-associated asthma is uncertain. There are no prospective studies that have assessed the difference in IL-17A expression in airways and blood.

Objectives: We investigated the expression of IL-17A in bronchoalveolar lavage (BAL) and peripheral blood mononuclear cells (PBMCs) in asthmatic subjects with ABPA, SAFS, severe asthma-non fungal sensitised (SANFS), mild asthma (MA) and healthy control subjects (NH).

Methods: Airway lymphocytes and PBMCs were characterized by multiparameter flow cytometric analysis to determine the percentage of CD4+ cells expressing IL-17A (% CD4+IL-17A+): CD4 (APC-Cy7), IL-17A (PerCP-CY5.5). Peripheral blood eosinophils, neutrophils, total and specific fungal IgE, fungal culture positivity and lung function were evaluated.

Results: 59 patients were analysed (ABPA=16, SAFS=15, SANFS=11, MA=6, NH=11), mean age 49 yrs (19-75). Patients with severe asthma had significantly higher CD4+ IL-17A expressing cells in both BAL (p<0.001) and PBMCs (p<0.001) compared to non-severe groups or NH. The % of CD4+ lymphocytes expressing IL-17A (%CD4+ IL-17A) in ABPA, SAFS and SANFS were similar. The %CD4+ IL-17A correlated positively with serum neutrophil counts in severe asthma in both BAL and PBMCs (p=0.015 and 0.007 respectively). Fungal culture positivity was associated with higher % CD4+IL-17A expressing lymphocytes in BAL (p=0.004) as well as in PBMC (p=0.001). %CD+IL-17A were not different between BAL and blood.

Conclusion: The number of CD4+ IL-17A lymphocytes is higher in those with severe asthma regardless of phenotype and correlates with FEV1, fungal culture and peripheral neutrophilia. Circulating lymphocyte numbers may reflect airway inflammation, and provide an alternative method of assessing airway IL-17A expression.

Key words: Th-17 cell, IL-17A, Th2, allergy, cytokines, severe asthma, allergic sensitisation
2.2 INTRODUCTION

Asthma affects about 300 million people worldwide of which about 5-15% have asthma that is difficult to treat. High treatment burden including systemic oral corticosteroids, frequent exacerbations, a high morbidity and excessive health care usage/costs are features of severe asthma (SA) [254]. Severe asthma, SAFS and allergic bronchopulmonary aspergillosis (ABPA) are complex and heterogeneous diseases with high morbidity and mortality whose pathogenesis is unclear. Airway inflammation characterized by infiltration of inflammatory cells is typical [255-257]. However, multiple immunopathological processes have been documented in each group and the mechanisms involved in the initiation and persistence of airway inflammation are potentially quite diverse and incompletely understood. There is a strong link between asthma severity and fungal sensitisation. Asthma patients sensitised to fungi often have a poor quality of life and patients with poorly controlled severe asthma have frequent exacerbations requiring multiple hospital admissions [4] including a proportion requiring admission to the intensive care unit [138]. Other problematic developments, especially in ABPA, include bronchiectasis and bronchial wall thickening [258], progression to chronic pulmonary aspergillosis (CPA), pulmonary fibrosis, hyper-attenuated mucus impaction, chronic respiratory failure and premature death. The underlying pathogenesis is partially understood with cellular proliferation, cytokine activation, mucus gland hyperplasia and airway smooth muscle cell hypertrophy all implicated. Remodelling is characterised by thickening of the airway mucosa, sub-epithelial hypertrophy and mucous gland hyperplasia [259, 260].

When A. fumigatus spores are inhaled by ABPA patients (with atopic asthma or cystic fibrosis (CF)), they germinate in bronchial mucus releasing allergens, including proteases resulting in an exaggerated Th2 CD4+ allergic inflammatory response in the airway of patients. The underlying susceptibility to ABPA is incompletely understood and complex, but multiple genetic links have been implicated.

SAFS is characterised by severe asthma, raised total IgE (but less than that of ABPA), eosinophilia and allergic sensitisation to one or multiple fungal allergens. The immunopathogenesis of SAFS is uncertain, but may arise from an aberrant response to repeated exposure to or colonisation/low grade infection with multiple fungal allergens [261]. The mechanisms underlying the pathogenesis of severe asthma, SAFS and ABPA is incompletely understood and attempts to use molecular and biological therapeutic targets have yielded conflicting results coupled with incomplete studies. Traditionally, the Th2
paradigm through the production of cytokines IL-4, IL-5 and IL-13 has provided a framework for understanding the pathological processes of asthma [177], but is incomplete.

About 40% of asthmatics, particularly those with severe disease, do not display a classical Th2 or eosinophilic airway inflammation and do not respond well to glucocorticoids [262]. Thus, severe asthma is now being classified into two broad molecular phenotypes: Th2-high (eosinophilic) that is steroid responsive and the Th2-low (neutrophilic) that is steroid resistant [78, 263]. The recent identification of a steroid-insensitive pathogenic Th17/IL-17 pathway in severe steroid resistant disease is therefore of major interest [264]. For such patients, the precise description of their disease and their categorization into well-characterized subpopulations could facilitate the development of stratified and targeted therapies [263]. IL-17 is a pro-inflammatory cytokine, originally discovered in the early 90s [265] that has provided new insights into the pathogenesis of lung inflammation [266], severe asthma [267, 268] and fungal lung disease [269]. It is produced predominantly by CD4+Th17 lymphocytes [270], although other cells such as CD8+ IL-17-producing T cells [69], gamma delta T cells (γδT cells) [271], natural killer T (NKT) cells [272], neutrophils [273], basophils, mast cells and monocytes may also produce IL-17 under certain circumstances [270].

Several studies have suggested a central role of IL-17 (also called IL-17A) in allergic airway disease. In murine models of allergic asthma, IL-17 orchestrates neutrophilic airway influx following allergen inhalation [274]. IL-17 expression is up-regulated after allergen challenge and has been proposed to modulate airway inflammation [275]. Further, in vitro allergen stimulation of T cells from atopic asthmatic patients can enhance IL-17 production [276]. In severe steroid resistant asthmatics, IL-17A expression is significantly increased in bronchial airway tissues [277], implicated in structural alterations of epithelial cells and smooth muscle contraction [264], induction of steroid resistance [278] and up-regulation of GR-beta and steroid resistance steroid hypo-responsiveness in severe asthmatics [279]. Emerging studies, most of them non-human suggest a role of IL-17 in fungal disease, but its role in allergic fungal lung disease associated with human asthma is unclear. It is said to play role in modulating antifungal immune resistance [280] and plays a protective role and resistance to bacterial, mycobacterial and fungal pathogens [281]. New insights suggest that IL-17 may have a role in Aspergillus-related airway inflammation. Observations from studies in mice using an A. fumigatus intratracheal model system demonstrated a role for IL-17 in driving Th-2-type inflammation to repeated inhalation of fungal conidia [282]. Further, repeated exposure to A. fumigatus conidia following intranasal challenge of C57BL/6 mice with A. fumigatus conidia leads to the development of chronic pulmonary inflammation and the co-
evolution of Th1, Th2, and Th17 responses in the lungs [283]. More recently, antigen-specific stimulation with *Aspergillus* spp. conidia in PBMCs in dogs with sino-nasal aspergillosis revealed an uncontrolled pro-inflammatory reaction driven by Th17 cells [284]. Little is known regarding the role of IL-17 in airway inflammation in human fungal related asthma, ABPA or SAFS. It's also not clear whether the population of IL-17 expressing cells in the airway differs from that of blood in humans.

**2.3 HYPOTHESIS**

We hypothesised that the percentage of CD4+IL-17 expression cells would be different between ABPA, SAFS, SA, MA and NH.

**2.4 AIMS AND OBJECTIVES**

1. The primary objective of this study was to investigate the number of airway and PBMC CD4+IL-17 expressing lymphocytes (CD4+IL-17+ cells) and association with features of asthma severity in patients with fungal asthma.

2. The secondary objectives were (i) to ascertain whether there was a difference in the number of CD4+IL-17+ cells between BAL and PBMCs and (ii) to investigate whether there was a relationship between fungi and markers of disease severity.

**2.5 MATERIALS AND METHODS**

**2.5.1 Study design and setting**

This was a prospective clinical and laboratory study conducted at the National Aspergillosis Centre (NAC) and the North West Severe Asthma Centre (NSAC) based at the University Hospital of South Manchester (UHSM), Manchester, UK between November 2011 and November 2013.

**2.5.1.1 Power calculations**

It wasn't possible to do power calculation as this was an exploratory study. We had no existing data on predicted levels of IL-17A expression in ABPA and SAFS and as such a power calculation could not be created apriori.
2.5.2 Ethics statement & Ethical approval

All parts of the study were designed and conducted in accordance with good clinical practice (GCP) principles. Ethical approval was granted from the North West-Greater Manchester research ethics committee (Ref: 11/NW/0175). The institutional review board for human studies at Wythenshawe Hospital approved the protocols and written consent was obtained from the subjects. The study was reviewed, approved and sponsored by the University of Manchester, research and ethics department under the research Governance Framework for Health & Social Care and Medicines for Human Use (Clinical Trials) Regulations 2004 (S12004/S12004/1031).

2.5.3 Study Subjects

The study subjects were 59 adults aged between 22-75 years old with ABPA (n=16), SAFS (n=15), severe asthma (not sensitised to fungal sensitised (SANFS), n=11), mild asthma (n=6) and healthy subjects (n=11), between November 2011 and November 2013.

2.5.3.1 Subject recruitment

ABPA, SAFS and severe asthma-non fungal sensitised (SANFS) patients were recruited from a cohort of patients attending the NWSAC and NAC clinics. Mild asthmatics and normal healthy subjects were recruited by myself from among hospital and university members of staff, general public and students following print and local advertisements. We used clinical databases at the NWSAC and NAC to select potential research participants. ABPA, SAFS and SANFS were predominantly recruited by myself, assisted by my supervisors. Subjects who expressed willingness to take part in the study were sent invitation letters, patient/subject information sheets (PIS) and copies of the consent forms. Those who still wanted to take part in the study were invited to attend visit 1 (consent, screening visit) and visit 2 (bronchoscopy plus blood collection).

Visit 1 - Consent/screening visit

The details of the study was fully discussed with the participant and the participant was given the opportunity to ask any questions. Those who decided to participate were offered to sign the consent form.

- A full history and examination was done to assess the suitability to undergo bronchoscopy,
- Lung function testing if necessary,
- If we did not have data for fungal allergy status, a skin prick allergy testing was done.
Note: for details of visit 2-bronchoscopy and blood collection visit (see bronchoscopy details; section 2.5.6).

All patients and controls who took part in the study gave informed written consent. We included patients who fulfilled the inclusion criteria.

2.5.3.2 Inclusion criteria
The inclusion criteria included: men or women, age (18-75 years old), lifelong non-smokers (<10 pack years) who had a physician diagnosis of asthma (or no asthma for healthy controls), ABPA, or SAFS; had no coexisting inflammatory chronic lung disease (except bronchiectasis in ABPA, SAFS and SA) and had an FEV1 ≥1 L/sec taken within the preceding 3 months. All patients with asthma underwent reversibility testing and confirmed by post-bronchodilator (PBD) increase in FEV1 ≥12% and or ≥200 mL compared with baseline [29] (except in some cases of chronic asthma who might have fixed airflow obstruction [30, 31], in the absence of features suggestive of COPD). Patients with symptoms suggestive of asthma but with no evidence of PBD reversibility features of asthma (provided no fixed airflow obstruction) underwent airway hyper-responsiveness testing using methacholine challenge test (MCT) [32]. In these patients, a diagnosis of asthma was based on the maximum provocation/concentration/dose of methacholine that was able to drop FEV1 by 20% or more from baseline on a 5-breath dosimeter protocol (PC20 or PD20) of <8mg/ml [36-38]. A positive test was defined as a PC20 <8mg/ml. Patients with a positive MCT and typical symptoms of asthma were diagnosed as asthma [33, 34].

Subjects also needed to have had stable disease with no recent flare-ups (within 4 weeks) and met the safety criteria to have a bronchoscopy including oxygen saturations whilst breathing room air of >90%. Normal healthy subjects had no evidence of any long term lung condition and no chest infection in the preceding 4 weeks.

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

2.5.4 Definitions

Patients were defined as reversible asthma if they demonstrated evidence of reversible airflow obstruction [59]. Patients with fixed airflow obstruction and a long standing history of asthma, with no clinical radiological or physiological features were included, but referred to as asthma with fixed airway obstruction.

Severe asthma was defined according to the BTS criteria (treatment steps 4 or 5) i.e. poorly controlled asthma on high dose of inhaled steroid (ICS) (≥800 mcg beclomethasone dipropionate, BDP daily, or equivalent) plus long acting B2-agonist (LABA), continuous use of oral corticosteroids (OCS) or frequent use of OCS (step 5) to provide disease control [70].

The diagnostic criteria for ABPA included asthma (of any severity), total serum IgE ≥1000 KIU/mL, before any antifungal therapy was commenced, bronchiectasis and any evidence of aspergillus sensitisation as demonstrated by either a positive skin test to aspergillus or raised specific IgE (sIgE) to aspergillus [150].

Diagnosis of SAFS included all of i) severe asthma, ii) total IgE <1000 KIU/mL and iii) positive skin test or raised specific IgE to any fungus [37].

Patients were included whether on or off anti-fungal therapy at the time of therapy.

2.5.5 Pulmonary function testing

All patients underwent full pulmonary function testing (PFT) at least one week before bronchoscopy. Lung function was carried out in accordance to the ATS protocol [285, 286].

2.5.6 Bronchoscopy and sample collections

Airway lymphocytes were obtained by flexible bronchoscopy at the UHSM using local guidelines and BTS standard procedures [287]. The tip of the bronchoscope was wedged into the orifice of a sub segmental bronchus of the lobes with significant radiological abnormality and high volume lavage (HVL) was collected. Where there were no radiological abnormalities such as in SAFS, most asthmatics or healthy individuals, the samples were collected from the right lower lobe.

As opposed to sampling the right middle lobe (RML) which is the commonly chosen lobe for BAL (because it is easy to wedge the bronchoscope), we specifically chose the lobes with
significant radiological findings as we anticipated that the presence of fungi would be higher in areas of the lungs with maximum damage. We found it easy to wedge the right lower lobes (RLL) in most SANFS and SA and so we deliberately chose this lobe for patients with no radiological abnormalities to standardise the sample collection method.

A maximum of 4 × 60mL (240 mls) aliquots of pre-warmed sterile 0.9% sodium chloride (0.9% NaCl, saline) solution was instilled into each chosen lobe. Aspirated BAL were immediately stored on cold ice at (-20°C) and transferred immediately to the laboratory for processing.

Due to ethical restrictions, we did not carry out endobronchial biopsies or brushings. At the outset of the study, because bronchoscopy was going to be done also in MA, NH as controls, the addition of biopsy produced some ethical restrictions during the ethical application and interview with the research ethics committee (REC).

About 200 µl was analysed for fungi using the BSOP57 protocol (HPA) [41] and 100 µl for standard bacterial culture using the BSOP57 protocol (HPA) [41]. On the same day as the bronchoscopy procedure, peripheral blood samples were collected from subjects for flow cytometry, IgE and PBMCs.

2.5.7 Isolation of lymphocytes from BAL

Aspirated BAL fluid was stored on ice before filtration (100-µm filter, BD Biosciences; Oxford, England). BAL was centrifuged (400g for 10 min at 4°C) and the cell pellet was washed in RPMI 1640 culture medium (Sigma; Dorset, England) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Viable cell counts were determined by trypan blue exclusion (Neubauer haemocytometer) and cells aliquoted at 2x10⁶ /ml per cryovial for staining (fig 2.1A, Appendix A2.1).
2.5.7.1 **Isolation of PBMC from whole blood**

20ml blood samples were collected from each participant and stored at room temperature and processed within 3 hours. PBMCs were obtained using Ficoll-hypaque (GE Healthcare, UK) separation medium techniques according to the manufacturer’s instruction (fig 2.1B, appendix A2.2) and cells aliquoted at $2 \times 10^6$ /ml per FACS tube for staining.
Fig 2.1 (B): **SOP for Isolation of PBMC from whole blood using ficoll separation media technique**

Blood collected in 20 ml EDTA tubes

layer blood gently onto ficoll separation media contained (50ml falcon tube)

Spin at 400g with no brake setting on centrifuge for 30 min

Pippet out the middle layer (PBMC)

Wash PBMC with RPMI (no supplement ) and pellet cells by spinning at 400g for 10 mins

Resuspend cells in 5ml RPMI (supplemented with pen/strep) and perform cell viability count

Perform cell viability count with trypan blue 50:50 mix (use MAAS calculation) for storing cells at $2 \times 10^6$ cells per tube (3 if possible)

Spin cells at 400g for 10min to pellet, remove supernatant for waste and resuspend in appropriate volume of cryopreservation media or wash medium, pipette up and down gently

---

**Fig 2.1 (B). Separation of PMBC (middle layer in tube B) using ficol separation technique** according to the manufacturer’s instruction.
2.5.8  **Cell staining and flow cytometric analysis**

We labelled harvested cells in a series of combinations with Allophycocyanin-cyanin 7 (APC-CY7) anti-CD4 conjugated antibodies (BD Biosciences, Oxford, England) for surface staining. Labelled cells were incubated at room temperature in the dark for 30 minutes and later washed with FACS wash (PBS with 1% FCS) and centrifuged at 400g, 4˚C for 10 minutes.

We stained cells for both IL-17A and regulatory T-cells (FoxP3 and other markers) (see suppl) in the same tube. Cells for IL-17A and Fox P3 staining were prepared for intracellular (IC) staining with a “Fix and Perm” kit (E-bioscience; Hatfield, England) following the manufacturer’s instructions. After permeabilization and washing, cells were labelled with PerCP-Cy™5.5 anti-Human IL-17A antibodies for IL-17A intracellular (IC) staining. At the same time, cells for Tregs were labelled with appropriate markers. Appropriate IgG isotype and colour-matched control (BD Biosciences, Oxford, England) were included.

Labelled cells were incubated in the fridge at 4˚C for 30-60 minutes and washed with 2mls of x1 perm wash twice. 300μl of 1% PBS + 100μl of 4% paraformaldehyde (PFA) were added to each FACS tube. Fixed cells were analysed using BD Canto II with Diva software (BD Biosciences).
Fig 2.2: Surface and intracellular (IC) staining procedure for IL-17A

Harvested cells ready for staining

2 × 10^6 cells (tube A) → 5uL of CD4+APC-Cy7 (tube A)

Cells incubated at room Temperature, dark (30-60 mins)

Add 1ml Fix/Perm buffer (fresh)

2ml x1 perm buffer added

5uL of IL-17 (PerCP-Cy™5.5) added

Was 2ml x1 perm buffer

300 uL 1%PBS, 100 uL of 4% PFA added

Ready for FACS

2 × 10^6 cells (tube B) → 5uL of CD4+APC-Cy7 (tube B)

As in A

FACS wash, centrifuge

Incubation in fridge, temp (4°C), 30-60 mins

As in A

5uL of IgG isotype control (PerCP-Cy™5.5) added

As in A

300 uL 1%PBS, 100 uL of 4% PFA

Ready for FACS

Fig 2.2: surface and intracellular (IC) staining for IL-17A.

APC-Cy7 = Allophycocyanin-cyanin 7; PBS = phosphate buffered saline; PFA = paraformaldehyde; PerCP-Cy™5.5 = Peridinin Chlorophyll Protein-Cy5; FCS = foetal calf serum.
2.5.8 Gating strategy for FACS, Data Recording, & Analysis

We gated labelled cells and analysed them using BD FACS CANTO11 with Diva software (BD Biosciences, Oxford, England) (figure). Lymphocyte gating was done using PBMC forward scatter (FSC) and side scatter (SSC) profiles and a Diva “auto compensate” tool was used to adjust fluorescence FL 1-6 compensation using single-labelled PBMCs. We applied these settings for acquisition of 10,000 gated CD4 events (where enough cells were available). IL-17A expression was quantified against the flow (A) minus one control (B) using fluorescence-activated flow cytometry plots (supplement fig 2.3). Data was exported & backed up using BD FACS CANTO11 with Diva software (BD Biosciences, Oxford, England).

2.5.8.1 Th2 cytokines

We did not measure the Th2 cytokines due to the maximum colour limit reached with our FACS CANTO 11 which had a maximum of 7-Color Option with 2 Lasers. This is because the gating was set to analysis cells stained for IL-17A and Tregs (fig 2.3E and F) and could not accommodate other markers for Th2 cytokines: IL-4, IL-5 and IL-13. In addition, we did not have enough BAL samples to stain separately and set up separate FACS acquisition panels.
Fig 2.3: Gating and FACS plots. Plot (A) Lymphocyte were gated using FSC vs. SSC profiles for acquisition of 10,000 lymphocyte events (12,922 in this example) gated CD4 events (where enough cells were available). In (B), the proportion of lymphocytes that were CD4+ were acquired (SSC vs. APC-Cy7). In C and D, the proportion of CD4+lymphocytes expressing IL-17A was obtained (APC-Cy vs. PerCp-Cy5-5). IL-17A expression was quantified against the flow (C) minus one control (D). Note that plot (C) and (D) also included gating for Tregs (CD25, CD4+FoxP3, CD25bright) and IL-10-different project. Treg cells are excluded from this thesis. PerCP-Cy5.5 - a tandem fluorochrome composed of peridinin chlorophyll protein (PerCP) coupled to the cyanine dye Cy5.5; APC-Cy7 - a tandem fluorochrome composed of Allophycocyanin (APC) coupled to the cyanine dye Cy7.
2.5.9 STATISTICAL ANALYSIS
All analyses were performed with the software SPSS 20.0 (IBM USA). For all comparisons involving severe asthma (SA), we included all patients with SA without fungal sensitisation (SANFS) and those with SAFS. ABPA subjects with severe asthma features were excluded for the purpose of this comparison. Descriptive statistics was used to summarise the data. Pearson’s Chi-square test was used to evaluate categorical variables or Fisher’s exact test where appropriate. Normally distributed data were analysed using t-test or analysis of variance (ANOVA). Non-normally distributed data were analysed using Mann-Whitney (2 sample) or Kuskal-Wallis (>2 sample) tests. For all comparisons, P values <0.05 were considered statistically significant.

2.6 RESULTS

2.6.1 Demographic characteristics
Seventy five subjects were recruited into the study (Fig 2.4), 59 of whom were included in the final analysis (ABPA=16, SAFS=15, SANFS=11, mild asthma=6, NH=11; Table 2.1). ABPA patients tended to be older (p=0.01). NH and MA subjects were significantly younger than ABPA, SAFS or SANFS patients. ie median Age (yrs); 60 (range 19-75), 50 (range 30-68), 47 (range 19-65), 25 (range 22-62) and 26 (range 22-58) for ABPA, SAFS, SANFS, MA and NH respectively.
As expected, lung function was better in healthy and mild asthma compared to other groups. Similarly, both total IgE (tIgE) and fungal allergen specific IgE (sIgE) were higher in ABPA than SAFS or SANF (fig 2.5, table 2.2). Patient demographic characteristics are described in table 1.
Fig 2.4: Patient recruitment, exclusions and drop-outs

75 patients were recruited of which 10 were excluded from the study while another 6 were excluded from analysis. Reasons for exclusions from the study were: withdraw of consent (n=3), holiday at the time of appointment (n=1), acute viral illness (n=2), COPD other than asthma (n=4).

ABPA=allergic bronchopulmonary aspergillosis; SANFS=severe asthma-non fungal sensitised SAFS=severe asthma with fungal sensitisation; BAL=bronchoalveolar lavage.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ABPA (n=16)</th>
<th>SAFS (n=15)</th>
<th>SANFS (n=11)</th>
<th>Mild Asthma (n=6)</th>
<th>Normal Healthy (11)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs.), median (range)</td>
<td>60 (19-75)</td>
<td>50 (30-68)</td>
<td>47 (19-65)</td>
<td>25 (22-62)</td>
<td>26 (22-58)</td>
<td>0.01</td>
</tr>
<tr>
<td>Gender: Male (%)</td>
<td>11 (64.7)</td>
<td>5 (33.3)</td>
<td>4 (40)</td>
<td>5 (83.3)</td>
<td>6 (54.5)</td>
<td>0.463</td>
</tr>
<tr>
<td>BMI (kg/m²), mean (sd)</td>
<td>27.3 (5.3)</td>
<td>27.1 (5.5)</td>
<td>30.4 (5.9)</td>
<td>25.4 (2.2)</td>
<td>28.2 (7.4)</td>
<td>0.499</td>
</tr>
<tr>
<td>FEV1% , mean (sd)</td>
<td>62.5 (19.1)</td>
<td>74.8 (20.2)</td>
<td>60.1 (23.8)</td>
<td>92.2 (13)</td>
<td>97 (4.9)</td>
<td>0.01</td>
</tr>
<tr>
<td>FVC %, mean (sd)</td>
<td>90.4 (15.7)</td>
<td>97.6 (17.8)</td>
<td>80.1 (15.1)</td>
<td>84.6 (44.2)</td>
<td>102 (20.2)</td>
<td>0.093</td>
</tr>
<tr>
<td>FEV1/FVC (%), median (range)</td>
<td>67 (30-85)</td>
<td>65 (13.2)</td>
<td>59 (33-92)</td>
<td>83 (54-86)</td>
<td>80 (75-88)</td>
<td>0.031</td>
</tr>
<tr>
<td>FEF25-75%, mean (sd)</td>
<td>28.6 (15.7)</td>
<td>37 (20.7)</td>
<td>41 (49.25)</td>
<td>72.8 (30)</td>
<td>74.2 (16)</td>
<td>0.004</td>
</tr>
<tr>
<td>DLCO, median (range)</td>
<td>6.9 (5.3-9.6)</td>
<td>7.3 (2.31)</td>
<td>7.6 (2.03)</td>
<td>9.9 (9.3-15.3)</td>
<td>9.5 (7.3-65)</td>
<td>0.01</td>
</tr>
<tr>
<td>Duration of asthma (yrs), mean (sd)</td>
<td>41.4 (19)</td>
<td>41 (17.6)</td>
<td>27.6 (20)</td>
<td>24 (16)</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>Bronchiectasis, n (%)</td>
<td>16 (94.1)</td>
<td>12 (80)</td>
<td>6 (60)</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Obesity, n (%)</td>
<td>3 (20)</td>
<td>3 (60)</td>
<td>4 (40)</td>
<td>0</td>
<td>2 (18)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>ABPA (n=17)</td>
<td>SAFS (n=17)</td>
<td>SANFS (n=17)</td>
<td>SA (n=17)</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
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<td>-------------</td>
<td>--------------</td>
<td>-----------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>Atopy, n (%)</td>
<td>12 (70.6)</td>
<td>12 (80)</td>
<td>5 (50)</td>
<td>1 (16.7)</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rhinitis/sinusitis, n (%)</td>
<td>12 (70.6)</td>
<td>10 (66.7)</td>
<td>3 (30)</td>
<td>1 (16.7)</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nasal polyps, n (%)</td>
<td>6 (35.3)</td>
<td>6 (40)</td>
<td>3 (30)</td>
<td>0</td>
<td>0</td>
<td>0.066</td>
</tr>
<tr>
<td>Antifungal therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>current, n (%)</td>
<td>8 (50)</td>
<td>4 (26.6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>previous, n (%)</td>
<td>4 (25)</td>
<td>2 (13.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>never, n (%)</td>
<td>4 (25)</td>
<td>9 (60)</td>
<td>11 (100)</td>
<td>6 (100)</td>
<td>11 (100)</td>
<td>0.97</td>
</tr>
<tr>
<td>Total IgE, median (range) ku/l</td>
<td>1200</td>
<td>195</td>
<td>43</td>
<td>96.3</td>
<td>54.3</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Blood eosinophils, median (range)</td>
<td>0.185(0.2-2)</td>
<td>0.38 (0.1-0.63)</td>
<td>0.165(0.1-0.8)</td>
<td>0.4 (0-0.8)</td>
<td>0.14 (0.7-0.24)</td>
<td>0.111</td>
</tr>
</tbody>
</table>

**Table 2.1: Demographic characteristics and spirometry data.**

ABPA=Allergic bronchopulmonary aspergilosis; SAFS=Severe asthma with fungal sensitization; SANFS=Severe asthma—no fungal sensitisation; SA=Severe asthma (SAFS+SANFS) tIgE =total IgE, Af=Aspergillus fumigatus; BMI=basal metabolic rate; Ht=height; SD=standard deviation; FEV1=forced expiratory volume in the first second; FVC=forced vital capacity; % pred=percentage of the predicted value; L=litres; KUa/L=kilounits of allergen per litre; ku/L=kilo units per litre; %L/s=percent litre per second. All current or previous smokers have < 10 pack years.
Fig 2.5: Concentrations of total IgE (A) and slgE to fungal allergens

Fig 2.5: Median concentrations of levels total IgE (A) and slgE according to subject groups. Levels to A. fumigatus (B), alternaria (C), trichophyton (D), candida (E) and cladosprium (F) within subject groups. Median slgE to all (except alternaria) were significantly higher in ABPA compared to SAFS and there statistical differences across the five groups. ABPA=Allergic bronchopulmonary aspergillosis; SAFS=Severe asthma with fungal sensitization; Af=aspergillus fumigatus. IgE=immunoglobulin E. slgE=specific IgE.

Severe asthma here refers to severe asthma without fungal sensitisation (SANFS).
<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients (n=59)</th>
<th>ABPA (n=16)</th>
<th>SAFS (n=15)</th>
<th>SANFS (n=11)</th>
<th>Mild asthma (n=6)</th>
<th>NHC (n=11)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>tIgE (ku/L, median (range))</td>
<td>240 (7.6-9300)</td>
<td>1200 (42.2-9300)</td>
<td>195 (53.9-680)</td>
<td>43 (8.6-500)</td>
<td>96.3 (30-580)</td>
<td>54.2 (7.6-600)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Af (KUa/L, median (range))</td>
<td>9.2 (0-74)</td>
<td>25.1 (1-74)</td>
<td>6.8 (2-20)</td>
<td>0</td>
<td>0.0 (0-0)</td>
<td>0.0 (0-0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IgGaf (kua/L, median (range))</td>
<td>35 (2-101)</td>
<td>42.5 (3-101)</td>
<td>33 (2-45)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Alt (KUa/L, median (range))</td>
<td>1.4 (0-35)</td>
<td>2.4 (1-35)</td>
<td>0.6 (0-2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.004</td>
</tr>
<tr>
<td>Trich (KUa/L, median (range))</td>
<td>1.2 (0-19)</td>
<td>1 (0-19)</td>
<td>0.7 (0-3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>Candida (KUa/L, median (range))</td>
<td>2.0 (0-18)</td>
<td>2 (0-18)</td>
<td>3.2 (1-4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.003</td>
</tr>
<tr>
<td>Clado (KUa/L, median (range))</td>
<td>1.2 (1-5)</td>
<td>0.93 (</td>
<td>0.6 (0-2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.142</td>
</tr>
<tr>
<td>Pen (KUa/L, median (range))</td>
<td>3.5 (0-27)</td>
<td>4.25 (1-27)</td>
<td>1.55 (0-4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 2.2. Total and specific IgE levels.

*tlgE =total IgE, Af = aspergillus fumigatus; Pen= penicillium; Alt= alternaria; trich= trichophyton; Clado= cladosporium; ku/L= kilo units per litre; KUa/L= kilo units of allergen per litre; ABPA= Allergic bronchopulmonary aspergilosis; SAFS= Severe asthma with fungal sensitization; Af= aspergillus fumigatus; NHC= normal healthy control*
Characteristics of BAL volume and cells from the study population

The total cell number per volume of BAL recovered significantly higher in patients with ABPA, SAFS and SANFS compared to MA and NH subjects (Table 2.3).

