The contribution of outdoor fungal spores to child and adolescent asthma hospitalisations; lung function and airway inflammation

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Melbourne School of Population and Global Health

The University of Melbourne
Dedication

To Lynda, Suu Kyi, Nelson and Shan
Thesis Abstract

Outdoor fungal spores are among the most common aerobiological particles in the air we breathe. Although a limited number of outdoor fungal species are recognised as exacerbating agents of a number of allergic and respiratory conditions, their contribution towards asthma exacerbation is unclear, particularly among children and adolescents. Moreover, we have limited understanding of the impacts that inhaled fungal spores have on lung function or airway inflammation, which may be pre-clinical signs of asthma exacerbation. Therefore the aim of my doctoral research is to examine whether there are associations between common outdoor fungal spores and child and adolescent asthma hospitalisations, and also to explore if short term exposure to ambient fungal spores is associated with lower lung function or airway inflammation.

In Chapter 2, a comprehensive literature review highlights that there are significant knowledge gaps in the contribution of outdoor fungal spores to child and adolescent asthma hospitalisations, lung function and airway inflammation. In order to address some of these gaps, my specific research objectives of this doctoral research were to: (a) systematically synthesise the current evidence as to whether outdoor fungal spores were significant triggers of child and adolescent asthma exacerbations resulting in health service attendances; (b) investigate if there were associations between short term exposure to outdoor fungal spores and child and adolescent asthma hospitalisations; (c) explore if any of these associations were modified by air pollutants, grass pollen, age group, sex, presence of human rhinovirus infection, or fungal sensitisation status; (d) investigate if there were associations between outdoor fungal spores and lower lung function or airway inflammation, and (e) explore if any associations were modified by air pollutants or pollen, age group or fungal sensitisation status.

In Chapter 3, my systematic review found that only a small number of studies have been conducted, predominantly in countries located in the northern hemisphere. Children with fungal sensitisation appeared to be at greater risk of asthma hospitalisations. Severity of asthma exacerbation may vary between fungal spore taxa. There were inconsistent findings, possibly due to the lack of accounting for other significant triggers of asthma exacerbations.
In Chapter 5, my ecological case-crossover study of child and adolescent asthma hospitalisations in south-west Sydney found that there were associations with *Coprinus, Periconia, Chaetomium, Ganoderma* and *Cerebella*, with same day and lagged effects. There was evidence of effect modification by sex, with girls demonstrating stronger associations with *Cladosporium, Coprinus* and *Chaetomium* than boys. Age also acted as an effect modifier with older adolescents, demonstrating stronger associations with *Coprinus* and *Ustilago/smuts* than those aged under 14 years.

In Chapter 6, my case-crossover study of child and adolescent asthma hospitalisations in Melbourne found associations between ambient *Alternaria, Coprinus, Drechslera* and *Leptosphaeria*, with signs of same day and lagged effects. These associations were independent of having a human rhinovirus respiratory infection. There was some evidence that *Cladosporium* sensitisation acted as an effect modifier.

In Chapter 7, my cross-sectional study of a high allergy risk cohort comprising of children, adolescents and adults found differing associations between outdoor fungal spores and lower lung function and presence of airway inflammation. Fungal sensitisation acted as an effect modifier with some fungi and parameters of lung function and markers of airway inflammations.

Overall, this research has contributed to filling some of the gaps in our current understanding of the contribution of outdoor fungal spore exposures to child and adolescent asthma hospitalisations, lower levels of lung function and airway inflammation. This research demonstrates that the contribution of outdoor fungal spores may be under-estimated. Importantly, species that have not been previously found to be associated with asthma exacerbations, but are genetically related to well-known fungal triggers of asthma exacerbation, may warrant further investigation. Future research needs to improve the modelling of dispersion and distribution of outdoor fungal spores on spatial and temporal levels. The development of reliable and standardised fungal reagents for detection of allergic sensitisation is needed. The presence of fungal spores in the air is important for the ecosystem, and may not be controlled on a large scale, but understanding how to prevent their effects on respiratory health will benefit public health.
Declaration

This is to certify that

I. The thesis comprises only my original work towards the PhD, except where indicated in the Preface and Acknowledgements.

II. Due acknowledgment is made in the text to all the other materials used.

III. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

………………………………………

Rachel Tham

20 April 2017
Preface

Within this thesis I have included three published first author papers which, I published with multiple co-authors, as thesis chapters. In addition to these publications, I authored and co-authored 10 peer-reviewed papers and one peer-reviewed book chapter during my PhD. These are listed on pages vi to vii.

I initiated and wrote the first drafts of all the papers included as Chapters in this thesis. I led the writing of all revisions in these published papers. I designed and performed the systematic review and the statistical analyses in each of the papers. My co-authors provided assistance with primary data collection in two studies: (1) Melbourne Air Pollen and Child and Adolescent Health (MAPCAH) Study; and (2) the Melbourne Atopy Cohort Study (MACS). My co-authors provided the fungal spore and pollen grain data. My co-authors also contributed to improving the analytical design, interpretation of the results and editing of the manuscript drafts. Hence, I have provided signed authorisation forms from each co-author of each published chapter, in addition to declarations of thesis with publications from my principal supervisor. All work included in this thesis was undertaken after my enrolment as a higher degree research student.

I declare that this work contains no material which has been submitted or accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously written or published by another person, except where due reference has been made in the text.

My PhD scholarship was jointly funded by the National Health and Medical Research Council Australian Government Research Training Program Scholarship (APP1055754); and the National Health and Medical Research Council Centre for Research Excellence, Centre for Air Quality and Health Research and Evaluation (CAR) Top-up Scholarship. The MAPCAH Study was funded by the National Health and Medical Research Council (Project ID: 541934). The MACS was funded by Nestlé Australia, the National Health and Medical Research Council and the Asthma Foundation of Victoria.
Acknowledgements

Firstly I would like to sincerely acknowledge and deeply thank my principal supervisor, Associate Professor Bircan Erbas, and my co-supervisors, Professor Shyamali Dharmage and Dr Adrian Lowe for their encouragement, guidance, and support along each stage of the PhD journey. I could never have completed this journey of discovery without their critical input into the study designs, analytical approaches, interpretation of the results, writing, and knowledge communication and translation. I also acknowledge and thank my Advisory Panel, Professor Jodie McVernon and Associate Professor Geoff Morgan, for their insights and guidance. This PhD research has included numerous intersectoral collaborators who have provided input at different times along the way, and without whom, I could not have brought this research together and published aspects of it in leading academic journals in this field. In particular I thank Professor Michael Abramson, Dr Philip Taylor, Professor Connie Katelaris, and Dr Don Vicendese. I extend my gratitude to Professor Ed Newbigin, Emma Lewis and Pamela Burton for providing many years of ambient fungal and pollen data that has taken much effort and expertise to collect, to identify and to count. I am deeply honoured and humbled to have worked alongside and learnt from such dedicated experts. My supervisors and co-authors were always responsive to my questions and providing input into my manuscript drafts and revisions which enabled me to publish three PhD chapters in leading journals in their respective fields.

I was privileged to study with talented and supportive postgraduate students who also became special friends. I particularly thank those in the Allergy and Lung Health Unit, and those with whom I shared a basement office for nearly four years. I especially acknowledge and applaud my PhD colleagues at other universities who supported me through the ups and downs with smiles and understanding: Dr Don Vicendese and Dr Nina Hinko-Najera.

I sincerely thank the National Health and Medical Research Council for awarding me a PhD Research Training Scholarship. I deeply thank the Centre for Air Quality and Health Research and Evaluation (CAR) for endowing me with top-up scholarships for three and a half years, providing me with the opportunity to participate in a range of symposia and training workshops, and funding a travel scholarship for me to attend the International
Society for Environmental Epidemiology Conference 2016 in Rome, Italy. I thank the University of Melbourne for awarding me a Melbourne Abroad Traveling Scholarship to attend the European Respiratory Congress 2015 in Munich. I also thank Melbourne School of Population and Global Health for awarding me PHIRST (Population Health Investing in Research Students’ Training) travel scholarship to attend the International Society for Environmental Epidemiology Conference 2016 in Rome, Italy.

I would not have undertaken this PhD research without the inspiration afforded to me by Associate Professor Tina Bell and Associate Professor Peg Levine, who always demonstrated such faith in me. I would like to thank Dr Mary-Faeth Chenery for listening and providing a non-epidemiological perspective to my research findings.

When I was 15 years old, I lost a school friend, Belinda Phillips. Her life was tragically cut short by a severe asthma exacerbation. I could not make sense of this immensely sad loss at the time, but I hope Belinda has continued to be by my side and feels that this research is making a difference to our understanding of asthma and asthma exacerbations.

My heartfelt thanks go to my parents, Vui Ling and Kathy, for always encouraging me to be the best I could be and never give up. I am deeply sad that Mum is not alive to share this step of my life. My PhD journey started with me finding Mum’s birth roots and uncovering some truths – this has kept me inspired and resilient.

My deepest and heartfelt gratitude is reserved for my life partner, Lynda Poke, who has been tireless in her love, commitment to our family, and immense support throughout – I could never have done this without her.
Publications, presentations, research contributions and grants

The publications, conference presentations, grants and research contributions resulting from the course of this PhD are listed as follows:

First author publications arising from this thesis

Peer-reviewed research publications


Other co-authored publications during the PhD

Peer reviewed journal publications


**Peer reviewed book chapter**


**Conference presentations**


**Conference abstracts submitted**

Grants

2016: Population Health Investing in Research Students’ Training (PHIRST) Grant - $1200

2016: NHMRC Centre of Research Excellence - Centre for Air quality and health Research and evaluation (CAR) Travel Grant - $1500

2014: Melbourne Abroad Travelling Scholarship – The University of Melbourne $1500

Teaching and supervision

Clinical teacher to undergraduate (3rd, 4th & 5th year) dental students in Bachelor of Health Sciences in Dentistry/Master of Dentistry at La Trobe University.

Teaching Associate to Bachelor of Medicine and Bachelor of Surgery 3rd year students – Evidence Based Clinical Practice and Environmental Medicine.

Clinical and Viva Examiner for 3rd year dental students at La Trobe University.

Other research activities and contributions


Reviewer for CAR Seeding Grant Applications 2013-2017

Chaired a oral presentation session at the Cleaner Air, Better Health: CAR Annual Symposium Sydney, 5 May 2016, Australia

Invited key speaker for the College of Community Dentistry in Colombo, Sri Lanka, October 2016 “Getting started in systematic reviews and meta-analysis as applied to public health and health related disciplines”.

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<td>Australian Institute of Health and Welfare</td>
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<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
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<tr>
<td>CAR</td>
<td>Centre for air quality and health research and evaluation</td>
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<tr>
<td>CFU/m³</td>
<td>Colony forming units per cubic metre</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>CRE</td>
<td>Centre of Research Excellence</td>
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<tr>
<td>DALY</td>
<td>Disability adjusted life years</td>
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<tr>
<td>EBC</td>
<td>Exhaled Breath Condensate</td>
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<tr>
<td>ECRHS</td>
<td>European Community Respiratory Health Survey</td>
</tr>
<tr>
<td>ERS</td>
<td>European Respiratory Society</td>
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<tr>
<td>FEF₂₅%-₇₅%</td>
<td>Mid expiratory flow</td>
</tr>
<tr>
<td>FeNO</td>
<td>Fractional exhaled Nitrogen Dioxide</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
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<td>GINA</td>
<td>Global Initiative for Asthma</td>
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<td>HRV</td>
<td>Human rhinovirus</td>
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<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
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<td>ISAAC</td>
<td>International Study of Asthma and Allergies in Childhood</td>
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<td>MACS</td>
<td>Melbourne Atopy Cohort Study</td>
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<tr>
<td>MAPCAH</td>
<td>Melbourne Air Pollen Child and Adolescent Health</td>
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<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
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<tr>
<td>NO</td>
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<td>O₃</td>
<td>Ozone</td>
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<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PEFR</td>
<td>Peak expiratory flow rate</td>
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<td>PHIRST</td>
<td>Population Health Investing in Student Training</td>
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<tr>
<td>PM</td>
<td>Particulate matter</td>
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<tr>
<td>PM₁₀</td>
<td>Particulate matter with aerodynamic diameter &lt;10um</td>
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<tr>
<td>PM₂·₅</td>
<td>Particulate matter with aerodynamic diameter &lt;2.5um</td>
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<tr>
<td>ppb</td>
<td>Parts per billion</td>
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<tr>
<td>ppm</td>
<td>Parts per million</td>
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<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RV</td>
<td>Residual volume</td>
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<tr>
<td>SE</td>
<td>Standard error</td>
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<tr>
<td>TAHS</td>
<td>Tasmanian Health Study</td>
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<tr>
<td>TLC</td>
<td>Total lung capacity</td>
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<tr>
<td>UK</td>
<td>United Kingdom</td>
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<tr>
<td>μL</td>
<td>Microlitre</td>
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<tr>
<td>μm</td>
<td>Micrometres/microns</td>
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<tr>
<td>μMol</td>
<td>Micromoles</td>
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<tr>
<td>USA</td>
<td>United States of America</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>YLD</td>
<td>Years of life living with a disability</td>
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Chapter 1
Introduction

1.1 Rationale

Outdoor fungal spores are among the most common foreign aerobiological particles we breathe in daily (1). The interaction of fungal spores in the respiratory system is complex and their effects include mediation of allergic reactions in addition to the release of mycotoxins that stimulate pathological changes in the airways (2). Although a limited number of outdoor fungal species are recognised as exacerbating agents of a range of allergic and respiratory conditions (3-5), the role of outdoor fungal spores in asthma exacerbations is unclear, especially in children and adolescents (6). Moreover, we have limited understanding of the impacts that fungal spore inhalation has on lung function and airway inflammation (7-9) which are important markers of lung health status (10) and may be pre-clinical signs of asthma exacerbation (11).

The aim of this doctoral research is to investigate if outdoor fungal spores are associated with child and adolescent asthma hospitalisations, lower lung function or airway inflammation.

This introduction will briefly cover the current understanding of asthma, asthma exacerbations leading to hospitalisation, airway inflammation and lung function; biology of outdoor fungi; airway responses to inhaled fungal spores; and the four research questions proposed for this research.

1.2 Asthma

Asthma is a complex heterogeneous chronic inflammatory syndrome affecting the respiratory system and is characterised by episodic symptoms that include recurrent wheezing, breathlessness, chest tightness, and coughing. These symptoms result from widespread but variable airflow limitations due to a range of pathological processes occurring in the airways. These processes may include airway smooth muscle contraction, airway inflammation, mucous secretion and bronchial hyper-responsiveness (12). Asthma is often reversible, either spontaneously, or with treatment (13).
Asthma can start early or much later in life. It may be a chronic stable condition that has minimal impact on morbidity or quality of life. However, it may present as asthma exacerbations that may be mild or severe. These exacerbations can vary in terms of triggers for onset and response to therapeutic interventions (13).

1.3 Asthma is a significant public health problem

Over the past 20 years or so, the prevalence of asthma has been increasing significantly to epidemic proportions in both developed nations, and countries transitioning to developed economic status (14). It is a significant global public health problem affecting an estimated 330 million people of all ages globally (13). The global prevalence of asthma in children is estimated to be 11% in the 6 to 7 year age group and 13% in the 13 to 14 year age group (13).

The countries with the highest estimated overall prevalence of clinical asthma include Australia, New Zealand, and the United Kingdom (15). In Australia, the prevalence of current asthma among children (aged 0 to 15 years) is estimated to be 10.4% and among adults is estimated to be 9.8% (16).

Asthma has substantial impacts on the quality of life of people living with this condition. It can affect participation in activities of daily living, particularly physical exercise (17); be associated with impaired breathing during sleep (18) or increase symptoms of sleepiness (19); disrupt attending school and/or work (20); affect weight (17) and height development (21); and exacerbations often require pharmacological or medical intervention (22).

It is clear from these concerning statistics that asthma creates substantial health and economic burdens on individuals, families and communities locally here in Australia, and globally. Hence asthma is recognised as a significant and increasing public health problem and is classified as a National Health Priority Area by the Australian government (23).

1.4 Asthma exacerbations, triggers and risk factors

Asthma exacerbations are airway obstruction with sudden onset symptoms, often requiring treatment with systemic corticosteroids, medical treatment in an emergency
department, or hospitalisation (24). In Australia, sudden-onset severe asthma exacerbation is a major cause of hospitalisation for children and adolescents (25, 26).

The suggested triggers for asthma exacerbation are numerous and include respiratory viral infections, especially human rhinovirus (HRV) (27, 28); allergens, such as house dust mite (29), pollen (30, 31), or some fungi (32, 33); air pollutants (34, 35); tobacco smoke (36), thunderstorms (in conjunction with high levels of respirable aeroallergens, particularly pollen, and particulate matter) (37, 38); medications such as non-steroidal anti-inflammatory drugs (39), hormones (menopause asthma) (40); exercise (41); stress (42). Given the heterogeneity of asthma it is very likely that different triggers will be more important for some asthmatics than others and their sensitivity to the exposures may also vary over time. Child and adolescent asthma exacerbations appear to be most vulnerable to HRV infections, allergic reactions to allergens (particularly pollen, house dust mites), thunderstorms, air pollutants and exercise (22, 43).

In addition, the ‘two-hit hypothesis’ may act in some situations where (44) some exposures may modify the effect of other exposures and there may be synergistic interactions that may lead to a more severe asthma exacerbation (45). An example of this is the possible interactions between respiratory viruses and exposure to allergens that may lead to increased inflammatory responses in the airways and severe asthma exacerbation (46). It has been proposed that viral infection causes airway inflammation that primes the airway for increased allergic response to exposure to allergens, such as fungal spores or pollen grains (24). There is increasing evidence that some aeroallergens can interact with other environmental components, such as high levels of air pollutants, leading to varying levels of asthma exacerbations in people of different ages and gender (45, 47, 48). Some individual characteristics have been suggested as potential effect modifiers. Individuals that are sensitised to specific allergens will express more severe allergic reactions and so their asthma exacerbation risk may be higher (49). Age and gender have been suggested as a potential effect modifier possibly related to biological differences in lung and airway size, volume and growth rates (50), changes in levels of sex hormones (51), or to socially-linked variations in exposures to physical activity, cosmetic products, cigarette smoking (52) or obesity (48, 53-55).
Pollen (56, 57) and indoor fungi (58, 59) have been the focal aeroallergens studied to date, and only limited numbers of studies have examined the contribution of outdoor fungal spores (2).

1.5 Airway inflammation and lung function

Airway inflammation and lung function are important markers of lung health. The presence of elevated levels of airway inflammation biomarkers can be a signal of respiratory system inflammation and is thought to be a contributor to asthma pathogenesis (11). Airway obstruction associated with asthma is detected by reductions in lung function parameters. Most studies that have examined associations between environmental exposures and lung function and airway inflammation have focussed on exposure to tobacco smoke (active, passive and in utero), and air pollutants, particularly traffic-related air pollution. Few observational studies have been published that examined associations between exposure to fungal spores and lung function in children (32, 60-63) and adults (64-66). The results of these studies indicated adverse effects, but the level of evidence is weak. To date, no epidemiological studies have been published that examined associations between outdoor fungal spores and airway inflammation in humans. As long-term impaired lung function can lead to chronic lung conditions such as asthma it is important to assess whether exposure to outdoor fungal spores is associated with increased airway inflammation and/or reduced lung function.

1.6 Outdoor fungal spores

Fungal spores are unicellular organisms that are ubiquitous in the atmosphere and people are exposed to them in both indoor and outdoor environments. It has been estimated that there are approximately 3.5 to 5.1 million species of fungi on our planet (67) of which approximately 97330 have been described in Kirk’s *Dictionary of the Fungi* (68). Fungi have the ability to degrade dead organic material for their energy source and for cellular synthesis that enables them to grow and reproduce. Most fungi have cell walls and some contain proteins that have been identified as allergenic. In addition, fungi metabolism involves the secretion of enzymes into their surrounds and absorption of the broken down products (1). Some of these enzymes have been identified as allergenic (3). Many fungi have adapted to maximise their reproductive chances by producing spores that are well adapted for airborne dispersal. As a result, their spores are very small, the majority being sized <10 µm in diameter. The combination of small size, allergenic surface and secretion
of allergenic enzymes contributes to their ability to be easily inhaled deep into the respiratory airways and lungs (69, 70). Based on these characteristics it is plausible that they may set off an allergic response and/or produce damaging organic by-products (mycotoxins) thereby possibly contributing to airway inflammation or obstruction, thereby inducing lower lung function and/or asthma exacerbation.

Counts of ambient fungal spores fluctuate widely between different climatic conditions, geographic regions and between indoor and outdoor environments (1). Outdoor fungal spore counts are generally more abundant than indoor fungal spores, and outdoor fungal spores commonly enter indoor environments via open windows and doors (71); however, it is less common for indoor fungal spores to be found in abundance in outdoor settings (72). The primary sources of outdoor fungal spores are plant materials and it is generally regarded that sources of these spores are seasonal and influenced by climatic variations (73-75). Although there are ecological and climatic differences between geographic regions and seasonal variation, the fungal genera that tend to dominate outdoor air include: *Cladosporium, Alternaria, Ustilago/smutts, Leptosphaeria, Coprinus, Penicillium*, and *Aspergillus* (71, 74, 76, 77).

**1.7 Exposure to outdoor fungal spores may be a risk factor for child and adolescent asthma hospitalisations, impaired lung function and increased airway inflammation**

Whilst exposure to outdoor fungal spores could plausibly trigger asthma exacerbations, contribute to reduced lung function or trigger airway inflammation, there is limited evidence to support this theory, especially in children and adolescents. As most evidence has been based on population studies that have been ecological time-series or small cross-sectional studies in design, there are methodological challenges in interpreting the evidence related to their findings. Few studies have accounted for the potential confounding effects of simultaneous exposure to ambient fungal spores, pollen grains, air pollution and meteorological factors (8, 47, 48, 60, 78-80). Fewer studies have examined the interaction with age or gender (48) or have accounted for potential effect modification by important individual factors such as whether participants were sensitised to fungal spores (81). To date no asthma hospitalisation studies have accounted for concurrent HRV respiratory infections which have been suggested as key triggers of severe asthma exacerbations in children and adolescents resulting in their hospitalisation.
(82). Hence any findings reported may be over- or under-estimated due to residual confounding or the effects of other important contributors.

1.8 Relevance and Importance

Australia has high prevalence of asthma and asthma exacerbation is a major cause of child and adolescent asthma hospitalisation. However, to date no Australian research has been published that has examined the associations between a range of allergenic outdoor fungal spores and asthma hospitalisations or lung function. Moreover, there is currently no published research that has examined associations between exposure to outdoor fungal spores and airway inflammation.

There is currently no cure for asthma or effective primary prevention strategies, but there is evidence that good management can reduce the incidence of asthma exacerbations and risk of hospitalisation. Improving the understanding of the relationship between outdoor fungal spores and asthma exacerbations may help inform asthma exacerbation prevention strategies. At the individual level this might involve improving targeted management and treatment plans to address high risk trigger factors. At the population health level, if the evidence indicates that, like pollen, outdoor fungal spores play a significant role in asthma exacerbations then, like the pollen monitoring system that is reported during the pollen season, a fungal spore monitoring system may be considered. This could provide early warning advice to high risk asthmatics.

1.9 Aim and objectives

The aim of my doctoral research was to investigate the contribution of outdoor fungal spores to (1) child and adolescent asthma exacerbations; (2) lung function; and (3) airway inflammation.

My specific objectives were to:

1. Systematically synthesise the evidence as to whether outdoor fungi were significant triggers of childhood asthma exacerbations that resulted in attendance to a primary care service, an emergency department or admission to a hospital for medical care. (Research Question 1)
2. Investigate if there were associations between short term exposure to outdoor fungal spores and child and adolescent asthma hospitalisations and explore if any associations were modified by:
   a. high levels of air pollutants or grass pollen; (Research Questions 2 & 3)
   b. age group; (Research Questions 2 & 3)
   c. sex; (Research Questions 2 & 3)
   d. presence of Human Rhinovirus respiratory infection (Research Question 3);
   e. fungal sensitisation status (Research Question 3)

3. Investigate if there were associations between short term exposure to outdoor fungal spores and lung function and airway inflammation, and explore if any associations were modified by:
   a. high levels of air pollutants or grass pollen;
   b. age group;
   c. fungal sensitisation status (all contribute to Research Question 4)

1.10 Thesis statement

This thesis is based on original research conducted during my PhD candidature. Hospital admission data from south-west Sydney were obtained to explore whether there are associations between outdoor fungal spores and child and adolescent asthma hospitalisations from an ecological perspective. The Melbourne Air Pollen Child and Adolescent Health (MAPCAH) data have been analysed to critically examine whether outdoor fungal spores were associated with child and adolescent asthma hospitalisations while controlling for human rhinovirus (HRV) respiratory infections and fungal sensitisation status. The Melbourne Atopy Cohort Study (MACS) data were used to explore if there were cross-sectional associations between outdoor fungal spores and differences in lung function and markers of airway inflammation. The strengths of this doctoral research include its exploratory nature; the utilisation of three analytical approaches with three different datasets; the inclusion of daily fungal spore counts (by taxa), air quality, meteorological and grass pollen data; and the investigation of interactions by HRV infections, fungal sensitisation, sex and age groups to identify groups at high risk of asthma exacerbation or lung health changes when exposed to outdoor fungal spores.
1.11 Thesis overview

**Chapter 1** is a summary of the background, rationale, aim and objectives of my thesis.

**Chapter 2** is a literature review of outdoor fungal spores and their association with asthma exacerbations, lung function and airway inflammation.

**Chapter 3** comprises a systematic review that was published in a peer-reviewed journal (Pediatric Allergy and Immunology) “Outdoor fungi and child asthma health service attendances”. [Research Question 1]

**Chapter 4** provides detailed description of the methods and their associated strengths and limitations that are common to the four research questions in this thesis: measuring outdoor fungal spores and pollen; air pollutants and meteorological conditions. All other methods that are specific to a single chapter are outlined in that specific results chapter.

**Chapter 5** is original research published in a peer-reviewed journal (Environmental Research) titled “The role of outdoor fungi on asthma hospital admissions in children and adolescents: A 5-year time stratified case-crossover analysis”. This research is an ecological case-crossover analysis of child and adolescent asthma hospitalisations in south-west Sydney. This chapter includes the methods specific to this research question. [Research Question 2]

**Chapter 6** is original research published in a peer-reviewed journal (Journal of Allergy and Clinical Immunology) “Associations between outdoor fungal spores and childhood and adolescent asthma hospitalisations”. This research is a case-crossover analysis of the MAPCAH child and adolescent asthma hospitalisations. This chapter includes the methods specific to this research question. [Research Question 3]

**Chapter 7** is a traditional thesis chapter titled “Is ambient exposure to outdoor fungal spores associated with lower lung function or airway inflammation in a high risk allergy cohort?” This is a cross-sectional analysis of lung function and markers of airway inflammation in the MACS cohort at the 18 year follow-up. This chapter includes the methods specific to this research question. [Research Question 4]
Chapter 8 offers an overall discussion of the research reported in this thesis; and the strengths and limitations of my research.

Chapter 9 discusses how the findings from the four research questions contribute to the field of research. From this, I discuss implications and recommendations for public health policy, clinical management and future research and provide final conclusions.
Chapter 2

Literature review

2.1 Introduction

There has been increasing public health interest in fungal spore exposure as it is hypothesised that this exposure may be associated with adverse effects on respiratory systems due to the very small size of spores which makes them respirable, their potential to act as mediators of allergic reactions, and the release of mycotoxins that may affect the epithelial lining of the airways. These physiological actions may result in acute clinical presentations, such as triggering asthma exacerbation, or may lead to pre-clinical effects such as short-term impaired lung function or airway inflammation. In cases of severe asthma exacerbations, particularly in children and adolescents, hospitalisation is often required in order to manage life-threatening symptoms. Fungal spores that are produced in the outdoor environment are ubiquitous biological particles in the air that we all breathe. There is a growing need to understand the extent to which outdoor fungal spores are associated with child and adolescent asthma exacerbations that result in hospitalisations, reduced lung function or airway inflammation.

To date, epidemiological studies examining associations between exposure to outdoor fungal spores and asthma exacerbations, airway inflammation, and changes in lung function, have been limited by inconsistent findings. Most research has been undertaken across geographic regions of the northern hemisphere, but little has been reported from countries, such as Australia, where outdoor fungal allergens may be important triggers for these symptoms. Many of these studies also lacked important information on patient assessment as well as data on exposure to other atmospheric particles, such as pollen and air pollution, that may act as confounders or effect modifiers on the influence of outdoor fungal spores on respiratory conditions.

When investigating exposures to outdoor fungal spores associated with asthma exacerbations/hospitalisations, lower lung function, or airway inflammation, it is important to account for other types of exposures that are already known to be strongly associated with these outcomes in order to increase the accuracy of detecting any true
associations. Common factors that have been recognised as important triggers of these outcomes include respiratory viral infections, especially human rhinovirus (HRV) (27, 28); aeroallergens, such as pollen (30, 31), house dust mite (29), indoor fungal spores (9, 64, 83) and animal dander (83); air pollutants (34, 35); tobacco smoke (36); thunderstorms (in association with pollen and respirable particles) (37, 38); medications, such as non-steroidal anti-inflammatory drugs (39) and hormones (menopausal asthma) (40); exercise (41); stress (42); and obesity (84, 85). It is also necessary to identify any potential confounders that are known to affect both outdoor fungal spores (the exposure) and asthma exacerbations/hospitalisations, lung function or airway inflammation (the outcomes). Controlling for potential confounding factors is important to minimise bias and to increase the accuracy of attributing an effect to a particular exposure. Potential confounders of outdoor fungal spore exposure on asthma exacerbation include meteorological variables, such as temperature and relative humidity (86, 87), air pollutants (47, 57) and pollen (1, 57, 88). Effect modification or interaction is another important biological phenomenon which may be occurring. This relates to the exposure having a different effect across a variety of circumstances. If this occurs, the magnitude of the effect of the primary exposure on the outcome will differ depending on the level of a third variable. In this situation, estimating an overall effect estimate will be misleading. For example, with asthma hospitalisations being more frequent in boys during childhood, and more frequent in girls during adolescence (89, 90) it is possible that age and sex may act as potential effect modifiers. This may be related to a higher prevalence of asthma among boys in childhood, and among girls in adolescence (90); or hormonal changes at adolescence that coincide with lung function changes characterised by reduction in the ratio of relative airway size to alveolar size (91). It is biologically plausible that other individual factors, such as the presence of respiratory viral infections, or being sensitised to fungi, may also act as effect modifiers as they may alter the susceptibility of a person to experience respiratory symptoms when exposed to outdoor fungal spores. Currently there is limited understanding of the potential confounding or interactive relationships between outdoor fungal spores and individual patient characteristics, with other triggers of asthma exacerbation, lower lung function or airway inflammation. This review chapter will describe the existing evidence from the published research literature.
In this chapter, the definitions of asthma, asthma exacerbation, asthma hospitalisation, airway inflammation and lung function will be reviewed. Then, the prevalence and burden of asthma will be outlined, followed by a review of the common allergic and non-allergic triggers for asthma exacerbation. Next, the mechanisms by which outdoor fungal spores can trigger asthma exacerbation will be described, including the current understanding of fungal sensitisation. Then, the research literature on outdoor fungal spores and asthma hospitalisation, lung function and airway inflammation will be summarised. I will discuss the potential confounding factors or interactions with other allergic and non-allergic factors that contribute to triggering asthma exacerbation, airway inflammation or impact on lung function. Finally, I will outline the current gaps in our knowledge that require attention.

2.2 Asthma

The word ‘asthma’ originates form the Greek word ‘ἀσθμα’, meaning to exhale with open mouth or to pant. The earliest records of this term date back to Homer’s Iliad and was also used as a medical term in Corpus Hippocraticum (92).

Asthma is understood to be:

“a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway (bronchial) hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment” The Global Initiative for Asthma (GINA) (13)

Asthma is a complex and heterogeneous disease that can begin at any age but most commonly starts in early childhood (93). This may be a condition that persists into adulthood or it may remit only to relapse again (13).

The fundamental causes of asthma are not well understood although it is believed to be associated with a complex combination of genetic and environmental factors (94). Both population studies and twin studies have identified a number of genetic variants that influence asthma risk, particularly in children (95-98). The genes implicated in these studies are associated with the communication of epithelial damage to the adaptive
immune system and activation of airway inflammation (96). The environmental causes can be broadly classified into allergic and non-allergic aetiological factors. These aetiological factors form the basis of a classification system for asthma: allergic asthma and non-allergic asthma.

Allergic asthma is characterised by an excessive immunoglobulin E (IgE) mediated immune response to antigens (allergens) entering the body that causes airway inflammation, reversible airflow obstruction and bronchial hyper-responsiveness. Commonly recognised aetiological agents for allergic asthma include pollen (99), house dust mite (100), cockroaches, pet dander (101) and fungal spores (102). Allergic asthma is estimated to account for approximately 50% of all asthma cases (103). The onset of allergic asthma is usually at an earlier age and a large proportion of childhood asthma is attributed to allergic asthma (104).

Non-allergic asthma is airway inflammation that is not triggered by allergens nor has an excessive IgE response. Non-allergic aetiological agents may include respiratory viruses, especially the human rhinovirus (HRV) (27, 105, 106), exercise (107), tobacco smoke (108), non-steroidal anti-inflammatory medications (e.g. aspirin) (109), air pollutants (110), obesity (111, 112) and industrial and cleaning chemicals, such as solvents, cleaning products, dyes, aldehydes, acrylates (113). The mechanisms behind non-allergic asthma are not well understood (103). The onset of non-allergic asthma tends to occur later, during adulthood (114).

Hence it is proposed that the causes that underlie the onset of asthma, and subsequent asthma exacerbations, are likely to be multifactorial and involve both genetic predisposition in conjunction with exposure to causative agents (94, 98, 104).

2.2.1 Mechanisms of asthma
The inflammatory processes affecting the airways of the respiratory system that results in asthma involve several types of inflammatory cells (mast cells, eosinophils, T-lymphocytes, macrophages and dendritic cells) and multiple mediators (chemokines, cysteiny1 leukotrienes, cytokines, histamine, nitric oxide and prostaglandin D2) (115). This inflammatory process is strongly associated with airway hyper-responsiveness (a state whereby spasm of the airway is easily triggered by a stimulus that may be innocuous in another person) and asthma symptoms (116). Airway inflammation in asthma may be
persistent, regardless of the episodic symptoms, and affects all levels of airways from the upper respiratory tract through to the bronchioles (11). The major physiological effects are most prominent in the medium sized bronchi where inflammation, mucous secretion, and constriction of the muscles result in airway limitation or obstruction, and hence the symptoms of asthma (115).

### 2.2.2 Asthma exacerbation

Asthma exacerbation is the transient worsening of asthma symptoms which may occur as a result of exposure to “triggers”. According to the Global Initiative for Asthma (GINA) guidelines, asthma exacerbations are episodes of progressive shortness of breath, cough, wheezing or chest tightness, or a combination of these symptoms (13). A joint taskforce of the American Thoracic Society and European Respiratory Society has defined asthma as events characterised by a change from the individual’s previous status (117).

> "1. Severe asthma exacerbations are defined as events that require urgent action on the part of the patient and physician to prevent a serious outcome, such as hospitalization or death from asthma.

> 2. Moderate asthma exacerbations are defined as events that are troublesome to the patient, and that prompt a need for a change in treatment, but that are not severe. These events are clinically identified by being outside the patient’s usual range of day-to-day asthma variation.” (117)

There are numerous factors that may trigger an asthma exacerbation. These include allergens such as pollen (30, 31), fungal spores (33, 118), house dust mite (29) or animal dander (119, 120). Also non-allergenic triggers such as respiratory viral infections, especially human rhinovirus (HRV) (27, 28); air pollutants (34, 35); tobacco smoke (36), weather events such as thunderstorms (in association with pollens and fine particulate matter) (37, 38); medications such as non-steroidal anti-inflammatory drugs (39), hormones (40); exercise (41); synthetic and industrial chemicals (113) and stress (42).

Mild and moderate exacerbations are generally characterised by audible wheezing but the individual can walk and speak whole sentences or phrases (depending on language development age) in one breath. With severe exacerbations, individuals may be unable to speak in sentences or full phrases, are visibly breathless, breathing takes effort or their
oxygen saturation is around 90-94% when measured by a pulse oximeter (121). In life-threatening exacerbations, individuals may be drowsy, exhausted, look cyanotic, have collapsed, unable to breath or have oxygen saturation of <90% (122).

2.2.3 Asthma hospitalisation

When an asthma exacerbation is severe or life threatening, an asthmatic person may not respond to initial treatment with a short acting bronchodilator, such as salbutamol, and/or oxygen therapy (123). In these cases, urgent treatment in a hospital is required to prevent severe morbidity or fatality. These are the asthmatic cases that are the focus of three chapters [Chapters 3, 5 and 6] of this thesis. The methods used in diagnosing the asthmatic cases are detailed in their respective chapters.

Lung function and airway inflammation may be clinical or pre-clinical markers of active asthma or asthma exacerbation. In the next section, I will introduce and explain the outcome measures that are used in Chapter 7 of this thesis. The measures that assessed lung function were obtained using spirometry; and the presence of markers of airway inflammation and oxidative stress were identified using fractional exhaled nitric oxide (FeNO) and exhaled breath condensate (EBC).

2.3 Lung function

Lung function is one of the important markers of lung health. Reduced lung function can indicate that airway obstruction may be underway as a result of airway inflammation and internal remodelling (11). Lung function tests provide a non-invasive method of assessing lung function without physically examining the lungs. The tests are used to measure breathing problems and include quantifying the amount of air that is breathed in and out of the lungs, measuring how quickly it can be exhaled, how well the lungs deliver oxygen to the circulating blood and the strength of the breathing muscles. In this thesis, I only used data on how much air is inhaled and exhaled; and how fast it can be exhaled using flow spirometry.

Spirometry measures the volume of air over time and can be utilised to measure the movement of air in and out of the lungs. People are asked to inhale to their maximum and then exhale as quickly and as forcefully as possible. This is known as the forced vital capacity manoeuvre.
A number of measurements can be examined that provide information on lung function:

- Forced expiratory volume in the 1st second of expiration (FEV<sub>1</sub>) - measured in litres of air.
- Forced vital capacity (FVC) – measured in total litres of exhaled air.
- Maximal flow rate between 25% and 75% of the vital capacity (FEF<sub>25%-75%</sub>) – measured in litres/second. This provides important information about the function of the small airways. (Figure 2.1)

![Spirometry](http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/pulmonary/pulmonary-function-testing/images/pulmonary-function-fig1_large.jpg)

**Figure 2.1: Spirometry reading showing lung function parameters for a healthy seated adult undergoing a forced vital capacity manoeuvre.**

(Figure 2.1 obtained from [http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/pulmonary/pulmonary-function-testing/images/pulmonary-function-fig1_large.jpg](http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/pulmonary/pulmonary-function-testing/images/pulmonary-function-fig1_large.jpg))

In Figure 2.1 the x-axis indicates time and the y-axis indicates lung volume. These curves show maximal inhalation from tidal respiration (resting tidal volume) to total lung capacity followed by rapid forced exhalation to the fullest extent (forced vital capacity FVC) until no further volume is exhaled at residual volume. FEV<sub>1</sub> is the exhaled volume in one-second after the commencement of maximal expiration. FEF<sub>25-75</sub> is the volume of air exhaled between the 25<sup>th</sup> and 75<sup>th</sup> percentiles of the FVC manoeuvre.

Spirometry readings usually fit in one of four major types of lung health (124):
1. Normal: Normal readings vary according to a variety of factors such as age, sex, height, racial/ethnic group. Charts containing reference values have been developed to take these factors into account and are referred to when interpreting spirometry results (125).

2. Obstructive: Narrowing of the airways results in an obstructive pattern. This is typical in cases of asthma and chronic obstructive pulmonary disease. In these cases, the amount of air inhaled (total capacity of the lung) is usually normal, but the amount of air blown out quickly is reduced. This can be seen with reduced FEV₁, FVC and FEF<sub>25%-75%</sub>; or increased residual volume/total lung capacity (RV/TLC) ratio.

3. Restrictive: Lung capacity for age, sex and height is reduced compared to normal levels. A range of conditions that affect lung tissue or restrict the expansive capacity of the lungs cause this restriction. This is seen in reduced total lung capacity, reduced FEV₁ and more reduced FVC.

4. Combined obstructive/restrictive: Features of both abnormal patterns as a result of narrowed airways and reduced lung capacity.

2.4 Airway inflammation and oxidative stress

Airway inflammation and oxidative stress are major contributors to asthma pathophysiology (10). Nitric oxide (NO) is produced by cells residing in the airways and plays a critical role in regulating airway function, and has both beneficial and detrimental effects on airway function (126). The production of NO is complex – it is a gaseous signalling molecule that is generated by three types of iso-enzymes of NO synthase (NOS). Each of these enzymes is regulated differently, and plays different roles in the airways. Neuronal NOS (NOS1) is thought to play a regulatory role in neurotransmission and produces small amounts of NO. Endothelial NOS (NOS3) has a regulatory role in vascular flow and also produces a small amount of NO. Inducible NOS (NOS2) is induced by infectious and inflammatory exposures and produces large amounts of NO, which in turn may have a pro-inflammatory effect (126) and may mediate airway hyper-responsiveness (127). Hence exhaled NO can be regarded as an indirect marker of airway inflammation.

Oxidative stress is a complex and dynamic imbalance between increased oxidative sources and reduced anti-oxidant mechanisms. A common source of oxidative stress in
the airways is the recruitment of inflammatory cells that are produced after exposure to trigger factors, such as air pollutants, aeroallergens (for example fungal spores, pollen grains) and tobacco smoke (128). The oxidative stress pathway is complex and involves many local chemical and enzymatic reactions that result in the production of increased NO and organic acids that reduce the pH level of the airway surface liquid (129). Hence increased NO and acidic levels (reduced pH) in respiratory vapour may be useful as an indirect marker of airway inflammation and oxidative stress.

Detection of these pathological processes may be helpful in detecting early inflammatory airway disorders before clinical signs of airway obstruction and contribute to improved monitoring and management of asthmatic people (10, 126, 127). Ideally, it is preferable to use non-invasive systems that are relatively simple and cost-effective to accurately identify and measure levels of markers of airway inflammation and oxidative stress. I will define the non-invasive methods employed by the studies that I have analysed for this thesis, however detailed methods for each will be reported in the relevant chapter, Chapter 7.

2.4.1 Fractional Exhaled Nitric Oxide (FeNO)

Fractional exhaled nitric oxide (FeNO) is the amount of forced exhaled nitric oxide in the breath. In asthma, increased FeNO levels results from eosinophilic-mediated inflammation in the central and peripheral airways. It is measured non-invasively using specifically designed portable equipment that uses an electrochemical sensor to determine the levels of exhaled NO. The American Thoracic Society (ATS) has endorsed the use of FeNO as a non-invasive, safe and simple method of quantifying the amount of NO in exhaled air in order to estimate/measure airway inflammation (130).

FeNO is recommended as an option to (1) assist in diagnosing child and adult asthma if the history, clinical examination and lung function testing is indicative, but not diagnostic of, airway obstruction; and (2) support asthma management in asthmatic people who remain symptomatic despite the use of inhaled corticosteroids (130, 131).

Methods for obtaining and measuring FeNO are reported in Chapter 7.

2.4.2 Exhaled Breath Condensate (EBC)

Exhaled breath condensate (EBC) is another non-invasive method of measuring airway biomarkers. In the study reported in Chapter 7, I used information on EBC nitrogen
oxides (NOx) and acidity levels (pH) in breath exhalate that were available (132). Other compounds such as hydrogen peroxide, ammonia, leukotrienes and cytokines have been identified in EBC but my thesis is not examining these.

### 2.4.2.1 EBC Nitrogen Oxides (EBC NOx)

EBC NOx measures the nitrogen oxides, including nitrite (NO₂⁻) and nitrate (NO₃⁻), which are present in the respiratory tract epithelial lining fluid. These are biomarkers of airway inflammation.

### 2.4.2.2 EBC pH

Exhaled breath condensate pH (EBC pH) is a possible biomarker of airway inflammation and oxidative stress (133). Respiratory tract pH homeostasis is influenced by the production and release of acids and bases in the airways, and this balance is maintained by a number of different buffering systems (132). Low EBC pH value reflects acidic airway lining fluid. A recent systematic review reported that EBC pH levels in healthy subjects ranged between 6.1 and 8.0 and were consistently higher than the pH values in asthmatic subjects (134).

The methods for obtaining and measuring EBC are reported in Chapter 7.

Asthma is a significant public health problem due to its prevalence, impacts on health and quality of life, demand on the health care system and associated direct and indirect economic costs. In the next section, the comparative information is presented for the prevalence and burden of asthma globally and in Australia.

### 2.5 Prevalence of asthma

#### 2.5.1 Global prevalence of asthma

Over the past 20 years or so, the prevalence of asthma has increased to epidemic proportions in both developed nations and countries transitioning to developed economic status (14). It is estimated that asthma affects approximately 330 million people of all ages globally (13). The countries with the highest estimated overall prevalence of clinical asthma include Australia, New Zealand, and the United Kingdom (15, 103, 135).
The global prevalence of asthma in children has been identified by Phase III of the International Study of Asthma and Allergies in Childhood (ISAAC) as 11.1% to 11.6% in the 6 to 7 year age group; and 13.2% to 13.7% in the 13 to 14 year age group. There were variations between regions but the longitudinal trends indicated that prevalence of asthma in low to middle income countries has increased towards the rates found in developed countries and, although global disparities in asthma prevalence have decreased (103), overall prevalence has plateaued and not decreased.

The global prevalence of asthma in adults appears to be lower than in children. A population health survey in the mid-1990s by the European Community Respiratory Health Survey (ECRHS) of adults aged 20 to 44 years found prevalence of doctor diagnosed asthma to be 4.5% and wheeze to be 12.7% (136). The most recent survey of adults aged 18 to 45 years, conducted in 70 countries for 2002 and 2003 by the World Health Organization (WHO), found that doctor diagnosed asthma prevalence was 4.3% and wheezing prevalence had reduced to 8.6%. As with children, rates between countries varied by as much as 21-fold, with Australia reporting the highest doctor diagnosed asthma (21%) and wheezing (27.4%) (137).

2.5.2 Asthma prevalence in Australia

Asthma is the most common chronic medical condition affecting Australian children and is the major contributor to child hospitalisation. According to the 2007 to 2008 Australian National Health Survey (16) the overall prevalence of current asthma was estimated to be 9.9%, affecting approximately 2,049,086 people. The prevalence among children (aged 0 to 15 years) was estimated to be 10.4% and among adults (aged 16 to 45 years) was estimated to be 9.8% (16).

2.6 Health and economic burden of asthma

2.6.1 Burden on health and quality of life

Asthma has substantial impacts on the quality of life of people living with this condition and can affect participation in activities of daily life (123, 138), impair sleep (18, 19), disrupt school and work attendance (139), affect weight and height development (21), and exacerbations often require pharmacological or medical intervention at the primary care, emergency department or hospital level (138).
In the 1990 Global Burden of Disease study, in terms of disability-adjusted life years (DALYs) asthma was ranked 18th in the 1 to 4 year group, 8th for the 5 to 9 year group, 3rd for the 10 to 14 year group and 12th for the 15 to 19 year group compared to other causes (103). In the 2010 Global Burden of Disease study asthma was been ranked the 14th highest cause of years lived with a disability (YLD) for all ages and, when ranked among other non-communicable diseases, it is ranked 13th in the world (140). In terms of DALY rankings in 2010, asthma was ranked 29th globally for all ages, and in the top 10 causes of DALYs in the 5 to 14 year group (141).

Asthma exacerbations can be severe and require medical intervention from the primary care level through to emergency department attendance and hospitalisation to manage the symptoms. In Australia, sudden-onset severe asthma exacerbation is a major cause of hospital admissions for children and adolescents. In 2011, the rate of child and adolescent asthma hospitalisation was estimated to be 493 per 100,000 people. The significance of this rate is highlighted when compared to the estimates for adult asthma hospitalisation which were around 91 per 100,000 (16). More than half of the asthma hospital admissions involved children aged 0 to 14 years. Children aged 15 to 18 years were more likely to be admitted for longer periods (26).

### 2.6.2 Economic burden

It is clear from these statistics that asthma creates substantial health and economic burdens on individuals, families and communities. The economic burden stems from not only the direct medical costs associated with utilisation of health care services and medications, but also indirect costs associated with lost productivity in workplaces due to sick leave or carer’s leave and additional expenses not recorded in health economic data. In countries with a higher prevalence of asthma, it has been estimated that approximately 2% of the national health budget is spent on asthma associated costs (15).

In Australia, during 2008 and 2009, the estimated direct health expenditure attributed to asthma was $655 million (0.9% of total health expenditure), associated with direct hospital care (20%), prescription pharmaceuticals (50%), and out of hospital medical care (30%) (26). On top of these expenses there are the additional costs incurred by individuals, families and carers due to lost labour force productivity and unaccounted out-of-pocket expenses. As such, asthma is recognised as a significant and increasing
public health problem and is classified as a National Health Priority Area by the 
Australian government (23).

In the next section, I will describe the triggers that are most strongly associated with 
asthma exacerbations in children and adolescents and that may act as confounders or 
effect modifiers of outdoor fungal spore exposures. I will outline the key findings 
associated with respiratory viruses, pollen, and air pollutants. As outdoor fungal spores 
are the focus of this thesis, I will provide an in-depth review of the research literature in 
a separate section.

2.7 Triggers of child and adolescent asthma exacerbation

2.7.1 Respiratory viruses

Viral respiratory tract infections are common and, in otherwise healthy people, are 
generally limited in their morbidity and long-term health effects. However, due to the 
initiation of a complex series of inflammatory processes in the airways, these infections 
may trigger asthma exacerbations in asthmatic children and adults (106, 142). There are 
umerous respiratory viruses associated with asthma exacerbations including human 
rhinovirus (HRV), respiratory syncytial virus (RSV), metapneumovirus, parainfluenza 
virus and coronavirus (143-145). However, HRV has been identified as the predominant 
aetiological factor associated with asthma exacerbations. Some studies have reported that 
more than 80% of childhood asthma hospital admissions have been triggered by a HRV 
infection (105, 146), although allergic sensitisation may be an effect modifier in this 
relationship resulting in increased risk (45, 147-149). Infections with HRV have been 
found to occur year round (150), but some locations have noted seasonal peaks occurring 
in autumn (fall) and spring in temperate climates (82, 143). These peaks have been found 
to correlate with children returning to school after a holiday break (151). Infections with 
RSV and influenza have also been associated with increased asthma exacerbations during 
the winter period (105, 143).

To date, there are three genetically distinct HRV species: HRV-A, HRV-B and HRV-C, 
Within each of these species there are numerous subtypes: HRV-A has 74, HRV-B has 
26 and HRV-C has approximately 50 (82). Since the identification of HRV-C in 
approximately 2007 (152), HRV-C infections have been associated with more asthma
hospitalisations than other species of HRV (27, 153-155) and it has been suggested that this species may be responsible for substantial asthma exacerbations among children (105).

2.7.2 Pollen

Exposure to pollen grains has been found to be strongly associated with the exacerbation of asthma by causing closure of the airways and resulting in wheezing, cough and shortness of breath. Wind-pollinated pollen species have been found to be associated with early childhood wheezing (156) and asthma exacerbations (157) leading to asthma emergency department attendances (30, 158) and hospitalisations (31, 78, 88, 159) and increased FeNO (160).

Wind-pollinated pollen species most commonly associated with these asthma exacerbations are grasses, weeds and trees (57, 88, 158) mostly from vegetation originating from the northern hemisphere. In Australia, seasonal pollen emissions from introduced ryegrass (Lolium perenne) (Figure 2.2) has the strongest associations with child and adolescent asthma exacerbations resulting in emergency department attendances and hospitalisations (30, 31, 56).

