UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Division of Allergy and Inflammation Sciences

Bronchoalveolar Lavage Findings in Infant Wheeze

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Thesis submitted for the degree of Doctor of Medicine

March 2004

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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BRONCHOALVEOLAR LAVAGE FINDINGS IN INFANT WHEEZE

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Wheezing in infancy is common but the immune mechanisms underlying this process are not well understood. Some infants grow out of their symptoms whilst others persist. Persistent infant wheezing is associated with atopy and this would suggest that airway remodelling has occurred in a manner similar to atopic asthma. Matrix metalloproteinase (MMP)-9 and Tissue Inhibitor of MMP (TIMP)-1 have been implicated in airway remodelling. The aim of this study was to consider the role of atopy infant wheeze and its relationship to the persistence of symptoms by the investigation of bronchoalveolar (BAL) and nasal lavage (NAL) in infancy.

In an international multicentre study, 52 severe infant wheezers underwent clinical investigation by bronchoscopy with BAL and concomitant NAL. Differential cytology, ECP, IL-8, sICAM-1, MMP-9 and TIMP-1 concentrations were measured in the lavages. A subgroup of the study was followed up by questionnaire at a mean age of four years to look for persistence of symptoms and other allergic diseases.

A high proportion of wheezers had bacterial infection of BAL and lavage neutrophillia of both upper and lower respiratory tract as well as MMP-9 TIMP-1 molar ratio imbalance. Previously, viral but not bacterial infection has been associated with wheeze. Atopic wheezers did not show either an eosinophillia or MMP-TIMP imbalance, but correlations in BAL suggested that atopic wheezers had inflammation linked to epithelial cell shedding. In infants with Allergic Rhinitis (AR), eosinophils were raised in NAL and also in BAL with signs of epithelial shedding once more. This would suggest that airway remodelling was occurring in these children. NAL was sufficiently different from BAL not to provide a useful surrogate for this invasive procedure. Persistent wheeze was associated with clinical AR, BAL TIMP-1 and serum sICAM-1 concentrations in infancy.

This study suggests that persistent infant wheezers have airway remodelling, which involves airway fibrosis via TIMP-1, occurs early in the disease, and is linked to nasal symptoms in a manner similar to adult allergic asthma.

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AUTHOR'S DECLARATION

The clinical data from the bronchoscopy study was taken from a multicentre study on the action of cetirizine in infant wheeze. The author was co-investigator for the Southampton site.

The laboratory and follow up components of this work represents my own work except for the MMP-9 and TIMP-1 analysis, which was performed by Dr Sarah Dobson and Dr Puja Kochhar in the laboratory of my supervisor Dr Jane Warner.

UCB Pharma SA sponsored both BAL and Questionnaire Follow Up study and paid for my salary as a research fellow.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr Jane Warner and Prof John Warner for their advice and support during the preparation of this manuscript.

I would also like to thank and acknowledge the assistance of the following people:

Mrs Wendy Wilcocks and Mrs Linda Hopkins who cheerfully provided secretarial support.

Dr Gary Connett (Southampton), Dr Petr Pohunek (Prague), Prof Isidore Dab (Brussels) and Prof Jacques de Blic (Paris) recruited subjects to the lavage study. I would like to thank Dr Petr Pohunek especially for his for enthusiasm for the follow up study. Dr Isabella Annesi-Maesano provided the French questionnaire. Dr Ann Mander, Dr Jude Holloway, Dr Jackie Madden, Miss Eleanor Popplewell, Dr Laurie Lau and Dr Susan Wilson taught me the techniques and gave advice on the laboratory aspects of the study. Dr Stefan Ahlstedt (Pharmacia, Uppsala, Sweden) kindly donated the ECP radioimmunoassays used in this study. Dr Sarah Dobson and Dr Puja Kochhar performed the MMP-9 and TIMP-1 analysis during their intercalated BSc projects as undergraduates under the supervision of Dr Jane Warner. Dr Christian Agboton, Dr Sandra Staellert, Dr Marc de Longueville, Ms Christina Verkest and Ms Danielle Gerard of UCB Pharma SA, Belgium provided the clinical data from the other centres. Dr Linda Hunt, (Senior Lecturer in Medical Statistics, University of Bristol) for her assistance with statistical analysis.

Last but by no means least, Dr Catherine Powell, Dr Gill Vance, Dr Mark Beattie and Dr Margaret Fletcher for their advice and support with this work during the various phases of this study.

ABBREVIATIONS

ABPA	Allergic Bronchopulmonary Aspergillosis
AD	Atopic Dermatitis
APC	Antigen Presenting Cell
AR	Allergic Rhinitis
ARIA	Allergic Rhinitis and its Impact on Asthma
BAL	Bronchoalveolar Lavage (second and subsequent lavages)
BW	Bronchial Washing (first lavage)
CD	Cluster Differentiation molecule i.e. CD40, CD14 etc…
EBM	Epithelial Basement Membrane
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
ELF	Epithelial Lining Fluid
EPX	Eosinophil Protein X
ETAC	Early Treatment of the Atopic Child study
ETS	Environmental Tobacco Smoke
FcεR I	High Affinity IgE receptor
FEV1	Expiratory flow at 1 second (PEFR substitute)
FOB	Fibre Optic Bronchoscopy
FMLP	*fmet-leu-phe.* The bacterial signal amino acid sequence recognised by the immune system as proinflammtory
FRC	Functional Residual Capacity
GAG	Glycosaminoglycan
HDM	House Dust Mite
ICAM-1	Intercellular Adhesion Molecule 1
ICS	Inhaled Corticosteroids
IFNγ	Interferon gamma
IgE	Immunoglobulin E
IL	Interleukin
IL-8	Chemokine interleukin 8 (CXCL-8)
ISAAC	International Study of Asthma and Allergy in Childhood
JAK	Janus Kinase
kDa	Kilo Daltons (molecular weight)

KWT	Kruskal Wallis Test
LFA	Leucocyte Functional Antigen
LPS	Lipopolysaccharide
LRT	Lower Respiratory Tract
MHC	Major Histocompatability Complex
MMP	Matrix Metalloproteinase
MWU	Mann Whitney U test
NAL	Nasal lavage
NBL	Non-bronchoscopic Lavage
OCS	Oral Corticosteroids
PEFR	Peak Expiratory Flow Rate
RANTES	*'Regulated upon Activation Normal T cell Expressed and Secreted'*. Chemokine CCL5.
RAST	Radio Allergosorbent Test
RLL	Right Lower Lobe
RSV	Respiratory Syncitial Virus
RUL	Right Upper Lobe
sICAM-1	Soluble Intercellular Adhesion Molecule 1
sIL-2 R	Soluble IL-2 Receptor
SPT	Skin Prick Test
STAT	Signal Transducers and Activators of Transcription
TCR	T cell Receptor
TGF-β	Transforming Growth Factor β
Th $_{1/2}$	T helper cell cytokine battery 1 or 2
TIMP	Tissue Inhibitor of Metalloproteinase
TLR	Toll Like Receptor
TNCC	Total Nucleated Cell Count
TNF	Tumour Necrosis Factor
URTI	Upper Respiratory Tract Infection
VCAM-1	Vascular Cellular Adhesion Molecule 1
V_{max}FRC	Maximum flow at FRC (a surrogate for PEFR in infant lung function studies)

DEDICATION

This work is dedicated with love to my beautiful family

Carole, Isabelle, Juliette, Josephine
and to Gabriel, our new arrival

Also to the memory of my father

Carl Farrington René Erlewyn-Lajeunesse

who died during the preparation of this thesis.

