Does airway pathology in severe preschool wheezers predict childhood asthma?

Submitted for the degree of Doctor of Medicine (Research) in accordance with the regulations of Imperial College London 2012

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Abstract: Does airway pathology in severe preschool wheezers predict childhood asthma?

Although one third of all preschool children wheeze, only half of these will have persistent symptoms and go on to have asthma at school age. Pathological changes characteristic of asthma, including eosinophilic inflammation and increased reticular basement membrane thickness, were evident in endobronchial biopsies from severe recurrent wheezers aged 2-3 years when compared with age matched controls. However, at the time of endobronchial biopsy it was not known which children would persistently wheeze and develop asthma at school age.

The work of this thesis follows up this group of children, both preschool wheezers (n=47) and non-wheezing controls (n=21), aged between 6-11 years and establishes the presence or absence of school age asthma, relating this to pre-school airway pathology. Children (n=51) were followed up at school age, and those who attended for the research visit (n=39) were characterised in terms of atopic status, lung function (spirometry, lung clearance index) and airway inflammation (exhaled nitric oxide) at school age. Forty percent (15/37) of preschool wheezers had developed asthma at school age. Although increased airway smooth muscle is an established pathological feature of asthma in school age children, nothing is known about airway smooth muscle in preschool wheezers, hence airway smooth muscle, smooth muscle mast cells and reticular basement membrane tenascin-C were measured in the endobronchial biopsies taken at preschool age. Next, airway remodelling (increased airway smooth muscle and increased reticular basement membrane thickness) and airway inflammation at preschool age were related to the presence or absence of asthma at school age.

Sixty two percent (42/68) of children had one or more evaluable biopsies for airway smooth muscle assessment. Although reticular basement membrane thickness and submucosal eosinophils were significantly higher in preschool wheezers compared with controls, they did not discriminate the children who developed asthma by school age, suggesting these airway pathological features may be associated with current symptoms rather than future asthma risk. In contrast preschool airway smooth muscle was increased in those severe preschool wheezers who went on to develop
school age asthma (n=8, median age 8.2 [6-10.4] years, median ASM 0.12 [0.08-0.16]) when compared with those who did not develop asthma (n=24, median age 7.3 [5.9-11] years, median ASM 0.07 [0.02-0.23]), p=0.007. These data suggest that future studies investigating the mechanisms underlying the persistence of preschool wheeze and its development to asthma should have a primary focus on airway smooth muscle.
Acknowledgements

I was delighted to be given the opportunity to take on this project and am very grateful for all the help, patience, encouragement and mentoring that I have been given along the way from my three supervisors Sejal Saglani, Andrew Bush and Peter Jeffery. I have always felt very supported and have grown both as a person and as a doctor over the past 3 years under their supervision.

I am grateful to Asthma UK for providing the funding for the project and to the children and their parents for their time and cooperation in agreeing to take part in the project. I am particularly grateful to those families who travelled very long distances to attend for a research visits.

I am really grateful to Louise Fleming who answered most of the questions at the start of my research project that I would have been too embarrassed to ask anyone else, Samantha Irving for teaching me about multiple breath washout and Sarah Donovan for her assistance on technical issues with spirometry. Dr Sejal Saglani, Dr Jie Zhu and Mr Tim Oates taught me the practical aspects of endobronchial biopsy measurements and the use of the microscope. Mr Winston Banya helped me with statistical advice and data analysis.

As always I am grateful to my family, particularly my mother for pushing me and motivating throughout my life always to achieve more. Finally, to my lovely husband Ben who supported me when I often despaired with this thesis over the past years and is looking forward to having a kitchen table that is not covered in journal papers.
Declaration

I confirm that I have performed the work described in this thesis. Where colleagues have been involved, their contribution is acknowledged below. I have consulted all cited references. The work of this thesis has not been submitted elsewhere for a higher degree.

Contribution of work to this thesis attributable to Ruth O’Reilly and that performed by other colleagues

Chapter 1
1. Figure 1.4 replicated from Lloyd et al, Nature Medicine 2010 with permission.

Chapter 2
1. Children were initially recruited at preschool age by Dr Sejal Saglani. At school age all children were followed up by Ruth O’Reilly. All measurements and analysis of lung function, airway inflammation and atopy were performed by Ruth O’Reilly. For a subgroup of children multiple breath washout was performed and analysed in conjunction with Ms Samantha Irving.
2. Figure 2.3 was adapted by Dr Samantha Sonnappa and replicated from her thesis with permission.
3. Analysis of salivary cotinine was performed by Principal Biochemist, Ms Jackie Donovan, Royal Brompton Hospital

Chapters 3-7- Evaluation of preschool endobronchial biopsies
1. Flexible bronchoscopy and endobronchial biopsy was performed by Professor Andrew Bush, Dr Mark Rosenthal, Dr Ian Balfour-Lynn, Dr Jane Davies, Royal Brompton Hospital, London at the time of preschool recruitment
2. Biopsy fixation and processing was performed by Department of Histopathology, Royal Brompton Hospital, under the supervision of Professor Andrew Nicholson
4. Immunostaining was performed by Dr Jie Zhu and Dr Zhuo Wang, Lung Pathology, NHLI and Mr Timothy Oates, Leukocyte Biology, NHLI
4. All biopsy counts and measurements were performed by Ruth O’Reilly except for proliferating cell nuclear antigen smooth muscle counts which were measured by Mr
Timothy Oates. Dr Nicola Ullman was the second observer for inter-observer repeatability of airway smooth muscle measurements. RBM thickness and inflammatory cell counts had been measured by Dr Sejal Saglani at preschool age but were repeated at school age by Ruth O’Reilly.

Chapter 6
Helsinki Infant study
1. Recruitment of infants by Dr Kristiina Malmstrom, Dr Anna Pelkonen, Department of Allergology, Helsinki University Central Hospital, Helsinki
2. Infant lung function performed by Dr Pekka Malmberg, Department of Allergology, Helsinki University Central Hospital, Helsinki
3. Rigid bronchoscopy and endobronchial biopsy performed by Mr Harry Lindahl, Consultant Paediatric Surgeon, Hospital for Children and Adolescents, Helsinki
4. Biopsy fixation and processing performed by department of Histopathology, Skin and Allergy Hospital, Helsinki University Hospital, Helsinki
5. Biopsy measurements (reticular basement membrane thickness and inflammatory cell counts) were performed by Dr Sejal Saglani
Publications and Abstracts that have resulted to date from the work performed in this thesis

Publications

Increased airway smooth muscle in preschool wheezers who develop school age asthma

Ruth O’Reilly, Nicola Ullmann, Samantha Irving, Cara Bossley, Samantha Sonnappa, Jie Zhu, Timothy Oates BSc, Winston Banya, Peter K Jeffery, Andrew Bush, Sejal Saglani

Journal of Allergy and Clinical Immunology 2013 April;131(4):1024-32

Abstracts

1. More airway smooth muscle in preschool children increases risk of future asthma

R O’Reilly¹, T Oates, J Zhu², A Bush¹, PK Jeffery², S Saglani¹

1. Respiratory Paediatrics, 2. Lung Pathology, NHLI, Imperial College London

- Oral Presentation, European Respiratory Society September 2011

2. Increased reticular basement membrane thickness but NOT airway smooth muscle in endobronchial biopsies of severe preschool wheezers

R O’Reilly¹, J Zhu², A Bush¹, PK Jeffery², S Saglani¹

1. Respiratory Paediatrics, 2. Lung Pathology, NHLI, Imperial College London

Thorax 2010;65:A2-A3

- Highly commended BTS/BLF/BALR Young Investigator’s Prize Dec 1st 2010- Oral Presentation

3. Comparison of three methods to quantify Tenascin C expression within the bronchial reticular basement membrane of paediatric endobronchial biopsies

R O’Reilly¹, J Zhu², A Bush¹, PK Jeffery², S Saglani¹

1. Respiratory Paediatrics, 2. Lung Pathology, NHLI, Imperial College London


- Poster Discussion, American Thoracic Society, May 2010
- Oral presentation, British Thoracic Society Winter Meeting, 2009
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AHR</td>
<td>airway hyperresponsiveness</td>
</tr>
<tr>
<td>ALSPAC</td>
<td>Avon Longitudinal study of Parents and Children</td>
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<tr>
<td>ASM</td>
<td>airway smooth muscle</td>
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<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>COAST</td>
<td>Childhood Origins of Asthma</td>
</tr>
<tr>
<td>COPSAC</td>
<td>Copenhagen prospective study on Asthma in Childhood</td>
</tr>
<tr>
<td>CoV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>CW</td>
<td>confirmed wheezer at preschool age</td>
</tr>
<tr>
<td>EB</td>
<td>endobronchial biopsy</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ERS</td>
<td>European Respiratory Society</td>
</tr>
<tr>
<td>ETAC</td>
<td>Early Treatment of the Atopic Child Trial</td>
</tr>
<tr>
<td>FeNO</td>
<td>exhaled nitric oxide</td>
</tr>
<tr>
<td>FeNO$_{50}$</td>
<td>exhaled nitric oxide at 50ml/s</td>
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<tr>
<td>FEV$_1$</td>
<td>forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FRC</td>
<td>functional residual capacity</td>
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<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>GINA</td>
<td>Global Initiative for Asthma</td>
</tr>
<tr>
<td>ICC</td>
<td>intraclass correlation coefficient</td>
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<tr>
<td>ICS</td>
<td>inhaled corticosteroids</td>
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<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ISAAC</td>
<td>International Study of Asthma and Allergy in children</td>
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<tr>
<td>LCI</td>
<td>lung clearance index</td>
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<tr>
<td>MAAS</td>
<td>Manchester Asthma and Allergy Study</td>
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<tr>
<td>MAS</td>
<td>Multicentre Allergy Study (German)</td>
</tr>
<tr>
<td>MBW</td>
<td>multiple breath washout</td>
</tr>
<tr>
<td>MCT</td>
<td>mast cell tryptase</td>
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<tr>
<td>MCTC</td>
<td>mast cell chymase tryptase</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
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<tr>
<td>NPV</td>
<td>negative predictive value</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PEAK</td>
<td>Prevention of Early Asthma in Kids</td>
</tr>
<tr>
<td>PC</td>
<td>provocation concentration</td>
</tr>
<tr>
<td>PIAMA</td>
<td>Prevalence and Incidence of Asthma and Mite Allergy</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive value</td>
</tr>
<tr>
<td>RAST</td>
<td>radioallergosorbent test</td>
</tr>
<tr>
<td>RBH</td>
<td>Royal Brompton Hospital</td>
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<tr>
<td>RBM</td>
<td>reticular basement membrane</td>
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<tr>
<td>ROC</td>
<td>receiver operator curve</td>
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<tr>
<td>RO'R</td>
<td>Ruth O'Reilly</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
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<tr>
<td>RW</td>
<td>reported wheezer preschool age</td>
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<tr>
<td>$S_{\text{acin}}$</td>
<td>acinar airways inhomogeneity</td>
</tr>
<tr>
<td>$S_{\text{cond}}$</td>
<td>conducting airways inhomogeneity</td>
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<tr>
<td>SF$_6$</td>
<td>sulphur hexafluoride</td>
</tr>
<tr>
<td>SIII</td>
<td>phase 3 slope</td>
</tr>
<tr>
<td>SnIII</td>
<td>normalised phase III slope</td>
</tr>
<tr>
<td>SPT</td>
<td>skin prick tests</td>
</tr>
<tr>
<td>SS</td>
<td>Sejal Saglani</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TCRS</td>
<td>Tucson Children's Respiratory Study</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue-specific inhibitors of metalloproteinase</td>
</tr>
<tr>
<td>$T_H$</td>
<td>T Helper cell</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>$T_{\text{reg}}$</td>
<td>T lymphocyte regulatory cell</td>
</tr>
<tr>
<td>VOC</td>
<td>volatile organic compound</td>
</tr>
<tr>
<td>$V_{\text{max}}$ FRC</td>
<td>maximal flow at functional residual capacity</td>
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Chapter 1
Preschool Wheeze

1.1 Introduction
Wheeze is a polyphonic, musical, whistling sound produced in the airways during breathing. Around 1 in 3 children will have at least one episode of wheeze prior to their third birthday\(^{(1)}\) and by age 7 years 40% of children report at least 1 episode of wheezing\(^{(2)}\). It is estimated that children aged 1–5 years with wheeze cost the United Kingdom (UK) health service a total of £53 million with the greatest expenditure being £34 million in primary care\(^{(3)}\).

Wheeze in preschool children is often associated with respiratory viral infection and children may be completely asymptomatic between infections\(^{(4)}\). Although most cases of persistent asthma begin in early life, spontaneous resolution of wheezing will occur in many young children\(^{(5)}\). Only half of children with wheeze in preschool age will go on to have asthma at school age\(^{(6)}\). Those children who remain symptomatic and develop asthma are the focus of this thesis. The diagnosis of asthma is a clinical one and there are no standardised diagnostic criteria\(^{(7)}\). Most definitions include symptoms of wheeze, breathlessness, chest tightness or cough coupled with physiological evidence of variable airflow obstruction. More recent descriptions of asthma in children and in adults have included components of airway hyper-responsiveness and airway inflammation. In particular the Global Initiative for Asthma (GINA) guidelines define asthma as ‘a common chronic disorder of the airways that is complex and characterised by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation’\(^{(8)}\). However, how airway inflammation and airway hyper-responsiveness (AHR) relate to each other and how they contribute to the clinical manifestations of asthma is unclear. In many cases, neither will actually be measured, and the evidence implicating eosinophilic inflammation in wheezing in infancy is scanty.

The work described in this thesis is the follow up at school age of a previously studied cohort of severe preschool wheezers (‘preschool’ is defined in this thesis as aged between 3 months and 5 years)\(^{(9)}\). The aim was to determine if airway
pathology, including airway inflammation and remodelling at preschool age can predict school age asthma. There is no current treatment that is disease modifying, namely which prevents the progression of preschool wheeze to asthma, but determining which wheezers will have school age asthma may be the first step in helping to identify which children to target for further investigation of the mechanisms leading to the development of asthma. This chapter will focus on the early preschool years, specifically in relation to the difficulties associated with correctly identifying true wheeze, wheeze phenotypes and the usefulness of currently proposed clinical asthma predictive indexes. Airway pathology that is characteristic of asthma will also be reviewed.

1.2 Difficulties in assessing wheeze prevalence

There is considerable overlap between different preschool wheeze phenotypes. One of the difficulties in untangling the different preschool wheeze phenotypes is the reliance in longitudinal epidemiological studies on a parental report of wheeze to assess prevalence, table 1.1\(^{1;10-15}\). This is dependent on parental understanding of the term “wheeze”, but parental and physician perception of wheeze is often very different. One factor responsible for the poor prediction of which preschool children with wheeze develop future asthma is the use of the word “wheeze” to describe many disparate sounds.

At least 30% of parents use other words for wheeze and 30% of parents have labelled other sounds as "wheeze" in research studies using video questionnaires as the ‘gold standard’\(^{16;17}\). In particular, incorrect labeling of upper airway noises such as stertor, stridor or snoring as wheeze in young children may lead to an overestimate of both prevalence and frequency of wheeze at preschool age\(^{16;17}\). In a large questionnaire based study of over 4000 children aged 6-10 years, only 30% of families exclusively used the word “whistling” to describe wheezing, with a large proportion of parents describing other sounds as wheeze, most commonly rattly breathing (43.1%), followed by wet cough (20%) and noises from the nose or throat (20%) and least commonly snoring (3.2%)\(^{17}\). Frequent attacks of reported wheeze, maternal history of asthma and maternal education were significantly associated with a correct identification of wheeze as a whistling / squeaking sound, while incorrect identification was commoner in families of South Asian ethnicity, parents whose first
language was not English and families living in areas of social deprivation\textsuperscript{(17)}. Interestingly, “whistling” was only mentioned by 11% of 200 parents of children with ‘asthma’, when asked what they understood by wheeze, although it features in most wheeze epidemiology questionnaires\textsuperscript{(16)}. Video questionnaires of upper and lower respiratory noises in parents of children with asthma or other respiratory conditions show that the correct labelling of wheeze was 59% and 47% for other sounds such as stridor, snoring, stertor\textsuperscript{(18)}. Conversely, the prevalence of wheeze may also be underestimated, in one study 19% of children labelled as cough by parents were diagnosed with wheeze on auscultation by physicians\textsuperscript{(19)}. Physicians were able to reliably judge the presence and severity of wheeze, when compared with wheeze analysed by acoustic techniques, whereas nurses and parents were much less reliable\textsuperscript{(20)}.

The prevalence of preschool wheeze varies internationally between 17-50%, table 1.1. There were different definitions of ‘preschool’, which may contribute to some of the variability in reported prevalence. Importantly, children with parentally reported, but unconfirmed wheeze, have no difference in pulmonary function when compared to those who never wheezed, whereas children with physician confirmed wheeze have significantly poorer lung function\textsuperscript{(21)}. Table 1.1 summarises the main cohort studies that report both preschool wheeze and school age asthma prevalence.
### Table 1.1: Birth cohort studies from different regions report different prevalence’s of wheeze at preschool age and subsequent school-age asthma

<table>
<thead>
<tr>
<th>Author</th>
<th>Birth Cohort</th>
<th>Country</th>
<th>Year of publication</th>
<th>Number of children</th>
<th>Subject Retention</th>
<th>Preschool wheeze prevalence</th>
<th>Wheeze</th>
<th>Prevalence of school age asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudri et al(^{(10)})</td>
<td>PIAMA</td>
<td>Rotterdam, Netherlands</td>
<td>2009</td>
<td>3963</td>
<td>88%</td>
<td>Age 1-4 years 55%</td>
<td>Parental report</td>
<td>Age 7 years 11%*</td>
</tr>
<tr>
<td>Henderson et al(^{(11)})</td>
<td>ALSPAC</td>
<td>Avon, UK</td>
<td>2008</td>
<td>6265</td>
<td>54%</td>
<td>Age 1-5 years 40%</td>
<td>Parental report</td>
<td>Age 7 years 50%*</td>
</tr>
<tr>
<td>Jackson et al(^{(22)})</td>
<td>COAST</td>
<td>Wisconsin USA</td>
<td>2008</td>
<td>289</td>
<td>89%</td>
<td>Age 1-3 years (20-50%)</td>
<td>Doctor diagnosed</td>
<td>Age 6 years 28%*</td>
</tr>
<tr>
<td>Bisgaard et al(^{(23)})</td>
<td>COPSAC</td>
<td>Denmark</td>
<td>2007</td>
<td>411</td>
<td>79%</td>
<td>Not assessed</td>
<td>Doctor diagnosed</td>
<td>Age 5 years 14%*</td>
</tr>
<tr>
<td>Lodrup Carlsen et al(^{(14)})</td>
<td>Environment and Childhood Asthma (ECA)</td>
<td>Oslo, Norway</td>
<td>2006</td>
<td>803</td>
<td>77%</td>
<td>Age 0-3 years 21.4%</td>
<td>Parental report</td>
<td>Age 10 years- 11% Age10 years -61%#</td>
</tr>
<tr>
<td>Kurukulaaratchy et al(^{(12)})</td>
<td>Isle of Wight</td>
<td>Isle of Wight</td>
<td>2003</td>
<td>1456</td>
<td>71%</td>
<td>Age 1-4 years 33%</td>
<td>Parental report</td>
<td>Wheeze over past year Age 10 years 37%*</td>
</tr>
<tr>
<td>Lau et al(^{(24)})</td>
<td>German MAS</td>
<td>Berlin Germany</td>
<td>2003</td>
<td>1314</td>
<td>71.5%</td>
<td>Age 1-7 years 17.4%</td>
<td>Parental report</td>
<td>Age 7 years -10% current wheezing -6.1% DD asthma#</td>
</tr>
<tr>
<td>Oddy et al(^{(13)})</td>
<td>Perth</td>
<td>Perth, Australia</td>
<td>1999</td>
<td>2187</td>
<td>91%</td>
<td>Age 1-6 years 22.5%</td>
<td>Parental report</td>
<td>Age 6 years DD asthma 30.9%*</td>
</tr>
<tr>
<td>Martinez et al(^{(1)})</td>
<td>Tucson</td>
<td>Tucson, Arizona, USA</td>
<td>1995</td>
<td>1246</td>
<td>66%</td>
<td>Age 0-3 years 48.5%</td>
<td>Parental report</td>
<td>Age 6 years 40%*</td>
</tr>
</tbody>
</table>

\(^*(\%) = \%\) of asthma in whole cohort at school age, \(#(\%) = \%\) of preschool wheezers with asthma at school age)
1.3.1 Preschool wheezing – classification of phenotypes

The roots of most cases of asthma are in early childhood and it is likely some children are predisposed to asthma even before birth\(^5\). Longitudinal cohort studies from birth to adulthood have provided insight into the natural history of asthma and have described several different wheeze phenotypes at preschool age. A phenotype is defined in this thesis as a cluster of clinical or pathological features which tend to be associated, and which are useful in some way, such as in managing the child or understanding the mechanisms of a disease\(^{25}\). Approaches to describing phenotypes may be subjective, whereby children are divided into subsets based on a hypothesis by a clinician (for example, induced sputum patterns of cellularity), or can be objective, where phenotypes are mathematically derived in an unbiased manner (for example, latent class analysis and systems biology approaches). The latter avoids the constraints of investigator imposed classifications which are vulnerable to bias. Latent class analysis is a form of cluster analysis in which subjects are grouped according to similarities of response levels to a number of measured categorical variables. However, it should be noted that so-called ‘objective’ methods of analysis are also vulnerable to bias, in that the investigator has to determine which datasets are actually included in the analysis.

Epidemiological studies using unselected or selected high risk populations have demonstrated that different early wheeze patterns in terms of age of onset, frequency, severity and triggers (viral or bacterial) have different natural histories. Clinical phenotypes have also been described based on temporal wheeze patterns (episodic viral and multiple trigger wheeze). However, there is overlap between some of these described phenotypes. These can likely only be resolved by a large multicentre, long-term study with antenatal recruitment and repeated objective assessments using biomarkers (for example, exhaled nitric oxide (FeNO), exhaled breath condensate or induced sputum) as proxy measurements of inflammation to identify clinically useful phenotypes and early predictors of disease progression. Systems biology is one such innovative research technique which looks at biology as a group of connected pathways, using genetic, molecular, histopathology and physiological data to create a mathematical or computational model using principal component analysis that can then be used to define asthma phenotypes. The U-BIOPred consortium is currently undertaking a large international study in asthma.
using this approach\(^{(26)}\). The next section of this thesis describes the main cohort studies in which children were recruited either before or shortly after birth.

1.3.2 The Tucson Children’s Respiratory Study (TCRS)

TCRS was a landmark longitudinal epidemiological study which began in 1980 to investigate potential risk factors during the first 3 years of life for the future development of asthma\(^{(1)}\). It was one of the first cohort studies to recruit unselected infants at or shortly after birth. Its strengths include the large number of infants (n=1246), measurements made in cord blood before the onset of any respiratory illness and a long period of follow up with good retention of subjects. The TCRS has made a large contribution to our knowledge of preschool wheezing, describing three distinct wheezing phenotypes (transient infant wheezers, late onset wheezers and persistent wheezers)\(^{(1)}\). It was also the first longitudinal cohort study to develop an asthma predictive index in preschool wheezers\(^{(27)}\), discussed in section 1.6.

**Tucson Children’s Respiratory Study - wheezing phenotypes**

**Transient wheezers**

Transient wheezers were children who wheezed only during the first three years of life and who had stopped by age 6 years. Lung function as measured by maximal flow at functional residual capacity (\(V_{\text{max}} \text{FRC}\)) was lower in transient wheezers than their non wheezing peers\(^{(1)}\) shortly after birth and at age 6 years and they continued to have lower levels of forced expiratory flows at age 11 and 16 years\(^{(6)}\), figure 1.1. Their diminished lung function shortly after birth, before any postnatal respiratory insult, suggests the need to focus on antenatal factors which may predispose these children to wheezing. As the airways grow children are less likely to wheeze with respiratory viral infections. Risk factors for transient wheezing include prematurity, maternal smoking, maternal atopy, maternal hypertension and younger maternal age\(^{(1;28-30)}\).

**Persistent Wheezers**

Persistent wheezers referred to children who continued to wheeze throughout the first 6 years of life. Persistent wheezers had higher total serum immunoglobulin E (IgE) than non-wheezers at 9 months, despite no association between cord blood IgE and persistent wheezing at 6 years suggesting IgE mediated sensitisation may at
least start to develop during the first year of life. Almost all the persistent wheezers were sensitised to at least one aeroallergen by age 6 years. $V_{\text{max}}$FRC in infancy in persistent wheezers is similar to those children who subsequently never wheezed, but by 6 years of age, these children had reduced pulmonary function which subsequently tracks with time\(^{(1)}\). Children with persistent wheezing were more likely to have more severe symptoms, eczema, rhinitis apart from colds and a maternal history of asthma.

**Late onset wheezers**

Late onset wheezers are those who wheeze at age 6 years but do not wheeze in the first 3 years of life. Although late onset wheezers were as likely to remain symptomatic to 16 years as persistent wheezers they had no significant deficits in lung function. Children in the late onset wheezing group were more likely to be atopic than those children that had never wheezed, but atopy was less prevalent in this group than the persistent wheezers. Lung function tracks for all 3 phenotypes from the preschool years to adolescence at least, figure 1.1. This suggests that lung function deficits in children with asthma are already present by 6 years of age and remain stable (or at least do not improve) throughout adolescence. While the Tucson study has added greatly to our knowledge of the evolution of preschool wheeze, epidemiological classifications can only be applied retrospectively and do not contribute to the clinical management of the individual child. Furthermore, these phenotypes are largely self-fulfilling as children were only assessed at 3 fixed time points, and so could only have those 3 wheeze phenotypes.
Figure 1.1: Cross-sectional z scores of height-adjusted maximal expiratory flows at ages 2.4 months and 6, 11, and 16 years for the preschool wheeze phenotypes\(^{(6)}\) Changes in pulmonary function are established by 6 years of age, and track through to late adolescence

1.3.3 British Avon Longitudinal Study of Parents and Children (ALSPAC birth cohort)

Although the wheeze patterns as defined by the TCRS\(^{(1)}\) group were self-fulfilling, the findings were largely confirmed by the British Avon Longitudinal Study of Parents and Children (ALSPAC) birth cohort\(^{(6;11)}\). This was a longitudinal birth cohort study in which data on wheezing were available at 7 time points from birth to 7 years of age in 6,265 (45%) of the 14,062 unselected children\(^{(11)}\). Wheeze phenotypes were determined using latent class analysis (which allows phenotypes to emerge from the data, rather than be imposed on it as in the TCRS) and largely confirmed the temporal wheezing patterns and associated risk factors of the TCRS\(^{(1)}\). Two other phenotypes were also described; “prolonged early wheeze” characterised by wheezing in non atopic children from 6 to 54 months with low prevalence from age 69 months onwards and “intermediate onset wheeze” with onset between ages 18 and 42 months. These wheezing phenotypes were later confirmed in the Prevalence and Incidence of Asthma and Mite Allergy (PIAMA) Dutch birth cohort (also using latent class analysis) adding further evidence to the existence of an intermediate-onset phenotype with onset of wheeze after 2 years of age\(^{(31)}\). Unlike in the Tucson children\(^{(1)}\) the intermediate phenotype had a stronger association with atopy than the
persistent or late onset wheezers. Childhood wheeze phenotypes most associated with atopy and airway hyperresponsiveness (AHR) in this study had onset after 18 months. This cohort study suggests all wheezy phenotypes including the late onset wheezers are associated with impaired lung function by school age\(^{11}\).

### 1.3.4 German Multicentre Allergy study (MAS)

The German MAS was a birth cohort (n=1314) of healthy term infants (½ had 2 parents with atopy and increased cord blood IgE, and ⅔ had 1 parent with atopy) recruited in 1990 and were followed up until 13 years of age. The purpose of the study was to investigate the role of allergic sensitisation and allergen exposure in early life in the development of asthma. Children were followed annually including both clinical and lung function (body plethysmography) assessment, and also measurement of specific IgE to food and aeroallergens. The study showed that children with persistent or late onset wheeze have impaired pulmonary function at 7 years\(^{13}\), similar to the findings of the Tucson study. In children with wheeze, lung function tracked along values found at age 7, until age 10 and 13 years. However, the course of lung function differed significantly between 7-13 years in atopic and non atopic wheezers with impairment in all lung function indices only seen in atopic wheezers. The MAS cohort was the only study which measured childhood lung function at 10 and 13 years after bronchodilator treatment. This still showed reduced lung function in wheezing children, but the differences were less\(^{32}\).

In contrast to the TCRS study 90% of children with early wheeze, but no atopy, lost their symptoms at school age and had normal lung function by puberty\(^{32}\). This group may represent children who wheezed with viral infections at preschool age. Importantly, sensitisation to perennial allergens such as house dust mite, cat and dog hair that developed in the first 3 years of life was associated with a loss of lung function at school age. A positive family history of atopy, or early IgE sensitisation to aeroallergens were the strongest factors predicting whether children wheezing early in life will continue wheezing until puberty\(^{32}\).

### 1.3.5 British Manchester Asthma and Allergy (MAAS) cohort study

The Manchester Asthma and Allergy Study (MAAS) is an unselected, population-based study which recruited 1186 children antenatally\(^{33}\). Children were followed
prospectively, and attended review clinics at ages 1, 3, 5 and 8 years where skin prick tests were performed to aero- and food allergens. Lung function was measured using whole body plethsmography at age 3 (49%) and 5 (67%) years and showed reduced lung function in children with a history of transient and persistent wheeze similar to the TCRS and ALSPAC studies, and similar to the TCRS showed no difference in lung function between children who never wheezed and late-onset wheezers at age 5\(^{(34)}\).

Table 1.2: Comparison of preschool wheeze phenotypes and associated lung function in the Tucson Children’s Respiratory Study (TCRS), Avon Longitudinal Study of Parents and Children (ALSPAC) and Manchester Asthma and Allergy Study (MAAS)

<table>
<thead>
<tr>
<th>TCRS</th>
<th>ALSPAC</th>
<th>MAAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth</td>
<td>Age 6 yrs</td>
<td>Age 8-9 yrs</td>
</tr>
<tr>
<td>Never Wheezed</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Transient wheeze</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Prolonged early wheeze</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Intermediate Onset wheeze</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Late onset Wheeze</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Persistent Wheeze</td>
<td>N</td>
<td>↓↓</td>
</tr>
</tbody>
</table>

**TCRS:** Lung function was measured by \( V_{max} \) FRC at infancy and age 6 years\(^{(1)}\).

**ALSPAC:** Lung function was not assessed at birth and FEV\(_1\) was measured at 8-9 years. All wheeze phenotypes were associated with decrements of FEV\(_1\) when compared with the never wheezed group\(^{(1)}\). The greatest decrements were associated with prolonged early, intermediate onset and persistent wheezing\(^{(1)}\).

**MAAS:** Lung function measured by specific airways resistance at 3 and 5 years. *Children with transient wheeze had reduced lung function at 3 years when compared to non-wheezers but it was not statistically significant*\(^{(34)}\).

Similar to the German MAS the MAAS study also showed that early sensitisation to allergens (house dust mite, cat, dog, grasses, milk, egg) was associated with a decrement in lung function. Children who had never wheezed in the first 3 years of life had significantly higher specific airways resistance if they were atopic, or were at high risk of atopy (i.e., if both parents were atopic)\(^{(33)}\). The MAAS also adopted a machine based learning approach for latent classes of sensitisation analysis which suggested that IgE antibody responses do not reflect a single phenotype of atopy, but rather multiple different atopic vulnerabilities\(^{(35)}\). In particular children with multiple early atopic vulnerability were more likely to persistently wheeze\(^{(35)}\). This study may in part explain the global variations in asthma and atopy, as atopic
vulnerabilities may differ in each location consequent to differences in genetic predisposition and environmental exposures\(^{(35)}\). The importance of early environmental influences is highlighted by a study of asthma in South East Asian women living in Leicester\(^{(36)}\). Asthma prevalence in South East Asia is much lower than in the UK\(^{(37)}\). Women were born in South East Asia and immigrated to the UK in the first 4 years of life acquired the asthma risk of the UK, whereas women immigrating to the UK older than 5 years retained the South East Asian asthma prevalence\(^{(36)}\).

### 1.3.6 Perth study

The Western Australian pregnancy cohort study is a prospective birth cohort initially established (1989-92) which recruited 253 healthy full term infants\(^{(38)}\). The rapid thoracoabdominal compression technique was used to determine \(V_{\text{max}} FRC\) and AHR in infancy \((n=253)\) at 1 month, 6 months and 12 months of age. At 11 years of age study participants \((n=176)\) attended an assessment that included measurement of spirometry, AHR to histamine and skin prick reactivity\(^{(39)}\). However, contrary to the TCRS study, the Perth study suggested that persistent wheezers had abnormal lung function \((V_{\text{max}} FRC)\) at birth which did not deteriorate with time suggesting that impaired lung function preceded the development of persistent wheezy disorders and may predispose children to asthma\(^{(40)}\). The question of whether decreased lung function is a cause or consequence of asthma remains unresolved. The Perth study also reported that children who transiently wheeze have reduced \(V_{\text{max}} FRC\) at 4 weeks (similar to TCRS group), and reduced forced expiratory volume in one second \((\text{FEV}_1)\) at 6 years, but normal \(\text{FEV}_1\) by 11 years\(^{(41)}\).

In contrast TCRS transient wheezers have some lung function catch up with time but remain impaired when compared with non-wheezees, figure 1.1. However, given the much larger numbers in the TCRS study its results are more likely to be correct. Secondly, spirometry is a crude measure of lung function in asthma\(^{(42)}\) and other assessments of lung function may have detected some decrements. The Perth study also showed that children with house dust mite specific IgE titres of greater than 0.20 kU/L by age 2 years have a 12.7\% risk of persistent wheeze, increasing progressively to an 87.2\% risk with increasing numbers of severe lower respiratory
tract illnesses experienced\textsuperscript{(43)}. The role of AHR and the future development of asthma will be discussed in section 1.4.

1.3.7 Environment and Childhood Asthma (ECA)

In 1992 3754 unselected infants (with a birth weight greater than 2kg) born in Oslo, Norway were enrolled at birth into the Environment and Childhood Asthma (ECA) study. Information on the child’s health and environmental exposures was collected from parents by questionnaires at birth and when the child was age 0.5, 1, 1.5, 2, 4, and 10 years\textsuperscript{(14,44)}. Lung function was assessed shortly after birth (aged 2-3 days) in 802 infants while awake using tidal breathing techniques\textsuperscript{(45)}. Similar to the Perth findings, infants with abnormal tidal breathing patterns (the fraction of expiratory time to peak tidal expiratory flow to total expiratory time \([t\text{PTEF}/t\text{E}]\)) were more likely to have asthma at 10 years of age (77% follow-up)\textsuperscript{(45)}. The 10-year follow-up of the Oslo birth cohort also showed that doctor diagnosed asthma before the age of 10 years was positively associated with early life respiratory infections including croup, the common cold and otitis media as reported by parents at the 6 month and 12 month questionnaires\textsuperscript{(46)}.

1.3.8 Copenhagen Study on Asthma in Childhood (COPSAC)

The Copenhagen Prospective Study on Asthma in Childhood (COPSAC) is a single-center, birth cohort study of children (n=411) born to asthmatic mothers between 1998 and 2001. Infants had lung function measured using the raised volume thoracoabdominal compression technique, assessment of AHR using metacholine and FeNO measured at 1 month of age. Children were reviewed at 6 monthly intervals and followed up with a long-term diary card based on wheezy symptoms for the first 6 years of life (76% follow up)\textsuperscript{(47)}. FeNO levels (measured in 62% of the cohort) were increased in 1-month-old symptom-free babies who developed transient early wheeze but not persistent wheeze, but was unrelated to neonatal lung function\textsuperscript{(47)}. This suggests an early disease process unrelated to airway calibre contributing to the transient wheeze phenotype\textsuperscript{(47)}.

Perhaps a more interesting finding was that infants colonised in the hypopharyngeal region with \textit{S. pneumoniae}, \textit{H. influenzae}, or \textit{M. catarrhalis}, or with a combination of these organisms, at 1 month of age were at two fold increased risk for recurrent
wheeze and asthma early in life\(^{23}\). Blood eosinophil counts and total IgE at 4 years of age were significantly increased in children who were colonised as neonates\(^{23}\). Children in this study continued to have hypopharyngeal aspirates and nasopharyngeal aspirates taken every 6 months at routine follow up visits and also for any wheezy episode that lasted for 3 days or more\(^{48}\). Wheezy episodes were significantly associated with \textit{H influenzae}, \textit{M catarrhalis}, and \textit{S pneumonia}, similar to but independent of the association with virus infections\(^{48}\), figure 1.2. It is unknown whether the bacterial colonisation acts an environmental trigger in genetically predisposed children, or there is a subtle immunodeficiency in children who develop asthma leading to mucosal bacterial colonisation.

However, it is worth noting in this context that recent work has highlighted the importance of airway bacteria in normal and abnormal immunological development\(^{49}\). Once thought to be sterile, it is now clear that there is a rich bacterial flora in the human lower airway\(^{50}\), although not all groups have reproduced this finding\(^{51}\). From animal work, it is clear that absence of the normal lower airway flora leads to more pronounced T\(_{H2}\) inflammation\(^{52}\) and that the airway microbiome may modulate the response to inflammation\(^{53;54}\). Human data are more indirect. A recent manuscript linked increased environmental bacterial and fungal diversity to a reduced risk of asthma and atopic disease in children from farming and non-farming environments\(^{49}\). Furthermore, babies delivered by caesarean section are at increased risk of atopic disease\(^{55}\), and this has been linked to altered intestinal flora\(^{56}\). Infants of atopic parents who had a home but not hospital normal vaginal delivery had reduced risk of asthma and eczema at the same age\(^{57}\). The area of the interactions between the microbiomes of the intestine and the airway, and the development of disease, is rapidly evolving.
Figure 1.2: Risk of recurrent wheeze. COPSAC showed a strong association between colonisation of the airways with common pathogenic bacteria and development of recurrent wheeze and asthma\(^{(58)}\).

1.3.9 Childhood Origins of Asthma birth cohort study (COAST)

The Childhood Origins of Asthma (COAST) birth cohort study followed 289 children at high risk of asthma from birth, focusing on viral infections in the first year of life. At least 1 parent was required to have one or more positive skin prick tests to aeroallergens or a history of doctor diagnosed asthma. Nasopharyngeal specimens were taken during wheezy exacerbations during the first 3 years of life. The COAST study confirmed that 50% of children wheeze with respiratory syncytial virus (RSV) and 25% of children will wheeze with rhinovirus\(^{(59)}\). Rhinovirus wheezing in early life (0-3 years) was a better independent indicator of asthma risk at 6 years than RSV associated wheezing or aero-allergen sensitisation\(^{(22)}\). Nearly 90% of children wheezing with rhinovirus at age 3 subsequently developed asthma regardless of the presence or absence of aeroallergen sensitisation\(^{(22;59)}\). Interestingly, allergic sensitisation during early life predisposes children to more severe viral respiratory illnesses and wheezing, particularly with rhinovirus, but that wheezing respiratory illnesses do not increase the risk for the subsequent development of allergic sensitisation\(^{(60)}\).
1.3.10. Clinical wheeze phenotypes:- European Respiratory Society classification of preschool wheeze

A major limitation of epidemiological wheeze phenotypes is that they can only be applied retrospectively. Secondly, there is considerable overlap between phenotypes that have been described. Patterns of temporal wheeze in preschool children as described by the European Respiratory Society (ERS) Taskforce are multiple-trigger and episodic (viral) wheeze\textsuperscript{(61)}. This classification of wheeze phenotypes is clinical and can be applied by the physician prospectively at preschool age.

**Episodic (viral) wheezers** are children who wheeze during discrete time periods usually with a cold or other clinically diagnosed viral respiratory tract infection but who do not wheeze between episodes.

**Multiple-trigger wheezers** are children who wheeze both during exacerbations and between episodes. This group of children are often, but not always, also atopic.

Multiple-trigger wheezers have been shown to have pulmonary function abnormalities as measured by increased ventilation inhomogeneity, particularly of the conducting airways independent of atopic and current wheeze status and evidence of airway pathology changes similar to asthma when compared to episodic (viral) wheezers\textsuperscript{(62;63)}. Pulmonary function in episodic viral wheezers did not differ significantly from control subjects\textsuperscript{(62)}. Multiple trigger wheezers also had increased exhaled nitric oxide levels when compared to viral or episodic wheezers\textsuperscript{(62)}. However, these wheeze phenotypes are not fixed and children can move from one to the other. For example, episodic viral wheeze may evolve into a multiple-trigger pattern, and multiple trigger wheeze may become episodic over time or with treatment with inhaled corticosteroids. Multiple trigger and episodic wheeze phenotype stability was assessed every 3 months over a 1 year period and only remained stable in 50% of preschool children in a West Australian intervention study\textsuperscript{(64)}. However, frequent monitoring may have changed our view of natural history of wheeze in these children. It is possible that increased inhomogeneity of the conducting airways in the multiple-trigger preschool wheezers may reflect increased disease severity in a subgroup of preschool children who in response to viruses and environmental stimuli have more prolonged periods of persistent airflow obstruction or alternatively that multiple-trigger wheeze represent a different disease phenotype.
1.4 Early airway hyperresponsiveness

It is not clear whether increased premorbid AHR predisposes children to develop asthma or whether AHR develops as part of the pathophysiology of asthma. Three cohorts from London, Perth and Copenhagen have reported studies of AHR in early infancy, before the onset of symptoms and prior to the establishment of allergic airway inflammation\(^{(39,65-67)}\).

The London cohort measured AHR to histamine (expressed as the provocative concentration (PC) of histamine that induced a 30% (PC\(_{30}\)) decrease in \(V_{\text{max}}\)FRC) in healthy term infants (n=73) with at least 1 atopic parent by using the rapid thoracoabdominal compression technique\(^{(65)}\). AHR in female infants but not in male infants predicted subsequent wheezing in the first year of life. Children were followed up age 10 years with 89% follow up for wheezing history and 50% follow up with spirometry and methacholine challenge. An association was reported between neonatal AHR and transient early wheeze but not persistent wheeze at 10 years\(^{(66)}\). Neonatal AHR also significantly predicted subsequent FEV\(_1\) at 10 years but there was no correlation with subsequent AHR (PC\(_{20}\) the concentration of methacholine causing a 20% drop in FEV\(_1\))\(^{(66)}\).

The Perth cohort measured AHR to histamine (concentration provoking a 40% reduction in \(V_{\text{max}}\)FRC (PC\(_{40}\))) on 3 occasions at 1 month, 6 months and 12 months in 202 unselected infants using the rapid thoracoabdominal technique\(^{(68)}\). Children were followed up with assessments of lung function, AHR and asthma at age 6 and 11 years\(^{(39)}\). AHR in late infancy at 1 year but not at 1 or 6 months (when reduced \(V_{\text{max}}\)FRC was present) was associated with wheeze persistence at 11 years\(^{(39)}\). Unlike with the London cohort although AHR at 1 month was associated with asthma at 6 years, it was not at 11 years\(^{(39)}\). Conversely, reduced airway function (\(V_{\text{max}}\)FRC) present in early infancy is also associated with persistence of wheeze at 11 years of age, independent of the effect of increased AHR and atopy in childhood\(^{(40)}\). Neither the Perth nor the London cohort showed association between AHR in infancy and at school age\(^{(39,66)}\). However, the absence of increased AHR in both infancy and childhood was associated with a zero incidence of asthma and wheeze age 10-11 years in both studies.
The COPSAC birth cohort also studied AHR (PC$_{20}$ FEV$_{0.5}$) to methacholine in 402 neonates$^{(67)}$. The 17q21 gene variants (ORMDL3) were associated with increased AHR in infancy and at 4 years of age but not at 6 years of age, suggesting a genetic determinant of neonatal AHR$^{(67)}$. There was no associated risk of eczema, rhinitis, or allergic sensitization with the 17q21 gene variant.

The London and Perth cohort studies suggest that different factors drive AHR in infancy and later in life. Genetic factors such as β-adrenoceptor polymorphisms may influence AHR in the neonatal period$^{(66)}$, but other factors including early viral and bacterial infections or development of atopy may predominate after this. It should also be noted that measurements of AHR are very challenging in infants and increased AHR may not have been detected in some. On the other hand AHR may be detected in adults without respiratory symptoms. There is also a close link between upper and lower airways with 30% of adults with moderate-severe allergic rhinitis (perceiving only nasal symptoms) demonstrating AHR on methacholine challenge$^{(69)}$.

The 3 studies assessing AHR in infants used direct methods of assessing AHR with pharmacological stimuli such as histamine or methacholine causing constriction of the airway smooth muscle, leading to airway narrowing and airflow limitation. Indirect stimuli (allergens, cold air, exercise) act through one or more intermediate pathways, many (but not all) of which involve release of mediators from inflammatory cells. Bronchoconstriction from both direct and indirect airway challenges is reported to induce airway remodelling in patients with asthma$^{(70)}$.

1.5. Lung function and asthma: school age to adulthood

Both the TCRS and German MAS studies have shown deficits in lung function in those with persistent wheeze are present by 6 years and track through to adolescence$^{(6;32)}$. The longest running cohort studies, Melbourne$^{(71)}$ and Dunedin$^{(72)}$, have tracked deficits in lung function from time of recruitment at school age to adulthood in asthmatics.

The Melbourne asthma study was the first longitudinal asthma cohort study which followed randomly selected 7 year old school children at 7 year intervals until age 50
years between 1964 and 2007\(^{71,73}\). The term wheezy bronchitis was used in the 1960s to refer to wheezing in association with a respiratory tract infection (which would now be termed ‘episodic viral wheeze’). This cohort study is particularly interesting as children were recruited in 1967 before the routine use of inhaled steroids, β-agonists or sodium cromoglycate and helps to clarify the natural evolution of early childhood wheezing to adult asthma without modern interventions. At 42 years only 5% of the original wheezy bronchitis group had persistent asthma but 70% of the asthma group and 90% of the severe asthma group were still symptomatic. Most of the children with severe asthma had either a barrel chest deformity or reduction in FEV\(_1\) at recruitment, rarely seen now in clinical practice. Furthermore, children with severe asthma had more than 30 times the risk of developing adult chronic obstructive lung disease (COPD) as adults compared to children without asthma\(^{73}\).

Children from Dunedin (n=1139) were recruited shortly after the children from the Melbourne cohort study. The Dunedin cohort were born between 1972-1973 and followed from 9 years at 2 or 3 yearly intervals until age 26 years\(^{72}\). Subject retention was 59%. Persistence of wheeze was associated with smoking at 21 years (odds ratio (OR)=1.71), AHR (OR=3), female sex (OR=1.71) and sensitisation to house dust mite (OR=2.41) at 13 years. However, history of early childhood wheezing was retrospectively recalled when the children were 9 years old, which may have predisposed the study to maternal recall bias.