![Figure 2.2: Rye grass (Lolium perenne) pollen grain](image)

(Brightfield microscope image by Dr Philip Taylor, Deakin University)

2.7.3 Ambient air pollutants

The World Health Organization defines air pollution as “the contamination of the indoor or outdoor environment by any chemical, physical or biological agent that modifies the natural characteristics of the atmosphere” (161). Air pollutants can be transported long
distances from their source, are generally unavoidable, and all segments of the population can be exposed (162). It becomes a public health concern when it adversely affects the health of humans, animals and plants (161).

Common sources of air pollutants are associated with industrial facilities; motor vehicle emissions (163); household combustion devices such as wood/biomass fired heaters and stoves; (164) and vegetation fires (forest, bush, peat) (165). An air pollutant may be either directly emitted as a primary pollutant (e.g. carbon monoxide from a car’s exhaust); or may be formed in the atmosphere through the physical and chemical conversion of precursors resulting in a secondary pollutant (e.g. sunlight converting other pollutants into ozone and other oxidant species) (166).

Pollutants associated with asthma and asthma exacerbations include respirable particulate matter, nitrogen oxides, and ozone (167-171). Next is a description of each of these pollutants, their common sources, and a summary of their associations with asthma exacerbations, lung function and airway inflammation.

### 2.7.3.1 Particulate matter

Particulate matter is a wide range of solid and liquid materials suspended in the air, and these may travel long distances in air before depositing on surfaces. There are numerous sources of particulates including: dust, diesel exhaust, industrial combustion and emissions, bioaerosols, vegetation fires and smoke, and volcanic ash (172). Biomass burning of wood, leaves, peat, crops, and forests is often the largest contributor to atmospheric particulate matter in many regions (165).

Particulate matter (PM) are categorised according to their size: those with aerodynamic diameter of >10 µm are giant particles; <10 µm are PM<sub>10</sub> – coarse; <2.5 µm are PM<sub>2.5</sub> – fine; 0.1 µm are ultrafine PM (166). The smaller sized particles have the potential to be more damaging to human health as they are more likely to be inhaled as deep as the alveoli and impact the respiratory lining. Depending on their chemical composition, they can irritate and inflame the lining of the airways, deposit toxic chemicals into the tissues, or initiate allergic responses in the airway epithelium (173).
2.7.3.2 Nitrogen oxides

Nitrogen oxides are produced largely by industrial and vehicle combustion, and they can also be produced in the atmosphere by reactions between reactive oxygen molecules and nitrogen already present in the atmosphere. The form most commonly reported to be associated with asthma exacerbation is nitrogen dioxide (NO₂) (170, 174, 175).

2.7.3.3 Ozone

The ozone molecule consists of three oxygen atoms (O₃) instead of two oxygen atoms (O₂) that is needed by humans. In the stratosphere, ozone plays a very important role in shielding the Earth from the sun’s ultraviolet radiation; however, at ground level ozone can be toxic to humans (176).

2.7.4 Air pollution, asthma exacerbations and lung function

Numerous international studies have found that short term exposures to increased levels of outdoor PM₁₀, PM₂.₅, NO₂ and O₃ have been associated with increased asthma exacerbations, wheezing, asthma medication use, and asthma emergency department attendances and hospitalisation among children and adolescents (35, 57, 171, 177-180) and adults (164, 181).

Short term exposures to outdoor air pollutants have been associated with small but adverse effects on lung function in children, adolescents (182-185), and adults (186). One of the world’s largest air pollution research collaborations, the European Study of Cohorts for Air Pollution Effects (ESCAPE) analysed combined data (by meta-analysis) from birth cohort studies in Germany, Sweden, the Netherlands, and the United Kingdom, and measured lung function at 6 to 8 years of age (n = 5,921). They estimated annual average exposure to key air pollutants: nitrogen oxides (NO₂, NOx), PM₂.₅, PM₁₀ at the birth address and current address using land-use regression models. They found that NO₂, NOx and PM₂.₅ at the children’s current address were associated with small but significant decrease in lung function: decrease in FEV₁ ranged from -0.86% (95% CI: -1.48, -0.24%) for a 20ug/m³ increase in NOx; and -1.77% (95% CI: -3.34, -0.18%) for a 5ug/m³ increase in PM₂.₅ (185). The ESCAPE study also examined the long-term impact of ambient air pollutants on adult lung function (n=7613) and did not find an association between air pollutants and longitudinal lung function changes. However, they did find that a 10 μg/m³ increase in NO₂ exposure was associated with lower levels of FEV₁.
(−14.0 millilitres (ml), 95% CI −25.8 to −2.1) and FVC (−14.9 ml, 95% CI −28.7 to −1.1). An increase of 10 μg/m³ in PM₁₀, but not PM₂.₅, was associated with a lower level of FEV₁ (−44.6 ml, 95% CI −85.4 to −3.8) and FVC (−59.0 ml, 95% CI −112.3 to −5.6) (187). In Rice et al’s respiratory study of adults nested in the Framingham Heart Study, short-term exposure to moderate levels of air pollution were found to be associated with a 20.1ml lower FEV₁ for PM₂.₅ (95% CI −33.4, −6.9), a 30.6ml lower FEV₁ for NO₂ (95% CI −60.9, −0.2), and a 55.7ml lower FEV₁ for O₃ (95% CI −100.7, −10.8) compared with the low levels (186).

Clinical studies have provided some evidence that short-term exposure to elevated air pollutants can increase airway inflammation and/or oxidative stress in children. Patel et al’s study of 36 asthmatic and non-asthmatic adolescents identified a significant association between short-term exposure to outdoor NO₂ and decreased EBC pH, with no difference between the asthmatics and non-asthmatics (188). Short-term exposure to particulate matter (including PM₁₀ and PM₂.₅) has been found to be associated with significantly higher exhaled breath concentrations of nitric oxide in children (189, 190).

Another trigger for asthma exacerbation, and possibly decreased lung function or airway inflammation, is fungal spores and this is the focus of the next section.

2.8 Outdoor fungal spores

2.8.1 Fungal species, reproductive strategies and spore emissions

Fungi are among the most ecologically diverse and successful organisms on Earth, making up their own Kingdom. Fungi are ubiquitous eukaryotic organisms that produce large numbers of spores that are dispersed so effectively that they can be in almost all ecosystems. There are estimated to be more than 1.5 million species of fungi, of which fewer than 10% have been formally identified and described (67). They are not a homogeneous group of organisms, instead they vary in their morphology, physiology, and mode of reproduction.

Fungi that are commonly referred to as yeasts, are unicellular, and produce hyaline conidia into the atmosphere. However, most species of outdoor fungi produce thread-like hyphae at their tips and form mycelial networks in the soil, within plant tissues, or are saprophytic on decaying substrates. These hyphae range in size from 2 to 10μm in
diameter. The reproductive structures of fungi can be observed in the external environment as mushrooms, bracket fungi, plant rusts, smuts, puffballs, moulds or yeasts (191). Fungal spores range in size from a few microns to over 50 µm in diameter, and spore morphology is commonly used to identify fungi (1).

Fungi are ecologically very important as they decompose organic matter (191), are a food source, or are used in food production (192). They may also form symbiotic relationships with numerous plant and animal species (191). Some may act as pathogens if their colonisation causes disease in or death of the host. Some fungi have therapeutic properties and are used in the production of drugs, including antibiotics, vitamin preparations, cortisone, and cholesterol lowering medications (statins) (193). Fungi commonly have complex metabolism processes that may involve secreting enzymes into their immediate environment to solubilise substrates and then absorbing the broken down by-products. Some of these enzymes have demonstrated allergenic activity (194).

Fungal reproduction involves the formation of fungal spores (sporulation), and these spores are well adapted for airborne dispersal. The mode of sporulation is used to classify the two different phyla of fungi that are most frequently associated with asthma, allergic sensitisation, lung function and airway inflammation – the Ascomycota and Basidiomycota.

Fungi in the Ascomycota reproduce sexually and/or asexually. In sexual reproduction, non-motile spores called ascospores (Figure 2.3) are formed in a ‘sac-like’ microscopic structure called an ascus. These spores, along with liquid droplets, are ejected into the atmosphere by rain or high relative humidity (3). In asexual reproduction, conidia are formed on conidiophores and are passively released into the atmosphere by the action of wind or rain splash. Many fungal species that only reproduce asexually were previously classified as Deuteromycota, but were reclassified as Ascomycota or Basidiomycota upon genetic analysis (3). The relevance of this change will be highlighted when I discuss classification systems reported in previous research in this field. Well known examples of Ascomycota that mainly emit asexual spores (wind dispersal) are Alternaria (Figure 2.4), Cladosporium (Figure 2.5), and Aspergillus/Penicillium (Figure 2.6), whereas sexual spores are mainly emitted from Leptosphaeria (Figure 2.6).

In the phylum Basidiomycota, fungi commonly form sexual spores known as basidiospores (Figure 2.7). The ballistic emission of these spores is accompanied with liquid droplets. They are emitted from finger-like extensions on basidia during periods
of high humidity (3). Well known examples of this phylum are *Coprinus* and *Ganoderma*. A broad group that do not form basidiocarps (the fruiting body) are the *Ustilago* /smuts, and these are mainly wind dispersed (Figure 2.8). Some yeasts are also basidiomycetes.

Many fungi pose no threat to human health, but some cause diseases of plants, animals and humans (195). Of particular interest in my PhD research, are those allergenic fungal taxa that are known to be associated with allergic sensitisation, asthma exacerbation, airway inflammation and changes in lung function (63, 102, 196-198), and so my thesis will focus on these taxa.

**Ascospores**

![Ascospore with scale](image)

*Figure 2.3: Ascospore with scale*

(Photomicroscopy by Dr Philip Taylor, Deakin University)

![Alternaria alternata with scale](image)

*Figure 2.4: Well-known example of an allergenic ascomycete with asexual conidia: *Alternaria alternata* with scale*

Photograph obtained from [http://www.mycology.adelaide.edu.au/](http://www.mycology.adelaide.edu.au/) (199)
Figure 2.5: Well-known example of an allergenic ascomycete with asexual conidia: *Cladosporium cladosporioides* with scale

Photograph obtained from [http://www.mycology.adelaide.edu.au/](http://www.mycology.adelaide.edu.au/) (199)

Figure 2.6 Well-known examples of allergenic (a) sexual ascospores of *Leptosphaeria* spp; and (b) asexual conidia of *Aspergillus/Penicillium* with scale

(Photomicroscopy by Dr Philip Taylor, Deakin University)
**Basidiospores**

Figure 2.7: Basidiospores with scale

(Photomicroscopy by Dr Philip Taylor, Deakin University)

Figure 2.8: Well-known examples of allergenic basidiospores (a) *Coprinus* spp; (b) *Ganoderma* spp; and (c) teleospores of *Ustilago*/*smuts* spp with scale

(Photomicroscopy by Dr Philip Taylor, Deakin University)
2.8.2 Classification of outdoor fungal spores

The morphological identification of fungal spores has formed the basis for categorising exposure to outdoor fungal spores in epidemiological studies. Fungi are identified primarily by their method of spore production (1) and a simplified classification of fungi commonly found in south-eastern Australia is summarised in Table 2.1. In 2006, with advancements in genetic testing, the fungal phyla were modified with the incorporation of many Deuteromycetes (or Fungi Imperfecti) into the Ascomycota phylum (200) and thus making the Deuteromycete class defunct. Much of the previous research I will report on in this thesis will have examined associations with the “Deuteromycetes” but without specifying which fungal genera were included in this phylum. These changes are important as many of the allergenic fungal taxa, such as Alternaria, Cladosporium, Aspergillus, Penicillium and Drechslera, were included in this, now defunct, class. This has limited some of the comparisons between studies and with my own research.

The most recent taxonomic classification, based on DNA sequencing of the fungal phyla and genera associated with aeroallergen production, was reported by Levetin et al (3). The benefits of this new classification system include the specific IgE levels in fungal sensitised individuals appear to match their phylogenetic relationship closely. This, in turn, provides a more systematic approach to exploring potential cross-reactivity and permits the possibility for representatives from the same taxon to serve as a proxy for IgE to the genus (201).
Table 2.1: Classification of most common fungal species in south-eastern Australia relating to the major spore type and emission mechanism

<table>
<thead>
<tr>
<th>Fungal phylum</th>
<th>Sporulation type</th>
<th>Genus or taxon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota/</td>
<td>Predominately eject sexual ascospores into the air from asci, along with a large number of liquid droplets</td>
<td>Leptosphaeria</td>
</tr>
<tr>
<td>ascomycetes</td>
<td></td>
<td>Pleospora</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-Septate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xylaria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Didymella</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mildews</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other coloured</td>
</tr>
<tr>
<td>Basidiomycota/</td>
<td>Predominately eject sexual basidiospores into the air from extended basidia, as a result of high relative humidity</td>
<td>Coprinus</td>
</tr>
<tr>
<td>Basidiomycetes</td>
<td></td>
<td>Ganoderma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Agrocybe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other coloured</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other hyaline</td>
</tr>
<tr>
<td></td>
<td>Asexually produced teliospores are the main dispersal phase of smut fungi and are readily airborne. When the teliospores germinate, they give rise to smut basidiospores.</td>
<td>Smuts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rusts</td>
</tr>
<tr>
<td></td>
<td>Urediniospores – wind dispersed similar to many conidia</td>
<td></td>
</tr>
</tbody>
</table>
2.8.3 Sources of outdoor allergenic fungal spores

Aerobiological research has demonstrated that most outdoor fungal spores are from the Ascomycota and Basidiomycota phyla (33). The primary sources of outdoor fungal spores are plant materials and it is generally regarded that sources of these spores remain constant from year to year, but the release of fungal spores is seasonal and influenced by climatic conditions (1). However major changes in vegetation types are associated with variations in the fungal spores detected. For example, the fungal spore taxa found in a forest canopy differ greatly from those found associated with agricultural land growing crops and treated with fungal biocontrol agents (202). The mix of plant sources and weather patterns in the southern hemisphere is quite different to those in the northern hemisphere. Australia, in particular was colonised by European settlers approximately 230 years ago and as a consequence, the landscape contains a mix of native plants (predominantly Eucalypts *Myrtaceae*) and plant sources introduced from the northern hemisphere. In addition, Australia has some remnant native grasslands, mainly in rural areas and on the rural-urban interface, plus large swathes of pastoral grasslands and domestic grasses, that are predominantly introduced species. In general Australia has a temperate climate, but it also has highly variable weather patterns which will influence fungal growth and sporulation. Although there are ecological differences between regions and seasonal variation, the allergenic fungal genera that tend to dominate outdoor air include: *Cladosporium*, *Alternaria*, *Ustilago*/smuts*, *Leptosphaeria*, *Penicillium*, *Aspergillus* (71, 74, 76, 77).

High relative humidity is needed for basidiospores to be discharged, hence their concentrations are generally higher during, and following, rain events. Aerobiology research indicates that airborne concentrations of spores from the Basidiomycota are correlated with relative humidity rather than rainfall per se and that wind speeds have minimal effect on the release of these airborne spores, but is likely to influence dispersion, especially over long distance (203). Depending on the size of the rain drops, rainfall may also dislodge spores from fruiting bodies making them airborne. However prolonged rainfall may limit fungal spore dispersion as sporulation becomes difficult in these environmental conditions (204).

In contrast to these, although moisture (dew or rainfall) is required for sporulation of *Alternaria*, *Cladosporium*, *Aspergillus* and *Penicillium*, they are usually released under dry and windy conditions. These dry discharged spores are easily dispersed on the wind
currents and can be transported long distances, depending on the numbers clustered together, the shape of the spores, and the wind speed (203).

### 2.8.4 Fungal spore dispersion

Fungal spores are so small that they are easily transported by air currents and may affect people living away from the fungal spore sources. Closer proximity will lead to larger exposures (1) and little is known about the impact of long distance transport. Modelling of fungal spore dispersion is complex and still in the process of development for different environmental conditions. Monitoring stations have detected fungal spores that have been transported thousands of miles across seas, oceans and continents. However, very little is known about the vertical and distant transport of spores and other giant particles (>10 µm in diameter) (205).

The following section describes a range of methods used to sample and analyse outdoor fungal spores.

### 2.8.5 Sampling and analysing outdoor aeroallergens

A number of different air sampling techniques have been used to measure aeroallergen exposure (206). Aeroallergens are present in the atmosphere as airborne particles. Airborne particles can be sampled passively using gravity, as well sampled via airflow and impaction devices to enable volumetric analysis (207).

The earliest methods used gravity settling onto a coated microscope slide or open petri dish containing an agar medium placed in the area of interest for a defined period of time. This technique is regarded as a non-quantitative assessment of ambient concentrations of particles (for example spores or pollen grains) and the settlement of particles is affected by air movement and particle size. There is a bias toward larger and heavier particle sizes and so this form of sampling is not recommended despite being still available (207).

The most common instruments used for air sampling are impaction samplers. These samplers use inertia to separate particles out from the air stream and impact them onto an adhesive surface as the air stream is bent. The airstream is created by suction impactors or rotational arm impactors. The Burkard Volumetric spore trap (Burkard, Hertfordshire, England) is the most common example of a suction slit impactor. This slit impactor draws 10 litres of air per minute continuously through a 14mm by 2mm orifice onto a tape coated with adhesive which moves past the inlet at 2mm per hour over a 24-hour period.
This spore trap has a lower cut off of approximately 3.7 µm in particle size. The tape is transferred to a microscope slide and the fungal spores and pollen grains are identified and quantified using brightfield microscopy. Details of this ambient aeroallergen sampling system are outlined in Chapter 4 Methods, of this thesis.

The most common example of the rotational arm impactor is the Rotorod sampler. In this system particles adhere to silicon greased sample rods which rotate at 2400 rpm and collect for 1 minute from every 10 minute period over a 24-hour period (208). As for the suction slit impactor, the fungal spores and pollen grains are quantified using visual brightfield microscopic evaluation.

Although the Burkard Volumetric trap and the Rotorod sampling instruments appear to record similar relative changes in airborne particle concentrations, their particle recovery systems differ. The Burkard appears to be a superior instrument for sampling particles <10µm and the Rotorod appears to be equal or superior to the Burkard for collecting particles >10µm (209, 210). The selection of instrument can contribute to measurement bias as the majority of fungal spores are sized <10µm in both aerodynamic diameter and physical size (211, 212). The sampling rate can vary with differing wind speeds which can result in oversampling in high wind speed conditions (208). However, there are currently no alternative devices available that are more accurate for quantifying giant airborne particles.

A portable Anderson sampler uses culture plates with a variety of stages capturing different cut off levels. The culture plate samplers impact the airborne particles directly onto a Petri dish containing a culturable medium such as dichloran glycerol agar (DG18). Sampling times are usually one to five minutes with an average flow rate of 28.3 litres per minute. The plates need to be incubated and resulting colonies are analysed and counted. Results are reported as colony-forming units per cubic metre of air (CFU/m³). There is bias toward only those organisms that can be cultured and this method does not detect non-viable or difficult-to-germinate organisms collected from the air (207).

Air samples can be analysed using a range of methods that are selected on the basis of the sampling technique and the information needed from the sample. The methods commonly used include brightfield microscopy. This involves morphological analysis for identification and manual counting. This is time consuming, prone to human error and does not differentiate all species within the same genus, nor even within a family or
major group. Culturing allows for improved species identification but only of viable organisms that can grow on the selected medium. Biochemical methods have been used to identify mycotoxins and estimate total fungal biomass, as well as sugar types that are specific to fungi, such as mannitol and arabinitol. Advances in technology are leading to access and use of more sensitive techniques such as using immunoassays, molecular methods such as polymerase chain reaction (PCR) and genomic sequencing. But these methods generally require large samples (207). Image analysis, which is the extraction of classification information from digital microscopic images by means of digital image processing techniques, is also finding a place in air sample analyses along with automation, enhanced digital photography and image processing software [Personal communication, Dr Philip Taylor]. This technique has great potential to remove an enormous amount of labour out of manual counting of samples, enable rapid analysis and digital storage of the sample, and reduce the potential for human error, but no systems are currently available.

In the next section I will review the proposed mechanisms underlying exposure to fungal spores and inflammation in the airway tissues.

2.8.6 Mechanisms underlying outdoor fungal exposure that may lead to airway inflammation and asthma exacerbation

Multiple mechanisms have been proposed to explain the link between fungal spore exposures and airway inflammation. Most fungal spores and their associated hyphae are very small, with most sized from 2-10 µm in diameter (1). Fragmented hyphae have been detected in the air, and these may be of respirable size, but they only represent a small fraction of the mass released from fungal colonies (213). Fungal spores are readily dispersed by wind currents and their small size enables them to penetrate and lodge in the airways. The walls of fungal spores contain protein structures, some of which have been identified as allergenic (213). In addition to this characteristic, some fungal spores also have the ability to release damaging enzymes, or mycotoxins that can cause localised inflammation (33). The actions of these features can contribute to allergic sensitisation, allergic reaction, and induce airway inflammation and airway obstruction (102, 196-198). In addition to secreting enzymes, fungal spores may also release mycotoxins that can cause localised inflammation (33).
The precise mechanisms by which some fungal spores or hyphae cause adverse effects in the respiratory system have not been completely understood. Current understanding is that their small size and hydrophobic outer wall facilitates their deep penetration into the airways. Then, when in the airway, some spores have been found to germinate and secrete enzymes that directly affect the airway by degrading the integrity and barrier function of the epithelium, increasing inflammation at the site and allowing further penetration into the walls of the airway (Figure 2.9) (69). Another consequence of the lung epithelial breakdown is the hindrance of the maintenance of an effective anti-fungal fluid layer. In addition, fungal allergens can trigger the production of inflammatory cytokines which can exacerbate existing allergic inflammation and reduce tolerance to otherwise innocuous antigens present in the airway (Figure 2.10) (69). Fungi also release volatile organic compounds in a gaseous phase that may act as irritants, although allergens have not been detected in this phase (214).

The next section reviews allergenicity of fungi, fungal sensitisation, its association with asthma hospitalisation, how it can be detected, the issues with estimating its prevalence in the general population and the challenges in relation to cross-reactivity between fungal allergens.

Figure 2.9 Inhaled fungal spores penetrate to the terminal bronchioles due to size and hydrophobic coating. Upon germination, fungal spores expose their polysaccharide cell wall and release proteases which damage the epithelial cells barrier. Figure obtained from Roy et al 2013 (69)
2.8.7 Fungal allergenicity and sensitisation

2.8.7.1 Fungal allergenicity

Most fungi contain diverse and multiple allergens related to their cell wall structure, contents of their cytoplasm and/or their metabolic by-products (215). To date, only few fungal spore genera have been identified to be allergenic. According to the catalogue of fungal allergens approved by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies (IUIS) (216), of the approximately 100,000 fungal species that have been identified, currently only 111 fungal genera have been fully described and listed. The taxonomic relationships between these allergenic fungi are shown in Figure 2.11 (217). The majority of these allergenic fungi belong to the mitosporic species (produce spores by mitotic division) (215) of the Ascomycota. These include *Alternaria*, *Cladosporium*, *Candida*, *Aspergillus*, *Penicillium*, *Fusarium*, *Epicoccum*, *Trichophyton* and *Stachybotrys* (3, 218). Most allergen extracts used to test for fungal sensitisation are derived from these mitosporic fungi (219-221). The other major phylum of allergenic fungi associated with allergic airway conditions is the Basidiomycota phylum. Fungi in this group are meiosporic (produce spores via meiosis)
and release basidiospores, for example *Coprinus*; and those that release teliospores which are thick-walled spores that give rise to basidiospores, for example *Ustilago* /smuts (3). Currently there is a lack of commercial extracts for fungi in the Basidiomycota phylum to detect sensitisation to these potentially allergenic fungi but there is increasing evidence implicating this phylum in allergic diseases (33, 79, 215, 222).

### 2.8.7.2 Fungal sensitisation

Although humans are ubiquitously exposed to fungal spores throughout their life, not all develop fungal sensitisation, allergy or fungal-related pathology. The initial binding of antigen-specific immunoglobulin E (IgE) to mast cells or basophil cells that are associated with or mediate hypersensitivity or allergic reactions, sensitises these cells for future allergen exposure. Sensitisation is the development of elevated serum specific immunoglobulin E (IgE) against a certain agent (for the purposes of this thesis, fungal spores). Individuals with sensitisation to fungi do not necessarily develop allergic reactions when re-exposed to fungal spores or their associated by-products. However, sensitisation can evolve into symptoms of allergy, which is an inflammatory response caused by an exaggerated immune response, including symptoms of asthma (6).
Figure 2.11 Taxonomic relationships of allergenic fungi registered in the WHO/IUIS Allergen Nomenclature Subcommittee database (Figure obtained from Fukutomi et al (217))
2.8.7.3 Detecting fungal sensitisation

There are a number of methods used to detect allergen sensitisation in people. One method, skin allergen testing, confirms the presence of specific IgE antibody and establishes whether the antibody mediates an allergic response (223). It is a reliable method to detect sensitisation and can be used to help confirm diagnoses related to Type 1 IgE-mediated allergic diseases (224). In a positive skin test result, mast cells that are present in the skin release histamine which produces the wheal and flare response. There are two methods used to test the skin response: (1) prick and puncture (skin prick test using allergen reagent – SPT); and (2) intradermal (hypodermic injection of allergen reagent into the dermis). A review paper by the American Academy of Allergy, Asthma and Immunology reported that the SPT is more specific but less sensitive than the intradermal test. In addition, the SPT is minimally invasive, inexpensive, results are available within minutes and can be reproducible when administered by trained professionals (225). However there is the risk of a systemic allergic reaction to allergen reagents which must be planned for when undertaking these procedures (223). As such, the SPT is recommended as the primary test for detection of IgE mediated conditions, including sensitisation (223). In order to control for potential false negatives and false positive results, saline should be used as a negative control, and histamine should be used as a positive control (226). This procedure is described in the methods section of Chapter 6. Another method to detect allergen sensitisation is via the radioallergosorbent test (RAST). This involves analysis of allergen specific IgE levels in blood serum samples. Although these tests can be more specific than SPT and there is no risk of systemic allergic reactions, they are invasive to undertake, require more time to obtain a result, are lower in sensitivity, and can test only a limited range of allergens. I did not examine RAST results in this PhD research.

Currently there is no gold standard established for the diagnosis of fungal sensitisation, nor any method to assess the accuracy of a diagnosis (227). At this stage, sensitisation is assessed using crude reagents but there are issues with their reproducibility (194, 227). As a result there are currently no reference standards for fungal reagents despite the availability of a relatively wide range of extracts that are used in skin prick and serum IgE testing (228, 229).
The lack of standardisation of fungal extracts was highlighted in a large survey of 4962 subjects suspected of having respiratory allergy (aged 3 to 80 years) in Rome, Italy. Participants were tested for sensitisation to *Alternaria, Aspergillus, Candida, Cladosporium, Penicillium, Saccharomyces*, and *Trichophyton* using SPT (n=4962) and allergen-specific IgE tests (n=431) (228). The authors reported a comparative analysis of three commercial extracts used in SPT and IgE testing of a subset of fungal-sensitised participants. They found that the extracts exhibited high diversity among the allergenic protein contents and differing IgE reactivity patterns. They found discrepancies in the SPT response between extract types, and lower sensitivity for the IgE assay.

### 2.8.7.4 Fungal cross-reactivity

There are also challenges associated with cross-reactivity of fungal allergens with other antigens that complicates diagnosis of fungal sensitisation. Crameri and colleagues have succinctly described this complex immunological phenomenon. They explain that an allergen is a single protein that is able to induce a switch to IgE production in B cells resulting in the production of allergen-specific IgE antibodies. As a result allergens have the capacity to induce sensitisation and allergic symptoms, but not all IgE-binding proteins necessarily possess the capacity to induce allergic symptoms. However, all IgE-binding proteins that have at least two identical or different B cell antigenic attachments can potentially induce allergic symptoms via the cross-linking of specific IgE antibodies onto effector cells (mast cells, basophils) facilitating the release of histamine, leukotrienes, prostaglandins and cytokines, resulting in allergic disease (Figure 2.12) (194). Many fungal allergens exhibit cross-reactivity, but our current knowledge is limited with regard to cross-reactivity between species and the potential for a sensitised individual to react to a broader range of fungal genera than currently recognised (194).
2.8.7.5 Prevalence of fungal sensitisation

Most surveys of fungal sensitisation have been conducted in groups of people suspected of, or have, an allergic condition. Few studies have been conducted in the general population. Taking into account, the lack of standardisation of fungal reagents for testing sensitisation, varying estimates have been reported across risk groups and in different geographic locations.

2.8.7.5.1 General population

The prevalence of fungal sensitisation in the general population is not reliably known (218). In Australia, the Tasmanian Longitudinal Health Study (TAHS), which is a population based study, tested fungal sensitisation of 1157 participants at the 45 year old follow-up using SPT to *Alternaria tenuis*, *Cladosporium cladosporioides*, *Penicillium*
mix, and *Aspergillus fumigatus*. This study found that the weighted prevalence of fungal sensitisation varied between the fungal taxa: *Alternaria* (7%); *Cladosporium* (4%); *Aspergillus* (3%); and *Penicillium* (2%). Of these, the majority were poly-sensitised to multiple allergens. However about 10% of those sensitised to *Alternaria* were mono-sensitised to *Alternaria* (221). To date, this study provides best estimation of fungal sensitisation in the general Australian adult population.

Crameri et al’s 2014 review paper, using data originally sourced from Greece (Gioulekas et al (230)) and Kuwait (Ezeamuzie et al (231)), reported that prevalence of fungal sensitisation in the general population varied between different taxa: *Alternaria* (3.6-12.6%); *Aspergillus* (2.4%); *Candida* (8.5%); *Cladosporium* (2.5-2.9%); *Penicillium* (1.5%) and *Trichophyton* (1.9%) (218).

A study of 4962 subjects (aged 3 to 80 years) suspected of having respiratory allergy in Rome, Italy were tested for sensitisation to *Alternaria, Aspergillus, Candida, Cladosporium, Penicillium, Saccharomyces*, and *Trichophyton* using SPT (n=4962) and allergen-specific IgE tests (n=431) (228). Within this high-risk study sample, the results of the SPT estimated prevalence of sensitisation to at least one fungal extract to be 19% (n=621). Within this subset of the sample, the fungal reagents that accounted for positive SPT results were *Alternaria* (66%), *Candida* (44%), *Cladosporium* (13%), *Aspergillus* (13%), *Trichophyton* (10%) and *Penicillium* (8%) (228).

### 2.8.7.5.2 Children

A study of 1575 randomly selected school children aged 8 to 11 years was undertaken in two rural settings in Australia. One was located on the coast (Lismore) and one inland (Moree/Narrabri). Fungal sensitisation was assessed with SPT using *Alternaria tenuis* only. Sensitisation to *Alternaria* was influenced by geographic location, with prevalence in Lismore approximately 4%, and prevalence in Moree/Narrabri approximately 15% (the prevalence figures are reported in a graph and so estimates are reported here). The residential history indicated that those who had always lived inland had a higher sensitisation prevalence (16.8%) compared to those who had not (7.5%). Lismore children who had lived inland for more than 12 months had higher sensitisation prevalence (10.7%) than those who had not (2.8%) (29).
In a longitudinal study of 1246 infants in Tucson, Arizona in the USA total serum IgE levels were assayed and SPT were performed at the ages of 6 and 11 to identify allergen sensitisation. Fungal sensitisation was tested with *Alternaria alternata*. At age 6, 10.2% of controls (n=431) and 50% of doctor diagnosed asthmatics (n=80) had positive SPT to *Alternaria*. At age 11, 14.5% of controls (n=359) and 41% of doctor diagnosed asthmatics (n=106) had positive SPT to *Alternaria* (102).

### 2.8.7.5.3 Asthmatic population

The European Community respiratory health survey (ECRHS) obtained data on fungal sensitisation and asthma status from 1132 adults aged 20 to 44 years located in 30 centres throughout the United Kingdom (UK), Europe, Australia (Melbourne), New Zealand and Portland, Oregon in the United States. They assessed fungal sensitisation with SPT using *Alternaria alternata* and *Cladosporium herbarum* extracts. The study found that 11.9% of asthmatic adults were sensitised to *Alternaria*, and 5.8% were sensitised to *Cladosporium*. There were statistically significant regional differences for sensitisation to each fungus as shown in Table 2.2. They also found that sensitisation to fungi was significantly associated with the severity of asthma. For *Alternaria* sensitisation, in adjusted models, the odds ratio of having moderate vs mild asthma was 1.64 and for having severe vs mild asthma was 2.05 (95%CI 1.3 to 3.3). For *Cladosporium* sensitisation, the odds ratio was 3.2 (95%CI 1.7 to 5.9) for severe vs mild asthma (197).

<table>
<thead>
<tr>
<th align="left">Table 2.2 Proportions (%) of participants with asthma with fungal sensitisation in the ECRHS survey (Table adapted from Zureik et al (197)</th>
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<tr>
<td align="left">All</td>
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<td align="left">---</td>
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<tr>
<td align="left"><em>Alternaria alternata</em></td>
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<tr>
<td align="left"><em>Cladosporium herbarum</em></td>
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Sensitisation to a range of fungal genera has been associated with elevated risk of asthma exacerbation, severe asthma and asthma hospitalisations. The earliest evidence of an association between asthma hospitalisation and fungal sensitisation was in O’Hollaren et al’s 1991 retrospective study of 11 asthmatic children and young adults, aged between
11 and 25 years, between 1980 and 1989 hospitalised for respiratory arrest. They found that *Alternaria* sensitivity was associated with increased risk of respiratory arrest (232). Subsequent studies have built on this finding and extended the research to identify associations between sensitisation to other fungal genera (*Cladosporium, Aspergillus, Penicillium, Epicoccum, and Helminthsporium*) and asthma hospitalisations. (233-238).

In summary, prevalence of fungal sensitisation appears to be higher in children compared to adults. Monosensitisation is most often associated with *Alternaria*. Polysensitisation to a range of fungi is more common. Almost nothing is known about the prevalence of sensitisation to basidiospores or allergenic fungal taxa in the Basidiomycota phylum.

The following section discusses the associations between outdoor fungal spore exposure and asthma exacerbations, asthma hospitalisations, lung function and airway inflammation.

### 2.9 Outdoor fungal spores and child and adolescent asthma exacerbation

The probability that the exacerbation of asthma may be associated with environmental exposure to outdoor fungal spores has been hypothesised for more than 40 years (239). Children are more vulnerable to environmental exposures compared to adults because of their small lungs, and developing respiratory and immune systems. They tend to spend more time outdoors engaged in physical activity than adults, which increases the risk of exposure to outdoor fungal spores. Physiologically, children breathe more air per unit of body weight compared to adults which may expose them to a greater volume of fungal spores (240). Together, these factors potentially make children more vulnerable to the adverse health effects of outdoor fungal spores.

A birth cohort study of 514 children born to low-income, predominantly Mexican women in an agricultural region of California, were followed to 2 years of age. Researchers found that early life exposure to elevated outdoor fungal spores were associated with the onset of early life wheezing (odds ratio 3.1, 95% CI 1.3 to 7.4) independently of other seasonal factors including ambient levels of PM$_{2.5}$ and lower respiratory tract infections (156). Early childhood wheezing is associated with the development of childhood asthma and decline in lung function (241).
A panel study of 12 asthmatic school children over 6 weeks in the North American spring season found that 1-day lagged total fungal spore exposure was associated with increased asthma symptom scores and as-needed bronchodilator inhaler use. Participants were tested for *Alternaria*, *Cladosporium*, *Helminthsporium*, *Aspergillus* and *Penicillium* sensitisation with SPT. Regression models were adjusted for maximum temperature and relative humidity; and tests for day-of-week trends were applied. Interestingly they noted that in the sub-group of fungal sensitised participants (n=10) increased asthma symptom scores (coefficient = 0.03 standard error SE = 0.0068 p<0.001) and increased as-needed bronchodilator inhaler use (coefficient = 0.04 SE = 0.013 p=0.005) were most strongly associated with exposure to higher levels of unidentified fungal species that made up 41% of the total fungal spore counts (242). In another panel study of 13 children (aged 10 to 15 years) and 9 adults (aged 24 to 47 years), over 2 months in the North American spring-summer season, the same research group found that significantly increased as-needed bronchodilator inhaler use was associated with increased *Cladosporium* (coefficient = 0.45 95%CI 0.25 to 0.65), *Periconia* (coefficient = 0.23, 95%CI 0.1 to 0.36) and unidentified spores (coefficient = 0.33 95%CI 0.17 to 0.49) levels from their minimum to their 90th percentile (65). They also reported that increased asthma symptoms scores were significantly associated with increased total fungal spores (coefficient = 0.36 95%CI 0.26 to 0.46), *Alternaria* (coefficient = 0.27 95%CI 0.17 to 0.37), *Cladosporium* (coefficient = 0.36 95%CI 0.22 to 0.5), *Helminthsporium* (coefficient = 0.27 95%CI 0.17 to 0.37), *Coprinus* (coefficient = 0.2 95%CI 0.16 to 0.24), *Periconia* (coefficient = 0.3 95%CI 0.23 to 0.37), *Botrytis* (coefficient = 0.15 95%CI 0.09 to 0.21) and hyphae (coefficient = 0.28 95%CI 0.18 to 0.38). These models were adjusted for maximum temperature and relative humidity; and tests for day-of-week trends were applied to assess potential bias in the effect estimates. They identified more outdoor fungal spore taxa in this study than the previous one and reported that fungi other than the ones included in the sensitisation test panels may have equal or greater associations with asthma severity. Notably these fungi were *Coprinus*, *Periconia* and other unidentified taxa (65). The findings from these studies are to be interpreted with caution due to their small sample sizes. There is the risk that the results of analyses with a small sample may be spurious (due to chance alone) and the null hypothesis that exposure to higher levels of outdoor fungal spores are not associated with asthma symptoms is incorrectly rejected (Type I statistical error). Also, the results of a small
study sample in a single location may not be representative of the population. However the results give an indication that more research is needed on these types of exposures.

In a randomised controlled trial of environmental intervention to reduce asthma morbidity in New York City, Pongracic et al examined asthma symptoms and exacerbations that warranted an unscheduled visit to the doctor or hospital in a fungal-sensitised subset of the study sample. They found that increases in outdoor fungal spore levels (specifically *Alternaria, Aspergillus, Cladosporium* and *Penicillium*) were significantly associated with increased asthma symptoms, but only increased outdoor *Aspergillus* spores were associated with unscheduled visits to a health service for asthma management (81). It is noteworthy that *Aspergillus* and *Penicillium* are the most difficult species to detect with most outdoor impaction methods, and generally requires culture-based techniques. This study used a Burkard portable culture plate air sampler and there may have been some measurement bias as sampling times were generally short, the sampling involved low flow rate of air, and this method does not detect non-viable organisms or ones that are difficult to germinate.

In Australia, a prospective study of 399 school children in arid, inland rural New South Wales, Downs et al reported that airway hyperresponsiveness, wheeze, and bronchodilator inhaler use increased significantly in association with increased *Alternaria* spores concentrations. The increased airway hyperresponsiveness was greater in children sensitised to *Alternaria* than in other children (p-value = 0.01). The odds ratio for airway hyperresponsiveness in children sensitized to *Alternaria* was 1.26 (95% CI 1.14 to 1.39) after an increase in mean exposure of 100 spore/m$^3$/day over 1 month. They suggested that *Alternaria* allergens contributed to severe asthma symptoms in this region where exposure to the *Alternaria* spores was high (243). The authors stated that they adjusted for potential confounders but did not state what they were. They did state that they were unable to adjust for rye grass pollen as it was highly correlated with *Alternaria* spore counts.

A year-long time-series study of 14 asthmatic children and adolescents mono-sensitised to fungi (*Alternaria, Cladosporium, Penicillium* or *Aspergillus*) in Turkey found that levels of outdoor *Cladosporium* and *Alternaria* correlated with mean asthma symptom scores (32). It is difficult to interpret the correlations as the researchers did not use appropriate statistical methods to assess levels of association (risk) between increasing

48
levels of outdoor fungal spore taxa and the asthma symptoms. In addition, they did not control for potential confounding factors such as pollen or air pollution exposures, or respiratory illnesses that may have contributed to asthma exacerbation.

When asthma symptoms are exacerbated and become severe then medical intervention is often required to control the potential life-threatening effects of this condition. In Chapter 3, I report on the findings from a systematic review that I undertook to examine the reported associations between outdoor fungal spores and child and adolescent health service attendances for management or treatment of their asthma exacerbation. I will briefly outline the findings from this review and report on new research that has been published since that systematic review. The geographic distribution of studies is broader than the studies that have examined asthma symptoms.

Ecological studies have been the most common study design to investigate the associations between outdoor fungal spore levels and asthma exacerbations in children and adolescents that required attendance to a medical practitioner, emergency department or hospital. One time-series study conducted in Darwin (a regional city) in tropical northern Australia, limited fungal exposure identification to *Alternaria* only and no association was found (159). Ecological studies have been conducted in Canada (48, 78, 244-246) [26, 28, 39-42], USA (247, 248), Great Britain (79, 249), Mexico (250), Israel (251) and India (80) and have used varying methodologies for their analysis (i.e. correlation, time series). Overall, ecological studies that were conducted over a longer time period (48, 78, 246), with multiple fungal species data [27, 40], with larger sample size (48, 80, 244) and utilising time series analytical methods (48, 78-80, 244-247, 249) indicated that there may be a positive association between outdoor fungal spore exposure and asthma hospitalisation.

Pongracic et al’s cohort study nested in a randomised controlled trial (81) found that elevated levels of outdoor *Aspergillus* were associated with increased asthma health service attendances in children sensitised to fungal allergens. Two studies that analysed only correlational relationships between levels of outdoor fungal spores and asthma hospitalisations, one in Israel (251) and one in USA (248), in addition to an Australian time-series study (159) reported no association. These findings are to be interpreted with
caution as these studies reported only qualitative assessments of their data analyses and no data were provided; and so I did not consider this strong evidence of no associations.

Although time series methods have become more sophisticated and sensitive to enable fungal exposure to be analysed at the taxa or genera level rather than pooled at the aggregated level of phyla, group or total fungi, they appear to be limited as they were unable to analyse personal risk factors or individual characteristics such as infection with respiratory viruses, fungal sensitisation status, co-morbidities and often did not control for potentially confounding variables such as exposure to grass pollens. A strong finding from the studies that examined associations between outdoor fungal spores and asthma hospitalisations was that fungal sensitised children and adolescents appeared to be at much higher risk. This indicated that there is a need for research that comparatively examines asthma exacerbations and hospitalisations in children and adolescents who are either non-sensitised or sensitised to fungi, whilst also controlling for potential confounding factors that ecological studies have been unable to do.

Another apparent finding from this review is that, although Australia has a very high prevalence of asthma compared to the rest of the world, very little research has been undertaken to determine whether outdoor fungal spores may be contributing to the asthma exacerbation burden. To date, none has been reported from Melbourne, Australia. A recent severe thunderstorm asthma event in Melbourne (21 November 2016) resulted in nine fatalities and an unprecedented 8500 people attending Melbourne public hospitals and emergency departments in a 12-hour period. An extensive government review of the event is currently underway at the time of writing this thesis. Early reports from botanists and the pollen monitoring network did report extreme levels of rye grass pollen on that day but also there were high concentrations of fungal spores, especially smuts (252). Although this extreme event is rare, it is an indication that the contribution of ambient pollen and fungal spores to asthma and asthma exacerbation warrants further research.

Decreased lung function and increased airway inflammation may be clinical or pre-clinical markers of active asthma or asthma exacerbation. The following section provides a review of the current evidence of associations between outdoor fungal spore exposure and lung function and airway inflammation.
2.10 Outdoor fungal spores and lung function

A number of studies have been published that examined associations between exposure to outdoor fungal spores and changes in lung function. The results are mixed but tend toward indicating that increasing fungal spore levels are associated with decrements in lung function. The most common study design is the panel study with multiple readings over time, however one study was cross-sectional. I will start by discussing the cross-sectional study, then the panel studies.

In the USA, Nelson et al examined 1041 children aged between 5 to 12 years with chronic mild to moderate asthma symptoms. The children underwent SPT to identify possible sensitisation to *Alternaria tenuis*, *Aspergillus* species and *Penicillium* species. Fungal spore season, based on a published historical inventory of the presence of ambient *Alternaria*, was used as the proxy measure for exposure to any fungal spore type near their home. Spirometry was conducted at a single point in time to assess FEV\(_1\). Multiple linear regression models were adjusted for age, ethnicity, gender, clinic location, total eosinophil count, IgE levels, and duration since asthma diagnosis. Their results indicated that the *Alternaria* season did not significantly affect FEV\(_1\) in *Alternaria*-sensitised children (253), however there were a number of methodological issues which hampered this interpretation. Height is an important determinant of lung volume but this variable was not controlled for in the analysis. The ambient *Alternaria* spore levels were not validated using standardised ambient spore measuring methodologies. In addition, the models were not adjusted for potential confounders such as temperature, humidity.

Neas et al undertook a panel study in Pennsylvania, USA with 108 children with suspected respiratory problems; however any children who had used asthma medication in the previous 12 months were excluded. The children participated over one summer (June to August 1991) and kept diaries of respiratory symptoms and had four pulmonary function tests over this period using a peak flow meter to obtain morning and evening peak expiratory flow rate (PEFR). Fungal spore exposure was estimated using a Burkard volumetric spore trap at a single site and fungal genera were identified and counted. The fungal genera analysed were: *Alternaria*, *Epicoccum*, *Drechslera*, *Pithomyces*, *Stemphylium*, *Cladosporium*, *Coprinus*, *Ganoderma*, other coloured Basidiospores, and coloured Ascospores. The auto-regressive linear models were adjusted for average temperature, afternoon measurement and autocorrelation. The major finding in this study
was that increased levels of *Cladosporium, Epicoccum* and *Coprinus* were associated with deficits in mean morning PEFR (62). When the 24 hour average concentration of *Cladosporium* was above 10,000 spores/m³, the mean change in PEFR was -1.03 litres/minute (95%CI -1.86 to -0.2); when concentration of *Epicoccum* was above 60 spores/m³, the mean change in PEFR was -1.5 litres/minute (95%CI -2.83 to -0.18); and when concentration *Coprinus* was above 170 spores/m³ the mean change in PEFR was -0.78 litres/minute (95%CI -1.49 to -0.07). However, the models did not account for respiratory viral infection, fungal sensitisation status, concomitant pollen or air pollution levels, and so the results may be confounded. Pulmonary function testing was not supervised so there was the risk that the procedure was not undertaken or recorded correctly, possibly introducing some measurement and ascertainment bias.

Also in the USA, Delfino et al undertook a panel study with 22 asthmatic children and adults (aged 9 to 46 years) over one summer period (May to July 1994) which had been identified as a fungal spore season for that region. In this study, fungal sensitisation status was identified via SPT using the reagents of *Alternaria alternata, Cladosporium cladosporiodes, Helminihsporium interseminatum, Aspergillus* species mix; and *Penicillium* species mix. Fungal spore exposure was estimated using a Burkard volumetric spore trap at a single site. Fungal genera were identified and counted: *Alternaria, Cladosporium, Helminthsporium, Aspergillus-Penicillium*, total basidiospores, total ascospores, *Coprinus, Periconia, Botrytis, hyphae, Rusts,* and unidentified fungi. Daily morning and evening PEFR were recorded using a peak flow meter. All analyses were adjusted for maximum temperature and relative humidity. In the adjusted models increased total fungal spore levels from the minimum to the 90th percentile on the same day and up to one day lag were positively associated with reduced evening PEFR (-12.1 litres/minute, 95%CI -1.8 to -22.3) in those with positive SPT to fungi. When the associations were analysed by fungal genera, the largest PEFR effects were associated with the exposure to the category ‘unidentified spores’ (-10.6 litres/minute 95%CI -5.8 to -15.4), followed by higher levels of *Periconia* (-9.6 litres/minute, 95%CI -5.8 to -13.4) (65). The analyses were not stratified into children compared to adults. A limitation, but also strength of this study, was the inclusion of ‘unidentified fungi’ as this group represented about 18% of the total fungal spores detected. This raised the question of the contribution of other, less well identified, fungal spores to adverse respiratory effects. Self-recording of PEFR could have introduced some
measurement and ascertainment bias, but the researchers tried to overcome this by undertaking random observation of the procedure.

In Australia, Rutherford et al examined asthmatic children and adults in a panel study conducted in two sites (Brisbane and Ipswich) in sub-tropical south-east Queensland, Australia. The numbers of participants fluctuated during the 2-year study (1994 to 1995) due to holidays, withdrawals and recruitment of new participants. Participants underwent SPT and were classified as sensitised if they had a positive response to either a pollen or fungal allergen, but the panel of reagents tested was not reported. Only sensitised participants were included in the final analysis: 25 from Brisbane and 28 from Ipswich. Summary descriptive statistics of the participants from both sites were not clearly described. Participants were stratified into three age groups: <15 (n=17), 15 to 54 (n=20), and >54 years (n=16). The Brisbane group were predominantly aged >54 years and half of the Ipswich group were aged <15 years, however both groups were pooled for the final analysis. Fungal spore counts were estimated from two sites in different parts of Brisbane (capital city of Queensland) using the Burkard Volumetric spore trap. Participants recorded daily morning and evening PEFR using peak flow meters before they used any medications. Their key finding was that total outdoor fungal spores (Alternaria, Cladosporium, Ustilago/smuts, Epicoccum and ‘other fungi’ that were not identified) were associated with decreased standardised PEFR in adults aged >54 years (coeff = -0.019 litres/min, 95%CI -0.035 to -0.004) during wet periods of summer (peak fungal spore season) over the two year period. ‘Other fungi’ were associated with decreased standardised PEFR in adults aged >54 years (coeff = -0.022 litres/min, 95%CI -0.043 to -0.0006) and the total study sample (coeff = -0.026 litres/min, 95%CI -0.026 to -0.0007) during the same period. Ambient fungal spore levels were not significantly associated with changes in PEFR in children (66). The fungal spores held their significant associations in multi-pollutant models that included particulate matter and ozone. As with the other studies that relied on participant compliance with recording daily PEFR, without observation the results may be subjected to measurement and ascertainment biases. The inclusion of ‘other fungi’ also provides insights into the undetected contribution of other, less well recognised, fungal spore genera to lung function changes.

In Turkey, Inal et al undertook a panel study of 19 children aged 4 to 13 years who had been diagnosed with asthma or allergic rhinitis and were found to be sensitised to fungi only (Alternaria, Cladosporium, Penicillium, Aspergillus) by SPT for one year. Fungal
spore exposure was estimated using a single Burkard volumetric spore trap and they identified the fungal genera *Cladosporium, Alternaria, 1-septate Ascospores, Exosporium, Ustilago, Drechslera, Epicoccum, Pleospora, Periconia, and Leptosphaeria*. Participants recorded morning and evening PEFR, however the method to obtain this was not described. The researchers then analysed the associations between levels of total fungal spores and changes in PEFR using Spearman correlation. They reported that total outdoor fungal spore levels were significantly positively correlated with mean monthly asthma symptom scores ($r = 0.831, p = 0.001$); negatively correlated with mean monthly morning PEFR ($r = -0.741, p = 0.006$) and mean monthly evening PEFR ($r = -0.720, p = 0.008$) (32). Although they identified fungal genera they did not report correlations between the individual fungal genera and changes in PEFR. The statistical method has not analysed levels of associations between the fungal spores and the changes in lung function while controlling for any potential confounding variables so it was not possible to interpret these findings with confidence.