And as imagination bodies forth
The forms of things unknown, the poet's pen
Turns them to shapes, and gives to airy nothing
A local habitation and a name.

A Midsummer Night's Dream
William Shakespeare

Chapter 1: Introduction: Infant wheeze

1.1. What's in a name?

Wheezing in infancy is a common condition whose variable prognosis has been recognised for at least half a century (Korppi and Kotaniemi-Syrjanen 2003). Over the years there have been several terms attributed to the disorder including wheezy or eosinophillic bronchitis, bronchiolitis, viral associated (or induced) wheeze and also the concept of the happy wheezer, who has symptoms, but is troubled little by them. The terms have often been used interchangeably fuelling confusion around terminology.

The term *asthma* is now not often used in this age group, as practitioners do not wish to confuse infant wheeze, which is frequently a temporary condition, with the chronic chest disease of adulthood. Even the name *asthma* is a purely descriptive term, being the Greek for difficulty in breathing. As Silverman and Wilson elegantly state, all of these terms fall short of a definition:

'None of these terms adequately describe the range of symptoms and signs, and the varying natural history of the disease's progress through childhood.'

(Silverman and Wilson 1997a)

This is because the various labels for wheezing in infancy are merely descriptive terms for a disease whose underlying cause is not fully understood, and our lack of understanding is exemplified by its nomenclature. For example, *infant wheeze* is the equivalent of defining a fracture of the neck of the femur as a *painful hip*, where the symptoms are described without reference to the cause. It is by understanding the pathology of wheezing in infancy that progress in treatment and prevention can be made.

In this study the terms infant and preschool wheezing are used interchangeably. There is no accepted definition for infant wheezing. To be precise, infant wheezing should relate to children under a year and preschool wheezing to those less than five years old. However, as all infant wheezers grow into preschool wheezers quite

quickly the distinction is not always particularly useful. More recently the terms transient or persistent wheeze have been used (Martinez *et al.* 1995).

The introduction is divided into two chapters: the first part considers our knowledge of wheezing in the preschool child, its epidemiology and our ability to predict which children will go on to develop asthma. There is a particular focus on the role of paediatric bronchoscopy and methods of bronchoalveolar lavage. The second chapter discusses the concepts that encompass our current understanding of atopic asthma in adults and how these relate to preschool wheeze.

1.2. Aims and objectives

In order to provide an orientation to the work as a whole, the aims and objectives of the studies contributing to this thesis are considered at the outset. They were to investigate three areas of the pathogenesis of infant wheeze, with reference to the bronchoalveolar lavage findings.

The hypotheses examined were:

- That those infant wheezers with atopy will have a different inflammatory process in the lower airways than non-atopic wheezers and specifically that an imbalance of matrix metalloproteinases with their cognate inhibitors will occur.
- That this process will be mirrored by inflammation of the upper respiratory tract.
- That an imbalance in concentrations of matrix metalloproteinase and its cognate inhibitor in bronchoalveolar lavage will be related to this process.
- That nasal lavage, bronchial washings and serum provide a surrogate for bronchoalveolar lavage in infant wheeze.
- That the presence of eosinophillic inflammation in the airways of wheezy infants will predict the persistence of respiratory symptoms.

1.3. Clinical assessment of the wheezy infant

There is no diagnostic test for infant wheezing and as such it remains a clinical diagnosis (Scheinmann *et al.* 1998). Most infant wheezers are managed on clinical grounds without recourse to further investigations (de Vries *et al.* 2000). However, it is occasionally clinically necessary to investigate preschool wheezers.

1.3.1. Wheezing

Wheezing is both a symptom and a clinical sign. It is an onomatopoeia, described as musical or whistling sound occurring during breathing, and is the cardinal sign of obstructive airways disease. Wheeze is caused by airflow limitation through partially collapsed airways. This causes vibration with sound production in a manner similar to that of blowing the reed of a musical instrument, such as a clarinet or oboe (Bouchier and Morris 1982). Wheezing is related to both airflow velocity and the diameter of the airway, but total lung volume, and the compliance of the airway wall, may also be important in sound generation (Frey 2001). Parental reporting of recurrent wheeze should be considered in the context of a careful and thorough history (Young *et al.* 2002).

1.3.2. Causes of infant wheeze

Although the majority of infant wheeze is caused by an inflammatory disorder of unknown origin, a range of secondary disorders can cause wheezing, and these should be considered in the severe or atypical preschool wheezer. Chronic wheezing before six months old is more likely to be due to a secondary cause (Schellhase *et al.* 1998). Secondary causes are shown in Table 1 and can be divided into pulmonary and extra-pulmonary, congenital and acquired.

1.3.3. Investigation of infant wheeze

Careful assessment is required to identify these secondary wheezers from the more common inflammatory phenotypes. First line investigations include chest radiography, investigation for cystic fibrosis and other causes of increased susceptibility to infection including primary ciliary dyskinesia and

hypogammaglobulinaemia. Positive skin prick tests or raised serum IgE will augment a history of atopy. A therapeutic trial of bronchodilator or inhaled steroid may also be considered, although the role of inhaled corticosteroids in preschool wheeze remains controversial (Oommen *et al.* 2003a).

Pulmonary	Vocal cord palsy Laryngo and airway malacia Tracheal webs Tracheo-oesophageal fistulae Bronchogenic cysts Inhaled foreign body Cystic fibrosis Ciliary dyskinesia Bronchiectasis
Extrapulmonary	Gastro-oesophageal reflux disease Cardiac and great vessel abnormalities Cardiac failure Vascular rings Compression from a mediastinal mass

Table 1 Secondary causes of infant wheeze

This table shows many of the reported causes of infant wheeze (Carlsen 1997; Grimfeld 1997; Krieger 2002; Masters et al. 2002; Payne et al. 2000; Stewart et al. 2002).

Second line investigations include a lateral chest radiograph and the exclusion of significant gastro-oesophageal reflux. Further imaging of the chest such as computed tomography might be appropriate. Other investigations of the infant airway will depend upon local expertise (Nicolai 2001), and include infant lung function testing and bronchoscopy. Lung function testing in infancy requires sedation, whilst bronchoscopy requires intravenous access and deep sedation or general anaesthesia. As such both are not undertaken lightly. In spite of this, lung function and paediatric bronchoscopy are becoming routine investigations for infants with chest disease. In a recent series they were being performed in up to 10% of children referred to a US tertiary paediatric pulmonologist (Strunk 2002).

1.4. Paediatric bronchoscopy

Flexible paediatric bronchoscopes are fibre optic endoscopes that may be passed orally or via the nose, and can be used with or without prior endotracheal intubation depending on the clinical circumstances. The procedure is performed under deep intravenous sedation (Raine and Warner 1991) or general anaesthetic with topical anaesthetic applied to the airways to reduce coughing.