Table 2.3: Characteristics of BAL volume and cells from the study population

<table>
<thead>
<tr>
<th></th>
<th>ABPA (n=16)</th>
<th>SAFS (n=15)</th>
<th>SANFS (n=11)</th>
<th>MA (n=6)</th>
<th>NH (n=11)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol in , median (mls), range</td>
<td>45 (20-80)</td>
<td>50 (10-70)</td>
<td>45 (20-80)</td>
<td>75 (60-140)</td>
<td>60 (20-130)</td>
<td>0.005</td>
</tr>
<tr>
<td>Vol out (ml)</td>
<td>21 (4-56)</td>
<td>26 (3-58)</td>
<td>38 (4-65)</td>
<td>50 (41-80)</td>
<td>42 (10-86)</td>
<td>0.008</td>
</tr>
<tr>
<td>N cells (×10⁶), median</td>
<td>4.0 (1-8)</td>
<td>4.5 (1.8-13)</td>
<td>5 (2-14)</td>
<td>7 (4-13.6)</td>
<td>6 (2.2-10)</td>
<td>0.066</td>
</tr>
<tr>
<td>Cells/BAL vol (×10⁶/ml), median (range)</td>
<td>0.205 (0.07-0.63)</td>
<td>0.23 (0.07-0.6)</td>
<td>0.21 (0.07-0.5)</td>
<td>0.086 (-0.5)</td>
<td>0.117 (0.02-0.23)</td>
<td>0.034</td>
</tr>
</tbody>
</table>

Vol in = Volume instilled into sub segmental bronchus (mls), Vol out = BAL volume recovered, N cells = total number of cells, Cells/BAL vol = Total cell number per volume of BAL recovered
2.6.2 Percentage of CD4+ cells expressing IL-17A

Overall, there were significant differences across groups in the % of CD4+ lymphocytes that were expressing IL-17A (%CD4+IL-17+) in BAL (p<0.001) and in PBMC (p=0.01) (fig 2.6). However, ABPA, SAFS and SANFS were similar, but significantly higher than MA and NH (fig 2.6, table 2.4). The two fungal asthma groups (ABPA and SAFS) were not different (p=0.487; 0.862 for PBMC and BAL respectively). Similarly, the two severe asthma phenotypes (SAFS and SANF) were not different from each other (p=0.99; 0.412 for PBMC and BAL respectively), but were significantly higher IL-17A than MA and NH (fig 2.6, table 2.4).

**Fig 2.6:** % CD4+IL-17A expression within subject groups in BAL and Blood

![Graph A](image)

**Fig 2.6:** BAL % CD4+IL-17A (A) and PBMC% CD4+IL-17A (B) within subject groups. IL-17A= interleukin 17A; ABPA = Allergic bronchopulmonary aspergillosis; SAFS = Severe asthma with fungal sensitization; BAL = bronchoalveolar lavage; FACS = flow cytometry; SA = severe asthma, nonfungal sensitised; BAL % CD4+IL-17A = Percentage of CD4+ cells expressing IL-17A in BAL; PBMC% CD4+IL-17A = Percentage of CD4+ cells expressing IL-17A in blood.
Table 2.4: Multiple significance comparisons of %CD4+IL-17A expression in BAL between subject groups

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Comparative Group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPA</td>
<td>SAFS</td>
<td>0.862</td>
</tr>
<tr>
<td></td>
<td>SANFS</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>mild asthma</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Health control</td>
<td>0.000</td>
</tr>
<tr>
<td>SAFS</td>
<td>ABPA</td>
<td>0.862</td>
</tr>
<tr>
<td></td>
<td>SANFS</td>
<td>0.412</td>
</tr>
<tr>
<td></td>
<td>mild asthma</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Health control</td>
<td>0.000</td>
</tr>
<tr>
<td>SANFS</td>
<td>ABPA</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>SAFS</td>
<td>0.412</td>
</tr>
<tr>
<td></td>
<td>mild asthma</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>Health control</td>
<td>0.000</td>
</tr>
<tr>
<td>mild asthma</td>
<td>ABPA</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>SAFS</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>SANFS</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>Health control</td>
<td>0.824</td>
</tr>
<tr>
<td>Health control</td>
<td>ABPA</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>SAFS</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>SANFS</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>mild asthma</td>
<td>0.824</td>
</tr>
</tbody>
</table>

Table 2.4: Multiple comparisons for %CD4+ lymphocytes expressing IL-17A between subject groups in BAL. ABPA, SAFS and SANFS were similar but all were significantly higher than MA and NH. The two fungal asthma groups (ABPA and SAFS) were similar. Similarly, the two severe asthma phenotypes (SAFS and SANFS) were not different from each other but were different from MA and NH. ABPA=Allergic bronchopulmonary aspergilosis; SAFS=Severe asthma with fungal sensitization, SANFS=severe asthma-non fungal sensitised.
2.6.2.1 Comparison between BAL and PBMC in % CD4+IL-17A expression

When BAL and PMBC were compared within individual subject groups, there were no significant differences observed except for SANFS and NH subjects (fig 2.7, table 2.5).

Fig 2.7: Comparison between BAL and PBMC in %CD4+IL-17A expression within each subject group.

Severe asthma here refers to severe asthma without fungal sensitisation (SANFS)

Table 2.5: Comparison of CD4+ IL-17A expressing cells between BAL and PBMC

<table>
<thead>
<tr>
<th>Subject group</th>
<th>BAL</th>
<th>PBMC</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPA</td>
<td>74.4 (21.7-94.8)</td>
<td>76.9 (44-95)</td>
<td>0.281</td>
</tr>
<tr>
<td>SAFS</td>
<td>65 (21.9-81)</td>
<td>65 (21.9-81)</td>
<td>0.363</td>
</tr>
<tr>
<td>SANFS</td>
<td>52.9 (0.1-71)</td>
<td>67 (46-82)</td>
<td>0.016</td>
</tr>
<tr>
<td>MA</td>
<td>16.1 (9.6-23.2)</td>
<td>26.5 (13-49)</td>
<td>0.075</td>
</tr>
<tr>
<td>NH</td>
<td>3.8 (0-12.8)</td>
<td>12 (0-32)</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Table 2.5: Comparison of % CD4+IL-17A expression between BAL and PBMC within subject groups. IL-17A= interleukin 17A; ABPA= Allergic bronchopulmonary aspergilosis; SAFS= Severe asthma with fungal sensitization; SANFS= severe asthma-non fungal sesitised; NH= normal healthy; MA= mild asthma; tlgE = total IgE, Af= aspergillus fumigatus; BAL= bronchoalveolar lavage.
2.6.4 IL-17A and asthma severity

The percentage of CD4+ lymphocytes expressing IL-17A were significantly higher in subjects with severe asthma (SANFS plus SAFS combined) compared to MA and NH in both BAL and blood (<0.001) (table 2.6). Similarly, patients with SA had lower FEV1 than non-severe groups. In contrast, blood eosinophils and neutrophils did not differ across groups (p=0.117 and 0.138 respectively) (table 2.6). Interestingly, we did not observe any significant difference in IL-17A expression between the two severe asthma groups (SAFS vs. SA).

Table 2.6: Comparison between severe asthma and non-severe subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Severe asthma (SANFS+SAFS) (n=26)</th>
<th>Mild Asthma (n=6)</th>
<th>NH (n=11)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL CD4+IL-17A, median % (range)</td>
<td>59.7 (0.1-81)</td>
<td>16.15 (9.6-23.2)</td>
<td>3.8 (0-12.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PBMC CD4+IL-17A, median % (range)</td>
<td>67 (41.7-85)</td>
<td>26.5 (13-49)</td>
<td>12 (0.3-31.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV1%, mean (sd)</td>
<td>68.5 (28)</td>
<td>93.7 (12)</td>
<td>95.3 (15.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Blood Eosinophils, median (range)</td>
<td>0.235 (0.1-2.1)</td>
<td>0.04 (0.1-0.63)</td>
<td>0.14 (0.07-0.24)</td>
<td>0.117</td>
</tr>
<tr>
<td>Blood Neutrophils, mean (sd)</td>
<td>5.18 (2.19)</td>
<td>4.16 (3.1)</td>
<td>3.65 (0.92)</td>
<td>0.138</td>
</tr>
</tbody>
</table>

Table 2.6: Comparison between severe asthma and non-severe patients

Note: Severe asthma includes all SAFS plus SA subjects. ABPA subjects with severe asthma features were excluded for the purpose of this comparison.

IL-17=interleukin 17; SD=standard deviation; FEV1=forced expiratory volume in the first second; % pred= Percentage of the predicted value.
2.6.5 Correlation between IL-17A and and FEV1

There were negative correlations between %CD4+IL-17+ lymphocytes and FEV1 in all subjects ($r=-0.576$, $p<0.001$) (fig 2.8A and B). Higher CD4+IL-17A expression was associated with lower FEV1 in patients with severe asthma and ABPA (fig 2.8A and D).

Fig 2.8: Correlation between IL-17A and FEV1

IL-17A correlated negatively with FEV1 in both BAL and PBMC for all patients (A) and (B) respectively. (C) Correlation between IL-17A and FEV1. (D) Correlation between IL-17A and FEV1 in SA patients. ABPA=Allergic bronchopulmonary aspergilosis; SA=severe asthma; BAL=bronchoalvelar lavage; FEV1=forced expiratory volume in the first second; $r$=correlation coefficient.
2.6.6 IL-17A and blood neutrophils or eosinophils
The percentage of CD4+ cells expressing IL-17A (CD4+IL-17+ cells) in BAL correlated positively with blood neutrophil counts in the SA group (r=0.810, p=0.015), but not in other subjects (fig 9). Interestingly, no significant correlation was observed with blood eosinophil counts.

Fig 2.9. Correlation between BAL %CD4+IL-17A+ cells and blood neutrophil counts

![Graph showing correlation](image)

Fig 2.9: Correlation between %CD4+IL-17A and blood neutrophil counts in all patients (A) and those with SA (B). ABPA=Allergic bronchopulmonary aspergilosis; SA=severe asthma; BAL=bronchoalvelar lavage; FEV1=forced expiratory volume in the first second; r=correlation coefficient.

2.6.7 Fungal culture and IL-17A
Subjects with positive fungal cultures had significantly higher proportion of CD4+ IL-17A expressing lymphocytes than culture negative ones (p=0.01 and <0.001 for BAL and PBMC respectively) (table 4, fig 10). In addition, those patients with positive culture for fungi had lower FEV1 than negative culture individuals (p<0.001) (table 4). A full description of microbiome is described and discussed in chapter 4 of this thesis.
Fig 2.10: % CD4+IL-17A expression according to fungal culture positivity.

2.6.8 Antifungal therapy and IL-17A

Patients on current antifungals had significantly higher %CD4+IL-17A expression compared to patients who never antifungals group (16.5% vs 77%, p<0.001; 44% vs. 72%, p=0.002) for BAL or PBMC respectively. Similarly, findings were seen when comparing current treated with previous treated but the differences were much smaller than when compared to the never treated (16.5% vs. 65.5%) (table 2.7). However, there was no difference between previous and current antifungal groups (table 2.7).

Table 2.7: %CD4+IL-17A according to azole antifungal treatment status

<table>
<thead>
<tr>
<th>Variable</th>
<th>Never</th>
<th>Current</th>
<th>Previous</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL CD4+IL-17A, median % (range)</td>
<td>16.5 (0-79)</td>
<td>77 (47-78)</td>
<td>65.5 (21.9-74.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PBMC CD4+IL-17A, median % (range)</td>
<td>44 (0.3-8.2)</td>
<td>72 (46-94)</td>
<td>56 (54-92)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 2.7: %CD4+IL-17A according to azole antifungal treatment status. Never=never been treated with azole antifungal therapy; current=currently on azole antifungals; previous=previous received azole antifungal therapy.
2.7 DISCUSSION

This is the first study to evaluate the proportion of CD4+ lymphocytes expressing IL-17A (%CD4+IL-17+) in BAL and PBMC in fungal associated asthma. We found significant difference between severe asthma and non-severe asthma, but we did not find any difference between ABPA, SAFS and SANFS, suggesting that the difference seen in % CD+IL-17A expression may largely be attributed to the presence of asthma or its severity. This is also supported by our other findings. For example, when MA and NH groups were compared, proportion of CD4+ lymphocytes expressing IL-17A was significantly higher in MA patients compared to NH in both BAL (16.1 vs. 3.8, *p*=0.01) and PBMCs (26.5 vs. 12, *p*=0.07) respectively. Our findings support in part previous observations by Wong and colleagues in PBMC. These authors found higher PBMC IL-17 expression in asthmatic patients compared with control subjects (*p* < 0.01) [222]. However, none of their patients were tested for fungal allergy. There are no prospective studies that have assessed the difference in IL-17A expression in BAL and blood. We found no differences between airways and blood. Our finding is novel and suggests that circulating lymphocyte numbers may reflect airway inflammation, and provide an alternative method of assessing airway IL-17 expression.

2.7.1 IL-17A and asthma severity

We found the %CD4+ IL-17A producing cells to be significantly higher in patients with severe asthma compared to mild asthmatics or NH in both BAL and PBMC. Our finding support previous reports linking increased IL-17 with asthma severity from induced sputum [268], BAL [288], serum [289] and bronchial biopsies [277]. Immunocytochemistry testing of airway mucosal biopsies obtained from subjects with severe asthma, moderate asthma, mild asthma and health controls without asthma by Al-Ramli and colleagues demonstrated a significantly higher number of IL-17A+ cells in the severe asthmatics, and a slight increase in IL-17A+ cells in those with moderate asthma. Our findings also support previous findings by Agache et al. (2010) in 85 asthma patients [289].

In our study, we did not carry out endobronchial biopsies or brushings do to ethical restrictions. However, our findings in BAL are consistent with previous findings obtained from airway mucosal biopsies stained by immunocytochemistry testing among subjects with severe asthma, moderate asthma, mild asthma and health controls without asthma by Al-Ramli and colleagues [273]. On the other hand, these authors did not specifically test their
patients for fungal allergy and it’s unclear whether fungal allergy would have affected their results.

To our surprise, we did not find any significant difference in IL-17A expression between the different severe asthma groups. To date there has been no studies that have assessed the difference between SAFS and SANFS, but the lack of difference may signify that IL-17A is simply an independent risk factor for severe asthma, independent of cause [289].

2.7.2 IL-17A and blood neutrophils

An important point of discussion is the significance of neutrophils in our patients. We found that BAL %CD4+IL-17A expression correlated positively with blood neutrophil counts only in the SA group (r=0.867, \( p=0.002 \)), but not in other groups. A higher neutrophil count was associated with higher IL-17A expressing cells. The subgroup of severe asthmatics that were not sensitised to fungi or any other allergens (non-allergic severe asthmatics) had higher circulating neutrophil counts and IL-17A expression. Our findings support a possible underlying mechanism of the newly classified Th2 low severe asthma phenotype that displays predominant neutrophilic inflammation in which the Th17 pathway has been proposed to be involved in the neutrophilic inflammation and airway remodelling processes [290, 291]. We did not examine sputum neutrophils [268, 292, 293] in our patients, a limitation of our study. However, our findings suggest that circulating blood neutrophil numbers may reflect neutrophilic airway inflammation, and provide an alternative non-invasive method of assessing airway inflammation, even though affected by concurrent infection and corticosteroid use. It is also possible that IL-17A has a subdominant role in eosinophilic asthma, but a central role in non-allergic asthma neutrophilic asthma in which neutrophils play a critical role.

We found no significant correlation between IL-17A and blood eosinophil counts in all patient groups including fungal sensitised patients. In contrast, Murdock et al (2012) demonstrated that IL-17 drives pulmonary eosinophilia following repeated exposure to A. fumigatus conidia [294]. We found no significant difference between groups in eosinophil count. This may be attributed to the fact that we used blood eosinophils rather than airway eosinophils, and could not control adequately for corticosteroid usage. There is no relationship between eosinophil count and lung function or other immunological parameters [295]. Our findings are consistent with this.
2.7.3 IL-17A and fungi
The higher number of CD+IL-17A expressing cells in BAL and PBMC and fungal culture positivity that we found is intriguing and novel. There are no prospective human studies that have assessed the relationship between fungal airway infection/colonisation and IL-17A expression in asthma patients. A link between higher IL-17A levels in candidaemic patients in intensive care unit (ICU) may be indirectly relevant [296]. IL-17 may be important for mobilization of immune responses against microbial pathogens [269], however patients with invasive aspergillosis (IA) have very low IL-17 levels in BAL and serum [297]. More recently, there have been suggestions from studies in asthma models of rats that IL-17 may potentially affect the expression of pathogen recognition receptors and may attenuate cellular defence against *A.fumigatus* [259]. The ability of *A. fumigatus* to activate, suppress, or subvert host immunity has undergone considerable debate, which our data contributes to; it is possible that IL-17 plays a different role in allergic lung disease from IA.

Other findings
Multiple fungi have been implicated in fungal asthma [298]. We found positive correlations between CD4+IL-17 expression and levels of specific IgE (sIgE) to *A. fumigatus*, *Trichophyton*, *Candida* and *Penicillium* spp., but not with *Alternaria* or *Cladosporium* spp. The reasons for this are not clear, but could reflect the differences in exposure: *Alternaria* and *Cladosporium* do not colonise or infect humans with any regularity, unlike the other fungi we evaluated.

We also observed that the duration of asthma was significantly longer in patients with severe asthma compared to those milder forms. We also found that about 78.8% of patients with severe asthma had bronchiectasis, a significantly higher frequency compared to the non-severe phenotype. In addition, these severe asthma patients had more frequent atopy, rhinosinus disease and GORD. The clinical evaluation of severe asthma patients should include a holistic approach to co-morbidities [44].

2.7.4 Azole antifungal therapy and IL-17A
In this study, we found that patients who were currently on azole antifungal treatment had significantly higher %CD4+IL-17A expression compared to patients who were never treated with antifungals. However, there was no difference between previous and current antifungal groups in either BAL or PBMC. There are no prospective studies that have assessed effect of anti-fungals on IL-17A expression in humans. Our data may suggest the potential link
between azole antifungal metabolism or action and IL-17A, but this needs further investigations involving larger studies including molecular and metabolism studies.

2.7.5 Proportionally high %CD4+IL-17A
The % of CD+ IL-17+ found in this study is somewhat high in all subject groups (except normal health controls). There are no human studies that have assessed IL-17A expression in fungal asthma patients. In addition, previous IL-17 studies have utilised other methods of determining IL-17 such as immuno-polymerase chain reaction (IPCR) [299], ELISA [289, 300], quantitative RT-PCR [278]. The disease process and severity among these patients were different from our patient group. However one study analyzed the percentage of CD4(+) cells expressing IL-17 in twenty five stable asthmatic and twelve age-matched control subjects from BAL using flow cytometry [301]. The median percentage of CD4+IL-17+ cells found was 4.6% (1.2–14.4%) for asthma patients vs. 2.2% (0.5–7.7%) for normal healthy control. Both the fungal status and asthma severity in their study are not known. These workers also stimulated their cells and it’s unclear how this could have affected their results. We did not stimulate our cells. The sets of antibodies used in their study are different from ours as they used Alexa Fluor 647, whereas we used PerCP-Cy5.5 in our study. Despite these differences and despite the high % found in our study, this could not have affected our results or interpretation as antibody, gating and analysis was standardised and remain the same throughout the study for all patients.

Another factor that might have affected CD4+IL-17A levels in our patients is concomitant treatment with azole antifungals. As stated earlier, patients who were currently on azole antifungal treatment in this study had significantly higher %CD4+IL-17A expression compared to patients who were never treated with antifungals.

2.7.6 Apparent age difference between groups
In our NH and MA subjects were significantly younger than ABPA, SAFS or SANFS patients. This may explain the difference in measures such as lung function and might have been a source of bias with regard to such parameters. However, there are no studies that have assessed the influence of age on IL-17A expression in the lung of asthma patients and it is unclear whether this apparent age difference between groups could have affected the results in our subjects.
2.8 Study limitations
We recognise several limitations in this study. The low specificity of culture techniques and concomitant antifungal therapy may have affected our results as 50 % of ABPA and 26.6 % of SAFS were on antifungals at the time of the study. Some patients were also on oral and/or inhaled steroids and azithromycin. This could have affected the levels of IgE, eosinophils and IL-17 expression. IgE levels were used to allocate patients in the ABPA and SAFS groups and hence differences in total IgE observed in these 2 groups may simply a result of selection and allocation bias.

We did not measure other Th-17 related cytokine (IFN-gamma, IL-4, IL-22, IL-23 and IL-25) concentrations in plasma and BAL supernatants. We did not also measure Th-2 related cytokines (IL-4, IL-5 and IL-13) due to inadequate BAL samples as our study also involved using the same sample for other projects such as microbiome work and Treg cells. It is likely that future projects that measure these additional cytokines together with IL-17A could provide additional clinical and scientific information.

It is also difficult to classify severe asthma in ABPA patients based on the need for steroid use as many patients use steroids due to frequency of exacerbations. Some patients had few few cells (<2x10^6) per FACS tube because of low volume BAL collected in some of these patients due to variation in tolerance of the procedure. As a result we did not acquire the required 10,000 gated lymphocyte populations in 5 patients.

We did not examine sputum neutrophils and eosinophils, a limitation of our study. However, our findings suggest that circulating blood neutrophil numbers may reflect neutrophilic airway inflammation, and provide an alternative non-invasive method of assessing airway inflammation, even though affected by concurrent infection and corticosteroid use.

2.8.1 BAL versus mucosal airway biopsies
In this study, we did not carry out endobronchial biopsies or brushings do to ethical restrictions.

This has the potential to limit the quality of our cells and expressed cytokine. In animal models, IL-17 in bronchial epithelium does not necessarily mirror cytokine expression in BAL cells and hence, bronchial biopsy can serve as an invaluable additional tool for investigation of time-dependent changes in inflammatory process in animal model of asthma [277].
In humans, A concentration of biomarkers can be detected in lung biopsies in comparison to BAL [302]. High yield of good quality samples which can measure cytokine RNA with RNA integrity numbers can be obtained from bronchial brushings. It also allows obtaining reproducible genes measured across the samples. BAL is easier to obtain and carries lower procedure risks and less complications but the samples are of lower quality compared to tissue samples obtained by biopsy due to factors such as dilution and examination of BAL examines the components of the epithelial lining fluid (ELF) rather than tissue. With biopsies, we can obtain more detailed airway inflammation from mucosal, sub mucosal, epithelial and sub-epithelial areas of the airway and hence superior sensitivity to standard BAL for the measurement of biomarkers of inflammation. Future work will involve biopsy as well as BAL sampling.

Despite this limitation, our findings in BAL are consistent with previous findings obtained from airway mucosal biopsies stained by immunocytochemistry testing among subjects with severe asthma, moderate asthma, mild asthma and health controls without asthma by Al-Ramli and colleagues [273]. On the other hand, these authors did not specifically test their patients for fungal allergy and it’s unclear whether fungal allergy would have affected their results.

2.9 CONCLUSIONS AND DIRECTIONS FOR THE FUTURE
Our data are indicative of a central role for IL-17A in severe asthma, regardless of phenotype. The relationship between fungal culture positivity, peripheral neutrophilia and IL-17A is intriguing. Circulating lymphocyte numbers may reflect airway inflammation, and provide an alternative method of assessing airway IL-17A expression. Further larger prospective studies are needed to determine the cause effect relationship, and IL-17A blockade and/or antifungal therapy have synergistic potential in severe asthma.

2.10 ACKNOWLEDGEMENTS
The authors would like to acknowledge the following: Simon Stephan - chief lab manager, Leanne Archer - laboratory scientist/technologist, Maryam Safari - laboratory scientist/technologist for their role in helping out with laboratory work. Mrs Ann Bird and Mrs Chris Harris-respiratory medicine secretary for their role in collecting notes and arranging bronchoscopies, Ms Marian Denson -laboratory scientist for her role in helping out with FACS work, Mrs Julie Morris and Mr Philip Foden-medical statistics for their role in helping out with statistical and graphical work, Mr Paul Somerset and Mrs Helen Carruthers-Medical illustrations for their role in helping out with images and figures.
CHAPTER 3

Immunoglobulin (IgE) expression in severe asthma with fungal sensitisation and allergic bronchopulmonary aspergillosis
3.0 ABSTRACT

The University of Manchester
Dr Livingstone Chishimba
Submitted for the Degree of Doctor of Philosophy
December 2015

RATIONALE AND BACKGROUND: Immunoglobulin E (IgE) plays an important role in the pathophysiology of allergies and allergic asthma. However, the role of IgE in asthmatic patients with ABPA and SAFS is not completely understood.

OBJECTIVE: We sought to investigate whether total IgE (tIgE), specific IgE to Staphylococcus aureus enterotoxins (SE-IgE) or IgE to Aspergillus fumigatus (Asp-IgE) might be relevant to disease activity in adult asthmatic patients with ABPA and SAFS.

METHODS: Specific IgE antibody concentrations in serum against S. aureus enterotoxins (SE-IgE, including staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin C (SEC) and toxic shock syndrome toxin (TSST-1), Asp-IgE and tIgE levels were measured in adult cohorts of SAFS (n=34) and ABPA (n=48) using ImmunoCAP system. Patients were retrospectively reviewed for clinical characteristics, lung function and radiological features.

RESULTS: Median tIgE, Asp-IgE and SE-IgE expression were significantly higher in ABPA than SAFS patients (p=0.001, 0.01, <0.001 respectively). SE-IgE (but not Asp-IgE) was significantly associated with FEV1 (r=-0.277; p=0.028), FEF 25-75 (r=-0.290, p=0.022) and oral corticosteroid (OCS) doses (r=0.290, p=0.05) for all patients. Patients with bronchiectasis had significantly higher Asp-IgE values compared to those without bronchiectasis (6.5 vs. 2.8 KUa/L, p=0.025). Although, nasal polyps, rhinitis and hay fever were more common in SAFS than in ABPA patients, there were no associations observed for tIgE, Asp-IgE or SE-IgE. Patient with SAFS had better lung function than those with ABPA.

CONCLUSIONS: Our data suggest that there may be an association between SE-IgE with lung function and disease activity in adult asthmatic patients with ABPA and SAFS, whereas Asp-IgE may not. Large prospective studies are needed to determine the causal-effect relationship.

Key words: asthma, ABPA, SAFS, severe asthma, Staphylococcus aureus enterotoxins
3.1 INTRODUCTION

Severe asthma with fungal sensitisation (SAFS) and allergic bronchopulmonary aspergillosis (ABPA) are progressive lung diseases that cause significant morbidity and mortality, yet the pathogenesis is only partially understood. Exaggerated immunoglobulin E (IgE) responses to fungal allergens are implicated in the pathological and clinical manifestation of both diseases [139, 146, 212].

About 5-10% of asthmatics are said to have severe asthma that is poorly controlled despite standard therapy. Mould sensitisation is a major risk factor for severe asthma [298, 303] and is present in about 21% to 60% of the severe asthma population [135, 304]. ABPA is estimated to be present in about 0.7 -3.5% of asthma population [305-307] and 1–15% of patients with cystic fibrosis (CF) [166, 170, 308].

Immunoglobulin E (IgE) is a monomeric immunoglobulin that is produced by activated B-cells in response to an allergen and exists in minute amounts in the human body. It was first identified by a Swedish scientist, Johansen in 1966. The antibody is defined by the presence of the epsilon heavy chain. It induces activation of mast cells and basophils through the high-affinity cell-surface receptor Fc epsilon RI which is a specific receptor for IgE present at on tissue-resident mast cells and basophils (fig 1.1, 1.2, 1.3, 1.5) [193]. Cross-linking of the Fc epsilon RI-bound IgE leads to cellular activation, resulting in release of preformed granular components (histamine and tryptase) and subsequent production of inflammatory mediators (prostaglandins and leukotrienes) and cytokines (interleukin-4 and interleukin-5) (fig 1.1, 1.3, 1.5).

Sensitisation to particular allergens can be identified by skin prick test (SPT) or by measuring specific IgE (sIgE) to individual allergens from blood or serum. Specific IgE antibody assays measure the level of IgE antibodies present in serum that are directed against specific defined allergens. Such assays provide an objective, accurate measurement of the amount of specific IgE directed against the allergen of interest. Specific IgE tests are performed to confirm a suspected diagnosis of allergy based on a detailed clinical history and/or to identify specific precipitants. The two commonly used systems for measuring total (tIgE) and sIgE in blood or serum are radioallergosorbent test (RAST) and ImmunoCAP systems.
3.1.1 Specific IgE testing using the immunoCAP system

The immunoCAP is a fluorescent enzyme immunoassay (FEIA) that measures allergen-specific IgE in human serum. This assay detects antibodies present in serum by binding them to a specific target immobilised on a solid phase. The solid phase (ImmunoCAP) is a sponge with a very large surface area which enables specific antibodies present in the serum to bind to the allergen on the sponge surface [309].

Test principle

3.1.1.1 Specific IgE measurement by ImmunoCAP

The specific allergen of interest, covalently coupled to ImmunoCap cellulose carrier (sponge), reacts with the specific IgE in the patient serum sample. After washing away non-specific IgE, enzyme-labeled antibodies against IgE are added to form a complex. After incubation, unbound enzyme anti-IgE is washed away and the bound complex is then incubated with a developing agent. After stopping the reaction, the sponge is compressed and the fluorescence of the resulting eluate is measured. The higher the response value, the more specific IgE is present in the sample. To evaluate the test results, the response for the patient samples is compared directly to the response for the calibrators.

3.1.1.2 Total IgE measurement by ImmunoCAP

Anti-IgE, covalently coupled to ImmunoCap reaction vessel, reacts with the total IgE in the sample. After washing, enzyme-labelled antibodies against IgE are added to form a complex. After incubation, unbound enzyme anti-IgE is washed away and the bound complex is then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate is measured. The fluorescence is directly proportional with the concentration of IgE in the sample. To evaluate the test results, the response for the patient samples is compared directly to the response for the calibrators.

Compared to the RAST system, the immunoCAP provides reliable results, with better diagnostic capacity than RAST, but it must be quantified for each allergen because its results are not interchangeable [310]. CAP is more sensitive than RAST and non-specific adsorption of IgE immunoglobulin to the solid phase is less in CAP than in RAST. The sensitivity and specificity of the CAP system is around 94.2% and 87.3%, respectively [311].
IgE responses have been implicated in disease pathogenesis of SAFS and ABPA. The inhalation of fungal spores (either immediately or following Airway colonisation) triggers an immune response in a subset of asthmatics who may be genetically predisposed, and the continued or repeated exposure to fungi maintains and propagates the initial Th2 response that leads to specific IgE production by activated B-lymphocytes via IL-4 and IL-13 [261, 312] (chapter 1 of this thesis). This process leads to activation of mast cell degranulation and release of histamines, leukotrienes (LTs), tryptases, prostaglandins (PGDs) that cause bronchospasm and airway mucus production, hence symptoms of ABPA and SAFS. The other mechanism is environmental proteases (i.e. A. niger) which interfere with epithelial barrier and are also directly allergenic.

Thus, not only is IgE implicated in disease pathogenesis, but measurement of serum total IgE (tIgE) and specific IgE (sIgE) levels are used in the diagnosis, monitoring of therapeutic responses and predicting disease activity in allergic asthma, SAFS and ABPA [150, 151, 261, 313-317]. It has been suggested that the tIgE level is a marker of immunological activity in ABPA, and it is probable that the changes in tIgE levels especially the magnitude of fall is associated with relapse and remission [317, 318]. However, there is considerable debate on the value of tIgE in predicting disease activity or clinical outcomes.

Existing knowledge on the use of these IgE antibodies has been mostly limited by the inability to measure IgE-based sensitivity to all allergens which consequently has limited our understanding as to which of the allergen-specific IgE antibody (sIgE) might be useful for monitoring fungal allergic asthma.

Current guidelines for anti-IgE therapy (Omalizumab) for severe allergic asthma requires a total IgE level of between 30-1500 IU/ml and confirmation IgE mediated allergy to perennial allergen by skin prick test or RAST. More recently, anti-IgE has been used in the treatment of ABPA. However, a major problem with the use of tIgE concentrations for diagnosis or guide to disease activity is its significant variability over time [319]. During antifungal therapy, there may be marked heterogeneity in IgE responses with dramatic falls, most varying erratically and some rising within the same individuals [315]. Moreover, a decline in serum IgE levels in ABPA during follow up appears not to predict clinical outcomes [313] and the central role of an elevation of tIgE levels in asthma development has also been questioned [320].
Another problem is the lack of agreement and standardisation between different serological tests and there are questions as to whether IgE production also reflects factors other than allergy, possibly non-allergic inflammation [321].