In Taiwan, Chen et al conducted a longitudinal study of 100 school children attending five schools located within 2.5km of the air quality monitoring station between October 2007 and November 2009. They conducted lung function tests, using spirometry, 5 to 10 times during the study period. Overall they obtained 824 readings of forced vital capacity (FVC), forced expiratory volume in 1 second (FEV$_1$), forced expiratory flow at 25%, 50%, and 75% of FVC (FEF$_{25\%}$, FEF$_{50\%}$, and FEF$_{75\%}$), and mid-expiratory flow (FEF$_{25\%–75\%}$) from the 100 students. Fungal spore counts of 22 fungal spore genera were estimated from the central monitoring station using the Burkard Volumetric spore trap. They found that, out of 22 fungal spore taxa examined, only when levels of *Cladosporium* spores exceeded 1000 spores/m$^3$, that a doubling of 1-day lag *Cladosporium* level was inversely associated with reduced FVC (-0.25 litre, 95%CI -0.37 to -0.13) and reduced FEV$_1$ (-0.23 litre, 95%CI -0.35 to -0.11) (8, 60). Each lung function parameter was adjusted for multiple and comprehensive covariates: age, height, interaction term of age and height, upper respiratory infection, asthma/allergic rhinitis symptomatic attack, use of asthmatic/allergic rhinitis medicine, 1-day lag PM$_{2.5}$, 1-day lag PM$_{10-2.5}$, 1-day lag ozone, 1-day lag carbon monoxide, 1-day lag nitrogen dioxide, 1-day lag sulfur dioxide, temperature, relative humidity, day-of-week, gender, school, parental education, parental atopy, household water damage, and household walls with visible mould.
In Japan, Watanabe’s undertook a panel study of 339 school children with and without allergic diseases. The children recorded their morning PEFR, using a peak flow meter when they arrived at school, over one month in the Japanese winter (February 2015). Fungal spore counts were measured at the school using a detachable filter in a high-volume air sampler from which fungal spores were cultured on agar plates. Daily concentrations of outdoor fungal spores were expressed as colony forming units per cubic metre of air (CFU/m$^3$). The location of the air sampler within the school was not described. Multivariate models were adjusted for suspended particulate matter and PM$_{2.5}$. The researchers reported that an increase in 46.2 CFU/m$^3$ of outdoor fungal spores (taxa were not specified) were associated with reduced morning PEFR by 1.44 litres/minute in asthmatic children (61). The results were stratified by diagnosis of asthma in the study subjects but no interaction analyses were reported or discussed. Although the researchers collected data on sex, age, height, weight, and the presence of asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies, they did not account for the presence of respiratory infections that are common during winter. As this study was conducted in winter, it is likely that fungal spore levels would be lower than in warmer times, such as spring and autumn when there are peaks in their concentrations. This may have underestimated the effect of fungal spores on lung function at warmer times of the year.

These studies have demonstrated that assessing the contribution of outdoor fungal spore exposure to changes in lung function is methodologically difficult to undertake for a number of reasons. Measuring individual exposure to ambient fungal spores in addition to other potentially confounding environmental factors, such as pollen and air pollutants, is challenging. Despite obtaining counts of fungal spores at the genera level, only three studies reported analyses at these levels, and reported different levels of associations between fungal spore genera and changes in lung function. Analyses conducted at this level were more sensitive to the diverse effects different fungal spores may have on different people, particularly in relation their risk profile. From these results, there does not appear to be any consistent fungal spore taxa associated with decreased lung function. The contributions of ‘unidentified spores’ to reductions in lung function raise an important question in relation to the nature of these spores and the need to identify and examine them more closely. Although a number of studies obtained information on the
fungal sensitisation status of participants, no studies reported the potential interaction this risk factor has on exposure to different fungal spore taxa. This could provide some further insight on sensitisation as a risk factor that may warrant preventive interventions to prevent respiratory problems or asthma exacerbation. It may also provide information to assist in better understanding potential cross-reactivity between fungal species on allergic responses, particularly in relation to asthma exacerbation. Although these studies are not closely comparable due to differing methodologies in relation to exposure assessment, outcome measurement and analytical approaches, it does appear that children appear to be vulnerable to different fungal genera than adults and older adults. No studies examined age as a potential effect modifier. Another factor that arises from these studies is the differences in fungal spore types and concentrations between study locations and whether the findings are generalisable across geographic settings.

2.11 Outdoor fungal spores and airway inflammation

Airway epithelial cells form an active barrier along the airways that connect the lungs to the external environment. This active barrier is the first point of contact for fungal allergens and their associated toxins. It is the initial point that triggers an inflammatory response to inhaled fungal spores and hyphae to protect the airways and lungs. Furthermore, it is also the site where a dysregulated response in the airway epithelial cells to the inhaled fungal spores can result in triggering allergic airway disease, such as an asthma exacerbation (6, 69). Hence it is biologically plausible that exposure to some outdoor fungal spores may trigger short-term airway inflammation which may increase an individual’s risk of experiencing an asthma exacerbation or other obstructive respiratory condition. Based on multiple animal studies this appears to be reasonably well established (254-256). Despite this, I have been unable to find any studies that have specifically reported on associations between exposure to outdoor fungal spores and the presence of markers of airway inflammation in humans.

However, numerous studies have reported associations between aeroallergen (including fungal spores) sensitisation and the presence of markers of airway inflammation, particularly nitric oxide detected in fractional exhaled nitric oxide (FeNO) tests (257-260). Sordillo et al studied 12 year old children (n=430) and found that, in univariate models, Alternaria sensitisation was associated with increased FeNO readings but this
association did not hold in the multivariate models. Sample size may have been a limitation in detecting an effect as only 4 to 11% of the 430 study participants were sensitised to *Alternaria* or *Penicillium* (257). Jackson et al reported that children with aeroallergen sensitization, had significantly increased levels of FeNO compared with those not sensitized to aeroallergens (6 years, 10.9 vs 6.7 ppb, p-value < .0001; 8 years, 14.6 vs 7.1 ppb, p-value < .0001) (259). In a follow-up of a birth cohort of 864 young adults at 18 years of age, Scott et al found that increased FeNO was significantly associated with allergen sensitisation (common aeroallergens that included *Alternaria* and *Cladosporium*, grass and tree pollen, house dust mite, animal dander; and a range of food allergens) but the analyses were not stratified by allergen types (260). In a larger population study of 1338 adolescents and adults, Craig et al found that exhaled nitric oxide levels increased in those with increasing numbers of positive skin prick test responses to a panel of aeroallergens, which included *Alternaria*, *Penicillium* and *Aspergillus* (258).

Understanding whether exposure to outdoor fungal spores is associated with airway inflammation may contribute to identifying those at risk for an asthma exacerbation.

### 2.12 Potential effect modifiers and confounders of outdoor fungal spores

Understanding what triggers an asthma attack is complex due the multifactorial aetiological factors that are associated with the exacerbation of asthma. In real life, multiple simultaneous exposures are frequent and it is important to try to separate synergistic or additive effects in order to understand how respiratory health can be protected.

Fungal sensitisation appears to be an important and strong risk factor for asthma exacerbation (49, 236), persistent asthma (234, 235), asthma hospitalisations (233, 238) and decreased lung function (66, 261). A number of studies have demonstrated that increases in aeroallergen sensitisation over time can occur in childhood (262, 263). It has been postulated that sensitisation to allergens, such as *Alternaria*, is dynamic and can change across the young lifespan, depending on the local patterns of sensitisation (264). As such it is likely to be an important effect modifier of outdoor fungal spore exposure.
due to the differential risk between those who are not sensitised to fungi and those who are.

Ambient particulate air pollutants smaller than 10µm in diameter are able to enter deep into the respiratory system and, due to their inherent porous surfaces and electrostatic properties, appear to readily adhere to aeroallergens such as fungal spores, pollen grains or animal dander upon the point of impact (173). The particulate matter or gaseous pollutants may stimulate airway inflammation and promote airway sensitisation or hyper-reactivity and thereby interact with the aeroallergens and modulate their allergenicity. The airway mucosal damage and the impaired mucociliary clearance induced by air pollutants may facilitate the access of inhaled allergens to the immune system; this link could enhance the risk of atopic sensitisation and exacerbation of symptoms in sensitised subjects (265). Controlled laboratory studies have demonstrated the synergistic relationship between air pollutants and aeroallergens (266, 267) and this has been further supported by large epidemiological studies in Canada that have shown that there is an increased risk of asthma hospitalisation associated with ambient fungal spores on days of higher air pollution (47, 268). Australian cities generally have similar low levels of air pollution as reported from Canada (269) but no similar research has been conducted in Australia where asthma prevalence is slightly higher than in Canada (14).

Pollen is a well-documented trigger for asthma exacerbation and asthma hospitalisations (30, 31, 158) and airway inflammation (160, 257). The pollen season is generally well defined, occurring during spring and early summer, when pollen grains are released and dispersed during warm, dry and windy conditions. Some allergenic fungal spores, such as *Ustilago* and other smut fungi produce and release their spores when their host plant (commonly grasses, cereal crops and corn) pollinates or matures (3, 270) under similar meteorological conditions. This could establish a situation where pollen and fungi may confound or interact with each other when analysing their associations with allergic respiratory outcomes.

Furthermore it is known that respiratory viral infections, particularly human rhinovirus (HRV) infections, are well documented as triggers of asthma exacerbations (27) with over 80% of asthma admissions in school-aged children being triggered by a HRV infection (46, 105, 106, 146) although allergic sensitisation may play a synergistic role (45). A case control study with adults (not children) found that allergens and respiratory
viruses may act synergistically to exacerbate asthma but exposure to fungal spores was not assessed (46).

Epidemiological research has documented clear gender differences in relation to the prevalence of asthma. There is a predominance of boys before puberty, and then there is a predominance of girls after puberty (51). This trend is also observed with asthma hospitalisations in Australia (26); however, in Melbourne, Australia, younger girls appear to be at greater risk of asthma readmissions within 28 days (OR = 1.15 95%CI 1.001 to 1.32) or 12 months (OR = 1.11; 95% CI: 1.05-1.19) (89). There are numerous hypotheses proposed to explain this gender differential and switch at puberty which include hormonal changes at puberty, testosterone acting as an immunosuppressant, inflammatory action of female sex steroids, sex differences in lung development, risk of obesity, socio-cultural influences (for example cigarette smoking patterns, exposure to cosmetics, engagement in physical activity) (51). However to date, no hypothesis has been confirmed.

2.13 Summary of knowledge gaps in the literature

This literature review shows that there is conflicting evidence as to whether outdoor fungal spore exposure triggers severe asthma exacerbation, or is associated with lower lung function. There is no published research reporting on outdoor fungal spore exposure and airway inflammation. There are major gaps in our understanding of the role that outdoor fungal spores play in asthma exacerbations, lung function and airway inflammation.

Australia is among the countries with the highest asthma prevalence among children and adolescents in the world. Much research has been undertaken to examine the effects of pollen and air pollution on asthma exacerbations, but despite the international recognition that fungal spores have been associated with asthma exacerbations and decreased lung function, little research has been undertaken in this country. Most of the research referred to in this literature review has been conducted in the northern hemisphere – Canada, USA, Europe, UK and northern Asia. It is not clear if the outdoor fungal species found to be associated, or not associated, with asthma exacerbations in the northern hemisphere will have similar effects in the southern hemisphere due to differing climate, seasons, plants, grasses, land use and land management practices and levels of air pollution.
Despite the strong evidence of the contribution of HRV infection to child and adolescent asthma hospitalisations, there is currently no published research that has examined the combined role of outdoor fungal spores, HRV infection and fungal sensitisation on child and adolescent asthma exacerbations. It is not been established if there may be a synergistic, confounding or interactive relationships between these potential triggers of severe asthma exacerbation.

There is limited understanding of the potential interactions that individual and environmental factors may have on outdoor fungal spores and asthma hospitalisations, airway inflammation or lung function. It is possible that ambient pollen grains may confound or interact with the effect of outdoor fungal spores, but there is little research that has explored this. There is limited evidence of effect modification by common air pollutants on outdoor fungal spores and asthma hospitalisations, and lung function. There has also been limited exploration of sex and age as potential effect modifiers of outdoor fungal spores on asthma hospitalisations, but none on associations with lung function or airway inflammation.

Moreover, there is no published research that has examined the contribution of outdoor fungal spores, with air pollutants, pollen exposure, fungal sensitisation and HRV infection together, on child and adolescent asthma hospitalisations.

My doctoral work aims to address some of the gaps in knowledge identified in this literature review, with particular focus on the effects of outdoor fungal spores on child and adolescent asthma hospitalisations; effects of outdoor fungal spores on preclinical markers of asthma exacerbation, namely lung function and airway inflammation; and explore potential interactive effects of sex, age, fungal sensitisation, human rhinovirus co-infection, air pollutants and grass pollen.
Chapter 3

Contribution of outdoor fungal spores in child and adolescent asthma health service attendances

3.1 Introduction

This chapter consists of a peer-reviewed published paper of a systematic review that I undertook at the commencement of my PhD research that aimed to synthesise the current evidence of the associations between outdoor fungal spores and child and adolescent asthma health services attendances. As this results chapter is closely aligned to the literature review synthesis I have placed this chapter before the methods and results of the other research questions.

This paper and supplementary material is the first systematic synthesis of the associations between exposure to outdoor fungal spores and child and adolescent health services attendances. It provides a critical appraisal of the current evidence and highlights the inconsistent methods and findings between studies but also illustrates that there is a trend that asthmatic children appear to be susceptible to severe asthma exacerbations when exposed to outdoor fungal spores.

I led the development of this research question, developed the methodology, undertook all aspects of the systematic review, led the writing of the manuscript, was responsible for all edits and revisions, and submitted the manuscript to Pediatric Allergy and Immunology.

3.2 Research question

What is the current evidence of the role of outdoor fungal spores on child asthma health services attendances?

3.3 Aim

To systematically synthesise the evidence as to whether outdoor fungi were significant triggers of childhood asthma exacerbations that resulted in attendance to a primary care service, an emergency department or admission to a hospital for medical care.
3.4 Publication

Main document and supplementary data

Outdoor fungi and child asthma health service attendances

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aerallergen; asthma; child; emergency department; fungi; health services; hospital; mould; systematic review

Abstract

Asthma is a significant global public health issue. Severe asthma exacerbations can be triggered by environmental factors and require medical care from health services. Although it is known that fungal exposure may lead to allergic sensitization, little is understood about its impact on asthma exacerbations. This review aims to examine whether outdoor fungi play a significant role in child asthma exacerbations. Systematic search of seven electronic databases and hand searching for peer-reviewed studies published in English, up to 31 August 2013. Inclusion criteria were study population aged <18 yr, diagnosis of asthma, attended a health service; outdoor fungi exposure was reported. Quality and risk of bias assessments were conducted. Due to significant heterogeneity, meta-analysis was not conducted. Of the 1896 articles found, 15 were eligible. Findings were not consistent, possibly due to methodological variations in exposure classifications, statistical methods and inclusion of confounders. Cross-sectional studies found no or weak associations. All but one time series studies indicated an association that varied between fungal species. Increasing evidence indicates that asthmatic children are susceptible to asthma exacerbations when exposed to outdoor fungal spores. There is limited understanding of the contributions of different fungal species. Research is needed to investigate interactions of outdoor fungi with pollen, air pollutants and respiratory viruses.

Asthma is a significant global public health problem (1). It is the most common chronic condition affecting children and adults, and it is estimated that 235 million people are affected by asthma (2).

Persistent asthma can significantly impair the quality of life of affected individuals and contribute to significant financial burdens on individuals, families and the healthcare system (3, 4). The burdens incurred include limited active social engagement, healthcare expenses, loss of income and reduced productivity (5, 6). Poorly managed childhood asthma may have long-term health effects including increased risk of developing chronic severe asthma and chronic obstructive pulmonary disease in adulthood (7).

A sudden-onset severe asthma attack often requires attendance to a general medical practitioner (family physician), or at an emergency department, and/or admission to hospital. It is a major cause of hospital admissions for children (8-10). More than half of asthma hospital admissions involve children aged 0–18 yr. Children aged 12–18 yr are most likely to be admitted for longer periods (4, 10–12). It is known that these asthma events can be triggered by a number of factors. Amongst the most common are respiratory viral infections, especially human rhinovirus (HRV) (13); aerallergens, such as pollen (14, 15); and fungi (16, 17); air pollutants (18); and thunderstorms (19). The impacts of most of these trigger factors have been investigated in relation to asthma exacerbations in the past couple of decades but exposure to outdoor fungi has not received the same attention.

Exposure to fungi is associated with allergic sensitization which is an important risk factor for allergic asthma (17, 18, 20-22). Furthermore, airborne fungal spores can trigger asthma because many of their proteins are allergenic and their
small size (mostly 2–10 μm) allows them to penetrate and lodge in the airways and lungs and produce damaging organic by-products (23, 24).

Fungi are amongst the most ecologically diverse and successful organisms on earth and are classified in their own kingdom, which continues to evolve. There is estimated to be more than 1.5 million species of fungi, of which fewer than 10% have been formally identified and described (25). Fungi are identified primarily by their method of spore production (26) and a simplified classification of fungi most commonly associated with respiratory effects are: (i) Ascomycota (those that eject sexual ascospores with liquid droplets into the air, e.g. *Leptosphaeria*, *Pleospora*); (ii) Deuteromycota (those that release asexual conidia into the air, e.g. *Alternaria*, *Aspergillus*, *Cladosporium*, *Drechslera*, *Epicea*, *Periconia*, *Stemphylium*, *Torula*, *Botrytis*, *Aureobasidium*, *Penicillium*, *Helminthosporium*); and (iii) Basidiomycota (those that eject sexual basidiospores with liquid droplets into the air, e.g. *Coprinus*, *Ganoderma*).

In 2006, with advancements in genetic testing, the fungi sub-groupings were modified with the incorporation of Deuteromycota into Ascomycota and Basidiomycota phyla (27).

The fungal species *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Epicea*, *Helminthosporium* and *Penicillium*, are most frequently implicated in fungi-related allergic asthma exacerbations amongst adults in indoor and outdoor settings (23, 28, 29). In general, outdoor fungi are generally more abundant than indoor fungi (30). However, it has not been clearly established whether these fungal species are important determinants of childhood asthma exacerbations resulting in health service attendances and hospital admissions.

To date, no systematic reviews have been published that examine the role of outdoor fungal spores in severe childhood asthma exacerbations. The aim of this review was to examine whether outdoor fungal spores play a significant role in childhood asthma exacerbations that require attendance to a primary care service, an emergency department or admission to a hospital for medical care.

**Methods**

This review was conducted following PRISMA guidelines (31) for reporting systematic reviews.

**Eligibility criteria**

Inclusion criteria were an observational study, human study, published in English as at 31 August 2013. The study population was children and adolescents aged <18 yr who had a discharge diagnosis of asthma when they attended a primary care service/general medical practice, an emergency department (ED) or were admitted to a hospital. Studies must have reported on exposure to outdoor fungi and provided exposure measurements.

**Search strategy**

The literature was searched using bibliographic databases: Ovid Medline, PubMed, EMBASE, CINAHL, Plus, Web of Science, ProQuest Health and Medical Complete, Google Scholar and Monash University Meta-search. An extensive list of search terms was used as the term ‘fungi’ is a term that is interchangeable with other terms such as mould, mold, spores, mould/mold components and specific species or genera of the fungi kingdom. The search terms used were related to exposure (fungi*, mould/mold, *spores, Cladosporium, Penicillium, Aspergillus, or Alternaria*); outcome (asthma, wheezing, or respiratory); population (child* and were combined (exposure, outcome and population).

Further hand searches were conducted using citations from included publications and related review papers.

The abstracts of all identified papers were reviewed for initial inclusion; then, full papers were read by two researchers (RT and DV) if all inclusion criteria were met.

**Assessment of quality and risk of bias**

The two researchers (RT and DV) independently conducted a quality assessment of each study using the quality assessment framework developed by Zaza et al (32). The quality questions were a checklist, with a maximum score of 21, that examined the description of the study population and how they were selected; how exposure was measured and whether this was valid and/or reliable; whether the outcome measures were valid and/or reliable; the appropriateness of the statistical testing; the appropriateness of controlling for design effects, potential biases and confounders in the analysis; and whether problems with data analysis limited the interpretation of the results.

The assessment of risk of bias was guided by the GRADE system for rating the quality of the evidence of observational studies (33). The key criteria assessed the following:

1. **Differentials in measurement of exposure and surveillance**
   - For the outcome of interest; and
2. **Adequate control for confounding**
   - The study types included in this review were observational. T-tests were used to assess any differences in overall scores between two researchers using the Stata IC 12.1 (StatCorp, Texas, TX, USA) statistical package.

**Data extraction**

Data were extracted from each study: study design, study country and whether it was urban, suburban rural or mixed; age range of children, number of children in the sample; exposure definition and how exposure was measured; outcome definition and how the outcome data were obtained; effect estimate including 95% confidence interval (95% CI); confounders included in analysis; subgroup analysis; interactions; and findings.

**Assessment for meta-analysis**

Exposure definitions (fungal species, fungal phyla and unspecified fungi) and effect estimates (odds ratios, relative risks, % change in visits, mean % change in visits, associations, correlations and % of visits) were abstracted overall and, where available, from different age groups. Due to substantial heterogeneity across the studies (design, exposure definition and
measurement, population, outcome definition and measurements of effect estimates), formal meta-analysis was not performed. We have qualitatively described and compared the studies and provided tabular data to illustrate our interpretation.

Results
The electronic literature search and hand searching found 1806 peer-reviewed scientific articles after duplicate papers were removed. Of these, 1786 were excluded after reviewing the abstracts to assess relevance to inclusion criteria. Of these, 10 full-text articles were assessed and 15 papers were included in this review (Figure 1 [34]) and their key characteristics are summarized in Table 1.

Quality assessment and risk of bias
Quality assessment scores that were allocated independently by two researchers (RT and DV) varied by up to two points for three papers, however, a t-test of the difference between the overall scores was not statistically significant (p = 0.43) (Table S1).

Using the GRADE guidelines, most studies were subject to a number of potential biases and limitations. The main sources of bias arises from the methodological diversity of exposure measurements (measurement bias); the inclusion of children aged <2 yr with whom diagnosis of asthma is very difficult and prone to misclassification and selection bias; selective reporting of findings within a study (reporting bias); and the lack of controlling and adjustment for key confounders in the statistical analysis and modelling in a number of studies (confounding bias) (35) (Table S1).

Calculation of fungal spore exposure
A number of different measuring techniques and equipment have been used to calculate outdoor fungal spore exposure (Table 1). The most common instruments reported were (i) the Burkard Volumetric 7-day trap which draws 10.1 of air per minute continuously onto a tape coated with adhesive which moves past the inlet 2 mm per hour over a 24-hour period; and (ii) the rotational impact or Rotordor sampling. In this system, particles adhere to silicon greased sample rods which rotate at 2400 rpm and collect for 1 min from every 10-min period over a 24-hour period. Although these two instruments appear very similar, their particle recovery systems differ. The Burkard appears to be a superior instrument for sampling particles 40 μm and the Rotordor appears to be equal or superior to the Burkard for collecting particles >10 μm (36, 37). The selection of instrument can contribute to measurement bias as the majority of fungal spores are sized <10 μm in both aerodynamic diameter and physical size (38, 39).

Results from the cross-sectional studies
These results are summarized in Table 2. In the oldest study (30), child asthma hospital admissions demonstrated a strong correlation with a particular fungal spore subgroup, colourised basidiospores. However, these results may have been confounded by meteorological conditions or air pollutants which they did not control for. In two other studies (11, 19), the levels of airborne fungi did not correlate with child asthma presentations at hospital. However, neither study controlled for confounders, such as seasonality, meteorological conditions or air pollutants. The final study (16) was a cross-sectional study nested within a large randomized controlled trial in the USA. This study compared the effects of indoor and outdoor fungal spores on unscheduled visits to primary care practices and EDs for child asthma. This is the only study that collected outdoor fungi count data from the homes of the study participants. Outdoor fungal exposure was primarily associated with increased asthma symptoms defined as wheeze, chest tightness, cough, reduced activity and waking through the night due to asthma. However, indoor fungal exposure was more strongly associated with asthma exacerbations which were identified as unscheduled visits to primary care practices and EDs (OR 1.18, 95% CI 1.01–

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**Figure 1** Flow diagram of review.
<table>
<thead>
<tr>
<th>Author, study type and sample type</th>
<th>Location, setting and time period</th>
<th>Exposure definition</th>
<th>Calculation of fungal exposure</th>
<th>Outcome definition</th>
<th>Age group and sample size/mean attendance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porpacq et al. (2010) (10)</td>
<td>USA - Inner city - Residential</td>
<td>Alternaea, Cladosporium</td>
<td>Bircard Portable Culture Plate Air Sampler</td>
<td>Unscheduled visits (USA) - unscheduled primary care visits + emergency department (ED) visits &amp; hospitalisations for asthma</td>
<td>5-11 yr n = 489 (with fungal sensitization)</td>
</tr>
<tr>
<td>Wang et al. (2007) (19)</td>
<td>USA - Delaware - Children's Hospital 2000-2003</td>
<td>Outdoor mould (unspecified)</td>
<td>Not stated</td>
<td>Monthly rates of presentations for asthma: low (9) vs. high (17)</td>
<td>Not stated</td>
</tr>
<tr>
<td>Retrospective correlation - population</td>
<td>Israel - Tel Aviv - Paediatric ED January-December 1993</td>
<td>Alternaea, Cladosporium, Staphylococcus</td>
<td>Rotary impact sampler</td>
<td>Diagnosis of acute asthma in an ED</td>
<td>1-18 yr n = 1067</td>
</tr>
<tr>
<td>Time series</td>
<td>India - Kolkata - 2 hospitals January-December 2010</td>
<td>Alternaea conidia spp</td>
<td>Bircard Volumetric trap</td>
<td>Emergency hospital admission for asthma</td>
<td>5-18 yr n = 2706</td>
</tr>
<tr>
<td>Chakraborty et al. (2013) (48)</td>
<td>Canada - Montreal - 3 hospital EDs 1994-2004</td>
<td>Basidiomycetes spp, Ganedema, deuteromycetes spp, Cladosporium</td>
<td>Rotary impact sampler</td>
<td>ED visits for asthma - first visits and readmissions</td>
<td>0-8 yr n = 43,780</td>
</tr>
<tr>
<td>Rapcsak et al. (2010) (24)</td>
<td>Australia - Darwin - Hospital ED April 2004-November 2005</td>
<td>Total fungal spores; Alternaea spp</td>
<td>Bircard Volumetric trap</td>
<td>ED attendance for asthma</td>
<td>&lt;15 yr n = 110</td>
</tr>
<tr>
<td>Atkinson et al. (2009) (47)</td>
<td>Canada - 10 Canadian cities - Hospitals 1993-2000</td>
<td>Basidiomycetes, ascomycetes, deuteromycetes</td>
<td>Rotary impact sampler for asthma</td>
<td>Emergency hospitalisations</td>
<td>&lt;13 yr Total n = 356,656 but n (children) not stated</td>
</tr>
<tr>
<td>Author, study type and sample type</td>
<td>Location, setting and time period</td>
<td>Exposure definition</td>
<td>Calculation of fungal exposure</td>
<td>Outcome definition</td>
<td>Age group and sample size/daily mean attendees</td>
</tr>
<tr>
<td>-----------------------------------</td>
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</tr>
<tr>
<td>Lief et al. (2003) (7)</td>
<td>USA – Cincinnati – Children’s Hospital 1996-1997</td>
<td>Fungal spores – unspecified</td>
<td>Rotary impact sampler</td>
<td>Hospitalization – status asthmaticus ED attendance – asthma or reactive airway disease</td>
<td>Not stated Daily mean range (April–October) = 8.5–17.2</td>
</tr>
</tbody>
</table>
Table 2 Results and findings of included studies

<table>
<thead>
<tr>
<th>Author</th>
<th>Results (significant findings reported where p &lt; 0.05)</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porangio</td>
<td>UVs: Aspergillus PST OR = 1.18 (1.01–1.37)</td>
<td>Outdoor fungal exposure is primarily associated with increased asthma symptoms, only modest increase in risk of UVs</td>
</tr>
<tr>
<td>2010 (18)</td>
<td>For all PST, the numbers of excess symptom days per 2 wk associated with increase in outdoor fungal levels were all significant</td>
<td></td>
</tr>
<tr>
<td>Wang</td>
<td>Not reported or described – qualitative report given in findings</td>
<td>Mould levels showed no significant effects on ED presentations for asthma</td>
</tr>
<tr>
<td>2007 (19)</td>
<td></td>
<td>Levels of airborne mould did not correlate with the ED visits of asthmatic children</td>
</tr>
<tr>
<td>Garty</td>
<td>Data not shown – qualitative explanation reported</td>
<td></td>
</tr>
<tr>
<td>1998 (11)</td>
<td></td>
<td>Coloured basidiospores showed a strong correlation with hospital asthma admissions, other fungal species did not</td>
</tr>
<tr>
<td>Khot</td>
<td>Coloured basidiospores accounted for 16.5%</td>
<td></td>
</tr>
<tr>
<td>1998 (40)</td>
<td>of the variation in hospital asthma admissions</td>
<td></td>
</tr>
<tr>
<td>Time series</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chakraborty</td>
<td>Uni increasing in Alternaria conidal spore counts are significantly correlated with increased asthma emergency hospital admissions (estimate = 0.03%)</td>
<td>Alternaria conidal spores might have a positive significant impact on emergency asthma hospitalization in school-aged children in this Indian city</td>
</tr>
<tr>
<td>2013 (49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raffiz</td>
<td>First visits to ED increased when concentrations</td>
<td>Varying levels of associations found between different fungal species and hospital admissions</td>
</tr>
<tr>
<td>2010 (24)</td>
<td>of basidiomycetes, deuteromycetes and Cladosporium spores were increased 3 or more days beforehand. For basidiomycetes, there was also a lagged effect for readmissions</td>
<td>Negative associations were found between deuteromycetes and Ganoderma spores and first visits and readmissions to ED</td>
</tr>
<tr>
<td>Hanigan and Johnston</td>
<td></td>
<td>No association found</td>
</tr>
<tr>
<td>2007 (15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atkinson</td>
<td>Relative risks (RR) of increase in visits for asthma associated with increases in fungal spore concentrations:</td>
<td>Increased spore concentrations associated with increased ED visits and hospital admissions</td>
</tr>
<tr>
<td>2006 (47)</td>
<td>ED Visits: Basidiomycota Q3 RR = 1.14 (1.02–1.27), Ascomycota Q4 RR = 1.15 (1.02–1.29)</td>
<td>Some individual spore taxa were found to be associated with ED attendances and hospital admissions</td>
</tr>
<tr>
<td>Cakmak</td>
<td>% change in daily hospitalizations</td>
<td>Younger males and those in lower socio-economic families may be more vulnerable to fungal spores</td>
</tr>
<tr>
<td>2005 (51)</td>
<td>Deuteromycetes: Males 0–13 = 5.2 (0.2–10.2); Females &gt;13 = 5.3 (–3.0 to 13.6); Lowest education quartile = 12.1 (3.1–21.1); Income &lt;250, 112 = 14.7 (3.2–26.4)</td>
<td></td>
</tr>
<tr>
<td>Dales</td>
<td>Consistently positive associations between asthma and fungal spores in each city, some being statistically significant</td>
<td>Increases in outdoor fungal spores were associated with daily increases in asthma hospital admissions</td>
</tr>
<tr>
<td>2004 (44)</td>
<td>With all cities combined: % increase in daily hospitalizations with increase in fungal levels: Basidiomycetes 3.3% (2.3–4.1); ascomycetes 3.1% (2.8–5.7); deuteromycetes 3.2% (1.6–4.8)</td>
<td></td>
</tr>
<tr>
<td>Dales</td>
<td>% increase in ED visits with increase spore concentration</td>
<td>Fungal spores approximately doubled on thunderstorm days</td>
</tr>
<tr>
<td>2003 (8)</td>
<td>Equal in magnitude to its mean: Deuteromycetes Total = 1.79%; Cladosporium 1.64%; Penicillium/Aspergillus 1.75%; basidiomycetes Total 3.75%; ascomycetes 2.88%; Total spore 2.18%</td>
<td>Days-to-day increases in fungal spores were associated with asthma visits, irrespective of thunderstorms</td>
</tr>
<tr>
<td>Lied</td>
<td>No data shown – qualitative reporting only</td>
<td>Fungal spore counts were not a predictor of asthma visits either on the same day or lagged 1–3 days</td>
</tr>
<tr>
<td>2003 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dales</td>
<td>Fungal spores accounted for 8.8% of ED visits</td>
<td>Deuteromycetes, basidiomycetes and ascomycetes appeared to have independent effects on variations in ED visits</td>
</tr>
<tr>
<td>2000 (45)</td>
<td>Basidiomycetes had strongest effect at 3.5%; ascomycetes at 1.8%; deuteromycetes at 1%</td>
<td>Some evidence that exceptional rates of admission for asthma in children aged 0–14 yr tend to occur on days with high total mould spore counts, but no specific taxon was implicated</td>
</tr>
<tr>
<td>Newton</td>
<td>Positive association between hospital admissions and high fungal spore counts:</td>
<td></td>
</tr>
<tr>
<td>2000 (46)</td>
<td>Asthma epidemics were 9.92 times more likely on a day with high total fungal spore count (95% CI 1.4–109.9)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2 (Continued)

<table>
<thead>
<tr>
<th>Author</th>
<th>Results (significant findings reported where p &lt; 0.05)</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosas 1998 (48)</td>
<td>In the dry season, dermatophytes was the major factor in child asthma ED admissions, accounting for 18% of the deviance</td>
<td>Results suggest that children may have been more responsive to fungal spores than adults and seniors</td>
</tr>
</tbody>
</table>

1.37). Overall, they found only a modest increase in the risk of having an unscheduled visit when children who were sensitized to *Aspergillus* were exposed to fungal spores (OR = 1.18 [1.01–1.37]). Exposure to outdoor fungal spores did not have a significant effect on children sensitized to fungal spores. Again this finding may be limited as they did not report controlling for confounders described previously, nor did they differentiate on the basis of sex or age group.

Results from the ecological time series studies

These results are summarized in Table 2. The time series were the most common methods used to explore relationships between outdoor fungi and childhood asthma, which is a common ecological approach used in other studies of environmental exposure, such as the effects of air pollutants and pollen (44, 45). These studies were conducted in Canada (4, 24, 44, 45), USA (7), Great Britain (46, 47), Mexico (48) and India (49).

Rosas et al. (48) utilized generalized linear modeling to estimate the associations between air pollutants, fungal spores, pollen, weather factors and ED admissions for asthma. They stratified their sample into children (0–15 yr), adults and seniors and separated the time periods into wet season and dry season. They then fitted their model for each age group by each season. The model which found that dermatophytes were the major factor associated with asthma ED attendances was in children during the dry season (accounting for 18% of the deviance).

Dales and colleagues (45) studied children attending an ED for asthma. Their methodological approach accounted for potential confounding factors, including seasonal trends related to viruses, exposure to ozone and other aerosol pollutants such as grass, tree pollen and ragweed pollen. After adjustment for potential confounders, they found that fungal spores accounted for 8.8% of ED visits in the time period. They examined fungal exposure by spore taxa and found that basidiomycetes appeared to have the strongest effect, accounting for 3.5% of ED attendances.

Dales and colleagues (8) also examined the role that fungal spores play in thunderstorm asthma in Ottawa, Canada and found that fungal spores approximately doubled on thunderstorm days. Day-to-day increases in fungal spores were found to be associated with ED asthma visits in a children’s hospital, irrespective of the presence of thunderstorms. Unfortunately, the authors did not stratify the asthma attendances by age groups, so it was not possible to determine whether children were affected differently to adolescents. Basidiomycetes was found to have the strongest effect, accounting for 3.75% increase in ED visits, compared with all spores accounting for 2.18% increase in ED visits.

Dales, Cakmak and colleagues expanded their research to examine the effect of outdoor fungal spores across 10 major Canadian cities (44, 50, 51). In the two papers that met the inclusion criteria for this review (44, 51), they reported their findings stratified by age groups – those aged <13 yr and those 13 yr and older. In the earlier paper (44), they found consistently positive associations between asthma and fungal spores and emergency hospitalizations for asthma across the 10 cities. However, the effect of basidiomycetes was only statistically significant (p < 0.05) in Ottawa, Vancouver and Windsor; and the effect of dermatophytes was statistically significant in Halifax, Ottawa and Windsor. Age stratification did not change the direction of association between the fungal spores and hospitalizations. With data from all cities combined, they found increased asthma hospitalizations with increased fungal levels. Once again basidiomycetes had the strongest effect of 3.3%, but closely followed by dermatophytes (3.2%) and ascomycetes (3.1%) (44). The second study examined the role of gender and socioeconomic status across 10 Canadian cities (51). They found that short-term exposure to outdoor fungal spores and asthma hospitalization was modified by age and gender in an interactive way. Males aged <13 yr appeared to be more vulnerable. They also found that those adults and children (pooled data) living in lower socio-economic areas were also more likely to be hospitalized for asthma (51).

Also in Canada, Raphoz et al. (24) examined the impact of outdoor fungal spores on asthma first visits and revisit rates to an ED in Montreal in children aged 0–9 yr. First visits to ED increased when concentrations of basidiomycetes, dermatophytes and *Cladosporium* spores were increased three or more days before. There was also a lagged effect for readmission with increased levels of basidiomycetes only for 3 days or more. They reported that some associations reached statistical significance, but do not indicate which one provide data to allow calculation.

In the UK, Newsom et al. examined the impact of fungal spores on child asthma hospitalizations in one region at the fungi genera levels and taxa levels (30 elementary taxa and three summary taxa). They found evidence of increased asthma hospital admission rates on days with high total fungal spore counts (OR = 9.82, 95% CI 1.4–100.8, p = 0.0061), but no particular taxon was implicated (46).

In another British study, Atkinson et al. found that increased fungal spore concentrations were associated with increased ED visits and hospitalizations for children with asthma. Some individual spore taxa were found to be associated: Basidiomycete (ED: RR of Q3 fungal spore concentration = 1.14;
hospitalizations RR of O3 fungal spore concentration = 1.16) and Aspergillus (ED RR of O4 fungal spore concentration RR = 1.15) (47).

In contrast, in the USA, Lefr et al. reported that fungal spore counts were not a predictor of child asthma ED visits to a children’s hospital visits on the same day or lagged 1–3 days (7); however, no data were provided.

In Australia, one study was conducted in a northern tropical city by Hanigan and Johnston. They found that exposure to Alternaria spp fungal spores had no association with total respiratory emergency hospital admissions (n = 1287), of which 33% were children aged <15 yr. Analysis of the asthma subgroup of these respiratory attendances (children n = 110.4) found no significant association between Alternaria spp and hospital admissions (15).

The most recent study, which was conducted in India (49), examined the impact of Alternaria spores in conjunction with ozone and PM10 (particulate matter <10 μm in aerodynamic diameter) on school-aged child asthma ED attendances using generalized linear and additive models over a 12-month period. They reported correlational results indicating that Alternaria may have a significant impact on child asthma ED attendances, but they did not calculate or report the strength of association. These findings were subject to some methodological limitations as they examined serial correlation only using two statistical methods and did not adjust for seasonality, effects of lag time or gender.

Discussion

Overall, the findings from the studies included in this review that reported more rigorous methodology and analysis suggest that elevated outdoor fungal spores in the atmosphere were associated with increased health services attendances and hospital admissions for child asthma.

The findings from these international studies have been mixed due to methodological variations: (i) the pooling of all age groups vs. analysing by stratified age groups; (ii) varying upper and lower age cut-offs for children; (iii) varying classifications of the large number of spore species, that is, by ‘all fungi’, by ‘taxa’, or by ‘genera’; (iv) differing time periods that involve different seasons and sometimes extreme climatic conditions; (v) different statistical methods, that is, correlation statistics vs. regression estimates; and (vi) varying inclusion of potential confounding factors, that is, meteorological variables and air pollutants.

The cross-sectional studies that were included in this review varied in their methodological approaches and the depth of their reporting. Two of the four cross-sectional studies reported in this review found an association between exposure to outdoor fungal spores and child asthma exacerbations. Pogracic and colleagues’ observational study (16) indicated that outdoor fungi was more strongly associated with asthma symptoms not requiring visits to health services. This was the only study that sampled fungal spore counts outside homes; however, this was limited to a 6-monthly basis for 2 years and was of short duration. In addition, this study was conducted only with children who were identified as being sensitized to one or more common fungal allergens. Khot and colleagues’ (40) study was limited by attempting to identify significant correlations over a relatively short time period that captured only one peak period for fungal spores in the outdoor atmosphere.

The reporting in the other two cross-sectional studies which did not find any association between outdoor fungal spores and child asthma exacerbations was very limited: Wang and colleagues (9) did not report how fungal exposure was calculated or estimated, what age groups were being analysed nor the sample size. Garty and colleagues’ (11) study was limited by a relatively short time frame (1 year) and did not present the fungal spore data nor how these data correlated with the asthma ED attendances.

The cross-sectional studies did not examine lagged effects beyond 1 day and did not adjust for confounders such as air pollution or meteorological conditions nor did they differentiate by age group or sex. However, of the nine studies that utilized time series methodology, all but one indicated that there is evidence of an association between severe child asthma exacerbations and exposure to outdoor fungal spores. The differences in the levels of association between the cross-sectional and time series methodologies may be related to the fact that time series analysis can detect lagged effects; can adjust for other contributing factors such as important air pollutants (PM2.5, PM10, ozone and NO3) and meteorological factors (humidity, temperature and rainfall); and therefore may be more sensitive to investigating the role of outdoor fungal spores on child asthma.

The strongest evidence has emerged from the research conducted in Canada due to the number of studies conducted, the sample sizes used, the comparable methodologies and the geographic breadth (10 Canadian cities) of the research. This review highlights the lack of similar research in other countries that could be comparable with these findings and to assess whether the Canadian findings are transferable to other geographic settings.

It is important to note that current aeroallergen research has identified the emerging importance of the roles of gender and age when investigating interactions and respiratory health outcomes (52, 53). There is growing evidence of an increased risk of aeroallergen sensitization in infants with subsequent asthma and hay fever in later childhood (54). This review did not identify any studies that reported this perspective in their analysis when examining the role of outdoor fungal spores on child asthma exacerbations. There is a need for research to examine infant sensitization to fungal spores and how this might relate to severe asthma exacerbations in childhood.

Strengths

Our systematic search of the literature did not find any other reviews that focused on outdoor fungal spores, children and severe asthma exacerbations, so our findings are unique and cannot be directly compared. However, Tischler’s systematic review and meta-analysis of indoor domestic mould and mould components found a positive association between visible mould and child asthma (OR 1.49 [95% CI 1.26–1.72]) (55). Hence, this is the first systematic review that has examined the relationship with outdoor fungal spore exposure and severe child asthma.
It provides insights into the complexity of assessing the relationship between outdoor fungal spore exposure and childhood asthma and also highlights further research that is needed to adequately investigate this relationship.

Limitations

Some limitations must be considered when interpreting the results of this review. Firstly only 15 studies met the inclusion criteria for this review amongst a large body of literature that explored asthma and fungal spore exposure. Our review focused on child populations, whereas most studies have focused on adult populations or have aggregated children and adults together in the analysis. Fungal spore exposure is difficult to measure as the location of the monitoring sites may not be applicable to the population of children who have asthma exacerbations requiring hospitalization or attendance to primary care service or ED.

In this review, the classification of fungal spores sampled varies greatly between studies, with some studies reporting fungal genera (aggregated separately), others reporting specific taxa or species (aggregated and separately) and some studies not reporting data on fungal exposure at all. The change in fungal classification systems since 2006 (27), with the incorporation of Deuteromycota into Ascomycota and Basidormycota phyla (56), needs to be taken into account when comparing the exposure measurements of different studies. This is of particular significance as the majority of fungal species implicated in child asthma exacerbations were previously classified as Deuteromycota but are now classified as either Ascomycota or Basidomycota. Also, the different instruments used to collect fungal spores and calculate fungal exposure have different particle recovery systems and may impact which identifiable spores are collected. So a finding from one study may not be comparable to another. It should be noted that in clinical practice, only some species (e.g. Alternaria, Aspergillus, Cladosporium, Epicoccum) are tested for sensitization, and very little is known about the sensitization potential of the other fungal species.

As the evidence of the role of outdoor fungal spores in asthma exacerbations becomes stronger, there is a need to develop standardized exposure assessments, as is done with pollen and air pollutants. Also, efficient and non-invasive methods to assess individual outdoor fungal exposure would enable well-measured and controlled studies to be undertaken. Due to the heterogeneity of the methodologies employed in the included studies, we have been unable to conduct a formal meta-analysis that could have enabled us to quantify the risk of severe child asthma with outdoor fungal spore exposure.

Conclusion

This review has identified increasing evidence that asthma and sensitized children may be susceptible to asthma exacerbations when exposed to outdoor fungal spores and that the severity of exacerbation may vary with different fungal species. This review has also highlighted the small number of studies conducted in limited geographic locations. The effect of different fungal species in different locations requires further investigation. It is important to build on the current knowledge base to determine whether findings are transferable to other countries with different climatic conditions and fungal ecology. Beyond the Canadian research, we know little about the role of outdoor fungal spores in other countries and settings that are experiencing changing environmental conditions that include weather patterns, sporulation times and physiology of different ecosystems (57). This review highlights how little we understand about the interactions of fungal spores with other environmental triggers such as pollen, air pollutants and respiratory viruses and how these interactions can impact on children. It is important to understand these environmental changes and interactions and their role in child and adolescent asthma exacerbations to prevent and manage an increasing global public health issue.

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Conflict of interest

The authors declare no competing interests in conducting this systematic review. MA reports an investigator-initiated grant from Pfizer, personal fees (consultancy) from Asta-Zeneca, conference travel support from Boehringer-Ingelheim, outside the submitted work.

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Fungi and asthma in childhood


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52. Gouvea E, Govey DT, Masa G, Yehamme KMC, Doli E, Barsi FD. The influence of age and gender on sensitivity to aero


**Supporting Information**

Additional Supporting Information may be found in the online version of this article.

**Table S1.** Quality assessment (QA) and risk of bias.
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<table>
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<tr>
<th>Study</th>
<th>Agreed QA score</th>
<th>Possible biases or limitations</th>
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</thead>
<tbody>
<tr>
<td><strong>Cross-sectional studies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pongracic (16)</td>
<td>17</td>
<td>Sample is only sensitized children – no controls; no air pollution or weather interactions considered; not stratified by age or sex</td>
</tr>
<tr>
<td>Wang (19)</td>
<td>11</td>
<td>Method of exposure measurement lacks detail of where and how; Inclusion of children aged &lt;2 years**; no adjustment for seasonality or confounders</td>
</tr>
<tr>
<td>Garty (11)</td>
<td>11</td>
<td>Use of Rotorod*** and method of exposure measurement lacks detail of where it was located (measurement bias); inappropriate statistical tests for study design; inclusion of children aged 1–2 years**; no stratification by age or sex</td>
</tr>
<tr>
<td>Khot (40)</td>
<td>17</td>
<td>No stratification by age or sex</td>
</tr>
<tr>
<td><strong>Time series</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chakraborty (49)</td>
<td>14</td>
<td>No adjustment for confounders or limiting potential biases;</td>
</tr>
<tr>
<td>Raphoz (24)</td>
<td>17</td>
<td>Use of Rotorod *<strong>; Inclusion of children aged &lt;2 years</strong>; Do not report which associations are statistically significant</td>
</tr>
<tr>
<td>Hanigan (15)</td>
<td>18</td>
<td>Measured only one species of fungal spore; no stratification by age or sex</td>
</tr>
<tr>
<td>Atkinson (47)</td>
<td>17</td>
<td>Inclusion of children aged &lt;2 years**; No stratification by age or sex</td>
</tr>
<tr>
<td>Cakmak (51)</td>
<td>21</td>
<td>Use of Rotorod***; Inclusion of children aged &lt;2 years**</td>
</tr>
<tr>
<td>Dales (44)</td>
<td>17</td>
<td>Use of Rotorod***; Inclusion of children aged &lt;2 years**; no stratification by age or sex</td>
</tr>
<tr>
<td>Dales (8)</td>
<td>18</td>
<td>Use of Rotorod***; No stratification by age and sex; inclusion of children &lt;2 years**</td>
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<td>Author</td>
<td>Score</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Lierl(7)</td>
<td>16</td>
<td>Use of Rotorod***; inclusion of children aged &lt;2 years***; no stratification by age or sex;</td>
</tr>
<tr>
<td>Newson (46)</td>
<td>17</td>
<td>Inclusion of children aged &lt;2 years**; no air pollution or weather interactions considered; not stratified by sex</td>
</tr>
<tr>
<td>Dales (45)</td>
<td>17</td>
<td>Use of Rotorod***; Inclusion of children aged &lt;2 years**; no stratification by age group or sex</td>
</tr>
<tr>
<td>Rosas (48)</td>
<td>17</td>
<td>No control for confounders; no adjustment for seasonality; inclusion of children &lt;2 years**; no stratification by age and sex</td>
</tr>
</tbody>
</table>

* Maximum score = 21  ** Misclassification bias  ***Measurement bias:

Rotorod systems are less sensitive than Burkard systems in collecting particles <10 µm
Chapter 4

Methods for collection of environmental data

As my overarching aim was to examine the contribution of outdoor fungal spores to asthma exacerbations, lung function and airway inflammation using a number of epidemiological methods and data sources, in this chapter I will describe the methods that were used to obtain the environmental data that I used in my analyses. As each research question is examined using different data sources and study designs, I will provide detail of each of those methodologies in their individual chapters.

4.1 Sampling outdoor fungal spores

Daily ambient fungal spores were sampled, identified and counted using the same methodology in two different sites for three of my research questions (RQ 2, 3 and 4). Each site used a Burkard 7-day recording volumetric spore trap (Burkard, Hertfordshire, England) (see Figure 4.1) that operates on the principle of “impaction through suction”. It is a common and efficient instrument for sampling outdoor air and is a standard reference throughout the world (271).

![Burkard 7-day recording volumetric spore trap](image)

Figure 4.1: Burkard 7-day recording volumetric spore trap

(Photograph provided by Pamela Burton, Campbelltown Hospital)
The location of the sampler is important as this can directly affect the results. If the location is too high above the ground then there will be a predominance of only fungal spores released from tree canopies. If it is located too low then obstructions may interfere and only the immediate area is sampled. The recommended location for aerobiological data collection is a flat rooftop at a height above local treetops and away from local obstructions.

I have analysed fungal spore data obtained from two sites (1) Parkville, Melbourne and (2) Campbelltown, south-west Sydney. In Melbourne, the Burkard sampler was located on the rooftop of the Earth Sciences building at the Parkville campus of the University of Melbourne. This site is located approximately 2 kilometres from the Royal Children’s Hospital where the asthma hospitalisations, lung function and airway inflammation data were collected (see Chapters 6 and 7) (Figure 4.2). In Campbelltown, the Burkard sampler was located on the rooftop of the Campbelltown Hospital which was one of the sites where I obtained data on asthma hospitalisations (see Chapter 5) (Figure 4.3). This site is located approximately 10.4 kilometres from Camden Hospital; and 20.2 kilometres from Liverpool Hospital where asthma hospitalisations data for south-west Sydney were also obtained. Both sampling sites were 11 metres above the ground and clear of building and tree obstructions and met the guidelines of the World Allergy Organisation (271).
Figure 4.2: Map showing the location of the Royal Children’s Hospital, the Burkard spore trap and the meteorological and air quality monitoring station for the Melbourne studies.
Figure 4.3: Map showing the location of Camden, Campbelltown and Liverpool Hospitals; the Burkard spore trap; the meteorological and air quality monitoring stations for the south-west Sydney study.
The Burkard sampler has a vacuum pump that, when started, draws in 10 litres of air per minute continuously. The spores are sucked into the Burkard sampler through an orifice where they enter the sampler chamber and they impact and adhere to a microscope slide over a 24-hour period. The Burkard sampler contains a slide holding mechanism that allows an adhesive-coated and labelled slide to be placed inside the machine adjacent to an orifice that is 14mm by 2mm in size. The slide moves at 2mm per hour.

The slides were examined under a light microscope with a vernier scale by a trained technician. Scanning, identifying and counting an entire slide is extremely time-consuming. So, a longitudinal transverse with correction factor was used to calculate the average spore concentrations per cubic metre of air tested during the 24-hour period.

Spores were identified using accredited identification manuals (272) and reference slides. At the Melbourne site identifiable fungal spores were classified into taxa: *Alternaria, Cladosporium, Coprinus, Drechslera, Ganoderma, Leptosphaeria, Periconia, Pithomyces, Pleospora, Sporormiella, Stemphylium,* and *Ustilago/smuts*. Data were not provided on ‘other’ or ‘non-identifiable’ spores which have been previously reported in other Melbourne, Australia studies (77).