Flexible fibre optic bronchoscopy (FOB) is an increasingly popular (Godfrey *et al.* 1997) and safe (de Blic *et al.* 2002) tool for the investigation of paediatric respiratory disease. It has been used for the investigation of difficult asthma in childhood (Payne *et al.* 2001) and infant wheeze (Schellhase *et al.* 1998). Many of the secondary causes of wheezing can be detected by bronchoscopy, and it is where these diagnoses are suspected by both parent and physician that it becomes appropriate to investigate (Nicolai 2001). Thus FOB is reserved for the minority of infant wheezers whose first line investigations remain inconclusive, and whose symptoms are severe enough to warrant further investigation.

FOB provides several sources of clinical information (Schellhase *et al.* 1998). Firstly, it gives a direct view of the upper and lower airways including larynx and vocal cords, trachea and main bronchi. The anatomy of the respiratory tree can be identified and the presence of obstruction or inflammation of the mucosa directly observed. These pictures can be documented as digital images for later review.

Paediatric bronchoscopes above an external diameter of 3.5mm contain a suction channel in addition to a fibre optic 'light' channel. The suction channel can be used for therapeutic lavage of thick secretions such as those causing lobar collapse and alveolar proteinosis, and also allows access with bronchial brushes and endobronchial biopsy forceps. Although FOB can identify inhaled foreign bodies, they can only be removed using a rigid bronchoscope and for this reason, the latter is the preferred method of endoscopies when a foreign body is suspected.

1.4.1. Bronchoalveolar lavage techniques

The suction channel also permits the instillation and recovery of warmed saline into the airway. Bronchoalveolar lavage (BAL) provides a sample of the cells, microorganisms and epithelial lining fluid (ELF) (Figure 1) present in the terminal bronchioles and alveolar spaces. BAL is most frequently used to provide microbiological diagnosis of lower respiratory tract infection. It is a safe procedure (Schellhase *et al.* 1999), with self-limiting fever in the 24 hours following lavage being the most common consequence of lavage.

BAL can be performed under direct vision or blind using a non-bronchoscopic technique. With bronchoscopic BAL (de Blic *et al.* 2000), the endoscope is positioned in the right middle lobe (or other area in localised disease) and advanced until it becomes wedged against the bronchial walls. Warmed sterile saline in instilled into the airway before being suctioned into a trap via the suction channel.

Several aliquots of lavage are taken during BAL. They may be analysed separately or pooled. The first aliquot is known as the bronchial wash and second or subsequent aliquots are the bronchoalveolar lavage. Laboratory analysis of BAL involves microbiological, cellular and non-cellular components. Samples for microbiological analysis should be taken before any processing occurs to prevent accidental contamination.

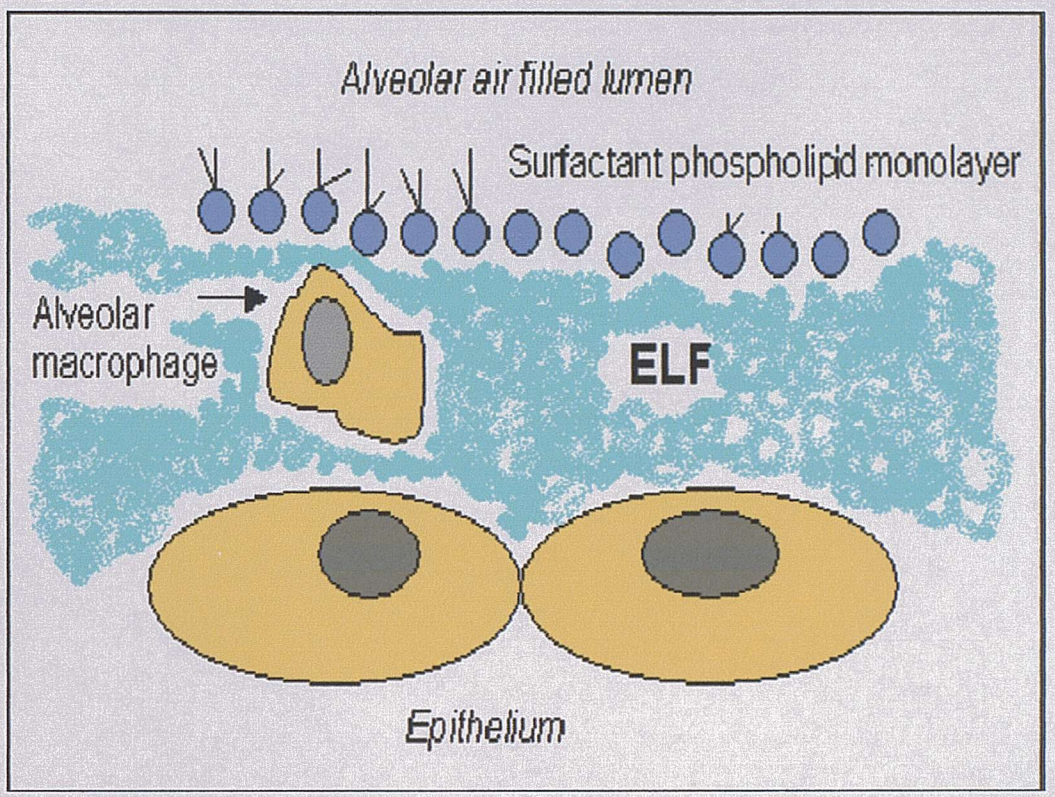


Figure 1 Epithelial lining fluid

The epithelial lining fluid (ELF) contains surfactant, cellular and non-cellular components, such as proteins and inflammatory mediators. The ELF forms the interface between the epithelium and the air space of the alveolus.

1.4.2. Lavage volume

Different lavage volumes have been used in paediatric bronchoscopy (de Blic *et al.* 2000). There is no set method for the calculation of BAL instillation volumes. Lavage can be performed using fixed volume aliquots, or volumes that reflect the size of the child (ml/kg) or their functional residual capacity (% FRC). Higher lavage volumes are required in infancy as the bronchoscopic wedge occurs at a more proximal point in the airway, reducing the volume of lavage return (Najafi *et al.* 2003).

1.4.3. Non-bronchoscopic lavage (NBL)

Non-bronchoscopic methods also permit safe lavage of the lower respiratory tract (Heaney *et al.* 1996). They have been performed on children attending day case

surgery for a variety of non-respiratory procedures. The method involves the passage of a suction catheter through the endotracheal tube of the anaesthetised patient until wedged in a distal airway. Control subjects have been recruited during day case surgery using this method, which has the advantage of being quick, safe and has been perceived as being less invasive than bronchoscopy (Stevenson *et al.* 1997).

1.5. BAL cytology in healthy children

Light microscopic examinations of BAL slide preparations reveals five main cell types: alveolar macrophages, lymphocytes, neutrophils, eosinophils and ciliated epithelial cells. Differential counts have been performed on bronchoscopic lavage from normal children and the results are summarised in Table 2. Ratjen *et al* (1994) recruited healthy children from non-respiratory surgical lists. Other studieshave considered that children with upper airway abnormalities such a laryngomalacia could provide normal data (Clement *et al,* 1987; Midulla *et al*, 1995; Riedler *et al*, 1995; Tessier *et al,* 1996; Mander, 1999). These data are summarised in the European Task Force on BAL in children (de Blic *et al* 2000). The degree to which lower respiratory tract pathology had been excluded in these 'normal' controls is variable. Riedler *et al* (1995) only enrolled children without lower respiratory symptoms who had also undergone rigorous chest investigations, the attention to detail in this study has not been matched by the others, and children in these studies may have had undiagnosed chest pathology. Heaney *et al* (1996) have considered the normal values in BAL using non-bronchoscopic lavage methods, which have not been shown so as to focus attention upon data from bronchoscopic BAL.