The correlation between fungal allergen sIgE levels and disease activity in ABPA and SAFS has not been fully demonstrated, though some emerging data from China involving 100 asthma patients (mild (n=52), moderate (n=24), severe (n=24)) suggests a correlation of asthma severity to sIgE to *Aspergillus, Penicillium* and *Candida albicans* allergens, but not with *Cladosporium herbarum* or *Alternaria* spp [322].

In the last decade, staphylococcal superantigens and staphylococcal enterotoxin (SE)-mediated mechanisms have been associated with asthma and other allergic airway diseases [323-326]. SE-IgE antibodies are more commonly found in patients with severe asthma than mild asthma [325, 326] and is an independent risk factor for asthma and asthma severity [327]. SE-IgE in nasal polyp tissues is associated with asthma [323]. More recently, a potential role for *S. aureus* in the pathogenesis of allergic fungal rhinitis has been demonstrated [328].

The mechanism is not clear, but it is likely that *S. aureus* superantigens amplify the local Th2 inflammation and may be involved in the modulation and stimulation of airway inflammation [323, 324]. Enterotoxins generated by *S. aureus* can act both as nominal antigens, stimulating specific IgE responses and as superantigens, promoting a polyclonal IgE response reflected by an increase in total IgE (tIgE) levels.

Little is known regarding the association between SE-IgE and SAFS or ABPA. Further, the appropriateness and clinical use of tIgE or specific IgE-Af in clinical setting in patients with ABPA or SAFS remains to be fully elucidated.

### 3.2 HYPOTHESIS

We hypothesised that IgE-Af and SE-IgE levels will be higher in ABPA than SAFS patients. We also hypothesised that SE-IgE will correlate with asthma severity and lung function in patients with ABPA and SAFS.
3.3 Aims and objectives: The aim of this study was to investigate whether total IgE, SE-IgE and Asp-IgE might be relevant to disease severity and activity in adult asthmatic patients with SAFS or ABPA. To address this, serum concentrations of these three IgEs were measured in adult cohorts of asthmatic patients with SAFS (n=34) and ABPA (n=48) and results matched with clinical parameters and lung function.

3.4 METHODS

3.4.1 Study design and Settings

We conducted an audit of adult asthmatics with ABPA and SAFS who were attending fungal allergic asthma outpatient clinics at the National Aspergillosis Centre (NAC) and North West Lung Centre Severe Asthma Service (NWSAS) based at the University Hospital of South Manchester, UK between August 2010 and December 2011 to investigate the relationship between serum IgE levels and clinical parameters. Laboratory work for IgE was carried out at the University Hospital Ghent, Upper Airways Research Laboratory (URL), De Pintelaan, Ghent, Belgium.

3.4.1.1 Power calculations

It wasn’t possible to do power calculation as this was an exploratory study. We had no existing data on predicted levels of IgE expression in ABPA and SAFS and as such a power calculation could not be created apriori.

3.4.2 Inclusion criteria

Men or women with asthma, age (18-70 years old); All patients had clear diagnosis of either SAFS or ABPA based on standardised diagnostic criteria [151, 153, 303].

3.4.3 Exclusion criteria

Patients who had concomitant diseases or conditions that are known to affect serum IgE concentrations such as strongyloidiasis, eosinophilic pneumonia, hyper-IgE syndrome, drug reaction with eczema, ongoing immunotherapy, and continuing treatment with omalizumab were excluded.

3.4.4 Ethical statement

As this was an audit, ethical approval was not specifically obtained from either the research ethics committee (REC) or the institutional review board (IRB). However, all new patients
attending our clinics at NAC or NWSAS give advanced written consent for their clinical samples to be used for research for studies being conducted at NAC and NWSAS.

**Definitions**

**Severe asthma** was defined according to the British Thoracic Society (BTS) criteria (treatment steps 4 or 5) [29, 329]. We used published criteria for the diagnosis of **SAFS** which included i) severe asthma ii) total IgE <1000 KU/L and iii) positive skin test or raised specific IgE to any fungus as described in chapters 1 and 2 of this thesis.

Patients were considered to have **ABPA** if they met (ii, iv and at least 2) of the following criteria ; i) asthma (of any severity), ii) total serum IgE ≥1000 KU/L, iii) positive skin prick tests (SPT) to *A. fumigatus* (*Af*) of >3mm compared to control or iv), elevated *A. fumigatus*-specific (IgE-*Af*) serum levels, v), positive *Af* serum precipitins , vi) a history of pulmonary infiltrates (transient or fixed), vii) central bronchiectasis (CB), viii) a history of expectoration of brown plugs or flecks and ix) isolation of *Af* from sputum [150, 151], of which ii) and iv) were essential.

### 3.4.4 Procedure and data collection

#### 3.4.4.1 Collection of clinical information

Patients were retrospectively reviewed for clinical characteristics, asthma control and severity, age of onset (early=onset age< 16, late =onset ≥16) and duration of asthma, lung function, smoking and atopic status, nasal polyps, rhinitis, OCS and ICS use, azole antifungal use, azithromycin use and presence or absence of bronchiectasis on high resolution computed tomography (HRCT).

**Bronchodilator reversibility** was considered to be significant (reversible) if, after 200 mg of inhaled salbutamol, the forced expiratory volume in the first second (FEV1) and/or forced vital capacity (FVC) increased by more than 12% and 200 mL.

**Airflow obstruction (AFO)** was defined according to the BTS and Global initiative for chronic Obstructive Lung Disease (GOLD) criteria as a post-bronchodilator FEV1/FVC <0.70 and an FEV1<80%. Information was entered into an excel database and later into the SPSS (IBM SPSS 20, USA) spreadsheet for statistical analysis.

**Blood collection and laboratory procedure**

Blood samples were collected from patients with ABPA and SAFS and frozen at -20°C in a freezer. The samples were later aliquoted in 250 microlitres per patient, packed in boxes.
containing dry ice and shipped to our partners at the University Hospital Ghent, Upper Airways Research Laboratory (URL), Ghent in Belgium.

3.4.4.2 Measurement of total and specific IgE
All laboratory work was carried out by trained immunology scientists at the University Hospital Ghent, Upper Airways Research Laboratory (URL), Ghent, Belgium. The Phadia immunoCAP 100 system (Phadia, Uppsala, Sweden) was used to measure total and specific IgE. The Phadia immunoCAP system is an automated system that measures large panel of standardized high-quality allergens tests.

Samples were thawed in a water bath set at approximately room temperature, and mixed before testing. All samples were entered onto worksheets prior to testing. Serum concentrations of tIgE, SE-IgE (including SEA, SEC and toxin shock syndrome toxin (TSST-1) and Aspergillus slgE (Asp-IgE) were measured using the automated ImmunoCAP 100 system (Phadia, Uppsala, Sweden) according to the manufacture’s instruction. ImmunoCAP 100 an auto-analyzer for total and specific IgE.

The ImmunoCAP 100 system is designed to perform dilution and transfers (both aspirating and dispensing) of reagents and/or samples for the automatic preparation of biochemical assays. It washes, reads and transmits the results automatically. The capacity of each series is 48 determinations and the total time taken is 2.5 hours. The lower limit of specific IgE detection was set at 0.1 kUA/L as recommended by the manufacturer.

3.4.4.2.1 Internal Quality Control Procedures
Internal Quality Control (QC) was achieved by running Specific ImmunoCAP IgE control sera, provided by Thermo- Fisher, containing IgE antibodies to a number of different antigens. The QC was tested for d1 (House Dust Mite) for positive control and e1 (Cat Dander) for negative control. Results for an individual run were accepted if the QC sample lay within +/-2SD of the mean of the previous results.

For Total IgE, High, Low and Medium quality controls were used. These controls were prepared and performed in the same manner as patient samples and analyzed as part of each run.
3.5 Statistical analysis

All analyses were performed with the SPSS 20.0 software (IBM Corporation, USA) for windows and Excel statistics. Descriptive statistics were used to summarize the data. Normally distributed data were analysed as appropriate using Student’s t tests, one-way Analysis of Variance (ANOVA) or Pearson Correlations. Skewed data were normalised using a natural logarithmic transformation (Ln) prior to any analysis. The back-transformed results of geometric means were presented. Continuous data which could not be normalised were summarised showing median, quartiles (25th, 75th percentiles) and range (minimum, maximum) and analysed as appropriate using Mann-Whitney U tests, Kruskal Wallis tests or Spearman Correlations. For all comparisons, p values 0.05 were considered statistically significant.

3.6 RESULTS

3.6.1 Patient characteristics

Of 82 patients, 48 were diagnosed with ABPA (22 males, 26 females; mean age 59.7 years, SD=11.6) and 34 with SAFS (13 males, 21 females, mean age 54.7 SD=12.1) (Table 3.1). Mean BMI (SD) for all patients was 27.33 (6.6) (kg/m²). BMI was similar in ABPA and SAFS (27.3 vs. 28.9, p=0.412). The median duration of asthma for all patients was 40 years (yrs). Duration of asthma was longer in ABPA than SAFS patients, though not statistically significant (42 vs. 36.5 yrs, p=0.482) and the ABPA group were about five years older. Severe asthma was present in 23 (47.9%) of ABPA and all SAFS patients. Nasal polyps, rhinitis and hay fever were more common in SAFS than in ABPA patients, whereas eczema was equally uncommon (9.8%). Of all patients, thirty six (43.9%) were on continuous daily OCS was (ABPA=20 (41.7%), SAFS=16 (47.1%) within the last 12 months. The proportion of patients on a daily OCS dosage of 1-9mg/day was 13 (35.1%), on 10-19mg/day were 7 (18.9%) and >20mg/day was 3 (8.1%). 55 (67%) of all the patients were on azole antifungal therapy (ABPA=33, SAFS=22): Itraconazole (45), voriconazole (6) and posaconazole (4).
Table 3.1: Baseline characteristics of the 82 patients with SAFS and ABPA

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients (n=82)</th>
<th>ABPA (n=48)</th>
<th>SAFS (n=34)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs), mean (SD)</td>
<td>57.7 (12)</td>
<td>59.7 (11.6)</td>
<td>54.7 (12.1)</td>
<td>0.064</td>
</tr>
<tr>
<td>Gender: Female (%)</td>
<td>47 (57)</td>
<td>26 (54.2)</td>
<td>21 (61.8)</td>
<td>0.508</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.33(6.6)</td>
<td>27.3 (7.3)</td>
<td>28.9 (6.37)</td>
<td>0.412</td>
</tr>
<tr>
<td>FEV1(L), mean (SD)</td>
<td>2.2 (2.93)</td>
<td>1.78 (0.52)</td>
<td>2.72 (0.71)</td>
<td>0.204</td>
</tr>
<tr>
<td>FEV1% pred, mean (SD)</td>
<td>67.7(20.6)</td>
<td>66.54 (18.17)</td>
<td>69.1 (23.5)</td>
<td>0.637</td>
</tr>
<tr>
<td>FEV1 PBD change (%), median (range)</td>
<td>12.5 (-6.0-99)</td>
<td>4 (-6-25)</td>
<td>10 (2-99)</td>
<td>0.047</td>
</tr>
<tr>
<td>FEV1/FVC %, mean (SD)</td>
<td>58.57 (12.6)</td>
<td>57.49 (11.8)</td>
<td>59.92(13.46)</td>
<td>0.446</td>
</tr>
<tr>
<td>FEF25-75 (%L/s) , mean (SD)</td>
<td>1.0 (0.68)</td>
<td>0.9 (0.51)</td>
<td>1.15 (0.84)</td>
<td>0.09</td>
</tr>
<tr>
<td>Continuous OCS, n (%)</td>
<td>36 (43.9)</td>
<td>20 (42)</td>
<td>16 (47.5)</td>
<td>0.824</td>
</tr>
<tr>
<td>OCS (mg/day), median(range)</td>
<td>10 (1-22.5)</td>
<td>7.5 (1-15)</td>
<td>10 (5-22.5)</td>
<td>0.024</td>
</tr>
<tr>
<td>ICS dose (mcg), median (range)</td>
<td>1000(200-2000)</td>
<td>800 (200-2000)</td>
<td>1000 (400-2000)</td>
<td>0.013</td>
</tr>
<tr>
<td>Severe asthma (%)</td>
<td>57 (69.5)</td>
<td>23 (47.9), n=48</td>
<td>34 (100)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of asthma (yrs), median</td>
<td>40 (1-68)</td>
<td>42.0 (2-68)</td>
<td>36.5 (1-60)</td>
<td>0.482</td>
</tr>
<tr>
<td>Rhinitis (%)</td>
<td>22 (26.8)</td>
<td>10 (20.8)</td>
<td>12 (35.3)</td>
<td></td>
</tr>
<tr>
<td>Hay fever (%)</td>
<td>16 (19.5)</td>
<td>7 (14.6)</td>
<td>9 (26.5)</td>
<td></td>
</tr>
<tr>
<td>Nasal polyps (%)</td>
<td>14 (8)</td>
<td>5 (10.4)</td>
<td>9 (26.5)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: Demographic characteristics for ABPA and SAFS patients.

**ABPA**=Allergic bronchopulmonary aspergilosis; **SAFS**= Severe asthma with fungal sensitization; **BMI**=basal metabolic rate; **SD**=standard deviation; **FEV1**=forced expiratory volume in the first second; **FVC**=forced vital capacity; **PBD**=post bronchodilator; **% pred**= Percentage of the predicted value; **L**=litres; **FEF25-75** (%L/s) = Forced expiratory flow.
3.6.2 Lung function
Lung function were similar between the 2 groups, though as expected, SAFS patients were more reversible than ABPA as demonstrated by post bronchodilator FEV1 % reversibility (4% vs. 10%, \( p=0.047 \)) patients (table 3.1).

3.6.3 IgE expression
All IgE measures were non-normally distributed. Median serum concentrations of tIgE, SE-IgE and Asp-IgE in the ABPA group were significantly higher compared to those of SAFS (649 vs. 111 ku/L, \( p=0.001 \); 0.32 vs. 0.17 kUA/L; 7.58 vs. 0.8 kUA/L, \( p=0.01 \);, \( p=<0.001 \) respectively) (table 3.2) (fig 3.1). SE-IgE was not detectable in four (8.3%) cases of ABPA and in one (2.9%) case of SAFS.

Table 3.2: Total and specific IgE levels in ABPA and SAFS patients

<table>
<thead>
<tr>
<th>IgE</th>
<th>All patients</th>
<th>ABPA</th>
<th>SAFS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>tIgE (Ku/L), median (range)</td>
<td>290 (2.6-8800)</td>
<td>649 (47.4-8800)</td>
<td>111.0 (2.6-572)</td>
<td>0.001</td>
</tr>
<tr>
<td>SE-IgE</td>
<td>0.25 (0-61.5)</td>
<td>0.32 (0-61.5)</td>
<td>0.17(0-3.65)</td>
<td>0.01</td>
</tr>
<tr>
<td>Asp–IgE</td>
<td>4.42 (0-511)</td>
<td>7.58 (0-511)</td>
<td>0.8 (0-25.8)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 3.2: Total and specific IgE in ABPA and SAFS patients.

\( tIgE = total \ IgE, \ SE-IgE=IgE \text{ against staphylococcal enterotoxin}; \ Asp-IgE = specific IgE antibody against aspergillus fumigatus; KUA/L=kilounits of allergen per litre; ku/L=kilounits per litre)
Fig 3.1: Distribution of total and specific IgE for ABPA and SAFS patients.

*(r= correlation coefficient, tlgE =total IgE, SElgE=IgE against staphylococcal enterotoxin; m3=IgE antibody against aspergillus fumigatus; KUa/L=kilounits of allergen per litre; ku/L=kilounits per litre).*
3.6.4 IgE correlations

There were significant positive correlations among all the three IgE measures (table 3.3, fig 3.2). We also observed significant positive correlation for tIgE levels in FE25-75 (r=0.2.24; P=0.05 (table 3.3). No significant differences were observed for the levels of Asp-IgE or tIgE with FEV1, atopy or smoking.

TABLE 3.3: Correlations of tIgE, Asp-IgE and SE-IgE with FEV1, FEF25-75 and OCS Use (SAFS and ABPA)

<table>
<thead>
<tr>
<th></th>
<th>Ln tlgE</th>
<th>Asp-IgE</th>
<th>SE-IgE</th>
<th>FEV1</th>
<th>FEF25-75</th>
<th>OCS dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ln tlgE</td>
<td>r=1.000</td>
<td>r=0.63;</td>
<td>r=0.69;</td>
<td>r=-0.2;</td>
<td>r=-0.224;</td>
<td>r=-0.106;</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.0001</td>
<td>p&lt; 0.0001</td>
<td>p&lt;0.12</td>
<td>P=0.05</td>
<td>P=0.05</td>
<td>p=0.346</td>
</tr>
<tr>
<td>M3</td>
<td>r=0.637;</td>
<td>r=1.000</td>
<td>r=0.405;</td>
<td>r=0.098;</td>
<td>r=0.078;</td>
<td>r=0.077;</td>
</tr>
<tr>
<td></td>
<td>p=0.000</td>
<td></td>
<td>p&lt;0.0001</td>
<td>p=0.445</td>
<td>p=0.548</td>
<td>p=0.493</td>
</tr>
<tr>
<td>SE-IgE</td>
<td>r=0.69;</td>
<td>r=0.405;</td>
<td>r=1.000</td>
<td>r=-0.277;</td>
<td>r=-0.290;</td>
<td>r=-0.218;</td>
</tr>
<tr>
<td></td>
<td>p=0.000</td>
<td>p&lt;0.0001</td>
<td></td>
<td>p=0.028</td>
<td>p=0.022</td>
<td>p=0.05</td>
</tr>
</tbody>
</table>

Table 3.3: Correlation of IgE with clinical and lung function parameters.

Af= aspergillus fumigatus, r= correlation coefficient; tlgE =total IgE; SEIgE=staphylococcal enterotoxin A, C, TSST-1 ; TSST-1=Toxic Shock Syndrome Toxin 1, m3=IgE antibody against aspergillus fumigatus; KUa/L=kilounits of allergen per litre; ku/L=kilounits per litre; FEV1=forced expiratory volume in the first second; FEF25-75= forced expiratory flow between 25 and 75% of FVC; %L/s= percent litre per second.
Fig 3.3: Correlations of IgE in ABPA and SAFS

Fig 3.3: Correlation between tlgE and SE-lgE.

Fig 3.3a: Correlation between tlgE and m3 concentration.

Fig 3.3b: Correlation between SE-lgE and m3 in patients with SAFS and ABPA.

**Fig 3.3: Correlation of IgE levels in ABPA and SAFS patients**

(a): Correlation for tlgE with SE-lgE,

(b) Correlation for tlgE with m3,

(b) Correlation for m3 with SE-lgE

r= correlation coefficient, tlgE = total IgE, SE-lgE = IgE against staphylococcal enterotoxin; m3=Asp-lgE=lgE antibody against aspergillus fumigatus; KUa/L=kilounits of allergen per litre; ku/L=kilounits per liter)
3.6.4.1 Extreme outliers within the data set
There were some extreme outliers within the data set. For example, one ABPA patient (subject A09) had tIgE of 3860 Ku/L, SE-IgE of 61.5 KUa/L and Asp-IgE of 16.20 KUa/L. Another ABPA patient (subject A40) had tIgE of 8800 Ku/L and Asp-IgE of 511 Kua/L. These 2 outlying data points for SE-IgE of 61.5 KUa/L and Asp-IgE of 511 Kua/L fell near where the regression line would normally fall have skewed our data set and might have increased the size of the correlation coefficient (fig 3.3). Values of total IgE were normalised using a natural logarithmic transformation (Ln) prior to any analyses (table 3.3). The summaries and analyses used non-parametric methods for ranked data, thus the actual recorded value for those patients would not have influenced the analysis results to a great extent.

3.6.2 Specific IgE to staphylococcus enterotoxins (SE-IgE)
There were significant correlations for SE-IgE concentrations with FEV1 (r=0.277, p=0.028), FEF25-75 (r=0.290, p=0.022) and OCS dose (r=0.290; p=0.05) (table 3.3, fig 3.2). Higher SE-IgE concentrations were associated with higher OCS doses, lower FEV1 and FEF25-75 values in all patients (ABPA & SAFS combined) (table 3.3, fig3. 3). In ABPA patients, serum SE-IgE levels were significantly associated with percent (%) reversibility in FEV1 after 2.5 mg salbutamol nebuliser (PBD FEV1).

Fig 3.2: Correlations between SE-IgE and FEV1 or FEF25-FEF75

![Fig 3.2 showing correlations between SE-IgE with FEV1 (a) and FEF25-FEF75 in SAFS and ABPA patients.]()
3.6.3 Serum specific IgE to A. fumigatus (Asp-IgE)
There was no significant correlation between concentration of Asp-IgE and FEV1, FEF25-75 or dosages of OCS/ICS use (table 3.3). On the other hand, patients with bronchiectasis had significantly higher Asp-IgE concentrations compared to non-bronchiectasis (r=0.245, p=0.028). Although not statistically different, patients with recurrent pseudomonas colonization tended to have a much higher Asp-IgE concentrations than those without (9.09 vs. 3.9 kUA/L, p=0.442). No correlation was observed for all IgE measures in azithromycin or azole antifungal use/dose or doses, atopy, eczema, rhinitis, nasal polyps, radiological abnormalities on HRCT.

3.7 DISCUSSION
Severe asthma with fungal sensitisation is a recently described asthma phenotype in patients with severe asthma sensitised with fungal allergens and is said to be closely related to ABPA in asthma [316]. However, how SAFS differs from ABPA in clinical characteristics is unclear. There are not enough data in literature comparing the clinical characteristic between SAFS and ABPA (Pasqualotto, 2009). In this study, we found a higher frequency of nasal polyps, rhinitis, and hay fever in SAFS than in ABPA patients suggesting that SAFS patients may be more atopic than ABPA asthmatic subjects. Several genetic polymorphisms have been found in ABPA [152, 330], whereas at least one is not present in SAFS - toll-like receptor 9 (Carvalho, 2010), perhaps suggesting underlying pathogenetic differences. A highly distinctive feature of ABPA in India is the presence of hyper-attenuating mucus, which is uncommon in the predominantly Caucasian population I have studied [331]. To our knowledge, hyper-attenuating mucus is not found in SAFS, and many of our patients with ABPA have significant problems with thick inspissated mucus, which is less marked in SAFS patients.

3.7.1 Lung function reversibility
In this study, we observed no significant difference in pre-bronchodilator spirometric measures (FEV1, FEV1/FVC %, FEF and FEF25-75) between SAFS and ABPA. However, statistical differences were observed with post-bronchodilator (PBD) values. We also observed that the frequency of airflow obstruction (AFO) was more common in ABPA than SAFS subjects in which the FEV1 % reversibility values were significantly higher. There is no data in literature comparing lung function between SAFS and ABPA, but a potential explanation for this may be that the airways of ABPA may be undergoing more airways remodelling than SAFS patients, may be due to higher population of fungal airway
colonisation. In support of this hypothesis are the findings by Agbetile and co-workers that isolation of fungi from sputum of patients with asthma is associated with impaired post-bronchodilator FEV1 which might be partly responsible for the development of fixed airflow obstruction in asthma [38]. Our study did not evaluate the range of fungi that might have colonized the airways in the 2 groups of patients, but some data from observation studies suggest a higher population of fungi airway colonisation in ABPA compared to SAFS patients.

We also found that both ICS dosage and daily OCS use were significantly higher in SAFS than in ABPA patients (1000 vs. 800, p=0.013; 10 vs. 7.5, p=0.024, respectively, table 3.1). This might be explained by frequent exacerbations and poor disease control that characterises severe asthmatics requiring the use of these therapies. The difference in the steroid usage might have contributed to the differences in lung function, further supporting previous suggestions that regular treatment with ICS may partly have a beneficial effect on airway remodelling in asthma [332], although results from chapter 4 of this thesis point to the increase in airway fungi with the use of steroids. The frequency of use of antifungals and azithromycin was not different between the groups, and is unlikely to be the reason for the difference. In contrast, serum concentrations of total IgE (tIgE), specific IgE to staphylococcal enterotoxin (SE IgE) and A. fumigatus were significantly higher in ABPA group compared to those of SAFS (table 3.1). Taken together, our findings support clinical heterogeneity in asthma and the need for new approaches for the classification of disease and selection of appropriate treatment.

### 3.7.2 IgE correlations

As expected, we found significant correlations among all IgE measures and as others have found in severe refractory asthma (SRA) [31]. The positive correlation for tIgE with Asp-IgE and SE-IgE probably reflects the contribution of these environmental allergens towards the tIgE concentrations. However, a significant and strong correlation of SE-IgE with total IgE has recently been demonstrated in patients with allergic fungal rhinosinusitis (AFRS) which was also correlated with the co-expression of Asp-IgE [333]. Very few studies have described specifically the correlation between tIgE and fungal allergen specific IgE (sIgE) in patients with SAFS or ABPA. Although nasal polyps (NP), rhinitis and hay fever were more common in SAFS than ABPA patients, we found no significant correlation between IgE measures (tIgE, SE-IgE, Asp-IgE) and these atopic manifestations. Our finding is consistent with the findings of Schiappoli et al. (2012) who demonstrated lack of detection of serum SE-IgE in patients with nasal polyposis (NP), compared with healthy subjects [334].
However, it appears from previous studies that there may be 3 distinct NP groups with one group demonstrating no measurable SE-IgE, another demonstrating selected specific IgE and the third group demonstrating a multiclonal specific IgE, including SE-IgE [323]. It is not clear which group our patients belonged to but, our data suggest that measuring SE-IgE may help better characterisation of our asthma patients and the association with SRA, high OCS/ICS use, poor lung function and possible high frequency of NP suggest that high SE-IgE patients may be a specific phenotype.

3.7.3 Effect of outliers
Outliers exist in this study (fig 3.3). Some patients have extremely high tlgE and slgE levels outside the usual range, reflecting the wide variability in IgE levels within individual patients. For example, one ABPA patient (subject A09) had tlgE of 3860 Ku/L, SE-IgE=61.5 and Asp-IgE of 16.20 KUa/L. Another ABPA patient (subject A40) had tlgE of 8800 Ku/L and Asp-IgE of 511 Kua/L. These outliers have skewed our data set. These 2 outliers for SE-IgE (61.5) for subject and Asp-IgE of 511 Kua/L fall near where the regression line would normally fall have increased the size of the correlation coefficient (fig 3.3). We checked the common potential causes of extreme scores in a data set such as data recording or entry errors, motivated mis-reporting, sampling errors but none of these seem to exist. Therefore, these were legitimate scores re-enforcing the variability in IgE measures. However, these outliers might have deleterious effects on statistical and graphical analyses. As the data is non-randomly distributed, it is possible that these outliers have decreased normality, bias or influence estimates our correlations. However, we reduced the effect of outliers by logging the data for tlgE. The summaries and analyses used non-parametric methods for ranked data, thus the actual recorded value for those patients would not have influenced the analysis results to a greater extent. Despite this, the results re-enforce the great variability of IgE measurements.

A major problem with the use of tlgE concentrations for diagnosis or guide to disease activity is its significant variability over time [319]. During antifungal therapy, there may be marked heterogeneity in IgE responses with dramatic falls, most varying erratically and some rising within the same individuals [315].

3.7.4 Total IgE levels and lung function
The relationship between tlgE and lung function in patients with SAFS and ABPA is poorly understood. Previous reports on the relationship between serum tlgE concentrations and pulmonary function in the healthy subjects [40, 41], COPD [335], young adults with current
asthma [336] and in allergic asthmatic children sensitized to house dust mites [44] have produced conflicting results. We found a correlation for FEF25-75, but not with FEV1 suggesting a possible link between tIgE and measures of small airways disease. The non-significant findings with FEV1 in this study are consistent with previous findings by Sacco and colleagues in allergic asthmatic children sensitized to house dust mite [337], however our study numbers are small and these findings need to be interpreted with caution.

3.7.5 IgE levels and airflow obstruction
Subgroup analysis of ABPA patients demonstrated a significant negative correlation for Asp-IgE levels with FEV1 and % predicted FEV1, but not for SAFS patients. The negative correlation between Af-IgE supports previous findings in non-asthma and asthma populations.

In a recent study by Bafadhel M and colleagues to investigate the frequency of filamentous fungal culture and IgE sensitisation to Aspergillus fumigatus and the relationship to clinical outcomes in COPD subjects, more than 10% of patients with chronic obstructive airways disease (COPD) were sensitised to A. fumigatus which in turn was associated with worse pulmonary function [338]. An increase in tIgE was associated with the presence of airflow obstruction in both ABPA and SAFS. Our data supports previous findings in Germany [339], India [335] and Japan [41], though the patient groups are different from those of our study population. In a cross-sectional analysis of 1505 Japanese adults (non-COPD and non-asthmatic subjects), annual decline in FEV1 and AFO was associated with higher levels of total serum IgE (P = 0.029) [41]. Further, data from analysis of COPD patients demonstrated an association between the serum levels of IgE and severity of airway obstruction [335]. A. fumigatus airway colonisation is associated with reduced lung function and development of fixed airflow obstruction in asthma may be a consequent upon the damaging effects of airway colonization with A. fumigatus [3], which could be more profound in ABPA.

3.7.6 Aspergillus sensitisation and bronchiectasis
We found a significant association between higher specific IgE to A. fumigatus serum concentration and bronchiectasis for both groups. Our findings reinforce previous evidence linking Aspergillus sensitisation with bronchiectasis in asthma [47, 48]. In a study by Menzies et al of severe asthmatics A. fumigatus sensitization was associated with a 2.01 increased hazard ratio (HR) of bronchiectasis (p = 0.005), and more obstructive spirometry (p = 0.001). In addition, Fairs et al in their study of 79 severe asthmatic patients observed
significantly lower lung function (FEV1 and airway reversibility) and a higher frequency of bronchiectasis (68% versus 35%; P < 0.05) in A. fumigatus-IgE sensitized asthmatics compared to nonsensitized asthmatics [3]. In cystic fibrosis (CF) ABPA, Aspergillus sensitization and persistent fungal carriage were significantly associated with a larger decline in FEV1 [49].

3.7.7 *Staphylococcus aureus* enterotoxins (SE-IgE)

In this study, we found a significant positive correlation between SE-IgE concentrations and higher oral corticosteroid (OCS) doses (p=0.05, r=0.290) (table 3.2). Our findings support recent findings by Bachert et al in their study of adult cohorts of 69 control subjects, 152 patients with non-severe asthma and 166 severe asthmatics [32]. These researchers found that OCS use and hospitalizations were significantly increased in patients with SE-IgE. The underlying mechanism explaining the link between staphylococcal enterotoxin (SE) and OCS is unclear but SE has been shown to induce T-cell activation that is poorly responsive to glucocorticoid regulation leading to corticosteroid insensitivity [51-53].

3.7.8 SE-IgE and lung function

We found a significant correlation between SE-IgE and both FEV1 and FEF25-75 (table 3.2, fig 3.3). An increase in SE-IgE was associated with low FEV1 and FEF25-75 values. Our findings reinforce previous findings in Poland [31] and Belgium [30, 32]. In an analysis of serum from a cohort of severe asthma (n=109) and non-severe asthma (n=101), SE-IgE was significantly associated with low respiratory function parameters (FEV1, FEV1/FVC and FEF 25-75) and increased airway reversibility [31]. An association of SE-IgE with lung function parameters and asthma severity in severe asthma was earlier described in 2003 and 20% of those patients were non-atopic late-onset asthmatics [325]. In our study, we found that 32 (39%) of all asthma patients and 21 (36.8%) of all severe asthmatics were late onset asthma. We did not observe any significant association between age of onset and SE-IgE. Despite this, our study reinforces and supports a strong association between SE-IgE and lung function.

The mechanism is not clear but staphylococcal enterotoxins have been shown to promote airway inflammation and bronchial hyperresponsiveness in animal models [55] and to strongly amplify airway inflammation in combination with allergen exposure after sensitization in humans [56].
3.7.9 IgE and antifungal or anti-inflammatory agents

In this study, we found no correlation between any of the IgE measures with azithromycin or azole antifungal use or doses. The reasons for this might be multiple; in particular, the intermittent use of these therapies by the subjects might have affected the correlation.