At the Campbelltown site identifiable fungal spores were classified into taxa: *Alternaria, Aspergillus/Penicillium, Cerebella, Chaetomium, Cladosporium, Coprinus, Curvularia, Didymella, Drechslera, Epicoccum, Fusarium, Ganoderma, Nigrospora, Periconia, Pithomyces, Polythrincium, Puccinia, Stemphylium, Torula,* and *Ustilago/smuts*. As for the Melbourne site, data were not provided on ‘other’ or ‘non-identifiable’ spores.

4.2 Sampling pollen grains

As pollen grains are generally produced at ground level and dispersed by prevailing winds, the location of the Burkard sampler was very important so that a representative sample of grass pollen grains was obtained. Hence Poaceae (grass) pollen grains were captured at the same time as the fungal spores and were identified and counted using the same methods.

Other allergenic pollen types are also in the air in Melbourne and Campbelltown which may act as confounders for the fungal spores. Although, in Melbourne, Poaceae (grass)
pollen has been shown to have the strongest associations with asthma exacerbations (30, 31), Cupressaceae (conifer) pollen is far more abundant (273). In Sydney, both Oleaceae and Cupressaceae pollen have been found to be more abundant than Poaceae pollen (273).

4.3 Outdoor air pollution

The 24-hour average daily concentrations of particulate matter up to 2.5 µm in diameter (PM$_{2.5}$) and up to 10 µm in diameter (PM$_{10}$)(µg/m$^3$), the daily maximum one-hour average nitrogen dioxide (NO$_2$) level (ppb), and the daily maximum four-hour average ozone (O$_3$) level (ppb) were obtained from Victorian Environmental Protection Authority fixed air quality monitoring stations in Alphington, Melbourne (274) (Figure 4.2) and from New South Wales Environment Protection Authority fixed monitoring station at Liverpool (275) (Figure 4.3).

4.4 Meteorological data

The Australian Bureau of Meteorology provided data on daily maximum and minimum temperatures (degrees Celsius), rainfall (mm), and daily relative humidity (percentage) for both study sites: Alphington, Melbourne (Figure 4.2) and Liverpool, south-west Sydney (Figure 4.3).
Chapter 5

Are there associations between outdoor fungal spores and child and adolescent asthma hospitalisations?

5.1 Introduction

This chapter consists of a peer-reviewed published paper and supplementary data of a piece of original research that investigated associations between a range of outdoor fungal spores and child and adolescent asthma hospitalisations in three public hospitals in the south-west region of Sydney, Australia over a 5-year period (May 2008 to May 2013). This research examined if any associations were on the same day as outdoor fungal spore exposure or lagged. This research also examined whether any associations were modified by sex, age, presence of air pollutants or grass pollen.

This paper finds some evidence that exposure to some outdoor fungal spore taxa were associated with increased risk of child and adolescent asthma hospitalisations especially in girls and older adolescents. Air pollutants and grass pollen did not appear to act as effect modifiers.

I am first author of this paper. I developed the research question, obtained all ethics approvals, obtained all data, undertook the data analysis, led the writing of the manuscript, was responsible for all edits and revisions and submitted the manuscript to Environmental Research journal.

5.2 Research question

1. Are there associations between outdoor fungal spores and child and adolescent asthma hospitalisations?
2. If so, are these associations modified by presence sex, age, air pollution or grass pollen?
5.3 Research aims

1. To investigate if there are instantaneous or lagged associations between outdoor fungal spores and child and adolescent asthma hospitalisations using a case crossover study design.
2. To investigate whether any associations were modified by sex or age.
3. To investigate whether air pollution or grass pollen modified any associations.

5.4 Ethics

Ethics approvals were granted by South West Sydney Local Health District Human Research Ethics Committee (LNR/13/LPOOL/189).

5.5 Publication and supplementary material

The role of outdoor fungi on asthma hospital admissions in children and adolescents: A 5-year time stratified case-crossover analysis

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\textbf{ABSTRACT}

Background: Some fungal spores can trigger asthma exacerbation but knowledge of which outdoor fungal spores contribute to asthma hospitalisation is limited.

Objectives: To examine the role of outdoor fungal spores in child and adolescent asthma hospitalisations.

Methods: We conducted a bi-directional time-stratified case-crossover study of child and adolescent asthma hospitalisations over 5 years. Conditional logistic regression assessed the role of 20 fungal taxa (Same day [SD] and lagged [L1-L3]) adjusted for maximum temperature, humidity and grass pollen. Stratified specific effects were explored if there was evidence of effect modification by age, sex, air pollutants or grass pollen. Non-linear effects examined with Generalised Additive Models.

Results: Of 20082 children hospitalised for asthma, 60% were boys; mean age was 5.5 ± 3.7 years. Fungal spore counts peaked during warm months. Regression models found weak associations with 

\textbf{I. Introduction}

Asthma is a significant global public health problem (Global Asthma Report, 2014). In Australia, it is the most common chronic condition diagnosed in childhood and is a national health priority (Australian Institute of Health and Welfare). Severe asthma attacks are major causes of childhood hospitalisations and more than half of asthma hospitalisations involve children aged 0–18 years (Australian Institute of Health and Welfare, 2013).

Airborne fungal spores are ubiquitous and are among a number of short-term environmental factors considered to be important in triggering childhood and adolescent asthma exacerbations that can result in hospitalisation (American College of Occupational and Environmental Medicine, 2003; Denning et al., 2006). The sources of outdoor fungal spores are predominantly fungi growing on trees, plants and grasses (Igora and Torpy, 2015), whereas the sources of indoor...
fungal spores are related to persistent damp in household structures and may also be outdoor spores that have entered through doors and windows (Tischer and Heinrich, 2013). Counts of outdoor fungal spores are consistently much higher than indoor fungal spores (Garrett et al., 1997). Other environmental triggers associated with asthma exacerbations include Human Rhinovirus (HRV) infection (Busse et al., 2010; Erbas et al., 2015), air pollutants (Erbas et al., 2005; Jalaluddin et al., 2008) (particulate matter, ozone and nitrogen dioxide) and grass pollen (Erbas et al., 2012a).

Previous observational research that examined associations between total outdoor fungal spore counts and child asthma hospitalisations found significant associations in the UK (Newson et al., 2000), but no associations in the USA (Lefk and Homung, 2003; Wang and Youssef, 2007). Some studies that categorised fungal spore taxa into phyla (Atkinson et al., 2006; Cakmak et al., 2005; Rapoah et al., 2010) have found increased risk of asthma hospitalisations but their findings were not comparable due to the lack of detail regarding the taxa types that were categorised in phyla. Few studies have examined fungal spores by individual taxa (Chakraborty et al., 2014; Dales et al., 2003; Harigun and Johnston, 2007; Newson et al., 2000; Pongracz et al., 2010) and these studies also reported inconsistent associations with Aspergillus (Dales et al., 2003; Pongracz et al., 2010), Alternaria (Chakraborty et al., 2014; Harigun and Johnston, 2007) and Cladosporium (Dales et al., 2003; Rapoah et al., 2010). Only one time series study has reported lagged effects of outdoor fungal spores on child asthma hospitalisations (Rapoah et al., 2010). Two longitudinal time series studies reported that age, sex and air pollution may potentially modify the effects of outdoor fungal spores on child and adolescent asthma hospitalisation (Cakmak et al., 2005, 2012). The findings from these previous time-series, cross-sectional and correlational studies have been limited by the lack of control groups for the cases.

In this study we aimed to build on the findings of previous research and overcome some limitations by examining the associations between a range of outdoor fungal spore taxa and child and adolescent asthma hospitalisations in south-west (SW) Sydney, Australia over a five-year period using a case-crossover design. The objectives were to investigate whether these associations were (a) on the same day as fungal exposure or lagged; and (b) whether associations were modified by sex, age, air pollution or grass pollen.

2. Methods

2.1. Study design

This study used a bi-directional time-stratified case-crossover design which has been shown to be well-suited for studying the effects of transient short-term exposures (fungal spore release, air pollution changes) on the risk of short onset events (asthma hospitalisation) in individuals (Qian et al., 2015). As cases serve as their own controls, there is less risk of confounding due to stable individual characteristics (i.e. age, sex, behavioural factors, genetic factors) (Jassal, 2003). The hospital admission date was set as the index case day and referent control dates were the same day of the week within the same month and year as the index case day (Erbas et al., 2012b). This approach reduces potential biases related to possible time or seasonal trends (James et al., 2005).

For each admission date (case) and referent control days we compared the daily level of fungal spores, grass pollen, air pollution and meteorological variables. We removed all readmissions within 1–30 days of the previous admission to avoid confusing the definition of case and control days in the case-crossover design.

2.2. Asthma hospitalisation data

Daily counts of asthma hospital admissions at Campbelltown, Camden and Liverpool Hospitals for children and adolescents aged 2–18 years between 29 May 2008 and 3 May 2013 (n = 1880 days) were obtained from New South Wales Health. Due to coding variations between the hospitals the diagnosis coding definitions included three classification systems: (1) ICD-10-AM (Australian Consortium for Classification Development, 2016); Asthma (J45); Status asthmaticus (J46); (2) SNOMED-CT-AU (NEHTA, 2016); Asthma (J19970071), Asthma NOS (J26605000); (3) ICD-9 (Australian Institute of Health and Welfare, 2016); Extrinsic asthma (490.0); Intrinsic asthma (493.1); Asthma unspecified (493.9); Chronic obstructive asthma (493.2). Other forms of asthma (493.8); Cough variant asthma (493.82).

These individual level data contained hospital, age, sex, date of admission, principal diagnosis, and if readmitted within 28 days.

2.3. Fungal spore data

Daily ambient fungal spores were measured using a Burkard 7-d Volumetric spore trap (Burkard Manufacturing Co. Ltd, Rickmansworth, Herts, England) in accordance with the guidelines of the World Allergy Organisation (Hassan et al., 2007). The trap was located on the rooftop of the Campbelltown Hospital which is approximately 11 m from the ground and free from obstruction. The collection involved drawing 10 l of air per minute continuously across a microscope slide that had been coated with adhesive. Airborne particles stick to the slide as it moved past the inlet at 2 m/s. The fungal spores were identified and counted by a trained technician using a microscope. The fungal spore count is expressed as the number of fungal spores per cubic metre of air (counts/m3) tested averaged over a 24 h period. Identifiable fungal spores were classified into 21 taxa (Grant Smith, 1990): Alternaria, Catenosporium, Aspergillus/ Penicillium, Epicoccum, Ganoderma, Chaetomium, Ustilago/mutisia, Polysphinctria, Torula, Didymella, Coprinus, Cerbeilla, Curvularia, Periconia, Puccinia, Drechslera, Stenphyllum, Fusarium, Nigrospora, Pithomyces.

2.4. Grass pollen, air quality and meteorological data

Daily grass pollen counts/m3 were measured using the same methodology as the fungal spores. We obtained air quality data from the nearest NSW Environment Protection Authority fixed monitoring station which was located at Liverpool (20 km from the Campbelltown Hospital): 24 h average daily concentrations of particulate matter < 2.5 and < 10 μm diameter (PM2.5 and PM10) (μg/m3), daily maximum one-hour average nitrogen dioxide (NO2) in parts per billion (ppb) and daily maximum four-hour average ozone (O3) in ppb. We obtained Bureau of Meteorology climate data from Campbelltown weather monitoring station: daily maximum temperature (°C), rainfall (mm) and average daily relative humidity (%) (Bureau of Meteorology, 2004).

2.4.1. Age groups

Participants were stratified into age groups 2–13 years and 14–18 years so that age group categorisation was comparable to other outdoor fungi and asthma hospitalisation research (Cakmak et al., 2005; Dales et al., 2004).

3. Statistical methods

We used a conditional logistic regression model for binary outcomes (asthma hospitalisation) (Navidi and Weinhard, 2002). We assessed the estimated effects of same day (Lag0) and lagged fungal spore exposure up to 3 days (instantaneous Lag1, Lag2, Lag3; and cumulative lag). Maximum temperature, relative humidity and grass pollen were included as a priori confounders in the regression models, as these factors have been shown to be associated with fungal spore production and dispersion (Burge, 2002) and asthma exacerbations (Li et al., 2010).
et al., 2014). In addition individual fungal spore and asthma hospitalisations models were adjusted for air pollutants (PM$_{2.5}$, PM$_{10}$, NO$_2$ or O$_3$), and those were retained in the model if they changed the estimated associations by > 10% or had p-value < 0.05. Spearman’s correlations were estimated to determine the levels of correlation between the fungal taxa detected.

We tested sex, age group (2–13 years; 14–18 years); air pollutants; and grass pollen as categorical variables (low c90th percentile vs high > 90th percentile) as interaction terms to identify possible effect modification. As the statistical power to test for significant interactions was lower than to test for the main effect, we report strata specific associations if the p-value for the interaction was < 0.1 to avoid missing any important interactions (Kirkwood and Sterne, 2003). Results are presented as odds ratios (OR) and 95% confidence intervals (95% CI) that can be interpreted as the association per increase from the 75th to 90th percentiles of fungal spore counts/m$^3$ calculated for each fungal taxa. In the time series graph, daily counts of asthma hospitalisations were smoothed using LOWESS - Locally Weighted Scatterplot Smoothing. All statistical analyses were performed using Stata IC 13.1 (StataCorp, College Station, Texas).

We conducted secondary analysis to assess potential nonlinear effects of fungal spore exposure. A Poisson regression model was used to model daily asthma admissions using generalized additive model (GAM) (Hastie and Tibshirani, 1986) where penalized regression splines were used to estimate smoothing spline. To accommodate the case crossover design, the GAM was applied with a mixed effects approach, entailing a random intercept for each participant (Wood, 2013). Smoothing parameters with degrees of freedom up to 10 were automatically determined by Generalized Cross-Validation (GCV). Weather variables and grass pollen were included in the models. The GAMs were explored in three stages: (1) each fungal spore taxa univariately; (2) Each fungal spore taxa with temperature and humidity as confounders; and (3) As in (2) but with grass pollen levels as a further confounder. Diagnostic plots were checked for model fit. All nonlinear analyses were performed using “mgcv” package (Wood, 2006) in R software version 2.13.2 (Available from http://www.R-project.org).

4. Results

There were 2069 children hospitalised once during the study period to any one of the three hospitals. Of these, 60% were male; and mean age was 5.5 ± 3.7 years. The highest number of admissions was at Campbelltown, followed by Liverpool then Camden (Table 1).

The most prevalent fungal taxa detected were Cladosporium, Aspergillus, Didybalea, Cepasporus, Periconia, Ustilago/smutis, and Alternaria (Table 2). The total fungal spore count varied greatly, with a range of daily counts between 19 and 10,365. The Spearman correlations indicated that there were weak to moderate correlations between the fungal taxa (see Supplemental Material Table S1); and between the fungal spore taxa and the meteorological variables (see Supplemental Material Table S2).

During the study period, fungal spore counts were relatively low for most of the year, but for most genera, spore counts peaked during the warmer seasons (September to March). Daily asthma hospitalisations and total fungal spore counts over the study period showed seasonal variation (Fig. 1). The lower fungal spore concentrations in the period September 2009–September 2010 were correlated with the end of a major drought (high maximum temperatures and low rainfall) that affected much of southeastern Australia (see Supplemental Material Fig. S1).

Few associations were seen in the regression models adjusted for maximum temperature, relative humidity and grass pollen; and those associations that were observed were relatively inconsistent across the lag periods examined. There was some evidence for an association between asthma hospitalisations and Cepasporus at Lag0, Lag1 and cumulative lag; Cheilanthum at Lag2; Cerellia at cumulative lag; and total fungi spores at cumulative lag (Table 3). Air pollutants did not change the effect estimates by more than 10%, so were not included in the final models.

Evidence of interactions with sex and age group were found. Among girls, Cladosporium was significant at Lag0 and cumulative lag; Cepasporus at Lag7, Lag1, Lag2 and cumulative lag; and total fungi spores at Lag6; Lag7; cumulative lag (Table 4).

Among those aged 2–13 years, only Cepasporus had significantly increased OR at Lag0 and cumulative lag. Among older adolescents (14–18 years), Cepasporus (Lag0, Lag1, Lag2 and cumulative lag) and Ustilago/smutis (Lag0) had significantly increased OR. Interestingly, Sclerophyllum, Paeonia and Polytrichum had significantly reduced OR (Table 4). We found no evidence of effect modification by any air pollutants or grass pollen.

Possible non-linear associations between fungal levels and hospital admission were investigated. Non-linearity was not detected. All fungal taxa seemed to be linearly associated with the odds of asthma hospitalisation.

5. Discussion

This study found evidence of weak associations between some taxa of outdoor fungal spores found in SW Sydney, namely Cepasporus, Periconia, Chaetomiurn, Gasteromera, Cerellia, and total fungal spores and the children and adolescents who were hospitalised for asthma in this region. There was evidence of same day and lagged effects for these taxa. There was also some evidence that sex and age group were effect modifiers. Girls demonstrated stronger associations with Cladosporium, Cepasporus, Alternaria and total fungal spores than boys. Older adolescents demonstrated stronger associations with Cepasporus and Ustilago/smutis than the younger age group.

The proportions of outdoor fungal taxa reported in this study are similar to other surveys in London, UK (Atkinson et al., 2006) and Melbourne, Australia (Maksukis and Guest, 2001; Tham et al., 2016) that reported fungal spore taxa counts using a similar methodology to this study with predominance of Cladosporium, Aspergillus, Didybalea, Cepasporus, Periconia and Alternaria. Lower proportions of Alternaria and higher proportions of Aspergillus have been reported in an outdoor fungal spore survey in Sydney (54 km from this study site) that used a different sampling methodology (Friso and Torpy, 2015).

The only comparable study from this region was conducted by some authors of this research in another Australian city, Melbourne. In that study of 644 children and adolescents, exposure to outdoor Alternaria, Leptosphaeria, Cepasporus, and Drechslera were significantly associated with asthma hospitalisations with lagged effects up to 3 days. Sex or age group were not significant effect modifiers (Tham et al., 2016). The results from studies conducted elsewhere are mixed. No associations between outdoor fungal spores and asthma hospital attendance have been found in northern Australia (Hanigan and Johnston, 2007), the USA (Lief and Hormung, 2003; Pongracic et al., 2010; Wang and...
Table 2: Fungal spore taxa, grass pollen, weather and air pollutants, 29 May 2008–3 May 2013 (1800 days).

<table>
<thead>
<tr>
<th>Fungal spore taxa/m³</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>25%</th>
<th>Median</th>
<th>75%</th>
<th>90%</th>
<th>Max</th>
</tr>
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<td>Cladosporium</td>
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<td>666.2</td>
<td>861.1</td>
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<td>203</td>
<td>429</td>
<td>814</td>
<td>1442</td>
<td>9153</td>
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<td>189.8</td>
<td>0</td>
<td>9</td>
<td>41</td>
<td>95</td>
<td>235</td>
<td>2266</td>
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<td>5</td>
<td>29</td>
<td>81</td>
<td>217</td>
<td>1263</td>
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<td>266.2</td>
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<td>31.2</td>
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<td>18</td>
<td>32</td>
<td>253</td>
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<td>4.8</td>
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Fig. 1. Daily smoothed asthma hospitalisation counts and daily counts of total fungi.

Yousef, 2007. Israel (Garti et al., 1998) or UK (Khot et al., 1988), however these studies were conducted over shorter time periods with smaller study samples and may have lacked power to detect effects; none of these studies examined lagged effects or stratified by age groups or sex; and only one study examined individual fungal taxa (Pongracic et al., 2010). Other studies have found associations between some fungal taxa in Canada (Dales et al., 2000, 2003, 2004; Rapho et al., 2010), the UK (Atkinson et al., 2006; Newsom et al., 2000), and India (Chakraborty et al., 2014). The closest comparators are studies that analysed individual fungal taxa rather than by phyla (a subdivision of the Fungal Kingdom that groups fungal taxa based on their reproductive processes, e.g. Basidiomyetes, Ascomycetes and Deuteromyetes).

In the UK, Newsom and colleagues (Newsom et al., 2000) found significant associations between asthma epidemics involving the age group 0–14 years compared to those aged ≥15 years (which included adults) and hyaline Basidiomycetes, Didymula, Leptosphaeria, other colonised Ascomycetes and Botrytis on the same day and one day lag. In addition they found no associations between total fungal spore counts in either of these two age groups. Also in the UK, Atkinson and colleagues (Atkinson et al., 2006) found spores mostly strongly and consistently associated with emergency department (ED) attendance and hospitalisation included Alternaria, Epicoccum, Botrytis, spores, hyaline Basidiomycetes and other coloured Ascomycetes. They also found Cognatus was consistently associated with child asthma emergency department attendance, hospitalisation and visits to the medical practitioner which is similar to our most significant finding. Cognatus may be an under-recognised fungal spore taxon in relation to asthma exacerbation that may warrant further investigation.

In an inner city study conducted in multiple cities in the USA, Pongracic and colleagues (Pongracic et al., 2010) detected that outdoor Alternaria, Aspergillus, Cladosporium and Penicillium were significantly associated with increased asthma symptoms; and indoor Aspergillus was significantly associated with unscheduled visits to the doctor or hospital ED in children aged 5–11 years.

In Montreal, Canada, Raphol and colleagues (Rapho et al., 2010) found significant associations between increased asthma emergency department attendances and Basidiomycetes (taxa not defined) and Cladosporium at 4 days. A Canadian study that examined the association between thunderstorms and asthma ED attendances found that increases in Cladosporium, Penicillium, Aspergillus, Ascomycetes and total spores were associated with increased asthma ED attendances regardless of whether there was a thunderstorm or not (Dales et al., 2003). Unlike a number of these studies, our study did not find significant associations between asthma hospitalisation and Cladosporium (except in girls), Alternaria or Aspergillus.

It is possible that some fungal taxa included in our study were used...
Models adjusted for maximum temperature, relative humidity and grass pollen bold numbers statistically significant * p < 0.05.

We did not include air pollutants in the final model as they did not change the effect estimates significantly or appear to act as confounding variables. This is consistent with some studies (Atkinson et al., 2006; Dales et al., 2000; Raflhoz et al., 2010) but not others (Cakmak et al., 2012). Cakmak et al.’s large study that pooled data from 11 Canadian cities over 13 years found that the relative risk of asthma hospitalisation associated with three fungal phyla increased when air pollutant concentrations were higher (Cakmak et al., 2012). Our five-year study period in one region that may experience lower levels of air pollution compared to Canadian cities may have had insufficient power to detect interactions between fungal spores and air pollutants.

The evidence of lagged effects cannot be definitively explained as we do not know whether the hospitalised children had some delay in experiencing serious asthma symptoms, which had previously been to a general medical practitioner, but their condition had deteriorated.

Adjusted for age, maximum temperature, relative humidity and grass pollen p-int interaction p-value bold numbers statistically significant * p < 0.05.
warranting high level care, or whether parents/caregivers delayed accessing the hospital for any number of reasons. Other possible explanations for the lagged effects may relate to different biological mechanisms that may be taking place. Inspiration of fungal spores into the respiratory system can elicit an allergic response and subsequent bronchoconstriction which is likely to occur rapidly after exposure. Or alternatively, fungal spores can release mycotoxins or volatile organic compounds onto the epidermal lining that cause airway inflammation which may have a cumulative effect over a number of days of exposure, leading to delayed bronchoconstriction (Demenig et al., 2006). Residual confounding from other individual or environmental variables may also contribute to the lagged effects. Further studies of the lagged effects are needed.

Previous research in south-eastern Australia has indicated that, in asthma children, prevalence of Alternaria sensitisation is approximately 4% in humid, coastal areas and 17% in dry, inland areas (Peat et al., 1993). In a metropolitan area, sensitisation to Alternaria is approximately 9%, and sensitisation to either Alternaria or Cladosporium is approximately 14% (Tham et al., 2016). Although we did not know the sensitisation rates for children in south-western Sydney, if the rates are similar to these other regions then overall fungal sensitisation was quite low and this might contribute to the weak associations we have observed.

We did not have data on the atopic status of each child or adolescent to assess whether atopy had modified the effect of fungal spore exposure on asthma hospitalisation. Understanding of the contribution of fungal allergy to asthma exacerbation is limited. Assessing fungal allergy for the wide range of potential allergenic fungi has been restricted to focussing on only a few fungal taxa, predominately Alternaria, Cladosporium, Aspergillus and Penicillium out of approximately 80 genera that have been shown to induce allergic reactions (Simon-Nobbe et al., 2008). The contribution of atopy as a risk factor for asthma severity is further complicated by the role of cross-reactivity between fungal spore allergens. Cross-reactivity is the ability of the immune system to recognise similarities between different allergens, such that antibodies produced against one allergen will also react against another similar allergen. Fungal allergens that have been identified as potentially cross-reactive due to their protein structures include: Aspergillus, Alternaria, Cladosporium, Coprinus and Penicillium. Fungal cross-reactivity and its clinical significance require further research using a wider range of fungal allergens (Cramer et al., 2014). Further studies are required to more coherently identify whether allergy to one fungal taxon can increase the risk of allergic sensitisation to other fungal taxa and to investigate other possible mechanisms by which fungal spores can trigger asthma exacerbations. Improving our understanding of these relationships and mechanisms may help to identify high-risk groups for targeted interventions to prevent exacerbations of allergic conditions, such as asthma.

Only one other study examining the contribution of outdoor fungal spores to child asthma hospitalisation analysed differences by sex, and found that boys aged less than 13 years and women older than 15 years were more vulnerable (Cakmak et al., 2005). This contrasts with our finding that in SW Sydney, girls appear to be more vulnerable. The demographics of that large 10-city population study (approximately 350,000) were not reported and hospital readmissions were not explicitly excluded, so our results may not be directly comparable.

6. Strengths and limitations

This study has analysed five years of daily fungal spore count data at the taxa level which is unique in the Australian region. The case-crossover design is well suited for studying the effects of transient short-term environmental exposures on the risk of asthma exacerbations in individuals. As cases serve as their own controls, there is reduced risk of confounding due to stable individual characteristics i.e. age, sex, fungal sensitisation or behavioural factors. The selection of bidirectional control periods allows adjustment for seasonal trends. This method is an improvement on previous studies that have utilised time-series and conditional designs that could not be adjusted for differences in individual characteristics. Our study was limited by lack of data on whether the children and adolescents hospitalised for asthma were also infected with any type of respiratory virus. It is well documented that human rhinovirus is strongly associated with child asthma hospitalisations (Basse et al., 2010; Erbas et al., 2015; Tham et al., 2016) and should be controlled for in analyses. However, our previous research in Melbourne found that asthma hospitalisations were associated with a number of fungal taxa independent of human rhinovirus infection (Tham et al., 2016).

Our study used generalised additive models (GAM) rather than linear generalised linear models (GLM) as the GAM allows a smooth non-linear functional form between the exposure and outcome compared to a GLM which specifically fits a linear combination of the explanatory variables (Guisan et al., 2002). Given the scarcity of evidence on these tested associations, we chose not to make assumptions about any potential linearity in the relationships. Some exposures could be specified as linear, but this is dictated from the data relationship between the exposures and the outcome. A GAM also has the advantage of allowing a combination of linear and smooth terms for the predictors.

Although our effect estimates do not appear to be strong, geographic and demographic variables unique to Sydney may have contributed. Firstly, Campbelltown, Liverpool and Camden are classified as a national growth area (http://www.nugas.org.au/), with the largest population group being aged < 20 years and increasing in size (Australian Bureau of Statistics, 2011). The overall socioeconomic status of Campbelltown and Liverpool is considered lower than the national average, but within this there are areas that are high and some very low (South West Sydney Local Health District, 2014b, 2014c). The overall socioeconomic status of Camden is considered higher (South West Sydney Local Health District, 2014a). In 2008 current child (aged 2–13 years) asthma rates in this region were the third highest in New South Wales after the Hunter-New England and Greater Western (lowest socio-economic area) regions with significantly more boys affected than girls (Centre for Epidemiology and Research, 2010). Additionally it is an area of urban development of farming land that is nested alongside a national park. The vegetation was a major source of the outdoor fungal spores recorded in this study. As Campbelltown is located 61 km and Liverpool 35 km from Sydney city centre, we expected that most children with asthma exacerbations would attend the nearest hospital. However, Sydney has two well established children’s hospitals, one of which is located west of Sydney at Parramatta. It is possible that some parents of asthmatic children were prepared to travel to this specialised hospital and these children were excluded from our study. Also our sample size being limited to one region of a large city may have limited our ability to detect stronger associations or we may be seeing a Type I statistical error resulting in some possible false positive associations. The lower socioeconomic status in parts of this region increases the risk of asthma exacerbation possibly related to exposure to other environmental triggers, such as indoor air pollution, environmental tobacco smoke, and building conditions.

We should also consider exposure misclassification as a contributor to these findings. We cannot be certain that each child or adolescent was exposed to the same levels of fungal spores, pollen or pollution exposures counted at this single site and it is therefore impossible to gauge the generalisability of exposure. However we did limit the inclusion of hospital admissions to within 30 km of the fungal spore trap to reduce the variability of outdoor exposures with changing vegetation types and densities and climatic variations. In a previous study in inland New South Wales, Mitsakakis and colleagues (Mitsakakis et al., 2000) reported that the levels of Alternaria spores counted in a Burkard trap located in similar position as this study — on the roof of a
rural hospital) were similar to those counted in personal air monitors. They also found that fungal spores and pollen were inhaled at higher levels when participants (adults and children) were outdoors or active indoors compared to periods of rest.

As we are considering outdoor exposure, misclassification of fungal exposure is likely to be non-differential, hence it should bias the ratio estimates towards null. The absence of indoor fungal spore measurements may limit our exposure assessment and the possible contribution of these spores to asthma exacerbation. The concentrations and diversity of indoor fungal spores appears to be related to mould presence inside and the levels of outdoor fungal spores that enter the indoor environment through doors and windows (Voigt et al., 2010).

To overcome potential confounding from outdoor fungal spores that migrated indoors we controlled for the climatic variables that most influence fungal spore production (maximum temperature and relative humidity). However, as we could not account for household mould some asthma exacerbations attributable to outdoor fungal spores may be over-estimated or the contribution of indoor fungal spores to asthma exacerbations may be underestimated. However, if indoor and outdoor fungal spores have the same effect on hospitalisation risk then this would most likely lead to non-differential misclassification and a bias toward the null.

7. Conclusion

Our findings indicate that there may be associations between some outdoor fungal spore taxa and child and adolescent asthma hospitalisations in this region. This calls for further research to explore whether these findings can be replicated; and to examine whether fungal sensitisation and/or human rhinovirus infections are associated with stronger effects. If these findings are replicated, then the need to develop predictive models for fungal spore distribution and levels may become more important.

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Competing financial interests declaration

M.J.Abramson holds investigator initiated grants from Pfizer and Boehringer Ingelheim for unrelated research. He has undertaken an unrelated consultancy for AstraZeneca. He has received assistance with conference attendance from Boehringer Ingelheim and Sanofi. All other authors declare no conflict of interest.

Ethics

Ethics approvals were obtained from South West Sydney Local Health District Human Research Ethics Committee (LNR/13/LPOOLI/189).

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2016.12.016.

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Supplemental Material

The role of outdoor fungi on asthma hospital admissions in children and adolescents: A 5-year time stratified case-crossover analysis

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\textsuperscript{b} Western Sydney University, Department of Medicine, Immunology and Allergy, Campbelltown Hospital, Campbelltown, New South Wales, Australia
\textsuperscript{c} School of Public Health, College of Science Health and Engineering, La Trobe University, Bundoora, Victoria, Australia
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\textsuperscript{e} Department of Epidemiology and Preventive Medicine, School of Public Health and Preventive Medicine, Monash University, Melbourne, Victoria, Australia
**Table S1: Spearman’s correlations between fungal spore taxa**

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<th>Alternaria</th>
<th>Cladosporium</th>
<th>Aspergillus</th>
<th>Epicoccum</th>
<th>Ganoderma</th>
<th>Cheatomium</th>
<th>Ustilago</th>
<th>Polythrinicum</th>
<th>Torula</th>
<th>Didymella</th>
<th>Corrinus</th>
<th>Cerebella</th>
<th>Curvularia</th>
<th>Posporea</th>
<th>Puccinia</th>
<th>Decaleon</th>
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<td>0.169</td>
<td>0.116</td>
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<td>0.318</td>
<td>0.291</td>
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<tr>
<td>Stemphylium</td>
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<td>0.069</td>
<td>0.037</td>
<td>0.198</td>
<td>0.091</td>
<td>0.118</td>
<td>0.059</td>
<td>0.026</td>
<td>0.083</td>
<td>0.064</td>
<td>0.250</td>
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<td>0.094</td>
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<td>0.288</td>
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<td>0.318</td>
<td>0.286</td>
<td>0.148</td>
<td>0.093</td>
<td>0.107</td>
<td>-0.017</td>
<td>0.089</td>
<td>0.224</td>
<td>0.155</td>
<td>0.222</td>
<td>0.249</td>
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<td>0.137</td>
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<td>1.000</td>
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<tr>
<td>Pithomyces</td>
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<td>0.256</td>
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<td>0.034</td>
<td>0.128</td>
<td>0.015</td>
<td>0.073</td>
<td>0.064</td>
<td>0.122</td>
<td>0.226</td>
<td>0.240</td>
<td>0.224</td>
<td>0.017</td>
<td>0.207</td>
<td>0.199</td>
<td>0.077</td>
<td>0.263</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.527</td>
<td>0.898</td>
<td>0.466</td>
<td>0.434</td>
<td>0.403</td>
<td>0.205</td>
<td>0.290</td>
<td>0.130</td>
<td>0.044</td>
<td>0.460</td>
<td>0.407</td>
<td>0.202</td>
<td>0.379</td>
<td>0.439</td>
<td>0.378</td>
<td>0.280</td>
<td>0.023</td>
<td>0.329</td>
<td>0.279</td>
<td>1.000</td>
<td></td>
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</table>

|         | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
Supplemental Figure Legends

**Figure S1:** Daily maximum temperature (°Celsius) and rainfall (mm) for SW Sydney May 2008 – May 2013
Chapter 6

Are there associations between outdoor fungal spores, human rhinovirus respiratory infections and air pollutants and child and adolescent asthma hospitalisations?

6.1 Introduction

This peer-reviewed published paper and supplementary data investigated associations between outdoor fungal spores and child and adolescent asthma hospitalisations at the Royal Children’s Hospital in Melbourne between September 2009 and December 2011 using the data obtained in the Melbourne Air Pollen Child and Adolescent Health (MAPCAH) study. This paper also examined if human rhinovirus infection, fungal sensitisation, sex or age acted as effect modifiers.

This paper provides some evidence that exposure to several outdoor fungal spore taxa were associated with increased risk of child and adolescent asthma hospitalisations especially in those sensitised to *Cladosporium* and independently of human rhinovirus infection. The presence of human rhinovirus infection, sex and age did not appear to act as effect modifiers.

I am first author of this paper. I developed the research question, undertook the data analysis, led the writing of the manuscript, was responsible for all edits and revisions and submitted the manuscript to the Journal of Allergy and Clinical Immunology.

This paper was selected for Editors’ Choice for the April 2017 Edition of Journal of Allergy and Clinical Immunology; and for the American Academy of Allergy, Asthma and Immunology (AAAAI) as a highlight in the September “Latest Research” section of their website (https://www.aaaai.org/). I authored these submissions and included them in the pages following the publication.
6.2 Research question

1. Are there associations between outdoor fungal spores and child and adolescent asthma hospitalisations?
2. If so, are these associations modified by presence of human rhinovirus infection, fungal sensitisation, sex or age group?

6.3 Research aims

1. To investigate if there are instantaneous or lagged associations between outdoor fungal spores and child and adolescent asthma hospitalisations using a case crossover study design.
2. To investigate if the presence of human rhinovirus respiratory infection modifies any associations.
3. To investigate if sensitisation to common outdoor fungal spores modifies any associations.
4. To investigate if sex or age modifies any associations.

6.4 Ethics approvals

Ethics approvals were granted by the Royal Children’s Hospital Ethics Committee, La Trobe University Human Ethics Committee and the University of Melbourne Human Ethics Sub-Committee (Health Sciences).

6.5 Publication and data supplement

Asthma and lower airway disease

Associations between outdoor fungal spores and childhood and adolescent asthma hospitalizations

Rachel Tham, MPH, MHSc,1 Don Vicendese, PhD,2 Shyamali C. Dharmage, PhD,3 Rob J. Hyndman, PhD,4 Ed Newbiggin, PhD,5 Emma Lewis, MSc,6 Molly O’Sullivan, B Appl Sc (Hons),7 Adrian J. Lowe, PhD,7,8 Philip Taylor, PhD,1 Philip Bardin, PhD,1 Mimi L. K. Tang, PhD,1,8,a,b Michael J. Abramson, PhD,1,8 and Bircan Erbas, PhD9
Melbourne, Bandoroa, Clayton, and Geelong, Australia

Background: Childhood asthma is a significant public health problem and severe exacerbations can result in diminished quality of life and hospitalization. Objective: We sought to examine the contribution of outdoor fungi to childhood and adolescent asthma hospitalizations. Methods: The Melbourne Air Pollen Children and Adolescent study is a case-crossover study of 644 children and adolescents (aged 2-17 years) hospitalized for asthma. The Melbourne Air Pollen Children and Adolescent study collected individual data on human rhinovirus infection and sensitization to Alternaria and Cladosporium and daily counts of ambient concentrations of fungal spores, pollen, and air pollutants. Conditional logistic regression models were used to assess associations with increases in spore counts while controlling for potential confounding and testing interactions. Results: Exposure to Alternaria (adjusted odds ratio [aOR], 1.07; 95% CI, 1.03-1.11), Leptosphaeria (aOR, 1.05; 95% CI, 1.02-1.08), Coptotrichum (aOR, 1.04; 95% CI, 1.01-1.07), Drechslera (aOR, 1.03; 95% CI, 1.00-1.05), and total spores (aOR, 1.05; 95% CI, 1.01-1.09) was significantly associated with child asthma hospitalizations independent of human rhinovirus infection. There were significant lagged effects up to 3 days with Alternaria, Leptosphaeria, Cladosporium, Sporormiella, Coptotrichum, and Drechslera. Some of these associations were significantly greater in participants with Cladosporium sensitization. Conclusions: Exposures to several outdoor fungal spore taxa, including some not reported in previous research, are associated with the risk of child and adolescent asthma hospitalization, particularly in individuals sensitized to Cladosporium. We need further studies to examine cross-reactivity causing asthma exacerbations, identifying sensitization to multiple fungal allergens in children with asthma could support the design and implementation of more effective strategies to prevent asthma exacerbations. (J Allergy Clin Immunol 2017;139:1146-7.)

Key words: Outdoor fungi, asthma, hospitalization, child, adolescent, case-crossover design

Asthma is a significant global public health problem, estimated to affect approximately 334 million people and 11% to 14% of the world’s children. The prevalence of asthma in Australia is comparable, with 9.9% of adults and 10.4% of children having current asthma. More than 50% of asthma hospitalizations involve children and adolescents aged between 0 and 15 years. Severe asthma exacerbations have adverse impacts on quality of life and long-term respiratory health and incur significant costs for families and the public health system. Environmental triggers of sudden-onset child asthma exacerbations that result in hospitalizations can include respiratory viruses (especially human rhinovirus [HRV]), pollen, indoor fungi (related to house mold/dampness), and air pollutants (such as particulate matter, ozone, and nitrogen dioxide). There has been limited research examining the contributions of outdoor fungi to asthma exacerbations. It is proposed that airborne fungi can trigger asthma exacerbation because of their allergenic protein structure and their small size (often 1-20 µm in diameter), which allows them to lodge deep in the airways and lungs, causing inflammation...
and allergic response. A number of ecological studies have examined the association between outdoor fungal spores and child and adolescent asthma hospitalizations and the predominant outdoor fungal taxa most often reported are Alternaria spp and Cladosporium spp. To date, only 1 study has examined this association at an individual level in children sensitized to fungi and no studies have controlled for respiratory viral infections (particularly HRV) in individuals, both being strong risk factors for child asthma exacerbations. It may be possible to improve asthma management care and create a better understanding of the contribution that fungal spores make to severe asthma exacerbations while accounting for potential confounders. We also need to understand potential interactions between exposures to outdoor fungal spores, being sensitized to fungi, and having HRV infection that may be associated with severe asthma exacerbations, to allow identification of high-risk groups.

Fungal exposure is ubiquitous, but the species (taxa) and numbers of fungi present in the air that we breathe depend on regional variations in vegetation, temperature, relative humidity, and seasonal changes. As a result, numbers can fluctuate within short time frames. This makes studying their effects on relatively rapid-onset events, such as asthma exacerbations, difficult. The case-crossover design has been used more broadly in environmental epidemiology to study the effects of short-term ambient exposures on the risk of rapid-onset events in individuals. There are no currently published studies that have used this approach to examine the association between outdoor fungal spore exposure and childhood and adolescent asthma exacerbations.

The aim of this case-crossover study was to examine the associations between outdoor fungal spores and asthma hospitalizations, and whether the associations were modified by sex, fungal sensitization status, presence of HRV infection, or age group.

**METHODS**

**Study design and population**

The Melbourne Air Pollen Children and Adolescent Health (MAPCAH) study is a case-crossover study of 644 children and adolescents (aged 2-17 years) with “incident” asthma admitted to the Royal Children’s Hospital in Melbourne, Australia, between September 2009 and December 2011. This study used a bidirectional time-stratified case-crossover design that has been shown to be well suited for studying the effects of transient short-term exposures (fungal spore release, air pollution changes) on the risk of short-onset events (asthma exacerbation requiring hospitalization) in individuals. Because cases serve as their own controls, there is less risk of confounding due to stable individual characteristics (i.e., age, sex, behavioral factors). The hospital admission date was set as the index case, and the referent control dates were the same day of the week within the same month and year as the index case. Bidirectional control sampling is valid because this approach reduces potential biases related to possible time or seasonal trends. For each admission date (case) and referent control days, we compared the daily level of fungi, grass pollen, air pollution, and meteorological variables.

This study was approved by the Royal Children’s Hospital Ethics Committee and the La Trobe University Human Ethics Committee, and all participating parents/guardians provided written informed consent.

**Definitions**

**Outcome.** The case definition for inclusion in the study was an asthma admission on a given day with a principal International Classification of Diseases, Tenth Revision diagnosis code of asthma (J45) identified through the admissions department and confirmed at discharge.

**Primary exposure: Ambient levels of fungal spores.** Daily ambient fungal spore counts were measured using a volumetric spore trap (Burkard, United Kingdom) located on the rooftop of the Earth Sciences building at the Parkville campus of the University of Melbourne, which is located approximately 2 km from the Royal Children’s Hospital and meets the guidelines of the World Allergy Organization. Briefly, collection involved drawing 10 L of air per minute continuously across a microscope slide that had been coated with an adhesive. Particles in the air stuck to the slide as it moved past the inlet at 2 mm/s. The fungal spores were identified and counted by a trained technician (E.L.) and related to the volumes of air sampled, giving concentrations per cubic meter of air, averaged over the 24 hours. Identifiable fungal spores were classified into taxa: Alternaria, Cladosporium, Geos- derma, Leptosphaeria, Pleospora, Sporormiella, Phialophora, smuts, Copri- nus, Drechslera, Stilbalemma, and Penicillia.

**HRV infection.** Viral respiratory illness was assessed using nasal throat swabs that were collected on admission. HRV was identified using multiplex PCR methods as described by Erbas et al.

**Fungal sensitization.** At admission, fungal sensitization was defined as a positive skin prick test result with a mean wheal diameter of 3 mm or greater to the fungi Alternaria or Cladosporium (Hollister-Stier, Spokane, Wash; Alyostal, Antony, France). Controls were defined as positive histamine (10 mg/mL) and negative saline.

**Outdoor air quality, pollen, and weather data.** The 24-hour average daily concentrations of particulate matter up to 2.5 µm in diameter (PM$_{2.5}$) and up to 10 µm in diameter (PM$_{10}$) (µg/m$^3$), the daily maximum 1-hour average nitrogen dioxide (NO$_2$) level (parts per billion), and the daily maximum 4-hour average ozone (O$_3$) level (parts per billion) were obtained from routine air quality monitoring stations in Melbourne. The Bureau of Meteorology provided data on daily maximum and minimum temperatures (degrees Celsius), rainfall (mm), and daily relative humidity (percentage) for the study period. Daily ambient pollen counts were collected and measured using the same method of capture as described for fungal spores. Pollen was considered here as they have been identified as triggers for respiratory admissions in Melbourne.

**Age groups.** Participants were stratified into age groups: (1) 2 to 5.6 to 10, 11 to 15, and 16 to 18 years and (2) 2 to 14 and 15 to 18 years.

**Statistical analysis**

Correlations between fungal spore taxa were assessed using Spearman rank correlation coefficients. We used conditional logistic regression models for binary outcomes to evaluate the association between the fungal taxa (continuous variable) and asthma hospitalization. Analyses were done for the same day (lag 0) and lagged fungal spore exposure (up to 3 days—multilag and cumulative).

Maximum temperature and relative humidity were included as a priori confounders in all adjusted models because these factors have been shown to be associated with fungal spore production and dispersion and asthma exacerbation. Models were adjusted for possible confounding variables: HRV infection (yes/no), grass, tree, and weed pollen (continuous), sensitization to Alternaria (yes/no), sensitization to Cladosporium (yes/no), and air pollutants: PM$_{10}$, PM$_{2.5}$, NO$_2$, or O$_3$ (continuous and stratified into low <75th percentile or high >75th percentile), and were retained in the model if they
TABLE I. MAPCAH participants' characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>n [%]</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
<td>644</td>
<td></td>
</tr>
<tr>
<td>Boys</td>
<td>407 (63.2)</td>
<td></td>
</tr>
<tr>
<td>Girls</td>
<td>237 (36.8)</td>
<td></td>
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<tr>
<td>Age (y), median ± SD (range)</td>
<td>5.2 ± 3.3 (2-17)</td>
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<tr>
<td>Age group (y)</td>
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<td></td>
</tr>
<tr>
<td>2-5</td>
<td>416 (64.6)</td>
<td></td>
</tr>
<tr>
<td>6-10</td>
<td>164 (25.5)</td>
<td></td>
</tr>
<tr>
<td>11-15</td>
<td>38 (9.0)</td>
<td></td>
</tr>
<tr>
<td>16-18</td>
<td>6 (1.9)</td>
<td></td>
</tr>
<tr>
<td>Age group (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-14</td>
<td>635 (98.6)</td>
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</tr>
<tr>
<td>15-18</td>
<td>9 (1.4)</td>
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<table>
<thead>
<tr>
<th>n tested</th>
<th>n [%] yes</th>
</tr>
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<td>Infected with HRV</td>
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</tr>
<tr>
<td>Sensitized to Alternaria</td>
<td>630</td>
</tr>
<tr>
<td>Sensitized to Cladosporium</td>
<td>630</td>
</tr>
<tr>
<td>Sensitized to Alternaria or Cladosporium</td>
<td>630</td>
</tr>
</tbody>
</table>

Unadjusted analyses

In unadjusted models, Alternaria (OR, 1.05; 95% CI, 1.03-1.08), Leptosphaeria (OR, 1.04; 95% CI, 1.02-1.06), Pleospora (OR, 1.12; 95% CI, 1.01-1.25), and Drechslera (OR, 1.02; 95% CI, 1.00-1.04) were significantly associated with increased odds of asthma hospital admission (Table III).

Adjusted analyses

In separate adjusted models for each fungus, 4 fungal taxa were significantly associated with asthma hospitalizations: Alternaria (adjusted odds ratio [aOR], 1.07; 95% CI, 1.03-1.11), Leptosphaeria (aOR, 1.05; 95% CI, 1.02-1.07), Coprinus (aOR, 1.04; 95% CI, 1.01-1.07), Drechslera (aOR, 1.03; 95% CI, 1.00-1.05), and total spores (aOR, 1.05; 95% CI, 1.01-1.09) (Table III). Because HRV infection was strongly associated with asthma hospitalization risk (OR, 5.1; 95% CI, 4.1-6.3; P < .001) and grass pollen is a potential confounder for asthma exacerbation, each model was adjusted for HRV infection and grass pollen in addition to the 2 a priori confounders: maximum temperature and relative humidity. None of the other covariates changed the association between the fungal spores and asthma hospitalization by more than 10%.

When stratified by sex, HRV infection status, sensitization to Alternaria or sensitization to Cladosporium, exposure to high versus low levels of individual air pollutants, and age group and assessed as effect modifiers in the adjusted models, we found that the associations between Alternaria, Coprinus, Drechslera, and Stemphylium and asthma hospitalizations were greater in participants who were sensitized to Cladosporium. Associations with Alternaria, Coprinus, Drechslera, and Saprolegnia in children sensitized to Alternaria were significant, but Alternaria sensitization fitted as an interaction term was not significant (Table IV).

When we stratified the data by presence of HRV infection at admission, we found that among the fungal species assessed, Saprolegnia, Ganoderma, and Pithomyces count were modified by HRV infection because stronger associations were seen with hospitalization in those with an HRV infection (interaction P < .05) (see Table E2 in this article’s Online Repository at www.jacionline.org). Air pollutants did not appear to act as effect modifiers. We also fitted multivariable regression models and found

Fungal spore distribution

The most prevalent fungi taxa detected were Cladosporium (44%), Leptosphaeria (14%), and Alternaria and smuts (11% each) of the total fungal spore count (Table I). The remaining fungal taxa accounted for 1% to 7% each. The daily fungal spore counts and daily participants enrolled in the MAPCAH study over the study period showed seasonal variation (Fig 1). During the study period, fungal spore concentrations were relatively low (median, 0-3) for most of the year, with Cladosporium, Leposphaeria, Alternaria, Pleospora, Sponomelia, and Periconia peaking during spring (September 1 to November 30); smuts, Coprinus, and Drechslera peaking in spring (December 1 to February 28); Ganoderma and Pithomyces peaking in autumn (March 1 to May 31); and none peaking during winter. Spring accounted for 54% and summer for 33% of the total fungal spore count for the study period. Most fungi taxa appeared highly correlated in this study except for Ganoderma and Saprolegnia (see Table E1 in this article’s Online Repository at www.jacionline.org).