In normal BAL, alveolar macrophages predominate with lymphocytes as the second most common cell type. Neutrophils are present in small numbers or are absent from healthy BAL. Eosinophils are infrequently found in normal BAL. Mast cells are also rare and require specific staining techniques for detection. The main cell types are shown in the photomicrographs (Figure 2). Ciliated epithelial cells are frequently encountered. In 'wet prep' slides they are often still viable and can be identified by the presence of motile cilia on their apical surfaces.

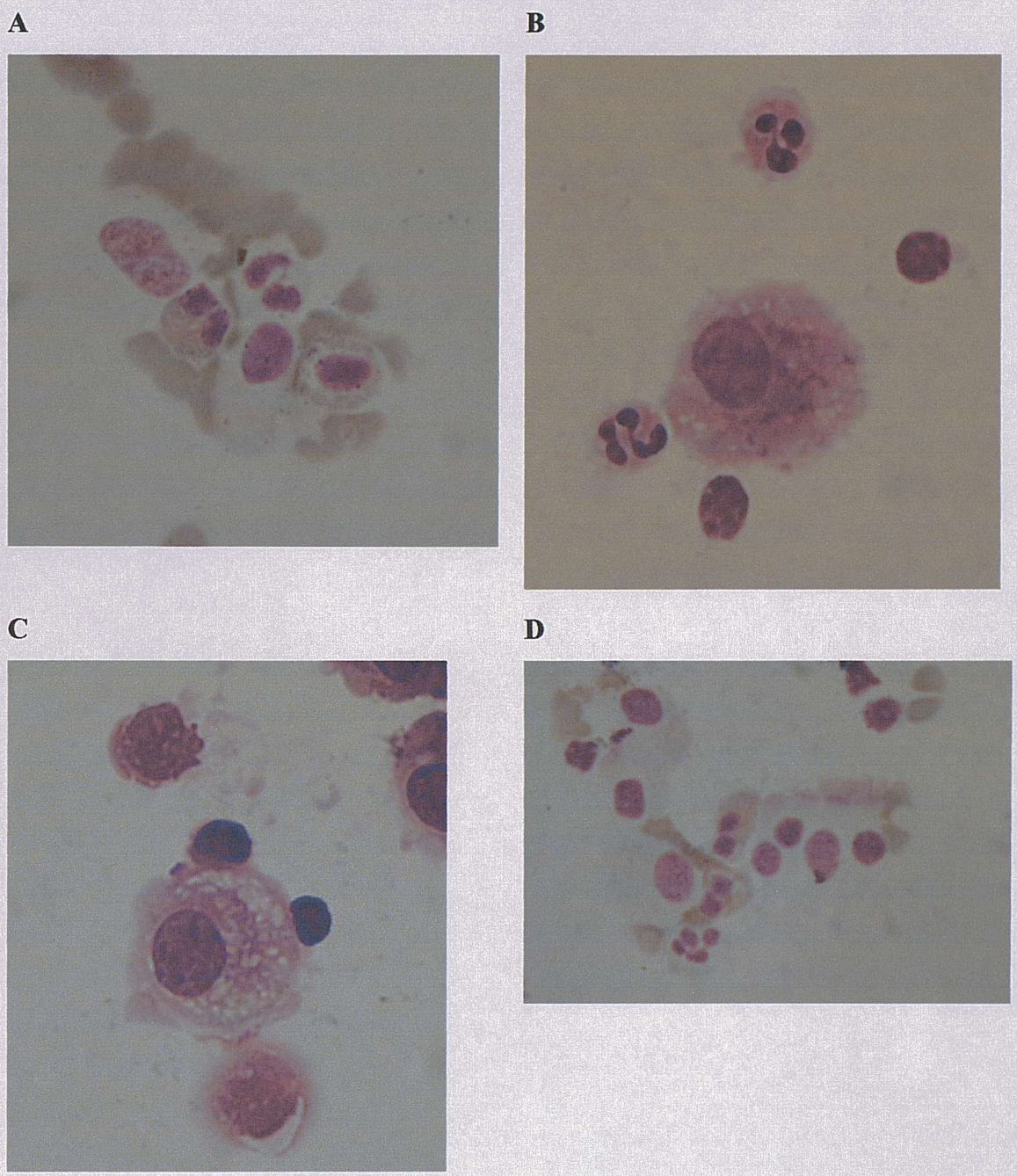


Figure 2 Photomicrographs of bronchoalveolar lavage cells

A BAL differential (X 600) showing from left to right alveolar macrophage, eosinophils, neutrophil, ciliated epithelial cell and another macrophage. ***B*** *BAL differential (x 600) showing the three major cell types of normal lavage, macrophages (centre), lymphocytes (down and right) and neutrophils (up and left).* ***C*** *BAL differential (x 600) showing lymphocytes adherent to the surface of a macrophage as is commonly seen.* ***D*** *BAL differential from asthmatic bronchial washing showing ciliated epithelial cells amongst eosinophils.*

	Clement *et al* (1987)	Ratjen *et al* (1994)	Riedler *et al* (1995)	Midulla *et al* (1995)	Tessier *et al* (1996)	Mander (1999)
n	11	48	18	16	11	29
Volume instilled	10% FRC	3ml/kg	3ml/kg	20ml	10% FRC	20ml
Mean recovery (%)	ND	58	62 (median)	43	70	57
Mean total cell counts (x10^5cells/ml)	2.55	1.03	1.55 (median)	5.99	3.51	2.77
Viable cells %	ND	ND	ND	ND	ND	89 (80-93)
Epithelial cells %	ND	ND	ND	ND	ND	1.7 (0-30.0)
Alveolar Macrophages %	90 (85 – 97)	84 (34.6-94)	91 (84.2-94)	87 (71-98)	92.5 (77-98)	83 (71-99)
Neutrophils %	1.3 (0 – 3)	0.9 (0-17)	1.7 (0.6-3.5)	3.5 (0-17)	1 (0-3)	1.3 (0-5)
Lymphocytes %	8.7 (1 – 17)	12.5 (2-61)	7.5 (4-12)	7 (2-22)	8 (2-22)	14 (0-24)
Eosinophils %	ND	0.2 (0-3.6)	0.2 (0-0.3)	0 (0-1)	0 (0)	0 (0 - 0.4)

Table 2 Differential cell counts from BAL in healthy children

*Data are from quoted references and with respect to Table 3 in ERS Task Force Document (de Blic et al. 2000) * Median values with 25th to 75th percentile ranges are stated in parenthesis. ND – not done. FRC functional residual capacity. See also text section 1.5*

1.6. BAL cellularity in infant wheeze and childhood asthma

Several studies have considered the bronchoalveolar lavage findings in infant wheeze, but only four have done so in comparison to a healthy control group (Azevedo *et al.* 2001; Krawiec *et al.* 2001; Le Bourgeois *et al.* 2002; Marguet *et al.* 2001). The results of these studies are tabulated in Table 3. All of these studies showed raised total nucleated cell count (TNCC) amongst infant wheezers. This indicates that there is persistent inflammation in the lower airway of infant wheezers causing cellular influx to the airway (Lemanske, Jr. 2002). The other findings are more variable and include raised neutrophil counts and reductions in lymphocytes (Le Bourgeois *et al.* 2002), which suggests that neutrophils might play an important role in viral induced wheezing.