### 1.6.1 Asthma Prediction Indices

Many preschool children with wheeze do not have asthma characterised by eosinophilic inflammation and are unlikely to respond to asthma treatment. At present there is no treatment able to prevent the development of school age asthma in preschool children with wheeze. Studies have shown that neither intermittent\(^{74}\) nor continuous\(^{75,76}\) inhaled corticosteroids (ICS) prevent the development of asthma in episodic wheezers. Intermittent ICS therapy in the first 3 years of life had no effect on the progression from episodic to persistent wheezing and no short-term benefit during episodes of wheezing\(^{74}\). Children at high risk of future asthma were enrolled in the Prevention of Early Asthma in Kids study (PEAK)\(^{77}\) to assess if chronic early therapy with ICS initiated in children aged 2 to 3 years of age at high risk of asthma.
can prevent the development of persistent asthma at 4 to 6 years of age. This hypothesis was subsequently disproven in this group of high risk children\textsuperscript{(76)}. Similarly, a subsequent study confirmed the early use of ICS in wheezy preschool children 1-2 years had no effect on the natural history of asthma, and nor did treatment prevent lung function decline or reduce airway reactivity by age 5 years\textsuperscript{(75)}. Other approaches to prevent the development of asthma have used anti-histamine treatment in high risk children with atopic dermatitis. The Early Treatment of the Atopic Child (ETAC) trial randomised infants aged 1-2 years to cetirizine or placebo for 18 months\textsuperscript{(78)}. There was no difference in wheeze prevalence for the group as a whole at the three-year follow-up (18 months after randomization was complete) but asthma was delayed in a subgroup of infants with atopic dermatitis sensitized to grass pollen and house dust mite\textsuperscript{(78)}. A second study, using L-cetirizine, was completely negative (Warner JO, personal communication).

Since loss of lung function takes place before 6 years of age, the preschool years represent a potential window for intervention to modify the natural history of asthma. Despite the body of literature regarding asthma and risk factors for its development, surprisingly few studies give ‘predictive indices’ for the development of asthma in young children with wheeze. Improved ability to predict which preschool wheezers are at high risk of persistent wheeze and school age asthma would be useful firstly to study mechanisms of conversion of episodic viral wheeze to asthma; and secondly as a tool to define high risk groups to allow the appropriate selection of children for future prevention strategies.

Cohort studies have been used to try to develop prediction indexes of the likelihood of later asthma in children with wheeze at preschool age. There are four main asthma predictive indices to date. The ‘Castro-Rodriguez\textsuperscript{27},’Isle of Wight\textsuperscript{12}, and PIAMA birth cohort\textsuperscript{10} indices are based on cohort studies with greater than 1000 patients and use asthma risk factors identified in epidemiological studies including parental history of asthma and atopy, and presence of other atopic conditions such as allergic rhinitis and eczema to develop a predictive index. The Oslo case control study\textsuperscript{79} is smaller and used a simple score based on frequency, persistence and severity of wheeze to predict later asthma.
1.6.2 ‘Castro-Rodriguez’ Asthma Predictive Index

As part of the Tucson longitudinal birth cohort study parents with children who wheezed had reported their severity on a scale (1-5, from 1= ‘very rarely’ to 5= ‘on most days’) at the year 2 and 3 surveys. Children with a score of greater than 3 were defined as frequent wheezers. To classify children at risk of asthma two indices, a ‘stringent index’ and a ‘loose index’ for the prediction of asthma were designed. For the stringent index the child had to be an early frequent wheezer during the first 3 years of life and meet one major or 2 minor criteria. For the loose index the children had to have wheeze at any time during the first 3 years and meet one major or 2 minor criteria, table 1.3. The ‘stringent index’ had good negative predictive value (NPV) (86%) and very poor positive predictive value (PPV) (42%) for diagnosis of asthma by 11 years. Conversely, a more 'loose index' which only required infrequent wheezing episodes plus the same combination of other risk factors still had high NPV (87%) but even lower PPV (27%). Sensitivity decreased with increasing age to 13 years, but specificity remained constant. The PEAK trial modified the published ‘Castro-Rodriguez’ asthma predictive index to include children age 2 years and markers of allergic sensitisation to improve future asthma prediction, table 1.3.

Table 1.3: Original and modified ‘Castro-Rodriguez’ asthma predictive index

Differences between the original and modified asthma predictive index are shown in italics

The child must have a history of 4 or more wheezing episodes with at least 1 confirmed by a physician plus

**Major criteria**
- Parental history of asthma
- Physician-diagnosed atopic dermatitis
- *Allergic sensitisation to ≥ 1 aeroallergen*

**Minor criteria**
- Wheezing unrelated to colds
- Blood eosinophils ≤4%
- Doctor Diagnosed Allergic Rhinitis
- *Allergic sensitisation to milk, egg or peanuts*
1.6.3 ‘Isle of Wight’ Asthma Predictive Index

A randomly selected birth cohort was established on the Isle of Wight in 1989 to prospectively study the natural history of childhood asthma\(^{12}\). Enrolment was at birth and information on family history of allergy (parental or siblings), household pets, smoking habit, social class, birth weight was recorded and cord blood IgE measured. Children were followed up at 1, 2, 4 and 10 years. Skin prick testing was performed on most children seen at 4 years (n=981). Follow up was 71% (n=1034) in children with information available from the 4 visits over the 10 year period. Risk factors including family history of asthma, atopy as measured by skin prick tests at 4 years, recurrent chest infections and recurrent nasal symptoms (which were shown to have a protective effect) were used to create a cumulative risk score (0-4) for wheeze persistence. A maximum risk score of ‘4’ was associated with prevalence for wheezing persistence of 83% at age 10 years while a ‘0’ score was associated with prevalence for transient wheezing of 80%. A limitation of this index is that scores at either extreme were only present in a few children. A score of 3 had a PPV of 68% and a NPV of 74%. The likelihood ratio, which refers to sensitivity/(1-specificity) and provides a direct estimate of how much the asthma predictive index will change the odds of having a disease, was 3 for an asthma predictive score of 3.

1.6.4 ‘PIAMA’ Asthma Predictive index

The PIAMA birth cohort followed 3,963 children for 8 years in the Netherlands. Between 0 and 4 years of age, 2,171 (55%) children reported “wheezing,” “coughing at night without a cold,” or both\(^{10}\). In these children possible predictors for later asthma were assessed at the age respiratory symptoms were first reported. Eight clinical parameters independently predicted asthma at 7 to 8 years of age: male sex, post-term delivery, parental education less than third level, any parental inhaled asthma medication use, wheezing frequency, wheeze / dyspnoea apart from colds, respiratory infections, and eczema. A clinical risk score was developed (range, 0-55 points) based on these 8 clinical parameters at the time preschool children first reported asthma. Eleven percent of children with symptoms at 0-4 years of age had asthma at 7-8 years of age. The area under the receiver operating characteristic curve (AUC) for this score is 0.74. The predictive power of this rule is not easily comparable with that of other studies because of differences in study design. As with all such studies, there was no second validation cohort. Symptomatic children with a
score of less than 10 points had a 3% risk of asthma, whereas children with a score of 30 points or greater had a 42% risk of asthma.

1.6.5 ‘Oslo’ Asthma Predictive Index
The ‘Oslo’ asthma predictive index was derived from a nested case control study within the ECA study\(^{(14)}\), comprising 233 2-year-old subjects with recurrent (>2 episodes) bronchial obstruction and 216 subjects without bronchial obstruction\(^{(79)}\). A severity score (0-12 points) at 2 years was calculated by frequency and persistence of bronchial obstruction and hospital admissions because of recurrent wheeze. Wheeze sufficiently severe to require hospitalisation had been shown to be a risk factor for the development of asthma\(^{(79)}\). A severity score of greater than 5 is predictive of asthma, PPV (44-51%) and NPV (78-87%). This score had a linear increase with an increase in OR of 1.35 for each point increase in the severity score. The OR for persistent wheeze increased from 3.7 to 9.8 as the severity score increased from 1-12. However, there were very wide confidence intervals at both extremes of the scale.

1.6.6 General Practice based Asthma Predictive Indices
Two general practice based studies also developed very simple asthma predictive indices using postal questionnaires to diagnosticate asthma at school age\(^{(80,81)}\). In a British study of children aged 0-4 years, only wheeze after exercise and history of eczema or hay fever at preschool age were predictive of wheezing 6 years after follow up\(^{(80)}\). Absence of these two factors reduced the likelihood of asthma at age 6 years by a factor of 5\(^{(80)}\). Limitations of this study include the small numbers with recurrent preschool wheeze (<150), poor subject retention and the lack of any objective confirmation of wheeze. The general practices were situated on a housing estate in Manchester with evidence of socioeconomic deprivation, which may limit generalisability.

Eyesink et al\(^{(81)}\), measured IgE in 654 children aged 1-4 years with persistent coughing for greater than 5 days who presented to primary care in the Netherlands. All children with a raised IgE (27%, n=96) but only a random sample of children (n=96) with a negative IgE were followed up until age 6. In contrast to the German MAS study\(^{(32)}\), addition of specific IgE (for house dust mite, cat and dog dander) to a
prediction model based on age, wheeze and family history of pollen allergy had little effect on the asthma predictive values. This study had several limitations including loss of 25% of the atopic children to follow up, history of wheezing which was retrospectively obtained from patient notes, and inclusion of children with isolated recurrent cough, a very non-specific symptom of asthma.

Summary
Current asthma predictive indices have better negative predictive values than positive predictive values. This suggests that the API models are better at excluding asthma rather than identifying the children who will develop asthma at school age. The PPVs for asthma predictive indices range from (27-68)% which is little better than flipping a coin in predicting which preschool wheezers will persistently wheeze and have asthma at school age. The four most clinically significant asthma predictive indexes are summarised in table 1.4.
Table 1.4: Asthma Predictive indexes for children with recurrent wheeze at preschool age have good negative but poor positive predictive values

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of children</th>
<th>Asthma Predictive Index</th>
<th>Predictive value for asthma at age 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castro Rodriguez JA, 2000(20)</td>
<td>1,246</td>
<td><strong>Major Criteria</strong> Parental DD asthma DD eczema <strong>Minor Criteria</strong> DD allergic rhinitis Wheezing apart from colds Peripheral blood eosinophilia <strong>Stringent Index</strong> Frequent wheeze plus One major or two minor criteria <strong>Loose index</strong> Any wheeze plus One major or two minor criteria</td>
<td>'stringent index' PPV (42%), NPV (86%) Specificity (97%) Sensitivity (16%) OR= 4.3-9.8 'loose index' PPV (27%) NPV (87%) Specificity (40%) Sensitivity (80%) OR 2.6-5.5</td>
</tr>
<tr>
<td>Kurululaaratchy RJ, 2003(12)</td>
<td>1,456</td>
<td><strong>Score 0-4</strong> Recurrent chest infections at 2 years Family history of asthma Atopic skin prick tests at 4 years Absence of nasal symptoms at 1 year <strong>Score of ≥ 3 increases likelihood of asthma by 3</strong> PPV (68%), NPV (74%) Sensitivity (52%) Specificity (84%) Likelihood ratio=3.4 Very few children with scores of either 0 or 4</td>
<td></td>
</tr>
<tr>
<td>Devulappalli CS, 2008(19)</td>
<td>449</td>
<td><strong>Score 0-12</strong> Frequency persistence of wheeze Persistence of wheeze Number of hospital admissions <strong>Scores &gt;5 are indicative of asthma</strong> PPV (43.9-51%), NPV (78-87%) Sensitivity (2-51%) Specificity (88-99%) 2 point score rise increased the OR by 1.82 (CI 1.57-2.11)</td>
<td></td>
</tr>
<tr>
<td>Caudri D, 2009(10)</td>
<td>3963</td>
<td><strong>Score 0-55</strong> Wheezing frequency Serious infections DD eczema Use of inhaled medications <strong>Score ≥ 30</strong> PPV 42%, NPV 91% Sensitivity 19% Specificity 97%</td>
<td></td>
</tr>
</tbody>
</table>

DD=doctor diagnosed; PPV=positive predictive value; NPV= negative predictive value
1.7 Airway pathology and asthma
Epidemiological studies have failed to provide us with a predictive index for preschool wheeze which has a good positive predictive value for asthma at school age. The pathology of asthma is characteristic and very different from the normal airway; hence pathological indices may be better positive predictors of asthma progression. The next section reviews what is known about the pathology of paediatric preschool wheeze and asthma.

1.7.1 Safety of endobronchial biopsies for assessing airway pathology
Early evidence for airway changes associated with asthma came from post mortem studies, often in adults who had died from fatal asthma exacerbations\(^{82,83}\). More recently routine use of bronchoscopy and endobronchial biopsy (EB) for purely research purposes in adults has advanced our understanding of the pathology of asthma, allowing the comparison of endobronchial tissue between asthmatic adults with normal healthy individuals\(^{84}\). There are guidelines on the most appropriate research methods to perform, process and analyse endobronchial biopsies, with specific guidelines for paediatric EB incorporated\(^{85}\). The safety of EB has now been reported for school aged and preschool children\(^{86-90}\). In children, bronchoscopy can only be performed if there is a clinical indication. However, it adds just 5 minutes to the bronchoscopy procedure to obtain three endobronchial biopsies in children\(^{91}\). The taking of additional samples for research at the time of such a procedure can be justified if the ethics committee approves, the carers consent, and the child gives age appropriate assent.

1.7.2 Airway pathology features typical of asthma
Typical pathological abnormalities seen in EB from asthmatic adults and older children include; airway inflammation (particularly eosinophils and T lymphocytes) and airway remodelling (defined as structural changes in the airway wall including increased reticular basement membrane (RBM) thickness, increased airway smooth muscle (ASM), epithelial shedding, angiogenesis and goblet cell hyperplasia, figure 1.3 & 1.4\(^{82-95}\). However, the role of airway remodelling and inflammation in disease persistence and progression is not clear. Furthermore, the onset and course of airway inflammatory changes and remodelling in children with asthma are not yet fully characterised. One aim of this thesis is to further characterise airway pathology
in severe recurrent preschool wheezers and its relationship to future school age asthma.

1.7.3 Airway inflammation in asthma
Airway inflammation in asthma involves multiple cells, particularly CD4+ T lymphocyte cells, eosinophils and mast cells. Stimulation of these cells leads to release of proinflammatory mediators and cytokines, which in turn cause vascular leakage, smooth muscle contraction, inflammatory cell infiltration, mucus hypersecretion and airway hyperresponsiveness.

T lymphocytes
T lymphocytes are particularly important in driving inflammation. When CD4+ T-helper cells are activated, they differentiate depending on the local cytokine milieu into distinct lineages of effector cells, called T helper (T_{H}) cell types including T_{H}1, T_{H}2, T_{H}9, T_{H}17 and T_{H}22, or regulatory T cells (T_{reg}). T_{H}1 cells contribute to protection against viruses and bacteria, and T_{H}2 cells protect against parasites and helminths. Children and adults with asthma typically show a predominant T_{H}2 immune inflammatory profile which develops after exposure to an inhaled aeroallergen, characterised by cytokines such as interleukin (IL)-4, IL-5 and IL-13\(^{96}\). However, asthma has several subphenotypes and this is perhaps an oversimplified approach. T_{H}17 cells are reported to be mainly involved in neutrophilic asthma phenotypes\(^{97}\). At birth, there is a presumed fetal T_{H}2 predominance, thought to be necessary to prevent immune rejection of the fetus by the mother, but as exposure to bacteria and viruses in infancy and early childhood drives a switch to a T_{H}1 phenotype, characterized by the release of cytokines such as interferon-\(\gamma\)\(^{96,98}\). The T_{H}1/T_{H}2 balance may not be fully developed in infant and preschool wheezers. Delayed maturation of T_{H}1 responses in the neonate may contribute to the development of atopy\(^{99}\). T_{reg} cells constitute 5–10% of the total CD4+ T cells and control T_{H}1 and T_{H}2 immune responses. It is likely that primary functions of T_{reg} cells in the airways is to limit the inflammatory consequences of infection and to maintain tolerance to harmless, inhaled aeroallergens\(^{96}\). Steroid naive wheezy children aged 8-20 months at a high risk of developing asthma (using the Castro-Rodriguez predictive index) had a significantly lower absolute number of T_{reg} cells and lower
expression of IFN-γ following in vitro stimulation with house dust mite compared with those at a low risk of future asthma\textsuperscript{(100)}. 

**Eosinophilic inflammation**

Eosinophils are the prominent cell type in many asthmatics\textsuperscript{(101)} and increased eosinophils have been found in blood\textsuperscript{(102)}, sputum\textsuperscript{(103)}, bronchoalveolar lavage (BAL)\textsuperscript{(104)} and endobronchial biopsy tissue\textsuperscript{(105)}. Although not all children with asthma show eosinophilic inflammation, adults and children with asthma who have tissue eosinophilia have worse airway remodelling and more acute exacerbations\textsuperscript{(93;106;107)}. 

**1.7.4 Airway remodelling in asthma**

Airway remodelling is a key pathological feature of asthma and is defined as the presence of persistent changes to the normal airway wall structure involving changes in the composition, organisation and function of structural cells including enhanced turnover of extracellular matrix components\textsuperscript{(108)}. Remodelling can occur both during normal lung growth and development and in response to injury and inflammation\textsuperscript{(109)}. In asthma, it is uncertain whether airway remodelling occurs as a result of tissue injury and repair or whether it is driven by (as yet undetermined) specific factor(s). Features of airway remodelling, such as increased RBM thickness and increased ASM mass in adult asthmatics are associated with persistent airflow limitation, reduced lung function and AHR\textsuperscript{(110;111)}. However, it is unclear which individual components of remodelling contribute to the clinical manifestations of asthma and in fact whether all remodelling is detrimental in asthma or whether some aspects may be protective. This thesis will focus primarily on the two most well-described aspects of remodelling which have been consistently associated with lung function abnormalities, increased RBM thickness\textsuperscript{(112)} and increased ASM\textsuperscript{(110)}. 


Figure 1.3: A paediatric endobronchial biopsy showing epithelium, reticular basement membrane (RBM), submucosa and airway smooth muscle

Reticular basement membrane
The RBM is visible as an acellular layer directly beneath the epithelium, figure 1.3. The RBM is not present in healthy airways of all species and its function and the reason for its presence in healthy humans remain unclear. Recently the natural history of RBM development has been described in humans\textsuperscript{(113)}. The RBM in children is visible 30 weeks post conceptional age and increases in thickness with age until birth, and subsequently increases rapidly until about 6 years of age\textsuperscript{(113)}. RBM thickness continues to increase after age 6 years, but at a slower rate, until about 17 years\textsuperscript{(113)}. However, there is considerable variability in RBM thickness measurements in healthy school age children and adults\textsuperscript{(113)}.

Increased RBM thickness is the hallmark of established asthma in adults\textsuperscript{(114)}, and is seen by school-age (6-16 years) in severe\textsuperscript{(115;116)} and mild-moderate\textsuperscript{(93;117)} asthmatics. The abnormal thickening of the RBM begins early in childhood\textsuperscript{(9;116)}, and is already maximally thickened in children aged 6–16 years with severe asthma\textsuperscript{(116;118;119)}. 
Airway smooth muscle

Increased ASM mass is thought to account for most of the functional contribution of airway remodelling to the pathophysiology of AHR\cite{109,120}. Increased smooth muscle mass in the airway wall of asthmatic children and adults is due to both hypertrophy and hyperplasia of smooth muscle cells\cite{110,121,122} and has been linked to both disease severity and duration\cite{110,123}. In particular, alterations in ASM are most consistently associated with abnormalities in lung function. ASM hypertrophy and hyperplasia in school aged children and adults with asthma have been significantly related to bronchodilator responsiveness\cite{110,121}. Furthermore, the proportion of ASM increases as airflow limitation worsens\cite{124,125}.

Epithelium and Epithelial Mesenchymal Trophic Unit

The bronchial epithelium acts as a barrier separating the external environment and the lung parenchyma. The epithelium is emerging as an area of interest as new research shows interactions between the epithelium and the immune system are crucial in driving asthma\cite{126}. Genetic studies also suggest that the epithelium plays a significant role in the allergic response. The protocadherin-1 gene on chromosome 5q31-q33, which is significantly associated with AHR, encodes an adhesion molecule expressed in airway epithelium, which at least suggests that a structural defect affecting the integrity of the airway epithelium contributes to the development of AHR\cite{127}. Epithelial cells from children with mild asthma are intrinsically different both biochemically and functionally compared with epithelial cells from children without asthma\cite{128}. Asthmatic epithelial cells from children with asthma also spontaneously produced significantly greater amounts of IL-6, prostaglandin E\textsubscript{2}, and epidermal growth factor, and equivalent amounts of IL-1β and soluble intracellular adhesion molecule-1, but significantly lower amounts of transforming growth factor (TGF) β\textsubscript{1}\cite{128}.

Early adult asthmatic endobronchial biopsy studies showed epithelial desquamation\cite{129}, but the implication of this is controversial as some evidence suggests that epithelial damage may be due to tissue processing\cite{130}. The presence of epithelial shedding in children is unclear. Children with mild-moderate asthma (aged 2-15 years) had evidence of epithelial shedding on EB\cite{94}. However, no differences in epithelial shedding was seen between atopic and non-atopic children.
with mild-moderate asthma. In contrast, school age children (aged 5-16 years) had similar epithelial shedding on EB when compared to nonasthmatic controls, but atopic children with severe asthma had more epithelial shedding than non-atopic children with severe asthma. It is possible that there is an increased influence of atopy on epithelial shedding as asthma severity increases.

The bronchial epithelium and underlying RBM have a close spatial and functional inter-relationship and are considered an epithelial-mesenchymal trophic unit (EMTU). Many types of challenges to the epithelium, including pathogens, allergens, environmental pollutants, cigarette smoke, and even mechanical forces, can elicit production of mediators by the epithelium, which can be translated into remodelling responses by the mesenchyme. Damage to epithelial cells is thought to stimulate fibroblasts which lie directly underneath the epithelium to differentiate into myofibroblasts that secrete extracellular matrix (ECM) proteins as well as proinflammatory mediators. In asthma, it has been suggested that abnormal mechanical susceptibility to injury and aberrant repair responses result in persistent activation of the EMTU leading to tissue remodelling and altered airway function.

**Angiogenesis**

Angiogenesis, the formation of new blood vessels from pre-existing ones, is recognised in both mild and severe asthma. Morphological analysis of the vascular bed in biopsies from adults with asthma report an increase in both total number of vessels and vascular area. Children with mild-moderate asthma and atopic children without asthma had an increase in lamina propria vessels when compared to normal non-atopic control children.

**Goblet cell hyperplasia**

Goblet cell hyperplasia has been established as a pathologic characteristic of mild, moderate, and severe asthma in adults. Hypersecretion of mucus into the airway lumen in asthmatic patients appears to be caused by both submucosal gland hypertrophy and goblet cell hyperplasia. Abnormalities in goblet cell number are accompanied by changes in stored and secreted mucin. Mild adult asthmatics have increased stored mucin in the airway epithelium, whereas adults with moderate asthma have both increased stored and secreted mucin. An
important factor causing the increased mucus production may be persistent allergen inhalation. Neonatal mouse pups had evidence of goblet cell hyperplasia after 3 weeks of inhaled allergen challenge, however evidence of goblet cell hyperplasia was no longer present 4 weeks after stopping allergen challenge. Goblet cell hyperplasia, epithelial shedding or angiogenesis in EB from preschool children are not discussed further in this thesis. In particular the epithelium was not intact in EB from both preschool wheezers and controls, the subjects of this thesis, and thought to be related to biopsy processing.

Figure 1.4: Interactions between the environment, airway remodelling and inflammation are involved in the pathogenesis of asthma (Reproduced with permission Llyod et al, Nature Medicine 2010)

1.7.5 Pathogenesis of asthma
Several models of the onset of asthma pathophysiology have been proposed. The initial assumption was that persistent chronic inflammation or recurrent episodes of acute inflammation result in the development of chronic airway remodelling over many years, and in a minority of patients resulting in irreversible airway obstruction. This suggests that airway inflammation is an early event and remodelling late in the pathogenesis of asthma. Recent findings challenge the hypothesis that inflammation is the primary driver of pathological change in asthmatic airways. Bronchoconstriction itself in adult asthmatics without additional inflammation has
been shown to induce airway remodelling\(^\text{70}\). A more likely hypothesis is that airway inflammation and airway remodelling occur in parallel driven by some ‘asthma factor’\(^\text{136}\). This is confirmed by animal data; neonatal mice exposed to inhaled house dust mite developed eosinophilic inflammation, airway remodelling, and AHR in parallel between 2-3 weeks of age\(^\text{135}\).

It has also been suggested that in asthma a structurally and functionally defective lower airways epithelium underlies abnormal responses to the inhaled environment leading to enhanced signalling between the EMTU and immune cells\(^\text{137}\). This in turn promotes a microenvironment that facilitates allergic sensitisation, supports different types of inflammation and predisposes the airways to exacerbations leading to persistence of asthma during childhood\(^\text{137}\).

1.8 What is known about airway remodelling and inflammation in preschool children?
The natural history of the airway pathology associated with preschool wheeze is not completely understood. School age children with mild asthma aged 4-12 years already had evidence of both airway eosinophilia and increased RBM thickness, suggesting that airway remodelling and inflammation began before age 4 years\(^\text{93}\). Infants aged 1 year recruited from Helsinki, Finland despite having severe wheeze and reversible airflow obstruction, did not have features of either increased subepithelial inflammation or increased RBM thickening in EB\(^\text{138}\). However, preschool children recruited from the Royal Brompton Hospital (RBH) London aged 2-3 years with confirmed severe, recurrent wheezing did have evidence of increased subepithelial eosinophilic inflammation and RBM thickening in EB when compared to age matched non-wheezing controls\(^\text{9}\). Allowing for the limitations (these were two separate cross sectional studies with different definitions of preschool wheeze), these two studies suggest that there may be a window between the onset of symptoms, and two years of age, during which features of airway remodelling and inflammation consistent with asthma develops. This suggests that pathological abnormalities characteristic of asthma become evident around 2 years of age, very similar to the timing of onset of abnormal lung function in the TCRS persistent wheezers and likely future asthmatics\(^\text{6}\).
Importantly, non-atopic children (median age 5 years, range 2-15 years) with multiple-trigger wheezing and bronchodilator reversibility had evidence of increased RBM thickness, suggesting non-atopic wheezing in children is also associated with this characteristic pathological features of asthma\textsuperscript{(107)}. In support of this, children who had EB at RBH (median age 2 years) were compared by multiple-trigger and episodic wheeze phenotypes at 4 years. Multiple-trigger wheezers had increased RBM thickness and eosinophils when compared with episodic wheezers\textsuperscript{(63)}. However, RBM thickness is not as great in the severe preschool school wheezers when compared school age children with difficult-to-treat asthma, suggesting that disease process may not be fully established at age 2-3 years, figure 1.5.

**Figure 1.5: Reticular basement membrane (RBM) thickness in preschool confirmed wheezers (CW) is increased when compared to non wheezing control subjects (Ctrl), but is significantly less than school-aged children with difficult asthma\textsuperscript{(9)}**

There are few data available on the school age respiratory outcomes for children who had EB for wheezing at preschool age. Follow up of the wheezy 1 year old infants from Helsinki at 3 years of age showed that individual values of RBM thickness at 1 year of age correlated with higher respiratory morbidity and more frequent purchase of ICS at 3 years, but follow up data at school age has not been reported\textsuperscript{(139)}. EB were performed in Czech children (n=27) aged 1-12 years without a diagnosis of asthma for recurrent or chronic respiratory symptoms and were followed up at variable time periods after EB (22–80 months) to determine whether or not they
had subsequently developed asthma\(^{(140)}\). Although, this study has several limitations children who developed asthma following EB had increased eosinophilia and RBM thickness suggesting pathological changes preceded the diagnosis of asthma\(^{(140)}\).

Follow up of the severe, recurrent wheezers recruited from the RBH aged 4-5 years showed that RBM thickness and subepithelial eosinophilia correlated with FeNO but not lung function\(^{(63)}\). However, neither at this time (aged 4-5 years) nor at the time the time of the original study, was it known which of the severe, recurrent preschool wheezers recruited from the RBH would have developed asthma by school age. Exploring the pathophysiology of severe recurrent wheeze in preschool children further may help establish at what age different components of airway remodelling appear and their relationship to future asthma. This is the first step in identifying the remodelling components that could be potential targets for any future interventional studies and at what point in the natural history treatment should be initiated to achieve the best results. It is likely that early diagnosis and prevention of airway remodelling has the potential to decrease disease severity and prevent disease expression. Some aspects of airway remodelling are beneficial, for example tissue repair following injury, so any future therapies will need to differentiate harmful and beneficial remodelling. This thesis will explore airway pathology associated with preschool wheeze further, and relate airway remodelling and inflammation at preschool age to the presence or absence of asthma at school age.

1.9 Airway pathology not previously explored in severe recurrent preschool wheezers

Airway pathology in children with severe recurrent wheeze is incompletely understood. Nothing is known about the role of ASM, mast cells or tenascin-C a component of the RBM in severe recurrent wheezers or their relationship to future asthma at school age.

**Tenascin -C**

RBM thickness detects a group rather than individual difference between wheezers and controls suggesting RBM thickness alone may not be a good marker of future asthma in the individual child\(^{(9)}\), figure 1.5. Tenascin-C (TN-C) is an extracellular matrix glycoprotein component of the RBM and is almost absent in normal adult
tissues, but expression is increased during organogenesis and fibrotic, neoplastic and inflammatory conditions. TN-C is expressed in both acute and chronic inflammatory conditions\(^{(141)}\) including increased expression in the RBM in asthma\(^{(142-144)}\). However, this has not previously been explored in the RBM of severe recurrent preschool wheezers and may be more specific in detecting individual children who will wheeze persistently and develop asthma. This will be discussed in detail in chapter 4.

**Airway Smooth Muscle**

It has previously been shown that moderate to severe asthmatic children as young as 8 years have increased ASM when compared to age matched controls\(^{(121)}\). Reversibility of bronchial obstruction in school age children with asthma has also been associated with increased smooth muscle mass\(^{(121)}\). However, although there is increased RBM thickness in 2-3 year old severe recurrent wheezers nothing is known concerning airway smooth muscle at pre-school age. Chapter 5 will discuss ASM in preschool wheezers.

**Mast cells**

Mast cells are one of the cell types critical in mediating the acute response in asthma. Amongst other mediators, mast cells secrete histamine, prostaglandin D\(_2\), and leukotriene C\(_4\), which are capable of inducing bronchoconstriction, mucus secretion, and mucosal oedema, all features of asthma\(^{(145)}\). Increased mast cells have been found in the epithelium\(^{(146)}\), submucosa\(^{(147)}\), and ASM\(^{(148-150)}\) in EB from adult asthmatics. In particular, mast cell infiltration of ASM distinguishes asthma from eosinophilic bronchitis in adults and correlates with airway hyperresponsiveness\(^{(148-150)}\). Mast cells are found adjacent to the blood vessels in the lamina propria in normal human airways but migrate to the ASM in adult asthematics\(^{(148)}\). Smooth muscle mast cells have been associated with increased IL-4 and IL-13 positive cells within smooth muscle\(^{(151)}\). Activation of mast cell β-tryptase in the ASM bundle promotes airway smooth muscle cell differentiation into a more contractile phenotype possibly by the autocrine release of TGF-β\(^{(152)}\).

Studies which reviewed the role of mast cells in children are limited. Children (median age 12 years) with severe therapy resistant asthma had no associated mast
cell myositis\textsuperscript{(119)}. Furthermore, children (aged 4-12 years) with mild-moderate asthma had no difference in submucosal mast cells when compared with atopic children without asthma and children with no atopy or asthma\textsuperscript{(93)}. Infants with reversible airflow obstruction (median age 1 year) from Helsinki had no increase in submucosal mast cells\textsuperscript{(138)}. The role of mast cells (both submucosal and smooth muscle) in children with severe recurrent preschool wheeze is unknown. Chapter 6 will discuss submucosal airway inflammation including both submucosal and airway smooth muscle mast cells.

1.10 Hypotheses, Aims & Objectives

In summary, a limitation of the previous study investigating the pathology of preschool wheeze\textsuperscript{(9)} is that at the time of biopsy it was not known which wheezers would go on to develop asthma by school-age. The purpose of this thesis is to study these children who are now at school age (6-11) years, establish current asthma status and relate this to early pathological findings. Other features of airway remodelling (increased ASM and RBM TN-C) and inflammation (mast cells) that have not previously been explored in children with preschool wheeze will also be investigated. In particular increased airway smooth muscle is a feature of established asthma in adults and children as young as 7 years, but nothing is known about ASM in preschool wheezers.

Hypotheses

1. School-aged children with asthma have evidence of airway remodelling (increased reticular basement membrane thickness and ASM) and eosinophilic airway inflammation in endobronchial biopsies taken while symptomatic at preschool age, when compared to school-aged children without asthma.

2. A combination of clinical and airway pathological characteristics at preschool age may be used to predict asthma at school age, better than clinical features alone.
Aims

1. To establish whether the preschool children (both wheezers and non-wheezing controls) who had a bronchoscopy and endobronchial biopsy between October 2002 and February 2005 (study number 02-061), and are currently aged 6-11 years have asthma.

2. To extend the previously made observations on airway wall changes in pre-school wheeze by quantifying tenascin-C, ASM and mast cells in endobronchial biopsies

3. To determine whether any or all of pulmonary function, exhaled nitric oxide, and airway hyperresponsiveness at school age relates to the airway pathology in the preschool years.

4. To determine the relationships between combinations of clinical characteristics and pathological markers on endobronchial biopsy at preschool age and a diagnosis of asthma at school age.

Specific Objectives

1. To collect information from the children now aged 6-11 years who had an endobronchial biopsy between 2002 and 2005 (study number 02-061) on current symptoms (history and examination), atopic status (skin prick tests), airway inflammation (exhaled nitric oxide, induced sputum cytology), lung function (spirometry, multiple breath washout, and hypertonic saline challenge).

2. To establish the most discriminative and repeatable method of measuring tenascin-C expression within the reticular basement membrane (comparing stereology and use of computer aided image analysis) in endobronchial biopsies archived in paraffin.

3. To quantify both RBM thickness and RBM tenascin-C in preschool endobronchial biopsies and relate the findings to any subsequent asthma diagnosis.
4. To quantify the proportion of ASM in the preschool endobronchial biopsies and relate this to the presence or absence of asthma at school age

5. To measure submucosal eosinophil volume density by stereology, and mast cell infiltration within the submucosa and ASM by total cell counts in pre-school bronchial mucosal biopsies, and relate the findings to any subsequent asthma diagnosis

6. To generate an asthma predictive index for school age asthma, based on a combination of clinical and pathological features in the pre-school years
Chapter 2
Clinical Research visit
Subjects, Methods and Results

2.1 Summary of study design
This was a second cross-sectional study of children who are now at school age who were originally prospectively recruited from the RBH between 2002 and 2005\(^9\). The group comprises preschool controls (children without lower respiratory symptoms) and children who had severe, recurrent wheeze aged 3 months to 5 years and had undergone bronchoscopy, EB and assessment of atopy at preschool age as part of their clinical investigation. These children were followed up at age 6-11 years to establish whether they had developed asthma. The children who agreed to return for a research visit at school age underwent assessment of lung function, airway inflammation and atopy (study number 6243). The investigator (RO’R) was blind to the biopsy pathology results during the clinical visits and to all clinical data during the airway pathology measurements.

2.2 Study details at preschool age

2.2.1 Patient characteristics of the preschool ‘wheezers’ group
These data have been reported before\(^9\). In brief, all of the children (n=47) included in the original study as wheezers had at least 3 severe episodes of wheeze (each episode with symptoms for more than 3 days) in the previous 6 months\(^9\). They had all been referred to the RBH for specialist advice regarding management of their respiratory symptoms. The majority of the children had at least 1 admission to hospital. Almost all of the children had also undergone a trial of inhaled corticosteroids (ICS) and had persistent symptoms despite use of prescribed medications including inhaled β-agonists.

Children were characterised into wheeze phenotypes, namely episodic (symptoms with viral colds, otherwise asymptomatic) and multi-trigger (acute exacerbations with viral colds and interval symptoms between colds). The following exclusion criteria had been applied at preschool age by Dr Sejal Saglani (SS) to ensure that those
included were not more likely to have a diagnosis other than asthma as an explanation for their wheeze:

i) children with isolated cough without associated noisy breathing

ii) children whose main problem was recurrent lower respiratory tract infection

iii) children who were on long term oxygen therapy

iv) children with chronic lung disease of prematurity.

2.2.2 Patient characteristics of the preschool ‘Controls’ group

Controls (n=21) for the original study were obtained in one of two ways\(^9\). The majority were recruited from patients referred for further assessment of troublesome stridor or to assess airway compression from a vascular ring. The remainder were recruited from patients undergoing a clinically indicated right sided diagnostic cardiac catheterisation and whose carers consented to a research bronchoscopy and EB before the cardiac angiogram. None of the controls had a history of wheezing. The ideal controls would have been truly normal children, but this would obviously not have been ethical.

2.2.3 Use of video questionnaire to identify wheeze in the preschool group

Parent and physician perception of wheeze may be different making it difficult to assess prevalence of asthma accurately\(^{16-19;153;154}\). It has been shown that there may be as little as 50% agreement between parents and clinicians’ findings of wheeze\(^{18}\). Therefore, all parents were shown a validated video questionnaire containing 4 clips each lasting 1 minute of pre-school children making different respiratory noises\(^{18;153}\). The first showed an example of wheeze (male, 6 years), the second stridor (male aged 6 months) and the other two clips showed different upper respiratory noises (both female aged 10 and 24 months). The first upper respiratory noise sounded like a child snoring, although she was awake and the second sound resembled that of loud mouth breathing. Parents were asked “Which if any of the noises in this video does your child make?” They were free to choose none, one or more video clips.

Children were then classified as

- **Confirmed Wheeze (CW) (n=27)**

  - Children whose parents reported wheeze as their child’s main symptom and also identified wheeze from the video questionnaire
- **Reported Wheeze (RW) (n=20)**
  - Children whose parents reported wheeze as their child’s main symptom but did not recognise it on video questionnaire.

- **Controls (n=21)**
  - Children whose parents reported stridor or no symptoms from video questionnaire.

### 2.2.4 Preschool atopic status
All children had atopic status assessed by measuring total IgE and radioallergosorbent tests (RAST) to three food (milk, egg, peanut) and five aeroallergens (grass and tree pollen, house dust mite, cat and dog). The sample of blood was taken while the children were anesthetised for bronchoscopy. Eczema was defined as a positive parental report of doctor diagnosed eczema at preschool age. Atopic status was defined at preschool age as the presence of either or both of eczema and at least one positive RAST to food or aero-allergens.

### 2.2.5 Fibreoptic bronchoscopy and endobronchial biopsy
Fibreoptic bronchoscopy and EB was performed under general anaesthetic at preschool age by one of four respiratory paediatric consultants. A size 2.8mm bronchoscope was used for children aged 3 months to 2 years, and a 3.6mm bronchoscope was used for older children. After evaluation of the airway up to 4 endobronchial biopsies were taken from the sub-carinae of the 3rd and 4th generation bronchi of the right lower lobe using rat tooth forceps (1mm, Olympus, Keymed, UK serial number FB-56D-1) from all children.
Table 2.1: Demographic details of all subjects and controls recruited at preschool age. (The children with confirmed wheeze were significantly older and heavier than the other groups.)

<table>
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<th>Confirmed wheeze n=27</th>
<th>Reported Wheeze n=20</th>
<th>Controls n=21</th>
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<td>12 / 8</td>
<td>16 / 5</td>
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<td>Age (months)</td>
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<td>15</td>
<td>0.005†</td>
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<td>(6-58)</td>
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<td>11.2</td>
<td>0.009†</td>
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<td>(6.16-19.6)</td>
<td>(5.45-18.7)</td>
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<tr>
<td>Atopic*</td>
<td>16 (60%)</td>
<td>11 (55%)</td>
<td>7 (33%)</td>
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<td>Eczema</td>
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<td>3.5</td>
<td>3.26</td>
<td>0.66</td>
</tr>
<tr>
<td>Range</td>
<td>(1.87-4.45)</td>
<td>(1.6-4.6)</td>
<td>(1.95-2.99)</td>
<td></td>
</tr>
<tr>
<td>Gestation (median weeks)</td>
<td>39</td>
<td>40</td>
<td>39</td>
<td>0.58</td>
</tr>
<tr>
<td>Range (weeks)</td>
<td>(30-42)</td>
<td>(32-42)</td>
<td>(28-41)</td>
<td></td>
</tr>
<tr>
<td>Main reported symptom</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheeze</td>
<td>16</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Wheeze and cough (equal)</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cough more than wheeze</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Barking cough</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Stridor</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Shortness of breath</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>4**</td>
<td></td>
</tr>
<tr>
<td>Family history of asthma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal history of asthma</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Paternal history of asthma</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Day care (&lt; 1year)</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pets (Cat/ Dog)</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal smoking</td>
<td>7</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Paternal smoking</td>
<td>12</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Maternal smoking during pregnancy</td>
<td>11</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Treated with ICS</td>
<td>14</td>
<td>10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Dose of ICS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mcg beclomethasone)***</td>
<td>800</td>
<td>400</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Range (mcg)</td>
<td>(200-2000)</td>
<td>(100-1000)</td>
<td>(200-500)</td>
<td></td>
</tr>
</tbody>
</table>

*eczema and / or one or more positive RASTs to aero or food allergens
**n=2 had cardiac angiogram, n=2 had bronchoscopy to assess severity of vascular ring
***Or beclomethasone equivalent

Significant difference between ICW and controls, ICW and controls & CW and RW
2.3 Aims of current study

The aims and objectives of this part of the study were to

1. To establish whether the preschool children (both wheezers (confirmed and reported, n=48) and non-wheezing controls (n=21)) who had bronchoscopy and EB between October 2002 and February 2005 and at the time of re-recruitment were aged 6-11 years had a diagnosis of asthma.

2. To characterise the preschool cohort at school age in terms of lung function, airway inflammation and atopy by collecting information on
   - current symptoms (history and examination)
   - atopic status (skin prick tests)
   - lung function (spirometry, multiple breath washout (SF₆), hypertonic saline challenge)
   - airway inflammation (exhaled nitric oxide, induced sputum cytology).

2.4 Difficulties in diagnosing asthma

One of the most difficult aspects of this thesis and a challenge in all asthma research is the absence of a gold standard diagnostic test for the disease. Reported asthma prevalence varies both with time and area partly due to the different criteria used to define asthma, table 2.2. Criteria most often used to define asthma include one or more of the following, wheeze, doctor’s diagnosis of asthma, asthma medication use and positive bronchial challenge. Asthma prevalence rates vary widely depending on the definition used for diagnosis. Prevalence rates of asthma within a cohort of children can vary from 15% using a diagnosis of doctor diagnosed asthma ever and wheeze in the last 12 months, to 50% using a definition of AHR and symptoms and/or use of medication in the last 12 months.

The term asthma, as used today, is likely more inclusive compared with previous physicians’ diagnoses, including many more children with milder symptoms who might not have been diagnosed with asthma in the past. Increased awareness of asthma as a disease both among parents and physicians is also likely to have increased reporting. Doctor diagnosed asthma is often used in epidemiological studies to qualify the diagnosis, but often there is little consensus on asthma diagnosis between physicians. Studies have shown that up to 30% of adults with a history of doctor diagnosed asthma did not have asthma after objective assessment.
of lung function and AHR\textsuperscript{(157)}. In many, but not all, countries there is avoidance of the term asthma until school age. Even the definition of the start of school age is variable, being defined as anything between 4 and 8 years.

It is widely accepted that parental and physician perception of wheeze is often different and in many cases parents may label other upper respiratory noises as wheeze (section 1.2). Furthermore, lower prevalence rates have been found based on written medical reports rather than the diagnosis of asthma based on parental report of wheeze in the last 12 months on the International Study of Asthma and Allergy in children (ISAAC) questionnaire\textsuperscript{(156;158)}. This may have been for several reasons including incompleteness of records, errors in parental recall or often physicians using asthma as an all embracing diagnosis for recurrent cough or general respiratory symptoms without confirming the diagnosis.

**Definition of current asthma in this study**

There is no consensus as to what definition of asthma at school age should be used. In the present study a diagnosis of asthma based on symptoms alone was made at school age. Current ‘asthma’ at school age was defined for the purpose of this research as a parental report of wheeze in the last 12 months and a parental report of doctor diagnosed asthma ever. This information was available for all children recruited at school age. Measures of lung function and lung inflammation were only available in the subgroup who attended for a research visit. All references to school age asthma in this thesis have used this definition.

The use of asthma medication was not part of the diagnostic criteria in this study. ICS and inhaled salbutamol are often prescribed to children for the treatment of general respiratory symptoms. Cough is a common complaint in childhood with 10\% of preschool and early school aged children having chronic cough without wheeze at some time\textsuperscript{(159)}. Although persistent cough in children without wheeze is much less likely to be asthma\textsuperscript{(160)}, children may often be prescribed ICS. Indeed, three of the original group of controls were treated with ICS at preschool age for stridor and / or a barking cough, although there is no evidence for efficacy in this group. Children with chronic wet cough may have bronchiectasis or persistent bacterial bronchitis but may also be prescribed inhaled corticosteroids\textsuperscript{(161)}.  

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Table 2.2: Different criteria used for diagnosing asthma

<table>
<thead>
<tr>
<th>Study</th>
<th>Age (years)</th>
<th>Criteria for diagnosing asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castro-Rodriguez Index</td>
<td>6-13</td>
<td>• asthma diagnosed by a physician with plus ≥ 1 episode of asthma during the previous year or • had &gt;3 episodes of wheezing during the previous year regardless of asthma diagnosis</td>
</tr>
<tr>
<td>AJRCCM 2000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isle of Wight</td>
<td>10</td>
<td>• &quot;Current wheeze&quot; was recorded as having occurred in the prior 12 months.</td>
</tr>
<tr>
<td>Eur J Resp 2003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eysink et al</td>
<td>6</td>
<td>• combination of both symptoms and/or use of asthma medication and impaired lung function. • Symptoms were defined as current complaints or complaints during the previous 12 months of wheezing and/or shortness of breath and/or recurrent coughing. • Use of asthma medication was defined as use of β₂ agonists or inhaled corticosteroids currently or during the previous 12 months. • Impaired lung function was defined as a positive histamine test, (PC20 &lt;8mg/ml). • Children with no wheeze in the last 12 months were not considered to have asthma.</td>
</tr>
<tr>
<td>BPGP 2005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oslo severity score</td>
<td>10</td>
<td>• Two of the following three criteria (1) Dyspnoea, chest tightness and/or wheezing from 0 to 3 years and/or from 4 to 10 years of age (2) Doctor diagnosed asthma (3) Used asthma medication (inhaled corticosteroids, β₂ agonists, sodium cromoglycate, leucotriene antagonists and/or aminophylline) at 0–3 years and/or 4–10 years • Current asthma was defined as asthma (see above) plus at least one of the following criteria positive: (1) Dyspnoea, chest tightness and/or wheezing during the past 12 months (2) Use of asthma medication during the past 12 months (3) Positive exercise test</td>
</tr>
<tr>
<td>Thorax 2007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frank et al</td>
<td>8</td>
<td>• wheeze reported in the ISAAC survey at age 8</td>
</tr>
<tr>
<td>BMJ 2008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudri et al</td>
<td>8</td>
<td>• Positive asthma diagnosis if children had ≥1 positive items at 7 years and ≥1 positive items at 8 years. (1) at least 1 episode of wheezing (2) inhaled steroids prescribed by a medical doctor (3) a doctor's diagnosis of asthma (a parental report of a doctor's diagnosis of asthma at any time and a parental report of asthma during the past 12 months).</td>
</tr>
<tr>
<td>JACI 2009</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.5 Rationale for choosing assessment methods

Non-invasive, age appropriate methods were used to assess atopy (skin prick tests) airway inflammation (FeNO and sputum eosinophilis), exposure to tobacco smoke, lung function (spirometry and multiple breath washout (MBW)), and AHR. The reasons for selecting these techniques are as follows:

- Skin prick tests are an easy and acceptable method of establishing the atopic status of a preschool child\(^{(163)}\). Ideally atopy would have been measured using both RAST and skin prick tests at school age as concordance for individual allergens is poor\(^{(164)}\). However, it was deemed not ethically appropriate to do a further blood test at school age for research purposes alone. Furthermore, inclusion of a blood test in the research protocol would certainly have decreased the number of children recruited to the study.