The lower fungal spore concentrations in the period September 2009 to September 2010 correlated with the end of a major drought (high maximum temperatures and low rainfall) that affected Melbourne. The period September 2010 to December 2011 was characterized by above-average rainfall and lower temperatures across Melbourne, which stimulated fungal spore growth (see Fig E1 in this article’s Online Repository at www.jacionline.org).
TABLE II. Summary statistics for fungal spore counts and other environmental factors from September 2009 to December 2011 in Melbourne

<table>
<thead>
<tr>
<th>Fungal spore species (number of spores/m³)</th>
<th>N (d)</th>
<th>Mean ± SD</th>
<th>Geometric mean</th>
<th>Minimum</th>
<th>25%</th>
<th>Median</th>
<th>75%</th>
<th>90%</th>
<th>Maximum</th>
<th>Frequency % of total</th>
</tr>
</thead>
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<td>Chalcosporium</td>
<td>852</td>
<td>12.6 ± 45.9</td>
<td>6.3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>26</td>
<td>907</td>
<td>43.8</td>
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<tr>
<td>Leptosphaeria</td>
<td>852</td>
<td>4.0 ± 13.4</td>
<td>4.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>203</td>
<td>14.0</td>
</tr>
<tr>
<td>Alternaria</td>
<td>852</td>
<td>3.3 ± 8.0</td>
<td>3.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>9</td>
<td>98</td>
<td>11.4</td>
</tr>
<tr>
<td>Smuts</td>
<td>852</td>
<td>3.2 ± 14.8</td>
<td>5.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>22</td>
<td>113</td>
<td>11.3</td>
</tr>
<tr>
<td>Coprinus</td>
<td>852</td>
<td>1.9 ± 6.3</td>
<td>2.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>58</td>
<td>5.9</td>
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<tr>
<td>Drechsleria</td>
<td>852</td>
<td>1.4 ± 4.7</td>
<td>2.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>58</td>
<td>5.0</td>
</tr>
<tr>
<td>Periconia</td>
<td>852</td>
<td>0.7 ± 1.9</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>19</td>
<td>25</td>
<td>2.3</td>
</tr>
<tr>
<td>Plectosphaeria</td>
<td>852</td>
<td>0.5 ± 1.5</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>25</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Gomphidium</td>
<td>852</td>
<td>0.4 ± 2.2</td>
<td>2.5</td>
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<td>0</td>
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<td>42</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Pithomyces</td>
<td>852</td>
<td>0.3 ± 1.2</td>
<td>1.7</td>
<td>0</td>
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<td>0</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td>0.9</td>
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<tr>
<td>Stempylidiella</td>
<td>852</td>
<td>0.3 ± 0.9</td>
<td>1.6</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>0.5</td>
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<tr>
<td>Total spores</td>
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<td>28.6 ± 65.5</td>
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<td>2</td>
<td>9.5</td>
<td>23.5</td>
<td>72</td>
<td>949</td>
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<table>
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<th>Meteorological variables</th>
<th>N (d)</th>
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<th>Geometric mean</th>
<th>Minimum</th>
<th>25%</th>
<th>Median</th>
<th>75%</th>
<th>90%</th>
<th>Maximum</th>
<th>Frequency % of total</th>
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</thead>
<tbody>
<tr>
<td>Maximum temperature (ºC)</td>
<td>851</td>
<td>21.2 ± 5.0</td>
<td>10.8</td>
<td>16.5</td>
<td>20.3</td>
<td>24.0</td>
<td>29.5</td>
<td>43.6</td>
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<tr>
<td>Rainfall (mm)</td>
<td>824</td>
<td>2.3 ± 6.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.4</td>
<td>11.8</td>
<td>82.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative humidity (%)</td>
<td>849</td>
<td>51.3 ± 15.9</td>
<td>8</td>
<td>41</td>
<td>49</td>
<td>61</td>
<td>73</td>
<td>96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>N (d)</th>
<th>Mean ± SD</th>
<th>Geometric mean</th>
<th>Minimum</th>
<th>25%</th>
<th>Median</th>
<th>75%</th>
<th>90%</th>
<th>Maximum</th>
<th>Frequency % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM2.5 (µg/m³)</td>
<td>851</td>
<td>49 ± 3.1</td>
<td>-0.8</td>
<td>2.9</td>
<td>4.2</td>
<td>6.2</td>
<td>8.8</td>
<td>20.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM10 (µg/m³)</td>
<td>852</td>
<td>17.7 ± 7.0</td>
<td>3.5</td>
<td>12.7</td>
<td>16.45</td>
<td>21.4</td>
<td>26.2</td>
<td>60.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrogen dioxide (ppm)</td>
<td>847</td>
<td>9.5 ± 4.1</td>
<td>0.4</td>
<td>6.5</td>
<td>9</td>
<td>12.4</td>
<td>15.1</td>
<td>23.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ozone (ppm)</td>
<td>816</td>
<td>13.7 ± 5.8</td>
<td>-0.2</td>
<td>10.25</td>
<td>13.5</td>
<td>17.05</td>
<td>20.3</td>
<td>32.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grass pollen</td>
<td>824</td>
<td>10.8 ± 27.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>34</td>
<td>207</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

that Alternaria and Leptosphaeria continued to be independently associated (see Table E3 in this article’s Online Repository at www.jacionline.org).

Lag analysis

We undertook a lag analysis up to day 3 including cumulative 4-day lags. In adjusted regression models, Alternaria (Lag1, Lag2, cumulative lag), Leptosphaeria (cumulative lag), Sporormiella (Lag5, Lags8), Coprinus (Lag1, cumulative lag), Drechslera (Lag1, Lag2, cumulative lag), Stempylida (Lag1, Periconia (Lag3)), and total spores (Lag3 and cumulative lag) were associated with increased odds of asthma admissions (Table V).

DISCUSSION

Using a case-crossover approach, we found that exposures to spores of several outdoor fungal taxa, including some that, individually, have not been reported in previous research, were associated with the risk of asthma exacerbations in children and adolescents. Alternaria, Leptosphaeria, Coprinus, and Drechslera spores were associated with asthma hospitalizations on the day of exposure. In addition, there were associations with Alternaria, Coprinus, Drechslera, and Stempylida 1 day after exposure, associations with Alternaria and Drechslera 2 days after exposure, and associations with Periconia and total spores 3 days after exposure. Associations were found with Alternaria, Leptosphaeria, Coprinus, Drechslera, and total spores after 4-day cumulative exposure. All these spores have been shown to be associated with aeroallergen production except Leptosphaeria.16

Our findings build on previous research that some ambient fungal spores are associated with child and adolescent asthma hospitalization. Alternaria is commonly documented in the childhood asthma hospitalization literature. It has been associated with bronchial hyperreactivity in children sensitized to Alternaria in inland rural Australia.6 Coprinus was associated with an increased risk of child asthma hospital admission.13 Individually, Leptosphaeria and Drechslera have not been commonly reported as being associated with child asthma hospitalizations. Nouwen et al14 found no significant association between Leptosphaeria and Drechslera and child asthma hospital admissions. However, their inclusion in fungal phylum (Ascomycetes) for analysis may provide some evidence of their effect. Atkinson et al15 grouped Leptosphaeria into the Ascomycetes and elevated counts of this phylum were significantly associated with child asthma emergency department attendances. Coprinus is in the Basidiomycetes and elevated levels of spores from this phylum were associated with child asthma emergency department attendances and hospitalizations.16 In Auckland, New Zealand, Hasnain’s study17 correlated elevated ambient Leptosphaeria levels with high regional prevalence of allergic respiratory diseases although asthma was not the specific outcome of interest. Leptosphaeria may be a significant and underidentified aeroallergen in temperate environments in the southern hemisphere.
Our study also found that the risk of asthma hospitalization was still significant when children were exposed to *Alternaria, Leptosphaeria, Coprinus, Drechslera, Stemphylium,* and *Periconia* over a range of lag periods. This is similar to findings from Raphoe et al. who found associations with increased child asthma emergency department visits and *Cladosporium* and *Drechslera* (possibly including *Alternaria, Coprinus, Drechslera,* and *Stemphylium* but these were not specified) at Lag3 to Lag5. Newson et al. also found associations with *Alternaria, Cladosporium,* and *Drechslera* at Lag1, but our study did not find any associations with *Cladosporium.*

Our findings that grass pollen and air pollutants (PM$_{2.5}$, PM$_{10}$, NO$_2$, or O$_3$) did not change the effect estimates by more than 10% have been reported in other epidemiological studies examining the effect of fungi spores on child asthma hospitalizations. However, to date, no comparable studies have accounted for individual factors such as HRV infection or fungal sensitization status that may interact with the effect of fungal exposure on asthma exacerbation. Our study examined potential confounding or effect modification by other individual and environmental factors. We included maximum temperature and relative humidity as *a priori* confounders because these were known to affect both spore production and were also associated with asthma exacerbations in children. The analysis of other covariates in our modeling highlighted that HRV infection was strongly associated with asthma exacerbations, so we controlled for this factor in our modeling to reduce error variance. We interpreted the finding of HRV infection modifying the effects of *Sponnoriella, Genodermat*, and *Pleurocytomyces* with caution because the relatively small number of participants with no HRV infection at admission and low fungal spore counts may contribute to these findings. Other individual factors such as age group, sex, or *Alternaria* sensitization status did not appear to act as effect modifiers. We also examined environmental covariates—ambient grass pollen, air pollutants (PM$_{2.5}$, NO$_2$, and O$_3$)—and found that they did not have significant confounding or effect modification. These analyses potentially reduced the possible bias in our findings.

Although we found significant associations between fungal spore taxa and children both sensitized and nonsensitized to

**TABLE III.** Associations between fungi taxa and asthma hospitalizations—unadjusted and adjusted models

<table>
<thead>
<tr>
<th>Fungi species</th>
<th>Unadjusted model, OR (95% CI) [N = 644]</th>
<th>Adjusted$^a$ model, aOR (95% CI) [N = 644]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cladosporium</em></td>
<td>1.01 (0.99-1.03)</td>
<td>1.02 (0.98-1.04)</td>
</tr>
<tr>
<td><em>Leptosphaeria</em></td>
<td>1.04 (1.01-1.06)</td>
<td>1.05 (1.02-1.07)</td>
</tr>
<tr>
<td><em>Alternaria</em></td>
<td>1.05 (1.03-1.08)</td>
<td>1.07 (1.03-1.11)</td>
</tr>
<tr>
<td><em>Spatula</em></td>
<td>0.99 (0.97-1.00)</td>
<td>0.98 (0.95-1.01)</td>
</tr>
<tr>
<td><em>Cephalospori</em></td>
<td>1.03 (1.00-1.06)</td>
<td>1.04 (1.01-1.07)</td>
</tr>
<tr>
<td><em>Drechslera</em></td>
<td>1.02 (1.00-1.04)</td>
<td>1.03 (1.00-1.05)</td>
</tr>
<tr>
<td><em>Periconia</em></td>
<td>1.03 (0.95-1.05)</td>
<td>1.00 (0.93-1.07)</td>
</tr>
<tr>
<td><em>Penicillium</em></td>
<td>1.12 (1.01-1.23)</td>
<td>1.05 (0.92-1.20)</td>
</tr>
<tr>
<td><em>Genodermat</em></td>
<td>0.96 (0.91-1.01)</td>
<td>0.98 (0.94-1.02)</td>
</tr>
<tr>
<td><em>Pleurocytomyces</em></td>
<td>1.01 (0.95-1.07)</td>
<td>1.02 (0.95-1.11)</td>
</tr>
<tr>
<td><em>Stemphylium</em></td>
<td>1.01 (0.94-1.08)</td>
<td>1.04 (0.96-1.13)</td>
</tr>
<tr>
<td><em>Spongillia</em></td>
<td>1.06 (0.97-1.18)</td>
<td>1.06 (0.96-1.18)</td>
</tr>
<tr>
<td>Total spores</td>
<td>1.03 (1.00-1.06)</td>
<td>1.05 (1.01-1.09)</td>
</tr>
</tbody>
</table>

*OR and aOR per increase from 75th to 90th percentile. Statistically significant results are in boldface.

$^a$Adjusted for HRV status, relative humidity, maximum temperature, and grass pollen. $^b$p < .001.

$^c$OR and aOR for unit increase per fungal spore when the change from 75th to 90th percentile is 6.
**TABLE IV.** Adjusted associations between fungal taxa and asthma hospitalizations stratified by *Alternaria* and *Cladosporium* sensitization status (yes/no) and with sensitization as an interaction term

<table>
<thead>
<tr>
<th>Fungal spore species</th>
<th><em>Alternaria</em> sensitivity (n = 530)</th>
<th><em>Cladosporium</em> sensitivity (n = 536)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n = 56), aOR* (95% CI)</td>
<td>No (n = 574), aOR* (95% CI)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cladosporium</em></td>
<td>1.19 (0.97-1.46)</td>
<td>1.02 (0.99-1.04)</td>
</tr>
<tr>
<td></td>
<td>1.02 (1.00-1.03)</td>
<td>1.02 (1.00-1.03)</td>
</tr>
<tr>
<td><em>Leptosphaeria</em></td>
<td>1.04 (0.88-1.22)</td>
<td>1.04 (1.02-1.07)</td>
</tr>
<tr>
<td></td>
<td>1.09 (0.85-1.40)</td>
<td>1.05 (1.02-1.17)</td>
</tr>
<tr>
<td><em>Alternaria</em></td>
<td>1.18 (1.08-1.29)</td>
<td>1.06 (1.02-1.10)</td>
</tr>
<tr>
<td></td>
<td>1.25 (1.07-1.48)</td>
<td>1.06 (1.02-1.15)</td>
</tr>
<tr>
<td><em>Smuts</em></td>
<td>0.83 (0.61-1.11)</td>
<td>0.98 (0.85-1.12)</td>
</tr>
<tr>
<td></td>
<td>0.62 (0.32-1.13)</td>
<td>0.98 (0.95-1.03)</td>
</tr>
<tr>
<td><em>Coprinus</em></td>
<td>1.65 (1.33-2.04)</td>
<td>1.02 (0.99-1.05)</td>
</tr>
<tr>
<td></td>
<td>1.15 (1.08-1.22)</td>
<td>1.03 (0.99-1.02)</td>
</tr>
<tr>
<td><em>Drechslera</em></td>
<td>1.14 (1.08-1.20)</td>
<td>1.02 (1.06-1.08)</td>
</tr>
<tr>
<td></td>
<td>1.24 (1.14-1.33)</td>
<td>1.02 (0.99-1.04)</td>
</tr>
<tr>
<td><em>Peronospora</em></td>
<td>0.70 (0.56-0.90)</td>
<td>1.03 (0.87-1.11)</td>
</tr>
<tr>
<td></td>
<td>0.90 (0.51-1.59)</td>
<td>1.07 (0.93-1.29)</td>
</tr>
<tr>
<td><em>Penicillium</em></td>
<td>0.98 (0.84-1.13)</td>
<td>1.00 (1.00-1.01)</td>
</tr>
<tr>
<td></td>
<td>1.00 (0.70-1.30)</td>
<td>0.98 (0.94-1.02)</td>
</tr>
<tr>
<td><em>Phycomycetes</em></td>
<td>1.17 (1.01-1.36)</td>
<td>1.06 (0.93-1.09)</td>
</tr>
<tr>
<td></td>
<td>0.73 (0.30-1.81)</td>
<td>0.83 (0.65-1.14)</td>
</tr>
<tr>
<td><em>Sporothrix</em></td>
<td>0.95 (0.83-1.11)</td>
<td>1.05 (0.87-1.21)</td>
</tr>
<tr>
<td></td>
<td>1.42 (1.06-1.90)</td>
<td>1.02 (0.94-1.59)</td>
</tr>
<tr>
<td><em>Sporespermella</em></td>
<td>1.50 (1.33-1.71)</td>
<td>1.05 (0.84-1.27)</td>
</tr>
<tr>
<td></td>
<td>1.64 (0.94-2.95)</td>
<td>1.06 (0.95-2.05)</td>
</tr>
<tr>
<td>Total spores</td>
<td>1.24 (0.94-1.63)</td>
<td>1.04 (1.04-1.08)</td>
</tr>
<tr>
<td></td>
<td>1.06 (0.90-1.14)</td>
<td>1.05 (1.01-1.14)</td>
</tr>
</tbody>
</table>

AOR per increase in fungal spores from 75th to 90th percentile. Statistically significant results are in boldface.

* Adjusted for HRV infection status, maximum temperature, relative humidity, grass pollen.

** P < .05.

† P < .001.

†AOR for unit increase per fungal spore when the change from 75th to 90th percentile is 0.

**TABLE V.** Adjusted associations between fungal taxa and asthma hospitalizations—Lags 1, 2, and 3 and cumulative lag (Lag0-Lag3)

<table>
<thead>
<tr>
<th>Fungi species</th>
<th>Lag1*</th>
<th>Lag2*</th>
<th>Lag3*</th>
<th>Cumulative lag*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cladosporium</em></td>
<td>0.98 (0.94-1.01)</td>
<td>1.01 (0.99-1.03)</td>
<td>1.03 (1.00-1.06)</td>
<td>1.00 (0.99-1.01)</td>
</tr>
<tr>
<td><em>Leptosphaeria</em></td>
<td>1.01 (0.99-1.04)</td>
<td>1.02 (0.99-1.04)</td>
<td>1.03 (1.00-1.05)</td>
<td>1.01 (1.00-1.02)</td>
</tr>
<tr>
<td><em>Alternaria</em></td>
<td>1.06 (1.02-1.11)</td>
<td>1.06 (1.02-1.09)</td>
<td>1.00 (0.99-1.01)</td>
<td>1.01 (1.00-1.04)</td>
</tr>
<tr>
<td><em>Coprinus</em></td>
<td>1.01 (1.00-1.02)</td>
<td>1.02 (1.00-1.05)</td>
<td>1.00 (0.99-1.01)</td>
<td>1.02 (1.01-1.04)</td>
</tr>
<tr>
<td><em>Drechslera</em></td>
<td>1.03 (1.00-1.05)</td>
<td>1.04 (1.01-1.08)</td>
<td>1.00 (0.99-1.01)</td>
<td>1.01 (1.00-1.02)</td>
</tr>
<tr>
<td><em>Peronospora</em></td>
<td>1.01 (0.99-1.03)</td>
<td>1.01 (0.99-1.03)</td>
<td>1.00 (0.99-1.01)</td>
<td>1.02 (1.01-1.04)</td>
</tr>
<tr>
<td><em>Penicillium</em></td>
<td>1.00 (0.98-1.02)</td>
<td>1.01 (0.99-1.02)</td>
<td>1.00 (0.99-1.01)</td>
<td>1.02 (1.01-1.04)</td>
</tr>
<tr>
<td><em>Sporothrix</em></td>
<td>1.00 (0.98-1.02)</td>
<td>1.01 (0.99-1.02)</td>
<td>1.00 (0.99-1.01)</td>
<td>1.02 (1.01-1.04)</td>
</tr>
<tr>
<td><em>Sporothrix</em></td>
<td>1.00 (0.98-1.02)</td>
<td>1.01 (0.99-1.02)</td>
<td>1.00 (0.99-1.01)</td>
<td>1.02 (1.01-1.04)</td>
</tr>
</tbody>
</table>

AOR per increase in fungal spores from 75th to 90th percentile. Statistically significant results are in boldface.

* Adjusted for HRV status, relative humidity, maximum temperature, and grass pollen.

** P < .05.

† P < .001.

†AOR for unit increase per fungal spore when the change from 75th to 90th percentile is 0.

**Alternaria** and **Cladosporium**, the effect estimates were stronger in sensitized children. Although *Alternaria* sensitization was not significant when fitted as an interaction term, our results suggest that *Alternaria* sensitization may be significant when children are exposed to *Alternaria, Coprinus, Drechslera, and Sporothrix*. Our finding that the associations between *Alternaria, Coprinus, Drechslera, and Scedosporium* exposure were stronger in individuals with *Cladosporium* sensitization does not appear to fit the current understanding of the mechanisms of allergic asthma and may be due to a number of factors. Accurately detecting fungal sensitization may be complicated by the lack of standardization of testing reagents used, possible variation in the wheat pattern with different reagents, and possible difference in reaction to reagents in the same person between testing periods. The possibility of some cross-reactivity between fungal allergens (proteins) may also contribute to these findings. Cross-reactivity is the ability of the immune system to recognize similarities between different allergens, such that antibodies produced against one allergen will also react against another similar allergen. As 25 fungal taxa in the Ascomycetes and Basidiomycetes phyla have been officially identified as allergenic by the Nomenclature Subcommittee of the World Health Organization/International Union of Immunological Societies (www.allergen.org), the phenomenon of cross-reactivity complicates the attribution of fungal sensitization and fungal exposure to asthma exacerbation. Recent reviews of the field by Cramer et al. and summarized prominent cross-reactive fungal allergen structures on the basis of evidence to date, suggesting that the importance of fungal cross-reactivity and its clinical significance required further in vivo and in vitro research using fungal allergens. Other factors to consider may include the following: differing severity of sensitization between fungal atopic children; accurate sensitization in very young children may be difficult to detect by skin prick testing; the children not sensitized to fungi may be sensitized to other allergens that may trigger an asthma exacerbation, such as pollen or house dust mites; or the small sample may limit adequate power to overcome possible statistical errors.
The fungi spore distributions are similar to those recorded by Mirakakis and Guest,\textsuperscript{38} at the same site in Melbourne in 1993, except that the proportions of Alternaria were higher in this sample than in 1993 (11.3\% compared with 1.6\%) and proportions of Coprinus lower (6.6\% compared with 15.5\%). The dominance of Cladosporium, Leptosphaeria, and Alternaria spores and low counts for many fungal taxa have been reported in other sites such as in the United Kingdom,\textsuperscript{25,26} Canada,\textsuperscript{35} and Sydney, Australia.\textsuperscript{36} The lack of association with Pleospora, Gaeodermatium, Pathomyces, Sporobolomyces, and smuts could be due to a number of reasons: they have not been found to be allergenic in clinical/ laboratory studies\textsuperscript{34} and/or they are present in very low numbers even during their maximum sporulation time and so their dose is not high enough to elicit an allergic reaction and/or the low counts lacked the statistical power needed to detect effects.

**Strengths**

This case-crossover design is well suited for studying the effects of transient short-term ambient exposures on the risk of rapid-onset events (i.e., asthma exacerbation) in individuals. Because cases serve as their own controls, there is little risk of confounding due to stable individual characteristics (e.g., age, sex, fungal sensitization, behavioral factors). The bidirectional selection of the control periods allows adjustment for seasonal trends. This is the first study in the Australasian region that has included daily measures of fungal spores and other environmental factors over a 2-year period. The sample was representative of the total daily child asthma admissions in Melbourne for that period; hence, it can be considered to be generalizable to the young Melbourne population.

**Limitations**

Comparison of findings in this current report with findings in other studies is limited by significant variations in defining “exposure to fungi.” Some studies report specific fungal taxa (aggregated and separately),\textsuperscript{11,23} some report fungal phyla (grouping them by their reproductive processes: Ascomycetes/ Basidiomycetes/Deuteromycetes),\textsuperscript{1,21,25} and some as simply “total fungi.”\textsuperscript{1,21,25} The significant change in the fungal classification system in 2006,\textsuperscript{27} with the incorporation of Deuteromycetes into Ascomycetes or Basidiomycetes phyla is important because some fungi species or taxa (e.g., Alternaria, Cladosporium, and Drechslera) implicated in child asthma exacerbations were previously classified as Deuteromycetes but are now classified as either Ascomycetes or Basidiomycetes.

Exposure misclassification is a major limitation because the assessment of exposure to outdoor fungi was extrapolated from a single site. We cannot be certain that each child was exposed to the same levels counted at this single site and it is therefore impossible to gauge the generalizability of exposure. Levels of ambient fungi vary according to vegetation types and climate variations and there are no validated models currently available that enable us to assess the generalizability of exposure at outdoor and indoor levels. However, misclassification of fungal exposure is likely to be nondifferential; hence, it should bias the risk estimates toward null. Exposure assessment may also be limited by the absence of data on simultaneous indoor exposure to fungal spores, which can independently contribute to asthma exacerbation.\textsuperscript{8,17} Indoor fungal spores constitute these produced within the indoor environment plus spores that enter the home through building openings. Previous research in homes in a nearby region found that spore counts were consistently higher outdoors during the warmer months, were higher indoors during the cold winter months, and were highly correlated.\textsuperscript{19} To overcome potential confounding from outdoor fungi that moved indoors, we controlled for the climatic variables that most influence fungal spore production (maximum temperature and relative humidity). However, because we could not account for household conditions in relation to existing damp or mold, some asthma exacerbations attributed to outdoor fungal spores may be overestimated or the contribution of indoor spores to asthma exacerbations may be underestimated.

Our skin prick testing was limited to the 2 fungal taxa commonly associated with allergic asthma, Alternaria and Cladosporium, so we cannot be certain whether fungal sensitization to taxa other than these may be an important effect modifier.

**Clinical implications**

Identifying sensitization to multiple fungal allergens in children with asthma may help to guide improved asthma management. These findings may also contribute to informing future clinical trials of fungal immunotherapy of childhood asthma. Daily monitoring and reporting of high outdoor fungal spore levels could help reduce the risk of asthma exacerbations in high-risk children particularly if exposure reduction strategies are incorporated in their asthma management plans.

**Conclusions**

Children and adolescents with asthma exposed to ambient Alternaria, Leptosphaeria, Coprinus, and Drechslera in Melbourne, Australia, are at increased risk of being hospitalized with asthma independent of having HRV infection. Alternaria sensitization, and pollen and air pollution exposure. There are also associations with Alternaria, Cladosporium, Coprinus, Drechslera, Stemphylium, and Periconia over a range of lag periods before being hospitalized. The evidence of associations with Leptosphaeria, Coprinus, Drechslera, Stemphylium, and Periconia is new for this geographic region because these taxa have not been investigated before. Detecting sensitization to multiple fungal allergens may be important for asthma management. We need further studies to better understand the role of ambient fungi, fungal sensitization, and cross-reactivity in the causal pathway of asthma exacerbations from early childhood through to adolescence. This may permit identification of high-risk groups, support development of a public health warning system when relevant fungal spore counts are high, and support the design and implementation of more effective strategies to prevent asthma exacerbations.

HRV identification was performed by the Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia.
FIG E1. Maximum temperature and rainfall in Melbourne during the period September 2009 to December 2011.
<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>Cladosporium</th>
<th>Leptosphaeria</th>
<th>Alternaria</th>
<th>Smuts</th>
<th>Coprinus</th>
<th>Drechlera</th>
<th>Periconia</th>
<th>Pleospora</th>
<th>Ganoderma</th>
<th>Pithomyces</th>
<th>Stemphylium</th>
<th>Sporormiella</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cladosporium</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
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</tr>
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<td>Alternaria</td>
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<td>0.1307</td>
<td>0.28</td>
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<tr>
<td>Coprinus</td>
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<td>0.4086</td>
<td>0.2686</td>
<td>0.0653</td>
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<td>Drechlera</td>
<td>0.4594</td>
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<td>0.4411</td>
<td>0.2147</td>
<td>0.2685</td>
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</tr>
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<td>0.2156</td>
<td>0.2857</td>
<td>0.2123</td>
<td>0.212</td>
<td>0.3019</td>
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<td>Pleospora</td>
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<td>0.1663</td>
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<td>0.2619</td>
<td>0.1448</td>
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<tr>
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<td>−0.0251</td>
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<td>0.1044</td>
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</tr>
<tr>
<td>Pithomyces</td>
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<td>0.2014</td>
<td>0.219</td>
<td>0.0878</td>
<td>0.1862</td>
<td>0.2384</td>
<td>0.1753</td>
<td>0.1225</td>
<td>0.0336</td>
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<td></td>
</tr>
<tr>
<td>Stemphylium</td>
<td>0.3159</td>
<td>0.177</td>
<td>0.2912</td>
<td>0.0855</td>
<td>0.1471</td>
<td>0.2841</td>
<td>0.258</td>
<td>0.1557</td>
<td>0.022</td>
<td>0.1125</td>
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<td>Sporormiella</td>
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<td>0.2054</td>
<td>0.1307</td>
<td>−0.0529</td>
<td>0.1759</td>
<td>0.1255</td>
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<td>0.0902</td>
<td>0.1453</td>
<td>0.0959</td>
<td>0.1641</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total spores</td>
<td>0.8286</td>
<td>0.6258</td>
<td>0.6652</td>
<td>0.4044</td>
<td>0.4952</td>
<td>0.5567</td>
<td>0.4128</td>
<td>0.38</td>
<td>0.1856</td>
<td>0.2392</td>
<td>0.3571</td>
<td>0.1978</td>
<td>1</td>
</tr>
</tbody>
</table>

Correlations of fungal spore taxa and P values.
TABLE E2. Adjusted associations between fungal taxa and asthma hospitalizations stratified by HRV infection status (yes/no) and with HRV infection status as an interaction term

<table>
<thead>
<tr>
<th>Fungal spore species</th>
<th>HRV infection present (n = 642), aOR (95% CI)*</th>
<th>P interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (n = 195)</td>
<td>Yes (n = 447)</td>
</tr>
<tr>
<td>Cladosporium</td>
<td>0.98 (0.91-1.06)</td>
<td>1.00 (0.97-1.23)</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>1.03 (0.97-1.10)</td>
<td>1.06 (1.01-1.11)†</td>
</tr>
<tr>
<td>Alternaria</td>
<td>1.05 (0.98-1.12)</td>
<td>1.12 (1.06-1.19)†</td>
</tr>
<tr>
<td>Senata</td>
<td>1.01 (0.97-1.06)</td>
<td>0.96 (0.92-1.01)</td>
</tr>
<tr>
<td>Capniruss</td>
<td>1.04 (0.98-1.10)</td>
<td>1.08 (0.95-1.23)</td>
</tr>
<tr>
<td>Drechslera</td>
<td>1.02 (0.97-1.06)</td>
<td>1.02 (0.97-1.07)</td>
</tr>
<tr>
<td>Pericona</td>
<td>0.92 (0.87-1.07)</td>
<td>1.03 (0.92-1.16)</td>
</tr>
<tr>
<td>Pilospora</td>
<td>1.01 (0.75-1.37)</td>
<td>1.06 (0.90-1.25)</td>
</tr>
<tr>
<td>Ganserdorma‡</td>
<td>0.82 (0.63-1.00)</td>
<td>1.07 (0.84-1.34)</td>
</tr>
<tr>
<td>Pithomyces</td>
<td>1.05 (0.97-1.14)</td>
<td>0.91 (0.77-1.07)</td>
</tr>
<tr>
<td>Stemplyum</td>
<td>0.86 (0.7-1.06)</td>
<td>1.13 (0.92-1.4)</td>
</tr>
<tr>
<td>Spomomila‡</td>
<td>0.79 (0.52-1.21)</td>
<td>1.35 (1.06-1.74)†</td>
</tr>
<tr>
<td>Total spores</td>
<td>1.01 (0.92-1.11)</td>
<td>1.08 (0.96-1.2)</td>
</tr>
</tbody>
</table>

aOR per increase in fungal spores from 75th to 90th percentile. Statistically significant results are in boldface.

*Adjusted for relative humidity, maximum temperature, and grass pollen.
† P < .001.
‡aOR for unit increase per fungal spore when the change from 75th to 90th percentile is 0.
### TABLE E3. Adjusted multifungal models

<table>
<thead>
<tr>
<th>Multifungal models*</th>
<th>OR (95% CI) (n = 644)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria</td>
<td>1.06 (1.02-1.11)</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>1.04 (1.01-1.07)</td>
</tr>
<tr>
<td>Alternaria</td>
<td>1.06 (1.02-1.12)</td>
</tr>
<tr>
<td>Coprinus</td>
<td>1.01 (0.97-1.06)</td>
</tr>
<tr>
<td>Alternaria</td>
<td>1.09 (1.03-1.16)</td>
</tr>
<tr>
<td>Drechslea</td>
<td>0.99 (0.95-1.03)</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>1.04 (1.01-1.07)</td>
</tr>
<tr>
<td>Coprinus</td>
<td>1.03 (0.99-1.07)</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>1.04 (1.01-1.07)</td>
</tr>
<tr>
<td>Drechslea</td>
<td>1.02 (0.99-1.05)</td>
</tr>
<tr>
<td>Coprinus</td>
<td>1.03 (0.99-1.07)</td>
</tr>
<tr>
<td>Drechslea</td>
<td>1.02 (0.99-1.05)</td>
</tr>
</tbody>
</table>

*OR per increase in fungi spores from 75th to 90th percentile. Statistically significant results are in boldface.

*Adjusted for RSV infection status, maximum temperature, relative humidity, and grass pollen.

1 P < .05
2 P < .001
6.5.1 Editors’ Choice, The Journal of Allergy and Clinical Immunology, April 2017

Burden of poor health conditions in adults with primary immunodeficiencies

Most children with primary immunodeficiencies (PIDs) now reach adulthood, and assessment of their long-term health status has become a major challenge. Bardina et al (p 1275) report the results of a multicenter prospective follow-up program addressing the physical health condition of patients with PIDs who reached adulthood and their quality of life. The study’s major findings are as follows:

- Among the 894 participants, 12% were adults, and 58 had undergone transplantation, with a mean age at study participation of 27 years.
- All but 12% of adults with PIDs had experienced a severe (grade 5) or life-threatening (grade 6) condition. Overall, 7.6% of the patients reported rehospitalization.
- Adults scored significantly lower for all domains of quality of life, which was strongly associated with the burden of health conditions.

Distinct natural killer cell subsets in the human lung revealed

Relatively little is known about the phenotype and function of lung natural killer (NK) cells. Analyzing tumor-free lung tissue from a total of 332 patients with lung cancer, Marquardt et al (p 1322) investigated lung NK cell differentiation, killer cell immunoglobulin-like receptor expression (KIR), and function.

- The study shows that pulmonary NK cells are composed of a major population of circulating CD56dimCD16+ NK cells and a minor subset of tissue-resident CD56brightCD16- NK cells.
- The CD56brightCD16- lung NK cell population expressed more KIR and was more sensitized compared to corresponding peripheral blood NK cells.
- The total human lung NK cell population was found to be markedly hyporesponsive to target cell stimulation.

Further detection of tissue-resident lung NK cells will likely provide insight into their role in lung disease, as well as potential use in targeted immunotherapies against human lung cancer.

Genetic dysregulation of nucleotide signaling contributes to asthma risk

Genome-wide association studies test single nucleotide polymorphisms (SNPs) for association with disease risk, typically considering 1 SNP at a time. Although successful, in some situations the power of this approach could be improved. In this issue, Ferrer et al (p 1149)

- Developed a gene-based approach (called EUGENE) that improves power over the standard SNP-based analysis when (a) the expression of a gene contributes to disease risk and (b) gene expression is regulated by multiple independent SNPs.
- Applied EUGENE to 2 asthma genome-wide association studies and identified 4 previously missed risk genes (H4HA12F3, C500G, P2RY13, and P2RY4) involved in nucleotide signaling, and
- Found that agonists of P2RY13 and P2RY4 induces IL-33 release and mast cell degranulation in mouse tissue.

These findings establish an association between asthma risk and genes involved in nucleotide signaling and demonstrate that activation of P2RY13 and P2RY4 can induce release of the Th2-inducing cytokine IL-33.
To transplant or not to transplant in patients with X-linked hyper-IgM syndrome

X-linked hyper-IgM (XHIGM) syndrome is a rare primary immunodeficiency caused by mutations in the gene encoding CD40 ligand. The disease can be life-limiting, but hematopoietic cell transplantation (HCT) is considered curative but may not be available for all patients. In this issue, de la Maza et al. (p. 1282) compiled the largest international series of 176 patients with XHIGM. The study was designed with statistical power to identify a difference in overall survival between transplanted and nontransplanted patients. In addition, Karasik’s Luminex scoring to assess current standards of care is described.

Key findings included the following:

- The life expectancy for patients with XHIGM is guarded, with a median survival of 25 years after diagnosis.
- No difference in survival was observed between patients treated with or without HCT from time of diagnosis for all years (1996-2013).
- A statistical survival benefit for the group undergoing transplantation was noted in the late 1990s, suggesting improvement in transplantation practice for contemporary cohorts.
- Survivors treated with transplantation had a better overall well-being, as measured by using Luminex/Lamy scores.

Innovation for trials in allergy: The GA³LEN chamber

Exposure to allergens, such as pollen, varies not only from day to day, region to region, and between indoor and outdoor activities but also in some cases from block to block in a city. This makes standardization of multicenter field trials virtually impossible. Trials in pollen exposure chambers have been used to overcome this, but they do not allow multicenter trials. In this issue, Zabrodets et al. (p. 1158) described a new chamber developed by GA³LEN, (Global Allergy and Asthma European Network), an international research consortium founded by the European Union with the following characteristics:

- Two standardized containerized chambers. They can be assembled or disassembled in less than 4 hours and quickly transported by truck or rail.
- The chamber has a novel allergen-delivering system and unique laminar airflow that allows exposure of up to 9 study subjects with placebo and serum individually in the same session.

Outdoor fungi associated with child and adolescent asthma hospitalization

Although some outdoor fungi can be associated with asthma hospitalization, little is known about potential interactions with human rhinovirus (HRV) respiratory tract infections or fungal sensitization. In this issue, Than et al. (p. 1149) investigated whether outdoor fungi were associated with child and adolescent asthma hospitalizations while accounting for individual fungal sensitization and the presence of HRV infection. Key findings included the following:

- Seasonality of outdoor fungi correlated with asthma hospitalizations.
- Four allergen-specific outdoor fungi (Alternaria, Leptosphaeria, Ophiostoma, and Drechslera) were associated with hospitalization independent of HRV infection.
- Delayed effects were found for fungal spore exposure up to 3 days before hospitalization.
- Associations with Alternaria, Ophiostoma, and Drechslera species were stronger in those sensitized to Cladosporium species. This might be related to cross-reactivity between fungal species.

This research provides increasing evidence that outdoor fungi can contribute to asthma hospitalization. Investigation of their role in conjunction with other environmental factors requires closer attention.
6.5.2 “Latest Research” section of American Academy of Allergy, Asthma and Immunology (AAAAI) website

OUTDOOR FUNGI ARE ASSOCIATED WITH CHILDHOOD ASTHMA HOSPITAL ADMISSIONS

Published Online: September 16, 2016

Fungal spores are ever-present in outdoor air, but the types and levels of different species vary depending on the geographic location and weather conditions. Little is known about the effects that outdoor fungal spores have on asthmatic children and adolescents. Some studies have suggested that outdoor fungal spore species may be associated with child and adolescent asthma hospitalizations. Fungal allergy may contribute to this effect but it is unclear if this is the major risk factor or if there may be interaction with other factors such as viral infection. Few studies have examined the effect of outdoor fungi and asthma admissions at an individual level and none have investigated this effect among children who are infected with human rhinovirus (HRV) which causes the common cold and is also strongly associated with asthma admissions.

In a research paper recently published in The Journal of Allergy & Clinical Immunology (JACI), Tham and colleagues examined whether outdoor fungal spores were associated with asthma hospitalizations in children and adolescents while accounting for individual fungal sensitivity and whether HRV infection was present at the time they were admitted to hospital. They examined data from 644 children and adolescents who were hospitalized for asthma and participated in the Melbourne Air Pollen Children and Adolescent Health (MAPCAH) study in Australia. Detailed information about allergies and respiratory infections in participants and daily outdoor fungal spores (classified into 14 major types), grass pollen counts and air pollution during the study periods was obtained. They examined associations between outdoor fungal spores and asthma hospitalizations while accounting for allergies, HRV respiratory infection, and levels of grass pollen and air pollution, on the same day and up to three days prior.

The authors found that four allergenic species of outdoor fungal spores (Alternaria, Leptosphaeria, Coprinus and Drechslera) were associated with asthma hospital admission independent of having HRV infection. Some effects were also found for fungal spore exposure
up to three days prior to admission. Associations with Alternaria, Coprinus and Drechslera were stronger in those who were allergic to Cladosporium which may be related to cross-reactivity between fungal species. These are new findings for this region.

Globally, asthma remains a significant public health issue and the outdoor environment is a major contributor to its aetiology. This research provides increasing evidence that outdoor fungal spores contribute to asthma hospital admissions. Investigation of their role in conjunction with other environmental factors requires greater attention.

*The Journal of Allergy and Clinical Immunology (JACI)* is an official scientific journal of the AAAAI, and is the most-cited journal in the field of allergy and clinical immunology.

**ADDITIONAL INFORMATION**

- **ASTHMA SYMPTOMS, DIAGNOSIS, TREATMENT & MANAGEMENT »**
Chapter 7

Is ambient exposure to outdoor fungal spores associated with lower lung function or airway inflammation in a high risk allergy cohort?

7.1 Abstract

Decreased lung function and airway inflammation may be clinical or preclinical markers of asthma or asthma exacerbation. Despite the ubiquitous presence of outdoor fungal spores in the air we breathe, few studies have examined whether exposure to outdoor fungal spores is associated with changes in lung function or airway inflammation. In this chapter I aimed to examine whether there were any associations between short term exposure to outdoor fungal spores and lung function and airway inflammation in a cohort of children, adolescents and adults at high risk of allergic disease. In addition I aimed to explore if any associations were modified by fungal sensitisation status or age group.

The study population was comprised of participants in the Melbourne Atopy Cohort Study 18 year follow-up, during the period from September 2009 to December 2011. At the follow-up all participants (probands, siblings and parents) were invited to undergo clinical testing which included skin prick testing for Alternaria, Cladosporium and Penicillium sensitisation. They had spirometry to assess lung function, and Fractional exhaled Nitric Oxide (FeNO) testing to assess whether there were markers of airway inflammation. Probands were invited to provide an Exhaled Breath Condensate sample for measurement of other markers of airway inflammation (EBC NOx and EBC pH).

Environmental exposure data used in the MAPCAH study (Chapter 6) were incorporated into this study: daily fungal spore counts, pollen grain counts, meteorological variables, and air pollutants. Generalised linear models assessed the effect of individual fungal spore taxa on lung function, levels of nitric oxide in FeNO readings, and airway acidification (pH) from EBC readings. Ordinal logistic regression was used to assess effect of the fungal spore taxa on higher levels of NOx in EBC readings. Models were adjusted for age, sex, height, relative humidity, maximum temperature, grass pollen and
PM$_{2.5}$, EBC NOx and EBC pH analyses were also adjusted for storage time to control for possible degradation of the samples. Alternaria, Cladosporium and/or Penicillium sensitisation; age group (≤18 years and >18 years; ≤25 years and >25 years) and sex were tested as interaction terms. Results are reported as coefficient change (generalised linear models) or odds ratios (ordinal logistic regression) per increase from the 75$^{th}$ to 90$^{th}$ percentile of the individual fungal spore counts/m$^3$.

The results were extensive and mixed so only the key results are described in this abstract. In the full sample (n=793) higher ambient levels of Ustilago/smuts at lag 0, lag 2 and cumulative 3-day lag; Drechslera at lag 2; and Pithomyces at lag 0 were associated with lower FEV$_1$, FVC and FEF$_{25-75}$. Ganoderma at lag 0 and lag 1 was associated with lower FVC. Leptosphaeria was associated with lower FEF$_{25-75}$. Ganoderma at lag 0 and lag 1 was associated with lower FVC. Leptosphaeria was associated with lower FEF$_{25-75}$. These associations were stronger than those observed in non-sensitised individuals (all p-values for interaction <0.05).

No associations were found between any fungal spore taxa and levels of NO in FeNO measurements. However, Ganoderma was associated with increased EBC acidification (lower pH) at lag 0 (coeff=-0.1, 95%CI=-0.2 to -0.01), and lag3 (coeff=-0.09 95%CI -0.16 to -0.01). The odds of higher levels of EBC NOx was associated with Cladosporium at lag 1 (OR= 1.4, 95%CI 1.1 to 1.6); Leptosphaeria at lag 2 (OR= 1.4, 95%CI 1.1 to 1.9); Alternaria at lag 1 (OR= 1.9 95%CI 1.2 to 2.9); and Ganoderma at lag 0 (OR=2.1, 95%CI 1.2 to 3.7).

In conclusion, these findings indicated that exposure to some outdoor fungal spores may be associated with lower lung function, and increased risk of higher EBC NOx, a marker of airway inflammation. Further research utilising longitudinal examination of larger samples in different geographic regions is required to establish if these results can be replicated, and to identify if there are threshold effects of outdoor fungal spores.
7.2 Introduction

Decreased lung function and increased airway inflammation may be clinical or pre-clinical markers of active asthma or asthma exacerbation. As long-term impaired lung function can lead to chronic lung conditions such as asthma it is important to assess whether exposure to outdoor fungal spores is associated with increased airway inflammation and/or reduced lung function.

Many outdoor fungal spores and their associated fragments and hyphae have median aerodynamic diameters of less than 10µm, which, when inhaled, may lodge in different parts of the respiratory tract depending on their size (212). Inhalation of fungal spores, fragments and hyphae may result in adverse respiratory health effects in predisposed individuals. A number of subpopulations who may be susceptible to fungal spores include children, as their respiratory system is developing; people who are at high risk of allergic diseases; people with pre-existing respiratory conditions that can be aggravated by exposure to further irritants; and immunocompromised people who are susceptible to infection. The mechanisms by which outdoor fungal spores or hyphal fragments may cause or aggravate respiratory conditions include localised allergic reactions sometimes with subsequent development of specific immunoglobulin E (IgE) to fungal antigens; inflammatory irritation of the respiratory mucosa by the release of mycotoxins; or infection of the respiratory system (2, 69).

A number of epidemiological studies have demonstrated associations between outdoor fungal spores and respiratory conditions such as exacerbations of allergic asthma and severe asthma (4, 276), allergic bronchopulmonary mycoses (33), and hypersensitivity pneumonitis (2). However, not all respiratory conditions will express themselves with acute clinical episodes. Fungal spore exposure may lead to short-term impaired lung function or airway inflammation.

Upper respiratory tract symptoms, cough, wheeze, and asthma exacerbations (276) in relation to indoor dampness, visible mould and mould odour are well documented (276, 277). There is some evidence that short and long term exposure to indoor damp and mould odour are associated with reduced lung function in healthy adults (64, 278) and asthmatic adults (9). However, relatively little is known about whether there is an association between exposure to outdoor fungal spores and lung function. Two recent studies of children aged 10-12 years found associations between outdoor fungal spores
and reduced lung function in children. In Chen et al’s longitudinal study of 100 school children tested 5 to 10 times over two years, Cladosporium levels above 1500 spores/m³ were associated with significantly reduced forced vital capacity (FVC) and forced expiratory volume in one second (FEV₁) at Lag 1 (60). In Watanabe et al’s panel study of 345 children assessed daily during one month, increases in total fungal spore counts during were significantly associated with reduced morning peak expiratory flow rate (PEF) (-1.18L/min) and this reduction was higher in asthmatic children (-1.45L/min) (61). Panel studies of asthmatic subjects (children, adolescents and adults) have reported some associations between outdoor fungal spore counts and decline in individual lung function over the study periods (66, 279) and conversely, associations with increased lung function (280). These studies indicated that there was increased risk of lung function decline when exposed to higher levels of fungal spores in the older age groups compared to younger age groups, however, none of these studies have investigated fungal sensitisation as an effect modifier.

Asthma and aeroallergen sensitisation are associated with markers of airway inflammation, particularly fractional exhaled nitric oxide (FeNO) (258, 280, 282). To date there have been no published studies that have reported on outdoor fungal spore exposure and markers of airway inflammation.

In this chapter my aim was to investigate the association between daily levels of outdoor fungal spores and lung function and markers of airway inflammation in a cohort of children, adolescents and adults at high risk of allergic disease; and whether any associations were modified by fungal sensitisation or age group.
7.3 Methods

The methods I describe here are specific to this chapter and add to the methods of assessing environmental exposures that I described in Chapter 4.

7.3.1 Study population and time period

The Melbourne Atopic Cohort Study (MACS) is a longitudinal, birth cohort study comprising 620 babies enrolled prior to birth by recruiting pregnant women living in Melbourne, Australia. MACS was established as a randomized controlled trial investigating the effect of infant milk formulas on allergic outcomes (283). Eligible babies had at least one first-degree family member with a history of eczema and/or asthma and/or allergic rhinitis and/or severe food allergy. The 620 probands were born between 24 March 1990 and 1 November 1994. These babies (probands) were followed up every 4 weeks from birth to 15 months; at 18 months; annually from 2 to 7 years; at 12 years; and at 18 years.

The analyses reported in this chapter are based on the 18 year follow-up of the MACS cohort which was conducted from September 2009 to December 2011. In this follow-up entire families, which included the probands, and their siblings and parents, were enrolled in the study and contributed information to these analyses. I chose to include the non-probands (siblings and parents) so that I could include and compare children, adolescents and adults; and to increase the sample size and statistical power of the analyses.

At this follow-up stage all participants were invited to undergo clinical testing which included spirometry and Fractional exhaled Nitric Oxide (FeNO) testing and skin prick tests (SPT) for 12 common allergens. Only probands were asked to provide an Exhaled Breath Condensate (EBC) sample for measurement of other markers of airway inflammation.

The MACS 18 year follow up questionnaire and the MACS procedure for lung function and airway inflammation testing are included in the Appendices.
7.3.2 Primary exposure: Ambient levels of fungal spores
I was granted access to daily ambient fungal spore and pollen counts that were measured and identified at The University of Melbourne, Parkville during the period September 2009 to December 2011 for the Melbourne Air Pollen Children and Adolescent Health (MAPCAH) study (Chapter 6). The methods for obtaining these data were described in Chapter 4. As outdoor fungal spore data were not routinely collected for the full period of the MACS clinical testing, my time analyses are limited to those participants tested between these dates.

7.3.3 Outdoor air quality, pollen and weather data
Air pollutants, extreme weather conditions and grass pollen may act as confounding variables. The methods for obtaining these data were described in Chapter 4.

In relation to the pollens, although in Melbourne it has been found that Cupressaceae (conifer) pollen can be more abundant than Poaceae (grass) pollen (273), in this analysis I considered only the grass pollen grains as they have been identified as a significant trigger for respiratory admissions in Melbourne (30, 31).

7.3.4 Age groups
Participants were stratified into two categories of age groups:

(1) ≤ 25 years and >25 years as, on average, lung function peaks at around 25 years of age. I selected this cut-off point as there is a decline in FEV₁ after 25-30 years of age (125);

(2) Participants were also stratified into ≤ 18 years and >18 years to enable some qualitative comparison with the findings from the other sub-studies in my PhD research which examined children and adolescents aged 2 to 18 years.
7.3.5 Fungal sensitisation
A trained research assistant tested fungal sensitisation with a skin prick test (SPT). They applied a single drop of allergen on the participant’s back, and the skin was pricked with a lancet. Histamine (1mg/mL) was used as a positive control and saline as a negative control. The SPTs were assessed at 10-15 minutes and wheal diameters were measured. Fungal sensitisation (atopy) was defined as a positive SPT with a mean wheal diameter of 3mm or greater to the fungal allergens *Alternaria*, *Penicillium* or *Hormodendrum* (synonym = *Cladosporium*) *cladosporoides* (Hollister-Stier, Spokane, WA, USA; Alyostal, Antony, France).

7.3.6 Outcomes

7.3.6.1 Lung function – Spirometry
Spirometry was performed according to standardized American Thoracic Society [ATS]/European Respiratory Society [ERS] guidelines for spirometric techniques (284) by trained respiratory scientists using the EasyOne Spirometer system (ndd Medical Technologies Inc., Andover, MA). Calibration checking was performed daily using a 3-litre syringe, and biological controls were used. Short-acting β-agonists (by metered dose inhaler) were withheld for 4 hours and long-acting bronchodilators for 12 hours before the test. Height was recorded at the time of testing, without shoes, to the nearest 0.1 cm. (Appendices 7 and 8)

There was a two stage method of checking for study inclusion. Initially, flow-volume loops were marked for inclusion/exclusion by respiratory scientists. Each test was reviewed by Dr Caroline Lodge for flow-volume loop selection and inclusion/exclusion. ATS/ERS 2005 criteria were used for test acceptance (283).

Lung function indices were described in detail in Chapter 2 (page 15). The key indices recorded and used in this study were all measured prior to the use of any bronchodilators (that is, pre-bronchodilator) (Figure 7.1):
Figure 7.1: Flow-volume loop

Forced Expiratory Flow at one second (FEV1) – the volume expired in the first second of maximal expiration after maximal inspiration. This is a useful measure of how quickly full lungs can be emptied.

Forced Vital Capacity (FVC) – the maximum volume of air exhaled or inspired during a maximally forced manoeuvre.

Mid Expiratory Flow (FEF25%-75%) – average expired flow over the middle half of the FVC manoeuvre. This provides a measure of small airways narrowing but may be difficult to interpret if FVC is reduced or increased.
7.3.6.2 Fractional exhaled Nitric Oxide (FeNO)

Exhaled air nitric oxide (NO) was measured (in parts per million [ppm]) using the Medisoft HypAir FeNO machine (Figure 7.2), an offline testing unit that uses a fuel cell based technology. Before each participant was tested the machine was turned on and atmospheric NO (ppm) was measured. Each participant was questioned about the timing of their last meal, exercise session, cigarette smoked, and inhalation of bronchodilator (in hours). The flow rate for the HypAir was set at 50ml/second. The mouthpiece was then inserted into the participant’s mouth and they were asked to breathe out maximally while sealing their mouth on the mouthpiece, then inhale NO free air via a NO scrubber, and then exhale for the sample collection. Values obtained for each blow were recorded and the test repeated until at least two acceptable blows with a FeNO value within 10% were obtained. (Appendices 9 and 10)

Acceptability of the test was assessed by examining whether the flow curve was in the target expiratory flow range of 40 to 60ml/second over 3 to 10 seconds; and the recorded confidence (%) that the NO analyser was working correctly.

Figure 7.2: Medisoft HYPAIR FeNO machine
7.3.6.3 Exhaled Breath Condensate (EBC)

The methodology for this section was kindly provided by University of Melbourne PhD Candidate, Fahad Aldakheel. Fahad undertook the analysis of the EBC as part of his PhD research during 2013 to 2016 (285).