All studies of infant wheeze BAL agree that eosinophils are notable by their absence. Stevenson *et al.* were the first to show that viral induced wheezers have different BAL cytology to atopic asthmatic children (Stevenson *et al.* 1997). Using non-bronchoscopic BAL from children with respiratory problems attending day case surgery, they showed that amongst children under five years old, atopics had higher concentrations of eosinophils in their lavage than their viral induced wheezing companions. This led them to the conclusion that there were two different forms of childhood asthma, those with eosinophils and those without. Raised eosinophils are found in school-aged children and adults with atopic asthma (Marguet *et al.* 1999; Stevenson *et al.* 1997).

The differences in BAL cellularity provide a pathological basis for the wheeze phenotypes described later (section 1.7.3), where atopic children are more likely to have persistent disease. However, no long term follow up studies have been performed of BAL findings in infancy and wheeze outcome.

Other studies have shown a significant difference in these cell types between infected and idiopathic wheezers. Their results are summarised in Table 3. The trend is toward an increase in neutrophils and eosinophils with a decrease in lymphocytes. The changes in cell composition are subtle and do not tend to increase the overall cellularity of BAL.

	Azvedo *(et al.)*	Marguet *(et al.)*	Le Bourgeois *(et al.)*	Krawiec *(et al.)*
BAL volume	4ml/kg	2-3ml/kg	2-3ml/kg	4ml/kg
Lavage site and preparation	FOB BAL Filtered	FOB Pooled Unfiltered	FOB BAL Unfiltered	FOB Pooled Unfiltered
Age (median years) Range	0.8 0.5 – 1.9	2.1 0.4 –2.5	0.9	1.2
N	36	30	83	20
Differential count	Resuspended	Resuspended	Resuspended	Resuspended
TNCC	7.0 ↑↑↑	7.0 ↑	6.4 ↑↑↑	2.1 ↑↑↑
Epithelial %	5.4 ↑	12 ↑	ND	4.0 ↔
Macrophages	84.5 ↔	47 ↓↓↓	82 ↔	74.6 ↔
Neutrophils	2.2 ↑	12 ↑↑↑	9 ↑↑↑	2.4 ↔
Lymphocytes	4.8 ↓↓↓	8 ↔	6.4 ↓↓↓	11.0 ↔
Eosinophils	0 ↔	0 ↔	0.1 ↔	0.4 ↔

Table 3 BAL cytology from infant wheezers compared to healthy controls

Cell counts are expressed as median percentage differential except for TNCC, which is shown as cells x 10^5/ml BALF. Methods of bronchoalveolar lavage, filtration of lavage fluid and slide preparation are shown. Comparisons to control data are shown as trends by single arrows (↑) whilst significant differences are shown as triple arrows (↓↓↓). FOB – Fibre Optic Bronchoscopy; Pooled – first and second lavage aliquots pooled together; Filtered – lavage filtered before processing; Resuspended - differential count performed following centrifugation to remove supernatant and cell pellet resuspended in a set volume of media. (Azevedo et al. 2001) (Marguet et al. 2001) (Le Bourgeois et al. 2002) (Krawiec et al. 2001)

1.7. Patterns and prognosis of preschool wheezing

Not all children who wheeze in infancy continue to do so throughout their childhood. Birth cohort studies have shown that there are several distinct temporal patterns to wheezing in childhood (Figure 3). A sample of 11,465 children born in 1970 was followed up at the ages of 5 and 10 years (Park *et al.* 1986). This showed that 21 % had experienced some wheezing in the first five years of life, most of which had

been variously labelled at that time as asthma, bronchitis, croup and wheeze. Of these preschool wheezers, 80% had outgrown their symptoms by ten years old.

The Poole study, of 100 infants recruited in 1976, also showed differences in the prognosis of wheeze according to the age of onset, and the development of atopy (Sporik *et al.* 1991). Twenty-two year follow up of this same cohort demonstrated that the majority of adult asthmatics had been sensitised to dietary allergens as infants, and had bronchial hyper-reactivity by mid-childhood (Rhodes *et al.* 2002).

The Tucson Respiratory Study followed a cohort of 1,246 children (recruited between 1980 and '84) from birth over the first six years of life using detailed environmental and genetic factors (Martinez *et al.* 1995). About half of the children (49%) experienced some wheezing during their preschool years. Parental smoking was a risk factor for all groups. At least three patterns of wheezing were defined in pre-school children: transient early wheezers, late onset wheezers and persistent wheezers. The study also considered serum IgE and lung function parameters in a subset, which provided insights into causes of these separate phenotypes. Wheezing phenotypes are a spectrum of disease, with some of each group represented in any sample of wheezy pre-schoolers (Silverman and Wilson 1997b). In fact wheeze throughout childhood appears to be a mixture of these phenotypes, and it is not until early teens that a more consistent atopic phenotype is observed (Wright 2002).

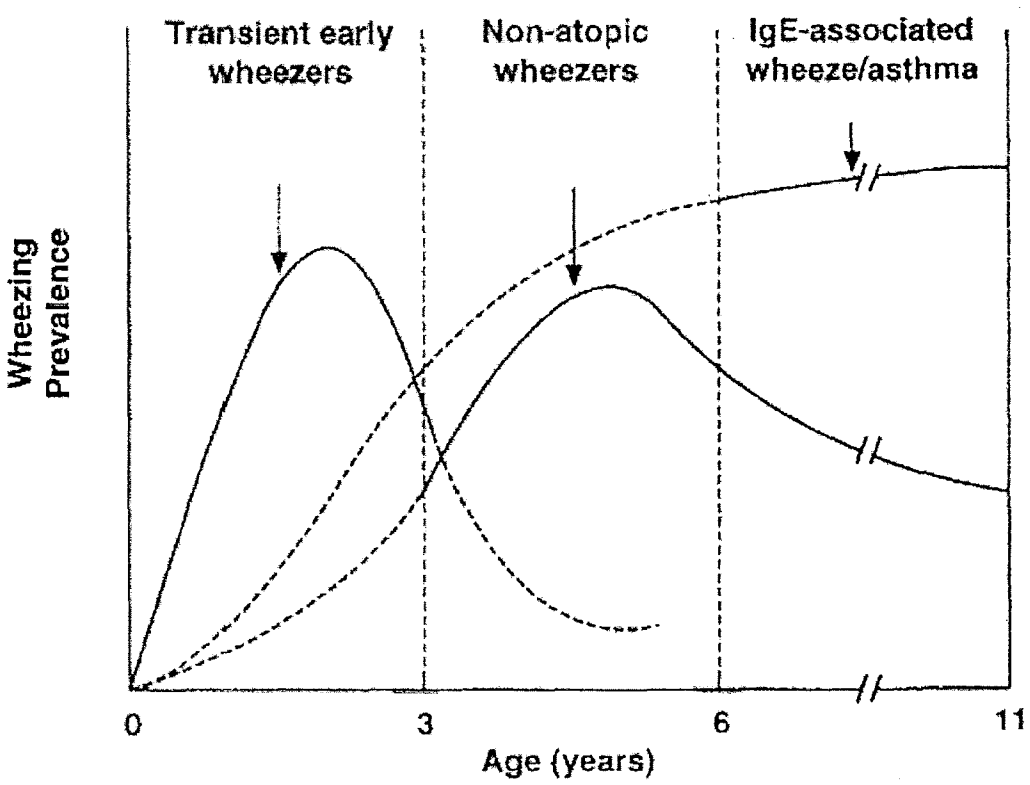


Figure 3 Patterns of wheezing in childhood.