- FeNO is increasingly used as a biomarker of eosinophilic airway inflammation in children and adults with asthma. FeNO is derived from endogenous nitric oxide (NO) production by inducible NO synthase in airway epithelium and inflammatory cells. FeNO can reliably be measured at 50ml/s in preschool and school age children (section 2.8.7). In children with asthma, FeNO shows some correlation with eosinophils in induced sputum\(^{(165)}\) and with increased submucosal eosinophils in EB tissue\(^{(63;166)}\) particularly in steroid-naïve children. However, raised FeNO is also associated with atopy in children irrespective of whether they have asthma\(^{(167)}\). Secondly, attempts to treat asthma using FeNO levels to guide anti-inflammatory treatment have had limited success\(^{(168)}\). The use of FeNO as an inflammatory marker does not reflect other inflammatory phenotypes in asthma such as neutrophilic airway inflammation which is why sputum induction was attempted.

- Exposure to passive smoking was assessed objectively as this is known to be associated with increased symptoms and decreased lung function in school children\(^{(169)}\). This was done by measuring salivary cotinine the principal metabolite of nicotine\(^{(170)}\).

- Lung function was measured using both spirometry and MBW. Spirometry although routinely performed to monitor children with asthma, is unlikely to detect subtle decrements in lung function\(^{(42)}\). MBW has been shown to be a more sensitive test in detecting ventilation heterogeneity in children with
asthma than spirometry\textsuperscript{(171;172)}. MBW has the advantage that unlike spirometry measurements normal values outside the first year of life are for practical purposes independent of age, gender and height\textsuperscript{(173;174)}. This will be discussed in more detail in section 2.8.3.

- Hypertonic saline was chosen to perform an indirect airway hyperresponsiveness challenge as it is acceptable to parents and in principal a combined sputum induction could be performed at the same time\textsuperscript{(175)}. The advantage in using an indirect challenge, over a direct challenge (e.g. histamine, metacholine) is that it is more physiological as most asthma stimuli in real life are indirect (exercise, allergen, cold air). A significant positive response suggests that inflammatory cells and their mediators are present in the airways in sufficient numbers and concentration to suggest that asthma is active at the time of testing\textsuperscript{(176)}. The corollary to this is that there can be a negative test in children with known asthma suggesting good control or mild disease\textsuperscript{(176)}. Induced sputum can be used to assess the inflammatory profile of the airways in children with asthma. In particular sputum cytology is used as a marker of airway inflammation in asthmatics\textsuperscript{(177)}.

2.6 Ethical approval for the present study (the work of this thesis)
Ethical approval was obtained from the Brompton, Harefield and NHLI NHS Research Ethics Committee (08/H0708/93). The original ethics submission was prepared by SS (the principal investigator). Ethical approval was given on 23/10/2008. The protocol was changed to include multiple breath washout as part of the assessment of lung function which was approved as a substantial amendment on 23/01/09 prior to beginning recruitment.

2.7 Recruitment for the present study
Between 2009 and 2010 children who had participated in the original study were identified and recruited for follow up. A study invitation letter and parent and child information leaflets were mailed to each family, appendix 1-3. The letters were followed after 10-14 days by a telephone call. The study was discussed at length on the telephone and parents were given the opportunity to ask questions. If parents had any concerns, no appointment was made and a second telephone call was made 2-3 months later. Those interested in participation were then given an
appointment for a clinical research visit. Written confirmation of the appointment time and advice to withhold ICS and long acting β-agonists for 24 hours and short acting β-agonists for 12 hours prior to the appointment was sent. Children with severe asthma on regular oral corticosteroids or immunomodulating steroid sparing agents (such as cyclosporin, azathioprine or methotrexate) were advised not to withhold treatment. Parents were advised not to withhold medication if their child was unwell. The family was telephoned 1-2 days prior to the research visit to remind them of the appointment time and that they needed to withhold ICS and β-agonists as above. If parents felt the child was unwell at time of telephone consultation the test was deferred for at least 2 weeks. For those families that could not be traced from hospital records, the general practitioner was approached for up to date contact details. If this failed, the local referring paediatrician was also contacted for further information. In all families contacted who did not wish to attend for a research visit a telephone questionnaire was administered where possible.

2.8 Research visit

The research visit lasted 2.5-3 hours. The procedures were done in the order listed below, and are discussed in more detail in subsequent sections.

I. Study consent
II. Height and weight
III. Lung clearance index
IV. Video questionnaire
V. Study questionnaire
VI. Salivary cotinine
VII. Exhaled nitric oxide (single expiratory flow rate 50ml/sec)
VIII. Spirometry
IX. Skin prick testing for common aero and food allergens
   (cat, dog, grass, tree, house dust mite, aspergillus, egg, milk, peanut)
X. Combined AHR and sputum induction using nebulised hypertonic saline (4.5%)\textsuperscript{175}.

2.8.1 (I). Study consent

All test procedures were explained and parents were asked to read and sign a consent form, appendix 4. The child’s general practitioner was also informed of the
child’s participation in the study, appendix 5. Informed consent was given to use the photographs in this chapter for illustration purposes.

2.8.2 (II). Height and weight
The child’s height without shoes was measured in centimetres to one decimal place by a calibrated wall-mounted Harpenden stadiometer. Weight was measured in kilograms to one decimal place with calibrated electronic scales, with the child wearing minimal clothing.

2.8.3 (III). Multiple breath washout (MBW)
Multiple breath washout (MBW) is a test of distal airway gas mixing efficiency which requires only passive co-operation, making it very attractive as a test in young children. It requires the subject to breathe tidally an inert tracer gas such as nitrogen (N2), helium (He), sulphur hexafluoride (SF6) or argon in a low concentration until equilibrium is reached. The child is disconnected from the tracer gas during expiration, and the tracer gas is then ‘washed out’ by continued tidal breathing of room air(174;178;179). The longer the wash-out time, the more inefficient is distal airway gas mixing. The use of this technique in children was first described in cystic fibrosis in 1985(180). Prospective longitudinal studies in children with cystic fibrosis have established that it is more sensitive to early lung disease than spirometry(181;182). MBW has also been shown to be a sensitive indicator of pulmonary function abnormalities in preschool wheezers and school age asthmatics(62;171).

MBW was measured in this study using the modified Innocor flow meter and side stream gas analyser (Innovision, Denmark) and pneumotachograph (Hans Rudolph, USA). This has been previously validated for use in adults and children as young as five years(173). The gas analysis unit is calibrated once per year by an Innovision engineer in line with manufacturer instructions. A calibration of the flowmeter and the flow-gas delay was performed once a day. Further calibrations were also performed if the gas sample line or the flowmeter screen was replaced. The flowmeter was calibrated before each study visit using a 3 litre syringe at low, medium and fast flow rates. The flow-gas delay was measured by assessing the carbon dioxide rise time by initially making slow expirations followed by very fast inspirations until the Innocor confirms measurement of the flow-gas delay. The inspirations have to be fast in
order to get a precise determination of the flow-gas delay. If one or two breaths failed the software automatically filtered these results. The flow-gas delay did not vary more than 20-40 ms from day to day in line with manufacturer instructions.

Children were seated with a nose clip and breathed 0.2% SF₆ in medical air, supplied from a compressed gas cylinder (BOC, Hertford, UK) via a mouthpiece and flow past (bias flow) system until an alveolar plateau had been reached, figure 2.1. The gas supply was then detached in expiration and patients breathed room air until the measured end-tidal SF₆ concentration was 1/40th of the starting concentration (0.005%), figure 2.2. Tidal breathing was used throughout the test, each taking 5–10 min to complete. Washouts were repeated three times at each stage to obtain mean values(183). Washouts were excluded if the measured functional residual capacity (FRC) differed by more than 10% from both other repeat washouts(183).

**Figure 2.1: Wash-in phase of multiple breath washout** *(Child seated with a nose clip breathing 0.2% sulphur hexafluoride (SF₆) in medical air).*

pneumotachograph

![Image of pneumotachograph with SF₆, Innocor, and Bias flow labels]
Figure 2.2: Multiple breath washout (SF₆) *The black trace represents flow and the green trace SF₆ concentration*

A - Gas wash-in until lung equilibrium reached (SF₆ concentration 0.2%)
B - Gas supply disconnected in expiration
C - SF₆ concentration falls with each expired breath

Gas wash-out is complete when the concentration of expired inert gas falls to 1/40 the original value, i.e. 0.005%

Many indices have been used to describe the wash-out curve, of which the most commonly reported is lung clearance index, for which functional residual capacity also needs to be determined[^184]. However, wash-out curves do not provide information on the sites of ventilation inhomogeneity, but this can be calculated from the MBW if the phase III slope (SIII) is calculated for each breath[^185;186]. The slope of phase 3 for each breath can then be used to calculate ventilation inhomogeneity measured by convection in the conducting airways ($S_{cond}$) and by diffusion-convection in the acinar airways ($S_{acin}$).

The following measures from MBW are reported in this study:

- Lung Clearance Index (LCI)
- Functional Residual Capacity (FRC)
- Acinar Airways Inhomogeneity ($S_{acin}$)
- Conducting Airways Inhomogeneity ($S_{cond}$)

**Lung Clearance Index (LCI)**

LCI is defined as the total cumulative expired volume (CEV) required to washout the tracer gas to 1/40th of the starting value, divided by the measured FRC (CEV/FRC).
**Functional Residual capacity (FRC)**

Functional residual capacity (FRC) was calculated from the total volume of expired tracer gas (SF$_6$) divided by the end-tidal SF$_6$ concentration at completion of washout$^{(187)}$. Washouts were excluded if there was a greater than 10% difference in FRC from the other 2 washouts. LCI, FRC, $S_{\text{cond}}$ and $S_{\text{acin}}$ are all calculated as the mean of at least two reproducible measurements out of three washouts$^{(171)}$.

**Calculation of phase III slope**

The calculation of the SnIII compares the concentration differences between lung units to the mean lung unit value. As the washout progresses this difference between lung units initially increases, but then remains constant as the concentration gradient between inspired gas and the resident gas in the two units falls. The progression of SnIII through the washout allows the observed ventilation inhomogeneity to be attributed to two separate mechanisms, convection dependent inhomogeneity and diffusion-convection dependant inhomogeneity, figure 2.3. $S_{\text{cond}}$ represents convection dependent inhomogeneity in the conducting airways and is predicted to increase linearly throughout the MBW test$^{(186)}$. $S_{\text{acin}}$ represents diffusion–convection related ventilation inhomogeneity generated in the acinar airways and only increases for the first 5 breaths or 1.5 lung turnovers$^{(186)}$. After the first 1.5 lung turnovers the increase in SnIII is diffusion independent and represents convection dependent inhomogeneity.

In this study each breath of the SF$_6$ concentration was plotted as a function of expired volume. A least-square fit of the slope of the alveolar phase (phase III slope, SnIII) was done. The margins of the regressed portion were manually adjusted to avoid phase II or phase IV elements, the influence of cardiogenic oscillations or signal noise obviously distorting the slopes. The SIII was then normalised by the mean tracer gas concentration over the phase III interval of interest, to account for dilution, giving the normalised phase III slope (SnIII) for SF$_6$$^{(173)}$. 

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Figure 2.3: The contribution of convection dependant inhomogeneity cdi ($S_{\text{cond}}$) and diffusion-convection dependant inhomogeneity dcdi ($S_{\text{acin}}$) to overall ventilation inhomogeneity$^{[174]}$(Adapted by Dr Sonnappa)

Conduction dependant inhomogeneity (CDI) has a linear relationship with expired volume when expressed as lung turnover (TO) Diffusion convection dependant inhomogeneity (DCDI) rises for the first 5 breaths, or 1.5 turnovers, and then is unchanged. The $S_{\text{cond}}$ index is calculated as the slope of the SnIII versus turnover CDI relationship. $S_{\text{acin}}$ is the DCDI component of the first breath SnIII, calculated by subtracting the CDI component (see inset equation).

**Calculation of $S_{\text{cond}}$ and $S_{\text{acin}}$**

$S_{\text{acin}}$ and $S_{\text{cond}}$ were calculated by the method described by Verbanck et al$^{[185;188]}$. The SnIII for each breath was plotted as a function of lung turnover. $S_{\text{cond}}$ and $S_{\text{acin}}$ are derived from the normalised slope curves. $S_{\text{cond}}$ is the normalised difference per unit lung turnover which is derived from lung turnovers 1.5-6 where only conductive airways are known to contribute to the rate of rise of normalised slope, figure 2.4. $S_{\text{acin}}$ is derived from the normalised slope of the first breath which is predominantly generated by the diffusion convection dependant ventilation inhomogeneity within peripheral acinar lung units, figure 2.4. $S_{\text{acin}}$ is determined by subtracting the slope of the first breath against lung turnover.
SnIII was not reported from any breaths that were not clearly visible, either because of inadequate breath volume or signal noise. If the first breath from a run could not be reported, or if more than one third of breaths over the $S_{\text{cond}}$ regression interval could not be reported, then that MBW was excluded from analysis. $S_{\text{cond}}$ and $S_{\text{acin}}$ are only reported if results from two or more MBW runs were available. Tidal volume was not restricted in the patients. Normalised phase III slope values were corrected by multiplying by the breath volume before calculating $S_{\text{cond}}$ and $S_{\text{acin}}$ to allow comparison of the data with published work in adults who were limited to breaths of 1 litre$^{(174;183)}$. In using this protocol, negative $S_{\text{cond}}$ values may be seen in both healthy volunteers and those with very mild disease. Although biologically this is impossible, negative values are seen in practice owing to variation in tidal volume throughout the washout and lack of progression of the normalised phase III slope$^{(171;174)}$.

**2.8.4 (IV). Video Questionnaire**

Parents were shown a modified video questionnaire with 4 clips; 1) wheeze (male 6 years), 2) nasal upper respiratory noises (female aged 10 months); 3) wet cough (male 7 years); 4) stridor (male age 4 years). Video clips 1&2 were shown at preschool and school age. The third and fourth video clips were changed from those used at preschool age which showed children age 6 and 10 months to a more age appropriate examples of stridor and wet cough. All video clips had been previously
Parents were again asked ‘Which if any of these noises does your child make?’. They were also asked ‘Has your child made any of these noises in the past?’ They were free to choose one or more video clips.

2.8.5 (V). Study Questionnaire

The study questionnaire used was a modified ISAAC core questionnaire for asthma age 6-7 years, and the ISAAC core questionnaires for eczema and allergic rhinitis age 6-7 years\(^{(158)}\). A second questionnaire recorded medication history including oral and inhaled corticosteroid use, total number of hospital admissions with wheeze, household pets, length of time spent in nursery and parental smoking. Parents were also asked if their child’s symptoms were mainly associated with colds or with allergy or if they were triggered by multiple stimuli. Although information such as family history of asthma, birth details and parental smoking during pregnancy had previously been recorded the answers were re-checked. Both questionnaires were interviewer directed and the answers were recorded by the interviewer. The same questionnaire was used for both the controls and wheezers, appendix 6.

2.8.6 (VI). Salivary Cotinine

Exposure to exhaled tobacco smoke is measured using the biomarker cotinine. Cotinine is a metabolite of nicotine that can be measured in urine, serum, saliva, and hair. Saliva was chosen as a non-invasive measurement of exposure to cigarette smoking and collected using plain (non citric acid) cotton salivettes. The child was asked to chew or suck the cotton salivette until it was saturated. Salivary cotinine analysis was performed using a salivary cotinine quantitative enzyme immunoassay analysis kit (Salimetrics®, USA). A salivary cotinine greater than 1.2ng/ml was defined as evidence of passive cigarette smoke exposure\(^{(170)}\).

2.8.7 (VII). Exhaled Nitric Oxide (FeNO) (single expiratory flow rate 50ml/s)

NO is formed in both the upper and lower respiratory tracts and diffuses into the lumen by gaseous diffusion down a concentration gradient\(^{(189)}\). Exhaled NO concentrations from the lower respiratory tract are significantly flow dependent, with a marked increase in exhaled FeNO concentration at low flows. This flow dependence is characteristic of a diffusion based process for NO transfer from airway wall to lumen and is explained by faster flows minimising the transit time of
alveolar gas in the airway, thereby reducing the amount of airway NO transferred\(^{(190)}\). The use of a constant expiratory flow of 50ml/s (FeNO\(_{50}\)) is recommended to obtain reproducible FeNO results which are comparable between groups\(^{(189)}\). Multiple exhalation flow FeNO has been previously used to calculate bronchial and alveolar nitric oxide production in older school age children with a median age of 14 (8-18) years\(^{(190)}\). FeNO measurements at multiple exhalation flow rates was not done as most of the children (median age 7 years) were unable to perform the manoeuvres at higher flow values\(^{(190)}\).

FeNO was measured using NiOX (Aerocrine AB, Solna Sweden). A constant temperature of 22°C was maintained in the room in which the analyser was stored to ensure optimal operating conditions. The ambient NO was recorded prior to each reading and was always <300ppb in accordance with the manufacturer's recommendations. Calibration of the analyser was performed every 14 days according to the manufacturer's instructions. External gases required for calibration were NO-free air and a calibration gas mixture of 1-200 ppb NO in N\(_2\) connected to the instrument rear panel. Calibration was automatically carried out by pressing a button on the keypad (one-button-calibration). In order to assure reliability of its results the analyser has an integrated calibration module for zero level and the NO reference gas. The flow head was calibrated daily prior to measurements using a 100ml calibration syringe. The equipment has an on-line display of flow control that allows the selection of good exhalations and the rejection of suboptimal exhalations. FeNO was always measured before forced expiratory manoeuvres such as spirometry because these may cause a decrease in FeNO levels\(^{(191)}\).

The procedure was explained and demonstrated to the child before beginning the test. All measurements were made with the child sitting holding the instrument held in front of them. A nose clip was not used. Children had several practise runs to optimise technique before proceeding to an actual measurement. An online method was used to measure FeNO where the expire is continuously sampled by the analyzer, and the resultant NO profile versus time or exhaled volume, together with other exhalation variables (e.g., airway flow rate), is captured and displayed in real time\(^{(189)}\). For the measurement, NO-free air (generated by the NiOX module internally from ambient air) was inhaled to near total lung capacity over a period of 2
to 3 seconds through the mouth-piece of the instrument. Then the child was asked to exhale slowly, being encouraged to maintain a constant flow by a visual display on a screen (blowing a little girl in a balloon between 2 cliffs), figure 2.5. There was automatic control of exhaled flow rate to a constant 50ml/s, making it easier for the child to comply. The NiOX analyser only accepts measurements as valid if the expiratory flow is between 37.5 and 62.5 ml/s at every instant. If the flow was not steady or decreased below 50ml/s the test was repeated. The exhaled NO value is the mean NO level during a 3 second NO plateau. Three acceptable readings (defined as agreement to within 10%) were performed with at least 30 second intervals between manoeuvres. FeNO$_{50}$ was calculated as the mean of the plateaus from three technically satisfactory exhalations.

Figure 2.5: Exhaled nitric oxide measurement with visual feedback incentive

2.8.8 (VIII). Spirometry performance and quality control

Spirometry was performed using the Vitalograph Compact (Vitalograph, Buckingham, UK). The spirometer was calibrated on the day of the study visit with a one litre syringe. As for many of the children it was the first attempt at spirometry a period of training was necessary. The child was familiarised with the equipment and a demonstration was given. The child was observed closely to ensure that there was no leak and that the manoeuvre was performed optimally. Manoeuvres were rejected
if the subject was thought to use sub-optimal effort, if a full inspiratory breath was not taken, if there was coughing during the first second, if a plateau was not reached on the volume-time curve, or if the blow was terminated prior to full expiration. The highest sum of forced vital capacity (FVC) and FEV\textsubscript{1} of the 3 manoeuvres was recorded as per American Thoracic Society (ATS) / ERS guidelines\textsuperscript{(192)}. If the child had difficulty performing the manoeuvre then interactive computerised incentive spirometry was used (Vitalograph Pneumotrac, Spirotrac\textsuperscript{®} IV software). As this machine was situated in another room and was often in use for busy outpatient clinics it was not possible to use it routinely. All spirometry results were compared to appropriate recent reference ranges\textsuperscript{(193)}.

2.8.9 (IX). Skin-prick testing for common aero and food allergens

SPT were performed for three common food allergens (egg, cow’s milk and peanut) and six common aero-allergens (cat & dog dander, grass & tree pollens, Aspergillus fumigatus and Dermatophagoides pteronyssinus) (Soluprick SQ, Alk Abello, Denmark)\textsuperscript{(163)}. A small drop of allergen extract was placed on the volar surface of the arm and the skin was pierced with a 1mm lancet (ALK, Denmark) in the centre of the droplet, ensuring a small amount of allergen penetrated into the skin, figure 2.6. A positive (histamine) and a negative control were always performed at the same time. The outer contour of the wheal reaction was outlined using a fine felt-tip pen, and the result expressed as the mean of the lengths of the longest diameter and the perpendicular line through its centre. The results were read 15 minutes after application. SPT was regarded as positive if the wheal size was at least 3mm greater than the negative control. The test was considered void if there was no reaction to the positive control. If a child was uncomfortable or had large wheals, after the test was read topical mepyramine maleate (Anthisan) was applied.
2.8.10 (X). Combined bronchial hyper-responsiveness and sputum induction

A combined airway hyperresponsiveness and sputum induction test was performed\(^{(175)}\). Children were asked to rinse their mouth and blow their nose to reduce squamous cell contamination before sputum induction was started. The children were instructed how to cough and clear the throat in order to obtain the best sample. The baseline FEV\(_1\) was recorded as the highest of the 3 manoeuvres from satisfactory spirometric curves (above). Children were seated comfortably and asked to inhale hypertonic (4.5%) saline for periods of increasing duration (0.5, 1, 2, 3 x 4 minute) using an ultrasonic nebuliser (De Vilbliss 2000, Somerset) as per the ISAAC protocol\(^{(163)}\). FEV\(_1\) was measured after each inhalation period and the higher of 2 readings were selected.

The initial 3 nebulisation periods of 30 seconds, 1 minute and 2 minutes were performed without encouraging sputum production, figure 2.7. After each of the three 4 minute nebulisation periods children were encouraged to cough and expectorate sputum into the falcon tube. If the FEV\(_1\) remained within 15% of the baseline value, nebulisation was repeated. If, after two repetitions, the FEV\(_1\) was less than 15%
below the baseline value, the duration of the inhalation period was doubled again according to the protocol. Clinical symptoms such as cough or wheeze were recorded at each stage.

The bronchial challenge was stopped if either the FEV₁ had fallen by 15% from baseline or the total inhalation period of 15.5 min had been reached. The saline canister was weighed on an electronic balance (Ohaus Scout pro, Switzerland) before the first and after the last inhalation period in order to measure the aerosol dose delivered. Children whose FEV₁ had decreased by more than 15% were given salbutamol (1000µg) through a large volume spacer and spirometry was repeated 10 minutes later to ensure return to baseline values before discharge. In children with a baseline FEV₁ of 75% of the predicted value, no bronchial challenge was performed.

Figure 2.7: Child inhaling 4.5% saline nebulised using an ultrasonic nebuliser (De Vilbliss 2000)

Different statistical approaches have been proposed for the analysis of stepwise provocation data such as a hypertonic saline challenge. AHR can be measured in terms of provocation time causing a decrease in FEV₁ (PT₁₅). Insufficient numbers of children in this study had a positive bronchial challenge and a PT₁₅ slope could not be calculated. In this study BHR was defined as a dichotomous variable (yes/no)
where a fall in FEV$_1$ $\geq$ 15% was considered positive and < 15% fall in FEV$_1$ was considered negative.

**Sputum processing**

Sputum samples were stored on ice until ready for processing. All samples were processed within 2 hours. The sample was poured into a petri dish, mucoid plugs selected and removed with a forceps. A selected rather than whole sample processing method was used to reduce salivary contamination (194) because this improves intra-observer repeatability of differential cell counts. The selected sputum was returned to a clean preweighed tube and weighed using a microbalance. One percent stock Dithiothreitol (DTT) (0.1%) was then added to the sputum in the ratio of 4 ml DTT to 1 g sputum. The mixture was gently aspirated with a 3 ml disposable wide bore plastic pipette and then gently agitated on a rolling mixer (Denley Spiramix 5, Denley Instruments, Colchester, UK) for 15 minutes. The sample was filtered using a 48$\mu$m pore nylon mesh filter. Phosphate buffered saline (PBS) (in the ratio of 4 ml PBS to 1 g original sputum weight) was used to wash out the tube and added to the filtrate (through the filter). The filtrate was centrifuged (Sorvall RT6000D, Thermo Fisher Scientific Inc, Massachusetts, USA) at 4°C for 10 minutes at 400g (1500rpm).

The remaining pellet was resuspended in 1 ml PBS and mixed by gentle vortexing (Vortex mixer, Vorsicht, Germany). 20$\mu$l of sputum cell suspension was added to 20$\mu$l trypan blue (1 in 2 dilution factor). 10$\mu$l of the mixture was placed under the light microscope (Leitz, Wetzlar, Germany) using a haemocytometer (improved Neubauer, BDH, Leicestershire, UK). All leukocytes (viable stained yellow and dead stained blue) and squamous cells were counted in the bottom left middle and top right quadrants of the haemocytometer grid. The total leukocyte count per ml was calculated using the following formula:

\[
\text{Total leukocyte count} = \text{mean number of viable and dead leukocytes} \times \text{trypan blue dilution factor} \times 10^4
\]

After calculation of the total leukocyte count the sputum cell suspension was adjusted to 200,000 cells/ml by diluting with PBS. Using 100 $\mu$l of cell suspension per slide, the mixture was centrifuged (Shandon Cytospin Preparation System, Thermo
Shandon, Cheshire, UK) at 450rpm for 3 min. If the total leukocyte count was less than 2 x 10^5/ml aliquots of 200µl or 400µl of sputum cell suspension were loaded. The 4 slides were left to air dry for 30 minutes and then fixed with methanol. The slides were then stained with a modified Wright Giemsa stain Reastain® Quick-Diff staining kit (Reagena Ltd. Toivala, Finland).

2.9 Completion of research visit
The children and parents were thanked for their time and participation. Study results were detailed and subsequently mailed to all participants in the clinical research visit. Travel expenses incurred in attending the RBH were reimbursed to the families.

2.10 School age atopy
Atopy was defined as either a parental report of eczema or allergic rhinitis in the ISAAC core questionnaires\textsuperscript{158} or the presence of 1 or more positive skin prick tests at school age. This definition has been used throughout this thesis. Current eczema was defined as yes to both of the questions ‘Has your child ever had an itchy rash which was coming and going for at least 6 months?’ and ‘Has your child had this itchy rash at any time in the last 12 months?’ Current allergic rhinitis was defined as a positive response to ‘In the past 12 months, has your child had a problem with sneezing, or a runny, or a blocked nose when he/she did not have a cold or the flu?’

2.11 Statistical analysis
The Mann Whitney U test was used to detect differences between 2 groups. Where more than 2 groups were present Kruskal-Wallis test was used, followed by Dunn’s post test to detect differences between groups. Categorical data was analysed using Chi-squared or Fisher’s exact test as appropriate. Spearman rank correlation was used to assess the relationship between numerical variables. Cohen’s kappa coefficient was used to measure the reliability or inter-rater agreement for qualitative items. Coefficient of variation (CoV) was used to measure repeatability between repeats of the same test on different occasions. Bland-Altman plots were used to assess agreement between two investigators. Sample size was opportunistic, determined by recruitment to the previous study and success of follow up at school age; in any case, there are no data to inform a power calculation. Analyses were
performed using MedCalc v12.1.0, GraphPad Prism version 5.02(OR) and SPSSv17.

2.12 Current Study: Recruitment

2.12.1 Children followed up at school age

Thirty nine children attended for a research visit, figure 2.8. Twelve families were either unable or did not wish to attend for a research visit but all subsequently agreed to answer a telephone questionnaire (see appendix). Total recruitment for either a full research visit or a telephone questionnaire was 51/68 (75%). Preschool and school age clinical characteristics for children followed up at school age are shown in table 2.3.

2.12.2 Co-morbid pathology at school age

Of the preschool CW group (n=21), two children had bronchiectasis at school age. One of the children with bronchiectasis attended for a research visit and the family of the second child answered a telephone questionnaire only, but the child’s clinical notes were obtained from the local hospital with permission from her parents. One child who completed the telephone interview had been removed from the care of his mother age 4 because of fabricated and induced illness. His father who had been his primary carer since then stated during telephone interview that he had never wheezed in his care. Two children were being treated for severe asthma in the Royal Brompton Hospital and were enrolled in a difficult asthma assessment during the study recruitment period (195) and a third child had been treated with cyclosporin for severe refractory asthma for several years in another hospital prior to assessment.

In the RW group (n=16) one girl was noted to have digital clubbing at the research visit, was referred for follow up and subsequently diagnosed with bronchiectasis. A boy from the RW group had a low FEV$_1$ at the research visit and was subsequently found to have evidence of air trapping and atelectasis in both lower lobes on high resolution computerised tomography (CT) of the chest. One girl had motor and learning difficulties and one boy had Carpenter’s syndrome and autistic spectrum disorder at school age making it difficult for them to participate in many of the lung function tests. Another boy was diagnosed in 2009 with cor triatriatum having had a long history of being breathless on exertion. One girl had scimitar syndrome with an
associated congenitally small right lung. One child from the original RW group was enrolled during the study period to our difficult asthma protocol\textsuperscript{(195)}.

Of the original group of preschool controls (n=14), 3 children had repaired complex congenital heart disease in early childhood and were very well. A fourth child had a Trisomy 14 mosaicism and a repaired Tetralogy of Fallot but was unable to attend for assessment. A fifth child in the control group had congestive cardiac failure secondary to a large ventricular septal defect at time of study recruitment; this was subsequently repaired in 2010. One boy had mild tracheal compression secondary to a right sided aortic arch. A girl who had a bronchoscopy at preschool age for investigation of cyanotic episodes was subsequently diagnosed with ulcerative colitis at age five, figure 2.8.
Figure 2.8: Study recruitment and alternative or co-morbid diagnosis at school age other than asthma

- Recruited at school age

- Controls n=21

- CW n=27
  - Research visit n=13
  - Telephone Q n=8

- RW n=20
  - Research visit n=14
  - Telephone Q n=2

Children with alternative or co-morbid diagnosis (other than asthma) at school age

- CW n=27
  - 2 children school age bronchiectasis
  - 1 child Fabricated and Induced Illness (diagnosed age 4)

- RW n=20
  - 1 child school age bronchiectasis
  - 1 child atelectasis and air trapping both lower lobes at school age
  - 1 child diagnosed Cor Triatriatum in 2009
  - 1 child had scimitar syndrome and a congenitally small right lung
  - 1 child had undiagnosed motor and learning disability
  - 1 child had Carpenter’s syndrome and autistic spectrum disorder

- Controls n=21
  - 3 children had congenital heart disease in infancy and remained well
  - 1 child had congestive heart failure secondary to an undiagnosed ventricular septal defect which was repaired in 2009
  - 1 child had Trisomy 14 mosaicism and Tetralogy of Fallot repaired in infancy
  - 1 child had tracheal compression from a right sided aortic arch
  - 1 child developed Ulcerative Coliitis at age 5
Table 2.3: Characteristics of children followed up at school age from 2009-2010 (Children with school age asthma had higher preschool IgE, and school age eczema and allergic rhinitis when compared to those without asthma at school age.)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Asthma</th>
<th>No asthma</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male / Female</td>
<td>11 / 5</td>
<td>21 / 14</td>
<td>0.54</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>3.41 (1.87-4.21)</td>
<td>3.38 (1.6-4.41)</td>
<td>0.57</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>39 (30-42)</td>
<td>40 (34-42)</td>
<td>0.51</td>
</tr>
<tr>
<td>Age at FOB (months)</td>
<td>28 (3-57)</td>
<td>17 (3-55)</td>
<td>0.01</td>
</tr>
<tr>
<td>Weight at FOB (kg)</td>
<td>13.35 (5.6-25)</td>
<td>11 (5.45-26.4)</td>
<td>0.043</td>
</tr>
<tr>
<td>Preschool IgE (IU/ml)</td>
<td>70 (1-635)</td>
<td>9 (1-432)†</td>
<td>0.04</td>
</tr>
<tr>
<td>Preschool ≥1 positive RAST, n (%)</td>
<td>6 (37.5)</td>
<td>10 (31.3)†</td>
<td>0.75</td>
</tr>
<tr>
<td>Age at first episode of wheeze (months)</td>
<td>14 (1.5-24)</td>
<td>6.5 (0.5-66)</td>
<td>0.21</td>
</tr>
<tr>
<td>Wheeze at preschool age</td>
<td>16</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Confirmed wheezers</td>
<td>11</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Reported wheezers</td>
<td>4</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>No wheeze</td>
<td>1</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Parental history doctor diagnosed asthma‡</td>
<td>13</td>
<td>17‡</td>
<td>0.04</td>
</tr>
<tr>
<td>Paternal history doctor diagnosed asthma</td>
<td>5</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>Maternal history doctor diagnosed asthma</td>
<td>5</td>
<td>7</td>
<td>0.28</td>
</tr>
<tr>
<td>Age at follow up (years)</td>
<td>8.2 (6-10.4)</td>
<td>7.3 (5.9-11)</td>
<td>0.1</td>
</tr>
<tr>
<td>Reported wheeze in last 12 months</td>
<td>16</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Wheeze pattern</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Episodic (with colds)</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Multiple trigger</td>
<td>13</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Frequency of wheeze</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>1-3 attacks of wheezing</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4-12 attacks of wheezing</td>
<td>8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>&gt;12 attacks of wheezing</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Exercise induced wheeze</td>
<td>13</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Dry cough at night§</td>
<td>14</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Number of hospital admissions with wheeze ever</td>
<td>17 (0-160)</td>
<td>3 (0-20)</td>
<td>0.005</td>
</tr>
<tr>
<td>Use of ICS at school age</td>
<td>13</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Dose of ICS (mcg beclomethasone) II</td>
<td>400 (200-1200)‖</td>
<td>300 (200-400)</td>
<td></td>
</tr>
<tr>
<td>Eczema</td>
<td>12</td>
<td>4</td>
<td>0.0001</td>
</tr>
<tr>
<td>Allergic rhinitis</td>
<td>7</td>
<td>9</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Data is reported as median and range.
†Results not available on 3 children
‡Family history was unknown in 2 children, as were both adopted at birth (previously in control group)
§Dry cough at night apart from a cough associated with a cold or chest infection
‖Or beclomethasone equivalent (2 parents did not know the prescribed ICS dose)
2.12.3 Children lost to follow up at school age

Seventeen children were lost to follow up. I was unable to contact 16/17 children despite contacting both the referring paediatrician and their General Practitioner at preschool age. One child who had a Trisomy 21 and complex congenital heart disease died shortly after recruitment to the preschool study at age 11 months from left ventricular infarction. There was no difference between children who participated in the study and those lost to follow up in terms of sex (p=0.77), or age at bronchoscopy and endobronchial biopsy (p=0.77) or preschool IgE (0.99), table 2.4.

Table 2.4: No difference in clinical characteristics of preschool children followed up at school age and those lost to follow up

<table>
<thead>
<tr>
<th></th>
<th>Followed up n=51</th>
<th>Lost to follow up n=17</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at biopsy*</td>
<td>19 (3-57)</td>
<td>15 (7-58)</td>
<td>0.77</td>
</tr>
<tr>
<td>Male/ Female</td>
<td>32/19</td>
<td>12/5</td>
<td>0.77</td>
</tr>
<tr>
<td>Wheezers/ Controls</td>
<td>37/14</td>
<td>10/7</td>
<td>0.36</td>
</tr>
<tr>
<td>Preschool IgE (IU/ml)</td>
<td>21 (1-635)†</td>
<td>18 (1-2605)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

*Data is reported as median and range.
†Results not available on 5 children

2.13. Clinical characteristics of children followed up at school age

2.13.1 Family history of asthma

Parents were asked if there was a history of maternal or paternal doctor diagnosed asthma. Two children had been adopted shortly after birth and their birth family history was unknown. Both of these children were in the ‘control’ preschool group.

Children with school age asthma were more likely to have a first degree family member with asthma (13/16 versus 17/33, Fishers exact test, p=0.043). Children with wheeze at preschool age (either reported or confirmed) and school age asthma were also more likely to have either a maternal or paternal (parental) history of asthma (Fischer’s exact test, p=0.038). Although a paternal history of doctor diagnosed asthma increased the likelihood of school
age asthma (p=0.01), a maternal history of doctor diagnosed asthma specifically did not (p=0.49), table 2.5.

Table 2.5: Contingency table of all children recruited comparing (i) parental history of asthma; (ii) doctor diagnosed maternal history of asthma; (iii) doctor diagnosed paternal history of asthma with school age asthma status (Children with asthma at school age were more likely to have a paternal history of asthma.)

(i)

<table>
<thead>
<tr>
<th>Family history asthma</th>
<th>School age asthma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Family history asthma</td>
<td>yes</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>33</td>
</tr>
</tbody>
</table>

*Fishers exact test, p=0.043

(ii)

<table>
<thead>
<tr>
<th>Maternal history asthma</th>
<th>School age asthma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Maternal history asthma</td>
<td>yes</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>33</td>
</tr>
</tbody>
</table>

*Fishers exact test, p=0.49

(iii)

<table>
<thead>
<tr>
<th>Paternal history asthma</th>
<th>School age asthma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Paternal history asthma</td>
<td>yes</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>33</td>
</tr>
</tbody>
</table>

*Fishers exact test, p=0.01
2.13.2 School age allergic rhinitis and eczema

Parents were asked to complete the ISAAC core questionnaires\(^{(158)}\) for eczema and allergic rhinitis. Children with school age asthma were more likely to have co-existing eczema (p=0.0001) and allergic rhinitis (p=0.001) than those without, table 2.6.

**Table 2.6: Contingency table comparing school age asthma and school age allergic rhinitis and eczema** (Children with school age asthma were more likely to have school age eczema and allergic rhinitis.)

<table>
<thead>
<tr>
<th>School age Asthma</th>
<th>School age Allergic Rhinitis*</th>
<th>School age Eczema**</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>No</td>
<td>9</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>No</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>No</td>
<td>1</td>
</tr>
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<td></td>
<td>Yes</td>
<td>Yes</td>
<td>12</td>
</tr>
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<td></td>
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<td>Yes</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>No</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>No</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>12</td>
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<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>51</td>
</tr>
</tbody>
</table>

Fishers exact test p=0.001*, p=0.0001**

2.13.3 School age asthma and preschool total IgE

There was no difference in preschool IgE between CW (n=26), RW (n=19) and controls (n=18), (p=0.22), figure 2.9 (i). Total IgE at preschool age was higher in those children who developed school age asthma (n=15) than those who did not develop asthma (n=31), (p=0.036), figure 2.9 (ii), however there is considerable overlap between the groups.
Figure 2.9: Total IgE at preschool age related to (i) preschool wheeze and (ii) school age asthma status

(i)  
No difference in total IgE at preschool age between confirmed and reported wheezers and controls

(ii)  
Increased total IgE at preschool age in children who developed future asthma

2.13.4 School age asthma status & preschool age RASTs
Blood samples were taken to measure RASTs and total IgE while children were anesthetised for bronchoscopy at preschool age. Atopic sensitisation was initially considered as a simple dichotomous variable. Sensitisation was considered positive if the RAST test had a value of 0.35 or higher. Of 48
children who had RASTs measured at preschool age for 3 common food and 5 aero-allergens, 16 children had one or more positive RAST test. There was no increased risk of asthma at school age in this group (Pearson’s Chi Square p=0.45). Further analysis excluding the subgroup of five children who had isolated positive RASTs to milk showed there was still no increased risk of asthma (Pearson’s Chi Square p=0.2). All 3 children had a positive RAST for egg at preschool age who were followed up at school age had asthma. Children with one or more positive aero-allergens (n=7) also did not have an increased risk of asthma at school age (p=0.15).

School age asthma status was also compared with quantified atopy in the preschool period, both as the sum of all specific IgE to both aero and food allergens and as the sum of all aeroallergens (house dust mite, cat, dog, grass and tree pollen)\(^{196}\). The sum of all allergens showed was no difference between those who developed school age asthma (n=14, median 2.72 (2.72-110.2)KU/L) and those who did not (n=30, 2.72 (2.72-11.64)KU/L). However, the sum of all aeroallergens was greater in those who developed asthma (n=14, 1.7 (1.7-109.2)KU/L) when compared to those who did not (n=30, 1.7 (1.7-8.3)KU/L), p=0.009, figure 2.10.

**Figure 2.10:** Sum of all aeroallergens is increased in those who developed school age asthma when compared with those who did not
2.14 Prevalence of asthma at school age

Population prevalence of asthma at school age varies with the diagnostic criteria selected\(^{(156)}\). The asthma prevalence in the current cohort of children using the definition of asthma given above (parental report of doctor diagnosed asthma ever and wheeze in the last 12 months) was compared with that using other definitions of asthma used in other longitudinal cohort studies, table 2.2 and table 2.7.

Prevalence of school age asthma in the group of preschool wheezers recruited from RBH between 2002 and 2005 varied from 38 to 57% depending on the criteria used to diagnose asthma, table 2.7. A report of wheeze in the last 12 months had the highest prevalence (57%) and an asthma definition that required wheeze and use of asthma medication in the last 12 months plus a parental report of doctor diagnosed asthma had the lowest (38%), table 2.7. The definition of asthma used in this thesis had a prevalence of 40.5%, table 2.7.

Prevalence of asthma at school age is higher as expected in the children with wheeze at preschool age (38-57)% than that of controls (29-45)%. Prevalence of wheeze reported in the UK using ISAAC questionnaire was 20.9% in children aged 6-7 years old\(^{(155)}\).
Table 2.7: Prevalence of asthma in preschool wheezers varies depending on definition used

<table>
<thead>
<tr>
<th>Original cohort</th>
<th>Preschool wheezers</th>
<th>All cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of children followed up at school age</td>
<td>n=47</td>
<td>n=68</td>
</tr>
<tr>
<td>n=37 (78.7%)</td>
<td>n=51 (75%)</td>
<td></td>
</tr>
</tbody>
</table>

Criteria for current asthma diagnosis at school age

<table>
<thead>
<tr>
<th>Criteria for current asthma diagnosis at school age</th>
<th>Preschool wheezers</th>
<th>All cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheeze in last 12 months (ISAAC questionnaire) (^{(12;80;158)})</td>
<td>21 (56.7%)</td>
<td>23 (45%)</td>
</tr>
<tr>
<td>Wheeze in the last 12 months and parental report of doctor diagnosed asthma ever (^{(156)})</td>
<td>15 (40.5%)</td>
<td>16 (31.4%)</td>
</tr>
<tr>
<td>Doctor’s diagnosis of asthma ever (^{(156)})</td>
<td>18 (48%)</td>
<td>20 (39.2%)</td>
</tr>
<tr>
<td>Wheeze in the last 12 months and one or both of (^{(10)})</td>
<td>19 (51%)</td>
<td>19 (37.3%)</td>
</tr>
<tr>
<td>• doctor diagnosed asthma ever</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• use of asthma medications* during the past 12 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheeze in the last 12 months and Parental report of doctor diagnosed asthma ever And use of inhaled corticosteroids during the past 12 months</td>
<td>14 (37.8%)</td>
<td>15 (29.4%)</td>
</tr>
</tbody>
</table>

*Asthma medication one or more of the following (inhaled corticosteroids, long or short-acting β-agonists, sodium cromoglycate, leukotriene antagonists and/or aminophylline)
2.15 Demographics of children who attended for a research visit
Thirty nine of the 51 children who answered the clinical questionnaire attended for a research visit for lung function, airway inflammation and atopy assessment, figure 2.11. Several children were less than seven years old or had learning or behavioural problems (discussed above) which made it difficult for them to complete all of the assessments.

Five children were unable to perform either exhaled FeNO$_{50}$ or spirometry
- 3 of the children were aged 5.9-6.1 years (one of whom also had learning disability)
- 1 child had behavioural problems and became very upset and refused to participate
- 1 child had learning disability and was unable to perform either test

A further child refused to do FeNO$_{50}$ but subsequently was persuaded to perform spirometry. Demographics of children who attended for the research visit are shown in table 2.8. All lung function analyses have been performed for all children (both preschool wheezers and controls) with and without school age asthma and both including children with other lower airway pathology and also as a separate subgroup analysis excluding them.

Table 2.8: Demographics of children who attended for research visit

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=12)</th>
<th>No Asthma (n=27)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male / Female</td>
<td>8/4</td>
<td>17/10</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>8.2 (6-10.4)</td>
<td>7.3 (5.8-11)</td>
<td>0.09</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>127.9 (110-157)</td>
<td>135.4 (109-146.1)</td>
<td>0.6</td>
</tr>
<tr>
<td>Height (z score)</td>
<td>-0.13 (-3.79-2.66)</td>
<td>0.06 (-3-2.16)</td>
<td>0.61</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>24.7 (21.5-48)</td>
<td>26 (16.7-37.1)</td>
<td>0.64</td>
</tr>
<tr>
<td>Weight (z score)</td>
<td>0.26 (-1.66-3.13)</td>
<td>0.35 (-2.07-2.81)</td>
<td>0.98</td>
</tr>
</tbody>
</table>
2.15.2 Video questionnaire at school age

Of those parents who reported that their child had wheezed over the past 12 months and were shown a video questionnaire (n=17) only 12/17 (71%) identified wheeze on the video questionnaire. There was no relationship between reported wheeze frequency and parental identification of wheeze on video questionnaire (Chi squared test, p=0.45) or between wheeze pattern (episodic or multiple-trigger at school age) and video questionnaire (Chi squared test, p=0.2). Furthermore, parents of children with current wheeze and a diagnosis of current asthma were not more likely to identify wheeze on video questionnaire than those who reported current wheeze but not a doctor’s diagnosis of asthma (Fisher’s exact test p=0.47).
5/17 children’s parents reported school age wheeze but did not identify it on video questionnaire. All of these children were attending the Royal Brompton Hospital for respiratory follow up. Two children were being followed with mild to moderate asthma, one child had scimitar syndrome and a congenitally small right lung, one child had Carpenter’s Syndrome with recurrent respiratory infections and reported wheeze only with colds and one child had bronchiectasis. Interestingly none of the parents recognised any other respiratory noises on video questionnaire including stridor, wet cough or stertor. Cohen's kappa coefficient showed moderate agreement between the video and written questionnaire (kappa 0.46, p=0.005).