7.3.6.3.1 Sample collection

EBC samples were collected following ATS/ERS guidelines (132, 286) using a glass-condensing chamber in wet ice (Figure 7.3). Participants were required to rinse their mouths with tap water prior to the collection of their EBC and were then asked to inhale deeply via their nose and exhale through their mouth via a mouthpiece with a one-way valve into curved glass tubing that was connected to a condenser container (thermal flask). A saliva trap was in place to avoid saliva contamination of the sample. The exhaled air droplets were converted to EBC in a test tube located inside the condensing chamber that was chilled by the wet ice. Each sampling session took approximately 10 to 20 minutes to obtain 0.4 to 3.0 mL of EBC.

Figure 7.3: Condenser device for the collection of exhaled breath condensate sample. (Image provided by Fahad Aldakheel)
7.3.6.3.2 Sample processing and storage

Each sample was divided into 120µL aliquots placed in Eppendorf tubes (number of sub-samples ranged from 5-12). The sub-samples were then de-aerated with argon gas for 20 seconds, and then immediately frozen and stored at -80 °C at the Genetic Epidemiology Laboratory (GEL), Department of Pathology, at The University of Melbourne. Prior to analysis, EBC samples were thawed at room temperature.

7.3.6.3.3 Laboratory analysis of EBC biomarkers

The EBC biomarkers were analysed and measured by Fahad Aldakheel. Fahad was blinded to the clinical status of all participants to reduce detection bias.

Measurement of EBC total nitric oxide (NOx) products

The total NOx in each EBC sample was measured using a standardised protocol of enzymatic reduction of nitrate using a fluorimetric modification of the Greiss reaction (spectrophotometric assays). The limit of detection of the NOx assay was 1.25 μMol (micromoles).

Measurement of EBC pH

A portable micro pH-meter (LAQUA Twin pH Meter, Spectrum Technologies, Inc., 3600 Thayer Ct, Aurora, IL 60504, USA) was used to measure pH (levels of hydrogen ions) of the EBC samples (see Figure 5.4). The pH meter was calibrated using standard two-point calibration solutions, PH4 and PH7 buffers, each day and was repeated for every 50 samples tested. Aliquots of 110 µL deaerated EBC samples were tested and the average of two readings was used in data analysis.
7.3.7 Statistical Analysis

Levels of correlations between each fungal spore taxa, meteorological variables, grass pollen and air pollutants were assessed using Spearman’s rank correlation coefficients (Table 7.2). To assess associations between fungal spore exposures and normally distributed, continuous scaled outcomes, I used generalised linear models. This included estimation of associations with lung function outcomes, FeNO readings and EBC pH.

As probands and non-probands from the same family were included in this study, each statistical model that involved the probands and non-probands included a cluster function to accommodate for the related nature of multiple members from the same family.

EBC NOx readings were not normally distributed. There was a strong right-skewed distribution and a large proportion of samples were below the limit of detection. To address this, I stratified these results into none (1.25 µMol), low (≥ 1.26-20 µMol) and high (≥ 20 µMol) and analysed using ordinal logistic regression. Since this model estimates one equation over all levels of the EBC NOx readings, I tested whether this one-equation model was valid using the Brant test of parallel regression assumption to assess if the proportional odds assumption had been violated (287).

I assessed estimated effects of the same day (Lag 0) and lagged fungal spore exposure (up to 3 days: Lags 1, 2, 3 and cumulative 3-day lag). As the fungal spore exposure is a continuous variable, the potential for non-linearity in the fungal spore exposure was explored with fractional polynomials.

7.3.7.1 Assessment of confounding

Lung function outcomes: Maximum temperature, relative humidity and grass pollen were included as a priori confounders in the models as these factors have been shown to be associated with fungal spore production and dispersion (1) and reduced lung function (87). In addition, models were adjusted for air pollutants (PM$_{2.5}$, PM$_{10}$, NO$_2$ and O$_3$) if they changed the estimated associations by >10% or had a p-value of <0.05. As age, sex and height are known to be key determinants of lung function outcomes, I adjusted for these covariates in the models to reduce residual error (125), rather than as true confounding variables.
EBC pH: Models were adjusted for age, sex, height, relative humidity, maximum temperature, storage time (to control for degradation of the sample), PM$_{2.5}$ and grass pollen.

EBC NOx: Models were adjusted for age, sex, height, relative humidity, maximum temperature, storage time (to control for degradation of the sample), PM$_{2.5}$ and grass pollen. The Brant test was used to assess if the proportional odds assumption had been violated.

7.3.7.2 Assessment for possible interactions

I tested *Alternaria, Cladosporium* and *Penicillium* sensitisation status (yes/no for each); age groups and sex as interaction terms to identify possible effect modification. As the statistical power to test for significant interactions was lower than to test for the main effect, I report strata specific associations if the p-value for the interaction was <0.1 to avoid missing any important interactions (288).

All results in the EBC NOx tables are presented as odds ratios (OR) and 95% confidence intervals (CIs). Other outcomes are reported as coefficient change with 95%CIs. The OR and coefficients can be interpreted as the association or coefficient change per increase from the 75th to 90th percentile of the fungal spore counts/m$^3$ calculated for each specific fungal taxa. The exception to this was in models where the change from 75th to 90th percentile was zero. In these models the coefficients or OR are expressed per single spore increase.

All statistical analyses were performed using Stata IC 13.1 (StataCorp, College Station, Texas).

7.3.8 Ethics

Ethics approvals were granted by the University of Melbourne Human Research Ethics Committee.

7.4 Results

7.4.1 Sample characteristics

This study sample was comprised of 936 participants of whom 54.7% were female. Mean age was 31.8 ± 16.9 years with approximately 55% aged 25 years and under and 37%
aged 18 years and under. Of these participants a varying number were tested for a range of lung function parameters and allergic sensitisation (Table 7.1).

Approximately 16% of all participants were sensitised to *Alternaria*, 10% were sensitised to *Cladosporium* and 7% were sensitised to *Penicillium*. With the possible exception of *Penicillium*, sensitisation to fungal allergens appeared to increase with increasing age of participants. Multiple fungal sensitisation levels appear to be higher in mothers than fathers. (Table 7.1)

Summary measures of lung function outcomes were: mean FEV1 was 3456 ml/sec; mean FVC was 4255 ml; and mean FEF25%-75% was 3512 ml.

Summary measures of FeNO were: Mean level of NO in FeNO readings was 43 ppm. Males had significantly higher level of NO (48ppm) in their FeNO readings compared to females (38ppm).

Summary measures of EBC were: Mean level of NOx in EBC was 16.5 µM. Females had higher EBC NOx readings than males but the differences were not significantly different. Mean EBC pH was 6.4 and there was no significant difference between males and females.
<table>
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<tr>
<th>Characteristic</th>
<th>All (N=936)</th>
<th>Proband (N=264)</th>
<th>Mother (N=238)</th>
<th>Father (N=160)</th>
<th>Sibling (N=274)</th>
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<td>N (%)</td>
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<td>264 (28.2)</td>
<td>238 (25.4)</td>
<td>160 (17.1)</td>
<td>274 (29.3)</td>
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<td>160</td>
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<td>Female n (%)</td>
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<td>137</td>
<td>238</td>
<td>-</td>
<td>137</td>
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<tr>
<td>Female (%)</td>
<td>(54.7%)</td>
<td>(51.9%)</td>
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<td>Age yrs ± SD (n=934)</td>
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<td>49.8 ± 4.1</td>
<td>52.2 ± 5.1</td>
<td>17.9 ± 5.9</td>
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<td>-</td>
<td>237 (57%)</td>
<td>159 (38%)</td>
<td>21 (5%)</td>
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<td>≤ 18 yrs</td>
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<td>-</td>
<td>-</td>
<td>161 (46%)</td>
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<td>164.2 ± 6.0</td>
<td>177.7 ± 11.8</td>
<td>167.6 ± 11.8</td>
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<td>Lung function test n (%)</td>
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<td>262 (30%)</td>
<td>218 (25%)</td>
<td>146 (17%)</td>
<td>244 (28%)</td>
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<td>FENO test n (%)</td>
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<td>222 (28%)</td>
<td>205 (26%)</td>
<td>139 (17%)</td>
<td>231 (29%)</td>
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<td>EBC test n (%)</td>
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<td>Alternaria + n (%)</td>
<td>147 (16%)</td>
<td>25 (10%)</td>
<td>47 (20%)</td>
<td>30 (19%)</td>
<td>45 (17%)</td>
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<tr>
<td>Penicillium + n (%)</td>
<td>61 (6.7%)</td>
<td>10 (4%)</td>
<td>17 (7%)</td>
<td>16 (6%)</td>
<td>18 (7%)</td>
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<td>Cladosporium + n (%)</td>
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<td>19 (8%)</td>
<td>20 (9%)</td>
<td>20 (14%)</td>
<td>27 (11%)</td>
</tr>
<tr>
<td>≥ 1 fungal reagent</td>
<td>191 (21%)</td>
<td>34 (18%)</td>
<td>58 (30%)</td>
<td>38 (20%)</td>
<td>61 (32%)</td>
</tr>
</tbody>
</table>

*Not all participants underwent skin prick testing.
 Alternaria SPT n=905; probands n=256; mother n=232; father n=157; siblings n=260
 Penicillium SPT n=907; probands n=257; mother n=233; father n=157; siblings n=260
 Cladosporium SPT n= 837 probands n=240; mother n=213; father n=142; siblings n=242
7.4.2 Fungal spore distribution

As I have used the same fungal spore data in Chapter 6, in this section I will provide a brief summary of these data and links to the previous tables.

The most prevalent fungi taxa detected were *Cladosporium* (44%), *Leptosphaeria* (14%) and *Alternaria* and *Ustilago/smutts* (11% each) of the total fungi spore count (page 97). The remaining fungal taxa accounted for 1-7% each. No data were provided on ‘other’ or ‘not-identifiable’ fungal spores. Most fungi taxa were low to moderately positively correlated in this study except for *Ganoderma* and *Sporormiella* (page 97). Most fungi taxa were low to moderately positively correlated to grass pollen. (Table 7.2)

7.4.3 Associations between fungal spore taxa and lung function outcomes

A range of fungal taxa were found to be associated with changes in lung function parameters.

7.4.3.1 Forced Expiratory Flow at one second (FEV₁)

*Ustilago/smutts* at Lag 0, Lag 2 and cumulative 3-day lag; *Drechslera* at Lag 2 and cumulative 3-day lag; and *Pithomyces* at Lag 0 were associated with reduced FEV₁ (Table 7.3). *Leptosphaeria* at Lag 1; *Alternaria* at Lag 3; *Pleospora* Lag 0; *Stemphylium* at Lag 1, Lag2 and cumulative 3-day lag; and *Sporormiella* at Lag 2 were associated with increased FEV₁ (Table 7.3).
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<tr>
<th></th>
<th>Alt</th>
<th>Clad</th>
<th>Gano</th>
<th>Lepto</th>
<th>Pleo</th>
<th>Spor</th>
<th>Pith</th>
<th>Smuts</th>
<th>Cop</th>
<th>Drech</th>
<th>Stem</th>
<th>Peri</th>
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<th>Grass pollen</th>
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<tr>
<td>Cladosporium</td>
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<td>Ganoderma</td>
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<td>Leptosphaeria</td>
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<td>Sporormiella</td>
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<td>Pithomyces</td>
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<td>Ustilago/smutts</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>Periconia</td>
<td>0.286</td>
<td>0.315</td>
<td>0.047</td>
<td>0.216</td>
<td>0.111</td>
<td>0.036</td>
<td>0.175</td>
<td>0.212</td>
<td>0.212</td>
<td>0.302</td>
<td>0.26</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total spores</td>
<td>0.665</td>
<td>0.829</td>
<td>0.186</td>
<td>0.626</td>
<td>0.380</td>
<td>0.198</td>
<td>0.259</td>
<td>0.404</td>
<td>0.495</td>
<td>0.557</td>
<td>0.36</td>
<td>0.413</td>
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<tr>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grass pollen</td>
<td>0.492</td>
<td>0.501</td>
<td>-0.007</td>
<td>0.162</td>
<td>0.056</td>
<td>0.074</td>
<td>0.068</td>
<td>0.290</td>
<td>0.202</td>
<td>0.375</td>
<td>0.19</td>
<td>0.243</td>
<td>0.507</td>
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</tr>
<tr>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td></td>
</tr>
</tbody>
</table>
Table 7.3: Adjusted associations between fungal spore counts and pre-bronchodilator FEV₁.

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>FEV₁ (ml) n=866</th>
<th>Lag 0</th>
<th>Lag 1</th>
<th>Lag 2</th>
<th>Lag 3</th>
<th>Cumulative Lag</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>coeff (95%CI)</td>
<td>coeff (95%CI)</td>
<td>coeff (95%CI)</td>
<td>coeff (95%CI)</td>
<td>coeff (95%CI)</td>
<td></td>
</tr>
<tr>
<td>Cladosporium</td>
<td>12.4 (-16.2, 41.1)</td>
<td>12.6 (-1.8, 27.0)</td>
<td>-8.9 (-31.0, 13.2)</td>
<td>20.7 (-16.3, 57.7)</td>
<td>4.5 (-5.6, 14.7)</td>
<td></td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>10.5 (-6.8, 27.7)</td>
<td>15.0 (5.1, 24.9) *</td>
<td>8.7 (-6.2, 23.7)</td>
<td>-14.3 (-32.9, 4.4)</td>
<td>2.5 (-3.4, 8.5)</td>
<td></td>
</tr>
<tr>
<td>Alternaria</td>
<td>-32.5 (-80.3, 15.3)</td>
<td>16.7 (-34.1, 67.5)</td>
<td>-14.5 (-72.8, 43.7)</td>
<td>31.7 (3.2, 60.2) *</td>
<td>3.9 (-16.7, 24.5)</td>
<td></td>
</tr>
<tr>
<td>Ustilago/smuts</td>
<td>-21.4 (-35.8, -7.0) *</td>
<td>10.7 (-11.5, 32.9)</td>
<td>-16.2 (-23.9, -8.6) **</td>
<td>1.8 (-61.8, 65.5)</td>
<td>-9.0 (-14.0, -4.0) **</td>
<td></td>
</tr>
<tr>
<td>Coprinus</td>
<td>1.2 (-52.2, 54.6)</td>
<td>-16.6 (-60.6, 27.3)</td>
<td>31.7 (2.3, 61.1)</td>
<td>23.0 (-10.0, 56.0)</td>
<td>5.7 (-8.1, 19.6)</td>
<td></td>
</tr>
<tr>
<td>Drechslera</td>
<td>-14.8 (-31.6, 2.1)</td>
<td>4.9 (-8.4, 18.2)</td>
<td>-18.3 (-28.5, -8.0) **</td>
<td>6.2 (-32.5, 44.9)</td>
<td>-7.7 (-14.4, -0.9) *</td>
<td></td>
</tr>
<tr>
<td>Periconia</td>
<td>-35.0 (-71.5, 1.5)</td>
<td>-17.7 (-81.5, 46.2)</td>
<td>-20.8 (-91.7, 50.0)</td>
<td>42.5 (-23.8, 108.9)</td>
<td>-6.2 (-29.2, 16.8)</td>
<td></td>
</tr>
<tr>
<td>Pleospora</td>
<td>97.3 (17.7, 176.8)</td>
<td>23.4 (-6.4, 53.2)</td>
<td>8.4 (-30.1, 46.9)</td>
<td>-10.3 (-53.5, 32.8)</td>
<td>16.4 (-2.7, 35.5)</td>
<td></td>
</tr>
<tr>
<td>Ganoderma</td>
<td>-18.2 (-49.1, 12.6)</td>
<td>-1.1 (-4.4, 2.2)</td>
<td>3.6 (-26.6, 33.7)</td>
<td>10.5 (-60.4, 81.4)</td>
<td>-1.2 (-5.2, 2.8)</td>
<td></td>
</tr>
<tr>
<td>Pithomyces</td>
<td>-39.8 (-67.4, -12.3) *</td>
<td>-12.4 (-45.3, 20.4)</td>
<td>7.4 (-30.7, 45.5)</td>
<td>-2.7 (-50.7, 45.2)</td>
<td>-7.8 (-23.0, 7.3)</td>
<td></td>
</tr>
<tr>
<td>Stemphylium</td>
<td>47.9 (-6.3, 102.2)</td>
<td>76.7 (37.5, 115.8) **</td>
<td>62.7 (12.3, 113.2) *</td>
<td>18.0 (-11.4, 47.4)</td>
<td>40.8 (21.0, 60.7) **</td>
<td></td>
</tr>
<tr>
<td>Sporormiella</td>
<td>20.4 (-24.5, 65.3)</td>
<td>-11.4 (-126.3, 103.6)</td>
<td>125.9 (60.5, 191.3) **</td>
<td>6.5 (-40.8, 53.8)</td>
<td>17.4 (-7.2, 42.1)</td>
<td></td>
</tr>
<tr>
<td>Total spores</td>
<td>-1.0 (-45.7, 43.8)</td>
<td>22.0 (-2.8, 46.7)</td>
<td>-29.6 (-61.9, 2.7)</td>
<td>20.3 (-30.9, 71.6)</td>
<td>-1.5 (-15.9, 12.9)</td>
<td></td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase from the 75th to 90th percentile in the fungal spore counts. *p<0.05 **p<0.001
7.4.3.2 Forced Vital Capacity FVC

*Ustilago* /smuts at Lag 0, Lag 2 and cumulative 3-day lag; *Drechslera* at Lag 2; *Periconia* at Lag 0; *Ganoderma* at Lag 0 and Lag 1; and *Pithomyces* at Lag 0 were associated with reduced FVC (Table 7.4).

*Cladosporium* at Lag 1; *Alternaria* at Lag 3; *Coprinus* at Lag 3; *Stemphylium* at Lag 2 and cumulative 3-day lag; *Sporormiella* at Lag2; and total spores at Lag 1 were associated with increased FVC (Table 7.4).

7.4.3.3 Mid Expiratory Flow FEF₂₅%-₇₅%

*Ustilago* /smuts (Lag 0, Lag 2 and cumulative 3-day lag); *Leptosphaeria* (Lag 3); *Drechslera* (Lag 2 and cumulative 3-day lag); and *Pithomyces* (Lag 0) were associated with reduced FEF₂₅%-₇₅% (Table 7.5).

*Cladosporium* (Lag 0), *Leptosphaeria* (Lag 1), *Pleospora* (Lag 0), *Stemphylium* (Lag1, Lag2 and cumulative 3-day lag) and *Sporormiella* (Lag 2) were associated with increased FEF₂₅%-₇₅% (Table 7.5)
Table 7.4: Adjusted associations between fungal spore counts and pre-bronchodilator FVC.

<table>
<thead>
<tr>
<th>FVC (ml)</th>
<th>Lag 0</th>
<th>Lag 1</th>
<th>Lag 2</th>
<th>Lag 3</th>
<th>Cumulative Lag</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>coeff (95% CI)</td>
<td>coeff (95% CI)</td>
<td>coeff (95% CI)</td>
<td>coeff (95% CI)</td>
<td>coeff (95% CI)</td>
</tr>
<tr>
<td><strong>Cladosporium</strong></td>
<td>10.6 (-21.8, 42.9)</td>
<td>15.5 (1.7, 29.4)*</td>
<td>2.0 (-21.8, 25.9)</td>
<td>30.4 (-11.4, 72.1)</td>
<td>7.1 (-3.1, 17.3)</td>
</tr>
<tr>
<td><strong>Leptosphaeria</strong></td>
<td>5.9 (-14.9, 26.7)</td>
<td>11.3 (-2.2, 24.8)</td>
<td>15.3 (-6.3, 36.9)</td>
<td>-1.4 (-23.5, 20.7)</td>
<td>4.2 (-3.3, 11.8)</td>
</tr>
<tr>
<td><strong>Alternaria</strong></td>
<td>-21.5 (-62.9, 19.9)</td>
<td>29.2 (-20.1, 78.5)</td>
<td>7.3 (-49.5, 64.2)</td>
<td><strong>42.9 (10.5, 75.3)</strong>*</td>
<td>12.7 (12.7, 34.1)</td>
</tr>
<tr>
<td><strong>Ustilago/smuts</strong></td>
<td><strong>-11.0 (-22.0, 0.0)</strong>*</td>
<td>17.2 (-8.5, 42.9)</td>
<td><strong>-8.8 (-14.7, -3.0)</strong>*</td>
<td>29.8 (-45.8, 105.4)</td>
<td><strong>-4.3 (-8.4, -0.2)</strong>*</td>
</tr>
<tr>
<td><strong>Caprinus</strong></td>
<td>-6.5 (-58.9, 45.9)</td>
<td>-0.6 (-37.4, 36.2)</td>
<td>33.5 (-1.3, 68.3)</td>
<td><strong>36.2 (1.3, 71.2)</strong>*</td>
<td>9.9 (-5.4, 25.1)</td>
</tr>
<tr>
<td><strong>Drechslera</strong></td>
<td>-9.4 (-21.4, 2.7)</td>
<td>9.3 (-5.6, 24.3)</td>
<td><strong>-8.8 (-17.3, -0.4)</strong>*</td>
<td>18.5 (-26.0, 63.1)</td>
<td>-3.0 (-8.9, 2.9)</td>
</tr>
<tr>
<td><strong>Periconia</strong></td>
<td><strong>-43.6 (-77.7, -9.4)</strong>*</td>
<td>-37.5 (-119.2, 44.3)</td>
<td>-29.1 (-102.8, 44.7)</td>
<td>30.8 (-48.5, 110.0)</td>
<td>-13.1 (-38.7, 12.5)</td>
</tr>
<tr>
<td><strong>Pleospora</strong></td>
<td>84.4 (-13.5, 182.3)</td>
<td>19.5 (48.7, 0.4)</td>
<td>13.1 (-17.7, 43.8)</td>
<td>15.2 (-29.3, 59.8)</td>
<td>18.5 (-0.2, 37.3)</td>
</tr>
<tr>
<td><strong>Ganoderma</strong></td>
<td><strong>-24.9 (-48.9, -0.9)</strong>*</td>
<td><strong>-4.7 (-8.2, -1.2)</strong>*</td>
<td>-9.0 (-45.2, 27.1)</td>
<td>2.7 (-73.4, 78.8)</td>
<td>-4.1 (-8.5, 0.2)</td>
</tr>
<tr>
<td><strong>Pithomyces</strong></td>
<td><strong>-36.5 (-64.0, -9.1)</strong>*</td>
<td>-11.1 (-33.5, 11.3)</td>
<td>0.0 (-28.5, 28.4)</td>
<td>-11.7 (-69.6, 46.2)</td>
<td>-8.5 (-19.9, 2.9)</td>
</tr>
<tr>
<td><strong>Stemphylium</strong></td>
<td>39.1 (-24.7, 102.8)</td>
<td><strong>74.8 (34.0, 115.5)</strong>**</td>
<td>43.5 (-14.3, 101.3)</td>
<td>29.4 (-10.6, 69.5)</td>
<td><strong>38.1 (16.4, 59.8)</strong>*</td>
</tr>
<tr>
<td><strong>Sporormiella</strong></td>
<td>25.0 (-25.2, 75.2)</td>
<td>-44.7 (-155.4, 66.0)</td>
<td><strong>144.7 (79.8, 209.7)</strong>**</td>
<td>-9.8 (-60.0, 40.5)</td>
<td>14.1 (-11.9, 40.1)</td>
</tr>
<tr>
<td><strong>Total spores</strong></td>
<td>2.9 (-41.7, 47.5)</td>
<td><strong>25.9 (2.4, 49.5)</strong>*</td>
<td>-7.4 (-38.6, 23.8)</td>
<td>47.2 (-8.5, 103.0)</td>
<td>5.0 (-8.5, 18.5)</td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001
Table 7.5: Associations between fungal spore counts and pre-bronchodilator FEF\textsubscript{25%-75%}.

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>n = 866</th>
<th>Lag 0</th>
<th>Lag 1</th>
<th>Lag 2</th>
<th>Lag 3</th>
<th>Cumulative lag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cladosporium</td>
<td>18.1 (17.0, 53.1)*</td>
<td>16.3 (-21.1, 53.7)</td>
<td>-13.4 (-65.1, 38.2)</td>
<td>4.9 (-54.9, 64.6)</td>
<td>4.7 (-13.6, 23.1)</td>
<td></td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>25.3 (-6.5, 57.0)</td>
<td>40.5 (18.7, 62.4)**</td>
<td>-0.7 (-27.4, 26.0)</td>
<td>-46.9 (-75.2, -18.7)*</td>
<td>2.2 (-10.3, 14.6)</td>
<td></td>
</tr>
<tr>
<td>Alternaria</td>
<td>-30.9 (-129.5, 67.6)</td>
<td>27.6 (-86.4, 141.6)</td>
<td>-18.1 (-153.5, 117.2)</td>
<td>19.7 (-27.5, 67.0)</td>
<td>2.1 (-33.6, 37.9)</td>
<td></td>
</tr>
<tr>
<td>Ustilago/smuts</td>
<td>-38.9 (-65.0, -12.8)*</td>
<td>-3.5 (-48.3, 41.2)</td>
<td>-31.6 (-45.5, -17.7)**</td>
<td>-43.6 (-163.7, 76.5)</td>
<td>-18.0 (-26.8, -9.3)**</td>
<td></td>
</tr>
<tr>
<td>Coprinus</td>
<td>19.3 (-81.2, 119.8)</td>
<td>-37.8 (-116.8, 41.2)</td>
<td>36.6 (-14.0, 87.1)</td>
<td>4.1 (-63.7, 71.9)</td>
<td>2.0 (-21.9, 25.8)</td>
<td></td>
</tr>
<tr>
<td>Drechslera</td>
<td>-20.0 (-52.0, 11.9)</td>
<td>0.4 (-29.9, 30.8)</td>
<td>-35.8 (-53.8, -17.8)**</td>
<td>-18.6 (-74.1, 36.9)</td>
<td>-15.1 (-26.8, -3.4)*</td>
<td></td>
</tr>
<tr>
<td>Periconia</td>
<td>-17.4 (-92.5, 57.7)</td>
<td>12.4 (-82.6, 107.3)</td>
<td>-24.6 (-146.3, 97.0)</td>
<td>75.9 (-9.2, 161.0)</td>
<td>5.6 (-29.8, 41.0)</td>
<td></td>
</tr>
<tr>
<td>Pleospora</td>
<td>177.2 (52.2, 302.2)</td>
<td>52.0 (-0.3, 104.3)</td>
<td>16.8 (-83.8, 117.3)</td>
<td>-38.0 (-153.7, 77.8)</td>
<td>31.1 (-12.3, 74.5)</td>
<td></td>
</tr>
<tr>
<td>Ganoderma</td>
<td>-28.9 (-89.9, 32.2)</td>
<td>6.4 (-1.1, 13.9)</td>
<td>13.1 (-33.4, 59.5)</td>
<td>22.5 (-95.1, 140.2)</td>
<td>3.4 (-4.0, 10.8)</td>
<td></td>
</tr>
<tr>
<td>Pithomyces</td>
<td>-53.5 (-102.2, -4.9)*</td>
<td>-5.7 (-102.6, 91.2)</td>
<td>33.2 (-71.2, 137.5)</td>
<td>27.7 (-65.4, 120.9)</td>
<td>-2.7 (-45.8, 40.5)</td>
<td></td>
</tr>
<tr>
<td>Stemphylium</td>
<td>75.0 (-40.3, 190.3)</td>
<td>121.3 (45.8, 196.8)*</td>
<td>137.0 (36.2, 237.8)*</td>
<td>-8.6 (-75.5, 58.4)</td>
<td>62.8 (19.2, 106.5)*</td>
<td></td>
</tr>
<tr>
<td>Sporormiella</td>
<td>17.9 (-77.6, 113.5)</td>
<td>57.5 (-241.8, 356.7)</td>
<td>153.3 (18.3, 288.2)*</td>
<td>35.5 (-61.8, 132.9)</td>
<td>30.8 (-24.8, 86.5)</td>
<td></td>
</tr>
<tr>
<td>Total spores</td>
<td>35.8 (-27.1, 98.8)</td>
<td>35.8 (-27.1, 98.8)</td>
<td>-58.3 (-119.9, 3.4)</td>
<td>-29.9 (-117.8, 57.9)</td>
<td>-7.4 (-32.9, 18.1)</td>
<td></td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001
7.4.4 Associations between fungal spore taxa and markers of airway inflammation

7.4.4.1 FeNO

No associations were found with any fungal spore taxa and increased exhaled nitric oxide using the FENO measures. However *Pithomyces* was associated with reduced exhaled nitric oxide at Lag 3 (Table 7.6).

7.4.4.2 EBC NOx

Interestingly, when I examined associations with exhaled nitric oxide products (NOx) in the exhaled breath condensate I found that higher levels of NOx were associated with a number of fungal taxa: *Cladosporium* at Lag 1 and cumulative 3-day lag; *Leptosphaeria* at Lag 2; *Alternaria* at Lag 1 and cumulative 3-day lag; *Drechslera* at Lag 0 and Lag 1 (although the lower bound of the 95% CI was 1); *Ganoderma* at Lag 0 and Lag 2 (although the small number of observations left this result to be unreliable); and total spores at Lag 1, Lag 2 and cumulative 3-day lag (Table 7.7).

7.4.4.3 EBC pH

Associations were found between *Ganoderma* and increased EBC acidification (reduced pH) pH at Lag 0, Lag 1, Lag 3 and cumulative 3-day lag (Table 7.8).

*Leptosphaeria* at Lag 0 and cumulative 3-day lag, *Alternaria* at Lag 3 and *Periconia* at Lag 3 were associated with small increases in EBC pH (Table 7.8).
Table 7.6: Adjusted associations between fungal spore counts and exhaled nitric oxide using FeNO measures.

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>coeff (95%CI)</th>
<th>coeff (95%CI)</th>
<th>coeff (95%CI)</th>
<th>coeff (95%CI)</th>
<th>coeff (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FeNO n=797</strong></td>
<td><strong>LAG 0</strong></td>
<td><strong>LAG 1</strong></td>
<td><strong>LAG 2</strong></td>
<td><strong>LAG 3</strong></td>
<td><strong>Cumulative Lag</strong></td>
</tr>
<tr>
<td>Cladosporium</td>
<td>0.6 (-0.6, 1.8)</td>
<td>0.4 (-1.0, 1.7)</td>
<td>0.8 (-0.7, 2.2)</td>
<td>0.8 (-0.7, 2.3)</td>
<td>0.3 (-0.2, 0.9)</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>0.3 (-1.3, 1.9)</td>
<td>-0.2 (-1.3, 1.0)</td>
<td>0.6 (-1.1, 2.3)</td>
<td>0.8 (-0.7, 2.3)</td>
<td>0.2 (-0.3, 0.7)</td>
</tr>
<tr>
<td>Alternaria</td>
<td>1.9 (-1.3, 5.1)</td>
<td>1.0 (-2.5, 4.5)</td>
<td>0.2 (-3.8, 4.3)</td>
<td>0.1 (-1.5, 1.8)</td>
<td>0.5 (-0.6, 1.5)</td>
</tr>
<tr>
<td>Ustilago/smut</td>
<td>0.9 (-0.2, 1.9)</td>
<td>1.1 (-0.9, 3.2)</td>
<td>0.5 (-0.1, 1.2)</td>
<td>3.6 (-2.5, 9.6)</td>
<td>0.4 (0.0, 0.8)</td>
</tr>
<tr>
<td>Coprinus</td>
<td>2.1 (-2.2, 6.4)</td>
<td>2.7 (0.0, 5.5)</td>
<td>1.2 (-1.4, 3.9)</td>
<td>1.0 (-2.1, 4.1)</td>
<td>0.9 (-0.2, 1.9)</td>
</tr>
<tr>
<td>Drechslera</td>
<td>0.6 (-0.4, 1.7)</td>
<td>0.8 (-0.5, 2.1)</td>
<td>0.6 (-0.2, 1.4)</td>
<td>-0.4 (-2.3, 1.4)</td>
<td>0.3 (-0.1, 0.8)</td>
</tr>
<tr>
<td>Periconia</td>
<td>-1.2 (-3.6, 1.2)</td>
<td>-0.3 (-2.6, 2.0)</td>
<td>-0.7 (-4.4, 3.1)</td>
<td>-1.5 (-4.4, 1.3)</td>
<td>-0.5 (-1.5, 0.5)</td>
</tr>
<tr>
<td>Pleospora</td>
<td>-1.0 (-4.6, 2.6)</td>
<td>-0.6 (-2.0, 0.9)</td>
<td>-1.2 (-3.3, 0.9)</td>
<td>1.8 (-1.8, 5.4)</td>
<td>-0.3 (-1.4, 0.7)</td>
</tr>
<tr>
<td>Ganoderma</td>
<td>1.5 (-2.5, 5.5)</td>
<td>-0.3 (-0.6, 0.1)</td>
<td>1.3 (-4.8, 7.3)</td>
<td>1.9 (-5.5, 9.3)</td>
<td>-0.1 (-0.5, 0.3)</td>
</tr>
<tr>
<td>Pithomyces</td>
<td>0.6 (-1.3, 2.6)</td>
<td>-0.3 (-1.5, 0.9)</td>
<td>-1.6 (-3.2, 0.1)</td>
<td>-4.8 (-8.7, -0.9)*</td>
<td>-0.3 (-1.0, 0.3)</td>
</tr>
<tr>
<td>Stemphylium</td>
<td>-1.1 (-4.8, 2.6)</td>
<td>-1.6 (-4.0, 0.8)</td>
<td>-2.0 (-6.3, 2.3)</td>
<td>-0.3 (-3.2, 2.5)</td>
<td>-1.0 (-2.5, 0.6)</td>
</tr>
<tr>
<td>Sporormiella</td>
<td>3.1 (-4.8, 10.9)</td>
<td>6.6 (-6.9, 20.1)</td>
<td>0.8 (-7.4, 9.0)</td>
<td>-3.5 (-8.3, 1.2)</td>
<td>0.9 (-2.5, 4.4)</td>
</tr>
<tr>
<td>Total spores</td>
<td>1.3 (-0.8, 3.4)</td>
<td>0.7 (-1.4, 2.8)</td>
<td>1.5 (-0.4, 3.5)</td>
<td>1.6 (-1.3, 4.5)</td>
<td>0.6 (-0.2, 1.3)</td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001
Table 7.7: Adjusted associations between fungi taxa and increased NOx in EBC (lower to higher)

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>EBC NOx (µM) n=231</th>
<th>Lag 0</th>
<th>Lag 1</th>
<th>Lag 2</th>
<th>Lag 3</th>
<th>Cumulative Lag</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95%CI)</td>
<td>OR (95%CI)</td>
<td>OR (95%CI)</td>
<td>OR (95%CI)</td>
<td>OR (95%CI)</td>
<td></td>
</tr>
<tr>
<td>Cladosporium</td>
<td>1.1 (0.9-1.3)</td>
<td>1.4 (1.1-1.6)*</td>
<td>1.2 (1.0-1.4)</td>
<td>0.9 (0.7-1.2)</td>
<td>1.1 (1.0-1.2)*</td>
<td></td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>0.9 (0.8-1.0)</td>
<td>1.0 (0.9-1.1)</td>
<td>1.4 (1.1-1.9)*</td>
<td>1.2 (1.0-1.5)</td>
<td>1.0 (1.0-1.1)</td>
<td></td>
</tr>
<tr>
<td>Alternaria</td>
<td>1.3 (0.9-1.9)</td>
<td>1.9 (1.2-2.9)*</td>
<td>1.2 (0.8-1.7)</td>
<td>1.0 (0.8-1.3)</td>
<td>1.1 (1.0-1.3)*</td>
<td></td>
</tr>
<tr>
<td>Ustilago/smuts</td>
<td>1.1 (1.0-1.2)</td>
<td>1.0 (0.7-1.7)</td>
<td>1.0 (1.0-1.1)</td>
<td>1.2 (0.7-2.2)</td>
<td>1.0 (1.0-1.0)</td>
<td></td>
</tr>
<tr>
<td>Coprinus</td>
<td>1.0 (0.7-1.5)</td>
<td>1.1 (0.9-1.4)</td>
<td>1.2 (0.9-1.6)</td>
<td>1.3 (0.8-1.4)</td>
<td>1.0 (1.0-1.2)</td>
<td></td>
</tr>
<tr>
<td>Drechslera</td>
<td>1.1 (1.0-1.3)*</td>
<td>1.4 (1.0-1.8)*</td>
<td>1.0 (1.0-1.1)</td>
<td>1.0 (0.8-1.4)</td>
<td>1.0 (1.0-1.1)</td>
<td></td>
</tr>
<tr>
<td>Periconia</td>
<td>0.9 (0.7-1.2)</td>
<td>1.1 (0.7-1.7)</td>
<td>1.3 (0.9-1.9)</td>
<td>1.0 (0.7-1.6)</td>
<td>1.0 (0.9-1.2)</td>
<td></td>
</tr>
<tr>
<td>Pleospora</td>
<td>0.6 (0.3-1.2)</td>
<td>0.9 (0.8-1.1)</td>
<td>1.1 (0.9, 1.4)</td>
<td>1.4 (1.0-2.0)</td>
<td>1.0 (0.9-1.2)</td>
<td></td>
</tr>
<tr>
<td>Ganoderma</td>
<td>2.1 (1.2-3.7)*</td>
<td>X</td>
<td>2.8 (1.4-5.6)*</td>
<td>1.0 (0.6-1.5)</td>
<td>1.3 (0.9-1.8)</td>
<td></td>
</tr>
<tr>
<td>Pithomyces</td>
<td>1.2 (0.9-1.5)</td>
<td>1.1 (1.0-1.3)</td>
<td>1.2 (0.9-1.5)</td>
<td>0.8 (0.4-1.4)</td>
<td>1.1 (1.0-1.2)</td>
<td></td>
</tr>
<tr>
<td>Stemphylium</td>
<td>1.2 (0.8-1.8)</td>
<td>1.3 (0.9-1.8)</td>
<td>1.2 (0.8-1.7)</td>
<td>1.0 (0.7-1.6)</td>
<td>1.1 (0.9-1.3)</td>
<td></td>
</tr>
<tr>
<td>Sporormiella</td>
<td>1.0 (0.6-1.8)</td>
<td>0.7 (0.2-2.3)</td>
<td>0.7 (0.4-1.1)</td>
<td>0.7 (0.3-1.6)</td>
<td>0.9 (0.7-1.1)</td>
<td></td>
</tr>
<tr>
<td>Total spores</td>
<td>1.2 (0.9-1.5)</td>
<td>1.5 (1.1-2.2)*</td>
<td>1.3 (1.1-1.5)*</td>
<td>1.1 (0.7, 1.5)</td>
<td>1.1 (1.0-1.2)*</td>
<td></td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models. Adjusted for age, sex, height, relative humidity, maximum temperature, storage time, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05  **p<0.001

The OR of higher category of NOx with spore increase from the 75th to 90th percentile is significant in those highlighted in red. X = OR could not be reliably determined due to inadequate observations. Brant test results were not significant (>0.05), so the proportional odds assumption was not violated.
Table 7.8: Adjusted associations between fungi taxa and EBC pH

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>Lag 0</th>
<th>Lag 1</th>
<th>Lag 2</th>
<th>Lag 3</th>
<th>Cumulative Lag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cladosporium</td>
<td>0.00 (-0.04, 0.03)</td>
<td>-0.01 (-0.08, 0.06)</td>
<td>0.00 (-0.02, 0.01)</td>
<td>0.01 (-0.02, 0.04)</td>
<td>0.00 (-0.01, 0.01)</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>0.03 (0.01, 0.04)*</td>
<td>0.01 (-0.01, 0.02)</td>
<td>0.03 (-0.01, 0.06)</td>
<td>0.03 (-0.02, 0.07)</td>
<td><strong>0.01 (0.00, 0.02)</strong>*</td>
</tr>
<tr>
<td>Alternaria</td>
<td>-0.04 (-0.10, 0.02)</td>
<td>0.08 (-0.01, 0.17)</td>
<td>0.04 (-0.03, 0.11)</td>
<td><strong>0.07 (0.03, 0.10)</strong>**</td>
<td>0.02 (0.00, 0.05)</td>
</tr>
<tr>
<td>Ustilago/smuts</td>
<td>-0.01 (-0.04, 0.02)</td>
<td>0.05 (-0.03, 0.12)</td>
<td>-0.01 (-0.02, 0.01)</td>
<td>0.09 (-0.04, 0.21)</td>
<td>0.00 (-0.01, 0.01)</td>
</tr>
<tr>
<td>Coprinus</td>
<td>-0.01 (-0.08, 0.05)</td>
<td>-0.02 (-0.08, 0.03)</td>
<td>0.03 (-0.03, 0.08)</td>
<td>-0.05 (-0.16, 0.07)</td>
<td>0.00 (-0.03, 0.02)</td>
</tr>
<tr>
<td>Drechslera</td>
<td>-0.02 (-0.04, 0.01)</td>
<td>0.02 (-0.02, 0.06)</td>
<td>-0.01 (-0.03, 0.01)</td>
<td>0.03 (-0.04, 0.09)</td>
<td>-0.01 (-0.02, 0.01)</td>
</tr>
<tr>
<td>Periconia</td>
<td>0.02 (-0.03, 0.07)</td>
<td>0.05 (-0.04, 0.15)</td>
<td>0.03 (-0.10, 0.17)</td>
<td><strong>0.06 (0.01, 0.10)</strong>*</td>
<td>0.02 (-0.01, 0.06)</td>
</tr>
<tr>
<td>Pleospora</td>
<td>0.06 (-0.05, 0.18)</td>
<td>-0.03 (-0.07, 0.02)</td>
<td>0.00 (-0.05, 0.06)</td>
<td>-0.02 (-0.07, 0.04)</td>
<td>-0.01 (-0.03, 0.01)</td>
</tr>
<tr>
<td>Ganoderma</td>
<td><strong>-0.10 (-0.20, -0.01)</strong>*</td>
<td><strong>-0.01 (-0.02, 0.00)</strong>*</td>
<td>-0.04 (-0.17, 0.10)</td>
<td><strong>-0.09 (-0.16, -0.01)</strong>*</td>
<td><strong>-0.01 (-0.02, 0.00)</strong>*</td>
</tr>
<tr>
<td>Pithomyces</td>
<td>0.04 (0.00, 0.09)</td>
<td>-0.01 (-0.05, 0.04)</td>
<td>0.01 (-0.06, 0.07)</td>
<td>0.05 (-0.18, 0.28)</td>
<td>0.01 (-0.02, 0.03)</td>
</tr>
<tr>
<td>Stemphylium</td>
<td>0.03 (-0.06, 0.11)</td>
<td>0.04 (-0.02, 0.10)</td>
<td>0.04 (-0.03, 0.11)</td>
<td>0.00 (-0.10, 0.10)</td>
<td>0.02 (-0.01, 0.05)</td>
</tr>
<tr>
<td>Sporormiella</td>
<td>-0.06 (-0.18, 0.07)</td>
<td>-0.17 (-0.48, 0.14)</td>
<td>0.03 (-0.04, 0.11)</td>
<td>0.03 (-0.10, 0.17)</td>
<td>-0.01 (-0.06, 0.05)</td>
</tr>
<tr>
<td>Total spores</td>
<td>-0.01 (-0.06, 0.05)</td>
<td>0.00 (-0.08, 0.07)</td>
<td>-0.01 (-0.05, 0.03)</td>
<td>0.05 (-0.02, 0.11)</td>
<td>0.00 (-0.02, 0.02)</td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models. Adjusted for age, sex, height, relative humidity, maximum temperature, storage time, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001
7.4.5 Interactions

7.4.5.1 Fungal sensitisation and lung function parameters

When I examined interactions with *Alternaria*, *Cladosporium* and *Penicillium* sensitisation status at Lag 0 I found inconsistent results with lung function parameters. (Table 7.9, Table 7.10, Table 7.11, Table 7.12)

7.4.5.1.1 *Alternaria* sensitisation

In only those with *Alternaria* sensitisation, I found *Ustilago/smuts* was associated with significantly reduced FEV1, FVC and FEF25%-75%; *Drechslera* was associated with significantly reduced FEV1 and FVC; and *Pithomyces* exposure was associated with significantly greater reduction in FEV1 than in individuals who were not sensitised to *Alternaria*. Some fungal taxa (*Cladosporium, Leptosphaeria, Pleospora* and *Stemphylium*) were also associated with significantly increased FEV1 and FVC, only in those with *Alternaria* sensitisation (Table 7.9).

7.4.5.1.2 *Penicillium* sensitisation

Sensitisation to *Penicillium* modified the associations between some fungal exposures and lung function outcomes. In only those with *Penicillium* sensitisation: *Periconia* and *Sporormiella* were associated with reduced FEV1; *Ustilago/smuts* exposure was associated with significantly greater reduction in FEV1 than in those who were not sensitised to *Penicillium; Ustilago/smuts, Periconia, and Sporormiella* were associated with significantly reduced FVC; *Periconia* was associated with significantly reduced FEF25%-75%. *Cladosporium* was associated with significantly increased FEV1 and FVC in only those with *Penicillium* sensitisation. In those not sensitised to *Penicillium*: *Pithomyces* was associated with significantly reduced FEV1 and FEF25%-75%; and *Pithomyces* was associated with significantly reduced FEF25%-75% (Table 7.10).

7.4.5.1.3 *Cladosporium* sensitisation

Similarly, some associations with lung function outcomes were modified by sensitisation to *Cladosporium*. In only those with *Cladosporium* sensitisation: *Ustilago/smuts* and *Periconia* were associated with reduced FVC; *Pithomyces* was associated with a significantly greater reduction in FVC than in those without sensitisation to *Cladosporium*. In only those not sensitised to *Cladosporium, Ustilago/smuts* was associated with significantly reduced FEF25%-75%. *Alternaria* was associated with...
significantly increased FEF$_{25\text{-}75\%}$ in those with sensitisation to *Cladosporium* (Table 7.11).

### 7.4.5.1.4 Sensitised to ≥1 fungal reagent

I also investigated whether there were significant interactions with being sensitised to one or more than one fungal reagents. In those sensitised to at least one fungal reagent *Ustilago*/*smuts* and *Drechslera* were associated with small but significant reductions in FEV$_1$ and FVC; *Pithomyces* was associated with significantly reduced FEV$_1$, FVC and FEF$_{25\text{-}75\%}$. These associations were not seen in those without fungal sensitisation. *Cladosporium* maintained associations with significantly increased FEV$_1$ and FVC in the sensitised group (Table 7.12).

### 7.4.5.2 Fungal sensitisation and FeNO, EBC NOx and EBC pH

Some associations with FeNO, EBC NOx and EBC pH were modified by fungal sensitisation. In those sensitised to at least one fungal reagent, *Cladosporium* and *Periconia* were associated with small increases in FeNO. Small decrease in FeNO was associated with *Periconia* in those not sensitised to any fungal reagents (Table 7.13).

There was one significant association between *Stemphylium* and higher levels of EBC NOx in those sensitised to fungi but the effect estimate was imprecise (Table 7.13).