Hypothetical yearly peak prevalence of wheezing according to phenotype in childhood. Asthma phenotypes reflect a heterogeneous group of conditions characterised by recurrent airway obstruction. Three of these phenotypes are shown. This classification should not imply that these groups are exclusive. Dashed lines suggest that wheezing can be represented by different curve shapes resulting from many different factors, including overlap of groups. Figure and legend reproduced with the kind permission of Professor F Martinez (Martinez 2002).

Each wheeze phenotype is discussed in detail below, illustrated using the Tucson longitudinal study (Martinez *et al.* 1995), but substantiated subsequently. At present there is no specific test, which can readily distinguish one phenotype from another.

1.7.1. Transient early wheezers

Twenty percent of children in the Tucson study had outgrown their symptoms by three years of age. These are the viral induced wheezers who mainly wheezed with colds in the first year of life. They were shown to have smaller airways than other wheezers, as demonstrated by $V_{max}FRC$ on infant lung function testing. Their lung function remained reduced for their age when retested at six years. Linkage analysis suggests that there is a heritable component to small airways separate from that of atopy (see section 2.3.1).

1.7.2. Late onset wheezers

Late onset wheezers developed wheezing after three years old. They made up 15% of preschool wheezers. Late onset of symptoms was associated with atopic sensitisation and a family history of asthma. Lung function was normal at both one and six years old.

Children who started wheezing after the age of two were more likely to have asthma at the age of ten. The number of attacks of wheeze was also related to persistence.

1.7.3. Persistent wheezers

Of those who wheezed in the first year of life, 14% continued to wheeze at six years old (Martinez *et al.* 1995). It seems as if a combination of both poor lung function and atopic sensitisation combine to give these children early onset and persistent symptoms. They had higher serum IgE concentrations than others at nine months old. Wheeze was associated with maternal asthma, symptoms of rhinitis and atopic sensitisation.

In Tucson, persistent wheezers had the lowest lung function levels at six years but normal V_{max}FRC at a year, suggesting that chronic inflammation has an impact on lung growth as well as function. Other studies disagree with these findings suggesting that persistent wheezers have lower lung function both on diagnosis between 2 to 4 years old and at follow up, at the age of four years, than those who grew out of their symptoms (Delacourt *et al.* 2001). Persistent wheezers show changes in lung function which predate their symptoms (Murray *et al.* 2002). Indeed, the Tucson group had earlier shown that low respiratory function as neonates was related to increased risk of wheezing illness (Martinez *et al.* 1988). Increased bronchial reactivity to histamine at a month old has also been shown to be associated with cough, low FEV1 and asthma at six years old (Palmer *et al.* 2001). As well as decreases in lung function, such persistent wheezers have more hospital admissions and use of steroid based medication than other wheezers (Kurukulaaratchy *et al.* 2003).

1.7.4. RSV induced wheezing

Transient viral induced wheezing may also be precipitated by RSV bronchiolitis, which is a further independent risk factor for the development of wheezing in childhood (von Mutius 2001). In a prospective study of 47 infants admitted with proven RSV bronchiolitis in infancy, asthma wheezing and atopy were more likely to be present at one, three and seven years old than in an age, sex and geographically matched control sample (Sigurs *et al.* 2000). This was in spite of similar frequencies of parental smoking, allergic and asthmatic families between groups. At seven years old, these ex-bronchiolitic children had increased T cell IL-4 responses to RSV and cat dander than their controls indicating an allergic response to common aeroallergens (Pala *et al.* 2002). The role of allergy and the infective agent are discussed later (2.3.2.2.).

Although RSV may predispose to wheezing in childhood the differences caused by this infection have disappeared by the teenage years (Stein *et al.* 1999). Within the original Tucson study some of these children would be considered as persistent wheezers at six years old.

1.7.5. Relationship to adult lung disease

Persistent wheezing through infancy and childhood is clearly associated with loss of lung function in adulthood. Pulmonary function testing showed that persistent wheezers have lower lung function than aged matched controls (Strachan *et al.* 1996). They also showed that at least part of this was due to pulmonary changes that did not reverse on the administration of $\beta 2$ agonists.

In contrast, transient wheezing in infancy is considered by many to be a self-limiting and benign condition. Over sixty percent of infant wheezers in Tucson had outgrown their symptoms by six years old (Martinez *et al.* 1995). Follow up of this cohort at eleven years old revealed lung function associations with wheezing at six, but not for wheezers at three years (Stein *et al.* 1997) and a similar study showed that transient wheezers had low-normal lung function by seven (Gern *et al.* 2002). This suggests that transient early wheezing does not cause lasting impairment of lung function.

Asthma may follow a more relapsing remitting course than has been previously thought (Sears *et al.* 2003). This study from New Zealand considered a prospective cohort up to their 26th birthday. They showed that only 27% of their complete birth cohort had never wheezed by this age. Nearly 10% had intermittent symptoms and 12% had relapsed following a remission of symptoms at some stage. Pulmonary function testing, which was first performed at 9 years old in this cohort, was persistently lower in the group who continued to wheeze. Von Mutius reviewed (von Mutius 2001) other studies that demonstrated that many children grow out of their symptoms during puberty only to regain them as adults.

Persistent wheezing in childhood is associated with adult respiratory symptoms. For this reason prevention of the lung damage caused by wheezing in infancy is paramount to respiratory health throughout life.

1.7.6. Can we predict who will continue to wheeze?

Identifying preschool wheezers who will continue to have symptoms into childhood may encourage the development of interventions to alter the course of the disease (Carlsen 1997). Studies of neonatal lung function have shown that diminished

indexes of lung function are associated with development of wheezing in childhood (Martinez *et al.* 1988; Murray *et al.* 2002). Neonatal bronchial hyper-responsiveness is also associated with development of wheeze (Palmer *et al.* 2001). Persistent wheezers have also been shown to have reduced lung function when compared to those who became asymptomatic, over a four year period from 2 years old (Delacourt *et al.* 2001). As changes in pulmonary function have been shown to occur in persistent wheezers, much research to date has focused on prevention of wheeze associated with atopy.

Although persistent wheezing is associated with atopy there is no single atopic marker that is able to predict persistence of symptoms. In children admitted with acute wheeze in infancy, the presence of atopic dermatitis, raised serum eosinophils, IgE, and positive skin prick tests predicted the persistence of wheezing at seven years old (Kotaniemi-Syrjanen *et al.* 2002). In particular they found that IgE to aeroallergens, egg and wheat significantly predicted childhood asthma (Kotaniemi-Syrjanen *et al.* 2003a). The combination of egg allergy and atopic dermatitis in infancy has also been associated with the development of asthma and aeroallergen sensitivity at four years (Tariq *et al.* 2000).