2.15.3 Comparison of RASTs at preschool age with skin prick tests at school age
Thirty four children had atopy measured by skin prick tests at school age, of whom 32 also had RASTS measured at preschool age. The skin prick tests performed at school age were the same 3 food allergens (egg, milk, peanut) and 6 aeroallergens (grass, tree, cat, dog, Aspergillus fumigatus and Dermatophagoides pteronyssinus). Aspergillus RAST was not measured at preschool age. Children with school age asthma (8/12) were more likely to have one or more positive RASTs for aero-allergens than those without asthma (3/22) (Fishers exact test p=0.005).

Of 23 children with no positive RASTs at preschool age, 5 (20%) had one or more positive skin prick tests at school age. Total IgE at preschool age in those with no positive RASTs at preschool age showed a trend for a higher IgE in those that subsequently had one or more positive skin prick tests (n=5, median IgE 42 (range 4-526)IU/ml) when compared with those who did not (n=18, median IgE 5 (range 1-292)IU/ml) (p=0.16). Of the children (n=5) with no positive RASTs at preschool age but with positive skin prick tests at school age, all 5 had school age eczema, and 3 had asthma and allergic rhinitis.
2.15.4 Tobacco smoke exposure at school age

Thirty six children had salivary cotinine measured successfully and 3 children refused to participate. Four samples had insufficient saliva collected. Of those children (n=32) who had salivary cotinine measured only 4 families reported one or more parents smoking. Five children had salivary cotinine levels greater than the upper limit of normal (1.2μg/l)\(^{(170)}\). One child had a salivary cotinine level of 10.3μg/l. Mean reported salivary cotinine in children where one or both parents smoked is reported as 3.7 (range 1.3-9.8)μg/l\(^{(170)}\). There was no difference in salivary cotinine from the homes where smoking was reported (n=4 median 1.8 (range 0.1-10.3)μg/l) and those where it was not (n=28, median 0.2 (range 0.1-1.8)μg/l), p=0.15, figure 2.12. There was no difference in salivary cotinine between children with asthma (n=13, median 0.2 (range 0.1-10.3)μg/l) and children without school age asthma (n=19, median 0.2 (range 0.1-1.7)μg/l), p=0.59.

Figure 2.12: Salivary cotinine at school age compared between houses where one or more persons and non smoking homes (There is no difference between the groups; the horizontal dotted line shows the upper limit of normal for salivary cotinine 1.2μg/l)

\[ p=0.15 \]
2.15.5 Spirometry

Spirometry was successfully performed in 35 children\(^{(192)}\). All children had 3 flow volume loops recorded after a period of practice. Repeatability as measured by the CoV between manoeuvres for FEV\(_1\) was 4.4% (standard deviation 2.9)% and for FVC 5.6% (standard deviation 3.6)%.

There was a significant difference in FVC measured between preschool CW (median z score -0.4) and RW (median z score -1.6), p=0.03, figure 2.13 (i). However, there was no difference in school age FEV\(_1\) z score (p=0.45) or the ratio of FEV\(_1\)/FVC (p=0.14) between preschool wheezers (confirmed (n=12) and reported (n=13)) and children with no lower respiratory symptoms at preschool age (n=10), figure 2.13 (ii) and (iii). Children with asthma (n=12) at school age had similar FEV\(_1\) (p=0.43), FVC (p=0.48) and FEV\(_1\)/FVC (p=0.84) z scores to those without asthma (n=23), figures 2.13 (iv)-(vi). A subgroup analysis of children with persistent wheeze (n=15) (i.e. wheezing during preschool years and reported wheeze in the previous 12 months) and children who did not report wheeze either at preschool age or in the past 12 months (n=9) again showed no significant difference in FEV\(_1\) (p=0.8), FVC (p=0.76) or FEV\(_1\)/FVC (p=0.18) z scores.

Four children with co-morbid lower airway pathology other than asthma known to decrease spirometry were excluded for the purpose of comparing lung function between asthmatics and non-asthmatics. The children excluded from the second analysis are as follows:
- a girl with bronchiectasis at school age (school age asthma; FVC z score= -0.3, FEV\(_1\) z score= -0.12)
- a boy with evidence of gas trapping and atelectasis on computerised tomography at school age (no asthma at school age; FVC z score= -2.64, FEV\(_1\) z score= -3.01)
- a girl with scimitar syndrome and a congenitally small right lung (no asthma at school age; FVC z score= -2.69, FEV\(_1\) z score= -2.08)
- a boy with evidence of upper airway obstruction caused by tracheal compression secondary to a right sided aortic arch (no asthma at school age; FVC z score= -0.01, FEV\(_1\) z score= -0.29).
After exclusion of the 4 children with other airway pathology (1 with school age asthma and 3 children without school age asthma) no difference was seen between children with and without asthma at school age for FVC (p=0.7), FEV\textsubscript{1} (p=0.8) and FEV\textsubscript{1}/FVC, (p=0.76), figure 2.14.
Figure 2.13: No difference in school age spirometry related to school age asthma status

School age spirometry related to preschool wheeze status

(i) Decreased FVC in RW compared to CW

(ii) No difference in FEV₁ between CW, RW and controls

(iii) No difference in ratio of FEV₁ to FVC in CW, RW or controls

School age spirometry related to presence of school age asthma

(iv) No difference in FVC between children with and without school age asthma

(v) No difference in FEV₁ between children with and without school age asthma

(vi) No difference in ratio of FEV₁ to FVC in children with and without school age asthma
Figure 2.14: Lung function measures compared between children with and without school age asthma after exclusion of children with comorbid respiratory diagnosis

(i) FVC (z score)

*p=0.7*

No difference in FVC between children with and without school age asthma

(ii) FEV₁ (z score)

*p=0.8*

No difference in FEV₁ between children with and without school age asthma

(iii) FEV₁/FVC (z score)

*p=0.76*

No difference in FEV₁/FVC between children with and without school age asthma
2.15.6 Multiple breath washout

Twenty two children had 3 acceptable washouts and 14 children had 2 acceptable washouts. Mean coefficient of variation between multiple breath washout measurements in the same child with 3 acceptable washouts was 4.9% (standard deviation 2.5%). Similarly mean FRC was 1.08L (standard deviation 0.28L) with the mean coefficient of variation between measures for the same child of 5% (standard deviation 2.5%). A randomly selected subset (n=14) of the multiple breath wash out studies were analysed by a second more experienced observer, Ms Samantha Irving, to ensure quality control and there was good correlation between the two observers (Bland Altman plot, standard deviation +/-0.19, bias 0.14), figure 2.15.

Figure 2.15: Difference between lung clearance index measurements by Dr R O'Reilly (measurement 1) and Ms S Irving (measurement 2) plotted against the mean of the 2 measurements (No significant bias seen)
2.15.6.2 Lung Clearance Index
There was no difference in LCI between the children either stratified by their preschool age wheeze status (n=36, p=0.44) or by the presence or absence of asthma at school age (p=0.5), figures 2.16 (i) and (iv) and table 2.9.

Three children with airway pathology other than asthma known to affect the distal airways were excluded from the MBW sub-analysis, bronchiectasis n=2, (LCI=10.99, LCI=8.17), and atelectasis and gas trapping n=1, (LCI=7.98). I included in this analysis the boy with tracheal compression (LCI=6.93) and the girl with a congenitally small right lung (LCI=7.27) as both had normal LCIs. There was still no difference between groups stratified either by preschool wheeze status (CW [n=12, LCI median 7.23 (6.34-10.99)], RW [n=11 LCI median 7.6 (6.2-7.75)] and controls [n=10, LCI median 7.4 (6.24-7.88)], p=0.72, or by school age asthma status (asthma [n=11, LCI median 7.25 (6.34-10.99)] versus no asthma [n=22, LCI median 7.13 (6.2-7.83)]), p=0.3.

There was no difference in LCI between children that persistently wheezed from preschool age and were still wheezing at school age (n=15, median LCI 7.37 [6.34-10.99]) and those who never wheezed (n=9 median LCI 6.94 [6.24-7.88]), p=0.15. Similarly there was no difference in LCI between parental reported wheeze phenotypes at school age, either multiple-trigger (n=10, LCI median 7.44 [6.34-10.99]) or episodic (viral) wheeze (n=6, LCI median 7.24 [6.65-7.78]) and children who did not report wheeze at school age (n=20, LCI median 7.12 [6.2-10.95]), p=0.33. FRC was similar in all groups, 1.05 (0.71-2.09) litres, table 2.9.

The upper limits of normality for LCI reported in another study are 7.42, range (7.25-7.59)\(^{62}\). If these limits are applied to this study in the absence of reference data from our own department, 12 children in this study had LCI greater than 7.42. However seven of these were just above this range (7.42-7.8). Of the remaining children (n=5) two had had evidence of mild to moderate bronchiectasis on CT (LCI: 8.17, 10.95), the third had evidence of gas trapping and atelectasis (LCI=7.98), and two children had no obvious reasons (LCI 10.99, 8.41).
2.15.6.3 $S_{\text{cond}}$ & $S_{\text{acin}}$

$S_{\text{cond}}$ (conductive airways inhomogeneity) and $S_{\text{acin}}$ (acinar airways inhomogeneity) were analysed in 29 children who had two or more technically acceptable washouts. Seven children were excluded because they did not have two or more traces from which a phase III slope could be reproducibly analysed.

There was no difference when $S_{\text{acin}}$ was compared in preschool wheeze (p=0.21), figure 2.16 (ii), or school age asthma status (p=0.97), figure 2.16 (v). Although CW (n=9, 0.032) showed a trend to higher $S_{\text{cond}}$ when compared with RW (n=11, 0.014) and controls (n=9, 0.015) it was not statistically significant (p=0.2), figure 2.16 (iii). Children with asthma at school age (n=7, 0.043) had a higher $S_{\text{cond}}$ when compared to those who did not (n=22, 0.014) p=0.049, figure 2.16 (vi). However on exclusion of the 2 children included in the analysis with co-morbid lower airway pathology (1 with a diagnosis of school age asthma and 1 without asthma) there was still a trend to higher $S_{\text{cond}}$ in the school age asthmatics (n=6, $S_{\text{cond}}$=0.039, range [0.003-0.061]) compared to those children without asthma at school age (n=21, $S_{\text{cond}}$= 0.014, range [-0.027-0.032]), but was no longer statistically significant, p=0.1. There was also no difference when children were compared by preschool wheeze groups episodic (viral) and multiple-trigger at school age.
Table 2.9: Lung clearance index, functional residual capacity, $S_{\text{cond}}$ and $S_{\text{acin}}$ related to school preschool age wheeze status and school age asthma status. ($S_{\text{cond}}$ is increased in those children who developed school age asthma)

<table>
<thead>
<tr>
<th>Preschool age</th>
<th>Confirmed wheezers n=13</th>
<th>Reported wheezers n=13</th>
<th>Controls n=10</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRC (l)</td>
<td>1.055 (0.75-2.09)</td>
<td>0.996 (0.71-1.61)</td>
<td>1.027 (0.75-1.43)</td>
<td>0.83</td>
</tr>
<tr>
<td>LCI</td>
<td>7.37 (6.3-10.99)</td>
<td>7.25 (6.2-8.17)</td>
<td>7.17 (6.24-7.88)</td>
<td>0.44</td>
</tr>
</tbody>
</table>

| S_{\text{cond}} Range | 0.032 (-0.003-0.06) | 0.014 (-0.027-0.044) | 0.015 (-0.027-0.023) | 0.2 |
| S_{\text{acin}} Range | 0.099 (0.028-0.5) | 0.13 (0.033-0.24) | 0.076 (0.034-0.62) | 0.21 |

<table>
<thead>
<tr>
<th>School age</th>
<th>Asthma n=12</th>
<th>No Asthma n=24</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRC</td>
<td>1.06 (0.75-2.09)</td>
<td>1.05 (0.71-1.61)</td>
<td>0.69</td>
</tr>
<tr>
<td>LCI</td>
<td>7.33 (6.1-10.99)</td>
<td>7.24 (6.28-10.95)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

| S_{\text{cond}} Range | 0.047 (0.003-0.06) | 0.014 (-0.027-0.03) | 0.0498 |
| S_{\text{acin}} Range | 0.08 (0.06-0.21) | 0.1 (0.028-0.62) | 0.97 |
School age multiple breath washout related to preschool wheeze status

(i) p=0.44

No difference in school age LCI when compared with preschool wheeze status

(ii) p=0.21

No difference in school age \( S_{\text{acin}} \) when compared with preschool wheeze status

(iii) p=0.2

No difference in school age \( S_{\text{cond}} \) when compared with preschool wheeze status

School age multiple breath washout related to school age asthma status

(iv) p=0.50

No increase in LCI in school age asthmatics

(v) p=0.97

No increase in \( S_{\text{acin}} \) in children with school age asthma

(vi) p=0.0498

Increased \( S_{\text{cond}} \) in children with school aged asthma

Figure 2.16: Increased \( S_{\text{cond}} \) in children with school aged asthma
2.15.7 Exhaled nitric oxide

Thirty three children successfully performed FeNO$_{50}$. CW (n=10, 11.95 [7.7-35] ppb) had higher FeNO$_{50}$ when compared to RW (n=13, 5.9 [2-20.6]ppb) but not controls (n=10, 7.33 [3.8-42.6]ppb), p=0.06, figure 2.17 (i). Of the 2 children from the original control group with raised FeNO$_{50}$ one had asthma, eczema and positive skin prick tests to cat, milk and peanut at school age and the second child had eczema and allergic rhinitis but no positive skin prick tests. Children with school age asthma (n=11, 13.3 [4.9-40.3] ppb) had higher FeNO$_{50}$ when compared to children without school age asthma 7.08 [2-42.6]ppb, p=0.02, figure 2.17(ii). FeNO$_{50}$ was greater in atopic (n=13, 16.3 [4.96-42.6]ppb) compared to non-atopic children (n=20, 7.15 [2-20.6]ppb) irrespective of current wheeze status (p=0.01), figure 2.17 (iii).

Figure 2.17: (i) FeNO$_{50}$ greater in confirmed rather than reported wheezers, (ii) Increased FeNO$_{50}$ in children with school age asthma when compared to children without asthma, (iii) Increased FeNO$_{50}$ in children with school age atopy

![Figure 2.17](image-url)
Increased exhaled nitric oxide in children with asthma at school age when compared to those without

FeNO 50ml/s (ppb)

Asthma (n=11) No asthma (n=22)

Increased exhaled nitric oxide in children with asthma at school age when compared to those without

FeNO 50ml/s (ppb)

Atopic (n=13) Non Atopic (n=20)

Increased exhaled nitric oxide in children with school age atopy

2.15.8 Hypertonic saline challenge

Hypertonic saline (4.5%) provocation testing was used to measure AHR. The test was considered complete if either the FEV₁ had fallen by 15% from baseline or the total inhalation period of 15.5 min had been reached. β-agonists were withheld for the previous 12 hours and inhaled corticosteroids for the previous 24 hours. Two children with severe asthma requiring maintenance daily oral prednisolone were asked not to withhold medication. Twenty six children had bronchial challenges: 22 were full studies and 4 studies had to be prematurely discontinued, table 2.10. Two children disliked the salty taste and were reluctant to continue, a third child spat pink liquid into the tubing and the fourth became bored and despite repeated
encouragement was not using maximal effort at spirometry. The median participation time in these children was 5.5 minutes (range 1.5-11.5 minutes).

In children (n=5) with FEV$_1$≤75% bronchial challenge was not performed. Three of the five children had bronchodilator reversibility (BDR) assessed 20 minutes after inhalation of 1000mcg salbutamol. The fourth child had a congenitally small right lung explaining her low lung function. The fifth child recently had surgery for Cor Triatriatum and the mother was reluctant to give him medication. Four children were unable to perform repeatable lung function and 4 children refused to participate, table 2.10. The reasons given were ‘fear of the nebuliser and dislike of the salty taste’.

Table 2.10: Acceptability and tolerance of bronchial challenge with hypertonic (4.5%) saline

<table>
<thead>
<tr>
<th>Assessment of bronchial hyper-responsiveness</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full bronchial challenges</td>
<td>22</td>
</tr>
<tr>
<td>Incomplete bronchial challenges</td>
<td>4</td>
</tr>
<tr>
<td>Unable to perform repeatable lung function</td>
<td>4</td>
</tr>
<tr>
<td>Refused test</td>
<td>4</td>
</tr>
<tr>
<td>Not performed as lung function ≤75%</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>39</strong></td>
</tr>
</tbody>
</table>

2.15.8.2 Amount of hypertonic saline inhaled

There was considerable variation in the amount of saline inhaled among children who completed the full inhalation period, figure 2.19. The median amount was 22.4g (range 2-31.1g). Only 5 children inhaled ≥ 23g of hypertonic (4.5%) saline. A further 5 children were just under this threshold value with amounts of inhaled saline between 21-23g. There was only moderate correlation between duration of time and amount of inhaled saline (Spearman r=0.59, p=0.004), figure 2.18. Two patients were nebulised for an additional 4 minutes until an inhalation dose of 23g was reached. The remainder of the children were reluctant to prolong the nebulisation period.
Figure 2.18: Variability of amount of inhaled hypertonic saline (4.5%) inhaled at end point time of 15.5 minutes

Spearman r=0.59, p=0.004

Duration of inhalation and amount of inhaled hypertonic (4.5%) saline

2.15.8.3 Airway hyper-responsiveness, multiple breath washout and exhaled nitric oxide

There was no relationship between either AHR or bronchodilator response to LCI or \( S_{acin} \). However, there was a trend towards higher \( FeNO_{50} \) in those with evidence of bronchodilator reversibility or AHR, table 2.11. \( S_{cond} \) was higher in those with reactive airways although numbers are very small (n=4).

2.15.8.4 Induced Sputum

All except 3 of the children had a dry non-productive cough when sputum induction was attempted (3/25, 12%). Several of the children on expectorating produced samples of saliva which were not analysed as there were no sputum plugs. All the children that produced a sample had a wet cough prior to induction despite parents reporting absence of symptoms over the preceding 3 weeks. One of the sputum samples was processed incorrectly and only 2 sputum results were obtained so no further analysis has been performed. Both sputums were predominantly neutrophilic (72% and 84%).
Table 2.11: No significant difference between children with evidence of bronchodilator response or airway hyperresponsiveness and those without at school age

<table>
<thead>
<tr>
<th>Evidence of Airway Hyper-responsiveness or Bronchodilator Reversibility</th>
<th>Positive n=5</th>
<th>Negative n=20</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male / Female</td>
<td>4/1</td>
<td>13/7</td>
<td>0.1</td>
</tr>
<tr>
<td>Age (range)</td>
<td>9.28 (7.15-10.4)</td>
<td>7.74 (6.8-9.8)</td>
<td>0.06</td>
</tr>
<tr>
<td>Symptomatic (wheezy, chest tightness)</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Change in FEV₁ ≥ 15%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDR ≥+15%</td>
<td>n=2</td>
<td>n=1</td>
<td></td>
</tr>
<tr>
<td>17.7%</td>
<td>9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHR ≥-15%</td>
<td>n=3</td>
<td>n=19</td>
<td></td>
</tr>
<tr>
<td>-17 (15-36) %</td>
<td>+2 (-14 to +17)%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheeze last 12 months</td>
<td>3</td>
<td>9</td>
<td>0.45</td>
</tr>
<tr>
<td>Asthma</td>
<td>3</td>
<td>9</td>
<td>0.38</td>
</tr>
<tr>
<td>LCI (Range)</td>
<td>7.74 (n=5) (6.88-8.41)</td>
<td>7.24 (n=20) (6.21-10.99)</td>
<td>0.29</td>
</tr>
<tr>
<td>Scond (Range)</td>
<td>0.0435 (n=4) (0.03-0.05)</td>
<td>0.009 (n=17) (-0.0275-0.061)</td>
<td>0.01</td>
</tr>
<tr>
<td>Sacin (Range)</td>
<td>0.1245 (n=4) (0.028-0.2357)</td>
<td>0.083 (n=17) (0.033-0.61)</td>
<td>0.89</td>
</tr>
<tr>
<td>FeNO (50ml/s ppb)</td>
<td>(n=5)</td>
<td>(n=19)</td>
<td></td>
</tr>
<tr>
<td>19.4 (8.7-38)</td>
<td>8.1 (3.8-42.6)</td>
<td>0.055</td>
<td></td>
</tr>
</tbody>
</table>

2.16 Summary of Results

- Seventy five percent of the original preschool cohort (51/68) were followed up at school age
- Forty percent of the original preschool wheezers that were followed up had a diagnosis of asthma at school age (defined as parental report of wheeze in the last 12 months and a doctor diagnosis of asthma ever)
- Children with pre-school video confirmed wheeze at preschool age had a higher risk of school age asthma compared with parental reported wheezers and controls.
- Children with school age asthma had increased prevalence of allergic rhinitis (p=0.001), eczema (p=0.0001), positive skin prick tests (p=0.006) and FeNO\textsubscript{50} (p=0.02) when compared to those without asthma.
- \(S_{\text{cond}}\) was higher in children in school age asthma and in children with airway reactivity, but no difference was seen for spirometry measurements, LCI or \(S_{\text{acin}}\).
- There was no correlation between AHR or bronchodilator reversibility and wheeze in the last 12 months or current asthma, but the numbers who were positive are too small for any conclusions to be drawn.

2.17 Discussion

2.17.1 Review of aims and objectives

The primary aims and objectives of this part of the study have been partly achieved

1) A diagnosis of asthma was established in 51/68 (75%) of the original preschool cohort.
2) A subgroup of this cohort 39/68 (58%) were characterised in terms of lung function and atopy.

2.17.2 Limitations of the study

The main limitation was that some children (18%) could not or would not attend for the research visit so pulmonary function and atopy measurements were available in all children. Final numbers followed up at school age were small and the preschool ‘control’ group while wheeze free at preschool age had other medical problems and were not truly ‘normal controls’. Several of the children had co-morbid or alternative diagnoses at school age. Importantly this group of preschool children represented a cohort with severe recurrent wheeze with only 6 children reporting no hospital admissions with wheeze at school age. Children had a median of 8 hospital admissions with wheeze over their lifetime at follow up. These children are not representative of preschool wheezers in the general population but rather a specific group with more severe disease. This also partly explains why some of the children had overlap with other respiratory pathology at school age.
2.17.3 Strengths of the study
Seventy five percent of the preschool children were followed up in one way or another at school age, figure 2.11. Other studies report similar subject retention of between 54-91\%\(^{(1;10-12;14;15)}\).

2.17.4 Asthma diagnosis
The absence of standardised criteria for the diagnosis of asthma in children was one of the most challenging aspects of this study. Seventy eight percent of the original preschool wheezers were followed up at school age. Asthma prevalence in this study varies from 29 to 45\% depending on the definition used, table 2.7. Although symptoms (wheeze, chest tightness) are sensitive for the presence of asthma, they may also be influenced by perception, culture and the interviewer. In order to prevent interviewer bias as much as possible all parents that attended for a research visit were shown the video questionnaire first followed by the written questionnaire and questions were asked in the same order each time. In all but 3 families, the parents that attended with the child for the research visit were Caucasian and English was their first language.

Parental identification of wheeze on video questionnaire at preschool age improved asthma prediction at school age (p=0.015). However, several of the parents still reported wheeze at school age but did not identify it on a validated video questionnaire, confirming that parental and physician perception of wheeze differs\(^{(16;153)}\). There was only moderate correlation between reporting wheeze in the last 12 months and identifying it as a respiratory symptom in the last 12 months on video questionnaire (kappa 0.46, p=0.005). A Canadian English speaking study has also shown limited agreement between the ISAAC questionnaire and video identification of wheeze\(^{(194)}\). In a more recent ISAAC study involving over 40 countries responses to a video questionnaire gave lower prevalence's of wheeze than with the written questionnaire, which was more apparent in English speaking countries\(^{(197)}\). Parental recall bias may also contribute to this discrepancy with two mothers who previously identified wheeze at preschool age no longer able to identify it on video questionnaire at school age despite reporting persistent wheeze.
The definition of asthma used in this study was parental report of wheeze in the last 12 months and a parental report of doctor diagnosed asthma ever. This definition has been used to assess wheeze and asthma prevalence in many studies\(^\text{156;198-204}\). The group of children with asthma at school age by this definition had higher parental history of doctor diagnosed asthma, higher IgE at preschool age, and a greater prevalence of allergic rhinitis, eczema and FeNO\(_{50}\) at school age when compared to those without, suggesting that this definition selected children likely to have asthma. Spirometry did not detect significant differences between those with and without school age asthma, however it is known to be a poor marker of asthma severity\(^42;205\).

Several longitudinal cohort studies have shown parental asthma to be an important factor in predicting school age asthma\(^\text{10;12;27}\). It is difficult to explain why paternal asthma was associated with school age asthma, rather than maternal asthma which has previously been shown to be a stronger predictor of school age asthma, table 2.5\(^\text{72;204;206;207}\). Importantly, an objective report was not obtained from health care providers for either maternal or paternal asthma. Paternal rather than maternal history of asthma is associated with AHR, an important determinant of disease severity in children with asthma\(^\text{203}\). However, given the small size of the study, which was not powered for or intended to study parental contributions to asthma in the offspring, it would be wrong to draw any conclusions from these data.

### 2.17.5 Lung function

Previous large epidemiological studies have shown that early wheezing associated with poor airway function in the late preschool years tracks with age and is associated with asthma in adulthood\(^6;208\). A limitation of this study was that preschool lung function was not available and thus it was not possible to compare preschool and school age lung function.

School age children who successfully performed spirometry were able to perform very repeatable manoeuvres with less than 5% variability between attempts comparable with other studies\(^\text{209;210}\). Interestingly there was no difference in lung function at school age as measured by spirometry between those with asthma and those without, figure 2.13 (iv-vi). The ratio of FEV\(_1\)/ FVC which has been shown to be
more sensitive in classifying asthma severity in children also showed no difference between the children with and without asthma at school age\(^{(42)}\). This may have been because the study was not powered to detect a difference in lung function between the two groups. Secondly, all children who attended for a research visit were symptom free at the time. The majority of children with persistent asthma have normal lung function during symptom-free periods with abnormal pulmonary function only during acute exacerbations\(^{(42,211)}\). Furthermore, spirometry is not a helpful marker for asthma severity in children \(\text{(above)}\)\(^{(42,205)}\).

The decision whether to include children in the lung function analysis with both bronchiectasis and asthma was difficult. Confusion of asthma and bronchiectasis is common and the diagnostic process is further complicated by the fact that the two may co-exist\(^{(161)}\). In adults with severe asthma, bronchiectasis is particularly common\(^{(212)}\). Secondly, it remains unclear whether bronchiectasis in patients with asthma is a comorbidity or whether it represents structural change or remodelling with natural progression of the disease\(^{(213)}\). However, in this study data were analysed both with and without these patients. When children with other airway pathology at school age were excluded there was no longer a difference in FVC between preschool RW and CW or between \(S_{\text{cond}}\) in children with or without school age asthma.

There was also no difference in LCI at school age by preschool wheeze phenotypes (confirmed and reported, multi-trigger and episodic) or by school age asthma status. This study showed a higher \(S_{\text{cond}}\) in seven year old school age asthmatics (0.043) when compared to children without school age asthma (0.014), \(p=0.049\). However, although there was still a trend towards higher \(S_{\text{cond}}\) in school age asthmatics (\(S_{\text{cond}}\) asthma 0.039, range \([0.003-0.061]\) versus non asthmatics 0.014, range \([-0.027-0.032]\)) this was no longer significant when children with lower airways disease not due to asthma at school age were excluded. This study was not powered to detect a difference between the two groups. The number of children necessary to detect a 10\% difference in MBW indices between children with and without school age asthma was calculated as 56 with 80\% power at the 5\% significance level\(^{(62)}\).
\(S_{\text{cond}}\) has been shown in other studies to be the most sensitive marker of small airways disease in asthma\(^{(62;172)}\). LCI in children with asthma, while often slightly higher than controls is still within the normal range and cannot be used as a useful indicator of disease in the individual child\(^{(62;171;172)}\). Children aged 4-5 years with multiple-trigger wheeze have been shown to have significantly higher LCI (multiple-trigger 7.4 (7.1-7.8) vs episodic 6.7 (6.5-6.9) vs controls 6.6 (6.5-6.7)) and \(S_{\text{cond}}\) (multiple-trigger 0.042 (0.030-0.058) vs episodic 0.014 (0.009-0.023) vs controls 0.010 (0.007-0.014)) when compared to episodic wheezers and healthy controls\(^{(62)}\). The differences from the current study likely reflect the greater numbers reported by Sonnapa et al\(^{(62)}\). Older children with well controlled asthma aged 10 years also had a higher LCI (6.69 (standard deviation (SD) 0.91) vs 6.24 (SD 0.47), \(p=0.02\)) and a trend towards a higher \(S_{\text{cond}}\) (0.026 (SD 0.02) vs 0.017 (SD 0.02)), \(p=0.06\)\(^{(171)}\). A Swedish study of asthmatic children with positive skin prick tests to aero-allergens aged 13 years showed that elevated \(S_{\text{cond}}\) correlated with increased levels of AHR and exhaled NO\(^{(172)}\). This study, although numbers were small (\(n=21\)), also showed increased \(S_{\text{cond}}\) in those children who demonstrated AHR or bronchodilator reversibility but no relationship to exhaled NO. This again suggests that disease of distal airways is an important part of asthma. It would have been useful to calculate alveolar NO and bronchial flux NO concentration in children as a marker of distal airway inflammation, but the children were not able to perform FeNO at higher flow rates\(^{(190)}\).

### 2.17.6 Atopic status and school age asthma

Children with higher total IgE at preschool age were more likely to develop asthma at school age, \(p=0.036\). Sensitisation to one or more aeroallergens was not predictive of asthma at school age, \(p=0.15\), but the cumulative sum of sensitisation to all aeroallergens was, \(p=0.009\)\(^{(196)}\). Of the 37 preschool wheezers followed up at school age over half had been atopic at preschool age (defined as the presence of eczema and or one more positive RASTs tests). There was no difference between the atopic preschool wheeze group when compared to the non-atopic preschool wheeze group in prevalence of current wheeze (Fishers exact test \(p=0.74\)) or current asthma (Fishers exact test \(p=0.73\)) at school age.
One third of the non atopic preschool wheeze group were still wheezing at school age. In contrast the much larger German MAS study showed that 90% of children with wheeze before age 3 years but no atopy lost their symptoms at school age\textsuperscript{(32)}. Sensitisation to perennial allergens (house dust mite, cat and dog hair) developing in the first 3 years of life was associated with persistent wheeze and loss of lung function at school age\textsuperscript{(155)}. The difference in results is likely explained by the small number of children in the current study and also that they represent a group of more severe wheezers.

In early life IgE antibodies are often first directed to food allergens and later to indoor and outdoor aero-allergens\textsuperscript{(214)}. As the median age of this group of children was just 2 years this would suggest that more children would become sensitised by school age. In fact four children had no positive skin prick tests at school age having previously had one or more positive RASTs at preschool age (3 children had positive RASTs to milk and 1 child had a positive RAST to dog). Conversely, two children who did not have a positive RAST at preschool age had one or more positive skin prick tests to aeroallergens at school age. It is important to remember in comparing RASTs that there is only limited agreement with skin prick tests and the extent of agreement also varies between allergens\textsuperscript{(164,215)}.

2.17.7 Airway hyper-responsiveness

Twenty percent of children (5/25) demonstrated either AHR or bronchodilator reversibility at the research visit. However, the bronchial challenge was poorly tolerated with 8/30 either not completing the challenge or refusing to participate. This may be attributed to the median age of the children involved being 7 years and bronchial challenge being performed at the end of the assessment period when children were more likely to be tired.

According to the protocol a complete bronchial challenge lasted 15.5 minutes and 23g of saline was nebulised\textsuperscript{(163)}. The correlation between amount of inhaled hypertonic saline (4.5%) and time of inhalation was only moderate (Spearman \( r=0.59 \)) suggesting that there is either not consistent output from the nebulisers or steady inhalation by the children. Similar lack of correlation between inhaled saline and length of inhalation have been found in other studies of 9-11 year olds in
Germany\textsuperscript{(216)}. Other studies have also found variation in the output in different models of the same nebuliser\textsuperscript{(217)}. Variable doses of hypertonic saline (2-31.1 g) were nebulised over the 15.5 minute period with (12/25) children achieving a nebulised concentration of 23g or just under of hypertonic saline. Similarly in a study of German children aged 9-11 years the investigators found it was not possible to nebulise 23g of hypertonic saline in 15.5 minutes in over half of the cohort\textsuperscript{(216)}. The nebulisation period was extended by 4 minutes to achieve 23g of saline in 2 of the children. However the remaining children declined to prolong the provocation test. Other studies have also found that increasing the inhalation time is not practical in this age group\textsuperscript{(216)}.

Previous epidemiological studies in children have shown that inhalation time as opposed to hypertonic saline dose does discriminate between those with asthma and those without\textsuperscript{(216)}. In contrast to this only 3 children in this study had a positive bronchial challenge (defined as a dichotomous variable). All 3 children were symptomatic with acute wheeze and chest tightness and also demonstrated ≥ 15% bronchodilator reversibility on administration of 1000µg salbutamol following the bronchial challenge. There was no relationship between AHR and presence of wheeze in the last 12 months (p=0.45) or asthma (p=0.38). This may have been because of the small numbers (n=22) that had a complete bronchial challenge. It is not possible to exclude the possibility that variations in nebuliser output and inhalation on the part of the child may also be partly responsible for the low numbers. A washout period of 24 hours may not have been sufficient for ICS and may have suppressed bronchial reactivity. Two children were also on maintenance oral corticosteroids during the challenge.

**2.17.8 Sputum induction**

There was very poor success with sputum induction in this study. Jones et al reported that it was more difficult to get an adequate sample of sputum using the combined challenge protocol with 65% of children producing the sample compared with a much higher rate of (92%) of children who had sputum induction alone\textsuperscript{(175)}. Of note the mean age of children involved in that study was 11 years as opposed to 7 years in the current study. Furthermore success of sputum induction is better in children older than 12 years\textsuperscript{(218)}.\n
2.18 Conclusion

There was reasonable subject retention for this preschool cohort at school age. A diagnosis of asthma has been confirmed in 75% of the original cohort, which is supported by higher levels of atopy, exhaled nitric oxide and $S_{\text{cond}}$ when compared to those without school age asthma. There were insufficient AHR data to draw any conclusions.

The next chapter will discuss the pathological changes in the preschool endobronchial biopsies which have previously been associated with asthma in adults and older children. In particular the next chapter will look at RBM thickness at preschool age and relate this to the presence or absence of school age asthma.
Chapter 3

Reticular basement membrane thickness in preschool wheezers and its relationship with childhood asthma.

3.1 Introduction

The bronchial epithelial basement membrane is an acellular matrix composed of two structurally distinct layers, the basal lamina (true basement membrane) and the RBM. The basal lamina can be visualised only by electron microscopy since its width (~80nm) is below the resolution of the light microscope. In contrast, the RBM is considerably thicker (~3-5µm in children)\(^9\) and can be seen using light microscopy. Abnormal thickening of the RBM is a feature of airway remodelling and is seen in several inflammatory airway diseases including cystic fibrosis\(^{219}\), COPD\(^{220}\), chronic cough\(^{221}\) and post lung transplant\(^{222}\) but is particularly characteristic of asthma\(^9\).

The relationship between RBM thickness and lung function remains uncertain. While some adult studies show that increased RBM thickness is associated with worse airway obstruction\(^{112}\), others show that RBM thickening is associated with reduced AHR\(^{223}\). RBM thickness positively correlated with the dose of metacholine needed to cause a 20% decrease in FEV\(_1\) and negatively correlated with the FVC/FEV\(_1\) ratio in adult patients (n=33) with asthma, allergic rhinitis or COPD\(^{223}\). In contrast adults with mild-moderate and severe asthma have demonstrated a relationship between bronchodilator responsiveness and increased RBM thickness\(^{224}\). However, in children no association has been found between RBM thickness and lung function\(^{116}\,^{219}\).

Preschool children, median age 2-3 years, recruited from the Royal Brompton Hospital from 2002-2005 with severe recurrent wheezing were found to have increased RBM thickness when compared with non-wheezing controls\(^9\). It was unknown at that time which of the children with severe recurrent wheeze at preschool age would persistently wheeze throughout childhood and develop asthma\(^9\). RBM measurements were repeated so there was consistency with only one observer making all pathology measurements throughout this study.
3.1.2 Hypothesis
The hypothesis to be tested in this chapter:
*Preschool wheezers who have asthma at school age have increased RBM thickness in preschool endobronchial biopsies compared to wheezy preschool children whose symptoms subsequently regressed.*

3.1.3 Aims and Objectives
1. To re-measure RBM thickness in preschool endobronchial biopsies (previously measured by Dr S Saglani) blinded to preschool and school age clinical status
2. To relate RBM thickness at preschool age to the presence or absence of asthma at school age

3.2 Methods: Measurement of reticular basement membrane thickness in preschool children

**Biopsy processing and evaluation**
At the time of bronchoscopy and EB up to 4 biopsies had been taken and fixed in 10% formal saline and processed to paraffin blocks 4-24 hours after fixation. Three 5μm thick sections were then taken at 25-50μm step intervals, dependent on biopsy size. These sections were stained using haematoxylin and eosin (H&E). Sections containing only glandular tissue or strips of epithelium and RBM without associated subepithelial mucosa were not used. Children had only 1 or 2 suitable biopsies for analysis. Biopsies had previously been assessed for quality and suitability of measurement of RBM thickness by 2 observers, Dr S Saglani (SS) and Dr D Payne. A formal clinical report had also been provided by Professor A Nicholson, Royal Brompton Hospital at the time of biopsy. For this thesis, sections were counted in random order by Dr R O’Reilly (RO’R) blinded to both the children’s preschool group (wheezers or control) and school age asthma status.

**Measurement of RBM thickness**
During the time period between 2005 and 2009 further sections had been cut and stained with H&E so it is likely that different sections were measured for a given child by SS and RO’R. Biopsies were considered suitable for RBM measurement if epithelium, subepithelium and RBM were present. RBM thickness was measured
using computer aided image analysis (NIH image 1.55: National Institute of Health, Bethesda MD) starting from the upper left corner of each biopsy and measured at 20µm intervals anticlockwise along a length of 1mm RBM for each biopsy. If the RBM could not be recognised it was not measured. Each measurement was made by drawing a line at a tangent to the luminal edge of the RBM (the luminal edge was adjacent to the epithelium) across its full length, figure 3.1. Forty measurements were made 20µm apart\(^{225}\). The geometric mean of the 40 measurements taken was used to represent the mean RBM thickness for that biopsy\(^{225}\).

**Figure 3.1:** Haematoxylin and eosin stained endobronchial biopsy section showing reticular basement membrane thickness measured at right angles to a tangent marking the outer perimeter of the reticular basement membrane at that point

3.2.2 Statistical analysis

This section describes the statistical methods used for chapters 3-6. Non-parametric tests were applied in all calculations as the numbers of subjects in all groups were small. Kruskal-Wallis was used to assess differences between 3 or more groups and Dunn’s post test used to detect differences between pairs groups. The Mann Whitney U test was used to detect differences between 2 groups. Repeatability for repeated biopsy measurements over time was assessed using the CoV. Variability of measurements within biopsy was also assessed using the CoV. Agreement between
2 observers was assessed using intraclass correlation coefficient and Bland-Altman plots. Sample size was determined by recruitment to the previous study and success of follow up at school age. However, for adult EB studies, 15 patients for inflammatory studies and 10 for RBM thickness in each limb are usually sufficient to give positive results\(^{(225)}\).

3.3 Results

3.3.1 Repeatability of RBM thickness measurements

Three sections were measured on 3 occasions at intervals of a week and a month by RO’R. The mean CoV for the 3 sections measured on 3 occasions was 5.7% (3.5%, 4.7% & 8.8%), table 3.1.

Table 3.1: Variability between measurements in the same section by the same observer at day 1, day 7 and day 30

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 30</th>
<th>CoV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Section 1</td>
<td>5.5</td>
<td>5.3</td>
<td>5.1</td>
<td>3.5</td>
</tr>
<tr>
<td>Section 2</td>
<td>5.1</td>
<td>4.8</td>
<td>5.2</td>
<td>4.7</td>
</tr>
<tr>
<td>Section 3</td>
<td>4.3</td>
<td>4.5</td>
<td>3.9</td>
<td>8.8</td>
</tr>
</tbody>
</table>

3.3.2 Comparison with previous reticular basement membrane thickness measurements

The values for RBM thickness measured by RO’R are similar to those reported by SS, in 2007\(^{(9)}\). The ICC for measurements in the same child compared between RO’R and SS is 0.67. There was no significant bias between the 2 observers shown by using a Bland-Altman plot, figure 3.2.
Figure 3.2: A Bland-Altman plot showing variability between measurements as reported by observer 1 (RO’R) and observer 2 (SS). No significant bias is detectable.

3.3.3 Reticular basement membrane thickness related to preschool clinical status

Biopsies were suitable for measurement of RBM thickness in 37 wheezers. Children had been further divided into 2 groups using the video questionnaire at preschool age into CW (n=21) and RW (n=16). There were 16 controls. The RBM was significantly thicker in all wheezers both confirmed and reported (median thickness 4.5µm [1.41-7.3µm]) compared to controls (median 3.58µm [1.2-5.99 µm]), p=0.009, figure 3.3. The RBM was significantly thicker in CW (median 4.76µm [3.1-7.3µm]) compared to controls (median 3.58µm [1.2-5.99µm]), p=0.02, but there was no difference between both CW and RW (median 4.19µm [1.4-5.77µm]), or RW and controls, figure 3.4.
Figure 3.3: Increased reticular basement membrane thickness in endobronchial biopsies from preschool wheezers compared to age matched controls (RO’R measurements)

\[ p=0.009 \]

**Figure 3.4:** Increased reticular basement membrane thickness in preschool confirmed wheezers compared to controls (RO’R measurements)

(There was no difference between confirmed and reported wheezers or reported wheezers and controls)

\[ p=0.02 \]
3.3.4 Relationship of reticular basement membrane thickness at preschool age to asthma at school age

Thirty nine children with RBM thickness measured at preschool age were followed up at school age. There was no difference between children with RBM measurements who were followed up at school age and those not followed up in terms of age at EB (p=0.4), RBM thickness (p=0.54) or sex (p=1). Preschool RBM thickness was similar in children with school age asthma (median 4.77µm [range 3.14-7.02µm]) when compared to those without asthma (median 4.14µm [range 1.2-5.99µm]), p=0.15, figure 3.5.

**Figure 3.5: No difference in preschool reticular basement membrane thickness when compared with presence or absence of asthma at school age (RO’R measurements – all children included)**

On examining the children who wheezed at preschool age (excluding preschool controls), there was a trend towards increased RBM thickness in preschool wheezers who developed childhood asthma (n=11, median 4.79 [3.38-7.02]µm), when compared to those preschool wheezers who stopped wheezing (n=18, median 4.17 [1.4-5.72]µm) but it was not statistically significant, p=0.12, figure 3.6. There was a significant difference between age at EB of those preschool wheezers who developed school age asthma (42 [24-57] months) and the preschool wheezers who did not (15.5 [7-55] months), p=0.002.
Figure 3.6: No difference in reticular basement membrane thickness in severe recurrent preschool wheezers when compared with presence or absence of asthma at school age- (RO’R measurements-preschool wheezers alone)

\[ p=0.12 \]

3.3.5 Comparison of Dr Saglani’s reticular basement membrane measurements with asthma status at school age

SS’s measurements of RBM thickness in preschool children showed a significant difference between groups classified by asthma status at school age, in that those children who developed asthma (\( n=11 \), median 5.1\( \mu \)m [range 3.1-8\( \mu \)m]) had increased RBM thickness when compared with those who did not (\( n=28 \), median 3.94\( \mu \)m [range 2-5.44\( \mu \)m]), \( p=0.02 \), figure 3.7. However, individual measurements for children showed no significant bias between the 2 observers. Further sections had been cut and stained with H&E since the original study so one child with asthma at school age had not had RBM measured by SS but had by RO’R.
Figure 3.7: Dr S Saglani’s measurements showing increased reticular basement membrane thickness at preschool age in children with school age asthma (all children followed up)

\[ p=0.02 \]

Preschool wheezers compared with school age asthma status

On examining just the children who had wheeze at preschool age there was again significantly greater RBM thickness in those who developed school age asthma \( (n=10, \text{median } 5.25 \ [3.4-8]\ \mu m) \) compared with those who did not \( (n=18, \text{median } 4.15 \ [2.12-5.44] \ \mu m), p=0.02, \text{figure 3.8.} \)

Figure 3.8: Dr S Saglani’s measurements showing increased reticular basement membrane thickness in severe recurrent preschool wheezers who developed school age asthma when compared to preschool wheezers who did not develop asthma (preschool wheezers alone)

\[ p=0.02 \]
3.3.6 Comparison of preschool reticular basement membrane thickness with clinical findings at school age

All RBM thickness measurement reports are as measured by RO’R unless otherwise stated. RBM thickness at preschool age correlated with the number of overnight hospital admissions as reported by the child’s carer at school age over that child’s lifetime ($r=0.56$, $p=0.001$), figure 3.9. However there was no association between wheeze frequency at school age or reported medication use at school age, and RBM thickness at preschool age, table 3.2.

Figure 3.9: Positive correlation with number of lifetime overnight admissions to hospital with wheeze reported at school age and reticular basement membrane thickness at preschool age

\[ r=0.50, p=0.008 \]
Table 3.2: Clinical findings at school age related to reticular basement membrane thickness at preschool age

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>No Asthma</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBM (µm)</td>
<td>n=12</td>
<td>n=27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.77(3.14-7.02)</td>
<td>4.14(1.2-5.99)</td>
<td>0.15</td>
</tr>
<tr>
<td>Age at asthma diagnosis (years)</td>
<td>8.6 (6-10.4)</td>
<td>7.2 (5.9-11)</td>
<td>0.09</td>
</tr>
<tr>
<td>Age at FOB (months)</td>
<td>38.5 (3-57)</td>
<td>17 (6-55)</td>
<td><strong>0.005</strong></td>
</tr>
<tr>
<td>Male/ Female</td>
<td>8/4</td>
<td>16/11</td>
<td></td>
</tr>
</tbody>
</table>

**Symptoms at school age**

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>No Asthma</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheeze in the last year</td>
<td>n=17</td>
<td>n=22</td>
<td></td>
</tr>
<tr>
<td>RBM (µm)</td>
<td>n=12</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.55 (3.1-7.02)</td>
<td>4.11 (1.2-5.99)</td>
<td>0.41</td>
</tr>
<tr>
<td>Wheeze 4-12 times/ last year*</td>
<td>n=5</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td>RBM (µm)</td>
<td>n=12</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.63 (3.38-7.02)</td>
<td>3.73 (1.2-5.99)</td>
<td>0.16</td>
</tr>
<tr>
<td>Wheeze &gt; 12 times/ last year*</td>
<td>n=5</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td>RBM (µm)</td>
<td>n=5</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.07 (3.38-7.02)</td>
<td>3.73 (1.2-5.99)</td>
<td>0.44</td>
</tr>
<tr>
<td>ICS use at school age</td>
<td>n=13</td>
<td>n=16</td>
<td></td>
</tr>
<tr>
<td>RBM (µm)</td>
<td>n=13</td>
<td>n=16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.76 (3.38-7.02)</td>
<td>4.19 (1.4-5.7)</td>
<td>0.39</td>
</tr>
<tr>
<td>Positive skin prick tests school age</td>
<td>n=9</td>
<td>n=18</td>
<td></td>
</tr>
<tr>
<td>RBM (µm)</td>
<td>n=9</td>
<td>n=18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.5 (1.4-4.97)</td>
<td>4.07(1.2-7.02)</td>
<td>0.37</td>
</tr>
</tbody>
</table>

*When compared to children who did not wheeze either at preschool or school age
(All reported values are Dr R O’Reilly’s measurements)
3.3.7 Comparison of preschool reticular basement membrane thickness with measurements of airway inflammation and lung function at school age
There was no correlation between spirometry, LCI or $S_{\text{cond}}$ and preschool RBM thickness, table 3.3. Similarly there was no relationship between FeNO$_{50}$ and RBM thickness. There was a negative correlation between $S_{\text{acin}}$ and RBM thickness at preschool age ($r=-0.53$, $p=0.008$), figure 3.10.