3.8 LIMITATIONS

This study has a number of limitations. The retrospective nature of clinical data might have affected the results as clinical data was only based on recordings by individual clinicians. Asthma classification in ABPA is also difficult as most of ABPA patients do take frequent OCS which may reduce IgE results. Therefore use of continuous use of steroid as criteria for disease severity is usually inappropriate in this group of patients. As a result, the authors did not attempt any correlations with asthma severity. OCS therapy and doses was based on clinician preferences and prescribing varied slightly. This is likely to have altered the correlations and significance data. Concomitant antifungal therapy in 67% of our patients may have altered the natural history of the disorders, as it usually reduces IgE levels [315]. Finally, we assigned study groups (ABPA or SAFS) based on the highest total IgE serum level which might have affected results of tIgE distribution between disease groups. This is imperfect as a single means of distinguishing patients, especially those with severe asthma. Likewise the BTS guideline re step 4/5 is based on OCS, but this is required in some ABPA patients because of mucus obstruction, not asthma control. Numbers of ABPA and SAFS patients were unequal and therefore correlations sought comparing ABPA and SAFS may have resulted in different findings due to reduced power, because of smaller numbers in the SAFS population. The non-random/sequential nature of our patients could as well have affected our results.

3.9 CONCLUSIONS

Our data suggests that SE-IgE is relevant to airflow obstruction and Asp-IgE to bronchiectasis in adult asthmatic patients with ABPA and SAFS. We have also demonstrated that asthmatic patients with SAFS may be a distinctive asthma phenotype, although with a number of similar clinical characteristics with those of ABPA, further supporting clinical heterogeneity in asthma and the need for new approaches for the classification of disease and selection of appropriate treatment. Our data also suggests that measuring SE-IgE in asthma patients may provide a mechanism of further phenotyping severe asthma. Larger prospective cohort studies are needed to determine the causal-effect relationships.
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CHAPTER 4

Airway microbiota in allergic bronchopulmonary aspergillosis, severe asthma with fungal sensitisation and severe asthma
4.0 ABSTRACT

BACKGROUND: Emerging data suggest that bacterial lung microbiota may contribute to pathophysiologic processes of chronic airways disease including asthma. However, this data are almost exclusively bacterial, but the diversity and role of microbiome in patients with fungal associated asthma is unknown.

OBJECTIVES: The main objective of this study was to investigate the range of microbiome composition in airways of patients with ABPA, SAFS, severe asthma-not fungal sensitised (SANFS) and mild asthma (MA) compared to normal healthy (NH) individuals. We also compared microbiome yields from BAL using the Health Protection Agency standard culture method (BSOP57) and microbiome quantitative real time polymerase chain reaction (qPCR) plus sequencing.

METHODS: Airway microbiome composition were evaluated from bronchoalveolar lavage (BAL) of 59 patients with ABPA (n=16), SAFS (n=15), SANFS (n=11), MA (n=6) and NH (n=11) using (i) standard culture and (ii) molecular techniques (bacterial (16S & 23S), fungal (ITS1-5S & 28S)) followed by Illumina sequencing. Results were compared to known databases using QIIME.

RESULTS: There was a greater diversity of pathogenic and non-pathogenic microorganisms in all patients. Haemophilus spp. were present in all (health and disease), although were most abundant in ABPA and SAFS. There was a massive preponderance of A. fumigatus over all other fungi in asthmatic lungs, with severe asthma having the highest levels. There was a great diversity of isolates in the A. fumigatus group: Tens and probably hundreds of variant strains were found in each pair of lungs sampled. Individuals with severe asthma, whether sensitised to fungi or not (as in SANFS), had a significantly increased proportion of A. fumigatus in their mycobiome. Fungal colonised patients had worse FEV1 than non-colonised (p<0.001), a more airflow obstructive picture (p=0.001) and higher frequency of bronchiectasis (p=0.05). Steroid therapy increased the airway fungal burden in all patients. Culture-independent techniques were better and more sensitive than culture methods.

CONCLUSIONS: This data suggests that the airways of patients with ABPA, SANFS and SAFS contain a wide diversity of bacteria and fungi. Fungi and bacteria are probably more common in severe asthma population and may be linked with asthma severity and poor lung function. Steroid therapy increases fungal burden. Further studies need to be done to determine the causal effect relationship and also distinguish between pathogenicity and colonisation.
4.1 INTRODUCTION

Allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitisation (SAFS) and severe asthma (SANFS) are serious airways diseases whose pathogenesis is partially understood. Our current concept of these diseases is that fungal conditions such as ABPA and perhaps SAFS are caused by excessive and persistent fungal colonisation of lung airways. Emerging data suggest a link between the presence of microbiota and control of inflammation in humans [340-342] and there is growing evidence regarding the potential impact of the pulmonary microbiome on immunopathogenesis of Aspergillus-related lung disease [343]. Current dogma suggests that an individual’s microbiome may be associated with disease progression in asthma or severe asthma patients or any other lung condition, and is distinctive for these conditions. However, little is known regarding the range of microbiome population and their potential role in the pathogenesis and disease severity in ABPA, SAFS and SANFS.

Recent advances in culture-independent techniques based on deep sequencing have significantly improved our knowledge on the microbial communities, providing new insight into diversity of those microorganisms and the pathogenesis of many conditions. It is now known that microbiome has a great impact on human health [344, 345] and any imbalance in their composition may lead to disease [344, 346, 347]. However, it is remarkable that we do not know which species of fungi or bacteria are present in healthy individuals, how this microbiome is different in lungs of ABPA, SAFS patients and what conditions or microbiome composition could be associated with pathophysiological processes of chronic airway diseases.

Diseases caused by an opportunistic pathogen Aspergillus fumigatus are usually life-threatening, but there has been recent recognition of a much greater role of Aspergillus-associated asthma and allergic diseases such as ABPA and SAFS primarily in adults, but also children [348-350]. This awareness has generated numerous questions about pathogenesis. ABPA is characterized by an exaggerated Th2 CD4+ allergic inflammatory response to A. fumigatus allergens in the bronchial airway of atopic asthma and cystic fibrosis (CF) patients when A. fumigatus spores are inhaled and germinate in bronchial mucus releasing allergens, including proteases [351, 352]. The disease processes of ABPA and SAFS often involve chronic airway colonization by bacteria and fungi, and these
infections may cause irreversible lung damage. Although *Aspergillus* spp. has received
greatest attention in ABPA in the past, other fungal and bacterial spp. may play a role in
pathogenesis. However, there is little data describing which microbial species are present,
their relative and absolute abundances, and how antimicrobial or steroid therapies impact
these microbial communities [343]. Moreover, the vast majority of what we know at present
has been generated from smaller studies and in most cases, utilising sputum specimens
which have a potential for contamination with upper airway commensals making it difficult to
distinguish between pathogenic and none pathogenic [353] microorganisms. In addition,
most studies have focused upon characterizing the bacterial family of the airway microbiome
in the lungs, but little is known about the fungal constituents.

Our ignorance of the lung microbiome in disease stems from several factors: former dogma
that the lung is a sterile organ [354, 355], dependence on culture methods for fungi, which in
many cases are both insensitive and incomplete [356-358] and lack of comprehensive
population studies on lung microflora. Moreover, most of the microbiome studies performed
to date have focused on the easily accessible gastro-intestinal tract, oral, skin and vaginal
bacterial microenvironment with lung microbiome being a relatively unrecognised topic [359-
361].

There is growing evidence that the lung harbours vast numbers of microbial taxa, which
have a significant impact on clinical outcome of respiratory diseases. The role of the
bacterial microbiome has been reported in a variety of lung diseases including chronic
obstructive airways disease (COPD) [359], asthma [362], cystic fibrosis (CF) [363] and after
lung transplantation [364]. However, the fungal microbiome has been largely neglected
especially in conditions such as ABPA, SAFS, asthma and severe asthma (SANFS).

The relationship between asthma pathogenesis and the lung microbiome has been
suggested for many years. Such mechanisms include airway hyper-responsiveness in
patients with suboptimally controlled asthma [360]. How bacterial and fungal communities
modulate the immune system, interact with each other and change following antimicrobial
therapy or steroid treatment in these diseases is a subject of great interest and promise for
patient health improvement. It has been previously shown that an imbalance in bacterial
microbiome in the gut (e.g. by dietary change or use of antibiotics) modulates immune
responses and eventually may lead to development of asthma [362, 365]. In some cases
prolonged antibiotic usage may alter the composition of bacterial communities in the lung

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and possibly directs the immune responses towards *Aspergillus* and other fungi involved in fungal sensitised asthma. On the other hand the presence of fungi in the lung could change the local microbiome composition leading to specific immune responses and hence development of fungal related diseases. Moreover, steroid treatment may lead to even more profound consequences. Therefore it becomes apparent that both the gut and the lung commensal communities may greatly contribute to the pathophysiological processes associated with chronic airway diseases or to protection against those diseases.

More recently, there has been an increased use of macrolide antibiotics in patients with severe asthma with good responses in some cases, but whether the response seen is due to reduction in microbial population or purely due to the anti-inflammatory nature of macrolides is unclear. However, there is ongoing debate with regard to the causal-effect relationship between microorganisms and asthma [366]. In a retrospective study using culture techniques in Germany to determine the association between microbial population and asthma from tracheal secretions of asthmatics (n=296) obtained by BAL, bronchial aspirates and sputum Yayan and co-workers could not establish a causal relationship between microorganisms and asthma (P = 0.893) [366]. Thus a proper understanding of the relationship between airway microbiome presence and dominance may help direct alternative treatment approaches.

Traditionally, culture methods have been used to investigate airway microbiome composition, but these methods are limited by a number of factors such as low diagnostic yield, specificity and sensitivity. Modern molecular genetic technology allows complete microbiome identification, based on a sequence determination of conserved genetic regions. These studies have shown a previously unappreciated complexity to the bacterial and fungal microbiome of the respiratory tract [367, 368]. These regions form the 16s-subunit ribosomal RNA in microorganisms of different bacterial species [367, 369] and the 18s-subunit for fungal species [368]. These genes are detected by sequencing markers characteristic of individual microorganisms and their phylogenetic groups, and allow performing detailed analysis of the microbiota in health and patients with lung disease. Relative count number for specific sequences provides quantitative insight into microbial burden and can be associated with the commensal or pathogenic status of the microorganism.

An understanding of the relationship between airway bacterial and fungal presence and dominance in severe asthma may help direct alternative treatment approaches. Thus, in this...
study we investigated microbiome composition in the lungs of ABPA, SAFS, severe asthma-non fungalsensitised (SANFS) and or healthy individuals in order to improve our understanding of these diseases. We applied molecular techniques but also standard microbiological culture methods (for Asperillus spp. and pathogenic bacteria).

4.1.2 HYPOTHESIS
We hypothesised that the airway microbiome population will be higher in patients with fungal-associated asthma and that the microbiome density will be related to asthma severity. We also hypothesised that steroid use will increase the fungal population and diversity in ABPA, SAFS and SA (SANFS).

4.1.3 Aims/Objectives
The main objective of this study was to investigate microbiome composition in the lungs of ABPA, SAFS, asthma and severe asthma individuals or healthy in order to improve our understanding of these diseases.

The second objective was to compare microbiome yields from BAL using the Health Protection Agency standard culture method (BSOP57) and microbiome quantitative real time polymerase chain reaction (qPCR) plus sequencing.

4.2 MATERIALS AND METHODS

4.2.1 Study design and setting
This was a prospective clinical and laboratory study conducted between November 2011 and November 2013 at the National Aspergillosis Centre (NAC) and the North West Severe Asthma Centre (NSAC) based at the University Hospital of South Manchester (UHSM), Manchester, UK.

4.2.1.1 Power calculations
It wasn’t possible to do power calculation as this was an exploratory study. We had no existing data on predicted levels of for microbiome in ABPA and SAFS and as such a power calculation could not be created apriori.

4.2.1.2 Ethics statement & Ethical approval
All parts of the study were designed and conducted in accordance with good clinical practice (GCP) principles. Ethical approval was granted from the North West-Greater Manchester
research ethics committee (Ref: 11/NW/0175). The institutional review board for human studies at Wythenshawe Hospital approved the protocols and written consent was obtained from the subjects. The study was reviewed, approved and sponsored by the University of Manchester, research and ethics department under the research Governance Framework for Health & Social Care and Medicines for Human Use (Clinical Trials) Regulations 2004 (S12004/S12004/1031).

4.2.2 Study Subjects
The study subjects were 59 adults aged between 22-75 years old with ABPA (n=16), SAFS (n=15), SANFS (n=11), mild asthma (n=6) and healthy subjects (n=11), between November 2011 and November 2013.

4.4.2.1 Subject recruitment
ABPA, SAFS and severe asthma-non fungal sensitised (SANFS) patients were recruited from a cohort of patients attending the NWSAC and NAC clinics. Mild asthmatics and normal healthy subjects were recruited by myself from among hospital and University of Manchester (UoM) members of staff, general public and students following print and local advertisements. All subjects were predominantly recruited by myself, assisted by my supervisors.

We used clinical databases at the NWSAC and NAC to identify potential research participants. Subjects who expressed willingness to take part in the study were sent invitation letters, patient/subject information sheets (PIS) and copies of the consent forms. Those who still wanted to take part in the study were invited to attend visit 1 (consent, screening visit) and visit 2 (bronchoscopy plus blood collection).

Visit 1 - Consent/screening visit
At visit 1, a detailed discussion concerning the study was discussed with the participant at which the participant was given the opportunity to ask any questions. Those who decided to participate were invited to sign the consent form.

• A full history and examination was conducted to assess the suitability to undergo bronchoscopy and the eligibility according to the inclusion criteria below.
• Lung function test –
• If we did not have data for fungal allergy status, a skin prick allergy testing was done.

Visit 2 (bronchoscopy and blood collection visit (see bronchoscopy details; section 4.2.4).
All patients and controls gave informed written consent.

### 4.2.3 Inclusion criteria
The inclusion criteria included: men or women, age (18-75 years old), lifelong non-smokers (<10 pack years) who had a physician diagnosis of asthma (or no asthma for healthy controls), ABPA, or SAFS; had no coexisting inflammatory chronic lung disease (except bronchiectasis in ABPA, SAFS and SANFS) and had an FEV1 ≥1 L/sec taken within the preceding 3 months. All patients with asthma underwent reversibility testing and confirmed by post-bronchodilator (PBD) increase in FEV1 ≥12% and or ≥200 mL compared with baseline [29] (except in some cases of chronic asthma who might have fixed airflow obstruction [30, 31], in the absence of features suggestive of COPD). Patients with symptoms suggestive of asthma but with no evidence of PBD reversibility features of asthma (provided no fixed airflow obstruction) underwent airway hyper-responsiveness testing using methacholine challenge test (MCT) [32]. A diagnosis of asthma was considered when there was a significant bronchial responsiveness demonstrated by ≥ 20% fall in the FEV1 with methacholine challenge concentration of <8mg/dl (PC20 or PD20) on a 5-breath dosimeter protocol [33, 34] (see chapter 1 and 2 of this thesis).

Subjects also needed to have had stable disease with no recent flare-ups (within 4 weeks) and met the safety criteria to have a bronchoscopy including oxygen saturations whilst breathing room air of >90%. Normal healthy subjects had no evidence of any long term lung condition and no chest infection in the preceding 4 weeks.

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

The diagnostic criteria for ABPA included; i) asthma (of any severity), ii) total serum IgE ≥1000 KIU/mL, iii) immediate cutaneous reaction to A. fumigatus of > 3mm compared to control (patients with ABPA were not specifically tested for immediate skin reactivity to Aspergillus; so a raised serum specific IgE was regarded as equivalent), iv) elevated A. fumigatus-specific serum IgE levels (RAST),v) precipitating or IgG antibodies (ImmunoCap, Phadia, Uppsala, Sweden) to A. fumigatus in the serum, vi) central bronchiectasis (CB), vii) a history of expectoration of brown plugs or flecks and ix) isolation of A. fumigatus from sputum [150, 151], of which ii) and iv) were essential.

Asthma was defined according to international guidelines and definitions (chapter 1 and 2). Severe asthma was defined according to the BTS criteria (treatment steps 4 or 5) i.e. poorly controlled asthma on high dose of inhaled steroid (≥800 mcg beclomethasone dipropionate, BDP daily) plus long acting B2-agonist (LABA), continuous use of oral corticosteroids (OCS) or frequent use of OCS (step 5) to provide disease control [70].

SAFS was defined according to the published Denning criteria which included i) severe asthma, ii) total IgE <1000 KIU/mL and iii) either a positive skin test or raised specific IgE to any fungus [316].

4.2.6 Bronchoscopy and sample collections

We obtained airway lymphocytes by flexible bronchoscopy at the UHSM using local guidelines and BTS standard procedures [287]. The tip of the bronchoscope was wedged into the orifice of a subsegmental bronchus of the lobes with significant radiological abnormality and high volume lavage (HVL) was collected. Where there were no radiological abnormalities such as in SAFS, most asthmatics or healthy individuals, the samples were collected from the right lower lobe.

As opposed to sampling the right middle lobe (RML) which is the commonly chosen lobe for BAL (because it is easy to wedge the bronchoscope), we specifically chose the lobes with significant radiological findings as we anticipated that the presence of fungi would be higher in areas of the lungs with maximum damage. We found it easy to wedge the right lower lobes (RLL) in most SAFS and SA (SANFS) and so we deliberately chose this lobe for patients with no radiological abnormalities.
A maximum of 4 x 60mL (240 mls) aliquots of pre-warmed sterile 0.9% sodium chloride (0.9% NaCl, saline) solution was instilled into each chosen lobe. The volume instilled was judged by the clinician based on the patients’ clinical condition and current oxygen saturation. Between 2% and 20% of the instilled volume was recovered during the procedure from most of the patients. After the procedure all collected samples were immediately placed on ice and transferred to the laboratory and processed as in figure 4.1.

Figure 4.1: BAL sample processing for culture and Illumina sequencing.

Fig 4.1: BAL sample processing for culture and Illumina sequencing.
LABORATORY WORK

1.4.7.1 BACTERIAL AND FUNGAL CULTURE
Samples were cleaned from mucus plugs using a 100 µm Falcon cell strainer (Corning). Approximately 90% of the flown-through was used for T lymphocyte analysis. The remaining 10% was mixed with the mucus plugs and about 100 µl was analysed for bacteria using Health Protection Agency (HPA) standard culture method (BSOP57) protocol (HPA) (fig 4.1) [41].

We plated approximately 200 µl of BAL on Sabouraud dextrose agar supplemented with chloramphenicol for fungi using the BSOP57 protocol (HPA) [41] and incubated for 5 days at 30°C. The remaining BAL volume was subjected to DNA extraction, PCR and Illumina sequencing. After 5 days, the plates were examined macroscopically and those that showed microbial growth were examined microscopically by trained microbiologists in the fungal lab for identification of the species.

1.4.7.2 DNA EXTRACTION, PCR AND ILLUMINA SEQUENCING
In order to determine the microbial composition in the BAL, each sample was subjected to DNA extraction, PCR and Illumina MiSEQ/Nextera XT sequencing. Upon arrival in the laboratory and initial processing (above), we transferred samples to high performance 50 ml Falcon tubes (VWR) and centrifuged at 10000 x g for 20 min at 4°C. We extracted DNA on both the pellet and supernatant (500 µl) using a CTAB method previously described [370]. The DNA was resuspended in 100-300 µl sterile distilled water (dH₂O) and quantified using a spectrophotometer and stored at -80°C. Only DNA obtained from the pellets was used for PCR and sequencing.

1.4.7.2.1 Details of DNA extraction protocol and method
 Tubes containing sample pellets were suspended in 1ml of CTAB extraction buffer (100mM Tris, 1.4M NaCl, 10mM EDTA, 2% CTAB). The suspension was transferred to a 2 ml tube containing 600 µm glass beads and vortexed for 10 minutes at max speed using a GENE II vortex. Subsequently the tubes were incubated at 60°C for 10 minutes. The vortexing and heating steps were repeated and the tubes were centrifuged for 2 minutes at 13000 rpm. The supernatant was transferred to a new tube and mixed with 3µl of RNA. The tubes were subsequently incubated for 15 minutes at 37°C and then mixed with 700µl chloroform. We then centrifuged the tubes at max speed for 2 minutes and the aqueous phase was transferred to a new tube. The DNA was precipitated with 0.6 volumes of isopropanol,
centrifuged for 2 min at max speed and washed with 70% ethanol. We then resuspended DNA pellets in 50µl sterile dH2O and quantified using a spectrophotometer. Only DNA obtained from the pellets was used for PCR and sequencing.

1.4.7.2.2 Polymerase Chain Reaction (PCR)

We designed PCR to target the (i) bacterial 16S [367, 369] and 23S, (ii) fungal ITS1-5S and 28S [371] and (iii) archaeal 16S rDNA (table 4.1) ie 5 reactions per each BAL sample. PCRs were carried out in accordance with the ’16S Metagenomic Sequencing Library Preparation’ protocol (Illumina) with modifications and using the KAPA HiFi Hotstart Polymerase (Peqlab) in a total volume of 25 µl per sample. In the first PCR step, approximately 100 ng of total DNA obtained from the pellet from each BAL sample was used with the above primers containing specific Illumina overhang adapters (table 4.1). For bacterial and fungal PCR, 5 mM of a forward and a reverse primer was used. Five different forward archaeal primers at the concentration of 1 mM each were mixed with 5 mM of the reverse primer.
Table 4.1: Primers used to amplify bacterial, fungal and archaeal rDNA genes in the first amplification reaction

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’→3’</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial 16S:</strong></td>
<td></td>
<td>Klindworth et al. 2013</td>
</tr>
<tr>
<td>Bact334F</td>
<td>cctacgggnggcwgcag</td>
<td></td>
</tr>
<tr>
<td>Bact799R</td>
<td>ggaactchvgggtatctaatcc</td>
<td></td>
</tr>
<tr>
<td><strong>Bacterial 23S:</strong></td>
<td></td>
<td>Van Camp et al. 1993</td>
</tr>
<tr>
<td>Bact188F</td>
<td>ggaactgaacacatctaagta</td>
<td></td>
</tr>
<tr>
<td>Bact463R</td>
<td>cggtactggttcactatcgg</td>
<td></td>
</tr>
<tr>
<td><strong>Archaeal 16S:</strong></td>
<td></td>
<td>Siam et al. 2012</td>
</tr>
<tr>
<td>Arch517_1F</td>
<td>gcctaaagcatccgtagc</td>
<td></td>
</tr>
<tr>
<td>Arch517_2F</td>
<td>gcctaaarcgtcgtagc</td>
<td></td>
</tr>
<tr>
<td>Arch517_3F</td>
<td>gtctaaaggtgtcgtagc</td>
<td></td>
</tr>
<tr>
<td>Arch517_4F</td>
<td>gtctaaangtgcgtagc</td>
<td></td>
</tr>
<tr>
<td>Arch517_5F</td>
<td>gtctaaarcgygcgtagc</td>
<td></td>
</tr>
<tr>
<td>Arch958R</td>
<td>ccggcgttgantccttatt</td>
<td></td>
</tr>
<tr>
<td><strong>Fungal ITS1:</strong></td>
<td></td>
<td>White et al. 1990</td>
</tr>
<tr>
<td>fungITS1</td>
<td>tccgtaggtgaacctgcgg</td>
<td></td>
</tr>
<tr>
<td>fungITS2</td>
<td>gctgcgtctctctcatcgtgc</td>
<td></td>
</tr>
<tr>
<td><strong>Fungal 28S:</strong></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>fungD1</td>
<td>gcatacaataacggagga</td>
<td></td>
</tr>
<tr>
<td>fungD2</td>
<td>cctacttctcgcgtatcgg</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Primers used to amplify bacterial, fungal and archaeal rDNA genes in the first amplification reaction. Each primer had an Illumina specific adaptor attached to the 5’ end (forward primer: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, reverse primer: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG)

We carried out initial touchdown PCR of 5 cycles (95°C for 30 sec, 45-50°C for 30 sec and 72°C for 30 sec) before 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. After the PCR all 5 reactions per BAL sample were pooled and purified using Agencourt AMPure XP magnetic beads as per manufacturer’s instructions. The quality of the reactions was tested by electrophoresis on a 1.5% agar gel. Subsequently 5 µl of each purified pooled sample was used for the indexing PCR using the Nextera XT Index Kit (Illumina) as per
manufacturer's instructions adding unique indices to each sample and all reactions were bead purified as above. Subsequently all samples were normalised to 10 nM and sequenced using MiSeq. Results were analysed using QIIME.

4.3 STATISTICAL ANALYSIS
All analyses were performed with the software SPSS 20.0 (IBM USA) for Windows and Excel. Descriptive statistics was used to summarize the data. Pearson's Chi-square test was used to evaluate categorical variables (qualitative variables), or Fisher's exact test where appropriate. Normally distributed data were analysed using t-test or analysis of variance (ANOVA) where appropriate. Non-normally distributed data were analysed using Mann-Whitney (2 sample) or Kuskal-Wallis (>2 sample) tests where appropriate. For all comparisons, P values < 0.05 were considered statistically significant. Results of PCR and sequencing were analysed using QIIME.

4.4 RESULTS

4.4.1 Demographic characteristics
There were 59 subjects (ABPA=16, SAFS=15, SANFS=11, mild asthma=6, NH=11; males=31 (52.5%), median age 49 (19-75) (Table 2.1, chapter 2). The duration of asthma was significantly longer in fungal-sensitised patients than those without fungal sensitisation group (41.7 yrs. versus 15.2 yrs.; p=0.049) (tables 2.1). Demographic characteristics are described in table 2.1.

4.4.2 Fungal culture results
A variety of pathogenic and non-pathogenic cultured microorganisms were observed. Fungal isolates were cultured in 17 (28.8%) of all patients; *Aspergillus* spp. (11%) and *Candida* spp. (11.9%) were the most common isolates (table 4.2, fig 4.2). A positive fungal culture was significantly more common in patients with severe asthma (SA+SAFS) than non-severe group (16 vs. 4, p=0.025); remarkably *Aspergillus* spp. were mostly abundant in SAFS, but less frequent in ABPA (table 4.2). Fungal colonised patients had worse FEV1 than non-colonised (p<0.001), a more airflow obstructive picture (p=0.001) and higher frequency of bronchiectasis (p=0.05) (table 4.2) whereas no fungal isolates were found in mild asthma and normal healthy patients, two (9%) of SANFS patients had positive fungal cultures in BAL (fig 4.1, table 4.1). These patients had normal total IgE and RAST for sIgE to all tested fungi were negative.
Table 4.2: Frequency of fungal isolates in BAL culture

<table>
<thead>
<tr>
<th>Isolate</th>
<th>All patients</th>
<th>ABPA (n=16)</th>
<th>SAFS (n=15)</th>
<th>SANFS (n=11)</th>
<th>MA (n=6)</th>
<th>NH (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No isolate</td>
<td>42 (71.2)</td>
<td>9 (56)</td>
<td>6 (40)</td>
<td>9 (81)</td>
<td>6 (100)</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>All isolates, n (%)</td>
<td>17 (28.8)</td>
<td>6 (37.5)</td>
<td>9 (60)</td>
<td>2 (9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Aspergillus sp, (%)</td>
<td>7 (11.9)</td>
<td>3 (18.8)</td>
<td>5 (33)</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Candida spp, n (%)</td>
<td>7 (11.9)</td>
<td>2 (12.5)</td>
<td>2 (13)</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Penicillium spp, n (%)</td>
<td>3 (5.1)</td>
<td>1 (6.25)</td>
<td>2 (13)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Table 4.2: Frequency of fungi isolates from BAL culture
SANFS=severe asthma-non fungal sensitised, MA=mild asthma, NH=normal healthy, ABPA= allergic bronchopulmonary aspergillosis;

Fig 4.2: Frequency of fungi isolates from BAL culture.

Fig 4.2: Frequency of fungal isolates from BAL in ABPA, SAFS and other severe asthma patients. ABPA= allergic bronchopulmonary aspergillosis; SAFS=severe asthma with fungal sensitization; SA=severe asthma (SANFS). Bars represent number/frequency of isolates in each subject group. No isolates were grown from mild asthma patients and healthy controls.
Fig 4.3: Fungal isolates from 2 patients with Severe Asthma-non fungal sensitised.

Macroscopic photograph from patient (A) showing colonies of A. fumigatus. Microscopic image of Penicillium spp cultured from patient (B). Both patients were SANFS as confirmed by negative SPT and sIgE. Patient (B) had multiple hospital admissions, frequent OCS use due to exacerbations. SANFS=severe asthma–non fungal sensitised; SPT=skin prick test; OCS=oral corticosteroids; sIgE=specific IgE.

Table 4.3: Comparison between fungal culture positive and fungal culture negative subjects.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Fungal culture positive (n=20)</th>
<th>Fungal culture negative (n=39)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL IL-17A (%), median (range)</td>
<td>77 (0.1-94)</td>
<td>23.2 (0-71.2)</td>
<td>0.01</td>
</tr>
<tr>
<td>PBMC IL-17A (%), median (range)</td>
<td>74.6 (42-95)</td>
<td>49 (0-93)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV1 (L), median (range)</td>
<td>1.51 (0.8-2.8)</td>
<td>2.6 (1.1-4.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV1%, median (range)</td>
<td>59 (31-86)</td>
<td>84.5 (66-89)</td>
<td>0.01</td>
</tr>
<tr>
<td>FVC (L), median (range)</td>
<td>3.1 (1.6-4.7)</td>
<td>4.24 (2.5-9)</td>
<td>0.03</td>
</tr>
<tr>
<td>FEV1/FVC, median (range)</td>
<td>56.5 (30-97)</td>
<td>75 (26.8-92)</td>
<td>0.001</td>
</tr>
<tr>
<td>Bronchiectasis, n (%)</td>
<td>17 (85%)</td>
<td>17 (33.6%)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 4.3: Comparison between fungal culture positive and fungal culture negative subjects. FEV1=forced expiratory volume in the first second; FVC=forced vital capacity; % pred= percentage of the predicted value; BAL=bronchoalveolar lavage; PBMC=peripheral blood mononuclear cells; BAL IL-17A= % of cells expressing IL-17A.
4.4.3 PCR and sequencing

Sequencing results revealed a greater diversity of microorganism than that determined by culture method (fig 4.3). One NH was reported of having pathogenic bacteria. *Haemophilus* spp. was present in all groups although they were mostly abundant in ABPA and SAFS (fig 4.5). In ABPA patients, a great number of *Pseudomonas* spp. were also observed. Pseudomonas and Haemophilus are significantly expanded in ABPA using mild asthma and healthy controls. *Veillonella* spp., *Streptococcus* spp. and *Prevotella* spp. were present in all groups. Plant pathogenic *Phoma* spp. was present in all groups except SA. A striking observation was the diversity of isolates in the A. fumigatus group. Tens and probably hundreds of variant strains were found in each pair of lungs sampled.

Fig 4.4: bacterial and fungal taxonomic variations from sequencing and QIIME analysis

Fig 4.4: Bacterial and fungal taxonomic variations from sequencing and QIIME analysis showing a wide variety of microbiome. Height of bar represents Levels of fungi or bacteria as assessed by read count. Note presence of organisms in disease and health.
Fig 4.5. Mycome and microbiome composition of BAL from individuals with allergic disease.

Aspergillus fumigatus, N. Fischeri, Candida spp, Unknown Basidiomycete, Unknown Dothidiomycete.

Fig 4.5: Myco-bio-me and microbiome composition of BAL from individuals with allergic disease. (A). Total fungal counts per sample, (B). Total fungal counts per sample limited to a maximum of 2000 reads counts. (C). Proportion of fungal species in each sample. (D). Proportion of bacterial species in each sample. (E). Total bacterial counts per sample limited to a maximum of 4000 reads counts. (F). Total bacterial counts per sample. HC - healthy control, SAFS - severe asthma with fungal sensitisation, SA - severe asthma, ABPA - allergic bronchopulmonary aspergillosis, MA - mild asthma. AF – antifungal therapy, red; never on therapy, green; currently on therapy, blue; previously on therapy. ST – steroid use, red; no current steroid therapy, green; current steroid therapy. AZ – azithromycin therapy, red; no current therapy, green; current therapy.
4.4.3.1 Aspergillus abundance

One striking thing is, Aspergillus spp. were mostly abundant in SA (SANFS) and SAFS patients and less in ABPA (fig 4.7). Interestingly, Aspergillus spp. were also present in MA patients and NH, but with lower frequency. A striking observation was the diversity of isolates in the A. fumigatus group. Tens and probably hundreds of variant strains were found in each pair of lungs sampled.