The Tucson study group used their cohort of 1246 children to construct predictive factors for persistent wheeze (Castro-Rodriguez *et al.* 2000). They found that frequent wheezing in the first three years of life, atopic dermatitis and a parental history of asthma increased the likelihood of developing physician diagnosed asthma to an odds ratio of 5.7 (confidence intervals 2.8 – 11.6) at 13 years old. Physician diagnosed asthma may be an underestimate of disease burden in the community.

Clough *et al.* (Clough *et al.* 1999) showed that persistence of wheezing, in the twelve months from diagnosis, was associated with age at presentation and the serum soluble Interleukin-2 Receptor (sIL-2 R) concentration, a marker of T cell activation. They used a logistic regression model for their calculations on a sample of children who had at least one atopic parent. These variables gave a positive predictive value of 76.5% compared to infant atopy alone with a value of 70.1%.

Products of activated eosinophils such as Eosinophil Cationic Protein (ECP) and Eosinophil Protein X (EPX) have come under scrutiny. Serum ECP values greater than 20 ng/ml (μg/l) in wheezers under one year have been associated with persistent symptoms (Koller *et al.* 1997; Villa *et al.* 1998). Nasal ECP levels in neonates were also able to predict the presence of wheeze at six months suggesting that both nasal disease and the trend to wheezing occur early in life (Frischer *et al.* 2000). Similarly urine EPX was raised in acute atopic asthma but no different from non-atopic children or controls in chronic asthma (Oymar and Bjerknes 2001). Although these markers are raised in acute exacerbations, in older children with atopic asthma, nasal and urine ECP and EPX were not significantly different from baseline and they concluded that they did not usefully predict exacerbation (Wojnarowski *et al.* 1999). The overlap between urine EPX between transient and persistent wheezers further reduces the sensitivity of this test (Tauber *et al.* 2000).

Whilst these studies show that there are early differences in lung function and immunological response amongst those who remain symptomatic, there is no reliable test in infancy for the prediction of persistent wheeze.

Chapter 2: Concepts in allergic asthma

Current understanding of the pathogenesis of asthma is extremely complex. It can be considered as a triad of causality between genetic predisposition, allergens / infective agents, and the environment (Figure 4). All of these factors interact within the temporal framework of the developing lung and immune system. Combinations of factors that may be critical at a particular time-point in development may be harmless at other times, depending on the plasticity of developing tissue. This chapter considers the concepts in the pathogenesis of asthma that underpin our current understanding.

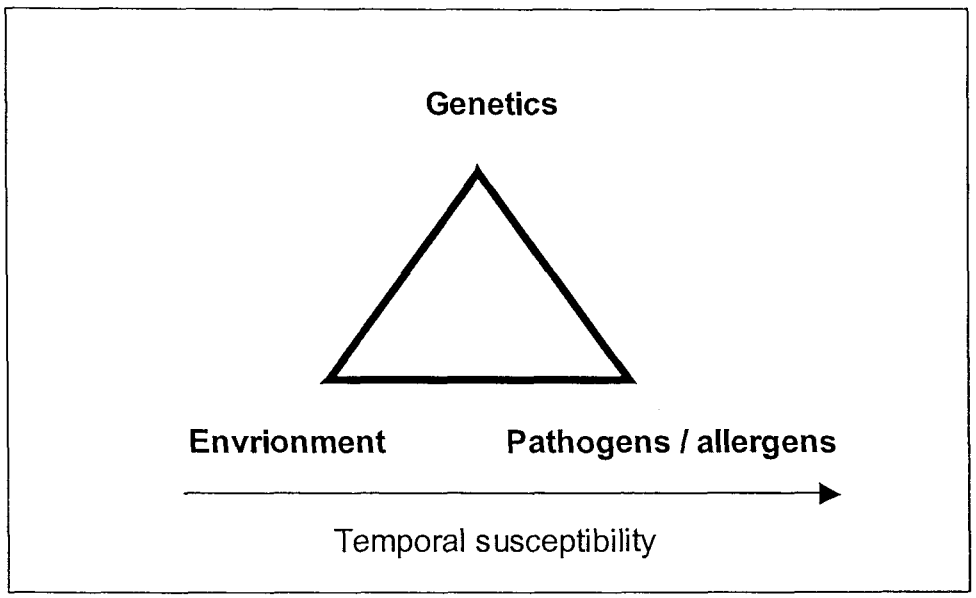


Figure 4 The pathogenesis of asthma

The triangle represents relationships between the various factors implicated in the pathogenesis of asthma.

2.1. Definitions for asthma

Without a known cause, providing a definition for asthma that will withstand the rigours required for sound epidemiology is virtually impossible, as definitions based on symptoms alone are too vague (von Mutius 1998). Also with asthma, none of the associated atopic or pulmonary signs are sufficiently specific to be used as a definitive test (Holloway *et al.* 1999). As an example of these difficulties, asthma has

been defined as '*A variable degree of airflow obstruction, bronchial hyper-responsiveness, and airway inflammation*' (Busse and Lemanske, Jr. 2001) which provides a summary of our understanding of the disease process but is also sufficiently vague to be of little practical use.

The Global Initiative for Asthma (GINA) have defined the condition as '*... a chronic inflammatory disorder of the airways. Chronically inflamed airways are hyperresponsive; they become obstructed and airflow is limited (by bronchoconstriction, mucus plugs, and increased inflammation) when airways are exposed to various risk factors*' (GINA 2002). This continues the use of descriptive terms for a disease whose underlying process is not understood. However, it seems a though inflammation of the airway is key to our understanding of asthma.

2.2. Epidemiology of childhood asthma

This section discusses the epidemiological aspects of wheezing in childhood. The economic impact for wheezing amongst preschool children has been estimated as 2.6 million pounds in the UK alone (Stevens *et al.* 2003).

Childhood asthma and wheezing are increasing in prevalence. This has been demonstrated by repeated sampling of specific age groups from the same geographical location. For instance there was an increase in asthma amongst 12 year old school children in Cardiff from 4 to 9% between 1973 and 1988 (Burr *et al.* 1989). This survey data was backed up by pulmonary function testing which showed a similar trend in both moderate and severe disease. A later survey of Nottingham primary school children indicated that asthma and current wheezing have continued to increase in prevalence (Venn *et al.* 1998). These trends have been confirmed in younger children. During the nineties a repeated survey of the parents of preschool children in Leicester showed an increase in the rates of current wheezing from 12 to 26% between 1990 and 1998 (Kuehni *et al.* 2001). The surveys showed an increase in all respiratory symptoms and diagnoses of asthma over this time period. These increases cannot be explained solely by changes in diagnostic labelling or increased awareness. However, more recently such studies have shown a plateau in the incidence of atopic disease (Toelle *et al.* 2004).

2.2.1. Global comparisons

The International Study of Asthma and Allergy in Childhood (ISAAC) was designed to assess the prevalence of symptoms between different communities (Asher *et al.* 1995a). This has enabled comparisons to be drawn between different environments. On average 8.0% of 13-14 year old children worldwide are affected by asthma (Asher and Weiland 1998). In this truly global study, involving more than 450,000 children in 56 countries, there was a 60-fold difference between the countries with the most, and those with the least prevalence. It showed that the United Kingdom has one of the highest prevalences of disease in the world.