Table 3.3: Correlation between lung function and airway inflammation at school age with reticular basement membrane thickness at preschool age

<table>
<thead>
<tr>
<th>RBM thickness (µm)</th>
<th>Correlation (Spearman)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV$_1$ (z score) n=27</td>
<td>0.18</td>
<td>0.38</td>
</tr>
<tr>
<td>FVC (z score) n=27</td>
<td>-0.1</td>
<td>0.6</td>
</tr>
<tr>
<td>LCI n=28</td>
<td>0.04</td>
<td>0.82</td>
</tr>
<tr>
<td>$S_{\text{acin}}$</td>
<td>-0.54</td>
<td>0.008</td>
</tr>
<tr>
<td>$S_{\text{cond}}$</td>
<td>0.22</td>
<td>0.29</td>
</tr>
<tr>
<td>FeNO (ppb) n=26</td>
<td>0.09</td>
<td>0.65</td>
</tr>
</tbody>
</table>

*Over child’s lifetime (with wheeze)*

Figure 3.10: Negative correlation between reticular basement membrane thickness (n=23) at preschool age and $S_{\text{acin}}$ at school age

Spearman $r=-0.53$, $p=0.008$
On comparing RBM thickness in the preschool wheezers alone there was a non-statistically significant trend with FEV₁ (n=20, Spearman r=0.42), p=0.07, figure 3.11 (i) and FeNO₅₀ (n=19, Spearman r=0.44), p=0.06, figure 3.11 (ii). There was no other positive correlation between preschool wheezers and lung function measurements.

**Figure 3.11 No correlation between RBM thickness in preschool wheezers and (i) FEV₁ and (ii) FeNO₅₀ at school age**

(i) Spearman r=0.42, p=0.066

![Graph showing RBM thickness vs. FEV₁ z score.]

(ii) Spearman r=0.44, p=0.06

![Graph showing RBM thickness vs. FeNO₅₀ (ppb).]
3.4 Summary of findings RBM thickness

Review of hypothesis

Preschool wheezers who persistently wheeze and have asthma at school age have increased RBM thickness on endobronchial biopsy compared to those wheezy preschool children whose symptoms subsequently regressed.

In summary:

- Measurement of RBM thickness by a second blinded observer (RO’R) confirmed previous findings showing increased RBM thickness in severe recurrent wheezers when compared with non wheezing controls.
- There was no difference in preschool RBM thickness as measured by RO’R when compared with presence or absence of school age asthma. The SS measurements showed increased RBM thickness in preschool wheezers who developed school age asthma. Both observers had similar coefficients of variation of RBM measurements, and this discrepancy (which likely arises because different sections were analysed by the two observers) makes RBM thickness unlikely to be a robust predictor of school age asthma in the individual child.
- RBM thickness at preschool age correlated with the number of lifetime hospital admissions with wheeze (at the time of the research visit).

3.5 Discussion:- Preschool RBM thickness and school age asthma

RBM thickness in severe recurrent wheezy preschool children, at least as measured by RO’R, did not discriminate which children developed school age asthma, suggesting RBM thickness in preschool wheezers may relate to current symptoms rather than future asthma risk. Even the SS measurements showed a marked overlap between the two groups. RBM thickness in severe recurrent CW (median 4.6 μm [3.1-7.3μm]) is greater than controls (median 3.5μm [1.2-5.99 μm]), but much less than older children with severe asthma (median 8.2μm [range 5.4–11.1μm]) and adults with asthma (8.1μm [5.8–10.0μm]). This suggests remodelling is not yet fully developed in the RBM of children with preschool wheeze which may reflect symptom duration. It would have been very interesting but unethical to repeat the
endobronchial biopsy at time of asthma diagnosis to correlate RBM thickness at preschool and school age.

There was a discrepancy between measurements of RBM thickness by RO’R and SS related to school age asthma status. The CoV for SS (mean 7%) for measurement of RBM thickness was very similar to RO’R (mean 6%). It is likely observers did not measure the same section for a given child and there can be between biopsy variability for the same child as reported by SS which may have contributed to the different results. The between biopsy variability for RBM thickness was assessed by SS for 6 patients who had 2 good quality biopsies. The results are shown in table 3.4.

**Table 3.4: Between biopsy variability of RBM thickness for 6 preschool children as measured by SS**

<table>
<thead>
<tr>
<th>Subject number</th>
<th>RBM thickness (μm)</th>
<th>Biopsy 1</th>
<th>Biopsy 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.0</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.9</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.9</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.3</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3.3</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6.0</td>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>

Unfortunately but unavoidably, there was also a significant difference between age of EB in children with RBM measured who developed asthma at school age (median age 38.5 months) and those who did not develop asthma (median age 17 months), p=0.002. The natural history of RBM development has been recently established and shows that RBM thickness increases during infancy, childhood and adolescence until age 17 years, figure 3.12\(^{(113)}\). In particular the rate that RBM thickness increase is greater in children less than 6 years old than in children older than 6 years\(^{(113)}\). Data suggests that RBM thickness should increase at least 1μm between age 1 year and 2.5 years, which is approximately the difference in RBM thickness and difference in age at EB seen between children with and without school age asthma as measured by SS. This suggests that the increased RBM thickness in the preschool years seen in those who went on to develop school age asthma may be related to age rather
than airway remodelling. However, a larger study with age-matched controls is required to confirm or refute this.

**Figure 3.12: RBM thickness related to age in post-mortem sections from children without known lung pathology (Adapted from Tsartsali et al)**

Severities of wheeze (as measured by the number of overnight hospital admissions with wheeze over the child’s lifetime to point of research visit) was related to RBM thickness at preschool age (Spearman r=0.56, p=0.001). This observation is vulnerable to recall bias over a 7-10 year time period. Other markers of severity including frequency of wheeze over the past 12 months or current use of ICS were not related to RBM thickness at preschool age. There is no obvious reason why S_{acin}, a measure of ventilation inhomogeneity in the acinar airways correlated with RBM thickness, measured in larger, very proximal airways.

RBM thickness did correlate weakly with FeNO_{50} at age 7 years, but only in the children who had wheezed at preschool age. This was similar to the findings when some of this group of wheezers were followed up at 5 years^{63}. There was also a non-significant association between FEV_{1} z score at school age and RBM thickness at preschool age in the preschool wheeze group, with those with increased RBM thickness having better lung function. It is unlikely this is related to a protective effect of RBM thickness on the airway as suggested by other studies^{223,226}, but rather that several of the wheezy children had other lower airway pathology at school age. Increased RBM thickness has been reported in adults with chronic cough^{221} and in
children with bronchiectasis (related to cystic fibrosis)\(^{(219)}\). Finally, the small numbers preclude definitive conclusions.

At preschool age there was a large overlap in RBM thickness between wheezers and controls suggesting that RBM thickness alone was unlikely to be a good predictor for future asthma. This was confirmed when preschool RBM thickness was compared with school age asthma status. The next hypothesis to be tested was that individual components of the RBM may be a more sensitive marker of future asthma in preschool wheezers.
Chapter 4
Components of the reticular basement membrane in preschool wheezers and their relationship with childhood asthma.

4.1 Components of the reticular basement membrane

The extracellular matrix (ECM) which includes the interstitial matrix and the RBM is composed of macromolecules that form the scaffolding of the airways. It consists of a complex network of proteins which affect many aspects of cellular behaviour including proliferation, differentiation, survival, adhesion and migration\(^{(227)}\). Increased thickness of the reticular basement membrane results from increased extracellular matrix protein deposition. There are many extracellular matrix glycoproteins including collagen I, III and V, fibronectin, laminan, versican and tenascin\(^{(227)}\). The quantity of ECM proteins is increased in asthmatic airways\(^{(227)}\). This may be due to increased \textit{de novo} protein synthesis, decreased activity of their degrading enzymes, i.e. matrix metalloproteinases (MMPs), or increased activity of tissue-specific inhibitors of metalloproteinase (TIMPs).

Tenascin-C (TN-C) is a particularly relevant ECM glycoprotein as its expression is increased in lung development and in inflammatory, fibrotic and neoplastic diseases but is almost absent otherwise. Five isoforms of tenascin (TN) have been characterised, namely TN-C, TN-R, TN-X, TN-Y and TN-W\(^{(228)}\). TN-R is found in the developing and adult nervous system, TN-X is primarily found in loose connective tissue, TN-W is found in the kidney and in developing bone and TN-Y in the developing skeleton of zebra fish\(^{(141)}\). TN-C was the first to be identified and it is the best studied\(^{(229)}\). It is TN-C that is referred to throughout this thesis. TN-C was selected for further investigation, in particular since its absence in health and presence in disease states in the airways suggested it may be a more accurate predictor of future asthma in preschool children than RBM thickness alone\(^{(141)}\). Limited biopsy tissue was available for staining and for this reason TN-C was the only component of the RBM that could be studied.
4.1.2 Tenascin-C expression during foetal lung development
During foetal development TN-C is expressed maximally at 12-23 weeks gestation, particularly at sites of airway branching during the canalicular phase (17-23 weeks) of development\(^{(230)}\). During the saccular phase (24-40 weeks) of development TN-C expression is weaker but still present below the alveolar and bronchiolar epithelium, and strongly expressed in the RBM\(^{(230,231)}\). It has been suggested that the increased expression of TN-C in basement membranes surrounding the glandular, acinar and vascular structures of the developing lung might have an important function in the morphogenesis of the dividing bronchial and vascular trees, and in determining whether specific cells will adhere to the matrix\(^{(231)}\). This is further supported by evidence from embryonic mice TN-C\(^{(-/-)}\) who had significantly decreased airway branching when compared to wild type mice\(^{(232)}\).

4.1.3 Tenascin-C expression in the lung during health and disease
TN-C expression is associated with tissue injury and repair\(^{(141)}\). TN-C has also been shown to be specifically and transiently upregulated during acute inflammation and persistently expressed in chronic inflammation\(^{(141)}\). In the lung increased TN-C expression in the pleura can be used as a marker of active pleural involvement in inflammatory and fibrotic diseases\(^{(233)}\). The most positive areas of TN-C immunoreactivity are newly formed areas of fibrosis\(^{(233)}\), suggesting it may be active in acute inflammation. TN-C concentration in the airway epithelial lining fluid is increased in patients with usual interstitial pneumonia, sarcoidosis, and extrinsic allergic alveolitis\(^{(234)}\). It is also widely expressed in the stroma of lung carcinomas\(^{(235,236)}\).

4.1.4 Tenascin-C expression in the airways in asthma
TN-C expression is also increased within the RBM in asthma\(^{(142-144)}\). TN-C expression within the RBM is elevated in adult asthmatics when compared with non-asthmatic, non-atopic controls\(^{(144)}\). TN-C thickness within the RBM was shown to be significantly thicker in atopic when compared to non-atopic adult asthmatics (7.6 vs 6.3µm)\(^{(143)}\). The thickness of TN-C correlated with eosinophils, T lymphocytes, macrophage counts and IL-4 positive submucosal cells only in atopic asthmatics\(^{(143)}\). Acute inhalational allergen challenge of atopic adult patients with asthma increased collagen synthesis and TN-C deposition within the RBM\(^{(237)}\). This was associated
with increased eosinophilic inflammation and evidence of IL-4 / IL-13 expression in the airway epithelium\(^{237}\). However, TN-C deposition within the RBM was only increased at 24 hours post allergen challenge, and had returned to baseline levels by 7 days\(^{238}\).

It has been shown that TN-C\(^{-/-}\) mice that had been sensitised and challenged with ovalbumin had decreased airway hyperreactivity, NF-kB activation and concentrations of monocyte chemoattractant protein-1, IL-5, IL-13, metalloproteinase-9 and IgE in the bronchoalveolar lavage fluid compared to their wild type counterparts\(^{239}\). The authors suggested that future work should explore TN-C in the pathogenesis of bronchial asthma\(^{239}\).

The next hypothesis tested was that TN-C expression
1) is greater in preschool children with recurrent wheeze when compared with age matched controls.
2) is greater in preschool children with recurrent wheeze who develop future asthma

**Aims and objectives**

1. To establish the most discriminative and repeatable method of measuring TN-C expression within the reticular basement membrane in endobronchial biopsies archived in paraffin.
2. To compare TN-C expression at preschool age between wheezers and controls and with presence or absence of asthma at school age

### 4.2.1 Methods- Tenascin–C immunohistochemistry staining

All previous studies in adult asthmatics quantifying TN-C expression in the RBM have used snap frozen endobronchial tissue and either immunofluorescence\(^{142-144}\) or immunohistochemistry\(^{220}\). All preschool endobronchial biopsies archived from 2002-2005 had been formalin fixed and paraffin embedded and there was no frozen tissue available. However, TN-C expression has been measured in paraffin embedded neoplastic breast tissue\(^{240}\), myocardium\(^{241}\), glomeruli\(^{242}\) and the developing fetal lung\(^{230;231;243}\). This is the first attempt at quantifying TN-C in the RBM of endobronchial biopsies processed in paraffin.
Details of endobronchial biopsy procedure, processing and evaluation are given in chapters 2 and 3. The preschool endobronchial biopsy tissue studied in this chapter was processed and stained by Dr J Zhu. Unstained tissue sections (5µm) from biopsy blocks were plated on poly-l-lysine-coated glass slides then deparaffinised in xylene and dehydrated. Next all sections were treated for 30 min with 1.5% hydrogen peroxide in distilled water to quench endogenous peroxidase activity. Sections were rinsed and bathed in phosphate buffered saline (PBS) twice for 5 minutes each time and then treated with 10% normal goat serum in PBS for 20 minutes. Slides were then incubated with mouse monoclonal antibody to tenascin-C (T2H5 ABCAM cat: 3970) dilution 1:40 with PBS at room temperature for 2 hours. Sections were again rinsed and bathed in PBS twice for 5 minutes each time. The sections were then incubated with HRP labelled polymers (which did not contain avidin-biotin complex) conjugated with secondary antibodies at room temperature for 40 minutes (Dako EnVision system, HRP, K4007). Rinsing and bathing with PBS twice for 5 minutes was repeated. The antigen–antibody reaction was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate chromagen which results in a brown coloured precipitate at the antigen site. The slides were then rinsed in distilled water and counterstained in Harris’ H&E stain, dehydrated and mounted.

4.2.2. Quantification of tenascin-C within the reticular basement membrane
It was important to establish a method that was both discriminative and repeatable in quantifying TN-C expression within the RBM of immunohistochemically stained sections. Three different methods were used -
1) measurement of TN-C thickness using computer aided image analysis
2) a semi-quantitative method using a picture grading system
3) measurement of TN-C using stereology techniques.

All biopsies were identified by number only. TN-C was measured in all sections that included recognisable epithelium, RBM and subepithelial mucosa. Leica DM2500 microscope, Leica DFC 300fx camera and LEICA Qwin version 3 software were used for all 3 methods.
4.2.2.1 Method 1- Measurement of tenascin-C thickness using computer aided image analysis

The mean of all TN-C thickness measurements at 20µm intervals along the whole RBM, using computer aided image analysis were calculated. This method has previously been used to measure total RBM thickness\(^{(225)}\), see chapter 3. The first measurement was taken at the upper right hand corner and the biopsy followed anticlockwise with subsequent measurements at 20 µm intervals for the whole length of the RBM. At each point a line perpendicular to the length of RBM was drawn, figure 4.1 (a), and TN-C was considered positive if any level of staining was present, figure 4.1 (b). ‘0’ was recorded if no staining for TN-C was present, figure 4.1 (c). The mean of all measurements was taken to represent RBM TN-C thickness for that biopsy.

**Figure 4.1: (a): Measuring tenascin-C thickness**

TN-C thickness is measured perpendicular to the length of RBM
Figure 4.1: (b): Tenascin-C positive staining in the RBM (magnification x400)

Figure 4.1: (c): Tenascin-C negative staining in the RBM (magnification x400)

4.2.2.2 Method 2- Semi-quantitative measurement of tenascin-C using a picture grading system
A semi quantitative scale was used to grade the TN-C positive proportion of RBM in the biopsy. Similar scales have been used to quantify TN-C expression in the myocardium of patients with myocarditis\(^\text{(241)}\), the glomeruli of adults with IgA nephropathy\(^\text{(242)}\) and within the RBM of adults with COPD and asthma\(^\text{(220)}\). Each
section was photographed at magnification X100 and re-numbered by an independent observer (Dr N Ullmann). Where more than one photomicrograph of a section was taken it was labelled with the same number and the pictures were reviewed together. The photographs were scored 0, 1 or 2 using a picture coded scale one week later, figure 4.2. Grade 0 represented <1/3 of the RBM stained positive for TN. Grade 1 represented 1/3-2/3 of the RBM stained positive for TN and grade 2 represented > 2/3 of the RBM stained positive for TN-C. They were assessed by a single blinded observer (RO’R) on a computer screen with the picture scale as reference. The same photographs were again reassessed by RO’R using the same method 1 week and 1 month later, in each case blinded to any previous measurements.

Figure 4.2: Reference pictures used to grade tenascin-C expression in the reticular basement membrane using a semi quantitative scale

- Grade 0: <1/3 of the total RBM stained positive for tenascin-C
• Grade 1: 1/3-2/3 of the RBM stained positive for tenascin-C

• Grade 2: ≥ 2/3 of the RBM stained positive for tenascin-C

4.2.2.3 Method 3- Measurement of tenascin-C using stereology
Stereology is the study of the three-dimensional properties of objects usually seen in two dimensions. Thus, by using stereology-based techniques, one can obtain quantitative three-dimensional information from measurements in the two dimensions of conventional histology\textsuperscript{(85)}. The biopsy section was examined using oil emersion at magnification x1000. The image was projected onto a computer screen and a virtual graticule containing 100 points each a fixed distance apart was overlaid on the top right hand corner of the biopsy at random but it was ensured that at least
one point landed on the RBM, figure 4.3. The graticule was then moved along the total length of the RBM. Care was taken not to overlap the graticule overlay by using biopsy landmarks.

**Figure 4.3: Tenascin-C staining overlayed by graticule** (magnification X1000)

![Tenascin-C staining overlayed by graticule](image)

Only TN-C positive and negative points within the RBM were counted. A point on the graticule was considered positive if it overlay an area stained positive for TN-C. Two quadrants or more, or the top right hand quadrant had to overlay an area stained with TN-C to be considered positive, figure 4.4. Similarly the same criteria applied to all points falling on the RBM. The number of points that were TN-C positive were expressed as a proportion of the total number of points on the RBM.
Figure 4.4: Criteria for tenascin positive and negative points.

A point was positive if it overlayed 2 or more quadrants or the upper right quadrant

A point was negative if it overlayed 1 or less than 1 single quadrant other than the upper right quadrant

4.2.3 Repeatability of measurements

The intra-observer repeatability of measurements was calculated by assessing the same biopsy 3 times at least a week and a month apart, and represented as the CoV. This was done for the same 4 sections using both methods 1&3. The mean of the 4 measurements of the CoV was used as a measure of repeatability for that method. The intraclass correlation coefficient was used to measure the repeatability of method 2 where all biopsies were assessed using a semi-quantitative scale a week and a month apart.

4.3 Results

4.3.1 Biopsy size and number

Biopsies of good quality to assess RBM TN-C were available in 48 preschool children, of which there were 31 wheezers (median age 24 months) and 8 controls (median age 19.5 months). Wheezers were subdivided into CW (n=15, median age 31 months) and RW (n=16, median age 15.5 months). Length of RBM was measured at magnification x100 using computer aided image analysis. There was no significant difference in the length of RBM measured between wheezers and controls (p=0.29). RBM thickness measured by RO’R and not that measured by SS in 2007\(^9\) was compared with RBM TN-C expression.
Table 4.1: Number of patients in each group with at least 1 biopsy of good quality for (i) tenascin measurement, (ii) reticular basement membrane thickness

<table>
<thead>
<tr>
<th></th>
<th>CW</th>
<th>RW</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients with biopsies available for TN measurement</td>
<td>15</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Number of patients with good quality biopsy for RBM and TN measurement</td>
<td>15</td>
<td>15</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Median length of RBM in (µm) (Tenascin immunostained biopsies)</td>
<td>1509</td>
<td>2130</td>
<td>1760</td>
<td>0.4</td>
</tr>
<tr>
<td>Range (µm)</td>
<td>729-6412</td>
<td>881-5248</td>
<td>998-2233</td>
<td></td>
</tr>
</tbody>
</table>

4.3.2 Comparing methods of quantifying TN-C

1. To compare 3 methods of quantifying TN-C expression within the RBM in endobronchial biopsies from preschool wheezers and controls to find the most discriminative and repeatable method for measuring TN-C

Method 1- Measurement of tenascin-C thickness using computer aided image analysis

There was no significant difference between the 3 groups at magnification X400 (p=0.07), figure 4.5 (a). The median TN RBM thickness did show a trend for higher values in CW (2.7µm) when compared with controls (0.89µm) and RW (0.85µm), p=0.07. The CoV for biopsies from 4 children measured on 3 occasions a week and a month later was 34%, 29%, 8.8% and 8.25% giving a mean of 20%.

Measurement of TN-C thickness at higher magnification (x1000) improved repeatability of measurements and again showed a trend for a difference between CW (2.35µm) and controls (0.96µm) (p=0.05), figure 4.5 (b). However, Dunns post test did not detect a difference between confirmed CW, RW or controls. Mann Whitney U test detected a significant difference between CW and controls (p=0.02). Repeatability was also improved at X1000, the coefficient of variation in the same 4 biopsies was 29%, 10.5%, 3% and 1.1%, giving a mean of 10.9%. RBM TN-C
measurements at magnification 400 and 1000 correlated well (Spearman $r=0.91$, (CI 0.84-0.95), $p<0.0001$).

**Figure 4.5**: Measurement of tenasin-C thickness within the reticular basement membrane of endobronchial biopsies at (a) magnification x400 and (b) magnification x1000

(a) Magnification X400

(i) $p=0.19$

(b) Magnification X1000

(i) $p=0.32$

(ii) $p=0.056$
Method 2 – Semi-quantitative method using a picture grading system
The scale completely failed to differentiate the groups, figure 4.6. Repeatability for this method was measured using the ICC, a week and a month later. All biopsies were assessed on each occasion. The ICC was 0.93 (p<0.001) and 0.87 (p<0.001) at 1 week and 1 month respectively.

Figure 4.6: Tenascin-C expression quantified by a semi quantitative scale.

Method 3: Measurement of tenascin-C using stereology techniques
The third method assessed the proportion of the RBM that stained positive for TN-C. This showed significant difference between CW (median 83%) and controls (median 38%) (p=0.04), figure 4.7(ii). Although the Kruskal Wallis test was significant (p=0.04), Dunns post test did not detect a difference between the 3 groups. Mann Whitney U test detected a difference between CW and controls (p=0.04). Repeatability for this method was assessed measuring the same 4 biopsies on 3 occasions a week and a month apart. CoV were 23%, 12.5%, 1% and 2.5% respectively, mean of 9.75%.
Figure 4.7: Proportion of the RBM occupied by tenascin-C measured using stereology techniques

Summary
Intra-observer repeatability (CoV) was more reliable at higher magnification (x1000) for method 1 (measuring TN-C thickness) and method 3 (measuring proportion of TN-C occupying the RBM), table 4.2, as it was easier to distinguish TN-C positive and negative areas of the RBM at higher magnification. Both methods also showed TN-C expression to be increased in CW when compared with controls when quantified as TN-C thickness within the RBM and proportion of the RBM occupied by TN-C. Method 2 (semi-quantitative scale) had excellent repeatability (ICC= 0.87) but very poor group discrimination. Either method 1 or 3 was therefore suitable for quantifying TN-C at magnification 1000, as both have similar repeatability. It is these results that are reported throughout the rest of the analysis.

Table 4.2: Repeatability of measuring of tenascin-C thickness at x400 & 1000, and of measuring the proportion of tenascin-C occupying the reticular basement membrane using stereology x1000.

<table>
<thead>
<tr>
<th>Coefficient of Variation (%)</th>
<th>TN thickness x400</th>
<th>TN thickness x1000</th>
<th>Stereology x1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsy 1</td>
<td>34</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>Biopsy 2</td>
<td>29</td>
<td>10.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Biopsy 3</td>
<td>8.8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Biopsy 4</td>
<td>8.25</td>
<td>1.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Mean</td>
<td>20</td>
<td>10.9</td>
<td>9.75</td>
</tr>
</tbody>
</table>
### 4.4 RBM Tenascin-C compared by viral and multiple-trigger wheeze phenotypes

There was no difference in TN-C thickness \( (n=6, 1.59(1.04-2.64)\mu m \text{ vs } n=25, 1.66(0-7.2)\mu m, \ p=0.98) \) or proportion of RBM TN-C \( (n=6, 82.5 (43.5-92.9)\% \text{ vs } n=25, 57 (0-100)\%, \ p=0.26) \) between children with viral or multiple-trigger wheeze at preschool age, figure 4.8.

**Figure 4.8:** No difference between (i) RBM tenasin-C thickness and (ii) proportion of RBM tenasin-C between viral and multiple-trigger wheezers at preschool age

![Graph showing no difference in TN-C thickness and proportion of TN-C between viral and multiple-trigger wheezers](image)

### 4.5 Inhaled corticosteroids and RBM tenasin-C

There was no difference in RBM TN-C thickness between those treated with ICS \( (n=16, \text{median } 1.44(0-7.2)\mu m) \) at time of EB and those who were not treated with ICS \( (n=15, \text{median } 2.28 (0-4.25)\mu m), \ p=0.62, \text{figure 4.9 (i).} \) Similar results were seen for proportion of RBM TN-C. Four of the children not on ICS had been treated with oral corticosteroids in the month prior to biopsy. However there was still no difference when children on oral corticosteroids were included in the analysis.
Figure 4.9: No relationship between inhaled corticosteroids and (i) RBM tenascin-C thickness and (ii) proportion of RBM tenascin-C

Table 4.3: Summary of reticular basement membrane tenascin-C composition in preschool wheezers compared with preschool controls. There is an increased proportion of RBM tenascin-C in preschool wheezers when compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>Wheezers n=31</th>
<th>Controls n=8</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (months)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW n=15</td>
<td>24 (7-58)</td>
<td>19.5 (11-42)</td>
<td>0.9</td>
</tr>
<tr>
<td>RW n=16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/ Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW=7/8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW=10/6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhaled corticosteroids*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median BDP (mcg)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Range mcg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW n=8</td>
<td>800 (200-2000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>900 (500-2000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW n=8</td>
<td>500 (200-500)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 (200-1000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnification x400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN-C thickness (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW=2.74</td>
<td>1.89 (0-7.47)</td>
<td>0.895 (0-2.77)</td>
<td>0.19</td>
</tr>
<tr>
<td>(0-7.47)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW=0.85</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0-5.03)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnification x1000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TN-C thickness (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW=2.35</td>
<td>1.66 (0-7.2)</td>
<td>0.97 (0-2.7)</td>
<td>0.07</td>
</tr>
<tr>
<td>(0.29-7.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW=1.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0-4.25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnification x1000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of RBM TN-C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CW=82.9</td>
<td>61.5 (0-100)</td>
<td>41.2 (0-92)</td>
<td>0.25</td>
</tr>
<tr>
<td>(12.6-100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW=37.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0-94.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.6 Relationship between reticular basement membrane tenascin-C expression and reticular basement membrane thickness

There was a weak correlation between RBM TN-C thickness (method 1) and total RBM thickness (Spearman \( r=0.34 \), CI= (0.012-0.61), \( p=0.04 \), figure 4.10 (a). However, comparing RBM TN-C (method 3) and RBM thickness (Spearman \( r=0.33 \), \( p=0.04 \)), the confidence interval included zero (-0.001-0.60) showing it is not significant, figure 4.10 (b).

Figure 4.10: Relationship between tenascin-C and total reticular basement membrane thickness when measured as (a) tenascin-C thickness within the reticular basement membrane, and (b) as the proportion of tenascin-C occupying the reticular basement membrane.

(a)  
(b)

Spearman \( r=0.34 \), \( p=0.04 \)

Spearman \( r=0.33 \), \( p=0.04 \)

4.7 Preschool reticular basement membrane tenascin-C and school age asthma

RBM TN-C thickness (method 1) at preschool age was similar in children who developed school age asthma (n=9, median 2.27 [0.29-7.2]µm) and children who did not develop asthma (n=20, median 1.35 [0-3.67]µm), \( p=0.1 \), figure 4.11 (a). The proportion of the RBM occupied by TN-C was also similar in those that did and did not develop asthma (method 3) (median 61.5 [12.6-100]% vs (median 50.4% [0-94.7]%), \( p=0.11 \), figure 4.11 (b). When preschool wheezers alone were considered, there was no difference in RBM TN-C thickness between those who developed asthma (median 2.27µm [range 0.29-7.2]µm) and those who did not (median 1.99µm
[range 0 -3.67]µm), p=0.18. Similarly, there was no difference in the proportion of RBM TN-C between those preschool wheezers who developed school age asthma (median 61.5% [range 12.6-100]%) and those who did not (median 62.3% [range 0-94]%), p=0.19. There was however a significant difference between age at EB in children who had RBM TN-C measured who developed school age asthma (42 [24-57] months) and those who did not (17.5 [7-55] months), p=0.003.

**Figure 4.11:**

a) No difference in reticular basement membrane tenasin-C thickness (method 1) at preschool age in children who developed school age asthma when compared with those who did not

![Graph showing no difference in reticular basement membrane tenasin-C thickness](image)

b) No difference in the proportion of reticular basement membrane tenasin-C (method 3) at preschool age between children who developed asthma by school age and those who did not

![Graph showing no difference in the proportion of reticular basement membrane tenasin-C](image)
4.8 Tenascin-C expression within the reticular basement membrane and age

While TN-C expression is absent in developed lungs, the age at which normal developmental TN-C expression ceases in the lungs is unknown. There was no relationship between RBM TN-C expression and age in the controls, figure 4.12. However, the numbers studied (n=8) are very small. It was not possible to assess the relationship of TN-C expression within the RBM with age further because of subsequent difficulties with immunostaining described below.

Figure 4.12: Relationship between age and (a) tenascin-C thickness within the reticular basement membrane and (b) the proportion of tenascin-C occupying the reticular basement membrane in control preschool children

(a) spearman r=0.33, p=0.43

(b) spearman r=0.4, p=0.32

n=8
4.9 Difficulties with tenascin-C staining in paraffin embedded tissue

The sections from the severe preschool wheezers and age matched controls were stained successfully in 2 batches in 2007. The immunohistochemistry run was kept constant on both occasions. It had been planned to explore the role of TN-C in older children with severe asthma and also the natural history of TN-C expression in the RBM of infants and children. However, subsequent immunohistochemical staining for TN-C expression in the RBM of paraffin embedded tissue from adult resected neoplastic lung tissue, and from endobronchial biopsies from older children with severe asthma failed on several occasions in 2009 and 2010. Endobronchial tissue archived in paraffin both before and after 2005 was used. The antibody initially used in 2007 had been used up. The experiment was repeated by Dr J Zhu using two further antibodies for TN-C from batches of mouse monoclonal antibody from the original source (T2H5 cat no. abcam ab3970) using the previously successful method, but still no TN-C staining was found on the previous positive controls of tonsillar tissue.

A second investigator, Mr T Oates also tried staining the paraffin embedded endobronchial tissue by changing reagents and the initial protocol in stages with no success. The changes to the initial experiment are listed below.

1. The first change made was to dewax the slides from Histoclear® through alcohol to PBS and this was done for all subsequent experiments.
2. After dewaxing sections an antigen retrieval step was added. The sections were microwaved in pre-heated 10mM sodium citrate (2.941g in 1 litre milliQ water) on 3 occasions for 3 minutes. After each 3 minutes of heating a small amount of citrate liquid was poured out and topped up.
3. The incubation period with mouse monoclonal antibody to tenascin-C (T2H5 ABCAM cat: 3970) was increased to 12 hours.
4. The sections were incubated with an avidin-biotin complex using biotinylated mouse IgG (Vectastain Immunoperoxidase Elite ABC kit Cat No. PK6102, Vector Laboratories) instead of labelled polymer HRP (Dako EnVision+ system, HRP, K4007).
After these changes to the original protocol failed, a second TN-C mouse antibody (Clone T2H5 Monosan, Cat No. MON7025) from a different manufacturer was used throughout all the following experiments (numbers 5-7).

5. After dewaxing the sections the slides were left for 1 hour in PBS containing 1% saponin to permeabilise the cell membranes. The same protocol was followed as in the original experiment except PBS containing 1% saponin was used throughout all incubations and washes. The slides were incubated with tenascin mouse antibody (Clone T2H5 Monosan, Cat No. MON7025) overnight.

6. Antigen retrieval was performed by incubating the sections with pronase (1mg/ml) at room temperature for 10 minutes (Sigma Aldrich, Cat No. 81748).

7. Finally, antigen retrieval was tried using Tris ethylenediaminetetraacetic acid (EDTA) after dewaxing slides. The sections were treated with pre-heated Tris-EDTA plus 0.05% Tween (pH 8.0) and heated for 4 times for 5 minutes topping up with more Tris-EDTA after each 5 minutes. Slides were left to cool for 20 minutes after treatment. This initially stained positive for tenascin-C on the tonsillar positive control with completely clear negative IgG controls, figure 4.13 (a). However on repeating the experiment 3 days later there was no tenascin-C staining of the same tonsillar controls, figure 4.13 (b). This suggests that TN-C monoclonal antibody is unreliable in paraffin embedded tissue.

Figure 4.13:
(a) Tenascin-C positive staining of the tonsillar tissue
Part of the difficulty in achieving consistent runs with the antibody may have been because the endobronchial tissue had been processed in paraffin. In order to compare TN-C measurement in paraffin sections and frozen tissue, 2 pieces of adjacent lung tissue from excised solid lung tumours (n=5) were immersed in formalin and preserved in paraffin, or snap frozen (-80C). The biopsies were snap frozen by submerging in thawing iso-pentane, which had previously been frozen solid in liquid nitrogen after mounting in optimum cutter (Bayer UK Ltd, United Kingdom) on a cork base. They were then stored at -80C. 5μm sections were cut from the frozen biopsies and were stained using the immunoperoxidase protocol (Vectastain Immunoperoxidase Elite ABC kit Cat No. PK6102, Vector Laboratories). The sections were incubated for 1 hour at room temperature with tenascin mouse antibody (Clone T2H5 Monosan, Cat No. MON7025). Three of the 4 sections with RBM stained strongly positive for TN-C despite no staining having been evident in repeated experiments where immediately adjacent tissue had been processed in paraffin.

The conclusion is TN–C staining using immunohistochemistry is not consistent in paraffin processed tissue. It is unclear despite repeated experiments whether this is due to length of tissue fixation or to a change in tenascin antibody since 2005. For this reason a decision was made not to proceed with TN-C in further paraffin embedded tissue sections.
4.10 Summary of findings

**Review of hypothesis**

School-aged children with asthma have evidence of airway remodelling and airway inflammation in endobronchial biopsies taken while symptomatic at preschool age, when compared to school-aged children without asthma.

*TN-C within the RBM was greater in preschool children with severe recurrent wheeze when compared with age matched controls, but did NOT discriminate which children developed future asthma.*

In the initial, successful staining runs:

- Measurement of TN-C thickness was both discriminative and repeatable using stereology techniques at magnification X1000. Higher magnification aided discrimination of TN-C positive areas within the RBM. TN-C staining in some biopsies extended from the RBM into the surrounding stroma making it difficult to quantify at lower magnification.
- TN-C expression within the RBM is increased in CW compared with controls, \( p=0.04 \)
- There was no difference in RBM TN-C at preschool age between children that developed asthma and those that did not.
- However, TN-C staining in paraffin embedded tissue was not repeatable in subsequent runs.

4.11 Discussion: RBM tenascin-C expression

**Strengths**

This is the first study to stain for TN-C in the RBM of preschool wheezers and to relate this to the presence or absence of school age asthma. Although RBM TN-C expression was increased in preschool confirmed wheezers when compared to preschool controls, \( p=0.04 \), it did not discriminate between those children who developed asthma at school age and those who did not develop asthma. This suggests that TN-C expression, similar to RBM thickness, may relate to current symptoms rather than future asthma prognosis. Importantly, there was no difference in age between wheezers and controls at the time of EB for this part of the study,
suggesting the differences in RBM TN-C expression directly related to wheezy symptoms. However, the reported thickness of TN-C within the RBM (6-7µm) measured in adult studies\(^{(143,144)}\) was significantly greater than in the preschool wheezers (2.35µm). There are three possible reasons for this; 1) different methodology used in adult studies, immunofluorescence in frozen sections rather than immunohistochemistry in paraffin sections\(^{(142-144)}\); 2) development of TN-C within the RBM continues throughout childhood similar to RBM development\(^{(113)}\); 3) longer duration of symptoms in adults when compared to preschool children. The data reported here are not able to distinguish between these possibilities.

This is the first study to relate TN-C within the RBM to total RBM thickness. Previous adult studies did not measure TN-C as a proportion of the RBM or relate TN-C expression in the RBM to its thickness\(^{(142-144)}\). The role of TN-C in contributing to RBM thickening is unknown. TN-C knockout mice sensitised with ovalbumin had decreased airway hyper-reactivity and airway goblet cells when compared with wild type mice\(^{(239)}\). However, other markers of airway remodelling such as peribronchiolar collagen deposition were not measured. Adult asthmatics showed no increase in collagen III (the predominant collagen in the RBM) but an increase in TN-C 24 hours post allergen challenge, but after 7 days the reverse was shown, TN-C levels had returned to baseline and collagen III levels significantly increased\(^{(238)}\). This suggests that increased RBM TN-C expression and RBM thickening may occur via different mechanisms.

**Limitations**

The biggest limitation of this study is that TN-C staining is not consistent in paraffin embedded tissue. Children in whom TN-C was quantified within the RBM, and who developed asthma were older at time of EB when compared to those who did not (42 vs 17.5 months). The natural history of TN-C expression in early childhood is unknown and it was not possible to characterise the relationship of RBM TN-C with age further because of difficulties with immunohistochemistry staining as previously described. TN-C is absent in adult lungs but present at birth\(^{(230)}\). It is likely that it is still present in the lungs during the alveolar phase (where terminal saccules, alveolar ducts, and alveoli increase in number), and it is not known when this is complete.
Although the control subjects in the current study (median 41%, range 0-92%) had significantly less TN-C within RBM when compared with CW (median 83%, range 12.6-100%), only 1 control child had no detectable RBM TN-C. The thickness of the RBM TN-C layer was significantly less in the RBM of controls compared to CW (0.77µm versus 2.35µm). Interestingly, there was no correlation between RBM TN-C and age in controls. This may have been because of the small number of patients (n=8), and the likely natural biological variability of TN-C expression in healthy controls. However, given the small sample size this study is not able to answer this question.

4.11.2 RBM TN-C and corticosteroid use

RBM TN expression was decreased in atopic adults with seasonal asthma after 4-6 weeks of treatment with inhaled budesonide (400µg) twice a day\(^{(144)}\). In addition, in vitro studies have shown fluticasone decreases TN-C expression in human lung fibroblasts\(^{(244)}\). In the present study, there was no difference in TN-C expression between wheezers treated with either inhaled or oral corticosteroids at time of endobronchial biopsy and those not on steroid treatment. However, information on length of treatment with inhaled corticosteroids and adherence to prescribed therapy, prior to endobronchial biopsy is not available, and it is not possible to draw any firm conclusions.

4.11.3 Conclusion

There is considerable overlap between total RBM thickness and RBM TN-C expression between preschool wheezers and controls, and neither total RBM thickness nor TN-C within the RBM are predictive of asthma at school age. It is possible that both RBM thickness and RBM TN-C are related to current symptoms rather than future asthma risk, and that neither will be useful in predicting outcome. Hence an alternative approach is needed.

Increased airway smooth muscle is another characteristic feature of airway remodelling in adults and older children with asthma but has not previously been explored in preschool children with severe recurrent wheeze. The next chapter will examine smooth muscle in preschool children and determine whether this feature of airway pathology is a more accurate predictor of school age asthma.
Chapter 5
Airway smooth muscle in preschool wheezers and the development of school age asthma

5.1. Introduction

ASM is now recognised as playing a pivotal role in the pathophysiology of asthma, in particular inducing airway narrowing (bronchoconstriction) in asthma after exposure to stimuli such as allergens, infection, exercise or environmental triggers (for example, cold air). The ASM cell is also pro-inflammatory and immunomodulatory by virtue of producing a variety of cytokines and chemokines and expression of cell surface receptors. Increased ASM is an established feature of chronic asthma in adults and school aged children. In particular, alterations in ASM are most consistently associated with abnormalities in lung function. Increased ASM in school aged children with asthma has been significantly related to reversible airflow obstruction and increased asthma symptoms. Bronchial thermoplasty, a therapy aimed to reduce ASM mass in the airways using thermal energy has been used in adult patients with severe asthma who continue to be symptomatic despite maximal medical treatment. Randomized controlled clinical trials of bronchial thermoplasty have suggested an improvement in quality of life and reduction in the rate of severe exacerbations in adults with severe asthma, but have not been able to show a reduction in AHR or change in FEV1.

5.1.2 Characteristics of increased airway smooth muscle in asthma

Increase in ASM mass in children and adults with asthma results both from smooth muscle hyperplasia and hypertrophy. Further ultrastructural analysis shows that the increase in bronchial muscle bundle mass in asthma is multifactorial and includes ASM cell hypertrophy, basal lamina thickening, increased extracellular matrix deposits, and collagen deposition within and around ASM bundles. However, not all authors agree that myocyte hypertrophy is present in all asthmatics and suggest it may only be a feature of increasing asthma severity. In a group of adults with mild-moderate asthma, Woodruff et al found that there was hyperplasia of airway smooth muscle myocytes, but that myocyte hypertrophy was absent.
However, Benyaoun et al suggested that the increase in ASM size was secondary to myocyte hypertrophy with the severest asthmatics having the largest myocyte size\textsuperscript{(110)}. Most recently it has been shown that hypertrophy occurs in adults with both non-fatal (history of asthma but died from other causes) and fatal cases of asthma, but hyperplasia is present predominantly in cases of fatal asthma and involves large and small airways\textsuperscript{(249)}. In school age children with moderate-severe asthma both hypertrophy and hyperplasia contributed to increased ASM\textsuperscript{(121)}. It is likely that increases in ASM mass are secondary to both cell hypertrophy and hyperplasia, and that this varies both with asthma duration, severity and phenotype.

5.1.3 Asthma duration and airway smooth muscle
There are conflicting reports of ASM and asthma duration. A post-mortem study showed that ASM was four fold greater in adults aged 40-49 years than age matched controls when compared to younger adults (17-23 years) with fatal asthma where it was 2 fold greater than age matched controls\textsuperscript{(123)}. However a more recent autopsy study in fatal and non-fatal asthma suggests that ASM thickness (indexed to the perimeter of the basement membrane) in adults with an asthma duration of 7-27 years was not related to duration of asthma, age of onset of asthma, sex or smoking\textsuperscript{(125)}. However, these studies measured ASM in different ways which may account for the disparity in their results.

5.1.4 Asthma severity and airway smooth muscle
ASM increases with severity of asthma, with the proportion of smooth muscle in the airway increasing with worsening airflow limitation\textsuperscript{(124;125)}. In adult asthmatics with intermittent, mild-moderate and severe asthma fibroblast numbers and ASM cell size were negatively associated with pre and post bronchodilator spirometry\textsuperscript{(110)}. The thickness of the ASM layer has also been associated with asthma severity as determined by oral corticosteroid use, reported asthma hospitalisations and frequency of symptoms\textsuperscript{(125)}.

5.1.5 Functional effects of increased airway smooth muscle
Increased airway smooth muscle mass in asthmatics is thought to generate increased ASM shortening velocity, leading to increased AHR\textsuperscript{(250)}. Muscle contraction is triggered by phosphorylation of myosin, catalyzed by calcium /
calmodulin-dependent myosin light chain kinase (MLCK), which in turn is activated as calcium increases. Airway smooth muscle cells from mild-moderate adult asthmatics isolated from endobronchial biopsies showed significant increases in maximum shortening capacity when compared with controls, which was associated with increases in smooth muscle types of MCLK\textsuperscript{(251)}. Myocyte hypertrophy was also associated with an increase MCLK in adults with asthma\textsuperscript{(110)}, which was further amplified in patients with severe persistent asthma\textsuperscript{(110)}. Contractile proteins, associated with increased ASM shortening velocity, including myosin isoforms, transgelin and MCLK are also increased in endobronchial biopsies from adults with mild asthma\textsuperscript{(252)}. However, in contrast there was no evidence of increased gene expression levels of contractile proteins, MCLK or α-smooth muscle actin in adults with mild-moderate asthma with ASM hyperplasia but not hypertrophy\textsuperscript{(122)}. This may suggest that variations in gene expression levels of contractile proteins considered markers of a hypercontractile phenotype may vary with disease severity and duration.

5.1.6 Smooth muscle signalling pathways

Early research studies regarding ASM function in asthma focussed on its contractile properties, however a growing body of evidence suggests the ASM cell also displays proinflammatory and immunomodulatory functions\textsuperscript{(246)}. Smooth muscle may contribute to bronchial inflammation by secreting a range of mediators, recruiting and activating inflammatory cells, such as mast cells, T lymphocytes or eosinophils in response to a variety of triggers, including allergens, viruses or bacteria\textsuperscript{(245;246)}. In particular ASM cells participate in the inflammatory and remodelling process by expression of cellular adhesion molecules, receptors for cytokines (e.g., TNF-α), chemokines (RANTES, eotaxin, macrophage inflammatory protein 1a and IL-8), and Toll-like receptors\textsuperscript{(246)}.

Recent evidence suggests that airway constriction itself promotes remodelling, presumably via effects on ASM\textsuperscript{(70)}. Endobronchial biopsies from adult asthmatics showed similar increases in collagen III deposition in the RBM and epithelial TGF-β expression 4 days after methacholine (not pro-inflammatory) challenges and allergen (pro-inflammatory) challenges\textsuperscript{(70)}. ASM cells can release biologically active TGF-β, which is involved in various structural alterations such as epithelial cell apoptosis.
resulting in the detachment of epithelial cells, increased RBM thickness, mucus hypersecretion, goblet cell hyperplasia, and angiogenesis\(^{(253)}\). TGF-\(\beta\) is increased in the airways of patients with severe asthma compared with patients with less severe disease\(^{(253)}\).