In those who were sensitised to at least one fungal reagent, *Cladosporium* was associated with reduced EBC pH. In those without fungal sensitisation, *Ganoderma* was associated with reduced EBC pH (Table 7.13)
Table 7.9: Adjusted associations at Lag 0 between fungi taxa and lung function outcomes and stratification by *Alternaria* sensitisation

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>Not sensitised n=708</th>
<th>Sensitised n=134</th>
<th>p-int</th>
<th>Not sensitised n=708</th>
<th>Sensitised n=134</th>
<th>p-int</th>
<th>Not sensitised n=708</th>
<th>Sensitised n=134</th>
<th>p-int</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cladosporium</em></td>
<td>12.7 (20.7, 77.3)*</td>
<td>0.001</td>
<td>1.9</td>
<td>55.9 (25.2, 96.5)*</td>
<td>0.001</td>
<td>8.4</td>
<td>49.7 (25.2, 41.9)</td>
<td>(-2.6, 102.0)</td>
<td>0.131</td>
</tr>
<tr>
<td><em>Leptosphaeria</em></td>
<td>5.3 (31.4, 31.2)</td>
<td>-11.5, 36.8</td>
<td>0.026</td>
<td>73.8 (2.5, 145.1)*</td>
<td>0.057</td>
<td>19.4</td>
<td>55.7 (-15.1, 54.0)</td>
<td>(-51.4, 162.8)</td>
<td>0.528</td>
</tr>
<tr>
<td><em>Alternaria</em></td>
<td>-18.1 (-21.9, -57.2)</td>
<td>-149.8, 70.6</td>
<td>0.482</td>
<td>-45.6 (-41.2, 33.0)</td>
<td>0.431</td>
<td>-44.3</td>
<td>32.6 (-128.5, 39.8)</td>
<td>(-172.9, 238.1)</td>
<td>0.670</td>
</tr>
<tr>
<td><em>Pithomyces</em></td>
<td>-8.4 (-22.3, -66.2)</td>
<td>&lt;0.001</td>
<td>-4.3</td>
<td>-43.6 (-5.6, 14.2)</td>
<td>&lt;0.001</td>
<td>-74.5</td>
<td>126.3 (-49.4, 152.4)</td>
<td>(-308.8, 172.1)</td>
<td>0.024</td>
</tr>
<tr>
<td><em>Coprinus</em></td>
<td>13.1 87.2</td>
<td>0.257</td>
<td>8.5</td>
<td>105.9 (-47.1, 64.1)</td>
<td>0.213</td>
<td>25.9</td>
<td>-68.4 (-46.0, 15.0)</td>
<td>(-308.8, 172.1)</td>
<td>0.296</td>
</tr>
<tr>
<td><em>Drechslera</em></td>
<td>18.6 47.9</td>
<td>0.006</td>
<td>0.2</td>
<td>-51.9 (-2.5, 2.9)</td>
<td>&lt;0.001</td>
<td>-15.5</td>
<td>-34.4 (-46.0, 15.0)</td>
<td>(-308.8, 172.1)</td>
<td>0.384</td>
</tr>
<tr>
<td><em>Periconia</em></td>
<td>-23.1 -43.4</td>
<td>0.251</td>
<td>-32.3</td>
<td>-51.9 (-73.5, -13.3)</td>
<td>0.712</td>
<td>-6.3</td>
<td>-38.4 (-72.2, 59.6)</td>
<td>(-228.1, 151.2)</td>
<td>0.520</td>
</tr>
<tr>
<td><em>Pleospora</em></td>
<td>-58.2,120.0</td>
<td>&lt;0.001</td>
<td>-47.9</td>
<td>-121.0 (-73.0, 8.5)</td>
<td>0.206</td>
<td>-72.2</td>
<td>151.2 (-129.8, 34.0)</td>
<td>(-228.1, 151.2)</td>
<td>0.220</td>
</tr>
<tr>
<td><em>Ganoderma</em></td>
<td>-21.9 35.5</td>
<td>0.465</td>
<td>-29.4</td>
<td>-40.8 (-32.1, 7.8)</td>
<td>0.422</td>
<td>-30.4</td>
<td>-6.6 (-31.8, 285.6)</td>
<td>(-53.7, 413.0)</td>
<td>1.000</td>
</tr>
<tr>
<td><em>Pithomyces</em></td>
<td>-28.7 -91.5</td>
<td>-53.5,10.0</td>
<td>0.047</td>
<td>-75.4 (-55.7, -3.1)</td>
<td>0.144</td>
<td>-32.7</td>
<td>-147.9 (-73.1, 148.4)</td>
<td>(-252.5, 253.8)</td>
<td>0.035</td>
</tr>
<tr>
<td><em>Stemphylium</em></td>
<td>-34.9 170.4</td>
<td>-181.6, -1.3*</td>
<td>0.007</td>
<td>192.4 (-43.1, 91.4)</td>
<td>0.073</td>
<td>37.7</td>
<td>283.2 (-73.1, 148.4)</td>
<td>(-168, 583.2)</td>
<td>0.264</td>
</tr>
<tr>
<td><em>Sporormiella</em></td>
<td>-36.2,105.9</td>
<td>-43.1, 19.4</td>
<td>0.411</td>
<td>57.0 (49.0, 335.8)*</td>
<td>0.538</td>
<td>15.3</td>
<td>39.3 (-69.1, 99.7)</td>
<td>(-263.4, 454.1)</td>
<td>0.717</td>
</tr>
<tr>
<td>Total spores</td>
<td>-0.1 2.9</td>
<td>0.964</td>
<td>1.1</td>
<td>12.5 (-30.1, 81.3)</td>
<td>0.958</td>
<td>-6.3</td>
<td>2.6 (-61.7, 49.2)</td>
<td>(-156.6, 161.9)</td>
<td>0.972</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001
Table 7.10: Adjusted associations at Lag 0 between fungi taxa and lung function outcomes and stratification by *Penicillium* sensitisation

<table>
<thead>
<tr>
<th>Lag 0</th>
<th>FEV₁</th>
<th>FVC</th>
<th>FEF25%-75%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not sensitised</td>
<td>Sensitised</td>
<td>Not sensitised</td>
</tr>
<tr>
<td></td>
<td>n=784</td>
<td>n=60</td>
<td>n=784</td>
</tr>
<tr>
<td><strong>Fungal spore taxa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladosporium</td>
<td>8.2</td>
<td>46.3</td>
<td>0.009</td>
</tr>
<tr>
<td>(-13.4, 29.8)</td>
<td>(51.8, 76.7)*</td>
<td>(-18.1, 29.7)</td>
<td>(61.8, 84.7)*</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>10.7</td>
<td>6.9</td>
<td>0.529</td>
</tr>
<tr>
<td>(-6.4, 27.8)</td>
<td>(-9.6, 26.3)</td>
<td>(-13.6, 27.4)</td>
<td>(-123.4, 57.8)</td>
</tr>
<tr>
<td>Alternaria</td>
<td>13.4</td>
<td>-11.4</td>
<td>0.05</td>
</tr>
<tr>
<td>(-50.4, 23.6)</td>
<td>(-270.0, 40.2)</td>
<td>(-39.6, 29.7)</td>
<td>(-232.6, 50.2)</td>
</tr>
<tr>
<td>Ustilago/smutts</td>
<td>-12.4</td>
<td>-36.0</td>
<td>0.072</td>
</tr>
<tr>
<td>(-243, -0.5)*</td>
<td>(-712, -0.7)*</td>
<td>(-119.9, 6.9)</td>
<td>(-580, -1.5)*</td>
</tr>
<tr>
<td>Coprinus</td>
<td>5.7</td>
<td>0.8</td>
<td>0.484</td>
</tr>
<tr>
<td>(-44.8, 56.2)</td>
<td>(-167.1)</td>
<td>(-51.3, 52.9)</td>
<td>(-229.1, 155.0)</td>
</tr>
<tr>
<td>Drechslera</td>
<td>-7.3</td>
<td>-39.7</td>
<td>0.030</td>
</tr>
<tr>
<td>(-20.3, 5.7)</td>
<td>(-81.1, 1.8)</td>
<td>(-11.3, 5.7)</td>
<td>(-63.8, 2.7)</td>
</tr>
<tr>
<td>Periconia</td>
<td>-22.2</td>
<td>-104.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(-55.6, 11.2)</td>
<td>(-142, -66.5)**</td>
<td>(-66.5, 5.8)</td>
<td>(-275.3, -1.0)**</td>
</tr>
<tr>
<td>Pleospora</td>
<td>89.3</td>
<td>75.3</td>
<td>0.472</td>
</tr>
<tr>
<td>(-10.1, 188.8)</td>
<td>(-75.4, 226.0)</td>
<td>(-32.1, 209.9)</td>
<td>(-147.0, 197.3)</td>
</tr>
<tr>
<td>Ganoderma</td>
<td>-19.9</td>
<td>27.8</td>
<td>0.644</td>
</tr>
<tr>
<td>(-51.3, 11.4)</td>
<td>(-117.8, 291.6)</td>
<td>(-52.9, -2.8)*</td>
<td>(-105.2, 342.9)</td>
</tr>
<tr>
<td>Pithomyces</td>
<td>-38.3</td>
<td>-151.2</td>
<td>0.184</td>
</tr>
<tr>
<td>(-63.2, -13.3)*</td>
<td>(-339.6, 36.7)</td>
<td>(-61.5, -9.2)*</td>
<td>(-275.3, -1.0)*</td>
</tr>
<tr>
<td>Stemphylium</td>
<td>45.2</td>
<td>25.3</td>
<td>0.678</td>
</tr>
<tr>
<td>(-12.0, 102.4)</td>
<td>(-392.5, 341.9)</td>
<td>(-29.3, 109.0)</td>
<td>(-389.3, 354.1)</td>
</tr>
<tr>
<td>Sporormiella</td>
<td>42.5</td>
<td>87.8</td>
<td>0.001</td>
</tr>
<tr>
<td>(-0.4, 85.4)</td>
<td>(-158.5, -17.2)*</td>
<td>(-2.5, 94.3)</td>
<td>(-174.6, -11.9)*</td>
</tr>
<tr>
<td>Total spores</td>
<td>1.7</td>
<td>15.9</td>
<td>0.972</td>
</tr>
<tr>
<td>(-26.9, 30.3)</td>
<td>(-133.3, 165.0)</td>
<td>(-23.5, 32.4)</td>
<td>(-114.1, 156.7)</td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001
Table 7.11: Adjusted associations at Lag 0 between fungi taxa and lung function outcomes and stratification by *Cladosporium* sensitisation

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>Cladosporium</th>
<th>Ustilago/Smuts</th>
<th>Leptosphaeria</th>
<th>Alternaria</th>
<th>Coprinus</th>
<th>Drechslera</th>
<th>Periconia</th>
<th>Pleospora</th>
<th>Stenophyllum</th>
<th>Sporormiella</th>
<th>Total spores</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Not sensitised</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>n= 691</td>
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<tr>
<td>Sensitised</td>
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<td>n= 84</td>
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</tr>
<tr>
<td><strong>FEV1</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coeff (95%CI)</td>
<td>6.4 (6.0, 18.9)</td>
<td>(-31.1, 119.4)</td>
<td>7.6 (30.4)</td>
<td>(-11.7, 27.0)</td>
<td>17.7 (-71.3, 21.6)</td>
<td>-17.7 (-34.7, -0.6)*</td>
<td>3.5 (-52.3, 59.4)</td>
<td>-10.7 (-28.2, 6.7)</td>
<td>-23.2 (-58.4, 12.0)</td>
<td>-35.4 (-65.4, -5.5)*</td>
<td>51.5 (-8.5, 111.5)</td>
</tr>
<tr>
<td>p-int</td>
<td>0.458</td>
<td>0.221</td>
<td>0.776</td>
<td>0.932</td>
<td>0.090</td>
<td>0.093</td>
<td>0.425</td>
<td>-0.146</td>
<td>-0.396</td>
<td>-0.461</td>
<td>0.461</td>
</tr>
<tr>
<td><strong>FVC</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coeff (95%CI)</td>
<td>3.5 (-8.8, 15.8)</td>
<td>(-42.9, 30.3)</td>
<td>2.6 (-20.5, 25.8)</td>
<td>-6.3 (-42.9, 30.3)</td>
<td>-12.6 (-35.9, -2.3)*</td>
<td>-13.6 (-16.3, 4.4)</td>
<td>-4.0 (-54.0, 62.6)</td>
<td>-4.0 (-13.3, 5.3)</td>
<td>-6.8 (-65.2, 13.3)</td>
<td>-9.1 (-65.2, 13.3)</td>
<td>-18.4 (-184.7, 1.0)</td>
</tr>
<tr>
<td>p-int</td>
<td>0.458</td>
<td>0.776</td>
<td>0.025</td>
<td>0.932</td>
<td>0.090</td>
<td>0.932</td>
<td>0.425</td>
<td>-0.146</td>
<td>-0.396</td>
<td>-0.461</td>
<td>0.461</td>
</tr>
<tr>
<td><strong>FEF25%-75%</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coeff (95%CI)</td>
<td>45.2 (-47.1, 137.5)</td>
<td>(-96.7, 51.9)</td>
<td>53.9 (-43.7, 151.4)</td>
<td>-22.4 (-42.9, 30.3)</td>
<td>-19.0 (&lt;0.001, -14.9)</td>
<td>-35.9 (-35.9, -2.3)*</td>
<td>-15.2 (-209.8, 71.9)</td>
<td>-12.8 (-40.0, 9.7)</td>
<td>-72.8 (-122.0, -23.6)*</td>
<td>-72.8 (-122.0, -23.6)*</td>
<td>-23.2 (-60.6, -0.6)*</td>
</tr>
<tr>
<td>p-int</td>
<td>0.458</td>
<td>0.025</td>
<td>0.932</td>
<td>0.458</td>
<td>0.458</td>
<td>0.458</td>
<td>0.932</td>
<td>0.458</td>
<td>0.458</td>
<td>0.458</td>
<td>0.458</td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001
Table 7.12: Adjusted associations at Lag 0 between fungi taxa and lung function outcomes and stratification by sensitisation to ≥ 1 fungal taxa

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>FEV₁ (95%CI)</th>
<th>p-int</th>
<th>FVC (95%CI)</th>
<th>p-int</th>
<th>FEF25%-75% (95%CI)</th>
<th>p-int</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cladosporium</td>
<td>2.0 (22.0, 74.0)</td>
<td>0.005</td>
<td>0.0 (16.0, 78.0)</td>
<td>0.005</td>
<td>4.0 (14.0, 116.0)</td>
<td>0.120</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>3.0 (52.8)</td>
<td>0.134</td>
<td>2.4 (56.4)</td>
<td>0.243</td>
<td>17.4 (54.6)</td>
<td>0.552</td>
</tr>
<tr>
<td>Alternaria</td>
<td>-42.6 (-15.6)</td>
<td>0.651</td>
<td>-18.6 (-39.6)</td>
<td>0.249</td>
<td>-84.0 (102.6)</td>
<td>0.446</td>
</tr>
<tr>
<td>Ustilago/smuts</td>
<td>-14.4 (-34.8)</td>
<td>0.008</td>
<td>1.8 (-35.4)</td>
<td>&lt;0.001</td>
<td>-97.7 (120.9)</td>
<td>0.79</td>
</tr>
<tr>
<td>Coprinus</td>
<td>5.6 (-38.8)</td>
<td>0.246</td>
<td>0.4 (-53.6)</td>
<td>0.284</td>
<td>24.4 (-26.8)</td>
<td>0.285</td>
</tr>
<tr>
<td>Drechslera</td>
<td>-10.8 (-30.6)</td>
<td>0.015</td>
<td>-2.0 (-35.0)</td>
<td>&lt;0.001</td>
<td>-81.2 (120.9)</td>
<td>0.831</td>
</tr>
<tr>
<td>Periconia</td>
<td>-34.8 (-59.4, -10.2)</td>
<td>0.008</td>
<td>-9.6 (13.8)</td>
<td>(55.2, -15.0)</td>
<td>-119.2 (120.9)</td>
<td>0.447</td>
</tr>
<tr>
<td>Pleospora</td>
<td>73.2 (-119.2, 53.6)</td>
<td>0.359</td>
<td>-56.0 (169.6, 62.4)</td>
<td>0.284</td>
<td>-116.0 (129.6)</td>
<td>0.538</td>
</tr>
<tr>
<td>Ganoderma</td>
<td>-22.6 (30.8, 209.8)</td>
<td>0.377</td>
<td>-11.6 (180.9, 29.8)</td>
<td>0.397</td>
<td>-118.0 (129.6)</td>
<td>0.538</td>
</tr>
<tr>
<td>Pithomyces</td>
<td>-26.8 (-83.9)</td>
<td>0.011</td>
<td>-2.6 (-7.0)</td>
<td>0.418</td>
<td>-40.2 (148.4)</td>
<td>0.015</td>
</tr>
<tr>
<td>Stempphylium</td>
<td>-55.0 (140.7, -27.1)</td>
<td>0.042</td>
<td>-57.5 (125.4, -15.4)</td>
<td>&lt;0.001</td>
<td>-125.4 (120.9)</td>
<td>0.585</td>
</tr>
<tr>
<td>Sporormiella</td>
<td>42.2 (80.6)</td>
<td>0.92</td>
<td>34.2 (66.8)</td>
<td>0.993</td>
<td>44.1 (208.3)</td>
<td>0.81</td>
</tr>
<tr>
<td>Total spores</td>
<td>-9.3 (-14.0)</td>
<td>0.011</td>
<td>-1.9 (9.0)</td>
<td>0.83</td>
<td>-18.6 (46.5)</td>
<td>0.776</td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001
Table 7.13: Adjusted associations at Lag 0 between fungi taxa and exhaled nitric oxide using FeNO measures, exhaled nitric oxides using EBC and EBC pH and stratification by sensitisation to ≥ 1 fungal taxa

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>Lag0 FeNO</th>
<th>EBC NOx</th>
<th>EBC pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitised ≥ 1 fungal taxa</td>
<td>Sensitised n = 140</td>
<td>Not sensitised n = 632</td>
<td>Sensitised n = 25</td>
</tr>
</tbody>
</table>
| Cladosporium | -0.2 (95%CI) | 3.0 (95%CI) | <0.001 | 1.1 (95%CI) | 6.7 (95%CI) | 0.181 | 0 (95%CI) | 0 (95%CI) | 0.001 *p<0.05 **p<0.001
| Leptosphaeria | -0.1 (95%CI) | 3.6 (95%CI) | 0.019 | 1.0 (95%CI) | 0.7 (95%CI) | 0.966 | 0 (95%CI) | 0 (95%CI) | 0.019
| Alternaria | 1.8 (95%CI) | -1.8 (95%CI) | 0.998 | 1.4 (95%CI) | 16.8 (95%CI) | 0.561 | 0 (95%CI) | 0 (95%CI) | 0.019
| Ustilago/smuts | 1.2 (95%CI) | -0.6 (95%CI) | 0.248 | 1.2 (95%CI) | (1.01, 1.2) | *p<0.05 **p<0.001
| Coprinus | 0.8 (95%CI) | 3.2 (95%CI) | 0.238 | 0.8 (95%CI) | 1.7 (95%CI) | 0.513 | 0 (95%CI) | 0 (95%CI) | 0.019
| Drechslera | 0.8 (95%CI) | -0.4 (95%CI) | 0.958 | 1.2 (95%CI) | (0.8, 5.3) | 0.507 | 0 (95%CI) | 0 (95%CI) | 0.019
| Periconia | -3.2 (95%CI) | 3.0 (95%CI) | 0.027 | 1.0 (95%CI) | 1.7 (95%CI) | 0.346 | 0 (95%CI) | 0 (95%CI) | 0.019
| Pleospora | -1.4 (95%CI) | 5.2 (95%CI) | 0.952 | 0.7 (95%CI) | 0.1 (95%CI) | 0.363 | -0.1 (95%CI) | -0.1 (95%CI) | 0.019
| Ganoderma | 1.9 (95%CI) | -12.0 (95%CI) | 0.302 | 1.4 (95%CI) | X | 0.966 | (0.2, 0.05) | (0.2, 0.05) | 0.019
| Pithomyces | 0.5 (95%CI) | -1.2 (95%CI) | 0.307 | 1.3 (95%CI) | 1.4 | 0.784 | 0.1 | 0.1 | 0.019
| Stemphylium | -1.9 (95%CI) | 0.6 (95%CI) | 0.204 | (0.9, 2.0) | (1.3, 12467) | *p<0.05 **p<0.001
| Sporormiella | 2.7 (95%CI) | 5.6 (95%CI) | 0.365 | 0.8 (95%CI) | 2.6 (95%CI) | 0.135 | -0.2 (95%CI) | -0.2 (95%CI) | 0.019
| Total spores | 0.6 (95%CI) | 2.8 (95%CI) | 0.081 | 1.3 (95%CI) | 4.0 (95%CI) | 0.879 | 0 (95%CI) | 0 (95%CI) | 0.019

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001 X = OR could not be reliably determined due to inadequate number of observations.
7.4.5.3 Age groups and lung function, FeNO, EBC NOx and EBC pH

7.4.5.3.1 ≤25 years and >25 years
There was minimal evidence of interactions between any fungal spore taxa and age for lung function except for Cladosporium and increased FVC in those aged less than 25 years (Table 7.14). There was no evidence of interactions with the FeNO results. EBC results were not stratified in this way as all participants were aged less than 25 years.

7.4.5.3.2 ≤18 years and >18 years
There was no evidence of interactions between fungal spore taxa and age for FEV₁ and FEF₂₅%₋₇₅%. However, some of the associations between some fungal spore taxa with FVC, NO from the FeNO testing, EBC pH and EBC NOx appeared to be modified by age group (Table 7.15, Table 7.16, and Table 7.17). Drechslera was associated with reduced FVC in only the older age group; Periconia was associated with a greater reduction in FVC in the younger age group (Table 7.15). Ustilago/smuts was associated with increased NO in the FeNO (Table 7.16) and reduced EBC pH (Table 7.17) in the younger age group only. Conversely in the older age group Ustilago/smuts, Leptosphaeria and total spores were associated with slightly increased EBC pH (Table 7.17). Periconia was associated with reduced NO in the FeNO testing in the younger age group (Table 7.16).

The role of age as an effect modifier may be linked with fungal sensitisation as, in this cohort, the proportion of participants found to have fungal sensitisation increased in older age groups. To test this, I re-examined these age-stratified interaction models by controlling for fungal sensitisation to at least one of the fungal reagents in addition to age, sex, height, relative humidity, maximum temperature and grass pollen and found that (1) In those aged <18 years, Ustilago/smuts maintained significant associations with increased FeNO and reduced EBC pH (p-interaction <0.001 for both); (2) In those aged >18 years Ustilago/smuts maintained a significant association with increased pH (p-interaction <0.001); (3) Significant association held with Drechslera and reduced FVC in those aged >18 years (p-interaction <0.05). These findings indicated that age may act as an independent effect modifier.
7.4.6 Test for non-linearity

I explored non-linearity for each fungal spore taxa in separate fractional polynomial generalised linear models for each lung function outcome and marker of airway inflammation and found no evidence of significant non-linearity.
Table 7.14: Adjusted associations at Lag 0 between fungi taxa and lung function parameters and stratification by age group: ≤ 25 years and >25 years

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>Age group</th>
<th>FEV&lt;sub&gt;1&lt;/sub&gt;</th>
<th>FVC</th>
<th>FEF&lt;sub&gt;25%-75%&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ 25 years n = 488</td>
<td>&gt;25 years n = 382</td>
<td>≤ 25 years n = 488</td>
<td>&gt;25 years n = 382</td>
</tr>
<tr>
<td>Cladosporium</td>
<td>24.4</td>
<td>-8.1</td>
<td>0.114</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td>(1.1, 47.7)*</td>
<td>(-40.4, 24.2)</td>
<td></td>
<td>(2.5, 51.2)*</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>12.0</td>
<td>14.9</td>
<td>0.663</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>(-3.7, 27.8)</td>
<td>(-20.8, 50.5)</td>
<td></td>
<td>(-9.0, 32.1)</td>
</tr>
<tr>
<td>Alternaria</td>
<td>-8.1</td>
<td>-68.0</td>
<td>0.842</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>(-52.3, 36.1)</td>
<td>(-132.1, -3.9)*</td>
<td></td>
<td>(-19.7, 59.7)</td>
</tr>
<tr>
<td>Ustilago/smuts</td>
<td>-15.1</td>
<td>-27.9</td>
<td>0.414</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(-28.5, -1.7)*</td>
<td>(-44.2, -11.6)*</td>
<td></td>
<td>(-13.5, 16.0)</td>
</tr>
<tr>
<td>Coprinus</td>
<td>2.3</td>
<td>11.8</td>
<td>0.196</td>
<td>-2.7</td>
</tr>
<tr>
<td></td>
<td>(-52.0, 56.6)</td>
<td>(-53.6, 77.2)</td>
<td></td>
<td>(-59.3, 54.0)</td>
</tr>
<tr>
<td>Drechslera</td>
<td>-6.6</td>
<td>-27.6</td>
<td>0.983</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>(-12.3, 8.1)</td>
<td>(-47.7, -7.4)*</td>
<td></td>
<td>(-7.3, 15.5)</td>
</tr>
<tr>
<td>Periconia</td>
<td>-27.8</td>
<td>-34.8</td>
<td>0.487</td>
<td>-42.9</td>
</tr>
<tr>
<td></td>
<td>(-57.4, 1.8)</td>
<td>(-95.4, 25.8)</td>
<td></td>
<td>(-71.9, -14.0)*</td>
</tr>
<tr>
<td>Pleospora</td>
<td>122.5</td>
<td>45.6</td>
<td>0.327</td>
<td>106.5</td>
</tr>
<tr>
<td></td>
<td>(68.3, 176.6)**</td>
<td>(-74.5, 165.8)</td>
<td></td>
<td>(32.0, 180.9)</td>
</tr>
<tr>
<td>Ganoderma</td>
<td>-19.8</td>
<td>-15.6</td>
<td>0.753</td>
<td>-34.3</td>
</tr>
<tr>
<td></td>
<td>(-60.0, 20.5)</td>
<td>(-38.3, 7.1)</td>
<td></td>
<td>(-63.5, -5.1)*</td>
</tr>
<tr>
<td>Pithomyces</td>
<td>-24.4</td>
<td>-11.7</td>
<td>0.658</td>
<td>-45.8</td>
</tr>
<tr>
<td></td>
<td>(-52.0, 3.2)</td>
<td>(-75.3, 15.0)</td>
<td></td>
<td>(-45.8, 22.4)</td>
</tr>
<tr>
<td>Stemphylium</td>
<td>31.2</td>
<td>80.2</td>
<td>0.813</td>
<td>39.1</td>
</tr>
<tr>
<td></td>
<td>(-28.8, 91.1)</td>
<td>(11.1, 149.4)</td>
<td></td>
<td>(-30.3, 108.6)</td>
</tr>
<tr>
<td>Sporormiella</td>
<td>-0.3</td>
<td>45.8</td>
<td>0.286</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>(-44.3, 43.6)</td>
<td>(-24.8, 116.5)</td>
<td></td>
<td>(-5.1, 76.4)</td>
</tr>
<tr>
<td>Total spores</td>
<td>19.2</td>
<td>-35.8</td>
<td>0.182</td>
<td>32.9</td>
</tr>
<tr>
<td></td>
<td>(-18.7, 57.0)</td>
<td>(-90.1, 18.5)</td>
<td></td>
<td>(-3.1, 68.9)</td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05 **p<0.001
Table 7.15: Adjusted associations at Lag 0 between fungi taxa and lung function parameters and stratification by age group: ≤ 18 years and >18 years

<table>
<thead>
<tr>
<th>Age group</th>
<th>Fungal spore taxa</th>
<th>FEV1</th>
<th>FVC</th>
<th>FEF25%-75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 18 years</td>
<td>&gt;18 years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 332</td>
<td>n = 538</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>coeff (95% CI)</td>
<td>p-int</td>
<td>coeff (95% CI)</td>
<td>p-int</td>
</tr>
<tr>
<td>Cladosporium</td>
<td>19.0 (-10.2, 48.3)</td>
<td>0.269</td>
<td>22.8 (-9.4, 54.9)</td>
<td>0.299</td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>2.1 (-26.9, 31.0)</td>
<td>0.816</td>
<td>6.0 (-1.1, 43.1)</td>
<td>0.971</td>
</tr>
<tr>
<td>Alternaria</td>
<td>-34.6 (-99.8, 30.6)</td>
<td>0.793</td>
<td>6.5 (-53.6, 66.6)</td>
<td>0.603</td>
</tr>
<tr>
<td>Ustilago/smuts</td>
<td>-22.2 (-42.8, -32.8)</td>
<td>0.905</td>
<td>-4.2 (-20.2, 11.8)</td>
<td>0.263</td>
</tr>
<tr>
<td>Coprinus</td>
<td>-25.2 (-38.3, 32.8)</td>
<td>0.171</td>
<td>-1.7 (-83.2, 39.7)</td>
<td>0.503</td>
</tr>
<tr>
<td>Drechslera</td>
<td>-10.1 (-28.1, 7.9)</td>
<td>0.254</td>
<td>3.4 (-10.2, 16.9)</td>
<td>0.022</td>
</tr>
<tr>
<td>Periconia</td>
<td>-43.6 (-78.2, -9.0)</td>
<td>0.344</td>
<td>-61.0 (-96.6, -23.5)</td>
<td>0.011</td>
</tr>
<tr>
<td>Pleospora</td>
<td>105.6 (50.2, 170.9)</td>
<td>0.994</td>
<td>92.6 (-10.0, 195.3)</td>
<td>0.984</td>
</tr>
<tr>
<td>Ganoderma</td>
<td>-24.6 (-54.0, 4.8)</td>
<td>0.393</td>
<td>-35.2 (-64.0, -6.4)</td>
<td>0.200</td>
</tr>
<tr>
<td>Pithomyces</td>
<td>-19.3 (-49.0, 10.4)</td>
<td>0.832</td>
<td>-13.4 (-50.6, 23.9)</td>
<td>0.531</td>
</tr>
<tr>
<td>Stempthylium</td>
<td>44.0 64.0 (-39.9, 88.0)</td>
<td>0.154</td>
<td>20.6 (-65.9, 107.2)</td>
<td>0.301</td>
</tr>
<tr>
<td>Sporormiella</td>
<td>12.4 (-45.8, 70.5)</td>
<td>0.661</td>
<td>35.1 (-24.3, 112.6)</td>
<td>0.814</td>
</tr>
<tr>
<td>Total spores</td>
<td>3.8 (-37.1, 44.7)</td>
<td>0.569</td>
<td>21.4 (-19.7, 62.6)</td>
<td>0.384</td>
</tr>
</tbody>
</table>

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile; *p<0.05  **p<0.001
Table 7.16: Adjusted associations at Lag 0 between fungi taxa and exhaled nitric oxide using FeNO measures and stratification by age group: ≤ 18 years and >18 years

| Fungal spore taxa | FeNO |  
|-------------------|------|------|------|
|                   | coeff (95%CI) | coeff (95%CI) | p-int |
| **Lag 0**         | ≤ 18 years n = 286 | >18 years n = 510 |
| **Age group**     |      |      |
| ≤ 18 years        |      |      |
| >18 years         |      |      |
| **Fungal spore taxa** |      |      |
| Cladosporium      | -0.4 | 0.8  | 0.559 |
|                   | (-2.4, 1.7) | (-0.5, 2.1) | |
| Leptosphaeria     | -1.4 | 0.4  | 0.537 |
|                   | (-4.1, 1.3) | (-1.4, 2.2) | |
| Alternaria        | 4.2  | 0.1  | 0.251 |
|                   | (-1.4, 9.8) | (-3.1, 3.3) | |
| Ustilago/smuts    | 2.2  | -0.1 | <0.001 |
|                   | (0.6, 3.9)* | (-1.2, 1.0) | |
| Coprinus          | 0.6  | 2.4  | 0.717 |
|                   | (-4.4, 5.7) | (-2.3, 7.1) | |
| Drechslera        | 1.2  | 0.1  | 0.174 |
|                   | (-0.4, 2.8) | (-0.9, 1.2) | |
| Periconia         | -3.7 | -0.4 | 0.091 |
|                   | (-6.8, -0.5)* | (-3.4, 2.6) | |
| Pleospora         | -2.2 | -0.1 | 0.779 |
|                   | (-8.9, 4.4) | (-3.9, 3.8) | |
| Ganoderma         | 2.1  | 1.2  | 0.598 |
|                   | (-1.5, 5.6) | (-3.4, 5.9) | |
| Pithomyces        | 2.2  | 0.0  | 0.309 |
|                   | (-0.3, 4.6) | (-2.2, 2.3) | |
| Stemphylium       | -1.6 | -0.9 | 0.73  |
|                   | (-7.2, 3.9) | (-4.5, 2.6) | |
| Sporormiella      | 0.8  | 5.4  | 0.335 |
|                   | (-8.8, 10.4) | (-4.9, 15.6) | |
| Total spores      | 1.1  | 1.2  | 0.89  |
|                   | (-2.3, 4.6) | (-1.3, 3.7) | |

Associations estimated using generalised linear models, to account for clustering of exposures and outcomes within family units. Adjusted for age, sex, height, relative humidity, maximum temperature, and grass pollen. Coefficients can be interpreted as per increase in fungi taxa from the 75th to 90th percentile;

*p<0.05  **p<0.001
Table 7.17: Adjusted associations at Lag 0 between fungi taxa and EBC pH and EBC NOx and stratification by age group: ≤ 18 years and >18 years

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>Lag0 EBC pH</th>
<th>EBC NOx</th>
<th>p-int</th>
<th>OR (95%CI)</th>
<th>OR (95%CI)</th>
<th>p-int</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ 18 years (n= 170)</td>
<td>&gt;18 years (n = 61)</td>
<td>≤ 18 years (n = 170)</td>
<td>&gt;18 years (n = 61)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladosporium</td>
<td>Coeff (95%CI)</td>
<td>coeff (95%CI)</td>
<td>p-int</td>
<td>OR (95%CI)</td>
<td>OR (95%CI)</td>
<td>p-int</td>
</tr>
<tr>
<td></td>
<td>-0.01</td>
<td>0.08</td>
<td>0.043</td>
<td>1.0</td>
<td>1.7</td>
<td>0.368</td>
</tr>
<tr>
<td></td>
<td>(-0.05, 0.03)</td>
<td>(0.01, 0.15)</td>
<td></td>
<td>(0.9-1.2)</td>
<td>(0.7-4.0)</td>
<td></td>
</tr>
<tr>
<td>Leptosphaeria</td>
<td>0.05</td>
<td>0.02</td>
<td>0.074</td>
<td>1.1</td>
<td>1.0</td>
<td>0.611</td>
</tr>
<tr>
<td></td>
<td>(0.01, 0.09)</td>
<td>(0.01, 0.03)*</td>
<td></td>
<td>(0.8-1.3)</td>
<td>(0.9-1.2)</td>
<td></td>
</tr>
<tr>
<td>Alternaria</td>
<td>-0.09</td>
<td>0.01</td>
<td>0.285</td>
<td>1.8</td>
<td>1.2</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td>(-0.18, 0.01)</td>
<td>(-0.08, 0.11)</td>
<td></td>
<td>(1.1-3.1)*</td>
<td>(0.8-1.9)</td>
<td></td>
</tr>
<tr>
<td>Ustilago/smuts</td>
<td>-0.03</td>
<td>0.05</td>
<td>&lt;0.001</td>
<td>1.0</td>
<td>2.5</td>
<td>0.372</td>
</tr>
<tr>
<td></td>
<td>(-0.04, -0.01)*</td>
<td>(0.03, 0.08)*</td>
<td></td>
<td>(1.0-1.2)</td>
<td>(0.9-6.8)</td>
<td></td>
</tr>
<tr>
<td>Coprinus</td>
<td>-0.06</td>
<td>0.05</td>
<td>0.178</td>
<td>1.5</td>
<td>1.0</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
<td>(-0.15, 0.03)</td>
<td>(-0.07, 0.18)</td>
<td></td>
<td>(0.9-2.4)</td>
<td>(0.5-2.0)</td>
<td></td>
</tr>
<tr>
<td>Drechslera</td>
<td>-0.03</td>
<td>0.03</td>
<td>0.112</td>
<td>1.2</td>
<td>1.2</td>
<td>0.656</td>
</tr>
<tr>
<td></td>
<td>(-0.05, 0.00)</td>
<td>(-0.02, 0.08)</td>
<td></td>
<td>(1.0-1.3)</td>
<td>(1.0-1.5)</td>
<td></td>
</tr>
<tr>
<td>Periconia</td>
<td>0.03</td>
<td>0.01</td>
<td>0.495</td>
<td>1.0</td>
<td>1.1</td>
<td>0.891</td>
</tr>
<tr>
<td></td>
<td>(-0.06, 0.12)</td>
<td>(-0.05, 0.06)</td>
<td></td>
<td>(0.6-1.4)</td>
<td>(0.7-1.5)</td>
<td></td>
</tr>
<tr>
<td>Pleospora</td>
<td>0.03</td>
<td>0.21</td>
<td>0.224</td>
<td>0.7</td>
<td>0.4</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>(-0.09, 0.15)</td>
<td>(-0.06, 0.48)</td>
<td></td>
<td>(0.4-1.3)</td>
<td>(0.1-2.3)</td>
<td></td>
</tr>
<tr>
<td>Ganoderma</td>
<td>-0.11</td>
<td>-0.10</td>
<td>0.671</td>
<td>2.3</td>
<td>0.8</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>(-0.20, -0.03)*</td>
<td>(-0.28, 0.09)</td>
<td></td>
<td>(1.2-4.2)*</td>
<td>(0.3-2.4)</td>
<td></td>
</tr>
<tr>
<td>Pithomyces</td>
<td>0.04</td>
<td>0.07</td>
<td>0.87</td>
<td>1.3</td>
<td>1.0</td>
<td>0.705</td>
</tr>
<tr>
<td></td>
<td>(-0.01, 0.09)</td>
<td>(-0.29, 0.42)</td>
<td></td>
<td>(0.9-1.7)</td>
<td>(0.2-4.1)</td>
<td></td>
</tr>
<tr>
<td>Stemphylium</td>
<td>0.01</td>
<td>0.06</td>
<td>0.705</td>
<td>1.2</td>
<td>3.0</td>
<td>0.306</td>
</tr>
<tr>
<td></td>
<td>(-0.09, 0.11)</td>
<td>(-0.19, 0.31)</td>
<td></td>
<td>(0.8-1.8)</td>
<td>(1.0-9.5)</td>
<td></td>
</tr>
<tr>
<td>Sporormiella</td>
<td>-0.10</td>
<td>-0.15</td>
<td>0.428</td>
<td>1.1</td>
<td>0.5</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
<td>(-0.24, 0.04)</td>
<td>(-0.40, 0.10)</td>
<td></td>
<td>(0.6-2.3)</td>
<td>(0.1-1.6)</td>
<td></td>
</tr>
<tr>
<td>Total spores</td>
<td>-0.03</td>
<td>0.10</td>
<td>0.017</td>
<td>1.2</td>
<td>1.6</td>
<td>0.768</td>
</tr>
<tr>
<td></td>
<td>(-0.10, 0.03)</td>
<td>(0.02, 0.19)*</td>
<td></td>
<td>(0.9-1.6)</td>
<td>(0.9-2.8)</td>
<td></td>
</tr>
</tbody>
</table>

All models adjusted: age, sex, height, max temp, humidity, storage time and grass pollen. Probands only. Coefficients interpreted as per increase in fungi taxa from the 75th to 90th percentile. *p<0.05 **p<0.001
7.5 Discussion

This exploratory cross-sectional study found some evidence of associations between a range of fungal spore taxa and slightly reduced lung function parameters and increased airway inflammation. Some of these associations were lagged; and some modified by fungal sensitisation status and age. The only spore taxa found associated with a trend toward reduced lung function was *Ustilago* /smuts/ on the day of exposure and up to 3 days lag. No fungal spore taxa were found to be associated with changes in FeNO or EBC pH except where fungal sensitisation and age group were tested as potential effect modifiers. A number of fungal spore taxa were associated with increased risk of higher levels of EBC NOx at Lag 1, Lag 2 and cumulative 3-day Lag. The fungal spores with the strongest associations were associated with *Cladosporium*, *Alternaria*, *Leptosphaeria* and total spores. The association with *Alternaria* appeared to be stronger in the younger age group. I also found inconsistent associations between some fungal spore taxa (*Cladosporium*, *Alternaria*, *Leptosphaeria*, *Periconia*, *Pleospora*, *Stemphylium* and *Sporormiella*) and increased lung function parameters and reduced EBC pH at different lags.

The changes in lung volume in these models associated with fungal spore taxa exposure increasing from their 75th percentile to 90th percentile were in the range of <100ml which may not be clinically significant. However any reduction in lung function may contribute to increased respiratory morbidity.

7.5.1 Effect modification by fungal sensitisation status

Fungal sensitisation status is an important individual characteristic that may modify the effect of exposure to a potentially allergenic substance on the respiratory system. Few studies that examined the effect of outdoor fungal spore exposure on lung function have tested for such interactions, or reported stratified results (66, 253). In my analyses I found inconsistent associations when I explored fungal sensitisation as a potential effect modifier. For example, *Ustilago* /smuts/ was associated with small reductions in FEV\textsubscript{1}, FVC and FEF\textsubscript{25%-75%} at Lag 0. These associations were inconsistently held when the participants were stratified by *Alternaria*, *Penicillium*, or *Cladosporium* sensitisation status. The reduction in FEV\textsubscript{1} and FVC was stronger at Lag 0 in those sensitised to *Alternaria*, *Penicillium* or at least one fungal reagent. The reduction in FEF\textsubscript{25%-75%} was stronger but only seen in those sensitised to *Alternaria*. For those found to be sensitised
to *Cladosporium*, only an increased reduction in FVC was found. Interestingly, *Drechslera* was not associated with reduced lung function at Lag 0, but when *Alternaria* sensitisation was tested as a potential effect modifier, this fungal spore taxon was associated with reduced FEV₁ and FVC. The direction of results with *Cladosporium* was the opposite of what I expected, with associations with increased FVC at Lag 1 and FEF\(_{25\% - 75\%}\) at Lag 0. In the models stratified by fungal sensitisation these associations with increased FVC and FEF\(_{25\% - 75\%}\) remained present and, in fact, there were associations with increased FEV₁ in those sensitised to *Penicillium* and any one of the fungal reagents.

These findings add to the lung function results from Rutherford et al who examined a small sample (n=53) of asthmatic children and adults in a panel study conducted in south-east Queensland, Australia (66). They found total outdoor fungal spores (*Alternaria, Cladosporium, Ustilago, Epicoccum* and unidentified ‘other fungi’) were associated with decreased standardised peak expiratory flow rate (a person's maximum speed of expiration, as measured with a peak flow meter) in fungi sensitised adults aged >54 years during wet periods of summer (peak fungal spore season) over the two year period. In terms of comparing with the lung function parameters that I used, peak expiratory flow rate usually correlates well with FEV₁, however it has been reported that this correlation decreases in patients with asthma as airflow diminishes (289).

Fungal sensitisation has been shown to be associated with increased FeNO (257-260). My results indicated that FeNO levels were higher in those sensitised to at least one fungal spore taxa if exposed to *Cladosporium* and *Periconia*, demonstrating that in this study, fungal sensitisation acted as an effect modifier.

Sensitisation or atopy has also been shown to be associated with elevated EBC NOx (281) but my results were not consistent with this. The risk of having high levels of NOx in the EBC was higher in those who were not sensitised to the fungal reagents tested, but there was no evidence that fungal sensitisation itself was a statistically significant effect modifier. The finding with *Stemphylium* was not reliable, with the analysis generating imprecise estimates of associations.

Exposure to *Cladosporium* was associated with increased FeNO and reduced EBC pH in those with sensitisation to at least one fungal reagent; however I have not been able to find a similar study to compare this result with. I will further explore the role of *Cladosporium* later in this Discussion.
7.5.2 Effect modification by age group

Results from the age-stratified models indicate that age may be a potential effect modifier of the effects of fungal spore exposure. In those aged <18 years, *Ustilago*/smuts was associated with increased markers of airway inflammation: increased NO produced by FeNO and reduced EBC pH. In contrast, in those aged >18 years, *Ustilago*/smuts was associated with increased pH. *Drechslera* was associated with reduced FVC in those aged <18 years but *Coprinus* was associated with decreased FVC in those aged >18 years.

In this cross-sectional study ambient *Cladosporium* was associated with a trend toward increased lung function parameters even in those sensitised to the fungal reagents but, paradoxically, also associated with increased risk of high levels of EBC NOx. It is difficult to explain this inconsistency except that fungal sensitisation has been found to correlate with increased exhaled nitric oxide (258) and EBC NOx (280).

I tested if the role of age as an effect modifier may be linked with fungal sensitisation by controlling for fungal sensitisation status in age stratified models. My results indicated that, in this study, age acted as an independent effect modifier.

7.5.3 Lagged effects

Also, although in *in vitro* and *ex vivo* human studies and in *in vivo* animal studies, exposure to some fungal spores and hyphae have been found to promote airway inflammation and be associated with the onset of a range of respiratory diseases, the actual mechanisms by which fungal spores or hyphae cause adverse effects in the respiratory system have not been completely understood (69). The delayed onset of pathological changes after initial exposure may account for some of the lagged effects found in this study. The differing physiological responses between individuals may account for some of the variation in findings.

7.5.4 Exposure misclassification

Exposure misclassification should also be considered as a contributor to these findings. I cannot be certain that each participant was exposed to the same levels of fungal spores, pollen or pollution exposures counted at this single site and it is therefore impossible to gauge the generalisability of exposure. As I was considering outdoor exposure, misclassification of fungal exposure is likely to be non-differential, hence it should bias the risk estimates towards the null. The concentrations and diversity of indoor fungal
spores can be related to mould inside buildings and the levels of outdoor fungal spores that enter the indoor environment through doors and windows (290). The absence of indoor fungal spore measurements may limit exposure assessment and the possible contribution of indoor fungal spores on changes in lung function or airway inflammation and so may also be a source of exposure misclassification. However, this exposure is also likely to be non-differential and so will bias the results towards the null.

7.5.5 Other key determinants
In my analyses I attempted to control for key potential confounders and individual determinants known to affect lung function parameters and markers of airway inflammation to reduce potential errors in the estimation of the associations between outdoor fungal spores and the outcomes of interest. However there may be other potential effect modifying or mediating exposure variables that have not been taken into account such as recent or current history of respiratory illness and the aetiological agent of the illness; unmeasured exposure to ‘other’ or ‘not identifiable’ outdoor fungal spores; concurrent exposure to other pollen types; occupational exposures to outdoor fungal spores; exposures to indoor fungal spores produced by building mould or persistent damp; or time spent outdoors. Not accounting for these exposures may have resulted in over- or under-estimation of the true associations between the range outdoor fungal spore taxa and the outcomes.

7.5.6 Limitations of cross-sectional analysis
The cross-sectional approach of this analysis does not take into account day-by-day changes in lung function and airway inflammation which may provide stronger evidence of effects by fungal spore exposures. This may be of particular importance if exposure to elevated or threshold levels of fungal spores may be limited in duration and frequency.

7.5.7 Assessment of multiple exposures and multiple outcomes
Another general issue with all these analyses is that of utilising multiple comparisons. With 12 taxa being analysed, and multiple outcomes being assessed, there is a high risk of type 1 errors (rejecting the null hypotheses incorrectly). Also, I tested each taxon at up to 3 days lag and then examined possible effect modification and stratified the analyses by age (into two groups of age stratification) and fungal sensitisation status (four categories). As such, it is highly likely that there is at least one type 1 error present in these findings, and the results should be interpreted with some caution in light of this.
Nevertheless, this comprehensive analysis of lung function and markers of airway inflammation and outdoor fungal spore counts showed several positive associations with reduced lung function and increased airway inflammation, particularly with *Ustilago*/smuts, which might form a basis for future work that could incorporate a longitudinal methodology.

### 7.5.8 *Ustilago*/smuts

Overall, in this analysis, *Ustilago*/smuts appear to have the strongest and most consistent associations with reduced lung function parameters but not increased airway inflammation. Fungal sensitisation and age appear to act as effect modifiers of the associations between *Ustilago*/smuts and reduced lung function and increased airway inflammation. *Ustilago*/smuts is not commonly examined as a fungal spore taxon to be associated with changes in lung function and I have not been able to find comparable studies that specifically examined this fungal taxon. However, Atkinson and colleagues reported that *Ustilago*/smuts had a tendency to be associated with asthma emergency department presentations and hospital admissions in London, but these relative risks were not statistically significant. My research with the MAPCAH study (Chapter 6) did not find any associations between the children and adolescents hospitalised for asthma and *Ustilago*/smuts. However, significant associations were detected between *Ustilago*/smuts and the older adolescents (aged >14 years) hospitalised for asthma in the SW Sydney study (odds ratio = 1.25, 95%CI 1.03 to 1.5) (Chapter 5). The risk of outdoor fungi leading to adverse changes to the respiratory system may be higher in those who are older and those who are sensitised to fungal spores.

There is some evidence that *Ustilago*/smuts spore counts in outdoor air can be considerable as these fungal taxa are parasitic on grasses and thrive particularly well in pastures and on grains (270). Clinical studies have demonstrated that sensitisation rates in asthmatic patients are high and range between 14-52 % (291, 292). However, as sensitisation to this taxon was not tested in this study I cannot compare these rates with this high allergy risk population, nor assess whether *Ustilago*/smuts sensitisation may act as an effect modifier here. There is potential for cross-reactivity between smuts and other fungal spores that contain similar proteins (194, 217), which may account for the effect modification observed in the sensitised groups in this study.
7.5.9 *Cladosporium*

My results with *Cladosporium*, that demonstrated a trend toward being associated with increased lung function, were not consistent with the findings from the small number of studies that have examined associations between outdoor fungal spores and changes in lung function. Delfino et al found borderline significance (p<0.1) between *Cladosporium* spores and reduced evening peak expiratory flow rate PEFR (the maximum flow rate generated during a forceful exhalation, starting from full lung inflation) of -6.16 litres/minute per 1000 spore increase, in subjects that were sensitised to *Cladosporium*, over an 8-week period (279). However, these results were limited by a very small sample size (n=10) and these PEFR results are not necessarily comparable with the lung function parameters I analysed.

In Taiwan, Chen et al utilised a longitudinal follow-up approach over 10 months to examine associations between outdoor fungal spores and lung function change in 100 school-aged children. They found that *Cladosporium* spores, in higher concentrations (above 1500 spores per cubic metre) at 1-day lag were associated with decreased FVC (-0.25 litre 95%CI -0.37 to -0.13) and decreased FEV₁ (-0.23 litre, 95%CI -0.35 to -0.11) (60).

I found that *Cladosporium* was associated with a trend toward increased lung function parameters even in those sensitised to at least one the fungal reagents. Interestingly and somewhat paradoxically, I found that *Cladosporium* was associated with increased risk of high levels of EBC NOx in the total study sample but there was no evidence that fungal sensitisation acted as an effect modifier in this relationship. Also, *Cladosporium* was associated with increased FeNO and reduced EBC pH in those with sensitisation to at least one fungal reagent. A possible explanation for these divergent outcomes may be that the lung function measures used in this study were not sensitive enough to detect the airway flow changes associated with the airway inflammation.

7.6 Conclusion

The findings from these exploratory analyses are mixed but indicate that exposure to some allergenic outdoor fungal spores may be associated with changes in lung function and airway inflammation in Melbourne, Australia. Further research is needed to confirm if there are indeed associations between outdoor fungal spore exposure and airway
inflammation and changes in lung function. Future longitudinal research of larger samples is required that could identify whether there are threshold effects of the fungal spores while accounting for current sensitisation status and respiratory health status. In addition, the effect of fungal spores on lung health could vary by geographic location and population groups and so studies in different regions with a range of at-risk population groups is warranted.
Chapter 8
Overall discussion

8.1 Introduction

Asthma is a significant global public health problem. It creates a burden on health and well-being in addition to contributing to social and economic burdens on asthmatics, their families and health care systems. Poorly managed asthma in childhood and adolescence may have long-term adverse health effects that include increased risk of developing chronic adult asthma or other obstructive respiratory conditions.

Asthma exacerbations can be debilitating or life-threatening, and if severe and not responsive to self-administered medication treatment, will result in the need for medical care at the general practitioner, emergency department or in acute care in a hospital. It has been established that asthma exacerbations may be triggered by numerous environmental factors. Among the most common environmental factors that have been researched to date are respiratory viral infections, pollen, ambient air pollutants, house dust mites, tobacco smoke, and pet dander.

Limited research has examined the contribution of fungal spores as a possible trigger for asthma exacerbation, increasing airway inflammation or having adverse effects on lung function. Most research has focussed on exposure to indoor fungal spores related to building construction problems, chronic dampness in homes, and indoor moulds (58, 293, 294), particularly in relation to children’s respiratory health. Some of this focus seems to stem from the need to provide safe indoor environments in homes, schools, offices and other indoor public places (295).

However, the identifiable fungal taxa and concentrations found inside buildings usually differ from those found outdoors due to different sources and seasonal influences. So these findings from indoor fungal spores studies have not been generalisable across outdoor exposure settings. The fungal spores found outdoors grow on vegetation (grasses, bushes, trees) and so are ubiquitous, demonstrate high diversity, can be highly abundant during different seasons, and it is not possible to avoid inhaling them when spending time outdoors (1, 2, 75). Many fungal spores and their associated hyphae and
fragments contain allergenic proteins and are small enough to be inhaled deep into the respiratory airways, potentially mediating allergic reactions or releasing mycotoxins that affect the airway epithelium lining (296). These reactions may trigger airway inflammatory responses (69, 297), asthma exacerbations in vulnerable individuals (33) or may contribute to compromised lung function (33, 60). There are major gaps in understanding these relationships, especially in relation to assessing which taxa of outdoor fungi are the most important contributors to asthma exacerbations, whether the risks associated with these outdoor fungal spores vary between geographic locations, the individual risk factors that may modify potential effects of outdoor fungal spores, other environmental factors that may interact with outdoor fungal spores, and the timing of respiratory effects. A better understanding of their role in these respiratory effects could contribute to developing targeted strategies to prevent asthma exacerbations. Little is known about the mechanisms by which outdoor fungal spores may trigger asthma exacerbation. It is biologically plausible that fungal spore exposure may trigger airway inflammation as allergens triggering immune responses and also as chemical irritants. They may also contribute to lower lung function. These may be preclinical markers of airway obstruction associated with asthma exacerbation.

In my PhD research I aimed to address major gaps in our understanding of these complex relationships and provide evidence that may assist in informing future research, the development of appropriate and targeted management plans for individuals and possibly contribute to identifying if an outdoor fungal spore monitoring and warning system is needed.

My PhD research aimed to investigate the effect of exposure to outdoor fungal spores on: (1) child and adolescent asthma hospitalisations; (2) lung function; and (3) airway inflammation.

My specific objectives were to:

1. Systematically synthesise the evidence on outdoor fungal spores as triggers of childhood asthma exacerbations that resulted in attendance to a primary care service, an emergency department or admission to a hospital for medical care. (Research Question 1)
2. Investigate the associations between short term exposure to outdoor fungal spores and child and adolescent asthma hospitalisations, and explore if any associations were modified by:

- high levels of air pollutants or grass pollen; (Research Questions 2 & 3)
- age group; (Research Questions 2 & 3)
- sex; (Research Questions 2 & 3)
- presence of human rhinovirus respiratory infection (Research Question 3);
- fungal sensitisation status (Research Question 3)

3. Investigate if there were associations between short term exposure to outdoor fungal spores and lung function and airway inflammation and explore if any associations were modified by:

- high levels of air pollutants or grass pollen;
- age group; or
- fungal sensitisation status (Research Question 4)

I have discussed my findings in detail within each individual results chapter and summarised them in the concluding chapter (Chapter 9). In this discussion chapter a summary of the main findings within the context of current research will be presented. A summary of this thesis’ methodological strengths and limitations and review of general methodological and analytical considerations that may influence interpretation of my findings will be discussed.

8.2 Summary of thesis findings

8.2.1 Research question 1: Systematic appraisal and synthesis of the literature on associations between outdoor fungal spores and child and adolescent asthma health service attendances

In order to achieve my first objective, I undertook a systematic review (Chapter 3) of the current epidemiological evidence of the associations between outdoor fungal spore and asthma related health care service attendance. This is the only published systematic review on this topic at the time of writing this thesis. This systematic review found that there was strengthening evidence that asthmatic and sensitised children and adolescents may be susceptible to severe asthma exacerbations resulting in hospitalisation when
exposed to a range of outdoor fungal spores. This review also found that only a small number of studies, most of which were ecological in design, have been conducted in a limited range of geographic settings located principally in the northern hemisphere (Canada, USA, England). The diverse classifications of fungal spores used for exposure assessment contributed to heterogeneity of the included studies that precluded a meta-analysis to be undertaken.