**Fig 4.6: Aspergillus number of reads**

![Aspergillus number of reads](image)

**Fig 4.6. Aspergillus read numbers showing largest number of reads in severe asthma (SA) patients.** SA=severe asthma-non fungal sensitised (previously refered to as SANFS in chapter 2). HC=healthy control; MA=mild asthma; SAFS=severe asthma with fungal sensitisation. ABPA=allergic brochopulmonary aspergillosis.
4.4.3.1 Effect of antifungal therapy

Antifungal therapy had a dramatic impact on both microbiome and mycobiome. Patients who were currently on antifungal therapy had lower fungal load but, significant increase in bacterial of both *Pseudomonas, Staphylococcus* and *Haemophilus* relative to untreated individuals. The group with previous antifungal therapy was more similar to those never treated with a small but significant expansion of Haemophilus. For the mycobiome antifungal therapy was associated with reduced proportion and count of *A. fumigatus*. 
4.4.3.3 Steroid therapy and fungal burden

In all diseases, steroid treatment significantly increased fungal and *A. fumigatus* count. This was most noticeable in SAFS and SANFS with a 37 fold increase in fungal counts in the steroid treated group (fig 4.8).

**Fig 4.7: Effects of steroids on fungal load**

![Graph showing fungal load with and without steroids by disease]

**Fig 4.7: Fungal and bacterial microbiome load with steroid treatment**

(A) Analysis in individual subject groups (yes = patients on OCS, no = patients not currently on OCS). (B) All patients grouped into two treatment groups (1 = not on OCS; 2 = currently on OCS). Dark green bar represents density (counts) of fungal load and light green represents density of bacteria. Presence of OCS as at the time of the study (BAL sampling).

SA = severe asthma-non fungal sensitised (SANFS)
Fig 4.8: Per-individual average levels of fungus grouped by antifungal and steroid treatment

Fig 4.8: Per-individual averaged levels of fungus grouped by antifungal and steroid treatment. CN, Current antifungal treatment and no steroid treatment. NN, no antifungal treatment and no steroid treatment. PN, previous antifungal treatment and no steroid treatment. CY Current antifungal treatment and steroid treatment. NY, no antifungal treatment and steroid treatment. PY, previous antifungal treatment and steroid treatment. All individuals with previous antifungal therapy have SAFS or ABPA. The level of fungus in PY is significantly higher than PN (p=0.021) and CY is significantly higher than CN (p=0.035).

4.5 DISCUSSION

Changes in the airway microbiome may be important in the pathophysiology of chronic lung disease. However, little is known about the microbiome population in patients with ABPA, SAFS and SANFS. The relationship between the microbiomes and disease activity remains understudied. In this study we observed a wide variety of pathogenic and non-pathogenic microorganisms from BAL in all groups of patients (health and disease). For many of these organisms, it is not clear which are pathogenic or non-pathogenic or allergenic or non-allergenic. To our knowledge, this is the first study that has assessed the microbiome composition in patients with fungal-associated asthma using both culture and molecular techniques.

The most striking finding in this study is the massive preponderance of *A. fumigatus* over all other fungi in asthmatic lungs, of whatever asthma severity. Healthy controls have a greater diversity of fungi when using non-culture techniques [372], but *A. fumigatus* is in the minority.
None of the mild asthmatics or healthy controls was on therapies that could influence their lung mycobiome or microbiome, and so these findings reflect the ‘natural state’ of Caucasians in the NW of England. Individuals with severe asthma, whether sensitised to fungi or not (as in SAFS), have a significantly increased proportion of *A. fumigatus* in their mycobiome. This is surprising and in marked contrast to healthy controls, and even ABPA and mild asthmatics. The implications of this finding are not known. One hypothesis might be that *A. fumigatus* plays an important, even critical, but unsuspected role in the pathophysiology of asthma.

The second striking observation is diversity of isolates in the *A. fumigatus* group. Tens and probably hundreds of variant strains were found in each pair of lungs sampled. Several questions arise from this; Do lungs of individuals with excess mucus (a characteristic feature of asthma) act as efficient spore traps and simply accumulate fungal spores or airborne hyphae over time? Does the excess mucus ‘protect’ *A. fumigatus* from phagocytic attack? Or is the recently published work on galactosaminoglycan and adherence to galectin-3 expressed on epithelial cells, imply that mucus is irrelevant, and the lungs of asthmatics are ‘super-sticky’ for *A. fumigatus*? In either scenario, at least some of the inhaled spores and fragmented hyphae probably germinate forming mycelia and allergens in the airways [373]? Observations of mycelia intermingled with eosinophils and neutrophils in plugs of sputum from asthmatics do not distinguish between these different scenarios. It is not clear from our experiments whether the fungal nucleic acid detected represents live or dead mycelium or spores, but it is clear from other work that current culture methods for *A. fumigatus* are grossly sub-optimal [374][357], and so this question is challenging to answer currently. Our comparison of *Aspergillus* spp. PCR signal intensity based on Ct values and the number of *A. fumigatus* reads by sequencing was good, indicative of reliable quantification.

Other remarkable observations include very little *Candida* spp on sequencing, despite being frequently cultured in respiratory secretions, a substantial quantity of *A. fisherianus*, a sibling species to *A. fumigatus*, that is not pathogenic but may be allergenic, and substantial numbers of unknown species in the Dothidiomycete Class (Ascomycetes) and Phylum Basidiomycota. While a few allergens are described from individual species from the Basidiomycota [375, 376], and some occupational ‘allergic’ syndromes such as mushroom pickers’ lung are known, it is not clear what role such organisms play may play in asthma.
4.5.1 Fungal culture and asthma

The frequency of fungal isolates from patients with fungal lung disease varies depending on the nature of the sample, sampling, techniques used and exposure to anti-microbial agents. It has been proposed that about 60% asthmatics sensitised to A. fumigatus have Aspergillus growing in their sputum [377]. However, the range of fungi that may colonize the airways in asthma is not precisely known. In this study we found that seventeen (28.8%) of all patients were colonised with fungi, with Aspergillus spp. (11%) and Candida spp. (11.9%) being the most common cultured fungal isolates (table 4.1, fig 4.3). Our data is comparable to an earlier study by Fairs et al [378]. In their study to determine the range of filamentous fungi isolated from sputum of 126 asthma patients (94% moderate-severe, 18 healthy), Fairs and colleagues (2012) observed twenty-seven different taxa of filamentous fungi, with more than one species being detected in 17% compared with 3 (17%) healthy controls culturing any fungus (P < 0.01). These workers observed that Aspergillus species were most frequently cultured (usually in isolation) followed by Penicillium species. In our study, the most common fungi were Aspergillus and Candida spp. The high frequency of Candida species in our data may reflect a high frequency of antibiotics and steroid use in our patient population.

Our findings also support previous findings by Hilty et al [379] and Huang YJ [360] in asthma. In a study to evaluate the composition of the lung microbiome of asthma subjects compared to normal healthy controls, Hilty and colleagues found increased frequency of Proteobacteria when compared to controls [379]. In APBA patients a greater number of Pseudomonas spp. were observed than in other subjects. This could be explained by the presence of bronchiectasis in all our ABPA patients as Pseudomonas colonisation is common in patients with bronchiectasis.

4.5.2 Higher microbiome population in SAFS compared to ABPA

It is notable that ABPA patients had much less A. fumigatus in their airways compared with SAFS patients and those with severe asthma when evaluated by either culture or molecular techniques. This was unexpected. It partly reflects a higher frequency of antifungal therapy in these patients, but even within the ABPA group, a higher diversity of fungi were found, implying some real differences in the microbiomes in different groups of asthmatics. Further, there was more similarity between SAFS and SANFS, consistent with the suggestion that SAFS is simply a variant form of severe asthma with disproportionate sensitivity to fungi. Host genetic linkage to these different asthma endotypes (or phenotypes), will be required to
further address the differences. One other plausible explanation for this unexpected finding would be that concomitant antifungal therapy may have affected our results as 47.1% of ABPA and 9% of SAFS were on antifungals at the time of the study (table 2.1).

The isolation of fungi in patients with ABPA and SAFS supports the rationale for using antifungal agents in this group of patients and probably explains the clinical response observed in previous interventional studies [104, 232, 236, 237].

4.5.3 Fungal culture positivity and asthma severity
In this study, we found that the number of fungal culture isolates were significantly more common in patients with severe asthma than non-severe group (16 vs. 4, \( p=0.025 \)). Moreover, patients with positive fungal cultures had significantly lower lung function compared with culture negative subjects (table 4.3). There are no previous studies that have specifically assessed the relationship between fungal colonisation and lung function in patients with SAFS or ABPA. However, our findings support previous proposals that a positive airway fungal culture is associated with an impaired FEV1 and asthma severity (Agbetile, Fairs et al. 2012). In a study of 126 asthma subjects with moderate-severe disease (94%) and 18 NH by Agbetile and colleagues, post-bronchodilator FEV (L) (\% predicted) was significantly lower in subjects with a positive fungal culture compared to culture-negative subjects (71% vs. 83% \( P< 0.01 \)) [380]. Indeed, there is a link between microbiome population and AHR [343]. In the COPD population, airway fungi positivity has been linked with severity of COPD and frequency of exacerbations as seen in the FUNGI-COPD study in Spain [381].

Interestingly, we found that two (9%) of these patients had positive fungal cultures in BAL (fig 4.1, table 4.1). It is possible that there is probably a subgroup of severe asthmatics who though may have airway fungal infection or colonisation, but do not mount a Th2 response.

Few studies have reported the link between microbiome population and severity of airways disease. In COPD patients, microbiome diversity is linked with severity of airflow obstruction and severity of COPD [382]. Thus, the wide range of bacteria observed in BAL of SANFS, SAFS and ABPA subjects may partly explain the improvement in lung function observed after macrolide antibiotic therapy previously attributed to reduction in bronchial infection by specific bacteria.
4.5.4 Culture methods compared to culture-independent techniques (qPCR + sequencing)

In this study, sequencing results demonstrated great diversity of lung microbiome involving both pathogenic and non-pathogenic bacteria and fungi (fig 4.3). The microbiome population and diversity was higher in molecular methods compared to culture techniques. This finding supports previous findings by Fraczek et al (2014). These workers compared fungal yields from respiratory specimens and Aspergillus quantitative real time polymerase chain reaction (qPCR) and found that qPCR is much more sensitive than culture [356].

Although the lungs of healthy subjects were sterile when examined by culture-based techniques, this was not the case when molecular techniques were deployed (fig 4.1, 4.2). Our findings support previous findings and suggestions that the lungs, historically considered sterile in health, contains diverse communities of microbes (2013) [383]. It is clear from other work that current culture methods for A. fumigatus are grossly sub-optimal may underestimate fungal prevalence [353].

This result has clinical and research significance. The next generation sequencing approach to microbiome determination provides high resolving power and quantification for both bacterial and fungal populations. With appropriate adjustment of assignment algorithms the technique can assign fungal species with high accuracy. However fungal databases for such analysis lag somewhat behind bacterial databases and some desirable forms of statistical analysis are unavailable for the fungal databases. Additionally for fungi there are known weaknesses in determining taxonomy on the basis of 18S sequence alone.

UCLUST is a flexible program that is a high-performance clustering, alignment and search algorithm used in molecular biology that is capable of handling millions of sequences [384]. In this study, we initially failed to identify any archeal spp. It was found that significant errors occurred in the fungal microbiome analysis using UCLUST algorithm due to misalignment of short sequence matches to some unknown Ascomycete genera. The assignment algorithm was changed to BLAST and used with successively higher E-value cut offs until the spurious alignment was eliminated (E-50 cut off).

Another problem of the current molecular techniques is the difficult to distinguish between live microbiome or dead organisms which make it difficult to assign organisms fully to symptoms or disease. It is not clear from our experiments whether the fungal nucleic acid detected represents live or dead mycelium or spores. Nevertheless, fungal ITS1 is highly
divergent in fungi and capable of distinguishing between isolates of *A. fumigatus* which allows some resolution of the question of whether the lung is simply an efficient spore trap or whether colonisation arises from a single inoculum; multiple strains are the rule, at least in the context of asthma and healthy people.

### 4.5.5 Effects of steroids on fungal load

Inhaled corticosteroids are commonly administered to asthmatics. Those with severe asthma, and those with ABPA, receive intermittent or continuous oral corticosteroids. We found that oral corticosteroids without antifungal therapy substantially increased the total fungal load in the lungs. This result mirrors that in COPD patients based on sensitive culture techniques and high dose inhaled corticosteroids [338, 381]. Corticosteroids impair monocyte, macrophage and neutrophil phagocytosis of *A. fumigatus*. If the unproven assumption that the fungal load of *A. fumigatus* reflects allergen load, then this has important implications for management. *A. fumigatus* produces ~60 IgE binding proteins, including the ~20 approved allergens, so the potential for immune stimulation in the airways is massive, especially as corticosteroids have minimal impact on the TH17/IL17 immune axis.

### 4.5.6 Effects of antifungal treatment

Antimicrobial treatment has profound effects on the microbiome. As expected, antifungal therapy decreases the level of fungus in the lung. However it is striking that the level in individuals who have stopped antifungal therapy is actually significantly higher than in untreated individuals. Further, while oral azole antifungal therapy greatly reduces fungal load, and in particular *A. fumigatus* load, this suppression is not complete. This could imply antifungal resistance, which is demonstrable by molecular means in the absence of culture [358], might represent dead and dying fungi, ungerminated spores [385] or fungal cells sequestered inside biofilms, unavailable to azole attack. Individuals prescribed antifungals have worse asthma linked to fungal allergy in our practice and it is of great interest that they develop higher fungal loads in their lungs when they stop therapy in comparison to those who have never had therapy. This implies a direct relationship between fungal load and asthma control and symptoms.
4.6 LIMITATIONS
The low specificity of culture techniques and concomitant antifungal therapy may have affected my results as 50% of ABPA and 9% of SAFS were on antifungals at the time of the study (table 2.1).

Differences in sampling may have also contributed significantly to experimental variability, and the results from non-culture technique did not coalesce around standard ways to perform statistical comparisons. However, this emerging and exciting field of investigation is likely to lead to new ways of thinking about the lung and about lung disease.

The observation of increased proportions of *A. fumigatus* in asthma suggests a number of implications for treatment or aetiology of this disease. Our study was not large enough to distinguish all known asthma endotypes [81], but did include SAFS, not yet accepted as an asthma endotype. Partly for this reason, we relied on BTS scores for asthma severity to distinguish the asthma groups, as well as established definitions of ABPA. Further study is needed to distinguish associations with different asthma phenotypes and with more subtle difference in asthma severity. One obvious course of enquiry is to determine whether antifungal therapy has an effect on disease in this context.

4.7 CONCLUSIONS
Our findings suggest that the airways of patients with SANFS, SAFS and ABPA are colonised/infected by a wide diversity of organisms. Fungi and bacteria are probably more common in severe asthma population than previously thought and microbial population may be linked with asthma severity and poor lung function in ABPA and SAFS including SANFS patients. The non-culture molecular technique (qPCR) is more sensitive tool than the current HPA culture method. The use of steroids increases fungal burden in the lungs and should be considered carefully before being prescribed in these patients. Whereas, antifungal therapy has a dramatic impact on both microbiome and mycobiome, they appear to significantly increase both *Pseudomonas, Staphylococcus* and *Haemophilus* relative to untreated individuals. Further studies need to be conducted to determine the causal effect relationship and to distinguish between pathogenicity and colonisation.
ACKNOWLEDGEMENTS

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I would also like to acknowledge the external company in Liverpool that performed the sequencing work.
CHAPTER 5

Voriconazole and posaconazole improves asthma severity in Allergic Bronchopulmonary Aspergillosis and Severe Asthma with Fungal Sensitization

(Chishimba L. J Asthma. 2012 May;49(4):423-33)
ABSTRACT

Rationale and Objectives: Severe asthma with fungal sensitization (SAFS) and Allergic bronchopulmonary aspergillosis (ABPA) are progressive allergic fungal lung diseases whose effective treatment remains to be established. Current treatment with itraconazole is associated with a 40% failure rate and adverse events (AEs). We assessed the efficacy and safety of voriconazole or posaconazole as second and third line therapy.

Methods: We conducted a retrospective audit of efficacy and safety voriconazole or posaconazole therapy in adult asthmatic patients with either ABPA or SAFS. Clinical, radiological and immunological evaluation was used to assess response. We used the GINA criteria for asthma control and the BTS criteria for classifying asthma severity.

Results: There were 25 patients, ABPA (n = 20) or SAFS (n = 5), 10 males, median age = 58 yrs. All patients had failed itraconazole (n=14) or developed adverse events (AEs) (n=11). There were 33 courses of therapy analyzed, 24 with voriconazole and 9 with posaconazole. Clinical response to voriconazole was observed in 17/24 (70%) at 3 months, 15/20 (75%) at 6 months and 12/16 (75%) at 12 months, compared to 7/9 (78%) at 3, 6 and 12 months for posaconazole. Eighteen of 24 (75%) discontinued oral corticosteroids, 12 of them within 3 months of therapy. Asthma severity was downgraded from severe (BTS step 4/5) to moderate (step 2/3) (n=8) and moderate to mild (step 1) (n=1) asthma in 9 of 24 (38%) asthmatic patients. There was a marked reduction in OCS and SABA use, health care utilization due to asthma and improvement in overall health status. Furthermore, there was a statistically significant reduction in immunological markers appearing at 9 months (p=0.008) for total IgE and at 12 months for RAST IgE Aspergillus fumigatus (p=0.0056). Six of 23 (26%) patients on voriconazole had AEs requiring discontinuation before 6 months compared to none on posaconazole (p=0.15). Four relapsed (57%), one at 3 months and 3 at 12 months after discontinuation.

Conclusion: Both voriconazole and posaconazole are potentially effective alternative treatment options for SAFS and ABPA and may improve asthma control and reduce severity, though larger prospective studies are required to support these retrospective study findings.

Word count: 324

Key words: Allergic fungal pulmonary disease, Aspergillus, severe asthma, asthma control, fungal sensitisation, treatment.
5.1 INTRODUCTION

In recent years, there has been an increasing interest in the treatment options of allergic fungal airway diseases. Allergic bronchopulmonary aspergillosis (ABPA) and severe asthma with fungal sensitization (SAFS) are slowly progressive, debilitating allergic fungal diseases that affect the respiratory tract in asthma. The past decade has seen several studies of antifungal treatment improve patient quality of life, reduce corticosteroid requirements and may prevent lung damage.

The link between asthma severity and fungal allergy is strong [316, 386, 387]. Indeed sensitisation to fungal allergens is associated with the risk of severe asthma exacerbations requiring multiple hospital and intensive care admissions [136, 388].

ABPA is the most frequently recognized manifestation of allergic aspergillosis, with three published case series estimating that 0.7 - 3.5% of the referred asthma population have ABPA [305, 306]. It is characterized by exaggerated Th2 CD4+ allergic inflammatory response to A. fumigatus allergens in the bronchial airway of atopic asthma and CF patients when A. fumigatus spores are inhaled and germinate in bronchial mucus releasing allergens including proteases.

The clinical and pathological course of ABPA is variable including recurrent exacerbations or chronic persistent symptoms [153]. The disease may remit temporarily but is more commonly a progressive, unremitting disorder and untreated patients have a chronic course characterized by recurrent pulmonary consolidation and in many cases, progression to bronchiectasis or pulmonary fibrosis [153], bronchiolitis obliterans [154], granulomatous bronchiolitis [154], lung destruction [155], chronic cavitary aspergillosis (CPA) [156] and in rare cases, pleural effusion [157].

In recent years, a new asthma variant called ‘SAFS’ has been described by Denning & Niven et al [316]. These patients have severe asthma, are sensitized to one or more fungi but have normal or only slightly elevated total IgE concentrations [316]. The clinical and diagnostic manifestations of SAFS are thought to arise from an allergic response to multiple antigens expressed by A. fumigatus and possibly other fungi, colonizing the bronchial mucus [139].

Traditionally, treatment for allergic fungal diseases involves the use of systemic oral corticosteroids (OCS) [223-228]. Despite good efficacy, the chronic use of OCS has a number of adverse effects. In SAFS, even high dose, prolonged OCS courses may be only moderately successful. Similarly, in some asthmatics, corticosteroids do not prevent the development of ABPA, and that patients who develop the syndrome while on corticosteroids
may have a protracted course with poor response to the usually effective doses of corticosteroids [225]. In addition, the use of corticosteroids increases airway fungal burden in these patients (chapter 4 of this thesis).

5.1.1 Itraconazole

Since the introduction of itraconazole, the first oral antifungal agent with activity against *Aspergillus* species in 1991, some efficacy in ABPA has been documented [66, 229-232]. Randomized controlled trials show that treatment with oral itraconazole offers therapeutic benefit to approximately 60% of patients [234-236]. A marked beneficial effect on quality of life and other end points have been demonstrated among patients with SAFS treated with itraconazole [236, 237]. A retrospective study by Pasqualotto et al in 2009 showed that itraconazole therapy for 6-12 months in both SAFS and ABPA is beneficial by reducing immunological markers (total IgE and specific IgE for *A. fumigatus*), eosinophil count (P = 0.037), dose of oral steroids (P = 0.043), the number of courses of systemic steroids required by 57.4% (P = 0.041) and improvement in lung function (median FEV1 increased by 190 ml; P = 0.016) at 6 months although there were no statistically significant changes in these key variables at 12 months therapy compared with baseline [236].

However, a major problem with the use of itraconazole is the high failure rate and frequent adverse events (AEs) of around 39% of cases treated [389]. Such adverse events include nausea and vomiting (approx. 10%) diarrhea and flatulence (2%), constipation hyperlipidemia (1-9%), hypokalemia (6%) and liver enzyme elevations (2-7%) [238, 389], peripheral edema [237] and peripheral neuropathy [390].

Another major problem with itraconazole is the drug to drug interaction which normally results from its inhibition and sometimes inhibition of liver enzymes (chapter 1 of this thesis-sub-section 1.7.1). Most importantly, adrenal suppression (44%) especially when combined with some ICS is recognized [391]. Itraconazole is an inhibitor of cytochrome P450 3A4 [240, 392]. It increases exposure to methylprednisolone [245, 393], dexamethasone [392], budesonide [244] and probably fluticasone [394], but not prednisolone [245] or endogenous cortisol. Reversible adrenal suppression with inhaled corticosteroids and itraconazole in 50% of patients is a real concern [235]. No published study has addressed any interaction of itraconazole and beclomethasone. Occasionally, itraconazole has been associated with heart failure [395].

The optimal duration of therapy is unclear, given that short term (8 month) efficacy only has been demonstrated for itraconazole [234] and there are unanswered questions about the
safety of prolonged use of this therapy [389]. Furthermore, there is not yet any reliable evidence that this treatment ameliorates any progressive lung damage [234].

Recent developments have led to a renewed interest in finding more potent therapy. Some emerging data from studies of ABPA in cystic fibrosis indicate that voriconazole may be a useful adjunctive therapy for ABPA in CF [247, 248]. Little is known about the response rates or appropriateness of alternative antifungal treatment for those with ABPA or SAFS. In this study, we assessed the effect of voriconazole or posaconazole as second and third line therapy in SAFS and ABPA in asthmatics attending the National Aspergillosis Centre.

5.2 HYPOTHESIS
We hypothesized that both voriconazole and posaconazole would be effective and safe in SAFS and ABPA.

5.3 AIMS
The objectives/aims of this study were to assess the effect of voriconazole or posaconazole as second and third line therapy in SAFS and asthmatic ABPA patients.

5.4 METHODS

5.4.1 Study design and setting
We conducted a retrospective audit to assess the efficacy and safety of voriconazole or posaconazole. The audit population was 26 adults with ABPA (n= 21) and SAFS (n=5), all cases treated with either antifungal agent at National Aspergillosis Centre (NAC), based at the University Hospital of South Manchester, UK. Information from case notes, laboratory results, and radiological investigation were entered into an ACCESS database. A detailed review of all chest radiological images was made through PACS (Picture Archiving and Communication System).

2.5.1.1 Power calculations
It wasn’t possible to do power calculation because the numbers were too small in this retrospective study.
5.4.1.1 Ethical statement
This study was an audit and as such was considered as service evaluation and didn’t require ethical approval.

5.4.2 Inclusion criteria: All patients had previously received itraconazole but were discontinued because of either adverse events (peripheral neuropathy \(n=2\)), heart failure \(n=1\), hepatotoxicity \(n=3\), profound adrenal suppression \(n=2\), persistent nausea and vomiting \(n=5\) or lack of clinical efficacy \(n=11\), low serum concentrations \(n=1\) or itraconazole resistance in \(A. fumigatus\) \(n=2\).
The diagnostic criteria for ABPA included: i) asthma (of any severity), ii) total serum IgE \(\geq 1000\) KU/L, iii) immediate cutaneous reaction to \(A. fumigatus\) of \(> 3\) mm compared to control or iv) elevated \(A. fumigatus\)-specific serum IgE levels, v) precipitating antibodies to \(A. fumigatus\) in the serum, vi) a history of pulmonary infiltrates (transientor fixed), vii) central bronchiectasis (CB), viii) a history of expectoration of brown plugs or flecks and ix) isolation of \(A. fumigatus\) from sputum \([150, 151]\), of which ii) and iv) were essential.
Severe asthma was defined according to the BTS criteria (treatment steps 4 or 5) \([70]\). We used published criteria for the diagnosis of SAFS which included i) severe asthma, ii) total IgE <1000 KU/L and iii) positive skin test or raised specific IgE to any fungus.

Treatment and response
Voriconazole (300-600 mg/day) or posaconazole (800 mg/day) (adjusted by plasma level monitoring) was given for at least 6 months, if tolerated. The target plasma levels used were those used for invasive and chronic pulmonary aspergillosis, namely voriconazole pre-dose 1.3-5.7 mg/L \([396]\) and posaconazole random \(>0.7\) mg/L after steady state reached \([397]\).
Some patients received both agents at separate times, and each course is evaluated separately. Courses of therapy of at least 4 weeks were evaluated for efficacy \(n=34\) as done previously in the FAST study \([36]\). At least one spirometry measurement was done.
We evaluated response in clinical, immunological, lung function, peripheral blood eosinophilia, or radiological features. Among these improvement parameters, clinical improvement was considered as the most important parameter.

Clinical improvement was present if there was an improvement in symptoms and/or overall asthma control as defined by the GINA criteria that included reduction in dosage and courses of oral steroids (OCS), dosage of inhaled steroids (ICS), exacerbations, intercurrent infections, daytime or nocturnal symptoms; reduction in number of hospitalizations or emergency room visits for respiratory diseases, improvement in exercise tolerance, lung
function (FEV1), increase in energy or improvement in overall quality of life as defined by the patients perception of symptoms. Symptoms of breathlessness, cough, wheeze, chest tightness and nocturnal awakening and other clinical parameters were evaluated by the attending clinicians at each hospital attendance and retrospectively tabulated by the authors during the study. Each symptom was scored from 0 to 3 (0 = none, 1 = occasionally or mild, 2 = most of the time or moderate, 3 = all the time or severe. A patient was considered to have had symptomatic improvement if there was improvement in more than 50% of his/her overall total symptoms at the time of assessment by the time of assessment for the time point evaluated.

**Immunological improvement** was present if there was reduction in fungal serology as demonstrated by total IgE or RAST IgE concentrations.

**Radiological improvement** was considered present if there was any improvement in the extent of any of the abnormalities that included bronchiectasis (severity and/or distribution), fibrosis, consolidation, mucus plugging, lobar collapse, pulmonary nodules and/or cavitations. All image reports and images on PACS were reviewed. If any conflicting data or report was found, further review by one of 2 senior specialist chest radiologists was sought.

**Clinical deterioration** was considered present if intercurrent infection was absent but respiratory and constitutional signs and symptoms were progressive or there was a decline in asthma control as defined by the GINA criteria [42, 329, 398].

**Radiological deterioration** was considered present if i) increased severity of bronchiectasis, ii) appearance of new pulmonary nodules, cavitations, collapse etc, iii) increased size and severity of existing radiological abnormalities, iv) progressive pulmonary fibrosis, and/or v) progression to CCPA/CPA.

**Treatment failure** overall was defined by a lack of response, using the criteria above for response. Stability was defined as lack of deterioration or improvement.

**Relapse** after discontinuation of therapy was defined by deterioration in the same features conferring response after an initial response.

### 5.2.3 Data collection

Clinical data from 3- 6 months prior to the commencement of voriconazole or posaconazole (visit -1) were extracted from the clinic notes, and again collected at the start of treatment (visit 0), after 3, 6, 12 and 18 months of therapy. The data collected at visit -1 included reasons for discontinuing itraconazole, co-morbidities, weight, symptoms, spirometry and
immunological markers. This data was again collected at each subsequent visit including the number of hospitalizations, GP (general practitioner) or emergency room visits, exacerbations and dose of steroids, therapeutic drug levels (TDM), adverse effects and any new radiological findings. For the purpose of this study, GP records were not sought. Prednisolone was used as the reference steroid when comparisons of OCS were required. Similarly, beclomethasone dipropionate (BDP) equivalent dose was used as reference for ICS. Common ICS used in our centre include BDP, budesonide (1:1.5 dose equivalents) and fluticasone (1:2 dose equivalents with BDP).

Duration of therapy was calculated at the minimum time period, i.e. patients who had received less than 6 months of antifungal therapy at the time of this study were evaluated at 3 months only, those who had received between 6–12 months were evaluated up to 6 months etc.

5.3 STATISTICAL ANALYSIS
All analyses were performed with the software SPSS 15.0 for Windows and excel statistics. Descriptive statistics were used to summarize the data. To compare continuous variables before and after antifungal therapy, non-parametric Wilcoxon signed rank T test for related samples was used. For all comparisons, p values ≤0.05 were considered statistically significant.

5.6 RESULTS
5.4.1 Patient characteristics
There were 25 patients included in the study, 20 (80 %) of whom had ABPA and 5 (20 %) had SAFS (Table 5.1). Of all patients ten (40 %) were male and the median age was 58 yrs (range 20 – 80). ABPA patients had a higher mean age compared to SAFS (60 vs. 54 years).

Itraconazole had been given to all patients for a duration of between 6 months to 10 years previously but was discontinued because of lack of efficacy (n=11, 42%) or adverse events (n=12, 46%), persistently low serum concentrations (n=2, 7.8%) or itraconazole resistance in A. fumigatus (n=2, 7.8%). Demographic variables and co-morbidity prevalence are listed in table 5.1.
Courses of therapy
There were 33 courses of therapy analyzed, 24 with voriconazole (ABPA=19, SAFS=5) and 9 with posaconazole (all ABPA) (Table 5.1). Antifungal therapy with voriconazole was modified to posaconazole in 8 (32%) patients. Reasons for switch-over in these patients were side effects (n=5) or lack of response (n=3). Only one posaconazole course was not preceded by voriconazole due to in vitro resistance in *A. fumigatus*. The mean treatment duration of voriconazole was 16.7 months (range 2-48 months, SD=14.6) and 16.3 months (range 3-36 months, SD=14.7) for posaconazole. None of the SAFS patients received posaconazole.