### 5.1.7 Airway smooth muscle cell migration

Cell migration could be partly responsible for the pathogenesis of ASM cell hyperplasia and hypertrophy in asthma. TGF-\(\beta\) in the presence of platelet-derived growth factor, upregulates the expression of MMPs and TIMPs in ASM cells, thereby enhancing ASM cell migration toward the epithelium to form new bundles\(^{(254)}\).

### 5.1.8 Development of airway smooth muscle

Smooth-muscle cells are present in the human fetal trachea, primary, and lobar bronchi by the 6\(^{th}\)-8\(^{th}\) week of gestation\(^{(255)}\) and ASM can be identified by light microscopy in the small bronchi of fetal airways from 24 weeks gestation\(^{(256)}\). Early in gestation ASM becomes innervated and responsive to contractile and relaxing stimuli, where changes in intraluminal pressure are suggested to produce a mechanical signal, resulting in the release of growth factors, signalling molecules, or the regulation of gene expression\(^{(255)}\). It has been suggested that in particular ASM helps regulate early lung morphogenesis by fibroblast growth factor 10 production necessary for branching morphogenesis and pulmonary differentiation\(^{(257)}\). However, little is known about the natural history of ASM postnatally. A single post-mortem study measured ASM in the lungs from infants (\(n=20\)) without cardiopulmonary disease, who died aged 22 weeks postconceptional age to 8 months postnatal age and showed a linear increase in ASM when related to airway size\(^{(256)}\).

### 5.1.9 Airway smooth muscle in preschool wheezers

Although ASM has been extensively investigated in adult asthmatics, little is known about its role in children with asthma. Children with asthma as young as 7 years have evidence of increased ASM\(^{(121)}\), but nothing is known about ASM in preschool wheezers. Therefore, the next hypothesis developed in this thesis is that increased ASM may already be present in severe recurrent preschool wheezers and particularly in those children who go on to develop asthma at school age. This chapter will compare ASM in endobronchial biopsies at preschool age from severe
recurrent wheezers to age matched controls, and relate preschool ASM to school age asthma status.

5.1.10 Hypotheses
The overarching hypotheses of this thesis are:

1. School-aged children with asthma have evidence of airway remodelling and airway inflammation in endobronchial biopsies taken at preschool age when compared to school-aged children without asthma.

2. A combination of clinical and airway pathological characteristics at preschool age may be able to be used to predict asthma at school age, better than clinical features alone.

In this chapter, the contribution of ASM to remodelling and prediction of future asthma will be explored.

5.1.11 Aims
1. To quantify ASM in endobronchial biopsies from wheezy preschool children
2. To relate ASM at preschool age to the presence of asthma at school age in the Royal Brompton cohort

5.2 Methods

5.2.1 Characteristics of preschool wheezers
The clinical characteristics of preschool wheezers recruited from the Royal Brompton Hospital have been discussed in detail in chapter 2. Children had severe recurrent noisy breathing which had been assessed as wheeze during acute symptoms or from parental reports. Children were divided into confirmed wheezers (CW) and reported wheezers (RW) on the basis of parental identification of wheeze on video questionnaire. Exclusion criteria were children with isolated cough, those whose main problems were recurrent lower respiratory tract infections, chronic lung disease of prematurity and a history of long term oxygen therapy. Use of inhaled or oral steroids was not an exclusion criterion. Non-wheezing controls were children who mainly had bronchoscopy for evaluation of upper airway problems including stridor.
5.2.2 Evaluable biopsies
Details of the flexible bronchoscopy and EB processing are reported in chapter 2 and 3. Children with total area of evaluable biopsy tissue ≤0.2mm\(^2\) were excluded. Smooth muscle was quantified in biopsies fulfilling the following criteria:

i. presence of recognisable epithelium, RBM, subepithelial tissue and smooth muscle

ii. minimal crush artefact, oedema or blood within the biopsy, figure 5.1.

Figure 5.1: An evaluable biopsy for assessment of airway smooth muscle: endobronchial biopsy section stained with haematoxylin and eosin from a preschool child with recognisable epithelium, subepithelium, reticular basement membrane and smooth muscle.

![Biopsy section](image)

5.2.3 Measurement of airway smooth muscle
The area below the RBM and epithelium is referred to as the subepithelium and includes both ASM, mucosa (lamina propria) and submucosal glands (if present). Lamina propria is recognised as a highly cellular loose connective tissue, located beneath the RBM. Smooth muscle cells were recognised by their characteristic spindle shape of variable size with a longitudinal orientation and a centrally placed...
nucleus per cell. Clusters of smooth muscle cells are organised as smooth muscle bundles. ASM was measured in two ways.

Method 1:
Areas of ASM and subepithelial area were identified by morphologic examination. ASM area and total subepithelial area were measured at magnification x100 by drawing around the outer limits of the smooth muscle bundles and the subepithelial area of the biopsy\(^{221}\), figure 5.2. The subepithelial and ASM area was then calculated using computer aided image analysis (Leica DM2500 microscope, Leica DFC 300fx camera and Leica Qwin version 3 software, Olympus BH2, Japan). ASM mass was then expressed as a fraction of the total subepithelial area.

Figure 5.2: Smooth muscle bundles were traced using a computer mouse and then the area was calculated using computer aided analysis. (Scale not shown—image shown for illustration purposes)
Method 2:
ASM was measured using a stereology technique, point and line intersection counting, allowing three dimensional information from measurements in the two dimensions of conventional histology\(^{(85)}\). This is done by using a multipurpose test system, a Weibel M168 grid which combines area, point and lines which has the advantage of allowing a large amount of information to be collected from a single grid, figure 5.3\(^{(258)}\).

**Figure 5.3:** Smooth muscle volume fraction was quantified by overlaying a Weibel grid (M168) at x 200 magnification over the section and performing point counting. Length between 2 points (68µm).

A virtual Weibel (M168) grid was overlaid the section at random and moved systematically until the whole section had been measured. Point counting was used to measure ASM. Each end of the horizontal lines on the grid represented a point. A point was recorded as smooth muscle if it entirely fell on smooth muscle. Points falling on the total subepithelial area (submucosa, ASM and glands) were also recorded but those falling on empty space were not. ASM volume fraction was measured at magnification x200 and expressed as a fraction of the total number of points falling on smooth muscle over the total number of points falling on the subepithelium\(^{(118;121)}\). The formula used to calculate this was:

\[
V_v (\text{sm/subepithelium}) = \frac{(\Sigma \text{ points on ASM})}{(\Sigma \text{ points on subepithelial tissue})}.
\]
As there was considerable variation in biopsies between children, the hypothesis was that relating ASM to the RBM surface area may reduce variability, rather than indexing ASM to total subepithelial area. ASM volume fraction was indexed to the surface area of the RBM by counting the number of lines intersecting the apical surface of the RBM. The line had to completely cross the RBM to be counted. Lines that only partly crossed the RBM were not counted\(^{(121)}\). The following formula was used:

volume fraction of ASM indexed to surface area of RBM: \( V/S \text{ (sm/rbm)} = \frac{\sum \text{points on ASM} \times l(p)}{2 \times \sum \text{line intersections with RBM}} \) where \( l(p) \) denotes the length per point (68µm).

**Inter-observer repeatability**

ASM area-fraction was measured by a second observer (Dr N Ullmann) blinded to clinical and pathological details of the children using method 1. Measurements for ASM area-fraction by the 2 observers are represented by a Bland-Altman plot.

### 5.3 Assessment of smooth muscle proliferation

Children who had remaining EB tissue had anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody applied to paraffin sections to identify proliferating smooth muscle cells by Mr Tim Oates\(^{(118)}\). Paraffin sections were dewaxed in xylene and endogenous peroxidase activity was quenched by immersing sections in 3% \( \text{H}_2\text{O}_2 \) for 30 mins at room temperature. Sections were then incubated with primary mouse anti PCNA (Dako) followed by incubation with biotinylated mouse IgG (Vector Laboratories). Slides were counterstained with H&E. All nucleated PCNA positive smooth muscle cells were enumerated and expressed per mm\(^2\) smooth muscle\(^{(118)}\).

**Statistical analysis**

Data was analysed on a per individual as opposed to a per biopsy basis, e.g. the sum of all measurements obtained from all biopsies of a given child was taken as the value for this child\(^{(121)}\). Non parametric tests were used. Sample size was opportunistic as the number of children in this group was predetermined based on the cohort of preschool children recruited between 2002 and 2005. Any power calculations performed are retrospective and of limited value. However, power calculations showed that the groups (wheezers (n=28) and controls (n=14)) had 80%
power to detect a difference between means of 0.06 ASM area-fraction or ASM volume fraction with a significance level of p<0.05 (two-tailed t test).

5.4.1 Biopsy size and quantity in Brompton preschool wheezers
At least 1 good quality biopsy was available from 56 patients. 42/68 children were included in the preschool ASM analysis. Forty children had 1 evaluable biopsy and 2 children had 2 evaluable biopsies. Reasons for exclusion were: no evaluable biopsy (n=12), no identifiable ASM (n=7) and total cumulative biopsy area < 0.2mm$^2$ (n=7, 1 control and 6 wheezers), figure 5.4

Figure 5.4: Airway smooth muscle biopsy quantity

There was no difference between children with ASM on endobronchial biopsy and those without ASM in EB in terms of age at fibreoptic bronchoscopy, sex, preschool wheeze status and IgE at the time of EB, table 5.1. Similarly there was no difference between those children with ASM in EB who were followed up and the rest of the preschool cohort, table 5.2.
Table 5.1: No difference in clinical characteristics of children with and without ASM in evaluable endobronchial biopsies

<table>
<thead>
<tr>
<th></th>
<th>ASM n=49</th>
<th>No ASM n=7</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at biopsy</strong></td>
<td>19(3-58)</td>
<td>13(6-55)</td>
<td>0.39</td>
</tr>
<tr>
<td>Male/ Female</td>
<td>31/18</td>
<td>3/4</td>
<td>0.42</td>
</tr>
<tr>
<td>Wheezers/ Controls</td>
<td>34/15</td>
<td>4/3</td>
<td>0.67</td>
</tr>
<tr>
<td>Preschool IgE (IU/ml)</td>
<td>16 (1-635)†</td>
<td>17 (2-559)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*Data is reported as median and range. †Results not available on 3 children

Table 5.2: No difference in clinical characteristics of children followed up at school age with ASM in endobronchial biopsy and those who were either lost to follow up or did not have ASM in endobronchial biopsy

<table>
<thead>
<tr>
<th></th>
<th>Followed up n=32</th>
<th>Lost to follow up n=36</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at biopsy</strong></td>
<td>18.5 (3-57)</td>
<td>20 (3-58)</td>
<td>0.5</td>
</tr>
<tr>
<td>Male Female</td>
<td>19/13</td>
<td>25/11</td>
<td>0.45</td>
</tr>
<tr>
<td>Wheezers/ Controls</td>
<td>21/11</td>
<td>26/10</td>
<td>0.6</td>
</tr>
<tr>
<td>Preschool IgE (IU/ml)</td>
<td>28.5 (1-635)†</td>
<td>17 (1-2605) ‡</td>
<td>0.99</td>
</tr>
</tbody>
</table>

*Data is reported as median and range. †Results not available on 2 children ‡Results not available on 3 children

5.4.2 Repeatability of stereology and computer aided analysis methods

The CoV was calculated for ASM volume fraction and ASM area fraction measurement as a measure of variability. Each method was assessed by measuring 4 biopsies at 3 intervals with the following 2 measurements a week and a month later. The mean CoV for 4 biopsies measured using computer aided image analysis was 7.5% (3.6%, 6.1%, 9.6% and 10.8%). The mean coefficient of variation for ASM measured using stereology was higher at 15% (2.9%, 15.8%, 18% and 23.8%). The higher CoV associated with the stereology technique is probably because the average number of points counted per patient was low (median 79 (range 28-276)). ASM area-fraction was measured by a second observer showing good agreement between observers (Bland Altman plot, bias 0.0074, SD +/- 0.037, (-0.066-0.081)) figure 5.5. The mean CoV for V/S (ASM indexed RBM surface area) was very poor 121% (50.7%, 76.2%, 209.4%, 148.9%). The mean number of line intersections with
the RBM per section was low 3.85 (standard deviation +/-2.7) for all biopsies measured.

Figure 5.5: A Bland Altman plot showed good repeatability of ASM area-fraction measurements between observer 1 and observer 2

5.4.3 Comparing ASM volume-fraction measured using stereology and ASM area-fraction measured using computer aided image analysis
There was good correlation between ASM volume fraction measured using stereology and ASM area-fraction measured using computer aided analysis, Spearman r=0.75, p=0.001, figure 5.6. The ICC using computer aided analysis and point and line counting method to measure ASM was 0.78.

Figure 5.6: Correlation between measurements of airway smooth muscle volume fraction and airway smooth muscle area-fraction

Spearman r=0.75, p=0.001
5.4.4 Variability of ASM within and between biopsies

Fourteen children had 3 or more sections measured from the same biopsy. The within biopsy CoV for ASM was very high both for method 1 (ASM area fraction measured using computer aided analysis, median 37% (range 4-70%) and for method 2 (ASM volume fraction measured using stereology, median 57% (range 5-79%)). Within biopsy variability for the 12 children who had 2 sections measured by both methods is represented by a Bland Altman plot for method 1, figure 5.7 (i) and method 2, figure 5.7 (ii). Between biopsy variability is described for the 2 children who had two biopsies measured (ASM area-fraction (child 1; biopsy 1= 0.112, biopsy 2= 0.102) and (child 2; biopsy 1=0.1246, biopsy 2=0.08)).

Figure 5.7: Wide ASM variability within biopsy represented by a Bland Altman plot for (i) method 1 (ASM area fraction) and (ii) method 2 (ASM volume fraction). There is no significant bias.
5.4.5 Airway smooth muscle area fraction measured using computer aided analysis

There was no difference in ASM area fraction between preschool wheezers (n=28, median 0.08 (range 0.038-0.163)mm$^2$) and controls (n=14, median 0.077 (range 0.02-0.23)mm$^2$), $p=0.96$, figure 5.8 (i). Similarly there was no difference in ASM area fraction between CW (n=15, median 0.096 (range 0.04-0.16)mm$^2$), RW (n=13, median 0.075 (range 0.04-0.16)mm$^2$) and controls (n=14, median 0.077 (range 0.02-0.23)mm$^2$), $p=0.32$, figure 5.8 (ii).

Figure 5.8: Airway smooth muscle area fraction in endobronchial biopsies measured using computer aided image analysis compared between (i) wheezers and controls and (ii) confirmed and reported wheezers and controls

(i) $p=0.96$

(ii) $p=0.32$

No difference in airway smooth muscle between preschool wheezers and controls

No difference in airway smooth muscle between confirmed and reported wheezers, and controls
5.4.6 Airway smooth muscle measured using stereology techniques

There was no difference in ASM volume fraction between preschool wheezers (n=28, median 0.1088 (range (0.018-0.27)) and controls (n=14, median 0.099 (range 0.016-0.314)mm²), p=0.6, figure 5.9 (i). Similarly there was no difference in ASM volume fraction between CW (n=15, median 0.11 (range 0.018-0.27)), RW (n=13, median 0.076 (range 0.059-0.23)) and controls (n=14, median 0.099 (range 0.016-0.314)), p=0.77, figure 5.9 (ii).

Figure 5.9: Airway smooth muscle volume fraction in endobronchial biopsies measured using stereology compared between (i) wheezers and controls and (ii) confirmed wheezers, reported wheezers and controls

(i)

(ii)
5.4.7 Volume fraction of ASM indexed to the surface area of RBM

The volume fraction of ASM indexed to surface area of RBM (V/S) was also compared between wheezers (n=28, median 34.5 (range 4.25-340)) and controls (n=14, median 45.9 (range 5.7-374)), p=0.25, figure 5.10 (i). There was also no difference between subgroups, confirmed wheezers (n=15 median 34.95 (range 4.25-136)), reported wheezers (n=13, median 34, (range 17-340)) and controls, p=0.19, figure 5.10 (ii). There was also no correlation between RBM thickness and either ASM area fraction or ASM volume fraction.

Figure 5.10: No difference in volume fraction of airway smooth muscle indexed to surface area of reticular basement membrane between (i) preschool wheezers and controls and (ii) confirmed and reported wheezers and controls.
Table 5.3: Characteristics of preschool children with airway smooth muscle in endobronchial biopsy

<table>
<thead>
<tr>
<th></th>
<th>Wheezers (n=28)</th>
<th>Controls (n=14)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (months)</td>
<td>25.5 (7-58)</td>
<td>17.5 (4-42)</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(CW=31 (8-58) / RW=17 (7-57))</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Male/ Female</td>
<td>16/12</td>
<td>10/4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>(CW= 8/7 / RW= 9/4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhaled corticosteroids* (BDP (mcg)*)</td>
<td>14 (50%) (500 (200-2000))</td>
<td>2 (14%) 500</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>CW= 8 (53%) / RW=6 (46%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(CW 800 (200-2000) / RW 400 (200-1000))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biopsy area (mm²)</td>
<td>0.49 (0.26-1.27)</td>
<td>0.66 (0.27-1.54)</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>(CW=0.5 (0.29-1.15) / RW=0.49 (0.26-1.27))</td>
<td></td>
<td>0.52</td>
</tr>
<tr>
<td>ASM area-fraction</td>
<td>0.08 (0.038-0.163)</td>
<td>0.077 (0.02-0.23)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>(CW=0.096 (0.04-0.16) / RW=0.075 (0.04-0.16))</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>ASM volume-fraction</td>
<td>0.1088 (0.018-0.27)</td>
<td>0.099 (0.016-0.314)</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>(CW=0.11 (0.018-0.27) / RW=0.076 (0.059-0.23))</td>
<td></td>
<td>0.77</td>
</tr>
<tr>
<td>V/S</td>
<td>34.5 (4.25-340)</td>
<td>45.9 (5.7-374)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>(CW=34.95(4.25-136) / RW= 34(17-340))</td>
<td></td>
<td>0.19</td>
</tr>
</tbody>
</table>

All data expressed as median and range.
CW= Confirmed preschool wheezers
RW= Reported preschool wheezers
*BDP= Beclometasone dipropionate or equivalent
V/S volume fraction of smooth muscle indexed to surface area of reticular basement membrane

5.5 Atopic status and airway smooth muscle in preschool children

There was no correlation between ASM and total serum IgE at preschool age (Spearman r=-0.08, p=0.62), figure 5.11. There was also no difference in ASM between children who had one or more positive aeroallergens and children who were not sensitised to any aeroallergens, figure 5.12. Analysis of ASM volume fraction showed similar results.
Figure 5.11: No correlation between ASM area fraction and total preschool IgE in preschool wheezers

\[ r = -0.08, \ p = 0.62 \]

No correlation between airway smooth muscle and total preschool IgE

Figure 5.12: No difference in ASM area fraction in endobronchial biopsies from children sensitised to one or more positive aeroallergens compared with children not sensitised to any aeroallergens

\[ p = 0.15 \]

No increase in ASM in children sensitized to one or more aeroallergens at preschool age
5.6 Airway smooth muscle in different preschool wheeze phenotypes

Children were divided into clinical wheeze phenotypes viral (episodic) or multiple-trigger wheezers at preschool age\(^{61}\). Thirty four children had ASM in their biopsy, of these 6 were excluded because the biopsy size was less than 0.2mm\(^2\). There was no difference in ASM area fraction between the 2 phenotypes, viral (n=5, median ASM 0.096 (range 0.056-0.163)) and multiple-trigger (n=23, median ASM 0.077 (range 0.038-0.156)), p=0.33, figure 5.13. However, numbers of children with episodic or viral wheeze are small. Similar results were shown for ASM volume fraction.

Figure 5.13: Comparing airway smooth muscle area fraction in preschool children with viral and multiple-trigger wheeze

\[
p=0.33
\]

5.7 Inhaled corticosteroids and airway smooth muscle

Almost all of the preschool wheezers had a trial of ICS of varying duration before proceeding to bronchoscopy and endobronchial biopsy. There was no difference in ASM area fraction between those prescribed ICS (n=13, median 0.096 (range 0.039-0.156)) at the time of EB and those who not prescribed ICS (n=15, median 0.069 (range 0.038-0.163)), p=0.40, figure 5.14. Six of the children on daily ICS and 5 of the children not on ICS had been treated with oral corticosteroids in the month prior to biopsy. However there was still no difference when this was considered: (n=18, ICS and / or oral corticosteroids in the last month) and (n=10, no ICS / oral corticosteroids), p=0.48.
Figure 5.14: No difference in airway smooth muscle area-fraction compared between preschool wheezers on inhaled corticosteroids and those not on inhaled corticosteroids

5.8 School age asthma and preschool airway smooth muscle

Of 51 children followed up at school age, 38 children had ASM in their EB. Six children were excluded as they had a biopsy area of less than 0.2mm². The clinical characteristics of children with ASM in their endobronchial biopsy at preschool age and who were followed up at school age are reported in table 5.4.

Children with asthma at school age had increased ASM area fraction (n=8, median 0.12, (range 0.08-0.16)) when compared to those without (n=24, median 0.066, (range 0.024-0.23)), p=0.007, figure 5.15 (i). These findings were confirmed with increased ASM volume fraction in children with school age asthma (n=8, median 0.153, (range 0.094-0.266)) when compared with those without school age asthma (n=24, median 0.097, range (0.02-0.314)), p=0.016, figure 5.15 (ii). However, there was no relationship between ASM indexed to the surface area of the RBM between children who did and did not develop asthma at school age, p=0.15.
Figure 5.15: Increased preschool airway smooth muscle in children with asthma at school age measured using (i) area fraction (computer aided image analysis) and (ii) volume fraction (stereology point counting techniques).

When preschool wheezers were considered alone, those who developed asthma had increased ASM area-fraction ($n=7$, median 0.127, [range 0.083-0.156]) when compared with those who did not develop asthma ($n=14$, median 0.058, [range 0.038-0.163]), $p=0.006$, figure 5.16 (i). Similarly ASM volume fraction was increased in children with school age asthma ($n=7$, median 0.16, [range 0.094-0.266]) when compared with those who did not develop asthma ($n=14$, median 0.08, [range 0.033-0.227]), $p=0.006$, figure 5.16 (ii).

Figure 5.16: Increased preschool airway smooth muscle in preschool wheezers alone classified by the presence or absence of asthma at school age (i) area fraction and (ii) volume fraction.
Table 5.4: Clinical characteristics of children with ASM in endobronchial biopsy related to school age asthma status. (Children with asthma at school age had increased ASM, were younger at time of biopsy and had higher preschool IgE.)

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male/ Female</strong></td>
<td>5/3</td>
<td>10/14</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Age at FOB, months, median (range)</strong></td>
<td>45 (3-57)</td>
<td>17 (6-55)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Weight at FOB (kg)</strong></td>
<td>14.75 (5.6-25)</td>
<td>11.25 (7-18.7)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Birth Weight, kg, median (range)</strong></td>
<td>3.49 (2.72-3.8)</td>
<td>3.5 (2.55-4.99)</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>Gestation, weeks, median (range)</strong></td>
<td>40 (33-42)</td>
<td>40 (35-42)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Preschool group**

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confirmed wheezer</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Reported wheezer</td>
<td>1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>10</td>
<td></td>
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</tbody>
</table>

**Preschool wheeze phenotype**

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Multiple trigger</td>
<td>7</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><strong>Age at onset, months median (range)</strong></td>
<td>15.5 (3-24)</td>
<td>7 (0.5-66)</td>
<td>0.15</td>
</tr>
<tr>
<td>Admission to hospital</td>
<td>7</td>
<td>10/14</td>
<td></td>
</tr>
</tbody>
</table>

**Preschool inhaled/ Oral steroids, n (%)**

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental history of asthma</td>
<td>4</td>
<td>7*</td>
<td>0.41</td>
</tr>
<tr>
<td>Maternal asthma</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Paternal asthma</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Environment**

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal smoking in pregnancy</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Maternal / paternal smoking</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Day care &lt; 1 year</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Pets (Cat/ Dog)</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

**Preschool IgE (IU/ml)**

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1 positive RAST, n (%)</td>
<td>5 (82.5)</td>
<td>6* (25)</td>
<td>0.1</td>
</tr>
<tr>
<td>≥1 positive RAST aero-allergen, n (%)</td>
<td>3 (37.5)</td>
<td>3 (13.6)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Preschool eczema, n (%)**

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preschool eczema, n (%)</td>
<td>6 (75)</td>
<td>6 (25)</td>
<td></td>
</tr>
</tbody>
</table>

**Preschool ASM measurements**

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ASM area fraction</strong></td>
<td>0.12 (0.08-0.16)</td>
<td>0.066 (0.024-0.23)</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>Median biopsy area mm²</strong></td>
<td>0.81 (0.35-1.27)</td>
<td>0.52 (0.26-1.54)</td>
<td>0.043</td>
</tr>
<tr>
<td><strong>V/v – sm/ subepithelium</strong></td>
<td>0.153 (0.094-0.266)</td>
<td>0.097 (0.02-0.314)</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>V/S</strong></td>
<td>73.45 (28.3-127.7)</td>
<td>34 (9.3-374)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

**Clinical details at school age**

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frecuency of wheeze</strong></td>
<td>None</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>1-3 attacks of wheezing</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>4-12 attacks of wheezing</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&gt;12 attacks of wheezing</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Exercise induced wheeze</strong></td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Dry cough at night§</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Number of hospital admissions with wheeze</td>
<td>10 (1-160)</td>
<td>6 (0-20)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Asthma (n=8)</th>
<th>No asthma (n=24)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of children taking ICS at school age</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Dose of ICS (mcg)**</td>
<td>800 (400-1000)</td>
<td>400 (200-400)</td>
<td></td>
</tr>
<tr>
<td>Immunomodulating agent</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Family history unknown in 2 children, #IGE or Specific RASTs not known in 2 children
**mcg Budesonide or equivalent, §Dry cough at night apart from a cough associated with a cold or chest infection, V/v volume fraction of smooth muscle indexed to subepithelium
V/S volume fraction of smooth muscle indexed to surface area of reticular basement membrane
5.9 Age & preschool airway smooth muscle

When considering just children with ASM in EB, children with school age asthma (median 45 (range 3-57) months) were older than those who did not have school age asthma (median 17 (range 6-55) months) at time of biopsy, p=0.01. There was no relationship between age and ASM volume-fraction or ASM area-fraction in the controls, but the numbers were small.

To assess the relationship between ASM and age further, endobronchial biopsies from non-wheezing school-aged children had ASM volume fraction measured using point counting by a second investigator CB\textsuperscript{(119)}. These children had been recruited as controls for a study investigating airway remodelling and inflammation in children with severe therapy resistant asthma\textsuperscript{(119)}. Children (n=13) median age 10.5, range (5.9-14.2) years had clinically indicated bronchoscopy for evaluation of recurrent croup or a barking cough (n=6), noisy breathing (n=1), stridor (n=2) and shortness of breath (n=1). Three children had bronchoscopy for investigation of haemoptysis but had normal anatomical appearances on bronchoscopy. All parents consented to EB for research purposes. Biopsies were processed and stained in an identical manner to the preschool EBs. There was no correlation between age and ASM volume fraction in non-wheezing preschool children (n=14) and school age children (n=13), aged (0.25-14.2) years, Spearman r=0.027, p=0.89, figure 5.17.

**Figure 5.17: No relationship between age and ASM volume fraction in non-wheezing controls**
5.10 Airway smooth muscle hyperplasia

ASM proliferation was assessed by myocyte proliferating cell nuclear antigen in children with remaining EB tissue, and no difference was seen between wheezers (n=12) and controls (n=3) or those with school age asthma (n=3) and those without school age asthma (n=7), figure 5.18.

Figure 5.18: No difference in smooth muscle proliferation between wheezers and controls (figure A); No difference in smooth muscle proliferation between children with and without school age asthma (figure B).

A

B
5.11 Can airway smooth muscle in endobronchial biopsies from preschool wheezers help predict school age asthma?

The receiver operator curve (ROC) evaluates the discriminatory power of a test, in this case using ASM in endobronchial biopsies at preschool age to discriminate between preschool wheezers who developed school age asthma and those whose symptoms resolved. An area under the curve (AUC) of 1 would represent 100% sensitivity (no false negatives) and 100% specificity (no false positives). The area under the ROC curve in this case is 0.88 (standard error 0.08, CI (0.69-0.97), p=0.005) for ASM area fraction, figure 5.19, and similarly the AUC was 0.79 (standard error 0.08, CI (0.61-0.91), p=0.0008) for ASM volume fraction, figure 5.20. The ROC curve being based on sensitivity and specificity does not take any account of asthma prevalence. The PPV of ASM area fraction >0.1 at preschool age in preschool wheezers for asthma at school age was 75% (35-96%) and NPV was 92% (64-99%), table 5.5.

Figure 5.19: Area under the receiver-operator characteristics curve for airway smooth muscle area fraction (measured using computer aided analysis) in preschool wheezers in the diagnosis of future school age asthma was 0.88 (standard error 0.08, confidence interval (0.69-0.97)), p=0.005. (Confidence intervals are represented by broken line, area under the curve by the continuous line)
Figure 5.20: Receiver operator characteristics curve for airway smooth muscle volume fraction (measured using point counting) in preschool wheezers related to school age asthma (area under the curve 0.79, standard error 0.08, confidence intervals 0.61-0.91, p=0.0008). (Confidence intervals are represented by broken line, area under the curve by the continuous line)

Asthma predictive indexes

Preschool ASM was compared to two other asthma predictive indexes, the Castro-Rodriguez ‘stringent index’(27) and a wheeze severity score(79). Clinical data at preschool age was used to classify preschool wheezers using the Castro-Rodriguez stringent index, frequent wheeze plus 1 major or 2 minor criteria (major criteria; parental doctor diagnosed asthma or doctor diagnosed eczema in the child, minor criteria; wheezing apart from colds or peripheral blood eosinophilia)(27). A history of doctor diagnosed allergic rhinitis had not been recorded so wheezing apart from colds or peripheral blood eosinophilia were used as the minor criteria. All children had at least 3 episodes of wheeze at the time of EB, however the exact number was not recorded. For application of the wheeze severity score 3-4 episodes of wheeze plus the number of hospital admissions was used to approximate the score(79). Both clinical predictive indexes were compared with current asthma at school age. Of the children followed up at school age (37/47), data was available to apply the Castro-Rodriguez ‘stringent predictive index’ to 34 children, 22 satisfied the criteria (major (n=21) and minor (n=1))(27). This index did not significantly predict the development of asthma in this group of preschool wheezers, p=0.27, table 5.5. When the wheeze
severity score (wheeze frequency and hospital admissions) was compared with current asthma at school age, the area under the curve (AUC) was 0.70 (standard error 0.08, [CI 0.53-0.84], p=0.01), figure 5.21, and in this group of preschool wheezers a score >6 was predictive of asthma\(^{(79)}\). There was no significant difference between the AUC for ASM or for the wheeze severity score, p=0.13. As the study number was small all predictive indices and ROC curves have very wide confidence intervals.

**Figure 5.21:** Receiver operator characteristics curve for wheeze severity score related to school age asthma (area under the curve 0.7, standard error 0.08, CI 0.53-0.84, p=0.01). (Confidence intervals are represented by broken line, area under the curve by the continuous line)

![AUC=0.70](image)

**Table 5.5:** Sensitivity, specificity, positive and negative predictive values and likelihood ratios for a proportion of endobronchial biopsy greater than 0.1 ASM area-fraction, Castro-Rodriguez ‘stringent index’ and Devulapalli wheeze severity score in preschool wheezers

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
<th>p</th>
<th>Likelihood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASM area-fraction &gt; 0.1</td>
<td>86 (42-99)</td>
<td>86 (57-98)</td>
<td>92 (64-100)</td>
<td>75 (35-97)</td>
<td>0.003</td>
<td>6</td>
</tr>
<tr>
<td>Stringent index</td>
<td>79 (49-95)</td>
<td>45 (23-69)</td>
<td>50 (28-72)</td>
<td>75 (49-94)</td>
<td>0.27</td>
<td>1.6</td>
</tr>
<tr>
<td>Severity score &gt;6</td>
<td>60 (32-84)</td>
<td>77 (55-92)</td>
<td>64 (35-87)</td>
<td>74 (52-90)</td>
<td>0.03</td>
<td>2.6</td>
</tr>
</tbody>
</table>

*ASM measured using computer aided analysis as a proportion of the total subepithelial area*
5.12 Preschool ASM and lung function at school age

There was no correlation between preschool ASM area fraction and school age lung function; LCI (n=28, Spearman $r=0.12$, $p=0.54$), $S_{\text{acin}}$ (n=23, Spearman $r=0.04$, $p=0.87$), $S_{\text{cond}}$ (n=23, Spearman $r=-0.1$, $p=0.63$), FEV$_1$ z score (n=28, Spearman $r=-0.12$, $p=0.53$), FVC z score (n=28, Spearman $r=-0.18$, $p=0.35$), FEV$_1$/FVC (n=28, Spearman $r=0.09$, $p=0.35$). Similar results were seen for ASM volume fraction. When the preschool wheezers were considered alone there was a positive correlation between school age FeNO$_{50}$ and both preschool ASM area fraction (Spearman $r=0.58$, $p=0.01$), figure 5.22 (i), and ASM volume fraction (Spearman $r=0.44$, $p=0.08$). There was also a positive correlation between FEV$_1$/FVC and both ASM area fraction (Spearman $r=0.52$, $p=0.03$), figure 5.22 (ii) and ASM volume fraction (Spearman $r=0.49$, $p=0.04$).

Figure 5.22: Positive correlation between (i) FeNO$_{50}$ and (ii) FEV$_1$/FVC and ASM area fraction
Eighteen children with ASM in endobronchial biopsy had assessment of AHR using hypertonic saline (defined as positive airway challenge if there was a decrease in FEV₁ ≥ 15%). A further child with ASM on EB and lung function less than 75% had assessment of BDR (≥15% increase in FEV₁ 20 minutes post bronchodilator challenge). Two children were excluded because biopsy size was less than 0.2mm². There was no difference in ASM at preschool age between children who had either positive bronchodilator response (n=1) or a positive AHR challenge (n=2), [(n=3, median ASM= 0.127, range (0.05-0.156)], to those who did not at school age [n=14, median ASM 0.08, range (0.03-0.199)], p=0.57, figure 5.23. However, the numbers who were reactive were so small that no firm conclusion can be drawn.

Figure 5.23: Preschool airway smooth muscle is not increased in those with school age airway hyper-responsiveness or positive bronchodilator response
In summary
Although there was no difference in ASM at preschool age between wheezers and controls, preschool wheezers who went on to develop school age asthma had increased ASM at preschool age when compared to those who did not. One limitation of the study was that lung function at the time of the EB was not available. ASM has been significantly related to airflow obstruction in adults and children. In particular ASM in the airway wall increases as airflow limitation worsens in adults\(^{124;125}\). Furthermore, increased ASM has been related to reversible airflow obstruction in older school age children with asthma\(^{118;121}\). Infants with AHR at 1 year of age are more likely to have asthma at age 11 years, which might suggest ASM is already increased at this age\(^{39}\). A previous study showed no evidence of increased RBM thickness or subepithelial eosinophils in wheezy infants (median age 1 year) who had lung function measured at time of EB\(^{138}\). However, the next step further to explore the role of ASM was to study these wheezy infants with reversible airflow obstruction aged 1 year.

5.13 Airway smooth muscle measurement in the ‘Helsinki’ wheezy infants

5.13.1 Clinical description of infant cohort
The characteristics of the infant cohort have previously been reported. The infants were originally recruited by Dr K Malmström and co workers in Helsinki, Finland and all clinical assessments were performed there. Dr Sejal Saglani measured both reticular basement membrane thickness and quantified submucosal eosinophils in these infant endobronchial biopsies\(^{138}\).

Infants were recruited between 3 months and 2 years of age from 2 paediatric centres, The Skin and Allergy Hospital and the Hospital for Children and Adolescents, Helsinki where they had been referred for assessment for chronic dyspnoea, cough and wheeze. Children were referred for evaluation by bronchoscopy and lung function tests if clinically indicated in order to guide future management. Rigid bronchoscopy was routine clinical practice in both hospitals at that time and was performed under general anaesthesia, with a 3.0mm rigid bronchoscope, using biopsy forceps (No. 10378L; Karl Storz, GmbH and Co., Tuttingen, Germany) at the carina.
Infants all had clinically indicated bronchoscopy for wheeze and cough. Exclusion criteria were:
1. 3 days of oral corticosteroids in the previous 8 weeks
2. >400µg of inhaled corticosteroids within 4 weeks of the assessment and lung function
3. non-Caucasian ethnicity
4. birth at gestational age < 36 weeks or small for gestational age (birth weight <-2SD)
5. respiratory distress syndrome
6. bronchopulmonary dysplasia
7. respiratory infection in the 14 days preceding lung function
8. severe atopic dermatitis (>20% of area at the first visit)
9. obvious bronchomalacia or other anatomical malformation on bronchoscopy
10. specific airways conductance (sGaw)< 35% predicted for age combined with dyspnoea at rest

5.13.2 Infant Lung Function
Lung function was assessed with an infant body plethsmograph up to 3 weeks before the bronchoscopy (BabyBody, Jaeger GmbH, Wurtzburg, Germany) (259). Paired sets of measurements of FRC and sGaw were performed and between test variability assessed. FRC higher than the 95th centile (z score ≥ 1.65) and sGaw lower than that of the 5th centile (z score ≤1.65) of the reference range were considered abnormal. Bronchodilator response was assessed by repeating the measurements 15 minutes after inhalation of 600mcg (Ventolin® Glaxo, United Kingdom) delivered with a metallic spacer (Nebunette®, Astra, Sweden). Bronchodilator response was considered significant if there was increase in sGaw of at least 30% and if the increase exceeded 2 standard deviations of the variability (259).

Infants were divided into 3 groups based on the basis of their lung function

**Group A:** reduced airways conductance with bronchodilator response
**Group B:** reduced airways conductance, no bronchodilator response
**Group C:** normal lung function
5.13.3 Atopy
Atopy was defined in all subjects by a positive skin prick test (Solu-Prick Sq, Alk-Abello, Horsholm, Denmark) for at least one of the test allergens (birch, timothy, grass, cat, dog, Dermatophagoides pteronyssinus, latex, egg, cows milk).

5.13.4 Rigid bronchoscopy and endobronchial biopsy
All bronchoscopies were performed for clinical indications in Finland; however endobronchial biopsies were taken for research purposes under direct visualisation from the main carina. Up to 2 biopsies were fixed in 2.5% glutaraldehyde in 0.05-M sodium cacodylate buffer (pH=7.4) for a duration of 2 weeks to 3 months and transported to the Royal Brompton Hospital. Secondary fixation was in 1% osmium tetroxide in sodium cacodylate buffer for 1.5 hours. Biopsies were dehydrated and embedded in epoxy resin (Araldite). Plastic sections (1µm thick) were cut using a microtome (Reichert-Jung, Leica UK) and stained with toluidine blue (1% toluidine blue in 1% sodium tetraborate).

5.13.5 Quantification of RBM thickness
Sections stained with toludine blue that contained epithelium, RBM and subepithelium were used to measure RBM thickness by Dr Saglani using the methods described in chapter 3\(^{138}\). Briefly, RBM thickness was determined using computer aided image analysis as the mean of 40 measurements made at 20µm intervals\(^{225}\). These results have previously been reported\(^{138}\).

5.13.6 Quantification of smooth muscle
ASM area fraction was measured in evaluable biopsies stained with toluidine blue using computer aided analysis, figure 5.24. ASM is reported as a fraction of the subepithelial area. Results are reported as the mean of all tissue sections measured.
Figure 5.24: Evaluable endobronchial biopsy section from an infant stained with toluidine blue showing epithelium, RBM, submucosa and smooth muscle.

Results

5.13.7 Number of children with airway smooth muscle in endobronchial biopsy
71 sections from 53 infants with evaluable biopsies were reviewed. Of those, 33 sections from 24 children did not contain ASM. There was no difference in age (p=0.45) or atopic status (p=0.65) between children with ASM and without ASM on endobronchial biopsy. Twenty nine children had between 1-3 sections with ASM with a mean number of 1.3 sections per child. Median biopsy area measured 0.85 (range 0.21-1.71)mm².

5.13.8 Variability
Intra-observer repeatability was assessed by measuring 3 sections chosen randomly on 3 occasions a week and a month apart. The mean coefficient of variation for the 3 biopsies was 4%. The coefficient of variation values for each of the 3 biopsies were 1%, 4% and 7%. Only 5 children had more than 1 biopsy with ASM from which to assess between biopsy variability. Four children had 2 sections with ASM and 1 child
had three sections with ASM. The coefficient of variation between the 3 sections was very high at 89%. Furthermore a Bland-Altman plot of the 4 children with 2 sections also shows high variability, figure 5.25.

**Figure 5.25: Bland Altman plot of comparing interbiopsy variability of infant airway smooth muscle in the same child**

![Bland Altman plot](image)

5.13.9 **Airway smooth muscle in each infant lung function group**

Clinical characteristics of the Helsinki infants with ASM in EB are reported in table 5.6. Although these children were followed up at 3 years of age in Helsinki, this clinical data is not available to me. If anything, there was a trend towards decreased ASM area fraction in the infants with airflow obstruction and bronchodilator reversibility, group A (n=9, median=0.04, (range 0.008-0.18)), when compared with group B (n=11, median=0.099, (range 0.016-0.498)) and group C (n=9, median=0.099, (range 0.029-0.23)), but this was not statistically significant, figure 5.26.
Figure 5.26: No increase in ASM area fraction in wheezy infants (Helsinki) with airflow obstruction and bronchodilator reversibility when compared to infants with normal lung function.

Table 5.6: No difference in airway smooth muscle measurements between Helsinki infants with one or more biopsies

<table>
<thead>
<tr>
<th></th>
<th>Group A (n=16)</th>
<th>Group B (n=22)</th>
<th>Group C (n=15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median months)</td>
<td>11.6</td>
<td>12.36</td>
<td>11.52</td>
<td>0.57</td>
</tr>
<tr>
<td>Range</td>
<td>(3.8-18)</td>
<td>(5-25.9)</td>
<td>(3.36-24.2)</td>
<td></td>
</tr>
<tr>
<td>Sex (male)</td>
<td>8 (50%)</td>
<td>18 (82%)</td>
<td>17 (73%)</td>
<td>0.58</td>
</tr>
<tr>
<td>Age of onset of symptoms (months)</td>
<td>4.5 (0-16)</td>
<td>5 (0-14)</td>
<td>2 (0-13.5)</td>
<td></td>
</tr>
<tr>
<td>Parental asthma</td>
<td>8 (50%)</td>
<td>10 (45%)</td>
<td>3 (20%)</td>
<td></td>
</tr>
<tr>
<td>RBM (median μm)</td>
<td>4.35 (2.8-9.2)</td>
<td>4 (2.7-5.8)</td>
<td>3.8 (2.7-5.8)</td>
<td></td>
</tr>
<tr>
<td>No. of children with ASM</td>
<td>9 (56%)</td>
<td>11 (50%)</td>
<td>9 (60%)</td>
<td></td>
</tr>
<tr>
<td>Age (months)</td>
<td>7.3 (3.8-17.64)</td>
<td>12 (5-18.24)</td>
<td>16.08 (4.44-24.2)</td>
<td>0.07</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of children with atopy</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>0.44</td>
</tr>
<tr>
<td>Total IgE (median IU/ml)</td>
<td>16 (2-86)</td>
<td>20 (2-377)</td>
<td>17 (7-406)</td>
<td>0.2</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median biopsy (area mm²)</td>
<td>0.88 (0.22-0.92)</td>
<td>0.859 (0.3-1.71)</td>
<td>0.854 (0.215-2.8)</td>
<td>0.79</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ASM (median mm²)</td>
<td>0.04 (0.008-0.18)</td>
<td>0.099 (0.016-0.498)</td>
<td>0.099 (0.029-0.23)</td>
<td>0.06</td>
</tr>
</tbody>
</table>
5.13.10 Atopy and airway smooth muscle in wheezy infants (Helsinki)

There was no difference in ASM between atopic (n=9, median 0.08 (range 0.03-0.176)mm²) and non atopic infant wheezers (n=20, median 0.098 (range 0.008-0.49)mm²), figure 5.27. There was also no difference in total IgE between the 3 groups (p=0.2).

Figure 5.27: No difference in airway smooth muscle between atopic and non-atopic wheezers

5.13.11 Correlation of age and RBM thickness with smooth muscle in the wheezy infant cohort

There was no correlation between RBM thickness and fraction of smooth muscle (Spearman r=0.06. p=0.72), figure 5.28 (i). There was also no correlation between age of infants with normal lung function and airway smooth muscle, figure 5.28 (ii). Biopsies were taken from the carina in infants (Helsinki cohort) and from 3rd and 4th generation bronchi in the preschool group (RBH cohort), so ASM results may not be directly comparable. ASM distribution is not homogenous throughout the airways with the proportion of smooth muscle in the airway wall being relatively more in the peripheral airway walls (non cartilaginous, 10%) when compared with the central airways (cartilaginous airways, 2.8%) (260).
Figure 5.28: (i) Relationship between smooth muscle area and RBM thickness; (ii) Relationship between age and smooth muscle area fraction

(i)

Spearman r=-0.03, p=0.72

No correlation between RBM thickness and ASM

(ii)

Spearman r=-0.03, p=0.86

No correlation between ASM and age

5.14 Discussion

5.14.1 Statement of principal findings

Hypothesis

School-aged children with asthma had evidence of airway remodelling and airway inflammation in endobronchial biopsies taken at preschool age, when compared to school-aged children without asthma.

The main finding of this study is that airway wall smooth muscle area fraction or volume fraction, as measured in EB, discriminates to some extent in this highly
selected group between those severe preschool wheezers who will develop asthma at school age and those who will not. This strongly supports the hypothesis that evidence of airway remodelling is already present in those severe recurrent preschool wheezers who go on to develop asthma.

**Summary of findings in this chapter**

- No difference in ASM between preschool wheezers (RBH cohort, median age 2 years) and controls
- Increased ASM at preschool age in children who go on to develop school age asthma
- No difference in ASM between wheezy infants (Helsinki cohort, median age 1 year) with reversible airflow obstruction and those with normal lung function
- No correlation between age and ASM

**5.14.2 Strengths of study**

This is the first study to quantify ASM in endobronchial biopsies from preschool wheezers (RBH) and to relate pathological findings at preschool age with asthma status at school age. ASM in the biopsies was measured by a single blinded observer using two very different techniques, both giving the same results. Findings were confirmed by a second independent observer. ASM indexed to the surface area of RBM failed to show a difference between children with and without school age asthma however repeatability (CoV 121%) for this method was poor.

**5.14.3 Limitations of study**

- **Results may not be applicable to other wheezy preschool children**

An important limitation was that the preschool wheezers (RBH) enrolled in the original study were of necessity very severe otherwise they would not have undergone bronchoscopy. Numbers are relatively small, and only 32/42 with ASM in endobronchial biopsy could be followed up at school age and only 8 (25%) of these children had developed asthma. However, there was no difference in terms of age at biopsy, sex or atopy between those analysed at school age and those who were not suggesting drop-outs are not likely to have introduced a significant bias. Of the 7 children with preschool wheeze who had asthma at school age and ASM on their
endobronchial biopsy, 4 had been enrolled in the difficult asthma program RBH\(^{(195)}\) and were prescribed high dose ICS to control their symptoms (median 800mcg beclomethasone equivalent).