The Fungi Kingdom constitutes >100,000 species and is comprised of morphologically diverse organisms, of which, only about 80 species have been identified as allergenic (3, 217). This systematic review found that it was limiting to group fungal taxa together into phyla when examining potential associations for a number of reasons. Grouping fungal taxa on the basis of their sporulation mechanism (the traditional classification system used until 2006) (3) dismissed the differences in allergenic potential of the different fungal spore taxa, and the effect of one fungal taxon may have overridden the effect of another fungal taxon. Some studies did not specify which fungal taxa were included in each “grouping” or phylum and so comparing results was not possible. The studies which undertook analysis by fungal taxon found associations between some fungal taxa and asthma hospitalisations, but these effects were diminished when they grouped the taxa based on the older classification system (79, 249).

An important finding from my review was that little is understood about the potential interactions between outdoor fungal spores and individual characteristics such as age or sex; and other recognised triggers of asthma exacerbations such as respiratory viruses, air pollution and pollen.

Another key gap that I identified in this review and broader literature searching was that research reported from Australia was based on the hypothesis that the four major taxa of fungi identified in research from North America, Canada, United Kingdom and Europe (Alternaria, Cladosporium, Penicillium and Aspergillus) were likely to be also associated with asthma exacerbations or hospitalisations in Australia. However, given the different climate and vegetation types in Australia compared to the northern hemisphere, this presumption may be missing other fungal taxa which may be associated with asthma symptoms. In fact, in Australia very little is known about whether outdoor fungal spores contribute to asthma exacerbations at both the population and individual levels.
This leads to my second objective that aimed to investigate if there were associations between short term exposure to a range of allergenic outdoor fungal spores and child and adolescent asthma hospitalisations at both the population and individual levels. In the next section I will separate my second objective into two research questions that aimed to address this objective.

8.2.2 Research question 2: At the population level, are short term exposures to outdoor fungal spores associated with child and adolescent asthma hospitalisations?

For the first research question of my second objective, I explored whether there were any associations between a range of outdoor fungal spores and child and adolescent asthma hospitalisations in the south west region of Sydney. I used de-identified daily hospital records from a 5 year period that I examined using a bi-directional time-stratified case crossover analysis (Chapter 5). I examined potential confounding by grass pollen and air pollutants. I also explored a range of potential effect modifiers: age, gender, air pollutants and grass pollen. To the best of my knowledge, this is the first study to explore whether they were any associations between a range of allergenic outdoor fungal spores and asthma hospitalisations in Australia at the population level.

I found associations between some allergenic fungal spore taxa and child and adolescent asthma hospitalisations with evidence of some lagged effects. I found associations with *Coprinus* at Lag 0, Lag 1 and cumulative 3-day lag; *Chaetomium* at Lag 2; *Cerebella* at cumulative 3-day lag. These results are summarised in Table 8.1.

In these analyses, age group acted as an effect modifier with *Coprinus* and *Ustilago/smuts*. Significant positive associations with *Coprinus* were found among those aged 2 to 13 years with stronger associations with *Coprinus* among those aged 14 to 18 years. Significant positive association with *Ustilago/smuts* was found for those aged 14 to 18 years. Sex was also an effect modifier with significant associations with *Cladosporium*, *Coprinus* and total fungi among girls. Air pollutants and grass pollen did not act as effect modifiers in these analyses.
8.2.3 Research question 3: At the individual level, are there associations between short term exposure to outdoor fungal spores and child and adolescent asthma hospitalisations?

For the second research question of my second objective, I explored whether there were any associations between a range of outdoor fungal spores and a recruited sample of 644 child and adolescent asthma hospitalisations in Melbourne (298) using a bi-directional time-stratified case crossover analysis. I examined potential confounding by grass pollen and air pollutants. I also explored a range of potential effect modifiers: age, gender, presence of human rhinovirus (HRV) infection at time of admission, fungal sensitisation status, air pollutants, and grass pollen. This is the first published study to explore whether there were any associations between outdoor fungal spores and asthma hospitalisations at the individual level in Australia. It is also the first published study that has explored associations between outdoor fungal spores and asthma hospitalisations that accounted for HRV infection and fungal sensitisation status.

In this study there were significant associations with *Alternaria* at Lag 0, Lag 1, Lag 2 and cumulative 3-day lag; *Leptosphaeria* at Lag 0 and cumulative 3-day lag; *Coprinus* at Lag 0, Lag 1 and cumulative 3-day lag; *Drechslera* at Lag 0, Lag 1, Lag 2 and cumulative 3-day lag; *Stemphylium* at Lag 1; *Periconia* at Lag 3; and total spores at Lag 0, Lag 3 and cumulative 3-day lag. These results are summarised in Table 8.1.

In this study sensitisation to *Cladosporium* acted as an effect modifier. I found significant associations with *Alternaria, Coprinus, Drechslera* and *Stemphylium* in those who were found to be sensitised to *Cladosporium* (Chapter 6). These results are summarised in Table 8.1. Air pollutants, grass pollen and HRV infection did not act as effect modifiers in these analyses.

The results from these two studies provide some evidence that a number of outdoor fungal spores may be contributing to asthma hospitalisations in south-eastern Australia. The associations with *Alternaria* in Melbourne are supported by international research that has found ambient *Alternaria* levels to be associated with asthma exacerbations and hospitalisations (79, 80, 102, 299); however this finding was not replicated in the south-west Sydney study despite mean *Alternaria* counts being higher in this region. The lack
of association with *Cladosporium* in both studies (except for with girls in south-west Sydney) has been supported in other large time series studies, which analysed exposure by individual fungal taxa, in the UK (79) and Canada (78, 245) but not in other studies from Canada (244). The un-stratified associations with *Coprinus* are interesting and consistent between my two studies and have been supported by findings from research in London (79) but other supporting studies have not been published to date. It is possible that other studies may have included *Coprinus* in the phylum Basidiomycetes or Basidiomycota when they reported their findings as it is a prevalent spore taxa found in outdoor air, but the absence of explicit exposure definitions limited comparability of results.

Table 8.1: Summary of significant associations found in the SW Sydney and Melbourne studies

<table>
<thead>
<tr>
<th>Fungal spore taxa</th>
<th>SW Sydney Significant associations</th>
<th>Effect modification</th>
<th>Melbourne Significant associations</th>
<th>Effect modification</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alternaria</em></td>
<td>None</td>
<td>None</td>
<td>Lag 0, Lag 1, Lag 2, cumulative lag</td>
<td><em>Cladosporium</em> sensitisation</td>
</tr>
<tr>
<td><em>Cerebella</em></td>
<td>Cumulative lag</td>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Chaetomium</em></td>
<td>Lag 2</td>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Cladosporium</em></td>
<td>None</td>
<td>Girls</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><em>Coprinus</em></td>
<td>Lag 0, Lag 1, cumulative lag</td>
<td>Aged &lt;13 yr Girls</td>
<td>Lag 0, Lag 1, cumulative lag</td>
<td><em>Cladosporium</em> sensitisation</td>
</tr>
<tr>
<td><em>Drechslera</em></td>
<td>None</td>
<td>None</td>
<td>None</td>
<td><em>Cladosporium</em> sensitisation</td>
</tr>
<tr>
<td><em>Leptosphaeria</em></td>
<td>-</td>
<td>-</td>
<td>Lag 0, cumulative lag</td>
<td>None</td>
</tr>
<tr>
<td><em>Periconia</em></td>
<td>Lag 1</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><em>Stemphylium</em></td>
<td>None</td>
<td>None</td>
<td>None</td>
<td><em>Cladosporium</em> sensitisation</td>
</tr>
<tr>
<td>*Ustilago/<em>smuts</em></td>
<td>None</td>
<td>Aged &gt;13 yr</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*: not included in this study; * larger odds ratio than <13 yr
Asthma hospitalisation is the severe end of the asthma exacerbation spectrum. If outdoor fungal spores could trigger this outcome, then it is possible that this is due to induction of respiratory changes that are thought to contribute to asthma exacerbation. Inflammation in the airways is thought to be a contributor to airflow limitation, asthma pathogenesis and asthma exacerbation (11). The presence of elevated levels of airway inflammatory biomarkers may be a signal of inflammation in the respiratory system. To date, no published studies have examined outdoor fungal spore exposure and markers of airway inflammation.

Airway obstruction associated with asthma is detected by reductions in lung function parameters. Few observational studies have examined associations between exposure to outdoor fungal spores and lung function in children (60-62) and adults (66) and their results indicated a trend toward adverse effects. However no studies have accounted for potential effect modifiers such as fungal sensitisation status in non-asthmatic individuals.

In the next section summary findings from my study that explored whether short-term exposure to outdoor fungal spores was associated with markers of pre-clinical airway obstruction will be discussed.

8.2.4 Research question 4: Are there associations between short term exposure to outdoor fungal spores and lung function or airway inflammation?

In this study I found those higher ambient levels of *Ustilago*/smuts at Lag 0, Lag 2 and cumulative 3-day lag; *Drechslera* at Lag 2; and *Pithomyces* at Lag 0 were associated with lower FEV₁, FVC and FEF₂₅%-₇₅%. Higher levels *Ganoderma* at Lag 0 and Lag 1 was associated with lower FVC. Higher levels of *Leptosphaeria* were associated with lower FEF₂₅%-₇₅% at Lag 3.

In those with any fungal sensitisation, higher levels of *Ustilago*/smuts at Lag 0 were associated with lower FEV₁ and FVC. Higher levels of *Drechslera* were associated with lower FEV₁ and FVC. Higher levels of *Pithomyces* were associated with lower FEV₁. These associations were stronger in fungi sensitised individuals compared to non-sensitised individuals.

No fungal spore taxa were found to be associated with markers of airway inflammation (increased fractional exhaled nitric oxide (FeNO) or decreased exhaled breath condensate
(EBC) pH levels) except when fungal sensitisation status or age group were considered as effect modifiers. Exposure to *Cladosporium* was associated with higher FeNO levels and reduced EBC pH in those with fungal sensitisation. Exposure to *Ustilago* /smuts was associated with increased FeNO and reduced EBC pH in children and adolescents. There was some evidence of instantaneous and lagged effects with exposure to *Cladosporium* at Lag 0 and cumulative 3-day lag; *Drechslera* and Lag 0 and Lag 1; and *Ganoderma* at Lag 0 and Lag 2 and increased odds ratio of higher EBC nitric oxides (NOx). There were lagged effects associated with *Alternaria* at Lag 1 and cumulative 3-day lag; *Leptosphaeria* at Lag 2; and total spores at Lag 1, Lag 2 and cumulative 3-day lag and increased odds ratio of higher EBC nitric oxides (NOx).

### 8.3 Methodological strengths and limitations

The methodological issues relevant to each research objective were discussed in the relevant results chapters (Chapters 3, 5, 6 and 7). The overall strengths, limitations of my thesis in relation to methodology used in general will be discussed in this section.

To investigate Objectives 2 and 3 I undertook three separate research projects using three different data sources and study designs. As there is some overlap between the three studies I will discuss the overarching strengths and limitations in sections that relate to each individually and/or together. I will discuss the aspects of (1) study designs; (2) exposure assessment of outdoor environmental factors; (3) variations in definitions of exposures and outcomes; (4) residual confounding issues; (5) generalisability of the results.

#### 8.3.1 Study designs

When considering which study designs I could use to examine whether there are any associations between outdoor fungal spores and asthma hospitalisations I considered the ethics of the research, nature of the range of exposures I was examining, the types of study designs that had been previously used and how I could build on the knowledge base already established.
As I have described in my systematic review (Chapter 3), only a few published studies have described associations between outdoor fungal spores and child and adolescent asthma hospitalisations, of which, the majority were ecological time-series followed by observational cross-sectional studies and correlational studies that lacked control groups. As environmental epidemiology statistical methods have progressed with the case crossover design being used more widely in air pollution research, I used this design to test whether there were significant associations between short term exposure to outdoor fungal spores and child and adolescent asthma hospitalisations, and explore potential effect modifiers.

### 8.3.1.1 Strengths and limitations

In Chapter 5 I commenced my doctoral research with an ecological case crossover study utilising five years of data sourced from administrative hospital records, purposively collected aeroallergen samples and existing data routinely collected by meteorology and air quality monitoring stations. This study design had not been used previously in research examining outdoor fungal spores and asthma hospitalisations, so it was an important first step in exploring whether any associations may exist between a range of fungal spore taxa and child and adolescent asthma hospitalisations.

There are a number of strengths associated with this study design. As exposure to elevated levels of outdoor fungal spores is usually short-term, the case crossover design is well-suited for studying the effects of this exposure on a rare outcome, such as an asthma hospitalisation. The case crossover design meant that the cases served as their own controls thereby reducing the risk of confounding related to unknown differences between individual characteristics. The case crossover design also has the property of accommodating seasonal effects if properly specified. I specified a bidirectional time-stratified approach which controlled for bias due to time trend and confounding by season and day of the week (300, 301). The utilisation of pre-existing monitoring and administrative data that were originally collected for non-research purposes were not subject to recall or misclassification bias.

There were a number of limitations associated with this ecological case crossover study. These included the small number of study sites (hospitals and monitoring stations) over
a large geographic region which may have limited the power to detect significant associations between a wider range of fungal spore taxa and asthma hospitalisations. Although the cases acted as their own controls, the ecological nature of this study design lacked individual data (such as fungal sensitisation status, or presence of respiratory infection) which may have the potential to act as effect modifiers.

The results of this ecological study indicated that some taxa of outdoor fungal spores were weakly associated with child and adolescent asthma hospitalisations in this region of Sydney, however I did not examine individual level data. Therefore I established my next research question within the Melbourne Air Pollen Child and Adolescent Health (MAPCAH) case crossover study that I reported in Chapter 6. This study design was strengthened by the availability of individual level data on a sample of children and adolescents who were admitted to hospital with severe asthma exacerbation. To overcome possible detection bias in this study sample, participants were recruited after they had a doctor diagnosis of asthma. The recruitment process, however, may have been subject to selection bias but the representativeness of this study sample was examined by the study’s chief investigators and, although the participants were slightly older than the non-participants (mean age difference was 0.8 year), there were no differences by sex or the seasonal distribution of hospitalisations (302). The individual level data in this study included detailed analysis of the type of respiratory infection and fungal sensitisation status each participant may or may not have had at time of admission. This study also purposively collected daily counts on 14 fungal spore taxa and grass pollen. To the best of my knowledge, this is the first case crossover study published that has simultaneously examined associations between exposures to outdoor fungal spores and asthma hospitalisations to determine whether age, gender, presence of human rhinovirus infection, fungal sensitisation, air pollutants or grass pollen are effect modifiers.

My final study was nested in the Melbourne Atopy Cohort Study (MACS) which is a birth cohort study of children (probands) with a family history of allergic diseases, to investigate whether there were any associations between short-term exposure to outdoor fungal spores and the presence of airway inflammation or lower lung function (Chapter 7). Although MACS is longitudinal cohort study I used it for cross-sectional analysis
only. The MACS probands have been followed up frequently from birth to 18 years of age and so comprehensive data have been collected on each proband over time. This provided important historical data that enabled cross-checking for data cleaning and confirming familial linkages. At the 18-year follow-up, which was conducted during 2009 to 2011, probands, parents and siblings were invited to provide lung function, FeNO and allergic sensitisation data. This provided me with a large sample of nearly 900 participants for analysis. The availability of objective measurements of lung function, FeNO, EBC and skin prick tests has added another dimension to the analysis of the potential effects of outdoor fungal spores on asthma exacerbations.

This study was limited by the lack of outdoor fungal and pollen exposure data for lung function measures over multiple time points. Hence it is a cross-sectional analysis of a single point in time for each participant. Respiratory health status is in a dynamic state as it is exposed to changing conditions both externally and internally. Hence results would have been more reliable if multiple exposure measures alongside repeated lung function readings and measures of airway inflammation over longer periods had been obtained.

In summary, these studies have continued to build the epidemiological justification for the need for further research which should include controlled trials and longitudinal follow-up.

8.3.2 Exposure assessment of outdoor environmental factors: outdoor fungal spores, grass pollen, air pollutants, meteorological variables

Accurately assessing the exposure of human biological systems to outdoor environmental factors would be ideal when assessing associations between exposures and health outcomes in order to reduce exposure and spatial misclassifications, and subsequent introduction of bias or attribution errors. However, this is usually not economically or technically feasible, particularly at the population health level (206). In environmental epidemiology, particularly when examining outdoor environmental factors, most studies extrapolate exposure based on data from monitoring stations and/or sampling sites in one or more locations; use modelled exposures; or use proxy measures.
A strength of my doctoral research was the inclusion of daily outdoor fungal spore and grass pollen data over relatively long periods of time: 2 years in Melbourne and 5 years in Campbelltown in south-west Sydney, using similar aeroallergen monitoring methodology. Currently, in Australia, outdoor fungal spore data are not routinely collected as part of aerobiology monitoring and so the availability of longitudinal data is limited.

The fungal spore and grass pollen data in the three studies were all collected using the same type of measuring instrument, the Burkard volumetric trap. Both sampling sites were 11 metres above the ground and clear of building and tree obstructions and met the guidelines of the World Allergy Organisation (271). The use of similar measuring methodology reduces the risk of measurement bias between the two sites. The fungal spores and pollen grains were identified by trained botanical and mycological technicians at each site using accredited identification manuals (272) and reference slides which minimised ascertainment bias. The Burkard volumetric trap has been found to be a superior instrument for sampling particles less than 10μm in aerodynamic diameter than the rotary impact samplers) (208, 209). This is important as this is the size of particles that are most likely to be respirable deep into the airways and have effects on airway integrity (211). The selection of the appropriate instrument is crucial to reducing measurement and ascertainment bias (206, 207).

It is important to note that despite the similarities in the measuring methodology between the two sites, the fungal spore counts varied greatly. Although maximum temperature, rainfall and relative humidity were quite similar in scale, the fungal spore counts in Melbourne were much lower than those in Sydney; or indeed, compared to previous studies conducted by Mitakakis et al in the same sampling site in Melbourne (71). The lower counts could relate to the identification of fewer fungal spore taxa in Melbourne compared to Sydney. Also the data I used identified fewer fungal spore taxa compared to the 29 fungal spore taxa and 5 spore groups reported by Mitakakis et al in their fungal spore calendars for Melbourne in 1993 (71). Another contributing factor may be related to the lack of data on “other” or “not-identified” spores found in the samples. Also, these differences may be related to ascertainment or measurement errors in one or both sites. The low fungal spore counts in Melbourne could have limited the power to detect associations between fungal spore taxa and asthma hospitalisations, lung function or airway inflammation.
As has been discussed in individual chapters, there are limitations associated with applying measures from one daily measurement (24 hour average) obtained at one aeroallergen monitoring station to be representative of the ambient concentrations across the south-west Sydney and Melbourne metropolitan areas. There were marked daily fluctuations of outdoor fungal spores and pollen grains which may have originated from different types of vegetation, such as grasslands, trees, shrubs, or organic litter. The release of fungal spores and pollen grains into the atmosphere is altered by meteorological conditions (temperature, humidity, rainfall) and fungal spores and pollen grains are generally distributed by the prevailing winds (speed and direction) (1). The height of the source vegetation and relative mass of fungal spores may also affect their dispersion. Research has indicated that sampling height may influence the detection of different types of fungal spores. Ground samplers are more able to capture spores produced by low growing vegetation (for example, smuts, *Penicillium/Aspergillus*, basidiospores), whereas rooftop samplers may be biased toward fungal spores sourced from trees (for example, *Alternaria*, ascospores) (303). Daily averages may not reflect the fluctuations in fungal spore and grass pollen grain levels related to the changing meteorological conditions during a day. To date, modelling of the distribution of outdoor fungal spores and grass pollen grains is incomplete and not reliable (205, 304), so I could not apply predictive exposure models. In the absence of sound modelling data, it is possible that exposure to outdoor fungal spores and grass pollen may be non-differential but there may be a risk of exposure misclassification bias.

The absence of data on ‘other’ or ‘not-identified’ fungal spores in the samples could have further limited identification of potential associations with, or confounding effects of, fungal spore taxa not identified and not included in these analyses. Previous studies have reported associations between ‘other’ or ‘not-identified’ fungi with lower lung function (8, 66) and asthma hospitalisation (249). Future research should include these ‘other’ fungal spores in order to continually improve our understanding of the contributions of outdoor fungal spores to a range of health outcomes.

It would be ideal to determine exposure to outdoor environmental factors on an individual basis to minimise exposure misclassification. However, personal monitors for fungal spore exposure are expensive and intrusive. The analysis of daily individual data is highly resource intensive (305) and currently not feasible. Studies using personal monitors usually involve a small sample to examine associations with relatively common
outcomes or a small sample of high-risk individuals with frequent exposure (213, 306). These studies, conducted in rural areas of New South Wales (NSW) in Australia, compared personal air monitors to centrally located spore traps (positioned according to the World Allergy Organization guidelines) and reported differing results. In inland NSW Mitakakis et al found the spore counts to be comparable (306); while in coastal and subtropical NSW, Green et al detected significant differences between the sampling systems with personal inhalation between 2 fold and 10-fold higher than the estimates from the stationary sampling trap (307). The variations between the two sampling techniques were attributed to the differing operation, collection and quantification methods associated with each device type (307).

I did not have data on exposure to indoor fungal spores (for example, home, school or workplace) for this research and this may affect the individual exposure assessment. It is possible that the participants may have been unequally exposed to fungal spores. However if indoor and outdoor fungal spores have similar effects on asthma hospitalisation risk, then this misclassification is likely to be non-differential, hence it should bias the risk estimates toward the null.

I used data from one air quality monitoring station and meteorological monitoring station in each of the separate studies as the data from these stations were the most complete and located closest to the aeroallergen monitoring stations. The air quality and meteorological conditions of those sites may not have been accurate for all participants, but as for the aeroallergen exposure, the misclassification is likely to be non-differential and bias the results towards the null.

8.3.3 Analysis using multiple fungal taxa
A methodological strength of this research was the identification of multiple taxa of fungal spores by the trained botanical and mycological technicians. This enabled analysis of the contribution of outdoor fungal spores to asthma hospitalisations, airway inflammation and lung function from the perspective of individual taxa rather than grouped as phyla (such as Basidiomycota or Ascomycota) or analysed as “total fungi”. As not all fungal spores have the same pathophysiological or allergic potential, this has
reduced the chance that the effect of one fungal taxon overrides the effects of other taxa. It also means that these results can be interpreted from the perspective of associations with identified fungal taxa rather than associations with fungal phyla of which the constituent taxa have not been clearly described. However, due to the fungal classification having undergone substantial changes over the last two decades with recognition that the traditional grouping of fungi based on their sporulation patterns into phyla does not acknowledge the genetic similarities between fungal taxa (3), it has been challenging to compare my findings with other studies that have classified the fungal spores only into phyla. The differences in defining fungal spore exposure between studies could account for some of the variation in the reported findings.

The analysis of associations between multiple fungal taxa and multiple outcomes is not without its limitations. With 12 to 20 fungal taxa being analysed (across the three studies) with multiple outcomes and stratification analyses there is the elevated risk of incorrectly rejecting the null hypothesis (Type 1 statistical error) (287). With this consideration, some results needed to be interpreted with a degree of caution. I chose not to group the fungal spores into phyla in order to increase the power of the exposure variable, as I was concerned that this would mask the contribution of the individual taxa.

Nevertheless, in this exploratory work, the comprehensive analyses of a number of fungal taxa in each study provided several positive associations which might form a basis for future work that could incorporate larger sample sizes and longitudinal methodologies.

8.3.4 Definition of asthma hospitalisation

There were some variations in the definition of asthma hospitalisation between the two studies that explored whether there were associations between outdoor fungal spores and asthma hospitalisations due to coding differences between hospitals. In Chapter 5 of this thesis, I included data from three public hospitals in south-west Sydney. Interestingly, although they operated under the authority of the New South Wales Government Department of Health, different departments in the hospitals used three different diagnostic codes in their coding databases. The definitions for asthma hospitalisation was an asthma admission based on any one of the following three codes: (1) International Classification of Disease, 10th Revision Australian Modification (ICD10-AM) (308)
with a principal diagnosis code of Asthma (J45) or Status asthmaticus (J46); (2) SNOMED ® CT-AU (309) with a principal diagnosis code of Asthma (195967001) or Asthma NOS (266365004); (3) International Classification of Disease, 9th Revision (ICD-9) (310) with a principal diagnosis code of Extrinsic asthma (493.0); Intrinsic asthma (493.1); Asthma unspecified (493.9); Chronic obstructive asthma (493.2); Other forms of asthma (493.8); or Cough variant asthma (493.82). SNOMED codes were provided with ICD-10 AM codes for each hospitalisation episode to validate the asthma diagnosis. In Chapter 6 of this thesis asthma hospitalisation was defined as an asthma admission based on the International Classification of Disease, 10th Revision (ICD-10-AM) with a principal diagnosis code of asthma (J45)(308).

Most international studies have defined the asthma hospitalisation based on clinical coding in the databases using ICD-9 (48, 78, 79, 244, 246-248, 250), ICD-10 (159), or both ICD 9 and ICD-10 (47, 80) due to the overlapping the study timeframe and the introduction of the revised ICD-10 coding system. Others did not provide a definition other than ‘asthma attack’ (251), ‘acute asthma’ (311), ‘asthma admissions’ (249), or ‘self-report of asthma hospitalisation’ (81). In terms of asthma diagnosis codes there is no difference between ICD-10 and ICD-10-AM.

The strength of using the validated hospitalisation diagnosis codes is that all asthma admissions would have been captured as this is an important administrative database for hospital funding. However, there may have been some diagnostic misclassification which should have been non-differential. I also excluded children aged less than 2 years to reduce the risk of diagnosis misclassification bias related to the difficulty in accurately diagnosing asthma in this age group.

8.3.5 Detection of fungal sensitisation

Fungal sensitisation has been associated with elevated risk of asthma exacerbation, severe asthma and asthma hospitalisations and so it is likely to be an important effect modifier of exposure to outdoor fungal spores and respiratory health. A strength of the studies in Chapter 6 and Chapter 7 was the inclusion of fungal sensitisation data from skin prick testing for individual participants. In Chapter 6 MAPCAH participants were tested for sensitisation to *Alternaria* and *Cladosporium*. In Chapter 7 MACS participants were tested for sensitisation to *Alternaria, Cladosporium* and *Penicillium*. Very few studies that have examined outdoor fungal spores and asthma hospitalisations (81), lung
function or airway inflammation have included this important risk factor (6, 218, 238) and none to date have compared those sensitised with those not sensitised at the time of hospitalisation or respiratory testing.

Allergic sensitisation can be detected by both skin prick testing and serum specific-IgE. Some reports suggest that serum specific-IgE may be more sensitive to detecting clinically important sensitisation (119). However there is evidence that both methods are equivocal for detecting sensitisation, particularly in younger children, and that both methods may be complement each other when examining and understanding a person’s allergy status (224, 312). Moreover, skin prick testing is efficient and much less invasive than blood testing for serum specific IgE and some studies report that skin prick testing has a better positive predictive value (313-315). The lack of serum specific IgE measurements in MAPCAH and MACS may not have limited the detection of clinically relevant allergic sensitisation. However, testing for fungal sensitisation by skin prick tests or blood tests for specific IgE is challenging as there are currently no reference standards for the fungal reagents despite there being a wide range of extracts of a limited range of fungal allergens commercially available (215, 218). This may have limited standardised detection of allergic sensitisation within and between the MAPCAH and MACS studies, but the difference should be non-differential.

8.3.6 Testing for lung function and markers of airway inflammation

A strength of the MACS study was the collection of objective and validated lung function measures using spirometry. These parameters were comparable with those reported in other studies. Another strength of the MACS study has been the collection of objective measures of markers of airway inflammation using two non-invasive methods: fractional exhaled nitric oxide (FeNO) complemented by exhaled breath condensate (EBC) nitric oxides and pH. The FeNO is thought to reflect inflammatory conditions in the airway walls and alveolar compartments; and the EBC NOx and EBC pH provide markers of the presence of oxidative stress that plays an important part in allergic airway disease pathogenesis (316). To the best of my knowledge this is the first study that has examined outdoor fungal spore exposure and levels of markers of airway inflammation, so I have been unable to compare my findings.

A limitation of the MACS EBC samples was related to the long storage time as biomarkers in samples may degrade regardless of storage under conditions that are
8.3.7 Residual confounding

Although I adjusted for a range of biologically plausible confounding factors (meteorological conditions, air pollution and grass pollen) in the south-west Sydney, MAPCAH and MACS analyses, it is possible that some important unknown confounders may have been excluded in the regression models. Recent European research that analysed temporal and spatial variations in ambient *Alternaria* spores from 23 sites located in seven countries and across four major biogeographical regions reported that local climate, vegetation patterns, landscape management and annual meteorological variations are important parameters governing overall spore concentrations (317). Local characteristics, such as climate, landscape management, seasonal changes in vegetation types, may affect or influence an individual’s choice as to where they live, go to school, participate in outdoor activities or what occupation they undertake. As a result, it is possible that these local characteristics may affect the risk of asthma exacerbation, airway inflammation or lower lung function while also influencing the types and concentrations of ambient fungal spores. Hence some of these factors may act as unknown confounders. Any underlying residual confounding may have contributed to the inconsistent results between the studies reported in this thesis and other published research.

8.3.8 Cross-reactivity between fungal species

Cross-reactivity is the ability of the immune system to recognise similarities between different allergens, such that antibodies produced against one allergen will also react against another similar allergen. Although I did not examine cross-reactivity between fungal species specifically in this research, this may be an underlying factor that could contribute to some of the variable results between these studies and other research. Over the last five years a number of international reviews of fungal allergenicity and cross-reactivity (6, 194, 215, 217, 218) have highlighted the gaps in understanding in this area and the under-recognised contributions of fungal spores to allergic diseases, especially allergic airway diseases. Cross-reactivity between some fungal allergens has been demonstrated with *in vitro* research but the clinical relevance of this phenomenon requires further research (194). Within my own thesis findings, the potential for cross-reactivity between fungal species may have been observed in the findings from
MAPCAH and MACS analyses. In the MAPCAH study I saw stronger associations between exposure to ambient \textit{Alternaria, Coprinus, Drechslera} and \textit{Stemphylium} in those with \textit{Cladosporium} sensitisation than those not or those with \textit{Alternaria} sensitisation. In the MACS study, in general terms, I noted that exposure to ambient \textit{Ustilago}/smuts, \textit{Drechslera}, and \textit{Pithomyces} were associated with lower lung function parameters in those who were sensitised to \textit{Alternaria, Cladosporium} and/or \textit{Penicillium}.

\section*{8.4 Generalisability of the results}

The results of my three studies are based on data obtained in two different urban areas in south-eastern Australia and may not be generalisable to other regions which have different climates (daily and annual), land uses, land management practices, air pollution levels and other environmental and social factors.

In the next chapter I will present the salient findings of my thesis, demonstrate how they fill knowledge gaps and how they contribute to the field of outdoor fungal spore exposure and asthma exacerbations, lung function and airway inflammation. Within this context I will present clinical and public health implications, recommendations for future research and conclusions.
Chapter 9

Conclusions, implications and recommendations

This chapter provides a summary of the salient findings of my thesis, discusses how these results fill some knowledge gaps and how they contribute to the field of outdoor fungal spore exposure and asthma exacerbations, lung function and airway inflammation. Within this context, implications for clinical management and public health policy are presented. This is then followed by recommendations for future research and potential application and concluding remarks.

9.1 Chapter summaries and contribution to broader knowledge in the field

Asthma is a global public health problem and Australia is among the worst affected countries, especially among children and adolescents. Asthma exacerbation is a major cause of child and adolescent hospitalisations; and has significant physical, economic, and social impacts on individuals and communities. Currently, there is no cure for asthma, so it is essential that asthma management plans are responsive to significant triggers of asthma exacerbation to reduce the risk of exacerbation. Although some fungi are recognised as allergens that can trigger asthma exacerbation and affect lung function, there is uncertainty about the role that ambient outdoor fungal spores have on asthma hospitalisations, lung function or airway inflammation. There are major gaps in understanding these relationships, especially in relation to assessing which taxa of outdoor fungi are the most important contributors to asthma exacerbations, or whether the risks associated with these outdoor fungal spores vary between geographic locations. Furthermore, little is understood about the interactions between outdoor fungal spores and other asthma triggers and risk factors such as respiratory viruses, air pollutants, pollen and fungal sensitisation.

In my thesis I aimed to examine whether there are associations between a range of allergenic outdoor fungal spores and child and adolescent asthma hospitalisations; and child, adolescent and adult airway inflammation and lung function. My findings have contributed to the research literature on associations between outdoor fungal spore
exposure and asthma hospitalisations; airway inflammation and lung function. The key contributions are as follows:

9.1.1 Chapter 3 - Contribution of outdoor fungal spores in child and adolescent asthma health service attendances

9.1.1.1 Chapter summary

- My systematic review of the published research literature found that there is increasing evidence that asthmatic and sensitised children may be susceptible to asthma exacerbations that require health service attendance when exposed to outdoor fungal spores.

- The severity of exacerbation may vary with different fungal species.

- Overall, there have only been a small number of studies conducted in limited geographic locations, the majority were located in the northern hemisphere.

- Little is understood about the interactions between fungal spores and ambient air pollutants and grass pollen in asthmatic children and adolescents who are sensitised or not-sensitised to fungal spores.

- No research has investigated interactions between human rhinovirus infections and outdoor fungal spore exposure.

- Outdoor fungal exposure assessment between studies is inconsistent and non-standardised.

9.1.1.2 Chapter contribution

This is the first systematic review published examining the literature on outdoor fungal spore exposure and child and adolescent asthma health service attendances. This systematic review highlighted how little is understood about the contributions of outdoor fungal spores to asthma hospitalisations. It also drew attention to inconsistencies in exposure definitions and measurements of exposure between studies which limited comparability of the results. Very few studies examined interactive effects between outdoor fungal spores and common triggers of asthma exacerbation, such as, ambient air pollutants or grass pollen. No study included the presence of respiratory viral infection
in their analyses. All included studies were observational in design and were limited by lack of inclusion of control groups. Few studies investigated age or gender as effect modifiers (48, 250), despite these emerging as potentially important in the broader aeroallergen research (318, 319).

9.1.2 Chapter 5 - Are there associations between outdoor fungal spores and child and adolescent asthma hospitalisations?

9.1.2.1 Chapter summary

- This ecological case crossover study investigated the associations between 20 taxa of outdoor fungal spores and child and adolescent asthma hospitalisations in south-west region of Sydney, Australia.

- This study found associations between ambient *Coprinus, Periconia, Chaetomium, Ganoderma, Cerebella* and total fungal spores and increased risk of asthma hospitalisations. There was some evidence of same day and lagged effects for these taxa.

- There was also evidence of effect modification by age and sex. Girls had stronger associations with *Cladosporium, Coprinus, Chaetomium* and total fungal spores than boys. Older adolescents demonstrated stronger associations with *Coprinus* and *Ustilago*/smuts than those younger than 14 years of age.

9.1.2.2 Chapter contribution

This ecological case crossover study was an improvement on previous observational (time series and correlational) studies that examined associations between outdoor fungal spores and child and adolescent asthma hospitalisation for a number of reasons. Firstly, as the cases served as their own controls, the risk of confounding was reduced due to matched individual characteristics. Secondly, the use of bi-directional control periods allowed for adjustment for seasonal trends and day of the week effects. Thirdly, I was able to remove any hospital readmissions within 30 days of first admission from the analyses to avoid duplicating the same cases and confusing the definition of case days and matching control days.
This was the first study to be undertaken in this area of Australia where asthma prevalence in children and adolescents is high. There was evidence that different fungal taxa may be associated with asthma hospitalisations in south-eastern Australia that have not been consistently reported in the northern hemisphere research. This may be due to the different vegetation types found in Australia compared to North America and the United Kingdom.

9.1.3 Chapter 6 - Are there associations between outdoor fungal spores, human rhinovirus respiratory infections and air pollutants and child and adolescent asthma hospitalisations?

9.1.3.1 Chapter summary

- This case crossover study investigated the associations between 12 taxa of outdoor fungal spores and child and adolescent asthma hospitalisations in Melbourne, Australia.

- This study found associations between same day exposure to ambient *Alternaria*, *Coprinus*, *Drechslera*, *Leptosphaeria* and total fungal spores and increased risk of child and adolescent asthma hospitalisations.

- These associations were independent of being infected with human rhinovirus at the time of hospital admission for asthma.

- There were lagged effects up to three days with ambient *Alternaria*, *Cladosporium*, *Coprinus*, *Drechslera*, *Leptosphaeria*, and *Sporormiella* exposure.

- There was evidence that being sensitised to *Cladosporium* acted as an effect modifier of these associations.

9.1.3.2 Chapter contribution

This chapter builds on the findings from Chapter 5 by examining associations between short-term exposures to a range of fungal spore taxa and being hospitalised for asthma at the individual level. The case crossover methodology was strengthened in this study by the inclusion of pertinent individual data related to fungal sensitisation status and also
validated identification of whether a participant was infected with human rhinovirus virus.

This study demonstrated that exposures to outdoor fungal spores were associated with asthma hospitalisations independently of human rhinovirus infection. This provides the first epidemiological evidence that human rhinovirus has not confounded the effect of exposure to outdoor fungal spores, nor does it appear to act as an effect modifier.

This is the first study in this field to examine a cohort of children and adolescents with measurement of sensitisation to *Alternaria* and/or *Cladosporium* reagents. This study showed that only *Cladosporium* sensitisation acted as an effect modifier. This indicates that children and adolescents who are identified as “sensitised to fungi” are not a homogeneous group and that their risk of an asthma exacerbation could vary depending on the taxa they are sensitised to and the levels of ambient fungal spores they are exposed to.

**9.1.4 Chapter 7 - Are outdoor fungal spores associated with lower lung function or airway inflammation in a high risk allergy cohort?**

**9.1.4.1 Chapter summary**

- This cross-sectional study investigated the associations between 12 taxa of allergenic fungal spores and lung function and airway inflammation in participants residing in Melbourne, Australia.

- This study found differing associations between outdoor fungal spores and lung function and airway inflammation.

- Higher ambient levels of *Ustilago*/smuts at Lag 0, Lag 2 and cumulative 3-day lag; *Drechslera* at Lag 2; and *Pithomyces* at Lag 0 were associated with lower FEV$_1$, FVC and FEF$_{25\%-75\%}$. Higher levels *Ganoderma* at Lag 0 and Lag 1 was associated with lower FVC. Higher levels of *Leptosphaeria* were associated with lower FEF$_{25\%-75\%}$ at Lag 3.

- In those with fungal sensitisation, higher levels of *Ustilago*/smuts at Lag 0 were associated with lower FEV$_1$ and FVC. Higher levels of *Drechslera* were associated with lower FEV$_1$ and FVC. Higher levels of *Pithomyces* were associated with lower
FEV<sub>1</sub>. These associations were stronger in fungi-sensitised individuals compared to non-sensitised individuals.

- Higher levels of ambient *Cladosporium, Alternaria, Leptosphaeria* and total spores were associated with increased risk of higher levels of EBC NOx at Lag 1, Lag 2 and cumulative 3-day Lag.

- When assessed in the whole cohort, no fungal spore taxa were associated with differences in FeNO or EBC pH.
  - However, in those with fungal sensitisation, ambient exposure to *Cladosporium* was associated with higher FeNO levels and lower EBC pH, both markers of oxidative airway stress.
  - In children and adolescents aged <18 years, higher levels of ambient *Ustilago* /smuts was associated with higher FeNO and lower EBC pH.

### 9.1.4.2 Chapter contribution

No research has reported on associations between short-term exposure to outdoor fungal spores and airway inflammation; and few studies have examined associations between short-term exposures to outdoor fungal spores and lung function. Although, my study found mixed results between a range of allergenic fungal spore taxa and the parameters assessing lung function and airway inflammation, there is an indication that some outdoor fungal spores may be associated with lower lung function and increased markers of airway inflammation. These findings can contribute to the existing research that indicated reductions in lung function has been associated with *Cladosporium* (60, 62) and other unspecified outdoor fungal spore taxa (61, 66). This is the first study to examine if there were associations between exposure to outdoor fungal spores and markers of airway inflammation in EBC. The findings of associations between *Cladosporium, Alternaria, Leptosphaeria* and total spores and risk of higher levels of EBC NOx warrant further research to explore if these findings can be replicated. As these fungal spore taxa were associated with asthma hospitalisations in Chapter 6, which was in the same geographic region as this study, the identification of airway inflammation using non-invasive methods may contribute to identifying high-risk groups.
9.2 Implications and recommendations

9.2.1 Public health implications and recommendations

The public health significance of outdoor fungal spores in triggering severe asthma attacks in Australia may be underestimated. My research has shown that there is evidence to suggest that exposure to a number of ambient outdoor fungal spore species may trigger asthma exacerbations leading to hospitalisation, particularly in fungi sensitised individuals.

If these results can be replicated and sentinel fungal spore species associated with asthma exacerbations can be identified, then there may be cause for the inclusion of sentinel fungal spore taxa in existing aeroallergen monitoring systems. As fungal spores seasons differ from pollen seasons due to differing sources and climatic triggers for their release, high risk days associated with fungal spore levels could be identified and public health warnings issued, as is done for pollen via AusPollen (Australian Pollen Allergen Partnership) (320) and Deakin AIRwatch (321) in Australia.

Melbourne recently experienced a thunderstorm asthma epidemic event (21 to 22 November 2016) that was unprecedented in scale. It resulted in massive demands on emergency and health services and tragically resulted in nine deaths (322). Although there is uncertainty regarding the specific mechanisms of thunderstorm asthma, it is hypothesised that it is triggered by inspiration of respirable allergenic particles, such as pollen and fungal spores, carried by the storm’s downdrafts and outflows. Improved understanding of the contribution of outdoor fungal spores to asthma hospitalisations in Australia may contribute to refining future thunderstorm asthma prediction models and the development of appropriate and timely warnings to high-risk people.

There is growing research interest in the contribution of “greening” urban areas to improve public health (323-325). However, it is not known whether the increased vegetation may contribute to increased prevalence of allergic respiratory diseases (326-328) due to increased levels of outdoor fungal spores or pollen. Improved identification of the kinds of vegetation associated with allergenic fungal species is needed in order to support effective greening of urban spaces that will improve public health on multiple levels (73). The allergic effects of greening urban areas are subject to many confounders.
and methodological challenges to assess these associations in different geographic settings (329).

### 9.2.2 Clinical implications and recommendations

It is still too early to make specific recommendations at this time, and the results need to be replicated through extended methodologies. However, it is possible that identifying sensitisation to a broader panel of fungal allergens in children living with allergic conditions may help identify children and adolescents at higher risk of experiencing asthma exacerbations that warrant hospital care (217, 330).

Health professionals and patients may need to consider exposure to outdoor fungal spores as a potential trigger for asthma and consider appropriate medication use as a precautionary measure during seasons when fungal spores peak (218, 331, 332).

These findings may also be a factor that contributes to developments and advances in fungal immunotherapy for the prevention of child and adolescent asthma exacerbations (217, 218, 237).

### 9.2.3 Research implications and recommendations

My thesis identified further research gaps on aspects of outdoor fungal spore exposure and respiratory health:

- Modelling of outdoor fungal spore dispersion and distribution that captures spatial and temporal heterogeneity is lacking. As fungal spore exposure is ubiquitous and may be associated with asthma exacerbations and airway inflammation, future research should be undertaken to develop reliable and validated models which could be applied in epidemiological research.

- Assessment of exposure to outdoor fungal spores is inconsistent and each measuring method is subject to different types of measurement bias. To overcome these inconsistencies, future research should develop standardised measuring methods for outdoor fungal exposures.
• There is an emerging body of research that is investigating the mechanisms by which fungal spores, their hyphae and liquid aerosols trigger airway inflammation, impact on lung function and contribute to asthma exacerbations, the mechanisms are not clear (69). Future research focussed on elucidating these mechanisms can assist in understanding which components of fungal spores lead to adverse effects, thereby enabling the development of targeted therapeutic interventions that may reduce the risk of airway inflammation, reduced lung function or asthma exacerbation.

• Fungal sensitisation appears to be a significant effect modifier in my studies; however, reliable detection of sensitisation of a range of fungal allergens is limited by the lack of standardisation of fungal reagents. As allergenic sources, fungi are very complex, so fungal reagents are affected by a range of factors, including, their instability related to their protease content, time dependent release of IgE-binding components during fungal growth, culture conditions and medium used for growing the fungus, and reagent extraction procedures (218). Future research is required to tackle these challenges in order to improve detection of fungal sensitisation.

• Allergen-specific immunotherapy is a current approach to treating allergic diseases and has been used in clinical practice for approximately 100 years. There has been limited success with controlled immunotherapy trials with *Alternaria* and *Cladosporium* (218), however double-blinded, placebo controlled trials with large study samples are lacking. This gap in development links to the lack of fungal reagents for testing sensitisation, with the lack of development of standardised therapeutic agents to treat allergic diseases related to fungi.

• The role of fungal cross-reactivity between similar fungal proteins has been raised as an unresolved and under-estimated phenomenon in understanding the role of exposure to fungal spores in allergic diseases (194, 217). Technological improvements in genetic analysis of fungi have improved the identification of species-specific and cross-reactive allergic molecules from a range of allergenic fungal sources (3). However, currently, the data that verifies the clinical and diagnostic relevance of the IgE reactivity to these fungal allergens are insufficient. Further species-specific research is required in order to disentangle the cross-reactive impacts allergenic fungi have on allergic individuals.
9.3 Conclusions

At the commencement of my doctoral research I systematically synthesised the evidence of the associations between outdoor fungal spore exposure and child and adolescent asthma hospitalisations. Subsequently I undertook three studies using three separate datasets to investigate the associations between short-term outdoor fungal spore exposure and asthma hospitalisations, lung function and airway inflammation. I examined effect modification by a range of risk factors and other triggers of asthma exacerbations.

In summary I found the following:

- There was limited understanding of the contributions of outdoor fungal spores to asthma exacerbations, lung function or airway inflammation.

- There were inconsistent findings between studies possibly due lack of inclusion of other significant triggers of asthma exacerbations in analyses.

- A range of outdoor fungal spores were associated with child and adolescent asthma hospitalisations on the same day and up to 3-days lag.
  - In south-west Sydney these spores were *Periconia* (Phylum: Ascomycota – Order: Pleosporales) and *Chaetomium* (Ascomycota – Sordariales) and *Coprinus* (Basidiomycota - Agaricales)
    - In Melbourne these spores were *Alternaria*, *Drechslera* and *Leptosphaeria* which belong in the same phylum and order (Ascomycota - Pleosporales); and *Coprinus* (Basidiomycota – Agaricales).

- In south west Sydney, age interacted with *Coprinus* (Basidiomycota – Agaricales) and *Ustilago* /smuts (Basidiomycota), with older children at higher risk of asthma hospitalisation.

- In south west Sydney, sex (gender) interacted with *Coprinus* (Basidiomycota – Agaricales) and *Cladosporium* (Ascomycota - Capnodiales), with girls at higher risk of asthma hospitalisation.
In Melbourne, *Cladosporium* sensitisation interacted with *Alternaria, Coprinus, Drechslera* and *Stemphylium* fungal spore exposure, with those sensitised at higher risk of asthma hospitalisation.

In Melbourne, asthma hospitalisation was associated with exposure to *Alternaria, Coprinus, Drechslera* and *Leptosphaeria* independently of infection with human rhinovirus. Infection with human rhinovirus did not interact with exposure to outdoor fungal spores.

In Melbourne, *Ustilago* /smuts were associated with lower lung function parameters on the same day and up to 3-days lag.

In Melbourne, no fungal spore taxa were found to be associated with changes in FeNO or EBC pH except where fungal sensitisation and age group were tested as potential effect modifiers. FeNO levels were higher and EBC pH was lower in those sensitised to at least one fungal spore taxa if exposed to *Cladosporium* spores.

Air pollutants and grass pollen did not interact with outdoor fungal spores in any analyses.

In conclusion, my doctoral research has filled significant gaps in our understanding of exposure to outdoor fungal spores and asthma hospitalisations in children and adolescents, lower levels of lung function and airway inflammation. My research has demonstrated that a range of allergenic outdoor fungal spores may be contributing to asthma hospitalisations independently of recognised and significant triggers of asthma exacerbations, such as human rhinovirus infection, pollen and air pollutants. My findings indicate that contribution of outdoor fungal spores to severe asthma exacerbations have been under-estimated. Also, species that have not been previously found to be associated with asthma exacerbations, but are genetically related to well-known fungal triggers of asthma exacerbation, may warrant further investigation. Further research with large study samples, over longer periods of time with improved exposure assessment methods, and inclusion of important individual information that relate to respiratory infections and fungal sensitisation are needed to see if my findings are replicated. The findings of my research highlight the need for further research in the field of fungal allergens and allergic diseases. Future research needs to improve the standardisation of exposure assessment, and modelling the dispersion and distribution of outdoor fungal spores on spatial and
temporal levels. Technological advancements need to enhance the development of reliable and standardised fungal reagents for detection of allergic sensitisation and for therapeutic interventions. The presence of fungal spores in the air is important for the ecosystem, and may not be controlled on a large scale, but understanding how their effects on respiratory health can be prevented will benefit public health.
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Appendices
### Appendix 1: MACS lung function questionnaire

#### Lung Function Questionnaire and Body Measurements

<table>
<thead>
<tr>
<th>Date:</th>
<th>Participant ID (affix label)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operator:</td>
<td></td>
</tr>
</tbody>
</table>

1. Have you been hospitalized or undergone surgery in the last 6 weeks?  
2. Do you have a heart condition?  
3. Do you have an aneurysm?  

If participant answers yes to any of the questions above, obtain further details prior to proceeding with lung function testing.

4. Have you used a puffer or inhaler in the last 24 hours?  
   - Yes   
   - No
   - If YES → Which inhaler/s was/were used?  
     - When last used (hours)?

5. Have you taken other breathing medication in the last 24 hours?  
   - Y  
   - N
   - If YES → Which medications were used?  
     - When last used (hours)?

6. Have you smoked in the last 24 hours?  
   - Y  
   - N
   - If YES → How long ago (Hours)?

7. Have you taken any antihistamine or cough medicine in the last 72 hours?  
   - Y  
   - N
   - If YES → Which medication/s?  
     - When last taken (hours)?

8. Have you taken medication for high blood pressure or a heart condition or used eyedrops for glaucoma in the last 72 hours?  
   - Y  
   - N
   - If YES → Which medication/s?  
     - When last taken (hours)?

9. Have you had a respiratory infection in the last 3 weeks?  
   - Y  
   - N
   - If YES → How long ago did it end (days)?

10. How long since your last meal (hours)?

11. What was the first day of your last menstrual period?
## Appendix 2: MACS FeNO Recording Sheet

### Expired Nitric Oxide (FeNO) & Transepidermal Water Loss (TEWL) Recording Sheet

**Date:** [ ] [ ] [ ] [ ] [ ] [ ] [ ]  
**Operator:** [ ] [ ] [ ] [ ] [ ] [ ]  
**Participant ID:** [ ] [ ] [ ] [ ] [ ] [ ] [ ]

**Time (24hr):** [ ] : [ ]

#### FeNO Record:
- Atmospheric NO (ppm): [ ] [ ]
- Has participant smoked, had any food or drink or exercised strenuously in last hour? [ ] [ ]

If participant answers yes to any of the questions above, obtain further details prior to proceeding. Try to ensure one hour gap prior to testing.

- Does participant currently have URTI: Yes [ ] No [ ]
- Does participant currently have hay fever: Yes [ ] No [ ]

**Test Number:**
- [ ] [ ] [ ] [ ] [ ]

**NO Values (ppm):** [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ] [ ]

**Final NO:** [ ] [ ]

**Test Quality:** Poor [ ] Acceptable [ ] Uncertain [ ]

#### TEWL Record:

Ensure participant's forearm has been exposed to room air for at least 15 minutes.

- **Skin Temperature:** [ ] [ ]
- **Atmospheric Temperature:** [ ] [ ]
- **Atmospheric Humidity:** [ ] [ ]

**Test Number:**
- Skin Hydration: [ ] [ ] [ ]
- Tewl Values: [ ] [ ] [ ] [ ] [ ]

**Final TEWL:** [ ] [ ]