Radiology
Of all patients, 21 (80.1%) had baseline chest radiology done at the start of therapy either chest radiography alone (n=6, 28.6%), computed tomography (CT) (n=7, 26.9%) or both (n=8, 38%). Bronchiectasis was the most common radiological finding (n=17, 71.4%). Other common radiological findings are listed in table 4.1. Two (9.5%) of the 21 cases of ABPA analyzed had no bronchiectasis while one (20%) of the 5 cases of SAFS had mild bilateral lower lobe bronchiectasis.
Table 5.1: BASELINE DEMOGRAPHIC CHARACTERISTICS OF PATIENTS AND COURSES OF THERAPY (ABPA AND SAFS)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Patients (n=26)</th>
<th>ABPA (n=20)</th>
<th>SAFS (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All ABPA patients (n=20)</td>
<td>Voriconazole (n=19)</td>
</tr>
<tr>
<td>Demographic characteristic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years, (range)</td>
<td>57 (20 – 80)</td>
<td>60 (41-80)</td>
<td>61 (47 – 69)</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>11 (42)</td>
<td>8 (40)</td>
<td>11 (34)</td>
</tr>
<tr>
<td>Bronchiectasis, n (%)</td>
<td>16 (62)</td>
<td>12 (60)</td>
<td>13 (56)</td>
</tr>
<tr>
<td>Asthma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe, n (%)</td>
<td>18 (69)</td>
<td>12 (60)</td>
<td>17 (73.9)</td>
</tr>
<tr>
<td>Moderate, n (%)</td>
<td>2 (7.6)</td>
<td>2 (10)</td>
<td>2 (8.6)</td>
</tr>
<tr>
<td>Mild, n (%)</td>
<td>3 (11.5)</td>
<td>3 (15)</td>
<td>3 (13)</td>
</tr>
<tr>
<td>No asthma, n (%)</td>
<td>2 (7.6)</td>
<td>2 (10)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Current PSA, n (%)</td>
<td>3 (11.5)</td>
<td>3 (15)</td>
<td>2 (8.6)</td>
</tr>
<tr>
<td>Previous triazole therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itraconazole, n (%)</td>
<td>26 (100)</td>
<td>26 (100)</td>
<td>23 (100)</td>
</tr>
<tr>
<td>Voriconazole, n (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patients with baseline radiology, n (%)</td>
<td>21/26 (80.8)</td>
<td>16/20 (80)</td>
<td>19/23 (82.9)</td>
</tr>
<tr>
<td>Normal, n (%)</td>
<td>5/21 (23.8)</td>
<td>5/16 (31)</td>
<td>5/19 (23.9)</td>
</tr>
<tr>
<td>Bronchiectasis, n (%)</td>
<td>15/21 (71.4)</td>
<td>11/16 (68.7)</td>
<td>13/19 (68.4)</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Lobar collapse, n (%)</th>
<th>5/21 (23.8)</th>
<th>5/16 (31)</th>
<th>5/19 (26.3)</th>
<th>1/7 (14.3)</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper lobe fibrosis, n (%)</td>
<td>8/21 (38)</td>
<td>7/16 (43.7)</td>
<td>7/19 (36.8)</td>
<td>3/7 (42.9)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Pulmonary Nodules, n (%)</td>
<td>4/21 (19)</td>
<td>3/16 (19)</td>
<td>4/19 (21.1)</td>
<td>0</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Mucus impaction, n (%)</td>
<td>2/21 (9.5)</td>
<td>2/16 (12.5)</td>
<td>2/19 (10.5)</td>
<td>0</td>
<td></td>
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<tr>
<td>Immunology findings</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IgE, median (range) (kU/mL)</td>
<td>1,221 (64-7,200)</td>
<td>1,970 (550-7,200)</td>
<td>2,016 (64-2,500)</td>
<td>1,585 (610-2,500)</td>
<td>357.3 (76-760)</td>
</tr>
<tr>
<td>IgE Aspf, median (range) (KU/ml)</td>
<td>26.5 (0.6-64.8)</td>
<td>51 (7.3-76.1)</td>
<td>42.7 (7.3-76)</td>
<td>30 (22.7-54.2)</td>
<td>7.56 (1-20)</td>
</tr>
<tr>
<td>Spirometry</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Median FEV1, L (range)</td>
<td>(0.9-3.0)</td>
<td>1.65 (0.6-2.9)</td>
<td>1.6 (0.6-2.9)</td>
<td>1.7 (1.6-1.8)</td>
<td>1.47 (0.84-2.15)</td>
</tr>
<tr>
<td>Median FEV1% predicted (range), n</td>
<td>58.7 (20.7-92.7)</td>
<td>61.7 (20.7-92.7)</td>
<td>56.5 (27.8-92.8)</td>
<td>67 (59.7-86.9)</td>
<td>52.8 (27.8-75)</td>
</tr>
<tr>
<td>Median FVC, L (range) (n)</td>
<td>2.89 (1.8-4.4)</td>
<td>2.7 (1.8-4.4)</td>
<td>2.8 (1.52-4.2)</td>
<td>2.4 (2.3-2.6)</td>
<td>2.95 (1.52-4.2)</td>
</tr>
<tr>
<td>FEV1/FVC% (range) (n)</td>
<td>58.3 (28.3-77.3)</td>
<td>60.1 (28.4-77.3)</td>
<td>60.1 (28.4-77.3)</td>
<td>69.7 (62.0-77.3)</td>
<td>52.16 (28.4-69.4)</td>
</tr>
</tbody>
</table>

Table 5.1: BASELINE DEMOGRAPHIC CHARACTERISTICS BY PATIENTS AND COURSES OF THERAPY (ABPA AND SAFS).

*PSA=pseudomonas aerogenosa; IgEAspf=Specific IgE against Aspergillus fumigatus; KU/ml=kilo units per ml;*
5.4.2 Response to therapy

Clinical response at 3, 6 and 12 months of voriconazole or posaconazole treatment is summarized in Table 5.2 and 5.3. Overall clinical improvement to voriconazole treatment was observed in 17 (68%) (ABPA=13, SAFS =4) of 25 at 3 months, 15 (75%) of 20 at 6 months and 12 (70.6%) of 17 at 12 months, compared to 7 of 9 (78%) at 3, 6 and 12 months for posaconazole (table 5.2). On the basis of clinical parameters, treatment failure to voriconazole was observed in one of 20 (5%) at 3 months, none of 15 (0%) at 6 months and two of 15 (15%) at 12 months. No treatment failure was observed with posaconazole.

Table 5.2: Overall clinical response to therapy

<table>
<thead>
<tr>
<th></th>
<th>Clinical outcome of courses of therapy (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>3 months</td>
</tr>
<tr>
<td>ABPA</td>
<td></td>
</tr>
<tr>
<td>Voriconazole</td>
<td></td>
</tr>
<tr>
<td>Improved</td>
<td>13/20 (65)</td>
</tr>
<tr>
<td>Stable</td>
<td>2/20 (10)</td>
</tr>
<tr>
<td>Failure</td>
<td>1/20 (5)</td>
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<tr>
<td>Discontinued (AEs)</td>
<td>4/20 (20)</td>
</tr>
<tr>
<td>Posaconazole</td>
<td></td>
</tr>
<tr>
<td>Improved</td>
<td>7/9 (78)</td>
</tr>
<tr>
<td>Stable</td>
<td>2/9 (22)</td>
</tr>
<tr>
<td>Failure</td>
<td>0/9</td>
</tr>
<tr>
<td>Discontinued (AEs)</td>
<td>0/9</td>
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<tr>
<td>SAFS</td>
<td></td>
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<tr>
<td>Voriconazole</td>
<td></td>
</tr>
<tr>
<td>Improved</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td>Stable</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Failure</td>
<td>0/5</td>
</tr>
<tr>
<td>Discontinued (AEs)</td>
<td>0/5</td>
</tr>
</tbody>
</table>

Table 5.2: Overall clinical response to therapy at different times on treatment by disease groups. AEs = adverse events; ABPA=Allergic bronchopulmonary aspergillosis; SAFS=severe asthma with fungal sensitisation. () indicate %.
5.4.3 Asthma control and health related outcomes

There was a marked reduction in OCS and SABA use, health care utilization due to asthma and improvement in overall health status as subjectively perceived by individual patients in domains such as physical wellbeing, functioning, energy, increased exercise tolerance and reduction in patients’ overall symptoms (table 5.3).

There was a direct correlation between OCS use and other clinical parameters of clinical response.

Although the dosage of ICS was reportedly modified during antifungal therapy in some patients, it was difficult to quantify retrospectively, and overall was probably not markedly reduced.

There were 10 patients who had had frequent hospital admissions at the start of voriconazole therapy and no admissions were observed in 9 of them (90%) at 3 months. This benefit was maintained at 6 and 12 months of therapy. Voriconazole therapy reduced the frequency of recurrent chest infections or acute exacerbations in 17 of 24 (70%), 9 of 19 (47%) and 9 of 17 (52.9%) at 3, 6 and 12 months respectively (Table 5.3). Posaconazole therapy reduced the frequency of chest infections or acute exacerbations in 7 of 9 (78%) at 3 months and throughout the 12 months period (Table 5.3).
Table 5.3: Improvements in clinical parameters during voriconazole and posaconazole therapy at 3, 6 and 12 months (ABPA and SAFS patients combined)

<table>
<thead>
<tr>
<th>Clinical or healthcare utilization feature</th>
<th>3 months (%)</th>
<th>6 months (%)</th>
<th>12 months (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vori (n=25)</td>
<td>Posa (n=9)</td>
<td>Vori (n=19)</td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction in cough frequency (%)</td>
<td>17/24 (70)</td>
<td>7/9 (78)</td>
<td>15/19 (78)</td>
</tr>
<tr>
<td>Reduction in breathlessness (%)</td>
<td>10/24 (41)</td>
<td>5/9 (56)</td>
<td>12/19 (63)</td>
</tr>
<tr>
<td>Increased energy (%)</td>
<td>8/24 (33)</td>
<td>4/9 (44)</td>
<td>8/19 (42)</td>
</tr>
<tr>
<td>Reduced chest infections (%)</td>
<td>17/24 (70)</td>
<td>7/9 (78)</td>
<td>9/19 (47)</td>
</tr>
<tr>
<td>Medication use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction in oral antibiotics use (%)</td>
<td>16/24 (67)</td>
<td>7/9 (78)</td>
<td>11/19 (58)</td>
</tr>
<tr>
<td>Reduction in OCS use (%)</td>
<td>4/18 (22)</td>
<td>2/9 (29)</td>
<td>5/18 (28)</td>
</tr>
<tr>
<td>Discontinuation of OCS (%)</td>
<td>8/18 (33)</td>
<td>4/7 (57)</td>
<td>12/18 (67)</td>
</tr>
<tr>
<td>Reduction in SABA use (%)</td>
<td>12/25 (48)</td>
<td>6/9 (67)</td>
<td>8/19 (42)</td>
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<tr>
<td>Health care service use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction in hospital admissions (%)</td>
<td>9/10 (90)</td>
<td>3/2 (50)</td>
<td>9/10 (90)</td>
</tr>
<tr>
<td>Reduction in GP /emergency visits (%)</td>
<td>13/25 (52)</td>
<td>6/9 (67)</td>
<td>11/19 (58)</td>
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<tr>
<td>Quality of life</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Reduction inpatients’ overall symptoms (%)</td>
<td>18/25 (72)</td>
<td>7/9 (78)</td>
<td>13/19 (68)</td>
</tr>
<tr>
<td>Increased exercise tolerance (%)</td>
<td>7/25 (28)</td>
<td>4/9 (44)</td>
<td>6/19 (31)</td>
</tr>
<tr>
<td>Increased QOL (%)</td>
<td>18/25 (72)</td>
<td>7/9 (78)</td>
<td>13/19 (68)</td>
</tr>
</tbody>
</table>

Table 5.3: Improvements in clinical parameters during voriconazole therapy at 3, 6 and 12 months (ABPA and SAFS patients combined). OCS= Oral corticosteroid; SABA = short acting beta 2-agonist; QOL=quality of life; GP=General practice; ABPA=Allergic bronchopulmonary aspergillosis; SAFS=severe asthma with fungal sensitisation ;() indicate %. Vori=Voriconazole; Posa=posaconazole
5.4.4 Immunological response

Patient median total IgE values (KU/mL) over 12 months with ABPA only are summarised in fig 5.1. While modest falls were seen in some patients at 3 and 6 months, it was only at 9 and 12 months that sustained and statistically significant total IgE falls were seen. At 12 months, the median IgE decreased by 27.3% from baseline (median IgE 895, range 64-7,200) to a median IgE of 475 KU/L (range 51-3,100).

RAST to aspergillus fell, but was only significant at 12 months and beyond from 23.2 KUa/L (range 0.6-294) at baseline to 17.7 KUa/L at 12 months (range 0.6-57.4) (p=0.0056) (fig 5.1).
Fig 5.1: Percentage change in median total IgE (tIgE) for ABPA and SAFS patients during 12 months of treatment with voriconazole and/or posaconazole. Note: At 3 months (n=11, p=0.53), 6 months (n=15, p=0.57), 9 months (n=12, p=0.008), and 12 months (n=18, p=0.025), respectively. IgE=immunoglobulin E; ABPA=allergic bronchopulmonary aspergillosis; SAFS=severe asthma with fungal sensitization.
Fig 5.2 Percentage change in median RAST IgE to Af

Fig 5.2: Percentage change in median RAST IgE to Af for ABPA and SAFS patients at each time point assessment during 12 months treatment with voriconazole and or posaconazole, compared with baseline. IgE=immunoglobulin E, ABPA=allergic bronchopulmonary aspergillosis, SAFS=severe asthma with fungal sensitization. RAST=radioallergosorbent test, af= Aspergillus fumigatus. Note: At 3 months (n=10, p=0.17), 6 months (n=15, p=0.76), 9 months (n=12, p=0.65) and 12 months (n=18, p=0.0056) respectively.
We also observed marked heterogeneity in IgE responses with a few dramatic falls, most varying erratically through the course of therapy, and some rising. There was no consistent response between IgE and clinical impact and so decision to continue or discontinue therapy was based on clinical parameters (ie response & adverse effects). Total IgE antibody trend in ABPA by individual patient at baseline and on therapy at 3, 6, 9 and 12 months is shown in fig 5.1. None of the patients received Omaluzimub (Xolair).

5.4.5 Lung function
Overall, lung function improved throughout the treatment period. Of the 8 patients with spirometry values at both baseline and 3 months, there was a non-significant improvement of FEV1 5.5% from median baseline value of 1.64 ($P = 0.25$) and FVC improved by 16.9 % from a medium value of 2.67 ($P = 0.11$). There were no positive or negative statistically significant changes or trends witnessed beyond 3 months.

5.4.6 Radiological response
Of the voriconazole-treated patients who had baseline and follow up radiology (n=11), 5 of 10 (50%), 4 of 11 (36.4%) and 4 of 7 (57.1%) patients showed improvement in radiological abnormalities including bronchiectasis (severity and/or distribution, n=3 ), fibrosis (n=2), consolidation (n=4), mucus plugging (n=5), lobar collapse (n=3), pulmonary nodules (n=7) and/or cavitations (n=4) at 6, 12, and 18 months of therapy respectively. Two patients on voriconazole were noted to have worse radiological features at 3 months of therapy. Radiological deterioration was observed in 2 of 10 (20%) patients at 6 months and one of four (25%) at 12 months respectively. Both of these cases manifested with new pulmonary nodules. One of these completely cleared after 18 months of therapy, and may therefore be of uncertain significance.

One patient with ABPA developed extensive ground glass opacification (GGO) in the right upper lobe while on itraconazole (documented in vitro resistance to itraconazole and voriconazole) associated with clinical deterioration (fig 5.3A). There was significant resolution in the radiological lesions except for a residual small (17mm) cavitating lesion after 24 months of posaconazole therapy (fig 5.3B).

Another patient with ABPA who was intolerant to itraconazole subsequently developed right upper lobe consolidation while off antifungal therapy which cleared completely with voriconazole therapy (fig 5.4 A&B).
Fig 5.3: Radiological improvement in an ABPA patient treated with voriconazole

Figure 5.3: Computed tomography (CT) chest of a 63 year old woman with ABPA. A) Extensive ground glass appearances in the right upper lobe appeared while on itraconazole (documented in vitro resistance to itraconazole and voriconazole) associated with clinical deterioration (June 2008). B) Significant resolution in the radiological lesions except for a residual small (17mm) cavitating lesion after 24 months of posaconazole therapy (May 2010)

Figure 5.4. Chest radiographs (CXR) of a 69 year old lady with ABPA, intolerant to itraconazole (after 2 months of therapy) due to adverse effects. During posaconazole therapy, radiological improvement was seen in 1 of 4 (25%), 0 of 4 and 1 of 2 (50%) patients’ imaging at 6, 12 & 18 months of therapy respectively. Pulmonary nodules and consolidation showed the greatest improvement while fibrosis and bronchiectasis showed the least improvement.
5.4.7 Adverse events
Adverse events occurred in 11 (40%) patients taking voriconazole and 2 (22%) patients taking posaconazole. Voriconazole adverse events are shown in Fig 5.5. The most common were skin photosensitivity (n=11) and visual light flashes (n=10). Most of these side effects were transient and mild lasting less than a week except for some cases of photosensitivity and visual hallucinations. Significant adverse events resulting in discontinuation of treatment included; depression and sleep disturbance (1), photosensitivity (4), visual hallucinations (2) and peripheral neuropathy (1).

Posaconazole adverse events included insomnia (n=2), GI upset and mild liver impairment (n=1). No patient discontinued posaconazole because of adverse events. No patient with overt Cushing’s syndrome was noted during therapy with either posaconazole or voriconazole.

Two (8%) patients on voriconazole developed adrenal suppression thought to be induced by interaction between ICS (flixotide part of seretide) and voriconazole which has not been previously described. Subsequently, Seretide was changed to Serevent with ciclesonide and both patients started on regular oral hydrocortisone replacement after endocrinology review.
Fig 5.5: Adverse events seen in 25 patients treated with voriconazole evaluated for 2-48 months treatment. AE=Adverse events. Voriconazole AEs included GI upset (7), skin photosensitivity (11), blistering (4), visual light flashes (10), insomnia (2), visual hallucinations (2), depression (1), adrenal suppression (2), peripheral neuropathy (4), eye irritation (2), vivid dreams (1), dizziness (1) and headache (1) but most of them were transient and mild lasting less than a week except for some cases of photosensitivity and visual hallucinations.
5.4.7  **Relapse after discontinuation**
Among the seven patients who discontinued voriconazole, four relapsed (one at 3 months, 3 at 12 months).

5.4.8  **Exclusions**
We excluded one patient from analysis. This patient had hyper-IgE syndrome (male, age 21) who also was sensitized to multiple fungal allergens including *A. fumigatus* (voriconazole group treated for 14 months) and had mild bronchiectasis on HRCT. His baseline IgE was 25,000 KU/L and had less than 2 data points with respect to spirometry and radiology. His treatment was characterized by multiple disruptions due to repeated hospital admissions (ITU and general ward). He subsequently was started on azathioprine (AZA) as a steroid sparing agent to control his severe atopic asthma & eczema which stabilized his disease activity, hence classified as a voriconazole failure.
5.5 DISCUSSION

Numerous extrinsic factors are known to worsen asthma control, but the most common trigger is continuous exposure to allergens of which fungi are important factors [399]. Bandres Gimeno and Munoz Martinez in 2007 reported the first case of ABPA successful treated with voriconazole with clinical improvement and the reduction in IgE levels [400]. However, at the time of this study, there were no previously published studies to our knowledge which had directly assessed the effects of voriconazole or posaconazole therapy on asthma severity, health care service use and quality of life in individuals with SAFS or ABPA, although since this study was published, a blinded RCT with voriconazole and SAFS (EVITA3 study) has recently been published and did not show more benefit than placebo [401] [249].

We performed a retrospective audit of 25 patients who had previously been treated with voriconazole or posaconazole patients at the North West Lung Centre (NWLC) in Manchester, UK. In our cohort, we found that voriconazole therapy reduced frequent hospital admissions in 90% of the patients at 3 months, a benefit that was maintained at 6 and 12 months of therapy. We also demonstrated a reduction in the frequency of recurrent chest infections or acute exacerbations in 17 of 24 (70%), 9 of 19 (47%) and 9 of 17 (52.9%) at 3, 6 and 12 months respectively. The observed clinical response over time to voriconazole or posaconazole is a clinically significant finding that has implications for management of patients with poorly controlled severe asthma who are allergic to fungi and support previous suggestions linking mold sensitization with severe asthma attacks requiring hospital admission [136]. Our results differ from a double-blind, placebo-controlled, randomized trial published later by Agbetile and colleagues who did not show more benefit than placebo [249]. The possible reasons for this are explained at the end of this chapter.

5.5.1 Asthma control

The current goal of asthma treatment is to achieve and maintain optimal asthma control mostly using the GINA control criteria for day- and night-time symptoms, need for rescue medication, activity limitation and exacerbations and lung function [402, 403]. Since 1999 the British Guidelines on the Management of Asthma have been produced jointly by the British Thoracic Society (BTS) and Scottish Intercollegiate Guideline Network (SIGN) and achieving asthma control parameters is a key part of new versions of these guidelines [404].

In the present study, we demonstrated that identification and treatment of SAFS and asthmatic ABPA patients with voriconazole and posaconazole resulted in better asthma control as demonstrated by reduction in symptoms, improvement of patients’ perception of
QOL, and stabilization in lung function throughout the active treatment period (table 4.3). Our study supports the findings from the FAST study, a double-blind placebo-controlled randomized trial of itraconazole in patients with SAFS [237] and the retrospective study by Pasqualotto et al [236].

In our patients, the magnitude of voriconazole and or posaconazole effect on symptoms of asthma seen is encouraging. Better asthma control was achieved in over two thirds of patients during 12 or more months of therapy. Using the GINA definition of asthma control [402, 403], a marked improvement in parameters of asthma control including reduction in medication use (SABA, OCS), emergency health care use and exercise tolerance (ET) [236] was observed. Using the GINA criteria, we observed that at least 30% our patients could be reclassified from uncontrolled to partly controlled asthma [403]. We also observed a major reduction in cough frequency (>67%), reduction in breathlessness (>40%), and reduced exacerbations in (>75%) at 3, 6 and 12 months.

A major potential benefit of better asthma control is reduction in corticosteroid exposure. In our patients, over 30% of steroid-dependant asthmatic ABPA and SAFS patients could discontinue OCS completely with voriconazole or posaconazole therapy.

Not only are patients with severe asthma heavily burdened by disease, the health costs for these asthma sufferers individually and to health care services is enormous [199]. There appears to be a relationship between asthma control status and urgent health-care. In a recently published ‘Asthma Insights and Reality in Asia-Pacific Phase 2 (AIRIAP 2)’, a cross-sectional, community-based survey of 4,805 subjects looking at the relationship between control status derived from the GINA and urgent health-care utilization and the relationship with self-reported urgent health-care utilization related to asthma over the previous 12 months, Lai and colleagues found that each of the symptom criteria was significantly associated with urgent health-care utilization [405]. In our study, we have shown that with voriconazole or posaconazole therapy, there was significant reduction in health care service use with reduction in hospital admissions by 90% and 50% at 3 months and 6 months 90% and 100% respectively. This outcome was mirrored by reduction in GP or emergency visits by 72% and 78% at 3 months, 68% and 78% at 6 months and 58% and 78% for voriconazole and posaconazole respectively.
5.5.2 Monitoring improvement serologically and physiologically

There is very little research in the current literature with regard to serological response to voriconazole in patients with ABPA or SAFS although a retrospective review by Glackin L et al of CF patients with ABPA treated with voriconazole demonstrated significant drop in IgE levels post treatment [248]. In this study, we have demonstrated that treatment with voriconazole or posaconazole resulted in immunological improvement but statistically significant reduction in immunological markers started appearing at 9 months (p=0.008) for total IgE, although this was not mirrored by A. fumigatus specific IgE levels until 12 months of treatment (p=0.025) for both total and RAST (Aspergillus specific IgE) (figure5.1/ and fig 5.2). This raises an important question as to which immunological marker may be most useful for monitoring therapy and disease activity. These slow serological responses suggest reduction in fungal burden as a cause is likely, but more rigorous means of confirming this fact are required.

A major problem with the use of total IgE concentrations for monitoring or guiding therapy is its significant variability over time reflecting the many environmental and host factors influencing its concentration. In the German Multicenter Allergy Study, assessment of correlations of total IgE levels from birth to age 10 years in the birth cohort showed that in childhood, total IgE levels demonstrate remarkable variation over time even in the absence of atopy [406].

Another problem is that the inability to measure IgE-based sensitivity to all allergens has limited our understanding of what portion of asthma or other allergic disease is related to IgE. In our study, we observed marked heterogeneity in IgE responses with a few dramatic falls, most varying erratically through the course of therapy, and some rising within the same individual with little correlation with clinical parameters, though the numbers are small and the data not collected with precision.

Improvement or stability of lung function is one of the key objectives of asthma treatment and is one of the indices of the GINA asthma Control Criteria [403] and forms a major focus of asthma management by the BTS [29, 70, 407], ATS [199], ERS, Canadian Asthma Consensus Guidelines [408, 409] and other asthma societies. We did not demonstrate a statistically significant improvement in lung function in our patients. Several other studies have demonstrated a poor relationship between measures of asthma control and lung function in patients with asthma. The relationship between FEV1, asthma severity and symptom is poor especially in those with fixed airway obstruction and airway remodeling [410]. Our findings are similar to previous findings using itraconazole [234-236].
5.5.3 Other potential long term benefits of antifungal therapy

There is still ongoing debate as to whether antifungal treatment could ameliorate or reverse lung destruction in allergic fungal disease. We observed in our patients that in some cases, antifungal treatment did reverse some radiological abnormalities in 25-50% patients especially pulmonary nodular shadowing. The improvement in upper lobe cavitating mass and extensive surrounding ground glass opacity in one of our patient over 18 months of posaconazole therapy (fig 5.3) and the dramatic improvement of right upper lobe consolidation while on voriconazole therapy in another with ABPA (fig 5.4) is encouraging. Both of these patients were intolerant to itraconazole previously.

5.5.4 Adverse events

Several adverse effects have been reported with voriconazole use, including acute and chronic cutaneous adverse effects, mainly due to phototoxicity. According to safety data in studies assessing voriconazole effectiveness (primarily invasive aspergillosis), 8% of outpatients experienced phototoxic events [411]. More recently, some authors have reported that voriconazole was involved in the occurrence of multiple and often-aggressive cutaneous squamous cell carcinomas in immunocompromised patients if the treatment was maintained for a long time [412]. In our study, skin photosensitivity occurred in eleven (28%) patients (fig 5.5). We did not observe any skin cancers but this possibility is worth following up.

Other voriconazole side effects in our patients were gastrointestinal upsets (n=7), blistering (n=4), visual light flashes (n=10 (34%), insomnia (n=2), visual hallucinations (n=2), depression (n=1), adrenal suppression (n=2), peripheral neuropathy (n=4), eye irritation (n=2), vivid dreams (n=1), dizziness (n=1) and headache (n=1) but most of them were transient and mild lasting less than a week except for some cases of photosensitivity and visual hallucinations (fig 5.5). We observed that 5 (20%) developed significant adverse events to voriconazole requiring switch-over to posaconazole.

We did not observe any significant adverse events to posaconazole although some other authors have reported a variety of adverse events which appear to be unrelated to dose. In an evaluation of 18 healthy volunteers, Moton A and colleagues reported the frequency of adverse events when compared to placebo as : headache (17% vs. 13%), dry mouth (9% vs. 0%) and dizziness (6% vs. 2%) but there were no clinically significant changes in vital signs or laboratory test parameters except for transient, mild to moderate elevations in liver function test results [413]. These authors also demonstrated a minimal impact on QT interval
with posaconazole. Our study suggests that while voriconazole is efficacious, the possibility of serious adverse events is a strong possibility and both patient advice and close monitoring in the first few weeks of therapy is appropriate.

5.5.5 This study versus the EVITA3 study

Our results differ from a double-blind, placebo-controlled, randomized trial published later by Agbetile and colleagues who did not demonstrate improvement in QOL or reduction in severe exacerbations after 3 months of treatment with voriconazole [389]. These workers studied patients with asthma with A. fumigatus sensitization who had a history of at least 2 severe exacerbations in the preceding 12 months and treated them with a 3-month course of voriconazole, 200mg twice daily with a follow-up observation of 9 months. They did not demonstrate any statistically significant improvement in QOL or reduction in the number of severe exacerbations. The difference between the EVITA3 study and our study may be explained by a number of factors such as difference in study design (audit versus RCT), retrospective versus prospective and treatment period (12 months versus 3 months). In our study, we did demonstrate that treatment probably needed to be longer than 9 months for clinical benefit.

The EVITA3 study has a number of methodological limitations. First, after 9 months follow up, the primary end point was taken at 12 months even though the treatment was only given for 3 months.

Second, the treatment duration of 3 months is probably not adequate for this group of patients. In support of this, we have learned from the earlier randomized controlled trial of oral antifungal treatment for severe asthma with fungal sensitization using itraconazole: the Fungal Asthma Sensitization Trial (FAST) study in 2009 with SAFS patients treated for 32 weeks that improvement in Asthma Quality of Life Questionnaire (AQLQ) score was seen at 16 weeks of continuous treatment. In addition, clinical improvement was lost 4 months following cessation of therapy. It is possible therefore that the lack of improvement in the EVITA3 study is due to short duration of therapy.

The degrees of exacerbations are also different. Whereas EVITA3 study looked at severe exacerbations, we looked at all grades of exacerbations (mild-severe) and this may account for the difference between the two studies.

However, despite a number of limitations in our study such as the retrospective nature of the study, lack of randomization, subjective reporting by different clinicians and small sample
size, our findings are intruiging, novel and need further confirmation in a larger prospective study requiring more than 9 months duration of therapy as demonstrated by our findings. As opposed to the 3 months treatment period in the EVITA3 study, our data suggests that treatment in fungal allergic patients probably need longer than 9 months to achieve objective and measurable clinical benefit.

5.6 Study limitations
In this study, a number of limitations exist that include small sample size, its retrospective design/audit and subjective reporting, assessment and reporting by different individual doctors increasing the risk of variable observer bias [414]. In addition, some patients were treated with both agents and differing response parameters were necessarily used for ABPA and SAFS. Only 14 (54%) of our patients had both baseline and follow up imaging done; many were essentially normal at baseline.

4.7 CONCLUSIONS
Our study shows that both voriconazole and posaconazole are potentially effective alternative treatment options for SAFS and ABPA and may improve asthma control and reduce severity. However, larger prospective studies are required, including randomised studies with good power and requiring longer than 9 months treatment and follow up. The possibility of adverse events linked to voriconazole need to be taken into account and needs to be monitored. There is also a need to standardize the duration of antifungal therapy, which we suggest should not be less than 6 months, and 12 months may be preferable [400]. A major current limitation is drug acquisition cost but the high rate of efficacy shows that treatment with these agents as second line therapy is justified in certain patients.

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CHAPTER 6

Efficacy and Safety of nebulised amphotericin B (NAB) in Severe Asthma with Fungal Sensitization (SAFS) and Allergic Bronchopulmonary Aspergillosis (ABPA)

ABSTRACT

BACKGROUND AND RATIONALE: Antifungal therapy for severe asthma with fungal sensitization (SAFS) and allergic bronchopulmonary aspergillosis (ABPA) remains poorly studied. We assessed the efficacy and safety of nebulized amphotericin B (NAB) as second and third line therapy in SAFS and ABPA.

METHODS: 21 adult asthmatics with SAFS (n=11) and ABPA (n=10) who had either failed itraconazole (n=8), voriconazole proceeded by itraconazole (n=5) or developed adverse events (AEs) to either agent (n=7) were treated in an uncontrolled prospective study with 10mg of NAB (Fungizone) twice daily. We evaluated clinical and immunological response, using the Asthma Quality of Life Questionnaire (AQLQ-J) scores, asthma control, FEV1, healthcare utilization and IgE. Patients were followed up for 12 months.

RESULTS: 21 patients were treated (SAFS, n=11) and (ABPA, n=10), M: F= 8:12, median age 65 yrs (range, 24-78). The median duration of therapy was 30 days (0-1825). Clinical benefit was observed in three (14.3 %) in which overall mean AQLQ-J score improved by +2.9, mean FEV1 improved by 0.5L and there was improvement in overall asthma control. Seven (33%) failed initial dose (bronchospasm). Eleven (52.4%) discontinued within 12 months of therapy due to delayed bronchospasm (n=3, within 4 weeks), equipment problems (n=2, within 4 weeks) and lack of clinical benefit (n=4, within 16 weeks).

CONCLUSION: Our data suggests that the overall efficacy of NAB in this group of patients is poor and is associated with bronchospasm. However, the excellent response in 3 patients, suggest it may be considered when other alternatives have been exhausted. Overcoming the initial bronchospasm may improve tolerability.

Key words: Asthma, Asthma Pharmacology, Aspergillus Lung Disease, Allergic lung disease.
5.1 INTRODUCTION
Severe asthma with fungal sensitization (SAFS) and allergic bronchopulmonary aspergillosis (ABPA) are progressive and complex lung diseases whose treatment is not yet fully established. Not only are many patients with asthma desperately disabled by their disease, but, in the UK alone, asthma accounts for more than 1,000 deaths per year [415, 416]. The healthcare costs of these patients are enormous and any treatment option merits evaluation [417]. Epidemiological data estimates ABPA to occur in about 0.7 - 3.5% adult asthmatics [305] and 7.8% cystic fibrosis (CF) [418]. In the asthma population, severe asthma occurs in 5-10%, of which about 33% (25–50%) are estimated to have fungal sensitisation and therefore qualify for the diagnostic label SAFS, unless they have ABPA with severe asthma [419].