- **Lung function measurement**
  Unfortunately, lung function measurements were not performed in the preschool children prior to endobronchial biopsy, as standards for measuring lung function in preschool wheezers had not been established at the time of the original study\(^{(9)}\). No correlation was seen at school age between AHR or lung function and preschool ASM (RBH), but this is likely to be both due to the delay of several years between biopsy and assessing lung function and small sample size. However, wheezy infants (Helsinki) with reversible airflow obstruction showed no increase in ASM. This may suggest that ASM may not be the driving factor in AHR in this age group. However, the sample size is too small to draw any firm conclusions and these infant samples were taken at the bifurcation of the trachea where there is typically much less ASM (3\%) compared with more peripheral airway walls (10\%) from where the RBH biopsies were taken\(^{(260)}\).

- **Variability of ASM measurements**
  
  **Biopsy size**
  The bronchoscope used to biopsy preschool children (2.8 or 3.6mm) was much smaller than that used in adults and older children (>4mm). The biopsy channel was correspondingly smaller (1.2mm vs \(\geq\)2mm), and as a result the minimum amount of tissue measured (0.2mm\(^2\)) was much less than in studies in adults and older children (1mm\(^2\)).

  **Within and between biopsy variability**
  There is considerable variability in structural characteristics between endobronchial biopsies and between sections within biopsies. Within and between biopsy variability for the same child was up to 37\% (range 4-70\%) for ASM. The majority of children just had a single good quality biopsy for assessment. Previous studies have also shown that the thickness of airway smooth muscle relative to the airway diameter varied 10 fold within the same group of airways\(^{(260)}\). For this reason ASM was
analysed on a per child basis (the mean of the measurements obtained from all sections from the 1 or more biopsies available). However, not all sections from the preschool children that were assessed for ASM were consecutive as sections had also been stained for other inflammatory cell populations. A very superficial EB could also underestimate ASM.

**Inter and Intra-observer repeatability of ASM measurements**

ASM area fraction measurements showed good correlation between 2 observers. The variability for repeated measures of the same section by the same observer was higher for point counting than using the computer aided analysis program. The increased CoV is likely because of the small number of points counted per subject in the preschool biopsies (median 79 (range 28-276), median biopsy area 0.49mm$^2$) when compared with (571 (295–864), median biopsy area 1mm$^2$) points per subject in older children$^{(121)}$. However, there was good correlation between the 2 methods (ICC=0.78).

- **Age and airway smooth muscle**

Children with school age asthma were older at the time of EB when compared to children without school age asthma. Similarly the children with school age asthma also had increased endobronchial tissue measured when compared with controls. Data on normal ASM development is scanty. However there was no relationship between age and ASM in the preschool and school age non-wheezing controls. A post-mortem study showed that ASM increased linearly with airway diameter during infancy and childhood$^{(256)}$, but although adults have increased absolute amounts of ASM when compared to children, the proportion of ASM, which is what we quantified in the airway wall remains similar$^{(261)}$.

- **Quantifying smooth muscle further**

Previous studies have shown the distance between the submucosal limit of subepithelial basement membrane and the ASM layer is increased in asthmatics$^{(110)}$. However, it was not possible to assess this in the preschool or infant wheezers as the orientation of biopsies was very variable. Secondly, this study did not assess whether the increase in ASM in the subgroup of children who had asthma at school
age was secondary to cell hypertrophy or hyperplasia, or both. However, PCNA staining to assess smooth muscle hyperplasia was performed in a small number of children (n=10) followed up at school age, but showed no increase in PCNA positive cells in those with asthma (n=3).

5.14.4 Airway smooth muscle and asthma
The proportion of ASM in endobronchial biopsies from severe recurrent preschool wheezers is less than in older children and adults with asthma. The subgroup of children who developed asthma at school age had 12% (8-16)% of the subepithelium occupied by ASM, which is similar to the values reported in adults (16-18)% with mild-moderate asthma\(^{(110;122)}\). Further increases in ASM have been reported with increasing asthma severity, with 16-27% of subepithelial tissue occupied by ASM in school age children with moderate-to-severe asthma\(^{(118;121)}\), compared with 40% in adults with severe asthma\(^{(110)}\). The proportion of ASM occupying the subepithelial tissue in the preschool wheezers and controls who did not develop asthma was 6-9% (depending on the method used) similar to that reported for children aged 6-16 years without lower airway disease (4%)\(^{(121)}\), but less than that reported in adults with no respiratory symptoms (9-11.5%)\(^{(110;221)}\). ASM has traditionally thought to occupy approximately 10% of non-cartilaginous airways\(^{(262)}\). Larger biopsy forceps in adults would likely have taken larger and deeper biopsies containing more ASM.

5.14.5 Corticosteroid use and airway smooth muscle
There is little reported on the action of corticosteroids on smooth muscle of the asthmatic airway in adults, and none in young children. Half (14/28) of the preschool wheezers had been treated with regular ICS or a course of oral prednisolone in the month preceding the biopsy, but no difference was seen in the proportion of ASM in the subepithelial area. This is despite the children being treated with high doses of ICS (500mcg of budesonide or equivalent). In contrast animal studies have shown that ovalbumin sensitised female BALB/c-mice who were treated with intranasal fluticasone which was inhaled into the airways had decreased thickening of smooth muscle\(^{(263)}\).
5.14.6 Airway smooth muscle predicts school age asthma

Increased ASM was evident at preschool age in those children with school age asthma when compared to children without school age asthma. The AUC of the ROC was high (0.87), but numbers are small with wide confidence intervals. This is comparable with scores for wheeze severity age 2 years in children from Oslo predicting asthma at 10 years with an area under the ROC of 0.78, and when the score was applied to the RBH group of preschool wheezers AUC of 0.70\(^{(79)}\).

However, a higher severity score was needed to predict asthma in this group of preschool children compared to the Oslo cohort (>6 versus >5\(^{(79)}\)). The median age at follow up for the present study was 8 years. By comparison with the Castro-Rodriguez ‘stringent predictive index’ for asthma at 8 years, ASM biopsy proportion >10% had similar NPV (93% versus 88%) but improved PPV (72% versus 44%)\(^{(27)}\).

However, when the ‘stringent index’ was applied to this present group of children it did not significantly predict asthma. This is likely because the RBH group of children represented a much severer preschool wheeze phenotype and study numbers were much smaller. Secondly, some features of the index (i.e. doctor diagnosed allergic rhinitis) could not be applied to the present study group.

**In summary**

Although increased ASM in preschool wheezers increases the risk of school age asthma, the overlap in ASM between children who developed childhood asthma and those who did not is too great to be of any clinical prognostic value. However, this data suggests that future studies exploring the mechanisms underlying the persistence of preschool wheeze and its progression to asthma might profitably focus on airway smooth muscle.

Airway inflammation also plays an important role in asthma pathogenesis. However, its role in disease progression or disease persistence in preschool wheezers is unknown. The next chapter will relate airway inflammation at preschool age to school age asthma status.
Chapter 6

Airway Inflammation in Preschool Wheezers and its Relationship to School Age Asthma

6.1 Introduction

Airway inflammation plays an important role in the pathophysiology of school age asthma, and indeed the presence of inflammation is built into some definitions of the disease\(^8\). Currently anti-inflammatory therapy, particularly ICS, is central to long term asthma management. Several cell types are implicated in airway inflammation including eosinophils, neutrophils, CD4+ T lymphocytes and mast cells. In particular mast cells and eosinophils are thought to play a pivotal role in adult asthma.

Eosinophils

Eosinophils in sputum\(^{177}\), BAL\(^{264}\) and endobronchial biopsies\(^{93;94;265;266}\) are an established feature of asthma in school age children. Eosinophils may also be increased in non-wheezing children with atopy. BAL eosinophils and eosinophilic cationic protein, were increased in school age asthmatic children when compared to non-atopic controls but not in controls with atopy\(^{264}\). The presence of eosinophilic inflammation in endobronchial biopsies is well established in both mild-moderate\(^{93;94}\) and severe asthma\(^{119;265;266}\) in school age children.

The role of eosinophils in preschool children with wheeze is unclear. Wheezy children aged 3-6 years had increased BAL eosinophils (labelled asthmatic)\(^{264}\) when compared to age matched non-atopic non-asthmatic controls, however there was no difference in BAL eosinophils seen in children aged less than 3 years when compared with controls\(^{264}\). Asthmatic children aged <6 in the reported study likely represented multiple-trigger wheezers as they had repeated episodes of wheezing, breathlessness and cough, particularly at night and early morning, which were present apart from colds\(^{264}\). All ‘asthmatic children’ had to be responsive to bronchodilators by definition\(^{264}\). Similarly, wheezy infants (median age 1 year) with reversible airflow obstruction had no increase in subepithelial eosinophils in EB\(^{138}\), but children with severe recurrent wheeze aged 2-3 years recruited from the RBH did
have evidence of increased subepithelial eosinophils when compared to non-wheezing preschool controls\(^9\). In children with multiple-trigger wheezing aged 5 years, eosinophilic inflammation has been demonstrated in endobronchial biopsies irrespective of the presence or absence of atopy when compared with controls\(^{107}\).

Studies also suggest that airway inflammation is present prior to the onset of asthma diagnosis. EB performed in 27 children (median age 8 years) who were bronchoscopyed because of recurrent or chronic respiratory symptoms, subsequently followed up 2-6 years later, showed those who were later diagnosed with asthma had increased submucosal eosinophils at EB\(^{140}\). However, this study was limited by small numbers, only 3 children were less than 5 years at EB (age range 1.2-11 years), and there was variable time of follow up post EB. It is likely that several of the children had established asthma, reporting chronic cough and wheezing with viral infections, although not clinically diagnosed at time of EB\(^{140}\). A BAL study also suggested airway inflammation is present prior to the onset of asthma\(^{267}\). Children (n=81) with a median age 3.2 years who had never wheezed had a non-bronchoscopic BAL performed during elective surgery for non-pulmonary disease and were followed up 7 years later to ascertain whether any children had subsequently developed wheezing or other atopic disease (eczema, allergic rhinitis) using a modified ISAAC questionnaire\(^{267}\). Children who were clinically asymptomatic in terms of wheeze before the BAL and subsequently developed late onset wheezing in childhood had evidence of increased eosinophils on BAL when compared with children who did not develop late onset wheezing\(^{267}\). The increase in BAL eosinophils was not associated with elevated T\(_h\)2 cytokines as observed in children with established asthma. Furthermore, preschool atopy did not predict eosinophilic airway inflammation as there were no significant differences in blood and BAL eosinophils between those who became atopic but never wheezed and non-atopic children who did not develop wheeze\(^{267}\).

**Mast cells**

Mast cells are also thought to play an important role in asthma pathogenesis. Mast cell activation occurs in response to allergen presentation following IgE mediated crosslinking of the membrane bound IgE high affinity receptor (Fc\(\varepsilon RI\)) causing degranulation and the release of mediators (histamine, leucotrine C4, prostaglandin
D_2) and cytokines (IL-4, IL-5 and IL-13)\(^{268}\). Mast cells originate from pluripotent hematopoietic stem cells which circulate as CD34+ precursors until they migrate into tissues where they mature into long living effector cells\(^{268}\). In humans mast cell subtypes are classified by their protease content, mast cell tryptase (MCT) cells store primarily tryptase in their granules and are found predominantly in the lung and the intestinal muscosa. Mast cell tryptase chymase (MCTC) cells store both chymase, tryptase and carboxypeptidase in their granules and are found in the skin, lymph nodes and submucosa of the stomach and intestine\(^{268}\). Normally, MCT cells predominate in the lung, whereas MCTC cells represent less than 20% of lung mast cells. Mast cells are implicated in asthma pathogenesis, but their phenotype, location and activation varies with asthma severity\(^{147}\). In adults with asthma, mast cells migrate into the epithelium\(^{146}\), airway submucosa\(^{147}\) and ASM\(^{148;150;248}\).

**Epithelial mast cells**

The expression of mast cell tryptases and carboxypeptidase A3 (CPA3) in the airway epithelium of steroid naive adult asthmatic subjects (n=42) is increased when compared to healthy control subjects (n=28)\(^{146}\). Mast cell gene expression in the airway epithelium related closely to the expression of IL-13 signature genes and correlated positively with lung function improvements with subsequent ICS treatment\(^{146}\). The number of intraepithelial mast cells (measured only in those with intact epithelium) was higher in those adult asthmatics with high T_H2 cytokine profile when compared with those with a low T_H2 cytokine profile or healthy controls\(^{146}\). Intraepithelial mast cells were similar between asthmatics with a low T_H2 cytokine profile and healthy adults. Epithelial chymase positive mast cells were not reported in steroid naive asthmatics\(^{146}\), but epithelial MCTC have been reported in mild asthmatics using ICS\(^{147}\) and in severe adult asthmatics\(^{147}\).

**Submucosal mast cells**

The USA National Heart, Lung, and Blood Institute’s Severe Asthma Research Program investigated the expression of epithelial and submucosal mast cells from adults with range of asthma severity and normal control subjects\(^{147}\). Submucosal MCT cells were shown to be highest in adults with mild asthma not being treated with ICS and decreased with increasing asthma severity\(^{147}\). In contrast, in severe asthma the mast cell population is dominated by the chymase-positive phenotype.
(MCTC) in submucosa and in the epithelium\(^{(147)}\). A limitation of this study is that it did not quantify ASM mast cells.

**Airway smooth muscle mast cells**

The morphology of mast cells in the ASM of asthmatic patients seen on electron microscopy when compared to control groups are of smaller cells with fewer cytoplasmic pseudopods with partial or total degranulation\(^{(248)}\). Furthermore, *in vitro* studies have shown that ASM hyperplasia may be driven by mast cell derived chemokine ligand 19 via the activation of chemokine receptor 7 in ASM\(^{(269)}\). The relationship between mast cell myositis and asthma severity is unclear. Bequeret et al reported no relationship to asthma severity, as measured by AHR and infiltration of the ASM by mast cells\(^{(248)}\). In contrast, Siddiqui reported location of mast cells in the smooth muscle bundle but no other features of airway remodelling were associated with AHR in asthma\(^{(149)}\). Furthermore, mast cells localised in the smooth muscle bundle in contrast to mast cells localised in the submucosa expressed fibroblast markers that correlated strongly with the degree of AHR \((r=0.61, p=0.004)\)\(^{(150)}\). *In vitro* studies showed that these fibroblastoid mast cells have an increased expression of chymase and are in a heightened state of activation with increased release of histamine\(^{(150)}\). Mast cells and their relationship to both smooth muscle and submucosa have not previously been explored in preschool wheezy children. Epithelial mast cells in this current study were not quantified as the epithelium was not intact in either the RBH preschool wheezers or controls.

**T lymphocyte cells**

A predominance of subepithelial T\(_H\) cells (CD4\(^+\)) cells in endobronchial biopsies from children with well established severe asthma (median age 13 years) is associated with persistent airflow limitation\(^{(226)}\). Furthermore in two groups of adolescent asthmatic children (one with persistent symptoms and the second with few symptoms) there were increases in the T\(_H\)2 cytokines, IL-4 and IL-5 in bronchoalveolar lavage\(^{(115)}\). However, in the adolescent group with persistent asthma symptoms T\(_H\)2 type inflammation was associated with the presence of activated eosinophils in the bronchial epithelium, whereas in children with few asthma symptoms there was an increase in levels of the T\(_H\)1 cytokine IFN\(_\gamma\) and IFN\(_\gamma\)/IL-4
ratio, in bronchoalveolar lavage\textsuperscript{(115)}. However, in more severe asthma there is limited evidence for the role of $T_H2$ cytokines. A recent study showed that children with severe therapy resistant asthma were characterised by eosinophilic inflammation without increase in $T_H2$ cytokines (IL-4, IL-5 or IL-13) in most patients\textsuperscript{(119)}.

The pattern of airway inflammation is less clear in infants and preschool children with wheeze. Children (median age 8 (range 1-15) years) with asthma and atopy had evidence of ongoing airways inflammation (increased eosinophils and mast cells) on BAL collected during elective surgery while asymptomatic, when compared to children with virus associated wheeze or atopy alone\textsuperscript{(270)}. Significant T-cell-driven airway inflammation (IFN-$\gamma$, IL-2, IL-4, IL-5, and IL-10) is absent in mild or non-atopic children (mean age 5 years) with an episodic wheeze pattern who were asymptomatic at time of bronchoalveolar lavage\textsuperscript{(271)}. However, a BAL study of wheezy 1 year olds with persistent symptoms had significantly higher CD8+ lymphocytes (a marker for $T_H1$ response), in wheezers compared to controls, suggesting the contribution of viruses in the pathophysiology of wheezing in this age group\textsuperscript{(272)}. However, in this study controls were not free of lower respiratory symptoms and were older than the wheezers (mean age 2 years) which may also explain the $T_H1$ predominance\textsuperscript{(272)}.

**Neutrophils**

The role of neutrophils in childhood severe asthma is controversial. In adults with chronic severe asthma neutrophils have been consistently increased in BAL fluid, sputum and in both endobronchial and transbronchial biopsies\textsuperscript{(273,274)}. Measures of airflow obstruction, such as FEV$_1$, correlate with the degree of neutrophilia in sputum in adults with chronic severe asthma\textsuperscript{(274)}. School age children with severe asthma and reduced FEV$_1$ (mean 82%) had raised blood neutrophils\textsuperscript{(275)}, however it is possible that the apparent increase may be related to ongoing corticosteroid therapy. In contrast school age children with severe asthma did not have evidence of neutrophilia in bronchoalveolar lavage or EB\textsuperscript{(119)}.

Neutrophils have also been shown to have a role in infant and preschool wheezing. There are difficulties in interpreting these studies as neutrophilic inflammation in
studies that have used BAL may be confounded by coexistent viral or bacterial infection. In particular persistent bacterial bronchitis may mimic asthma. A BAL study in wheezy children aged 4-32 months, showed a higher neutrophil count but normal eosinophil counts in those with recurrent wheeze compared to controls (276). Over half of the infants with wheezing for whom bacterial analysis of BAL was performed had positive culture results (276). However, controls who did not wheeze, but did have other lower airway pathology did not show an increase in neutrophils despite having similar bacteriological growth frequency on BAL. In another BAL study in children aged 2.5 years with at least a 6 month history of wheezing, 80% of children had evidence of neutrophilic inflammation (277). However, half of this subgroup had raised bacterial counts suggesting infection. Furthermore, a weakness of all these studies is the lack of molecular data on the airway microbiome.

Current study
Severe recurrent wheezers recruited from the RBH had increased subepithelial eosinophils but no increase in subepithelial neutrophils, CD4+, CD8+ and CD45+ (a pan leucocyte marker of subepithelial inflammation) cell counts when compared to preschool controls (9). Airway inflammation will be related to asthma at school age in this chapter. Mast cells will be quantified for the first time in preschool endobronchial biopsies from the RBH.

6.2 Aims
1. To relate airway inflammatory cells (subepithelial eosinophils, neutrophils, CD4+, CD8+ and CD45+) from the preschool endobronchial biopsies to school age asthma status
2. To quantify ASM and submucosal infiltration by mast cells in severe recurrent preschool wheezers and relate these findings to school age asthma status

6.3 Methods
6.3.1 Immunostaining
Immunostaining: Leucocytes (CD45+), neutrophils, eosinophils, CD4+ & CD8+ cells
Immunostaining for eosinophils, neutrophils, CD4+, CD8+ and CD45+ cells had been performed previously (9). Inflammatory cells including CD45+ cells, neutrophils and
eosinophils within paraffin sections were identified using the EnVision-alkaline phosphatase (EV-AP) technique (DAKO Ltd, Cambridge, UK), and identification of CD4+ and CD8+ cells was performed using the EnVision Peroxidase staining method (DAKO Ltd, Cambridge, UK), according to the supplier's instructions. Monoclonal antibodies were applied which had been raised from mouse against human intracellular epitopes, neutrophil elastase (NE) (Dako, Ely) for neutrophils, human eosinophilic cationic protein and eosinophil protein X (EG2) (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) for eosinophils, and against the membrane-associated epitopes CD4+/CD45RO+ (OPD4) (Dako, Ely, UK) for CD4+ T-cells and CD8 (Dako, Ely, UK) for CD8+ cells. After dewaxing, sections were immunostained with the appropriate antibodies for 1 hour. Sections were then washed and incubated with labelled polymer, AP (pre-diluted secondary antibodies, rabbit anti-mouse/rabbit) (DAKO, Carpinteria, USA). After a further wash, bound alkaline phosphatase was detected as a red product following incubation with the chromogen, New Fuchsin substrate system. For CD4+ and CD8+ the sections were incubated with peroxidase-blocking solution. The sections were incubated with EnVision secondary antibody solution for 30 minutes (DAKO). After a further wash, sections were incubated with chromogen (liquid DAB and peroxide buffer). Stained antigen sites were detected as a brown product. Slides were counterstained with H&E to provide nuclear and morphological detail, and mounted in mounting medium. Antibody diluents without primary antibody were used as negative controls. Sections of resected human tonsillar tissue were used as positive controls.

**Immunostaining mast cells**

Endobronchial tissue was only available in small numbers of the original preschool cohort and in this study only MCT was stained. Sections were stained for MCT by Mr Tim Oates. Children with remaining endobronchial tissue archived in paraffin had single sections cut and dewaxed with xylene. They were rehydrated through decreasing alcohol concentrations (100%, 90% and 70%) to water. Sections were then microwaved in pre-heated 10mM sodium citrate (2.941g in 1 litre water). Sections were air dried and washed in Tris Buffer Saline. Biopsies were blocked with horse serum to reduce background staining (VECTASTAIN® Elite ABC, Vector Laboratories). Sections were then incubated with mast cell tryptase (Dako, Clone AA1, Mouse Monoclonal) diluted 1 in 200 in TBS containing 10% normal human
serum for 1 hour at room temperature. Sections were incubated with secondary biotinylated mouse IgG in blocking serum followed by incubation with avidin biotin complex (VECTASTAIN® Elite ABC, Vector Laboratories) to block endogenous biotin activity. MCT positive cells were visualised as red nucleated cells when stained with chromogen Fast Red. Levamisole was used to suppress endogenous phosphatase activity and reduce background staining. Sections were counterstained with H&E. Slides were mounted aqueous mountant (Faramount, Dako).

6.3.2 Quantification of neutrophils, eosinophils, and CD4+, CD45+ and CD8+ lymphocytes
Inflammatory cells had previously been quantified by SS\(^9\). Details of biopsy processing and evaluation are reported in chapter 3. Neutrophils, eosinophils, CD4+, CD45+ and CD8+ cells were assessed by point counting. Point counting is based on stereological principles, which is the study of the three-dimensional properties of objects seen in two dimensions\(^{278;279}\). Using point counting, the determination of volume density of objects in a reference volume can be estimated using the formula\(^{278}\):

\[
V_{v0, R} = \frac{P_O}{P_R}
\]

where \(V_{v0, R}\) denotes the volume of objects (O) contained in a reference volume (R). \(P_O\) is the number of points from a grid falling onto the structure of interest and \(P_R\) is the number of points falling into the reference space. \(V_v\) is the volume density and corresponds to the volume proportion that the structure of interest (the cell) occupies in the reference space (the tissue compartment).

In this study, point-counting was performed using a 100 point graticule and light microscopy (Leica DM2500 microscope, Leica DFC 300fx camera and LEICA Qwin version 3 software, Olympus BH2, Japan) at x400 magnification. The image was projected onto a computer screen and a virtual graticule (100 point) was moved systematically until all of that particular section had been measured. Points falling on smooth muscle, blood vessels, gland and artefactual spaces caused by the processing were not recorded. All points falling on positively stained cells were counted as positive and those and those falling on cells in other areas of the submucosa were counted as negative, figure 6.1. For all cell types, in each child the
value for volume density was calculated as the sum of all the positive points ($P_o$) in one section analysed for that subject, divided by the total number of points counted in the sections ($P_R$), and then multiplied by 100 to express the value as a percentage.

Figure 6.1: Nucleated cells stained with neutrophil elastase in an endobronchial biopsy section from a preschool child

6.3.3 Quantification of smooth muscle and submucosal mast cells

Mast cells were counted as total number of cells per unit area\(^{148}\). Areas of airway smooth muscle and submucosa (lamina propria) were identified by morphologic examination. The area of submucosa (excluding areas of smooth muscle, blood vessels, gland and artefactual spaces caused by the processing) was calculated by tracing around the submucosa with a computer mouse and the area was calculated by a computer analysis system (Leica DM2500 microscope Leica DFC 300fx camera and Leica Qwin version 3 software, Olympus BH2, Japan). The area of smooth muscle was calculated in the same way. Nucleated, immunostained cells were enumerated in the submucosal tissue and airway smooth muscle, and the number of cells was expressed as the number per square millimeter of submucosa and smooth muscle, figure 6.2 and 6.3. Cells were counted within the bundles of smooth muscle
but those in the adjacent areas were counted as submucosal mast cells. The total number of cells was counted for mast cells rather than volume density of cells to allow direct comparison with adult ASM and submucosal mast cell counts\(^{(148)}\).

**Figure 6.2:** Preschool endobronchial biopsy showing nucleated mast cells stained red with mast cell tryptase within airway smooth muscle

![Image of Figure 6.2](image1.png)

Legend: Both masts cells indicated here were counted as ASM mast cells

**Figure 6.3:** Preschool endobronchial biopsy showing nucleated mast cells stained red with mast cell tryptase in submucosa

![Image of Figure 6.3](image2.png)
6.3.4 Variability of inflammatory cell counts

The intra-observer variability of inflammatory cell counts, quantified using both methods (point counting and expressing total cell counts per unit area), was calculated from counts of the same section on 3 separate occasions, at least 3 days apart. As the tissue was limited and biopsies were relatively small, only a very limited number of biopsies (n=3) had more than one section stained per inflammatory cell for the original study\(^9\). Intra-biopsy variability was assessed in 3 biopsies, from 3 children, stained with EG2 for eosinophils. Inter-observer variability was assessed using Bland Altman plots and the intraclass correlation coefficient for cell counts performed by both SS and RO’R.

6.4.1 Results: Variability

Repeatability for eosinophils, represented by the CoV, for repeated measurements in the same section was 20% (20, 22 and 24%). The variability between sections from the same biopsy was also high with a coefficient of variation for 3 children 0% (biopsy with no eosinophils), 20.7% and 25.6%. On comparing measurements of subepithelial eosinophils between 2 observers, there was good agreement in children with cell volume density less than 1% but significantly more variability in cell volume densities greater than this, figure 6.4. The intraclass correlation coefficient for measurements of subepithelial eosinophils by SS and RO’R was 0.68.

Figure 6.4: Difference between measurements of subepithelial eosinophils in endobronchial biopsies measured by RO’R and SS plotted against the mean of the 2 measurements
6.4.2 Subepithelial eosinophils
Severe recurrent wheezers (n=29, 1.47 [range 0-3.33]%) had increased subepithelial eosinophils when compared to controls (n=8, 0 [range 0-0.8]%), p=0.01. Children with CW (0.78, [range 0-3.33]%) had no difference in subepithelial eosinophils when compared to RW (0.7, [range 0-2.8]%) but were increased when compared with controls, p=0.04. However, subepithelial eosinophils in EB were not increased in those children who went on to develop school age asthma, p=0.14, figure 6.5, table 6.1. There was no difference when preschool wheezers (excluding controls) who did and did not develop school age asthma were compared, p=0.43. There was no correlation between eosinophils and RBM thickness as measured by RO'R.

Figure 6.5: (i) Increased subepithelial eosinophils in severe recurrent preschool wheezers when compared to preschool controls. (ii) No difference in preschool subepithelial eosinophils between children who developed school age asthma and those who did not

![Graphs showing increased subepithelial eosinophils](image)

Preschool subepithelial eosinophils (measured by SS) were significantly different in children who developed school age asthma (n=8, 2.19 [range 0-3.52]%) compared to those who did not (n=19, 0.69 [range 0-1.85]%), p=0.02, figure 6.6.
Figure 6.6: Increased submucosal eosinophils as measured by SS when compared to school age asthma status

6.4.2.2 Relationship of subepithelial eosinophils and lung function and airway inflammation at school age

There was a positive correlation between school age FEV₁ z score (n=22, Spearman r=0.54, p=0.01) and FVC z score (n=22, Spearman r=0.49, p=0.02) and subepithelial eosinophils at preschool age measured by RO’R, figure 6.7. Similar correlations between subepithelial eosinophils in preschool wheezers (n=18) between FEV₁ z score (Spearman r=0.54, p=0.02) and FVC z score (Spearman r=0.47, p=0.05). No correlation was seen between eosinophils and FeNO₅₀ or MBW indices.

Figure 6.7: A positive correlation between preschool subepithelial eosinophils and (i) FVC z score and (ii) FEV₁ z score at school age
6.4.3 Other cell counts: neutrophils, CD45+, CD4+ and CD8+
There was no difference in neutrophil, CD45+, CD4+ and CD8+ cell counts either between severe recurrent preschool wheezers and non wheezing preschool controls or between children with or without school age asthma, table 6.1 and figure 6.8. The cells counts measured by SS also showed no difference. There was no difference for any of the inflammatory cell counts when wheezers were divided at preschool age into CW and RW. There was no correlation with lung function measurements and FeNO$_{50}$ at school age.

Table 6.1: Inflammatory cell counts in endobronchial biopsies taken at preschool age related to school age asthma status.

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>No asthma</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Submucosal eosinophils (n)</strong></td>
<td>8</td>
<td>19</td>
<td>0.14</td>
</tr>
<tr>
<td>Median (Range) %</td>
<td>1.47 (0-3.33)</td>
<td>0 (0-11)</td>
<td></td>
</tr>
<tr>
<td><strong>Neutrophils (n)</strong></td>
<td>8</td>
<td>13</td>
<td>0.48</td>
</tr>
<tr>
<td>Median (Range) %</td>
<td>0 (0-3)</td>
<td>0 (0-3.17)</td>
<td></td>
</tr>
<tr>
<td><strong>CD4+ (n)</strong></td>
<td>8</td>
<td>13</td>
<td>0.71</td>
</tr>
<tr>
<td>Median (Range) %</td>
<td>7.23 (0-22)</td>
<td>6.6 (0-33.8)</td>
<td></td>
</tr>
<tr>
<td><strong>CD8+ (n)</strong></td>
<td>8</td>
<td>12</td>
<td>0.35</td>
</tr>
<tr>
<td>Median (Range) %</td>
<td>7.375 (0-18.1)</td>
<td>10.9 (1.2-32)</td>
<td></td>
</tr>
<tr>
<td><strong>CD45+ (n)</strong></td>
<td>8</td>
<td>12</td>
<td>0.67</td>
</tr>
<tr>
<td>Median (Range) %</td>
<td>28.3 (3.6-53.6)</td>
<td>31.2 (8.9-49.8)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6.8: There was no difference in preschool subepithelial neutrophils, CD4+, CD8+ or CD45+ cell counts measured by RO’R when compared by preschool wheeze group or with school age asthma status.

Preschool age

- Neutrophils: $p=0.13$
- CD4+: $p=0.64$
- CD8+: $p=0.09$
- CD45+: $p=0.26$

School age

- Neutrophils: $p=0.48$
- CD4+: $p=0.71$
- CD8+: $p=0.35$
- CD45+: $p=0.67$
6.4.4 Mast cells in endobronchial biopsies from preschool wheezers

A subgroup of children (n=34), 25 wheezers and 9 controls, with remaining biopsy tissue had further sections stained for MCT. Two biopsies were excluded (2 controls) because of crush or distortion of the biopsy. There was very limited endobronchial tissue and MCT positive cells were only assessed in 1 section from each child. Repeatability, measured by the CoV, for repeated measurements in the same section was 5.3% (4.4, 5.4 and 6.2%).

6.4.5 Smooth muscle mast cells

Twelve children (12/32) did not have smooth muscle in the sections stained with MCT. There was a trend to higher mast cells per smooth muscle area in controls (n=5, median 88.5 (range 63.8-396.6)/mm²) when compared with wheezers (n=15, median 33.6 (range 0-239.3)/mm²) but this was not statistically significant, p=0.08, figure 6.9 (i). On analysis of subgroups there was no difference between CW (n=8, 33.4, (0-239.3)/mm²), RW (n=7, median 33.6 (0-206.4/mm²) and controls, p=0.19, figure 6.9(ii).

Figure 6.9: Mast cells per smooth muscle area (mm²) compared between (i) wheezers and controls and (ii) confirmed wheezers, reported wheezers and controls. There was a counter intuitive trend towards increased mast cells in the control group.

(i) (ii)
6.4.6 Submucosal mast cells

There was no difference in submucosal MCT positive cells between wheezers (n=25, median 139.3 (39.5-552.3)/mm$^2$) and controls (n=7, median 117.4 (61.3-278.6)/mm$^2$), p=0.41, figure 6.10 (i). Similarly there was no difference when the subgroups of wheezers with CW (n=13, median 139.3 (40.8-552.3)/mm$^2$) and RW (n=12, 135.4 (range 39.5-327.8)/mm$^2$) were compared with controls, p=0.6, figure 6.10 (ii).

Figure 6.10: Submucosal mast cells compared between (i) wheezers and controls and (ii) confirmed and reported wheezers and controls

6.4.7 Smooth muscle and submucosal mast cells & school age asthma

Irrespective of school age asthma status, there was no difference in preschool smooth muscle MCT positive cells, figure 6.11 and table 6.2. Similarly, there was no difference in submucosal MCT positive cells when compared with the presence or absence of asthma, figure 6.11 and table 6.2.
Figure 6.11: There was no difference in preschool smooth muscle mast cells when related to school age asthma status (i); or between preschool submucosal mast cells when related to school age asthma status (ii)

(i)

(ii)
Table 6.2: No difference in smooth muscle and submucosal mast cells between wheezers and controls at preschool age and those with and without asthma at school age.

<table>
<thead>
<tr>
<th></th>
<th>Wheezers</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submucosal Mast cells (mm²)</td>
<td>n=25</td>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td>CW n=13 / RW n=12</td>
<td>139.3 (39.5-552.3)</td>
<td>117.4 (61.3-278.6)</td>
<td>0.41</td>
</tr>
<tr>
<td>Smooth muscle Mast cells (mm²)</td>
<td>n=15</td>
<td>n=5</td>
<td></td>
</tr>
<tr>
<td>CW n=8 / RW n=7</td>
<td>33.6 (0-239.3)</td>
<td>88.5 (63.8-396.6)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

| Smooth muscle Mast cells (mm²) | n=7              | n=17             |      |
| Submucosal Mast Cells (mm2)   | 188.3 (40.8-552.3) | 129.5 (51.3-278.6) | 0.28 |

6.4.8 Airway smooth muscle and smooth muscle mast cells

There was no correlation between ASM area fraction and smooth muscle MCT positive cells (Spearman r=-0.01, p=0.94), figure 6.12. Similar, results were seen when MCT positive cells were correlated with ASM volume fraction (Spearman r=-0.03, p=0.86).

Figure 6.12: No correlation between ASM and smooth muscle mast cells
6.5 Discussion

6.5.1 Summary of Results

- Although there was a significant difference in submucosal eosinophil volume density between wheezers and non-wheezing controls at preschool age, no difference was seen in submucosal eosinophil volume density when compared by presence or absence of asthma at school age when measured by RO’R. However, SS’s eosinophil measurements in contradiction did show a significant increase in children who developed school age asthma, underscoring that this variable measurement is unlikely to be clinically useful.

- No difference was seen in neutrophils, CD4+, CD8+, CD45+ and mast cells in those who did and did not wheeze at preschool age or in those who did or did not have asthma at school age.

Strengths

This is the only study where tissue inflammatory cell counts in children with recurrent wheeze at preschool age have been related to asthma at school age. Furthermore, this was the first time that both ASM and submucosal mast cells were stained for and quantified in endobronchial biopsies from severe recurrent preschool wheezers.

Limitations

Study size

The number of preschool children who had sections stained for inflammatory cells and also the number of children who were followed up at school age was small. This study was not powered to detect a difference between groups at school age. A minimum sample size of 15 subjects per group has been suggested for studies of inflammatory cells in adult endobronchial biopsies\(^{(280)}\). Furthermore, a sample size of 13-48 subjects per group is needed to detect at least one doubling difference in cell number per 0.1 mm\(^2\) of tissue, for a particular inflammatory cell type, in a study with a parallel group design\(^{(281)}\). The majority of children in this study had only 1 section from a single biopsy stained per inflammatory cells. However inflammatory cell biopsy studies of adults with chronic obstructive airways disease suggest one biopsy taken from each of two airway generations, from each subject, should be examined
in order to maximise statistical power to detect differences between study groups (282).

Variability
The CoV for repeated measurements using the point counting method in this study was (0-22)%, similar to that reported by SS (0-17)%. Different factors which contribute to the overall variance for point counting have been estimated and include 70% variability between subjects, 20% between biopsy blocks, 5% between sections, 3% between light microscopy fields, and 2% between measurements (85). The CoV reported for measuring mast cells (expressed per mm²) was (4-6)%, comparable with inflammatory cell counts in adult studies (2.3%) (282).

Secondly, there was also considerable variability between observers (RO’R and SS) in measuring inflammatory cell counts. However, for consistency, all cell counts analysed in this study were performed by a single observer (RO’R) which eliminated any inter-observer bias that could have been introduced. Variability between biopsies was only assessed for eosinophils because of limited endobronchial tissue. Variability was similar between biopsies from the same child (20-25%) to variability between children with and without asthma at school age (15%).

Endobronchial biopsy size
It would have been useful to stain endobronchial tissue for both MCT and MCTC. However, this was not possible because there was no endobronchial tissue remaining.

One single measurement of inflammation
As in all chronic diseases, one single measurement of inflammation in an EB at a single point in time is unlikely to represent the full inflammatory picture. Other assessments of inflammation such as BAL inflammatory cell counts were not available in this group of children who had bronchoscopy at preschool age. Sputum inflammatory phenotypes were not stable in school age children with severe asthma (165), which may suggest that neither are submucosal inflammatory cell counts. Secondly, airway submucosa inflammation does not necessarily correlate with blood, sputum or BAL inflammation (283). This may be attributed to the fact that
sputum and BAL sample the airway lumen, whereas the cellular inflammation in the airway submucosa may be distinct. It is not known which compartment is more important.

6.5.2 Discussion of principal findings

Subepithelial eosinophils

Subepithelial eosinophils at preschool age did not predict wheeze persistence at school age when measured by RO’R. Although subepithelial endobronchial eosinophil measurements performed by Dr S Saglani did show a significant difference between children with and without school age asthma, the number of subjects was small and variability was large both intra-observer and between biopsy. A larger sample size with at least 2 good quality biopsies per child may conclusively answer this question. It is possible that increased eosinophils at preschool age may represent current symptoms rather than future asthma prognosis, but this study is unable to confirm or refute this. Interestingly, children aged 1-3 years with eosinophils in nasal secretions during an acute respiratory tract infection had significantly higher risk of wheezing over the following 2 months but not over the longer term, at 12 months(284).

Features of airway remodelling that are typical of asthma develop in asthmatic children even in the absence of a prominent eosinophilic infiltrate, indicating that other mechanisms, besides eosinophilic inflammation, may promote airway remodelling (increased RBM thickness and epithelial cell loss) early in life(285). There are conflicting reports about the correlation between subepithelial eosinophils and RBM thickness in children and adults with asthma. Adults with asthma showed a positive correlation between subepithelial eosinophils and RBM thickness(286). In contrast there was no correlation between subepithelial eosinophils in school age children with difficult-to-treat asthma(116) or children with mild-moderate asthma(285) and RBM thickness. Superficial eosinophils correlated with RBM thickness in the severe recurrent preschool wheezers(9), but repeat measurements by a second observer (RO’R) showed no correlation. Most of the preschool children were treated with ICS at time of EB, however airway inflammation and increased RBM thickness do not respond concordantly to ICS treatment(287). Adults with asthma (n=35) who
were treated with inhaled fluticasone and had BAL and EB at baseline, 3 months and 12 months showed a decreased in BAL inflammatory cells at 3 months but not in RBM thickness until 12 months after treatment with ICS\textsuperscript{287}.

Eosinophilic inflammation correlated positively with both FEV\textsubscript{1} and FVC z scores at school age. Children with no subepithelial eosinophils were more likely to have lower FEV\textsubscript{1} and FVC at school age, which is counter-intuitive. Several of these children had alternative diagnoses at school age unrelated to asthma (including scimitar syndrome and a small congenital right lung and repaired congenital heart disease) that likely accounted for this. However, although there was a correlation between eosinophilic airway inflammation in preschool wheezers and FeNO\textsubscript{50} when followed up age 5 years\textsuperscript{63}, there was no correlation with FeNO\textsubscript{50} at age 7 years.

**Mast cells within airway smooth muscle**

Infiltration of ASM by mast cells is thought to be responsible for the increase in ASM mass in adult asthmatics\textsuperscript{148}. There was no difference in MCT positive cells per ASM area between preschool wheezers and controls, or children with or without school age asthma. The number of MCT positive cells per ASM area is much higher for both wheezers and controls (median 64 (range 0-396) mast cells/mm\textsuperscript{2}) when compared to adult asthmatics (median 5.1 (range 0-33) mast cells/mm\textsuperscript{2})\textsuperscript{148}. However the absolute values for MCT positive cells seen in ASM were similar in children (median 2 (range 0-12)) and adults (median 2 (range 0-8))\textsuperscript{148}. Preschool children did however have a much smaller ASM area (median 0.02 (0.01-0.12mm\textsuperscript{2}) measured than adult patients (ASM area median 0.3 (0.16-0.97) mm\textsuperscript{2})\textsuperscript{148}. Although only cells within the bundles of smooth muscle were counted, serial sections were not available to confirm that mast cells were not juxtaposed from submucosal tissue onto smooth muscle owing to biopsy artefact\textsuperscript{148}. There was no relationship between total ASM and mast cells within smooth muscle. However, sample sizes are very small and any conclusions drawn are preliminary.

**Submucosal mast cells**

There was no difference in numbers of submucosal MCT positive cells between wheezers and controls or between children with and without school age asthma. However, mast cells quantified in the infant cohort recruited from Helsinki, Finland
with reversible airflow obstruction (median age 1 year) had a weakly positive correlation with symptom persistence as measured by asthma medication purchase at 3 years but school age follow up is not available to date\textsuperscript{(139)}. However, mast cell quantification in that study was performed using electron microscopy which recognises mast cells by their ultra-structural features and included all mast cells (chymase and tryptase), so a direct comparison is impossible\textsuperscript{(138)}.

Similarly, there was no difference in submucosal mast cells between school age children with mild-moderate asthma (most of whom were not treated with ICS) and atopic and non-atopic school age controls\textsuperscript{(93)}. However in a recent study submucosal MCT positive cells were shown to be highest in adults with mild asthma not being treated with ICS and decreased with increasing asthma severity\textsuperscript{(147)}. In contrast MCCT cells were the predominant mast cell phenotype in severe adult asthmatics\textsuperscript{(147)}. Children with severe therapy resistant asthma also did not have an increase in mast cells within smooth muscle or the submucosa\textsuperscript{(119)}. A limitation of the current study is that 9 wheezers were being treated with ICS and a further 3 wheezers with oral corticosteroids at the time of biopsy.

**Summary**

There was no relationship between inflammatory cell counts at preschool age and school age asthma. ASM mast cells were not related to ASM area fraction or volume fraction.
Chapter 7
Discussion and Final Summary

Principal findings reported in this thesis
The aim of this final chapter is to summarise and discuss (i) the implications of the results reported in this thesis with regard to the relationship between preschool airway pathology and school age asthma status and (ii) how the findings from this thesis suggest research directions for future studies.

The hypotheses tested in this thesis were:

- **School-aged children with asthma have evidence of airway remodelling and airway inflammation in endobronchial biopsies taken at preschool age when compared to school-aged children without asthma.**

This hypothesis was discussed in chapters 3-6 and found to be partly true. Although EB eosinophilia, RBM thickness (at least as measured by RO’R) and RBM TN-C (with all its questionable reliability) were significantly higher at preschool age in wheezers compared with controls, they did not discriminate between the children who developed asthma by school age and those who did not. ASM from preschool endobronchial biopsies to some extent discriminated those severe preschool wheezers who developed asthma at school age. However, there is significant overlap in ASM (expressed as the proportion of the EB area) between preschool wheezers who did and did not develop asthma at school age and also between controls who never wheezed. This suggests that ASM mass will not be a useful test in predicting children who develop asthma but rather points to ASM as a new focus to determine what drives the evolution of preschool wheeze to asthma. This study has only focussed on quantifying the amount of ASM in preschool biopsies, but has not assessed early function, and this may be an important determinant of future asthma. The numbers are small, and the results hypothesis generating, but this is the key novel finding arising from the work of this thesis, and forms the focus of much of this final chapter. Unfortunately
there was no further biopsy tissue available in which to perform mechanistic studies.

- **A combination of clinical and airway pathological characteristics at preschool age may be able to be used to predict asthma at school age, better than clinical features alone.**

Only 7 preschool wheezers who had asthma at school age had airway pathology measurements for RBM, ASM and eosinophils. Numbers were too small to develop a predictive score and test the hypothesis. Furthermore, there was not a second cohort available in which to validate any predictive score.

### 7.1 Strengths and novel data

This is the first study to quantify ASM, TN–C within the RBM and mast cell infiltration of smooth muscle and submucosa in EBs from severe recurrent preschool wheezers. All measurements were made by a single observer, blind to both preschool and school age clinical status. Secondly, this is the first study to relate pathological findings at preschool age with asthma status at school age.

ASM is increased in those preschool children with severe wheeze who go on to develop asthma at school age. Neither RBM thickness nor subepithelial eosinophils (as measured by RO’R) were significantly increased in those who developed asthma at school age. Although SS did show that RBM thickness was increased in those who developed asthma, this was likely related to the 12 month age difference in time of EB between those who did and did not develop asthma at school age. This could suggest that RBM thickness and subepithelial eosinophils at preschool age could be related to current symptoms rather than future prognosis, but even this must be considered as a preliminary, hypothesis-generating finding, given the variability of the measurements. The fact that two experienced observers obtained differences in results, which may be in part be due to counting different slides from the same patient, underscores the difficulty of using these methods. There was no relationship between school age asthma and any other inflammatory cell count at preschool age including submucosal and smooth muscle mast cells.
This thesis reports on the previously recruited wheezers and non-wheezing controls, who were well characterised at entrance to the study at preschool age, at aged 6-11 years. To my knowledge, this is the only prospective preschool group followed longitudinally who had bronchoscopy and EB at entrance to the study in the preschool years. Subject retention to school age (75%) was comparable to other studies (54-91%)(1;10;12;14;15). Wheeze had been confirmed at preschool age using a video questionnaire(9;153), and at school age confirmed wheeze at preschool age was a significant predictor of asthma. However, assessment of wheeze at school age showed only limited agreement between the video and written questionnaire (kappa 0.46, p=0.005). Failure of carers who reported wheeze to identify it on video questionnaire was more common in children with milder wheeze who may not have been as symptomatic as the child in the video questionnaire having an acute asthma exacerbation. The majority of children in this study had had at least 1 admission to hospital with wheeze and had wheeze confirmed by a doctor at least once.

7.2 Limitations of this thesis

- Small sample size

The most obvious limitation of this study was the small numbers with biopsies followed up at school age, figure 7.1. Only 8 children with ASM in their EB had asthma when followed up at school age. Secondly, not all children had complete data sets available for measurements of airway pathology at preschool age or measurements of airway inflammation and lung function at school age. Multiple comparisons were made in a small number of patients which could have generated spurious associations. In any case no second validation cohort was available so all findings must be considered hypotheses generating.
Figure 7.1: Follow up of preschool wheezers at school age related to airway pathology measurements

- **Wheeze at preschool age**

An important limitation was that the wheezers enrolled in the original study were of necessity very severe otherwise they would not have undergone bronchoscopy. Children with asthma at school age had many reported admissions with wheeze as the main symptom (median n=17). These children are not representative of preschool wheezers in the general population but rather a specific group with more severe disease and so the findings in this thesis may not be applicable to children with less severe wheeze. However, those early severe wheezers who go on to wheeze persistently have the largest early reduction in lung function, which is why this group is important in studying the factors driving the development of asthma\(^6\). Although recruiting children at or shortly after birth as in the Tuscon\(^1\) or COAST\(^59\) cohorts is preferable to preschool age, the numbers needed to capture enough wheezers severe enough to warrant bronchoscopy at preschool age would be impractical. Even by recruiting children with increased risk of wheeze (i.e. infants of asthmatic mothers) such as in the COPSAC\(^48\) cohort, the study numbers needed to capture those severe preschool wheezers who would warrant bronchoscopy is clinically not feasible. The number of children who have bronchoscopy for investigation of recurrent wheeze at preschool age is
likely less than 1% of all children, suggesting a sample size of 100 undergoing bronchoscopy would require a total recruitment cohort of 10,000.

- **Absence of truly healthy controls**
  A further limitation of this thesis is the absence of healthy, asymptomatic controls. Genuine, healthy asymptomatic controls could not be obtained in view of the invasive nature of the initial study. Unlike in adults, it is not ethically acceptable to perform bronchoscopy in infants or children solely for the purpose of research. Several of the control children had other pathology including congenital heart disease at preschool age and a diagnosis of ulcerative colitis at school age. This made it difficult to compare assessments of lung function at school age.

- **Absence of bronchoalveolar lavage at preschool age**
  At the time of the original study at preschool age BAL was not included in the research protocol. The main reason for this was logistic; there was no core facility for quantitative cell counting and storage of fluid at the time. Although BAL samples from the wheezers were done for clinical reasons at preschool age there was variability in methods used to obtain the lavage. However, subsequent cohort studies since recruitment of the original preschool RBH group have highlighted the importance of bacterial (COPSAC)\(^{(48)}\) and viral (COAST)\(^{(59)}\) infection in early wheezers in wheeze persistence and the development of asthma, and the absence of data on infection is an acknowledged weakness of the study.

- **Misclassification of asthma at school age**
  Defining asthma in young children is difficult\(^{(156)}\). In the present study, several children without asthma reported a dry cough at night (n=7), wheeze (n=7) and use of ICS (n=4) but none of these children had ever had a doctor diagnosis of asthma and many had a history of upper airway noises or co-morbid pathology. Of the children who did not have asthma at school age 4 had congenital heart disease, 1 had a vascular ring causing some mild airway compression, 1 had ulcerative colitis, 1 had non cystic fibrosis bronchiectasis likely related to an initial, severe viral bronchiolitis and 1 child had a diagnosis
of scimitar syndrome. The children with asthma who attended for clinical assessment had higher IgE at preschool age, were more likely to be atopic, have allergic rhinitis and eczema at school age recruitment, suggesting that a group likely to have asthma was selected. However, it is possible some children may have been misclassified as non-asthmatic at school age. The effect of this would have been to reduce the chances of showing differences between the groups

- **Possible confounding effects of ICS/ oral corticosteroids**

  The majority of preschool wheezers had been treated with either or both of ICS and oral glucocorticosteroids prior to bronchoscopy and EB. Despite this there was still a difference in RBM thickness and RBM TN-C expression and tissue eosinophilia seen between preschool wheezers and controls. No difference in ASM was shown between preschool wheezers and healthy controls when compared as groups. It is possible that treatment with ICS and/or oral steroids may have masked a difference between these 2 groups. However, in adult asthmatics ASM mitogenesis and growth are resistant to glucocorticoids\(^{(288)}\). *In vitro* studies suggest absence of CCAAT / Enhancer binding protein α (which binds to the glucocorticoid receptor to inhibit cell proliferation) in adult asthmatics is responsible for the enhanced proliferation of ASM cells and the failure of glucocorticoids to inhibit proliferation\(^{(288)}\). On the other hand RBM TN-C has been shown to decrease in adult asthmatics with ICS therapy\(^{(144, 244)}\). There have been contradictory reports on the effect of ICS on RBM thickness, with some studies showing a reduction with treatment with ICS in adult asthmatics over varying time periods from 6 weeks\(^{(289)}\) to 1 year\(^{(286)}\) to no treatment effect with ICS\(^{(290)}\). ICS are also known to reduce tissue eosinophils\(^{(287)}\) and mast cells\(^{(147)}\). It may be that the preschool children were not prescribed high enough amounts or a long enough duration of treatment with ICS, or more likely inadequate drug delivery or poor compliance in this young age group. A further weakness is that ICS compliance was not assessed prior to bronchoscopy and EB at preschool age.
**Age of endobronchial biopsy when compared with school age asthma status**

A major strength of the original study was that wheezers and controls were age matched\(^{(9)}\). However, when followed up at school age unavoidably children with school age asthma (median age 8.2 years) were older at the time of EB (28 months versus 17 months) when compared to children without school age asthma (median age 7.3 years). This means that age difference of EB between those who developed asthma at school age and those who did not may have affected the interpretation of some of the results.

The natural history of airway pathology in preschool wheeze has not been explored, but it is likely that other structural airway changes, like RBM thickness, will also evolve throughout childhood and adolescence. RBM TN-C developmental expression is unclear, it is known that there is a small amount present at birth but none in adulthood\(^{(141)}\), but it remains unknown when RBM TN-C disappears. RBM TN-C may disappear when RBM reaches adult thickness\(^{(113)}\). In this thesis, the age discrepancy at the time of biopsy may be a confounder, because those without asthma at school age were younger when biopsied, and may have had normal but increased RBM TN-C than the children who went on to develop asthma. In this regard, the failure of subsequent runs of TN-C staining meant that developmental changes in TN-C expression could not be explored.

Data on normal ASM development are scant. A post-mortem study showed that ASM increased linearly from 22 weeks post conceptual age to 8 months, and then two to four fold to early adulthood\(^{(256)}\). Although adults have more ASM in real terms when compared to children, the proportion of ASM in the airway wall remains similar\(^{(261)}\). Although the children with asthma were older than those without asthma, no correlation was found between ASM and age in non-wheezing preschool and school age children. This is likely because ASM was quantified as a proportion of the EB rather than the actual mass of ASM.
• **All data are from single time points**
  This thesis reports essentially two cross-sectional datasets, relying on retrospective recall of disease severity. In this study data are available on clinical symptoms (age 2-3 years and age 7-8 years), airway pathology (age 2-3 years) and lung function and non-invasive airway inflammation assessment (age 7-8 years) from single time points. It would have been very useful to repeat endobronchial biopsies at school age to compare preschool and school age airway pathology, but ethically impossible in clinically well children. An attempt was made to collect details of medication use between the two study time points; however, quite often parents were unable to recall their child’s prior medication history.

**Meaning of the study**
Early childhood may be an important time when disease modifying or preventative therapies could be applied before the persistent asthma phenotype becomes established. The purpose of this research was to try and understand what features of airway pathology changes might be related to wheeze persistence and the development of childhood asthma. The data suggests that a future focus on ASM may be key in helping understand what drives some children to develop school age asthma.

Study numbers were too small to allow identification of a specific endotype associated with increased ASM. However, those children with asthma (and ASM on their EB) had more severe disease at school age. Almost all children (7/8) with asthma at school age were still attending tertiary services for asthma management, had persistent asthma symptoms despite treatment with high dose ICS (6/8) and 4 children were classified as severe asthma at school age\(^{(195)}\). The data suggests that increased ASM is associated with severe preschool wheeze, suggesting that intrinsic abnormalities in ASM may predispose children to developing asthma and particularly severe asthma at school age. Longitudinal studies have suggested that children with severe wheeze early in life are more likely to have severe asthma later in life\(^{(71)}\).
Future work and unanswered questions

As there is no remaining endobronchial tissue from the preschool wheezers recruited between 2002 and 2005, future studies will need to recruit a second cohort of preschool wheezers. Although it would be interesting to recruit preschool children with a wider spectrum of wheeze severity, bronchoscopy and EB will be only clinically indicated in those children with severe recurrent wheeze and these are the group of children most likely to have pathological abnormalities. A future study would recruit recurrent preschool wheezers with assessments of lung function (measured by MBW and specific airways resistance) and BDR measured at entrance to the study, followed by clinically indicated bronchoscopy, bronchoalveolar lavage and EB. MBW is currently the most sensitive indicator of airway abnormality in young children with wheeze\(^{62,172}\) and bronchodilator reversibility has been successfully performed using both specific airways resistance and MBW in the preschool age group\(^{62}\). \(\text{FeNO}_{50}\) was assessed in the present study but more distal inflammation was not assessed as children were too young to perform \(\text{FeNO}\) at multiple flows. Preschool RBM correlated with \(\text{FeNO}_{50}\) at age 4-5 years\(^{63}\) and a non-significant trend with \(\text{FeNO}_{50}\) at school age in those with preschool wheeze. It would be interesting to follow up the RBH cohort at age 10 years or older to measure fractional exhaled NO to calculate alveolar and bronchial flux NO and relate this to preschool RBM thickness\(^{190}\). BAL should be assessed by bacterial culture and molecular studies (16s rRNA) and molecular techniques (including polymerase chain reaction) for common viruses. The BAL cytokine, microbiome and metabolome profile in preschool wheezers should also be measured. The airway microbiome is discussed in more detail in the ‘Novel areas for possible further exploration’ section which follows.

Exploring airway smooth muscle further in preschool wheezers

To date airway remodelling and airway inflammation has been quantified in preschool wheezers and compared to age matched controls, but no assessment has been made as to whether different structural components of the airway wall are inherently functionally different in preschool wheezers who develop asthma when compared to children who do not. In particular pilot data from this study suggests that a focus on ASM may yield some answers as to why some preschool children with wheeze develop asthma.
Although it was not possible to further characterise the mechanisms underlying ASM increase in this group of preschool wheezers as there was no remaining EB tissue, both smooth muscle hypertrophy and hyperplasia contribute to the increase in ASM in adults and school-age children with asthma\(^{(110;121;122;249)}\). There is an increasing body of evidence that ASM is fundamentally different in asthmatics compared to healthy controls suggesting that abnormalities in ASM cells may play a critical role in the development of the abnormal physiology in asthma and may contribute to the persistent airway inflammation\(^{(291)}\). Recent evidence suggests that ASM from adult asthmatics is intrinsically hyper-contractile and exhibits an increased oxidative stress burden when compared to healthy controls\(^{(291)}\). Nicotinamide adenine dinucleotide phosphate oxidase (NOX)\(_4\) expression is increased in asthma and its inhibition or siRNA knockdown normalises this airway smooth muscle hyper-contractility\(^{(291)}\). Cultured adult asthmatic (n=9) ASM cells in gel contraction assays have also shown an increased velocity of contraction in response to histamine when compared to adults without asthma\(^{(292)}\). It is possible but unproven that the ASM cells in those children with preschool wheeze who develop asthma are functionally distinct compared with children who do not develop asthma. ASM in preschool wheezers may also be intrinsically hypercontractile before children develop persistent wheeze and asthma. Future work needs to be undertaken to characterise ASM from preschool wheezers further, and will likely include the isolation of primary ASM bundles from EBs and culture of ASM cells\(^{(293)}\). ASM cell responses in preschool wheezers to allergen\(^{(70)}\), virus\(^{(59)}\) and bacterial exposure\(^{(23)}\) may be a fruitful source of future work since all have been implicated in the evolution of preschool wheeze into persistent wheeze and asthma.

ASM cells also express a wide variety of mediators, and in particular recent research highlights the innate cytokines thymic stromal lymphopoietin (TSLP) and IL-33 as mediators of AHR, with increased ASM expression of these mediators in severe adult asthmatics\(^{(294-296)}\). It may be that these or other ASM derived mediators are important in the switch from severe preschool wheeze to school age asthma. The identification of key mediators regulating the contribution of ASM to asthma may provide new opportunities for therapeutic intervention\(^{(297)}\).
However, the airway epithelium also plays a central role in the pathogenesis of asthma, and the role of the epithelium has not yet been explored in severe preschool wheezers who develop asthma at school age\(^{(126)}\). There is evidence that dysregulated epithelial repair originates in childhood asthma and is a critical determinant of disease progression into adulthood\(^{(128)}\). Bronchial epithelial cells from asthmatic children function abnormally even in the absence of inflammation with decreased production of TGF-β and the release of anti-inflammatory mediators\(^{(128)}\). There is also some in vitro evidence that an injured airway epithelium stimulates ASM cell hyperplasia, through IL-6, IL-8, monocyte chemotactic protein-1, and MMP-9 dependent mechanisms\(^{(298)}\). It is therefore possible that severe recurrent preschool wheezers who develop asthma at school age have inherently functionally different epithelial cells which thus respond abnormally to viral, bacterial or environmental stimuli leading to ASM hyperplasia. To address this hypothesis, a co-culture model of epithelial cells and ASM cells isolated from EB from severe recurrent wheezers could be compared with age matched non-wheezing controls\(^{(298)}\) to analyse epithelial production of chemokines, cytokines, and growth factors and identify what stimulates ASM hyperplasia\(^{(246)}\).

**Serially monitoring preschool wheezers**

It is likely that airway remodelling changes in preschool wheezers who develop school age asthma evolve gradually between preschool and school age. An important part of future studies will be to serially monitor airway remodelling, inflammation and ongoing symptoms. Most of the methods used to monitor remodelling require invasive procedures, use of a general anaesthetic in children or ionising radiation. High resolution CT has shown a positive correlation with RBM thickness in adult asthmatics, but overlap among the groups limits the diagnostic value in individual adult patients\(^{(112)}\). In children with problematic severe asthma there are conflicting reports on whether HRCT correlates with RBM thickness in children\(^{(299;300)}\). In any case, concerns regarding radiation exposure means this is unlikely to be a useful tool to monitor disease progression in wheezy preschool children through to school age\(^{(301)}\). Endobronchial ultrasound is a radiation free new technique that enables the assessment of bronchial wall layers\(^{(302)}\). In adults there was no significant difference in the measurements of total bronchial wall thickness between endobronchial ultrasound and high resolution CT scanning, suggesting it
may be a radiation free way of monitoring disease progression in adults. Furthermore, the thickness of the bronchial wall positively correlates with RBM thickness and negatively correlates with FEV$_1$ in adults with asthma$^{302}$. However, this technique in children while radiation free will still require a general anaesthetic and is unlikely to be an acceptable technique to use serially. Novel areas such as exhaled breath condensate analytes could be investigated further to see if they could provide a serial marker for airway remodelling.

Asthmatic symptoms in young children are due to a heterogeneous group of diseases. Although symptoms are the primary endpoint in both research and clinical management, there is no standardised method of symptom assessment. Assessment of ongoing disease is also important and wheeze severity has shown to be a risk factor for future asthma$^{79}$. Daily diaries have been used to assess symptoms and endotype asthma$^{303}$. A symptom algorithm based on a predefined number of days and episodes of troublesome lung symptoms rather than disease categorisation using qualitative characteristics or temporal patterns of wheeze showed a stronger relation to an underlying ORMDL3 genetic variant which is associated to asthma susceptibility in those of North European descent$^{303}$. In future cohorts a daily symptom diary which also records medication use and is submitted weekly either electronically or by telephone interview may help record disease persistence more accurately.

**Novel areas for possible further exploration**

There are several new areas of research which may be fruitful in exploring the development of asthma in preschool wheezers. Non-invasive methods of serially assessing airway inflammation and remodelling are an attractive way of improving our understanding of the progression of early wheezing to asthma. Exhaled biomarkers which could be used to serially monitor inflammation are volatile organic compounds (VOCs) and non-volatile organic compounds in exhaled breath condensate. Recent research has also suggested a focus on the airway microbiome may also be important.
**Volatile organic compounds**

VOCs are groups of exhaled biomarkers which are gaining interest as a method to serially monitor airway inflammation in preschool wheezers. Breath testing dates back to the early history of medicine, when ancient physicians tried to use the specific smell on the human breath to recognise illness such as diabetes or liver disease. VOCs are carbon-based chemicals that are volatile at room temperature and are formed during several pathophysiological processes, including airway inflammation\(^{(304)}\). During inflammatory processes free radicals are produced leading to lipid peroxidation and formation of volatile products including hydrocarbons such as ethane, pentane, hexanal, octanal, nonanal, propanol and butanol. Once VOCs are formed they enter the bloodstream and are excreted in the breath and can be measured by collection of exhaled breath and analysed by gas chromatography and mass spectrometry or the electronic e-nose. Hundreds of different VOCs are present in human breath, and their relative concentrations may alter in the presence of the disease. VOCs in exhaled air can be used to distinguish children with cystic fibrosis\(^{(305)}\) and asthma from healthy children\(^{(306)}\). In children and adults with cystic fibrosis (aged 8-29 years), the VOC pentane was much higher than in healthy controls, and higher in those with more severe lung disease or those having concurrent pulmonary exacerbations\(^{(305)}\). A total of 945 VOCs were screened to profile which hydrocarbons could differentiate between asthmatic and healthy children (aged 5-16 years)\(^{(306)}\). Eight VOCs discriminated between asthmatic and healthy children with a 92% positive predictive index, achieving a sensitivity of 89% and a specificity of 95%\(^{(306)}\).

One hypothesis is that the exhaled VOC profile is different in those severe recurrent wheezing preschool children when compared to healthy controls, and in particular in those wheezers who will go on to develop school age asthma. The e-nose technique creates a ‘breath-print’ of multiple different VOCs and is quick, easy to use and not very expensive, which suggests it could be used to serially monitor the exhaled VOC pattern in children over time. However, exhaled compounds can be affected by various factors including ambient VOCs, exhaled flow, and prior consumed food and before VOCs can be used in clinical practice standardisation and validation of VOC profiling is essential.
**Exhaled breath condensate**

Exhaled breath condensate analysis has also been successfully used in infant and preschool wheezers to monitor inflammation\(^{(307)}\). Exhaled breath condensate mainly consists of water droplets but also contains small amounts of non-volatile molecules. Airway turbulence during breathing is thought to cause the release of small aerosols of the epithelial lining fluid and to serve as a proxy method of monitoring airway inflammation. A modified gas chromatography system with a closed-glass condenser which enables recirculation of exhaled breath, increases the condensate collection 2 fold and enables collection in less than 10 minutes in young children\(^{(307)}\).

The exhaled breath condensate cytokine profile has been able to differentiate between recurrent wheezers and non-wheezing controls in the Asthma DEtection and monitoring (ADEM) study\(^{(307)}\). The ADEM study started in 2006 in the Netherlands and recruited 258 preschool children (aged 1.9-4.5 years), who were divided into children who never wheezed (n=57), children with 1-3 wheezing episodes (n=78) and children who experienced more than 3 wheezing episodes during life (recurrent wheezers, n=123). Recurrent wheezers had elevated T\(_{\text{H}2}\) cytokines (IL-4, IL-5, IL-13), a proinflammatory cytokine (IL-1a), a T\(_{\text{H}1}\) cytokine (IL-2), a chemokine (IL-8), an anti-inflammatory cytokine (IL-10), and an adhesion molecule (sICAM) when compared to controls\(^{(307)}\). The elevated levels of different types of cytokines fit the concept that in wheezing preschool children, more than one immune mechanism is involved. Children were followed up until age 6 years and those children with persistent wheeze had raised IL-2, IL-4, IL-8 and IL-10 at preschool age when compared to children who never wheezed\(^{(308)}\). However, exhaled breath condensate measurements at 2 and 3 years, did not differentiate between the transient and persistent wheezing phenotype\(^{(308)}\). The cytokine content of exhaled breath condensate could in the future be used to serially monitor inflammation in preschool wheezers, but more validation work is needed.

Several mediators of airway remodelling can be assessed in exhaled breath condensate including MMPs, TIMPs, cysteinyl leukotrienes and endothelins\(^{(309-311)}\). Endothelins mediate increased proliferation of ASM cells and thickening of the RBM\(^{(311)}\). Endothelin-1 was higher in exhaled breath condensate from adult
asthmatics, particularly in those with uncontrolled asthma, when compared with healthy controls\(^{(311)}\). Cysteinyl leukotrienes also increase ASM cell proliferation and a pilot study showed a correlation between cysteinyl leukotrienes and RBM thickness in school aged children with asthma\(^{(310)}\). However, ASM was not quantified in this study\(^{(310)}\). MMPs, particularly MMP-9, are also key mediators in airway remodelling (particularly increased RBM thickness), and have been investigated for their role in the pathogenesis of asthma. MMP-9 levels have been recently shown to be elevated in exhaled breath condensate from children with asthma and inversely correlated with lung function and had a positive correlation with other inflammatory markers such as IL-4 and IL10 in exhaled breath condensate\(^{(309)}\). Exhaled breath condensate could be used to measure mediators of airway remodelling, particularly of ASM hyperplasia and hypertrophy, including alpha-smooth muscle actin, endothelins, cysteinyl leukotrienes on the day of bronchoscopy and EB and relate this to quantified ASM. Serial measurements of exhaled breath analytes could then potentially be used as markers to monitor changes in ASM with wheeze persistence or remission.

The Airway Microbiome

Although the human airways were traditionally thought to be sterile, and there is now evidence that the respiratory tract harbors microbes including bacteria, yeasts, viruses and bacteriophages\(^{(50)}\). The airway microbiome refers to all microbes, their genomes, and environmental interactions in the lower airways. The respiratory tract is likely to be repetitively inoculated with the normal flora of the pharynx and the bacterial load in the airways has been proposed to be in the order of 2000 bacterial genomes per cm\(^2\) of surface area\(^{(50)}\).

Dysregulation of interactions between the immune system and commensal bacteria is one factor that underpins the development of many inflammatory diseases\(^{(312)}\). Several animal studies have investigated the respiratory microbiome and its relationship to the development of allergic diseases such as asthma. Pulmonary exposure of mice to *Escherichia Coli* results in protection against the development of allergic airways disease\(^{(54)}\). Secondly, mice who were intratracheally infected or treated with killed *Streptococcus pneumoniae* before, during or after ovalbumin sensitisation and subsequent challenge had suppression of hallmark features of
allergic airways disease with increased $T_{reg}$ which reduced T cell proliferation and cytokine release\(^{(53)}\). Gastrointestinal *Helicobacter pylori* infection protected ovalbumin stimulated mice from AHR, tissue inflammation, and goblet cell hyperplasia and prevented bronchoalveolar infiltration with eosinophils, $T_{H2}$ cells, and $T_{H17}$ cells\(^{(313)}\). This was more pronounced in those neonatally infected mice than adult mice\(^{(313)}\). In contrast germ free mice (that lack pathogenic and non-pathogenic organisms) develop worse allergic airways disease than those with a normal host flora\(^{(52)}\).

Further evidence for the role of the microbiome in the development of allergic disease is that children who grow up in environments with a wide range of microbial exposures, such as traditional farms, are protected from childhood asthma and atopy\(^{(49)}\). In two recent European cross-sectional studies, microbial diversity and the prevalence of asthma were compared in children living on farms and children who were not farm-dwellers. The PARSIFAL (Prevention of Allergy-Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle) study in Bavaria, Germany screened samples of the children’s mattress dust for bacterial DNA with the use of single-strand conformation polymorphism analyses to detect environmental bacteria that cannot be measured by means of culture techniques\(^{(49)}\). The GABRIELA (Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community) based in five rural areas in southern Germany, Switzerland, Austria and Poland evaluated samples of settled dust from children’s rooms for bacterial and fungal taxa with the use of culture techniques in five rural areas in southern Germany, Switzerland, Austria and Poland\(^{(49)}\). In both studies, children who lived on farms had lower prevalence’s of asthma and atopy and were exposed to a greater variety of environmental microorganisms than the children who did not live full time on farms\(^{(49)}\). Diversity of microbial exposure was inversely related to the risk of asthma in both the PARSIFAL and GABRIELA studies\(^{(49)}\).

School-aged asthmatic children have an abundant lower airway bacterial flora, which is different to that of controls\(^{(60)}\). Furthermore, a pilot study in adults with uncontrolled asthma, showed AHR correlated strongly with airway microbiota composition and diversity\(^{(314)}\). The influence of microbial exposures in infancy and their effects on
asthma development are now recognised\textsuperscript{(48,49,55)}.
The COPSAC cohort study highlighted the importance of bacteria in the development of asthma\textsuperscript{(48)}.
Neonates colonized in the hypopharyngeal region with \textit{Streptococcus pneumoniae}, \textit{Haemophilus influenzae}, or \textit{Moraxella catarrhalis} or with a combination of these organisms, are at increased risk for recurrent wheeze and asthma early in life\textsuperscript{(48)}.
However, more recent developments have demonstrated that the composition of bacterial communities colonising mucosal surfaces, rather than simply the presence of individual species, is important in the pathogenesis of disease\textsuperscript{(49)}.

The airway microbiota composition of preschool wheezers is unknown, but it is likely the microbial flora influences host innate immune responses to infection and allergens in preschool wheezers who develop future asthma. It is also possible that microbial flora will differ between those wheezers whose symptoms remit and those who go on to persistently wheeze. Conventional microbial cultures are inadequate to identify microbiota and only reveal part of the airway microbiome. However, bronchial epithelial brushings or BAL from children and adults with asthma have previously been used to profile the microbiome with high-density 16S ribosomal RNA microarray and parallel clone library-sequencing analysis\textsuperscript{(314)}. A trial of antibiotic therapy could then be initiated and microbial flora, inflammation and immune responses (\textit{T}_{H2}, \textit{T}_{H1}, \textit{T}_{H17}, \text{and} \textit{T}_{\text{regs}}) and mediators of airway remodelling assessed before and after antibiotic therapy. Key of course would be long-term outcome.

\textbf{Conclusion}

In summary, the work of this thesis has showed for the first time that ASM is increased in those preschool children with severe wheeze who go on to develop asthma at school age. The numbers are small and represent children with particularly severe wheeze at preschool age, such that the findings cannot be assumed to be applicable to children with less severe wheeze. However, the data indicate that future studies exploring the mechanisms underlying the persistence of preschool wheeze and its progression to asthma might profitably focus on changes in airway smooth muscle.
References


(26) http://www.ubiopred.european-lung-foundation.org/


(95) Jeffery P. Inflammation and remodeling in the adult and child with asthma. Pediatr Pulmonol Suppl 2001; 21:3-16.


Regamey N, Ochs M, Hilliard TN, Muhlfeld C, Cornish N, Fleming L et al. Increased airway smooth muscle mass in children with asthma, cystic fibrosis,


(238) Kariyawasam HH, Aizen M, Barkans J, Robinson DS, Kay AB. Remodeling and airway hyperresponsiveness but not cellular inflammation persist after allergen challenge in asthma. Am J Respir Crit Care Med 2007; 175(9):896-904.


Dear Mr & Mrs NAME,

I am writing to ask whether you would consider allowing NAME to take part in a research project at the Brompton Hospital.

You will remember that NAME had a camera test called a bronchoscopy at the Brompton Hospital when he was a baby. At that time you very kindly agreed for us to use any samples that were taken, and were left over after his clinical tests, for a research project looking at asthma in young children. We have now completed that study, and have found that overall children that were wheezy when they had their bronchoscopy did have features in their airway samples that suggested asthma. But at that time, we could not say for certain which children would have asthma when they got older and which would no longer be wheezy.

In order to see whether the samples that we took at that young age will help us to see whether a child will have asthma when they are older, we want to ask whether you would bring NAME back to the Brompton Hospital to answer a few questions about his breathing and to do some very simple blowing tests (no bronchoscopy). Details of all of the tests, how long they take and the reasons for doing this study are given in the information sheet enclosed with this letter.

We are very grateful to you for taking the time to read this information.

The doctor doing the research will contact you in a week or so to ensure you have received this information, and at the same time will be very happy to explain the research to you further and answer any questions that you might have. Please bear in mind that you are under no obligation to take part, and you may refuse without giving any reason.

Thank you again for your time and help in considering this information.

Yours sincerely

Dr Ruth O’ Reilly
Clinical Research Fellow
THANK YOU for taking the time to read this leaflet. We are doing a research study looking at breathing problems in children.

What is research?
Research is done to try and find out answers to important questions.

What is this research about?
By doing this research we hope to find out more about why some very young toddlers that have noisy breathing get asthma when they get older.

Why have I been asked to take part?
When you were a young toddler you had a special camera test of your lungs called a bronchoscopy at the Brompton Hospital. As you've had this special test, we now want to ask whether you would take the time to read on and consider taking part in our study.

What is wheezing?
Wheezing is a type of noise that comes from the chest. It can cause difficulty in breathing. Wheezing is very common and can sometimes make children very ill. Luckily, most children grow out of the problem, but some continue to wheeze as they get older and they can develop a problem called asthma. At the moment we can't tell which children will carry on wheezing and get asthma.

Why is this research important?
We want to know whether you have any noisy breathing or wheezing, and whether you might have asthma.

We want to know whether we can recognize who will have asthma at your age by looking at the results of the special bronchoscopy test that you and other children had when you were toddlers.
What happens if I agree to take part?

- We will ask you and your mum or dad to answer some questions about your breathing
- We will ask you to do 2 blowing tests
- We will do some special tests on your forearm to see if you have any allergies
- We will ask you to breathe in some misted air that tastes a bit salty, and then see if you can cough up some phlegm, and ask you to do some blowing tests after you've breathed in the misted air.
- We will ask you to sit still and breathe air mixed with a small amount of a harmless gas called a ‘tracer gas’ slowly from a mouth piece. This gas is not absorbed by your body at all. You may watch a DVD or read a book during this test so you don't get bored.
- We will explain all of the tests and the results to you
- All of the tests can be done in one visit to the hospital. You will be at the hospital for about 3 hours.

Do I have to take part?
You do not have to take part in the project. You do not have to say why not, either.

Will joining in with the research help me?
We cannot promise that taking part will help you, but the information that we get will help us to treat other toddlers and children with wheezing and asthma early and stop them from getting very ill.

What if I don’t have asthma?
- We would still like you to take part so that we can tell the difference between children with and without asthma

What if I already have asthma?
- You may already know whether you have asthma, and if so, we will be happy to give you all of the results from the tests and answer any of your questions.
- Taking part will not affect your asthma medicines, or the way your doctors treat you in any way

What do I do next?
If you would like to know more, or are interested in taking part in the study please tell your mum or dad and they will tell the doctors that are doing the study.

What if I change my mind and don’t want to do the research anymore?
If you agree to take part in the research, you can still change your mind and stop doing the research at anytime. This will not change your normal treatment in any way. You do not have to say why you have changed your mind.
EARLY AIRWAY PATHOLOGY PREDICTS LATER CHILDHOOD ASTHMA
Parent Information Sheet

THANK YOU for taking the time to read this leaflet. We are doing a study looking at children that have previously had a bronchoscopy at the Brompton Hospital. By doing this, we hope to find out more about which infants and toddlers will develop asthma. As your child had a bronchoscopy when he/she was under 5 years old, we wonder whether you would take the time to read on and consider taking part in this study.

What is wheezing?
Wheezing is a type of noise associated with difficulty in breathing, which is very common and can sometimes make children very ill. Luckily, most children grow out of the problem, but some continue to wheeze and get asthma when they get older. At the moment we are unable to tell which children will carry on wheezing. Also, we don’t know which is the best treatment for this problem. A lot of children are given strong medicines that they may not need, and these can cause many side-effects.

Why are we doing this study?
You will remember that when your child was younger, you came to the Brompton Hospital for your child to have a camera test to look at their airways (this was called a bronchoscopy). At the time, you very kindly agreed for us to use any samples (biopsies) that were taken for a research project in which we were looking for changes that suggest asthma. We have completed that study and found that children that were wheezing at the time of the bronchoscopy had some features suggestive of asthma in their airways. We would now like to confirm whether or not the children that had features suggestive of asthma in their biopsies early on have developed asthma in school age.

Why is this study important?
Understanding which infants and toddlers will carry on wheezing and get asthma is very important because if we could predict early which children will get asthma, we might use treatments to stop asthma developing or to minimize its severity.

No other studies have looked at biopsies in such young children to see if they help to identify children that will become asthmatic.

By doing this study we aim to:
1. See which children that were wheezing when they had a bronchoscopy now have asthma (at school age)
2. See if any children that were not wheezing when they had a bronchoscopy have developed asthma (by school age)
3. See whether changes in the biopsies that were taken during bronchoscopy were already present early in the children that now have asthma
What happens if you agree to take part in the study?

- We ask you to answer some questions about any wheezing or related problems, like eczema and hay fever in your family
- We ask you to answer some more specific questions about your child’s chest symptoms (if they have any)
- We would like your child to perform a blowing test so that we can assess how well their lungs are working (test takes 5 minutes)
- We would like your child to perform a second blowing test to see if they have inflamed airways (test takes 5 minutes)
- We would like to test your child for allergies to things like cat, dog, house dust and pollen using skin prick tests (takes about 20 minutes).
- We would like to see if your child has a tendency to be asthmatic by asking him/her to inhale a salty mist for 5 minutes and then doing the blowing test for lung function again. This would be repeated four times for a maximum of up to 20 minutes. During this test we would also see if your child can cough up a sample of sputum. This would then be used to look for inflammation in the airways. A small part of this sample would be frozen and stored for future tests to help identify asthma. (test takes about 40 minutes).
- We would like to do another test to see how the smaller airways in your child’s lungs are working by asking him/her to breathe in air with a small amount of a harmless ‘tracer gas’ while breathing normally. This gas is not taken up by the body at all, it is just breathed out again unchanged, and is harmless. After a few minutes the gas will be disconnected and we will continue to measure breathing while they breathe normal room air again. This would be repeated 3 times. This test is a measure of how well gases are mixed in the lungs and will tell us if there is any obstruction in the smaller airways that may not be picked up by the previous blowing tests. This test has been performed before in hundreds of babies, children and adults with no problems. (test takes about 45 minutes).
- We will be very happy to tell you the results once they are available.
- All information relating to your child will be completely confidential.

Where will the tests be done?

All of the tests will be done at the Brompton Hospital. They can all be completed in one visit lasting approximately 3 hours. We will reimburse your reasonable travel fare to and from the hospital.

What will happen once the tests have been done?

- Once the tests have been completed, we will look at the results to see whether your child has asthma.
- We will then go back to the biopsies that we took when they had a bronchoscopy. The biopsies will be looked at in detail under the microscope to see if they have features of asthma.
- Results from the current tests and previous biopsies will then be put together to see whether or not the early biopsies correctly predicted asthma in school age.
Will we be told the results?

We will be very happy to tell you the results of the study, once it has been completed.

If the tests show your child has asthma, or there are any other findings that you did not know about, we will tell you and will advise on any appropriate treatment that is needed. Your GP will also be informed of the results.

What happens next?

- If you would like to know more, or are interested in taking part in the study, please contact:
  Dr Ruth O'Reilly, Clinical Research Fellow, by phoning 077 72056622 or 020 73528121 Extension 2257
  email roreilly@imperial.ac.uk

OR

Dr Sejal Saglani, Clinical Senior Lecturer, by phoning 020 7594 3167 or email s.saglani@imperial.ac.uk

What if we change our mind?

If you and your child agree to take part in the study, but then either or both of you change your mind, you may withdraw at anytime. This will not change your child’s normal treatment in any way. You do not have to give any reason for withdrawing, or not taking part at all.

What if we don’t take part in the study?

It is fine to decline to take part. You do not have to give a reason. If you do not wish your child to take part in the study, this will not alter your child’s normal treatment in any way.

Imperial College London holds insurance policies which apply to this study. If your child experiences harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If your child is harmed due to someone’s negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way your child has been treated during the course of this study then you should immediately inform the Investigator (Dr Sejal Saglani, Tel: 0207 594 3167). The normal National Health Service complaint mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College Clinical Research Governance Office.

THANK YOU VERY MUCH FOR YOUR TIME AND PATIENCE IN READING THIS LEAFLET
STUDY INFORMATION & CONSENT FORM

Title of project: Early airway pathology predicts later childhood asthma

EXPLANATION OF PROJECT

We would like to invite you and your child to take part in this clinical study which we hope will help us to understand more about which wheezy infants will go on to develop asthma.

We know from previous research that although many children under 5 years old suffer from wheezing, only about one-third of wheezers develop asthma by school age. The aim of this study is to see if we can use information from tiny pieces of tissue (biopsies) taken from a child’s airways to predict which wheezy infants and preschool children will develop asthma. If we can find a predictor of future asthma, then we can look for treatments that can be used very early to either stop the development of asthma or minimise its severity.

You will remember that when your child was very young, you came to the Brompton Hospital for them to have a camera test to look at their airways (this was called a bronchoscopy). At the time, you very kindly agreed for us to use any samples that were taken for a research project in which we were looking for changes that suggest asthma. We have completed that study and found that children that were wheezing at the time of the bronchoscopy had some features suggestive of asthma in their airways. We would now like to confirm whether the children that had features suggestive of asthma early on are still wheezing and whether they have developed asthma in school age.

In order to do this, we would like to ask whether you would consider coming back to the Brompton Hospital with your child for the following tests:

i) We would ask you and your child some questions about their breathing, and how they have been since the bronchoscopy. This takes about 40 minutes

ii) We would like your child to perform a blowing test so that we can assess how well their lungs are working. This takes about 5 minutes

iii) We would like to perform a second blowing test to see if your child has inflamed airways. This also takes about 5 minutes

iv) We would like to test your child for allergies using skin prick tests. This takes approximately 20 minutes

v) We would like to see if your child has a tendency to be asthmatic by asking him/her to inhale a salty mist for 5 minutes and then doing the blowing tests for lung function again. This would be repeated four times for a maximum of up to 20 minutes. During this test we would also see if your child can cough up a sample of sputum. This would then be used to look for inflammation in the airways. A small part of this sample would be frozen and stored for future tests to help identify asthma.
vi) We would like to do another test to look at how well your child’s airways are working by asking him/her to inhale air containing a small amount of a harmless ‘tracer gas’ while breathing normally. We will then see how quickly the ‘tracer gas’ is removed from the lungs during continued normal breathing. This will be repeated 3 times and will take approximately 45 minutes.

All of this would be done on one day and would take approximately 3 hours. The tests will all be done by a children’s doctor. If any of the tests make your child feel uncomfortable, then they will be stopped.

The information obtained from the answers to the questions and the tests will allow us to work out whether or not your child now has asthma. We will then relate this back to the biopsies that we took from your child when they had their bronchoscopy to see if early information from the biopsies tallies with whether your child now has asthma or not.

Although taking part in this study will not benefit your child directly, it will allow us to help other children in the future. We will know whether information from airway biopsies taken early from wheezy preschool children can be used to identify which children will develop asthma.

Please take the chance to discuss this study fully, asking any questions that you need to, before deciding whether or not to take part. If you do not wish your child to take part, this will not affect his or her current or future treatment at the Brompton Hospital. If you and your child do agree to take part, but later decide you would like to withdraw from the study, you may do so at any time, and you do not have to give a reason and this will not affect your child’s treatment in any way. Thank you very much for your time.
CONSENT FORM FOR PARENTS & CHILDREN

Title of project: Early airway pathology predicts later childhood asthma

Name of researcher:

1. I confirm that I / my child have read and understand the information sheet dated 12th Jan 2009 v3 for the above study. I have had the opportunity to ask questions and have had these answered satisfactorily. ☐

2. I understand that my child’s participation is voluntary and that we as parents or he/she is free to withdraw at any time without giving any reason and without their medical care or legal rights being affected. ☐

3. I understand that sections of my child’s medical notes may be looked at by responsible individuals from the Brompton Hospital where it is relevant to my child taking part in this research. I give permission for these individuals to access my child’s records that are relevant to this research. ☐

4. I understand that samples from the study will be stored and may be used in future for similar tests. ☐

5. The compensation arrangements have been discussed with me. ☐

6. I agree to the child named below taking part in this study, and I am their parent / legal guardian. ☐

___________________________  ________________________  ______
Name of child               Signature                Date

___________________________  ________________________
Name of parent/legal guardian Signature                Date

___________________________  ________________________
Name of person taking consent Signature                Date
Dear Doctor

This is to inform you that your patient __________________________, DOB:____________
is taking part in the above research project being carried out at the Royal Brompton Hospital, London.

This study is following up children that have previously had a bronchoscopy at the Brompton hospital, aged 3 months to 5 years, between October 2002 and May 2005. We are looking at the current asthma status of the previously bronchoscoped children, and will relate this to pathological findings in airway biopsies that were taken during the bronchoscopy to see whether early airway pathology in the infant and preschool years predicts the development of asthma by school-age.

The children will undergo the following:
1. An interview / questionnaire with parents to assess current asthma symptoms
2. Skin prick tests to 5 common aero-allergens and 3 common food allergens
3. Spirometry
4. Measurement of exhaled nitric oxide
5. Combined sputum induction and assessment of airway hyperresponsiveness using 4.5% nebulised saline
6. Measurement of Lung Clearance Index (lung function test that gives a measure of small airways function)

All tests will be performed at the Brompton Hospital. Parents and children will be told the results. If any previously unknown results are found, we will write to you and let you know these, with our recommendations for future management and follow-up. There are no implications for the treatment of the child or long term follow-up.

If you have any questions relating to this study, please do not hesitate to contact the Principle Investigator:
Dr Sejal Saglani
Clinical Senior Lecturer & Honorary Consultant in Respiratory Paediatrics
Imperial College London & The Royal Brompton Hospital
374 Sir Alexander Fleming Building
Exhibition Road, London SW7 2AZ
Tel: 020 7594 3167
s.saglani@imperial.ac.uk

Thank you
Yours sincerely

Dr Sejal Saglani BSc, MBChB, MRCPCH, MD
## Appendix 6

**Date:**

<table>
<thead>
<tr>
<th>Name</th>
<th>Hospital No</th>
<th>DOB</th>
<th>Address</th>
<th>Telephone</th>
</tr>
</thead>
</table>

### Birth History:

- Gest _________
- Wgt_________
- Scubu/NICU Yes/ No
- Vent Days_______
- Cpap Days_______

### Smoking

- Smoking during pregnancy (mother)
- Smoking during pregnancy father (lived in house)
- Does anyone in the house smoke now?

<table>
<thead>
<tr>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Father</th>
<th>Mother</th>
<th>Other adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

### Day Care

- Did your child attend nursery?
- What age_______months
- Length of time_______ months

### Animals

- Do you live on a farm?
- Do you have pets at home?

<table>
<thead>
<tr>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</table>

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</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

### Medications in First 2 years of life

- Inhaled Corticosteroids ____________________
- Inhaled Salbutamol ________________________
- No of courses of Oral Steroids _____________
- Monteleukast Yes /No

### Medications Aged 2-5 years

- Inhaled Corticosteroids ____________________
- Inhaled Salbutamol ________________________
- Monteleukast Yes /No

<table>
<thead>
<tr>
<th>Number of courses of Oral steroid/year</th>
<th>Age 3</th>
<th>Age 4</th>
<th>Age 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 3</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Age 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age 5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Medications Age 5 to date

- Inhaled Corticosteroids ____________________
- Inhaled Salbutamol ________________________
- Monteleukast Yes /No
Number of courses of Oral steroid/year

Age 5-7 _________

Age 7+ __________

**ISAAC Core questionnaire wheezing module for 6–7 year olds**

1. Has your child ever had wheezing or whistling in the chest at any time in the past? Y/ N
   IF YOU ANSWERED "NO" PLEASE SKIP TO QUESTION 6
   When was the first time? ______________ Age months

2. Has your child had wheezing or whistling in the chest in the last 12 months? Y/N
   IF YOU ANSWERED "NO"
   When was the last time your child had wheeze __________ Age months
   Continue to question 6

3. How many attacks of wheezing has your child had in the last 12 months?
   None [ ] 1 to 3 [ ] 4 to 12 [ ] More than 12 [ ]
   On average how many attacks does your child have per year?
   None [ ] 1 to 3 [ ] 4 to 12 [ ] More than 12 [ ]

4. In the last 12 months, how often, on average, has your child's sleep been disturbed due to wheezing?
   Never woken with wheezing [ ] Less than one night per week [ ]
   One or more nights per week [ ]

5. In the last 12 months, has wheezing ever been severe enough to limit your child's speech to only one or two words at a time between breaths? Y/N

6. Has your child ever had asthma? Y/ N
   Please Circle      Doctor diagnosed      Parent diagnosed

7. In the last 12 months, has your child's chest sounded wheezy during or after exercise? Y/N

8. In the last 12 months, has your child had a dry cough at night, apart from a cough associated with a cold or a chest infection? Y/N

9. Does your child wheeze
   only with colds      multi trigger      Atopic

10. Any hospital visits with wheeze?_______________ Number
    If yes Age of last admission:_________ years

**Family History of Asthma**  yes/ No
   Who?

**Isaac Core questionnaire rhinitis module for 6–7 year olds**

11. Has you child ever had a problem with sneezing, or a runny, or a blocked nose when he/she DID NOT have a cold or the flu? Y/N
    IF YOU ANSWERED "NO" PLEASE SKIP TO QUESTION 16
12. In the past 12 months, has your child had a problem with sneezing, or a runny, or a blocked nose when he/she DID NOT have a cold or the flu?  
Y/N  
**IF YOU ANSWERED "NO" PLEASE SKIP TO QUESTION 16**

13. In the past 12 months, has this nose problem been accompanied by itchy-watery eyes?  
Y/N

14. In which of the past 12 months did this nose problem occur? (please tick any which apply)  
January [ ] February [ ] March [ ] April [ ] May [ ] June [ ] July [ ] August [ ] September [ ] October [ ] November [ ] December [ ]

15. In the past 12 months, how much did this nose problem interfere with your child's daily activities?  
Not at all [ ] A little [ ] A moderate amount [ ] A lot [ ]

16. Has your child ever had hay fever?  
Y/N

**Core questionnaire eczema module for 6–7 year olds**

17. Has your child ever had an itchy rash which was coming and going for at least 6 months?  
Y/N  
**IF YOU ANSWERED "NO" PLEASE SKIP TO QUESTION 22**

18. Has your child had this itchy rash at any time in the last 12 mths?  
Y/N  
**IF YOU ANSWERED "NO" PLEASE SKIP TO QUESTION 22**

19. Has this itchy rash at any time affected any of the following places: the folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears or eyes?  
Y/N

20. At what age did this itchy rash first occur?  
Under 2 years [ ] Age 2–4 [ ] Age 5 or more [ ]

21. Has this rash cleared completely at any time during the last 12 months?  
Y/N

22. In the last 12 months, how often, on average, has your child been kept awake at night by this itchy rash?  
Never in the last 12 months [ ] Less than one night per week [ ] One or more nights per week [ ]

22. Has your child ever had eczema?  
Y/N