Traditionally, treatment for allergic fungal disease has involved the use of systemic oral corticosteroids (OCS) to suppress inflammation and immunological activity [225]. Randomized controlled trials (RCTs) show that treatment with oral itraconazole offers therapeutic benefit to approximately 60% of ABPA patients [235]. Similarly, a marked beneficial effect on quality of life (QOL) and other end points have been demonstrated among patients with SAFS treated with itraconazole [237]. Recent data from our group suggest that treatment with voriconazole or posaconazole improves asthma severity, control and QOL in both SAFS and ABPA and leads to resolution of pulmonary consolidation and cavitation in ABPA patients [315], although a blinded RCT with voriconazole and SAFS did not show more benefit than placebo [401].

Despite improving asthma and ABPA control, the chronic use of OCS has a number of adverse effects (AEs) including predisposition to diabetes, osteopenia, thinning of the skin. In COPD, corticosteroids may predispose to serious bacterial pneumonia [420][299]. In asthma patients, in particular those fungal sensitised, steroid use may increase airway fungal load (chapter 4, sub-section 4.4.3 of this thesis). Itraconazole is associated with several drug interactions and AEs, including suppression of adrenal glucocorticoids synthesis especially when co-administered with inhaled corticosteroids (ICS) [391]. Voriconazole is associated with intolerable AEs in about 26% patients, notably hepatic toxicity, photosensitivity and skin cancer, peripheral neuropathy, impaired thought process and frequent relapses [315]. Posaconazole therapy is better tolerated, but has some AEs and substantial cost limitations for long term therapy. Thus, there is need to evaluate other potential therapeutic options.
Over the last decade, NAB has been proposed and used in heart and lung transplant and neutropenic patients for prevention and treatment of invasive fungal infections (IFI) [421]. More recently, some published case reports indicate that liposomal NAB (L-NAB) may have a role in the treatment of CF ABPA and in corticosteroid dependant ABPA [252]. A major benefit was demonstrated in 3 CF ABPA patients with improvement in hypoxemia, FEV1, reduction in serum IgE and precipitins to *A. fumigatus* [253].

Despite these promising results, data in asthmatic patients is scanty, safety remains uncertain and most data relate to single case reports with short follow up durations. In some cases treatment has been used in combination with nebulised corticosteroid (n=3) [253] or oral prednisone (n=2) [232] and it is unclear which of the two therapeutic agents contributed to the reported efficacy [253]. The optimal duration of therapy is unclear and there is lack of clarity regarding the safety of prolonged use of nebulized therapy. The vast majority of data relate to transplant [422], hematological [423] and CF patients [251-253]. Little is known regarding the appropriateness of NAB in asthmatic ABPA patients or SAFS. In this study, we assessed the efficacy and safety of NAB as second and third line therapy in SAFS and ABPA asthmatics patients attending the National Aspergillosis Centre (NAC) and the regional severe asthma service.

### 6.2 HYPOTHESIS

We hypothesized that nebulised amphotericin B (NAB) would be effective and safe in SAFS and ABPA. We also hypothesized that fungal airways disease could be treated without systemic corticosteroids in some patients.

### 6.3 AIMS

The objectives/aims of this study were to assess the effect of NAB as second and third line therapy in SAFS and asthmatic ABPA patients.

### 6.4 METHODS AND MATERIALS

#### 6.4.1 Study design and setting

We conducted an **uncontrolled prospective interventional study**. 21 adult asthmatics with SAFS (n=11) and ABPA (n=10) who were attending fungal allergic asthma outpatient clinics at the NAC and North West Lung Centre Severe Asthma Service (NWSAS), based at the University Hospital of South Manchester, UK, were treated with non-liposomal nebulized
amphotericin B (NL-NAB) (January 2011-May 2013). We audited clinical and immunological response.

6.4.2 Study endpoints
The primary measure was change in Juniper Asthma Quality of Life Questionnaire (AQLQ-J) scores. We also examined asthma control parameters, change in lung function (FEV1), reduction in oral corticosteroid (OCS) dosages, change in IgE (total and specific) and healthcare utilization.

6.4.3 Inclusion criteria
Men or women with asthma, age (18-70 years old), an established diagnosis of asthma (BTS asthma guidelines 2009) and either SAFS or ABPA based on standardised diagnostic criteria [151, 153, 303] were audited. All patients had either failed itraconazole (n=8), voriconazole proceeded by itraconazole (n=5) or developed adverse events (AEs) to either agent (n=7). All patients needed to be symptomatic or to have any one or markers of significant clinical or radiological deterioration at the start of treatment. All patients gave informed and written consent.

6.4.4 Exclusion criteria
Subjects were excluded if they did not fulfil the diagnostic criteria for SAFS or ABPA could not follow instructions and/or were unable to do the treatment. We also excluded patients with concomitant diseases or conditions that are known to affect serum IgE concentrations such as strongyloidiasis, eosinophilic pneumonia, hyper-IgE syndrome, and ongoing immunotherapy. Patients in whom more than 15% reduction in FEV1 following first NAB challenge was observed, were withdrawn immediately from the NAB therapy.

6.4.5 Ethics statement
This was an uncontrolled prospective interventional audit and was considered as part of service evaluation by the institutional local research and development (R&D) at Wythenshawe Hospital. It was an established out of licence treatment and patients previous patients have been on it for atleast 2 years within the service. The study was simply done to establish the evidence around an already existing treatment and hence didn’t require ethical approval. In addition, consultation was made to the local R&D team and the response was that we did not specifically need ethical approval and hence could do the intervention. However, all subjects gave written consent at the time of the study. In addition, all patients
attending the NWSA and the NAC give prior written consent to take part in clinical and laboratory studies including this study. These consent forms are filed in by each individual patient and patients were asked to re-confirm their consent at the time of the study.

**Definitions**

**Severe asthma** was defined according to the BTS criteria (treatment steps 4 or 5) [29]. We used published criteria for the diagnosis of SAFS which included i) severe asthma, ii) total IgE <1000 KU/L and iii) positive skin test or raised specific IgE to any fungus [303]. **ABPA** was diagnosed if patients met the following criteria (ii), (IV) and at least 2 others:- i) asthma (of any severity), ii) highest ever total serum IgE ≥1000 KU/L, iii) immediate cutaneous reaction to *A. fumigatus* of > 3mm compared to control or iv) elevated *A. fumigatus*-specific serum IgE levels, v) precipitating antibodies to *A. fumigatus* in the serum, vi) a history of pulmonary infiltrates (transient or fixed), vii) central bronchiectasis (CB), viii) a history of expectoration of brown plugs or flecks and ix) isolation of *A. fumigatus* from sputum [150, 151].

**6.4.6 Drug administration procedure**

We performed baseline spirometry in clinic immediately prior to drug administration followed by premedication with 2.5 mg nebulised salbutamol. 10mg amphotericin B (Fungizone) in 4ml water for injection was administered via a Pari Sprint nebuliser fitted with exhaust filter under direct physiotherapist supervision. Spirometry was performed immediately post dose. Rescue bronchodilators (nebulised salbutamol) with or without prednisolone were administered if bronchospasm (demonstrated by breathlessness, wheeze) or if more than 15% reduction in FEV1 following NAB challenge was observed. If the initial dose was tolerated, 10mg of NAB (Fungizone) was self-administered twice daily at home. Patients were given education regarding drug dilution, care and sterilization of equipment at time of challenge in clinic.

**6.4.6 Follow up, data collection and assessments.**

Patients were followed up and assessed at 1, 2, 6 and 12 months during which they were prospectively reviewed for clinical characteristics, immunological markers, GP or emergency room visits, lung function (FEV1) and medication use (including OCS use). The clinical data collected included percentage (%) of symptom-free days, number of exacerbations, hospitalizations per month and AEs.

Patients were given the AQLQ-J [424] for self-scoring and asthma control was assessed based on the GINA criteria. Data collection, assessments and follow up steps are shown in
fig 6.1. Retrospective scoring was also included for some patients whose scores were not available at the time of enrollment.

Prednisolone was used as the reference steroid when comparisons of OCS were required. Similarly, beclomethasone dipropionate (BDP) equivalent dose was used as reference for inhaled corticosteroid (ICS). Common ICS used in our center include BDP, budesonide (1:1.5 dose equivalents) and fluticasone (1:2 dose equivalents with BDP). The data collected was entered into an excel database and later into SPSS-version 20 for analysis.
Fig 6.1: Study flow chat for data collection, assessments and Follow up

Fig 6.1: Study flow chat for data collection, assessments and Follow up. OCS=oral corticosteroids; ICS=inhaled corticosteroid; Aes=Acute exacerbations; Eos=eosinophil; IgE=immunoglobulin E; NAB=nebulised amphotericin B; FEV1=forced expiratory volume in the first second; ACQ=asthma control questionnaire; AQLQ-J=asthma quality of life questionnaire – Junniper.

6.5 Statistical analysis

All analyses were performed with the software SPSS 20.0 (IBM, USA) for Windows and excel statistics. Descriptive statistics was used to summarize the data. We included analysis on data collected prospectively on 15 (71.4%) and retrospectively on 6 (28%) patients. Subjects whose data was unavailable were excluded from analysis. Because of the small sample size that completed the study, no significance tests were conducted.
6.6 RESULTS

6.6.1 Patient characteristics

There were 21 patients analyzed (SAFS, n=11) and (ABPA, n=10), M: F= 12:9, median age 65.5 yrs (range, 24-78). The mean BMI was 28.6 (SD=4.9). The median duration of therapy was 30 days (0-1825). Eight (38 %) of the patients took therapy for more than 4 weeks (4-20 weeks, n=5; > 20 weeks, n=3). The mean baseline FEV1 was 1.86 L (+/-0.53) for all patients. SAFS patients had slightly higher spirometry (FEV1, FEV1% predicted, FEV1/FVC % ) values than ABPA patients. Demographic data is shown in table 6.1.

6.6.2 Treatment outcomes

Clinical benefit was observed in three (14.3 %) patients and adverse events occurred in 12 (57.1%) (Fig 6.1). The commonest AE was immediate bronchospasm occurring in 7 (33.3%) patients and accounting for 58.3% of all adverse events (AEs) (fig 5.2). None of these were serious adverse events (SAEs). A further eleven (55%) patients discontinued therapy within 60 days of therapy due to delayed bronchospasm (n=3, within 4 weeks), equipment problems (n=2) and due to lack of clinical benefit (n=4) (fig 6.1, 6.2). The time to discontinuation of therapy and the proportion of patients on treatment in the study at each time point is illustrated in fig 6.3. There was no correlation between baseline lung function (FEV1) and the occurrence of adverse events.
Table 6.1: Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients (n=21)</th>
<th>ABPA (n=10)</th>
<th>SAFS (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs), median (range)</td>
<td>65 (25-78)</td>
<td>67 (40-78)</td>
<td>63 (24-73)</td>
</tr>
<tr>
<td>Gender: Male (%)</td>
<td>12 (57.1)</td>
<td>5 (50)</td>
<td>7 (63.6)</td>
</tr>
<tr>
<td>BMI (kg/m²), mean (SD)</td>
<td>28.6 (4.9)</td>
<td>26.2 (2.63)</td>
<td>30.4 (6)</td>
</tr>
<tr>
<td>FEV1 (L), mean (SD)</td>
<td>1.86 (0.53)</td>
<td>1.57 (0.54)</td>
<td>2.02 (0.46)</td>
</tr>
<tr>
<td>FEV1% pred, mean (SD)</td>
<td>62.1 (18.7)</td>
<td>55.9 (23.6)</td>
<td>69 (12.2)</td>
</tr>
<tr>
<td>FVC (L), median (range)</td>
<td>2.68 (1.61-6.1)</td>
<td>2.57 (1.61-6.05)</td>
<td>2.74 (1.69-3.44)</td>
</tr>
<tr>
<td>FEV1/FVC %, median (range)</td>
<td>56 (33-84)</td>
<td>55.5 (33-80)</td>
<td>60.6 (34-84)</td>
</tr>
<tr>
<td>tIgE (ku/L), median (range)</td>
<td>215 (32-6700)</td>
<td>1200 (100-6700)</td>
<td>150 (32-3500)</td>
</tr>
<tr>
<td>AflgE ( KUa/L)</td>
<td>10.7 (0.5-100)</td>
<td>34.24 (0.6-100)</td>
<td>3.0 (0.5-41.7)</td>
</tr>
<tr>
<td>Therapy duration (days), median (range)</td>
<td>30 (0-1825)</td>
<td>120(0-1825)</td>
<td>0 (0-540)</td>
</tr>
</tbody>
</table>

Table 6.1: Demographic characteristics for ABPA and SAFS patients treated with nebulised non-liposomal amphotericin. ABPA=Allergic bronchopulmonary aspergilosis; SAFS= Severe asthma with fungal sensitization; tIgE =total IgE, Af= aspergillus fumigatus; BMI=basal metabolic rate; Ht=height; SD=standard deviation; FEV1=forced expiratory volume in the first second; FVC=forced vital capacity; % pred= Percentage of the predicted value; L=litres; KUa/L=kilounits of allergen per litre; ku/L=kilounits per litre; %L/s= percent litre per second.
Fig 6.2: Clinical outcomes in SAFS and ABPA patients treated with NAB.

Fig 6.3: Showing frequency of adverse events among ABPA and SAFS patients treated with NAB. Commonest advent events were immediate bronchospams occurring within minutes of NAB challenge.
Fig 6.4: Proportion of patients remaining on treatment at each time point.

Fig 6.4. Caplan-meir curve showing proportion of patients continuing on NAB treatment at each time point. Only about 40% and 30% patients received treatment beyond 30 and 60 days respectively and 14.3% beyond 12 months.
6.6.3 Responders

Despite the large failure rate, the responses were excellent in three (14%) patients who took therapy for 12 months or more (ABPA, n=2, SAFS=1). The median duration of therapy for these 3 patients was 540 days (365-1825). Their overall mean AQLQ-J scores improved by +2.9 score (range, 2-6) at the end of 12 months therapy (fig 6.5a) and this improvement corresponded with improvement in asthma control. Similarly, there was a significant improvement in median FEV1 by +0.5 L (fig 6.4b). Two of these patients managed to return to work and one resumed her sporting activities.

There was a reduction in daily median OCS dosages from 18.5mg to 2.5 mg. We did not observe any reduction in ICS dosages. There were no significant changes observed in immunological outcomes.
Fig 6.5: Change in clinical end points. (6.5a) Trend in AQLQ-J in 3 patients treated with NAB over a period of 12 months. AQLQ-J = juniper asthma quality of life questioner. Patient 1 had ABPA, 2 had SAFS and patient 3 had ABPA. (6.5b) Trend in FEV1 in 3 patients treated with NAB over a period of 12 months.
6.6.3.1 Characteristics of the responders:
Overall, the 3 responders (ABPA, 2; SAFS, 1) had better lung function, less obstructive spirometry and higher Aspergillus specific IgE levels than the non-responders (table 6.2). In addition, the responders were relatively younger and all were never smokers. We could not do statistical testing due to the small number of the patient cohort.

Table 6.2: characteristics of the 3 patients who responded to NAB

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Responders (ABPA, 2; SAFS, 1)</th>
<th>None responders (n=18; ABPA=8, SAFS=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs), median (range)</td>
<td>49 (40-73)</td>
<td>65.5</td>
</tr>
<tr>
<td>Gender: Male, n (%)</td>
<td>1 (30)</td>
<td>7 (39)</td>
</tr>
<tr>
<td>BMI (kg/m²), mean (SD)</td>
<td>30.6</td>
<td>29.1</td>
</tr>
<tr>
<td>FEV1(L), mean (SD)</td>
<td>1.98 (0.1)</td>
<td>1.76 (0.59)</td>
</tr>
<tr>
<td>FEV1% pred, mean (SD)</td>
<td>93.8 (23.2)</td>
<td>72 (27.5)</td>
</tr>
<tr>
<td>FEV1/FVC %, median (range)</td>
<td>70 (56-84)</td>
<td>56 (33-80)</td>
</tr>
<tr>
<td>tIgE (ku/L), median (range)</td>
<td>1860 (222-3700)</td>
<td>1000 (230-1500)</td>
</tr>
<tr>
<td>AflgE ( KUa/L) (n), median</td>
<td>12.3 (6-87.4)</td>
<td>3 (1-10)</td>
</tr>
<tr>
<td>Duration of asthma (months), (mean)</td>
<td>43 (14.73)</td>
<td>46.89 (16)</td>
</tr>
<tr>
<td>Therapy duration (days), median (range)</td>
<td>540 (364-1825)</td>
<td>30 (0-210)</td>
</tr>
<tr>
<td>Pack yrs (n), median</td>
<td>0</td>
<td>2.31</td>
</tr>
<tr>
<td>Upper airway disease</td>
<td>0</td>
<td>2,</td>
</tr>
<tr>
<td>Atopy</td>
<td>2 (66)</td>
<td>10 (55%)</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>3 (100)</td>
<td>15 (83.3)</td>
</tr>
</tbody>
</table>

Table 6.2: Characteristics of the patients who responded to NAB.
No statistical testing was done due to the small number of the patient co-hort among the responders, so p-values are not included.
6.7 DISCUSSION

Current data regarding the efficacy and safety of NAB in ABPA are based on a few case reports, largely CF patients and no study has ever evaluated SAFS patients. Those few case reports suggest physiological and clinical efficacy along with the possibility of reducing OCS doses, although they did not consider non-liposomal formulations.

In this study, we assessed the efficacy and safety of non-liposomal nebulised amphotericin B (NL-NAB) in SAFS (n=11) and ABPA (n=10) patients. We observed major clinical benefits in 3 patients (ABPA, n=2; SAFS, n=1) (fig 6.1). We demonstrated major improvements in AQLQ-J scores by +2.9 and improvements in asthma control incorporating of daytime/nocturnal symptoms, reliever use and exacerbations within 12 months therapy. There are no previous studies that have assessed the efficacy of NAB in asthmatic ABPA or SAFS patients. However, our results support previous published case reports using liposomal nebulised amphotericin (L-NAB) in CF ABPA and in corticosteroid dependant ABPA patients [251, 252].

Previous case reports have reported major improvements with the use of NAB in the CF population. The use of NAB therapy in these cases involved different NAB regimens that varied in doses and durations of therapy. In a case report involving a 14-year-old female with end-stage CF lung disease associated with frequent hospital admissions due to progressing symptoms, very poor lung function and severe bronchiectasis (listed for lung transplantation 6 months earlier), treatment with NAB using 10 mg twice daily via a Pari LC PlusTM nebulizer resulted in improvements in cough, dyspnoea, hypoxia, 6 minute walk, reduction in OCS dosages and improvements in pulmonary function (FVC, FEV1) with no adverse events [251]. However, this patient received concomitant therapy with itraconazole during the first 3 months and the synergistic effect of both treatments may have played a part in the observed clinical and physiological improvements. In our study we have demonstrated major improvements in these 3 patients with NAB alone without concomitantazole antifungal therapy.

We did not observe any clinical benefit in those patients who took therapy for less than 6 months. Our data also supports previous observations observed in CF ABPA children reported in 2008 by Laoudi and co-workers [253]. These authors reported major clinical and physiological responses observed from the 6th month in three CF ABPA children (aged 7, 12 and 13 yrs) treated with NAB using 5 mg twice a day that resulted in improvements in
hypoxemia, FEV1, reduction of blood eosinophilia, total and specific serum IgE antibodies to *A. fumigatus* [253]. On the other hand, these reports did not specifically assess QOL. In our study, we have demonstrated for the first time that the use of NAB can result in a substantial improvement of measures of QOL and physiological parameters in selected patients who pass the initial dose challenge.

In France, Godet et al 2012 described a case of a 67-yr-old female with ABPA, previously unresponsive to itraconazole therapy which subsequently improved dramatically with a significant decrease in eosinophil count, precipitins, total and specific IgE levels complete resolution of consolidation (within 4 weeks) over time following 6 months therapy with liposomal NAB (l-NAB) [425]. These workers did not report any significant side-effects. In an article by Leon et al 1999, therapy with NAB showed good results for two ABPA patients but always in association with OCS [232]. Our study differs from prior literature in that co-administration of NAB with OCS, nebulized corticosteroids or azole antifungal was common [251, 253]. It is therefore unclear which of the duo or trio of agents was responsible for the observed clinical benefit. In contrast, we did not use any concomitant OCS, nebulized corticosteroids orazole antifungals. Our data provide support for the hypothesis that fungal airways disease can be treated without systemic corticosteroids, in some patients.

**Other outcomes**

This study also demonstrated improvements in other parameters. Of the 3 responders, 2 who had been sick off from work returned to work after 6 and 9 weeks respectively. Better diabetic control in one patient was also observed. The improvement in diabetic control observed in this patient may be as a result of complete cessation of OCS as a result of better control of her airways disease. No significant changes were observed in immunological and radiological outcomes.

### 6.7.1 Characteristics of the three responders

There are no prospective studies that have compared characteristics of patients that respond to NAB in fungal allergic asthmatic patients. In this study, we observed that overall, the 3 responders (ABPA, 2; SAFS, 1) had better lung function, less obstructive spirometry and more aspergillus sensitization than the non-responders (table 6.2). In addition, the responders were relatively younger and all were never smokers. We could not do statistical testing due to the small number of the patient cohort. However, this data somehow gives some idea of which patients might be candidates for clinical response with less adverse events in particular NAB.
6.7.2 Adverse events

We observed a significantly high frequency of adverse events (AEs) with NL-NAB occurring in 57% patients (fig 6.2). Immediate bronchospasm was the most frequent AE observed (33.3%). Other AEs included delayed bronchospasm occurring within 30 days of starting therapy (14.3%), headache (4.8 %) and fatigue (4.8 %) (Fig 6.3). We observed that bronchospasm was almost always associated with coughing.

There are no previous studies that have evaluated adverse events associated with the use of NAB in fungal sensitized asthma patients, but common adverse effects in previous studies in other diseases have been documented. These AEs approach 30%–40% with NL-NAB when used in neutropenic or post-stem cell or autologous transplant patients [426, 427].

The commonest documented AEs with NAB use are coughing and an aftertaste [426, 428]. Other reported adverse events include; nausea, vomiting, dysphagia, bad taste, epistaxis and renal insufficiency [426]. An RCT involving 65 patients receiving prophylactic NAB at a dose of 10 mg twice daily versus placebo demonstrated that coughing (54%), bad taste (51%), and nausea (37%) caused early cessation of treatment in 23% (15/65) cases [253].

The frequency and nature of AEs observed in our study differ from historical literature due to a high frequency of bronchospasm. Bronchospasm has not been widely reported with the use of NAB except for few incidences reported in lung transplant patients [428]. It is possible that the observed large frequency of bronchospasm in our patients may be due to the airway hyperresponsiveness which characterize most asthmatics. In some previous studies, all patients with severe asthma were excluded and may partly explain why those studies had less frequent bronchospasm compared to our patients [429]. Another possibility for the differences might be the presence of denervated lungs in transplant patients.

Effect of nebulizer types and dosages

Some of the main challenges involving the use of NAB lie in determining the correct type of nebuliser [430]. It is not clear whether the observed AEs in my study are due in part to differences in nebulizer type, pressure or dosages as compared to those used in previous studies. We used the Pari Sprint nebulizer for our study.
In contrast, different nebulisers and dosages have been used in many previous reports such as 12.5 mg twice weekly using an adaptive aerosol delivery system in neutropenic patients [428] the Pari LC plus nebuliser using 10 mg twice per day [251] and the Misty nebulizer using 30 mg daily [429]. The breath-actuated nebulizer (AeroEclipse®) has been shown to deliver 24mg of liposomal amphotericin B solution (AmBisome®) successfully without disrupting the liposomes and minimize drug loss with less nephrotoxicity [431, 432].

The pharmacokinetics of NL-NAB may also account for the observed difference in adverse events compared to previous studies. Previous studies have utilised amphotericin B lipid complex (ABLC) and very few utilised NL-NAB. In many respects, the study of NL-NAB or ABLC in lung transplant recipients is at a crossroads and there are pharmacokinetic differences between the 2 formulations. In animal models, amphotericin B (Fungizone) tends to foam in the nebulisation chamber [433]. This foam may be inhaled during nebulisation and may contribute towards cough and bronchospasm. Liposomes on other hand do not exhibit this characteristic and are well tolerated by the lung. This may partly explain the tolerability exhibited in previous studies using liposomal amphotericin B.

In human subjects, ABLC aerosols are better tolerated than is NL-NAB. In a prospective, randomized, double-blinded trial (n=100) comparing safety of aerosolized NL-NAB with ABLC in lung-transplant recipients, subjects receiving NL-NAB were more likely to have experienced an AE [434]. However, the nebulizer doses used in this study were different between the two treatment groups (25 mg and 50 mg daily, respectively) and it's unclear whether the observed differences in their study were due to the dose differences. Hence there remains a need to determine the most appropriate equipment and optimal dosages.

### 6.8 LIMITATIONS

This study has many limitations. Some of the data (28%) patients included is based on retrospective data for the patients who started treatment before the study started. Data on these patients therefore is likely to be less accurate due to subjective recording and reporting by clinicians. However, most of these patients did not pass the initial challenge (except for one) and therefore did not affect the follow up data. The number of measures and frequency varied from patient to patient and this might have affected data interpretation. Some had fewer data points compared to others.
7.9 CONCLUSIONS

Our data suggests that the overall efficacy of NAB in this group of patients may be poor and is associated with high frequency of adverse events. However, the responses were excellent in 3 (14%) patients. It is likely that factors such as lung function, smoking status, level and degree of fungal sensitization (as measured by levels of specific IgE) and young age may be contributing factors towards determining potential treatment candidates. Further studies need to be conducted to establish the optimal dose range (dose, frequency), nebulizer type, pressures and identification of patients who may respond. Antifungal treatment by inhalation could provide a new, simple therapeutic approach in patients unsuitable for azole antifungals or in whom there has been no clinical benefit with first line therapies, azole resistance or where it has been difficult to maintain adequate therapeutic drug levels with azole antifungal. Another advantage is that local administration of NAB to the airways also decreases the risk of drug interactions that is commonly observed with azole antifungals. It allows higher lung tissue deposition and concentrations and only limited absorption into blood, as previously illustrated by negligible serum concentrations post dose [421].
CHAPTER 7

FINAL DISCUSSION
Discussion

Not only are patients with severe asthma (SA), severe asthma with fungal sensitisation (SAFS) and ABPA disabled by their disease burden, the health costs attributed to their direct cost of care and loss of productivity as a result of sickness at work is enormous. It is therefore vital to understand the cause of these diseases and the optimal therapy which offer maximum efficacy with fewest adverse events and least cost. To date, the immunopathogenesis in these diseases remain poorly understood. Current available therapy is based on few case reports and few small studies. They have multiple adverse events and the safety of prolonged therapy is unclear. Therefore, there is an increasing need to further understand the immunopathogenesis for potential development of targeted therapy.

Asthma, in particular, severe asthma is increasingly being recognised as a syndrome with a heterogeneous presentation, consisting of multiple different pathogenetic subgroups with different cellular and molecular characteristics. Thus, the earlier classification of asthma is slowly being replaced by phenotyping of asthma for targeted treatment of particular subtypes [73, 78, 79]. In addition, ABPA and SAFS are heterogeneous diseases. For example, patients attending our specialist clinics at the NAC and North Lung Centre vary in terms of what triggers their symptoms, the symptoms experienced, severity of disease and response to therapy. Thus, the pathology, the underlying pathogenesis and the natural history and therapeutic strategy varies from one patient to another. Combination of strategies could be better than single ones in certain patients.

It is increasingly being recognised that sensitisation to fungi plays a major role in driving asthma symptoms. Emerging data suggest IL-17A, IgE and microbiome variants may be associated with the pathogenesis of asthma. However, several questions remain unanswered and need to be clarified. First, the role of fungi and the cause-effect relationship between IL-17A and allergic fungal asthma is unclear and there is paucity of data in this area. Secondly, much of what we know regarding the role of IL-17A in human fungal lung disease relates to anti-fungal immunity, but little data exist in allergic forms. Moreover, current insight relating to the role of IL-17A in allergic fungal disease is based on animal studies, whereas data on human studies is scanty. It is unclear whether animal data can be replicated in human models. Fourthly, in the case of severe asthma, most human studies have utilised PBMCs and very few have used airway samples. There are no prospective studies that have compared IL-17A expression in airways and blood.
Despite IgE being linked to pathogenesis of allergic asthma, little is known regarding the association between IgE against staphylococcal enterotoxin and disease activity in SAFS or ABPA. The range of microbial population and the effect on asthma severity in patients with fungal associated asthma is not fully understood. Little is known about the response rates or appropriateness of alternative antifungal treatment for those with ABPA or SAFS.

The current treatment of allergic fungal disease currently relies on itraconazole and OCS, both of which have multiple adverse events and do not prevent progressive lung damage. Little is known about the response rates or appropriateness of alternative antifungal treatment for those with ABPA or SAFS. Very few case series and mostly in haematological and heart and lung transplant have reported the use of NAB, but the role of NAB in patients with SAFS and asthmatic ABPA have not been fully studied. The safety profile for both short and long term use of NAB is not clear.

This thesis has evaluated the immupathogenesis and antifungal therapy of severe asthma, ABPA and SAFS. The role of IL-17A, IgE and lung microbiome in relationship to their association with disease severity and disease patterns have been evaluated. This thesis has identified IL-17A as a potential immunological target in clinical studies and potential therapeutic interventions. It has also identified lung microbiome population and specific fungal microbial genra as potential pathogenic elements in SANFS, SAFS and ABPA. It has identified an association of IL-17A, SE-IgE, and lung microbiome with asthma severity. In addition, this thesis has identified a potential association of steroid use and increase airway fungal load in fungal severe asthma, ABPA and SAFS. Furthermore, the findings of this thesis have helped to expand the current understanding of the role of alternative antifungal therapy and potential side effects. It has also identified potential areas for future research and intervention.

7.1 Summary of Thesis Studies

7.1.1 Chapter 2: IL-17A in severe asthma, ABPA and SAFS

This study has demonstrated that patients with severe asthma have significantly higher CD4+IL-17A expressing cells than those with less severe forms.

This study did not find any significant difference in IL-17A expression between the different severe asthma groups. This finding is novel and indicative of a central role for IL-17A in
severe asthma, regardless of phenotype. It appears IL-17A is simply an independent risk factor for severe asthma, independent of any link to fungal sensitisation [289]. The differences between severe and mild asthma are striking, suggesting that IL17A is not central to the pathogenesis of asthma, but implicated in severe disease. These observations need external confirmation.

With the similarity of IL17 expression between SAFS and SANFS, SAFS could be seen simply as a variant form of SA with disproportionate sensitivity to fungi. Host genetic linkage to these different asthma endotypes (or phenotypes), will be required to further address the differences. One other plausible explanation for this unexpected finding would be that concomitant antifungal therapy may have affected our results.

This study did not find any differences between airways and blood. This finding is novel and suggests that circulating lymphocyte numbers may reflect airway inflammation, and provide an alternative method of assessing airway IL-17A expression.

This study also found significantly higher %CD+IL-17A expressing cells in those subjects with positive fungal culture. Importantly, this group’s asthma severity was more severe than the group with negative cultures. This finding is intriguing and novel. This is the first study to demonstrate an association between fungal colonisation and IL-17A and provides one plausible mechanism via which fungi may worsen asthma severity and highlights the need for further studies to demonstrate the causal-effect relationship. It also provides a possible explanation for the improvement in asthma severity in previous studies following antifungal therapy.

An important point of discussion is the significance of neutrophils in this PhD thesis. A high neutrophil count was associated with higher IL-17A expressing cells only in a subgroup with severe asthma. The subgroup of severe asthmatics that were not sensitised to fungal or any other allergens (non-allergic severe asthmatics) was more neutrophilic and this neutrophil count correlated with IL-17A expression. This finding supports a possible underlying mechanism of the newly classified Th2 low severe asthma phenotype that displays predominant neutrophilic inflammation in which the Th17 pathway has been proposed to be involved in the neutrophilic inflammation and airway remodelling processes [290, 291].
The correlation of blood neutrophils with airway IL-17A found in this study has important implications and suggest that circulating blood neutrophil numbers may reflect neutrophilic airway inflammation, and provide an alternative non-invasive method of assessing airway inflammation, even though affected by concurrent infection and corticosteroid use. Assessing airway lymphocytes from BAL exposes patients to an invasive processure that is expensive and risky to patients and therefore the findings from this thesis will support the need for utilising cheaper and alternative ways of assessing airway inflammation in clinical practice and research.

This study demonstrated that patients who were currently on azole antifungal treatment hard significantly higher %CD4+IL-17A expression compared to patients who were never treated with antifungals. There are no prospective studies that have assessed L-17A expression in humans comparing antifungal treated versus antifungal naïve patients. This finding may suggest the potential link between azole antifungal metabolism or action and IL-17A, but this needs further investigations involving larger studies including molecular and metabolism studies.

7.1.2 Chapter 3: IgE expression in ABPA and SAFS

Immunoglobulin E (IgE) molecules play a crucial role in allergic diseases and may cause chronic airway inflammation in asthma, hence implicated in severe asthma pathogenesis [101, 435, 436]. Serum levels of immunoglobulin E (IgE) are used in the diagnosis and monitoring therapy for allergic asthma, SAFS and ABPA [150, 151, 261, 313-316], but the role of total IgE in disease pathogenesis and what allergens constitute total IgE is not precisely clear. The inability to measure IgE-based sensitivity to all allergens has limited our understanding of what portion of asthma or other allergic disease is related to total IgE and which of the allergen-specific IgE antibody might be useful for monitoring allergic disease. Thus far, it remains to be determined as to what extent t-IgE can be reliably used as a diagnostic parameter and which of the specific IgE would have pathogenic or prognostic role in asthma and ABPA.

In this Chapter 3, this thesis measured total IgE (tIgE), specific IgE to staphylococcal enterotoxins (SE-IgE). Specific IgE antibody concentrations in serum against S. aureus enterotoxins (SE- IgE), A. fumigatus (AF-IgE) and total IgE (t-IgE) in adult cohorts with SAFS and ABPA patients and correlated these with clinical variables. This study demonstrated that serum concentrations of total and staphylococcal enterotoxin (SEIgE) and A. fumigatus specific IgE are significantly higher in ABPA group compared to those of SAFS. In addition,
this study also demonstrated a significant positive correlation between SE-IgE concentrations and higher oral corticosteroid (OCS) doses. Whereas, sensitisation to staphylococcal aureus enterotoxins is relevant to airflow obstruction, sensitization to *Aspergillus* may be relevant to bronchiectasis in adult asthmatic patients with ABPA and SAFS.

We have also demonstrated that asthmatic patients with SAFS may be a distinctive asthma phenotype, although with a number of similar clinical characteristics to those of ABPA, further supporting clinical heterogeneity in asthma and the need for new approaches for the classification of disease and selection of appropriate treatment.

### 7.1.3 Chapter 4. Airway microbiome in SA (SANFS), SAFS and ABPA

Changes in the airway microbiome may be important in the pathophysiology of chronic lung disease, but the relationship between the microbiomes and disease activity remains understudied.

The most striking finding in this study is the massive preponderance of *A. fumigatus* over all other fungi in asthmatic lungs, of whatever asthma severity. Individuals with severe asthma, whether sensitised to fungi or not (as in SANFS), have a significantly increased proportion of *A. fumigatus* in their mycobiome. This is surprising and in marked contrast to healthy controls, and even ABPA and mild asthmatics. The implications of this finding are not known. One hypothesis might be that *A. fumigatus* plays an important, even critical, but unsuspected role in the pathophysiology of asthma.

Another striking observation in this study is the diversity of isolates in the *A. fumigatus* group. Tens and probably hundreds of variant strains were found in each pair of lungs sampled.

The third striking finding is the significant increase in fungal load and *A. fumigatus* count in patients on current inhaled steroid treatment. This finding is novel and intriguing and highlights the safety concerns around the use of OCS in these groups of patients.

The fourth striking finding is the significantly higher frequency of positive fungal cultures in patients with severe asthma compared to non-severe group. Moreover, patients with positive fungal cultures had significantly lower lung function compared with culture negative subjects. There are no previous studies that have specifically assessed the relationship between
fungal colonisation and lung function in patients with SAFS or ABPA, but these findings support previous proposals that positive airway fungal culture is associated with an impaired FEV1 and asthma severity by Agbetile et al in 2012. It also supports previous findings in the COPD population, where airway fungi positivity was linked with severity of COPD [381].

The other striking finding is the finding of fungal isolates in airway of two (9%) non-fungal sensitised (negative sIgE to fungal allergens) severe asthmatics is interesting. It is possible that there is probably a subgroup of severe asthmatics who though may have airway fungal infection or colonisation, but do not mount a Th2 response.

This study also found a great diversity of bacteria in the airways of all asthma groups, especially on those severe asthmatics with predominant blood eosinophilia. This finding may explain in part the clinical benefit observed with maintenance treatment with low-dose macrolides such as erythromycin and azithromycin in several chronic neutrophilic airway diseases, including neutrophilic severe asthma population [437, 438]. This finding needs to be confirmed in larger prospective studies.

7.1.4 Chapter 5: Role of Voriconazole and Posaconazole in SAFS and ABPA

In chapter 5, we assessed the effect of voriconazole or posaconazole as second and third line therapy in a retrospective audit of 26 adult asthmatic patients with ABPA (n=21) and SAFS (n=5).

The most striking finding in this study is the great improvement in asthma severity and QOL observed with voriconazole or posaconazole therapy. The study found marked reduction in OCS and SABA use, health care utilization due to asthma and improvement in overall health status. The observed clinical response over time to voriconazole or posaconazole is a clinically significant finding that has implications for management of patients with poorly controlled severe asthma who are allergic to fungi and support previous suggestions linking mold sensitization with severe asthma attacks requiring hospital admission. A major potential benefit of better asthma control is reduction in corticosteroid exposure. In our patients, over 30% of steroid-dependant asthmatic ABPA and SAFS patients could discontinue OCS completely with voriconazole or posaconazole therapy.

The results of this thesis differ from a double-blind, placebo-controlled, randomized trial published later by Agbetile and colleagues who did not demonstrate improvement in QOL or
reduction in severe exacerbations after 3 months of treatment with voriconazole [389]. The duration of treatment for their patients was 3 months. However, our study does demonstrate that the duration of treatment needed to be at least 6 months before clinical response is objectively observed. There is also a need to standardize the duration of antifungal therapy, which this study suggests should not be less than 6 months, and 8-12 months may be preferable in future studies if safety allows [400].

The other striking finding in this thesis is the improvement in radiological manifestations of disease in these patients with voriconazole or posaconazole. There is currently insufficient data assessing radiological benefit in ABPA patients treated by antifungals and it is not clear to date whether antifungal therapy could ameliorate or reverse lung destruction in allergic fungal disease. We observed in our patients that in some cases, antifungal treatment did reverse some radiological abnormalities in 25-50% patients especially pulmonary nodular shadowing. The improvement in upper lobe cavitating mass and extensive surrounding ground glass opacity in one of our patient over 18 months of posaconazole therapy (fig 5.3) and the dramatic improvement of right upper lobe consolidation while on voriconazole therapy in another with ABPA (fig 5.4) is encouraging. Both of these patients were intolerant to itraconazole previously.

However, a major concern is the wide range of adverse events associated with voriconazole therapy. The possibility of adverse events linked to voriconazole need to be taken into account and needs to be monitored.

7.1.5 Chapter 6: Efficacy and Safety of Nebulised Amphotericin B in SAFS and ABPA

NAB has been used in heart and lung transplant patients with some efficacy reported. Despite these promising results, data in asthmatic patients is scanty, safety remains uncertain and most data relate to single case reports with short follow up durations. In some cases treatment has been used in combination with nebulised corticosteroid (n=3) [253] or oral prednisone (n=2) [232] and it is unclear which of the two therapeutic agents contributed to the reported efficacy [253].

In this chapter, we conducted a prospective uncontrolled interventional study to assess the efficacy and safety of nebulised amphotericin B (NAB) in patients with SAFS (n=11) and ABPA (n=10). The most striking result of this study is the high frequency of bronchospasm occurring within minutes of NAB therapy occurring in 33.3% patients and accounting for 58.3% of all adverse events (AEs). A further eleven (55%) patients discontinued therapy.
within 60 days of therapy due to delayed bronchospasm (n=3, within 4 weeks), equipment problems (n=2) and due to lack of clinical benefit. This finding is intriguing and suggests that the overall efficacy of NAB in this group of patients is poor and is associated with bronchospasm.

The second most striking finding is that in those patients who pass the initial nebulizer challenge and go on treatment for at least 3 months, the efficacy is good as demonstrated by excellent responses in three (14%) of my patients. The median duration of therapy for these 3 patients was 540 days (365-1825). Their overall mean AQLQ-J scores improved by +2.9 score (range, 2-6) at the end of 12 months therapy and this improvement corresponded with improvement in asthma control. Similarly, there was a significant improvement in median FEV1 by +0.5 L (fig 6.4b). Two of these patients managed to return to work and one resumed her sporting activities.

It is not clear which patients are likely to respond. However, the characteristics of these 3 responders could provide some insight as to which patients might be candidates for clinical response with less adverse events in particular NAB. These 3 responders (ABPA, 2; SAFS, 1) had better lung function, less obstructive spirometry and more aspergillus sensitization than the non-responders (table 6.2). In addition, the responders were relatively younger and all were never smokers. Studies selecting patients with or without these characteristics could provide better understanding with regard to potential candidates for NAB.

If successful, antifungal treatment by inhalation could provide a new, simple therapeutic approach in patients unsuitable forazole antifungals or in whom there has been no clinical benefit with first line therapies,azole resistance or where it has been difficult to maintain adequate therapeutic drug levels with azole antifungal. Another advantage is that local administration of NAB to the airways also decreases the risk of drug interactions that is commonly observed with azole antifungals. It allows higher lung tissue deposition and concentrations and only limited absorption into blood, as previously illustrated by negligible serum concentrations post dose [421]

Further studies need to be conducted to establish the optimal dose range (dose, frequency), nebulizer type, pressures and identification of patients who may respond. Overcoming the initial bronchospasm may improve tolerability.

7.2 Thesis limitations

The limitations for this thesis are described at the end of each chapter.
7.3 Discussion of the main findings

7.3.1 IL-17A is has a central role in asthma severity, independent of cause. Circulating blood neutrophil numbers may reflect neutrophilic airway inflammation, and provide an alternative non-invasive method of assessing airway inflammation.

The high %CD4+IL-17A in severe asthma groups and correlation with serum neutrophils seen in this study provides opportunities for biological targets for treatment of severe neutrophilic asthma. These findings support previous reports linking increased IL-17A with asthma severity from induced sputum [268, 293], BAL [439] and bronchial biopsies [277]. It is not entirely clear as to whether the rise in CD4+IL-17A expressing cells is a cause or consquency of severe asthma. Previous studies have utilised airway neutrophils in association with airway IL-17 obtained from induced sputum analysis [268, 292, 293]. The correlation between blood neutrophils with airway %CD4+IL-17A suggest that the systematic changes may be associated with airway inflammation, hence providing an alternative method of assessing airway inflammation.

The association of %CD4+IL-17A+ cells with the presence of airway fungi seen in this study in the subjects with severe asthma provide a possible explanation for the link between fungi and asthma severity. More studies are needed to clearly understand this link.

The lack of difference in %CD4+IL-17A+ cells between SAFS and SANFS, together with a higher expression in severe asthma compared to non-severe subjects is indicative of a central role for IL-17A in severe asthma, regardless of phenotype, but not all asthma.

7.3.2 IgE against staphylococcal aureus (SE-IgE) is associated with reduced lung function with and increased OCS use. Sensitisation against staphylococcal enterotoxins is associated with asthma severity, whereas sensitisation against Aspergillus fumigatus is associated with high frequency of bronchiectasis.

In chapter 3, we have demonstrated that SE-IgE is associated with severe asthma in ABPA and SAFS. Traditionally, only Af-IgE and total IgE are currently used for diagnosis and monitoring therapy and disease progression in ABPA and SAFS. The study in chapter 3 highlights the need for widening IgE-based sensitivity testing and to increase the allegen testing panel and kits to capture other unknown allergens that may be relevant in disease
pathogenesis. Moreover, a decline in serum IgE levels in ABPA during follow up appears not to predict clinical outcomes [313] and the central role of an elevation of tlgE levels in asthma development has also been questioned [320].

7.3.3 **Aspergillus is more abundant in patients with severe asthma than previously thought. Steroids treatment increase airway fungal load. There is a possible link for airway microbiome with asthma severity.**

The study in chapter 4 has demonstrated that the microbiome community in ABPA, SA and SAFS are diverse. Fungi and bacteria are probably more common in severe asthma population than previously thought and microbial population may be linked with asthma severity. This study provides a possible explanation for the improvement in asthma severity in patients treated with anifungals in chapter 5. There are still unanswered questions regarding how to distinguish pathogenic from non-pathogenic organisms.

The use of steroids increase fungal burden in the lungs and should be considered carefully before being prescribed in these patients. Whereas, antifungal therapy has a dramatic impact on both microbiome and mycobiome, they appear to significantly increase Pseudomonas, *Staphylococcus* and *Haemophilus* relative to untreated patients.

The observation of increased proportions of *A. fumigatus* in asthma suggests a number of implications for treatment or aetiology of this disease.

This study has demonstrated that non-culture molecular technique (qPCR) is more sensitive tool than the current HPA culture method.

7.3.4 **Voriconazole &posaconazole improves asthma severity, QOL and radiological abnormalities in SAFS &ABPA. Voriconazole has a number of side effects, most of them mild.**

In chapter 5, the observed clinical response over time to voriconazole or posaconazole is a clinically significant finding that has implications for management of patients with poorly controlled severe asthma who are allergic to fungi and support previous suggestions linking mold sensitization with severe asthma attacks requiring hospital admission [136].

In this study, the magnitude of voriconazole and or posaconazole effect on symptoms of asthma seen is encouraging. However, the possibility of side effects need to be borne in
mind when considering voriconazole. Posaconazole is safer than voriconazole but is more expensive.

This study has also demonstrated that while voriconazole is efficacious, the possibility of serious adverse events is a strong possibility and both patient advice and close monitoring in the first few weeks of therapy is appropriate. Larger randomized studies need to be done to determine the optimal doses, duration of therapy and long term effect. Novel azole antifungals that minimize the adverse events observed in this study could be promising.

7.3.5 Overall efficacy of NAB in this group of patients is poor and associated with bronchospasm. NAB is effective in those that tolerate the initial bronchospasm.

The results of chapter 6 suggests that NAB is probably not safe and the lack of efficacy is largely due short duration of therapy due to intolerable side effects in about 60% patients. Immediate bronchospasm in about 33% cases was the main clinical challenge in this study. However, the excellent response in 3 patients, suggest it may be considered when other alternatives have been exhausted. Overcoming the initial bronchospasm may improve tolerability.

The frequency and nature of AEs observed in this study differ from historical literature due to a high frequency of bronchospasm. Bronchospasm has not been widely reported with the use of NAB except for few incidences reported in lung transplant patients [428]. It is possible that the observed large frequency of bronchospasm in our patients may be due to the airway hyperresponsiveness which characterize most asthmatics. In some previous studies, all patients with severe asthma were excluded and may partly explain why those studies hard less frequent bronchospasm compared to our patients [429]. Another possibility for the differences might be the presence of denervated lungs in transplant patients.

Antifungal treatment by inhalation if successful could provide a new, simple therapeutic approach in patients unsuitable for azole antifungals or in whom there has been no clinical benefit with first line therapies, azole resistance or where it has been difficult to maintain adequate therapeutic drug levels with azole antifungal. Another advantage is that local administration of NAB to the airways also decreases the risk of drug interactions that is commonly observed with azole antifungals. It allows higher lung tissue deposition and
concentrations and only limited absorption into blood, as previously illustrated by negligible serum concentrations post dose [421].

7.4 Directions for Future Work

7.4.1 IL-17A in ABPA, SA and SAFS

Our current conceptual framework for the pathogenesis of these diseases is that they are caused by an exaggerated Th2 CD4+ allergic inflammatory response. However, multiple immunopathological processes have been documented in each group and the mechanisms involved in the initiation and persistence of airway inflammation are potentially quite diverse and incompletely understood.

The findings in this study of the possible pathogenic role of IL-17A pathway and association with fungal airway colonisation and severe asthma is therefore of major interest.

- For such patients, the precise description of their IL-17A and fungal status and their categorization into well-characterized subpopulations could facilitate the development of stratified and targeted therapies. Future studies should be done on large patient populations with good power.

The 2 severe asthma groups (SAFS, SANFS) were similar. The lack of difference may signify that IL-17A is simply an independent risk factor for severe asthma, independent of cause.

- Further studies in large populations, looking at genetic and molecular differences may provide some more useful information to characterise these asthma groups and for further phenotyping and endotyping.

Results of the present study in chapter 2 are based on the majority of patients currently on antifungals, inhaled and oral corticosteroids. These drugs could have altered the results in this chapter.

- Studies involving patients not on antifungal agents could provide more useful results.
- Future work to design molecular targets against IL-17A could provide some breakthrough in the treatment of SA, SAFS and ABPA.
We did not study Th2 related cytokines. We did not measure other Th-17 related cytokine (IFN-gamma, IL-4, IL-22, IL-23 and IL-25) concentrations in plasma and BAL supernatants. We did not also measure Th-2 related cytokines (IL-4, IL-5 and IL-13) due to inadequate BAL samples as our study also involved using the same sample for other projects such as microbiome work and Treg cells. It is likely that future projects that measure these additional cytokines together with IL-17A could provide additional clinical and scientific information.

- Future studies that combine IL-17A and Th2-like cytokines could provide more useful information to further phenotype and endotype these patients for future targeted therapy.

In this study, we found that patients who were currently on azole antifungal treatment had significantly higher %CD4+IL-17A expression compared to patients who were never treated with antifungals. There are no prospective studies that have assessed IL-17A expression in humans.

- Our data may suggest the potential link between azole antifungal metabolism or action and IL-17A, but this needs further investigations involving larger studies including molecular and metabolism studies.
7.4.2 IgE expression in ABPA and SAFS.

The results of this study in chapter 3 highlights the need to measure IgE-based sensitivity to all allergens. Existing knowledge on the use of these IgE antibodies has been mostly limited by the inability to measure IgE-based sensitivity to all allergens which consequently has limited our understanding as to which of the allergen-specific IgE antibody (sIgE) might be useful for monitoring fungal allergic asthma. Another problem is the lack of agreement and standardisation between different serological tests and there are questions as to whether IgE production also reflects factors other than allergy, possibly non-allergic inflammation [321].

Thus, future work will aim to standardise serological tests and measure IgE-based sensitivity to all allergens in ABPA and SAFS. Such approaches could provide useful information to understand the role that IgE plays in the pathogenesis of these diseases. Further, larger prospective studies should be conducted to determine the cause effect relationship.

7.4.3 Lung microbiome in ABPA, SA and SAFS.

Further studies utilising high specific techniques on patients who are not on concomitant antifungal or corticosteroids therapy would provide better understanding of the pathogenic role of lung microbiomes.

It is not clear, whether the observed microbiome is pathogenic or not.

- Studies that aim to distinguish pathogenic from non-pathogenic commences will help to solve this problem and have clinical implication.
- The role of steroids in fungal asthma patients need to be studied further in larger and well characterised populations.

One striking observation in this study is diversity of isolates in the A. fumigatus group. Tens and probably hundreds of variant strains were found in each pair of lungs sampled. Several questions arise from this;

- Do lungs of individuals with excess mucus (a characteristic feature of asthma) act as efficient spore traps and simply accumulate fungal spores or airborne hyphae over time? Does the excess mucus ‘protect’ A. fumigatus from phagocytic attack?
- Or is the recently published work on galactosaminoglycan and adherence to galectin-3 expressed on epithelial cells, imply that mucus is irrelevant, and the lungs of asthmatics are ‘super-sticky’ for A. fumigatus?

Therefore, future studies should be done to answer these specific questions.
7.4.4 Role of voriconazole and posaconazole in ABPA and SAFS

The magnitude of voriconazole and or posaconazole effect on symptoms of asthma seen is encouraging.

- Larger prospective studies are required, including randomised, double blinded studies with good power.
- The possibility of adverse events linked to voriconazole need to be taken into account and needs to be monitored.

Our results differ from a double-blind, placebo-controlled, randomized trial published later by Agbetile and colleagues who did not demonstrate improvement in QOL or reduction in severe exacerbations after 3 months of treatment with voriconazole [389]. The duration of treatment 3 months is rather short, and exacerbations are probably not the best endpoint for such a study. However, our retrospective analyses demonstrate that the duration of treatment needed to be at least 6 months before clinical response was objectively observed.

- There is also a need to standardize the duration of antifungal therapy, which we suggest should not be less than 6 months, and 12 months may be preferable in future studies [400].

Despite a number of limitations in our study such as the retrospective nature of the study, lack of randomization, subjective reporting by different clinicians and small sample size, our findings are intriguing, novel and need

- Further confirmation in a larger prospective study requiring more than 9 months duration of therapy as demonstrated by our findings.
7.4.5 Nebulised Amphotericin B in ABPA and SAFS

In Chapter 6 of my thesis, patients who went beyond 6 months of treatment had significant benefit to NAB. It is not clear which patients are likely to respond or develop adverse events. Further randomized, placebo controlled, double blinded studies need to be conducted to establish the optimal dose range (dose, frequency), nebulizer type, pressures and identification of patients who may respond. There is also need to compare liposomal with none liposal formulations in a randomized controlled trial with respect to efficacy and adverse events.

It is not clear which patients are likely to respond. However, the characteristics of these 3 responders could provide some insight as to which patients might be candidates for clinical response with less adverse events in particular NAB. These 3 responders (ABPA, 2; SAFS, 1) had better lung function, less obstructive spirometry and more aspergillus sensitization than the non-responders (table 6.2). In addition, the responders were relatively younger and all were never smokers.

- Studies selecting patients with or without these characteristics could provide better understanding with regard to potential candidates for NAB.

If successful, antifungal treatment by inhalation could provide a new, simple therapeutic approach in patients unsuitable for azole antifungals or in whom there has been no clinical benefit with first line therapies, azole resistance or where it has been difficult to maintain adequate therapeutic drug levels with azole antifungal. Another advantage is that local administration of NAB to the airways also decreases the risk of drug interactions that is commonly observed with azole antifungals. It allows higher lung tissue deposition and concentrations and only limited absorption into blood, as previously illustrated by negligible serum concentrations post dose [421]

Further studies need to be conducted to establish the optimal dose range (dose, frequency), nebulizer type, pressures and identification of patients who may respond. Overcoming the initial bronchospasm may improve tolerability.
8. CONCLUSION

IL-17A, SE-IgE, and lung microbiome are associated with asthma severity. Steroid use in these patients may increase airway fungal load. Whereas voriconazole and posaconazole are efficacious, the use of NAB is associated with significant bronchospasm. Larger studies are needed.

The findings in this thesis are significant and have implications for clinical practice and research. It is hoped that the continuation of the work presented in this thesis to identify the role of IL-17A, lung microbiome and IgE in the immunopathogenesis of severe asthma, SAFS, ABPA including antifungal therapy will provide directions for greater understanding and treatment of these devastating chronic lung diseases.
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APPENDICES

A1.1: Asthma Control Test™
This survey is designed to help you describe your asthma and how your asthma affects how you feel and what you are able to do. To complete it, please circle the answer for each question.

In the past 4 weeks, how much of the time did your asthma keep you from getting as much done at work, school, or at home?
1. All of the time
2. Most of the time
3. Some of the time
4. A little of the time
5. None of the time

During the past 4 weeks, how often have you had shortness of breath?
1. More than once a day
2. Once a day
3. 3 to 6 times a week
4. Once or twice a week
5. Not at all

During the past 4 weeks, how often did your asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?
1. 4 or more nights a week
2. 2 or 3 nights a week
3. Once a week
4. Once or twice
5. Not at all

During the past 4 weeks, how often have you used your rescue inhaler or nebulizer medication?
1. 3 or more times per day
2. 1 or 2 times per day
3. 2 or 3 times per week
4. Once a week or less
5. Not at all

How would you rate your asthma control during the past 4 weeks?
1. Not controlled at all
2. Poorly controlled
3. Somewhat controlled
4. Well controlled
5. Completely controlled

(Adapted from with permission from the copyright owners)
A1.2 Mini-AQLQ-Juniper (adopted with permission from Prof E Juniper)

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

<table>
<thead>
<tr>
<th></th>
<th>Totally Limited</th>
<th>Extremely Limited</th>
<th>Very Limited</th>
<th>Moderate Limitation</th>
<th>Some Limitation</th>
<th>A Little Limitation</th>
<th>Not at all Limited</th>
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<tbody>
<tr>
<td>12. STRENUOUS ACTIVITIES (such as running, exercising, running up stairs, sports)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>13. MODERATE ACTIVITIES (such as walking, housework, gardening, shopping, climbing stairs)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>14. SOCIAL ACTIVITIES (such as talking, playing with pets/children, visiting friends/relatives)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>15. WORK-RELATED ACTIVITIES (tasks you have to do at work*)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>ANALYSIS</th>
<th>RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVERALL SCORE</td>
<td>Mean of all 15 responses Add all 15 responses together and divide by 15</td>
</tr>
<tr>
<td>SYMPTOMS</td>
<td>Mean of the responses to items 1, 4, 6, 8, 10 Add the responses to these 5 items and divide by 5</td>
</tr>
<tr>
<td>ACTIVITIES</td>
<td>Mean of the responses to items 12, 13, 14, 15</td>
</tr>
<tr>
<td>EMOTIONS</td>
<td>Mean of the responses to items 3, 5, 9a</td>
</tr>
<tr>
<td>ENVIRONMENT</td>
<td>Mean of the responses to items 2, 7, 11</td>
</tr>
</tbody>
</table>

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A1.2 Mini-AQLQ-Juniper (adopted with permission from Prof E Juniper)
A2.1 SOP for BAL collection and processing

BAL collected

Filter through 100μM filter in 50ml falcon

Spin filtrate at 400g for 10 min at 4°C

Re-suspend cells in 5ml Media (supplemented)

Perform cell viability count with trypan blue 50:50 mix (use MAAS calculation) for aliquoting cells at 2x10^6 cells per tube (3 if possible)

Leave on ice for collection – copy worksheet detailing patient info and cell number collected.

Do cell staining and FACS immediately. Put excess cells in freezing mix and store either in Mr Frost under liquid nitrogen or at -80°C
A2.2: SOP FOR BLOOD PBMC COLLECTION AND PROCESSING

- Blood collected in 2x 10ml green topped tubes
- Pipette blood onto ficoll / lymphoprep 2:1 blood:lymphoprep

Spin at 400g with no brake setting on centrifuge for 30 min

Harvest PBMCs from middle layer and dispose of remaining sample

Wash PBMC with RPMI (supplemented) and pellet cells by spinning at 400g for 10 mins

Resuspend cells in 5ml RPMI (supplemented) and perform cell viability count

Perform cell viability count with trypan blue 50:50 mix (use MAAS calculation) for storing cells at 2x10^6 cells per tube (3 if possible)

Spin cells at 400g for 10min to pellet, remove supernatant for waste and resuspend in appropriate volume of cryopreservation media or wash medium. Pipette up and down gently to mix cells. Stain and do FACS analysis

Aliquot extra cells into cryovials, place tubes into Mr Frosty, freeze at -80, and finally transfer to liquid nitrogen for long term storage after 24 hrs / weekend. Label tubes (and SLAB) with patient no / DOB / initials sample type and volume.
### Sample Recording Form

#### a) Visit /subject details

<table>
<thead>
<tr>
<th>Bronchoscopy date</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchoscopy Visit</td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sample source</td>
<td>(Bronchoscopy Study)</td>
</tr>
<tr>
<td>Category</td>
<td>HNC/SAFS/ABPA / SANFS</td>
</tr>
</tbody>
</table>

#### b) Sample details

**Appearance of BAL sample**

<table>
<thead>
<tr>
<th>BAL Sample</th>
<th>Clear sample</th>
<th>Bloody sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of BALF retrieved</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cell count</td>
<td>(A)</td>
<td></td>
</tr>
<tr>
<td>AM cell count</td>
<td>(B)</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte cell count</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM / Lymphs ratio of clear sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No of lymphs in bloody Sample. (Bloody AM count / ratio)</td>
<td>(C)</td>
<td></td>
</tr>
<tr>
<td>Total cell count: (A+B+C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC present?</td>
<td>Y / N</td>
<td>Over Ficol?</td>
</tr>
</tbody>
</table>

#### Paired blood taken

<table>
<thead>
<tr>
<th>Volume drawn (ml)</th>
<th>PBMCs isolated (x10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>Cell count/ml blood</th>
<th>FACs assays done</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>(b)</td>
<td>(b/a)</td>
<td></td>
<td>A B C D</td>
</tr>
</tbody>
</table>

Y N
A2.2: BAL sample analysis recording form: Recorded details included:- Visit /subject details (a), appearance of BAL sample (c) and assays performed on BAL. Because of multiple projects done on collected BAL sample, most of the subjects didn’t have cytospin done and cytospin is not included in this thesis.

A2.4  **SOP for Surface and intracellular staining protocol for IL-17A**

- Stain surface molecules with 5μl (APC-CY7 anti-CD4) conjugated antibodies for tube A and B.
- Incubate 30 mins in the dark
- Fill tubes with FACS wash (PBS with 1% FCS),
- Centrifuge, decant (tip and blot)
- Add 1ml Fix/Perm buffer (fresh)
- Incubate 4°C for 30-60mins
- Wash with 2ml 1x perm buffer, centrifuge + decant
- Add 7μl each of PerCP-Cy™5.5 -conjugated anti-Human IL-17A antibodies or IgG controls
- Incubate 4°C 30mins in the dark
- Wash twice with 2ml 1x perm buffer, centrifuge + decant
A2.5 Buffers during for IL-17A intracellular staining

Fix perm buffer
Fix perm concentrate (A)
Dilute 1 in 4 ie 1ml A +3ml diluents (B)

Perm wash buffer
Dilute 10x perm buffer with dH2O
I.e. 5ml C + 45ml water

Chapter 3 appendices

A3.1.1 Specific IgE testing using the immunoCAP system

Specific IgE antibody assays measure the level of IgE antibodies present in serum that are directed against specific defined allergens. The immunoCAP is a fluorescent enzyme immunoassay (FEIA) that measures allergen-specific IgE in human serum. It can measure IgE antibodies specific for animal, plant, fungi and other allergens such as silk, latex.

Principles of immunoCAP

Method Used: Fluorensyme Immunoassay.

This assay detects antibodies present in serum by binding them to a specific target immobilised on a solid phase. The solid phase, known as ‘ImmunoCAP’, is a sponge with a very large surface area which enables specific antibodies present in the serum to bind to the allergen on the sponge surface. Free allergen specific IgE molecules present in the serum sample are captured by anti-IgE antibodies that are covalently bound to the ImmunoCAP. After washing, enzyme-conjugated antibodies directed against human IgE are added, and become bound to the allergen specific IgE molecules captured from the serum sample. After incubation, unbound enzyme anti-IgE is washed away and the bound complex is then incubated with a substrate. The product formed from the action of the enzyme on the substrate is fluorescent. The reaction of enzyme upon substrate is stopped by the addition of a stop solution, the fluorescence in the separated solution measured. The fluorescence measured is proportional to the concentration of allergen specific IgE in the serum sample. Within every batch of patient samples, standards, controls and/or calibrators are run. A graph is drawn (electronically) of fluorescence values against the known IgE concentration in the standards, and the fluorescence values measured for patient samples are interpolated in
this graph in order to derive the values of total serum IgE concentration present in the initial patient samples. The fluorescence is proportional to the concentration of allergen specific IgE antibodies in the serum sample. To evaluate the test results, the response for the patient samples is compared directly to the response for the calibrators. The level of allergen specific IgE contained in the serum can then be quantified. With over 650 allergens and 90 components available, ImmunoCAP Specific IgE offers precise and comprehensive antibody testing.