2.2.2. Childhood asthma in the UK

To date five UK studies have used the parental administered ISAAC questionnaire to determine prevalence amongst the six to seven year old age group (Erlewyn-Lajeunesse and Edmondson-Jones 2002; Fielder *et al.* 1999; McCann *et al.* 2002; Ng Man *et al.* 2001; Shamssain and Shamsian 1999). The results are shown in Table 4. These studies are both temporally and geographically similar which enables a direct comparison of their results. Weighted averages of the responses show that one third (33.2%) of children had wheezed at some time by the age of seven, but that only 18.7% had symptoms in the last year. This indicates that half (56.4 %) have grown out of their symptoms by this age.

Study	1	2	3	4	5
n	1732	5011	3000	440	88
Diagnosis of asthma	24.2	29.7	22.7	20.0	28.4
Wheeze ever	-	35.8	29.6	30.0	31.8
Wheeze in last 12 months	18.9	19.4	18.0	17.0	18.2
Current use of inhalers	17.8	20.0	-	-	26.1
Attacks of wheeze	In the last 12 months				
1-3 attacks	17.3	11.6	10.8	-	6.8
4-12 attacks	8.2	7.4	5.1	-	6.8
>12 attacks	2.1		1.8	-	2.2
Limitation of speech	3.5	2.6	3.5	-	4.5
Night time sleep disturbance					
Any sleep disturbance	12.4	-	7.4	-	13.6
>1 night per week	4.2	8.2	5.7	-	6.8
Nocturnal cough*	25.3	-	-	25.0	26.1
Wheeze during or after exercise	12.9	4.4	13.0	-	15.9

Table 4 Asthma amongst six year olds in the UK

*The table shows a comparison of the results of the five parental administered ISAAC studies on symptoms amongst 6 to 7 year old children in the UK. Results expressed as percentages 1(McCann et al. 2002)2 (Ng Man et al. 2001) 3(Shamssain and Shamsian 1999) 4(Fielder et al. 1999) 5 (Erlewyn-Lajeunesse and Edmondson-Jones 2002) *Apart from that associated with a cold or chest infection. Missing data is where data was not reported.*

2.3. Pathogenesis of asthma

The relationship of these factors to infant wheeze remains for the most part speculative, and it is the aim of this thesis to consider how they may relate to the preschool child. The features of this triad are discussed in turn.

2.3.1. Genetics

Atopy and asthma carry a genetic predisposition (Dold *et al.* 1992). There is about a 50 to 60% heritability of asthma (Djukanovic and Holgate 1999). They are inherited in a multi-factorial manner, the details of which are not fully understood. Difficulties in linkage analysis are confounded by our inability to accurately define asthma and atopic phenotype (Holloway *et al.* 1999). Atopy is inherited more frequently if the mother is atopic than if it is the father who is atopic (Ruiz *et al.* 1992).

There are several genes that are known to play a role in the inheritance of asthma (Holloway *et al.* 1999). Linkage has been demonstrated in several areas of the genome including chromosomes 11q13 and the cytokine gene cluster on 5q. Both of these regions code for several cytokines and their receptors including FcεRI-β, IL-3, IL-4, IL-5, IL-9, IL-12 and IL-13. They clearly have a role in the modulation of the immune system, and the development of atopy. Polymorphisms in these genes may also alter the ability of the immune system to respond to infection, thus precipitating asthma (Vercelli 2003; Werner *et al.* 2003). However, mutations in these areas of the genome do not account for inheritance in all families. A mutation has been found in the cell surface metalloproteinase gene *ADAM 33* on chromosome 20p13 that links to the atopic asthma phenotype (Van Eerdewegh *et al.* 2002). Although the significance of genetic polymorphisms in this gene remain contentious, current evidence appears to reinforce linkage to the asthma phenotype, although its exact function remains unclear (Postma and Howard 2004).

Infant wheeze may involve other inheritance factors that are distinct from atopy and asthma. Christie *et al* have shown that airway diameter and lung function in older children and adults play a role (Christie *et al.* 1997). Genetic linkage studies, by this group, have linked parental wheezy bronchitis with improvement in their affected

offspring's chest symptoms as teenagers, marking these children out as transient wheezers regardless of the atopic status of the child (Christie *et al.* 1999). They consider that transient childhood wheezing has heritable traits that are separate from atopy, which may account for the inability of other studies to define persistent wheezing and asthma by atopic markers alone.

2.3.2. Precipitating foreign antigens

Foreign antigens stimulate an immune response, which may enhance or protect against the development of asthma and wheezing in childhood. These antigens include both infective agents and environmental allergens. The protective effects of infection are discussed later in this chapter when the hygiene hypothesis is discussed (Section 2.4), whilst their potential to enhance wheezing is considered here.

2.3.2.1. Allergen exposure

Allergens are environmental proteins that provoke an inappropriate immune response in the host. What makes some proteins allergenic is not known. The atopic arm of the immune system was previously deployed against legitimate parasitic infestation, and some consider that allergens are similar to hydrolase enzymes found in these parasites (Busse and Lemanske, Jr. 2001). Atopy is an allergic immune response to such allergens. Common allergens include house dust mite proteins, animal dander and tree and grass pollens.

In temperate climates, allergic sensitisation to house dust mite is associated with asthma and bronchial hyper-reactivity (Platts-Mills *et al.* 2000). However, the timing and mechanism of sensitisation remains elusive.

The evolutionary advantage of atopy remains unclear. It may be that the persistence of the allergic phenotype has never come under selection pressure, as most allergic individual's reproductive function is not impaired by this trait. This chapter has shown that allergy is a disease of the industrial environment and has increased specifically during the latter part of the twentieth century. As such, there have not yet been enough reproductive cycles, since the onset of disease in the population, to see a change in the industrial world's genotype toward or away from atopy.

2.3.2.2. Respiratory viruses

Viral lower respiratory tract infections in the first year of life and in particular Respiratory Syncitial Virus (RSV) bronchiolitis may predispose to recurrent wheezing in infancy (Sigurs *et al.* 2000). The timing of RSV infection may be the trigger for disease progression, with early life infection with RSV implicated in future lung disease (Culley *et al.* 2002). The relationship between RSV infection and predisposing risk factors for asthma remains contentious (Openshaw and Hewitt 2000).

Those infants with impaired lung function or immune predisposition may be predisposed to bronchiolitis. It is not known which comes first: the predisposition to wheeze or the precipitating RSV infection. Immune predisposition has been seen in infants with RSV bronchiolitis when compared to those infants who only had coryzal symptoms caused by RSV (Legg *et al.* 2003). RSV may precipitate wheezing and gain some evolutionary advantage by promoting an allergic Th2 response in the host, slowing viral clearance and thereby giving it valuable time in which to replicate (Openshaw and O'Donnell 1994). T cell IL-10 production is enhanced amongst ex-bronchiolitics who continue to wheeze, when no differences were noted in the acute phase of the illness, suggesting that RSV has an immuno-modulatory role (Bont *et al.* 2000).

Amongst infant wheezers and childhood asthmatics, acute attacks of wheezing are often triggered by viral upper respiratory tract infection. Other respiratory viruses such as rhinoviruses (Kotaniemi-Syrjanen *et al.* 2003b; Papadopoulos *et al.* 2002), and the recently discovered Human Metapneumovirus 9 (Jartti *et al.* 2002) may also be involved in precipitating wheeze.

2.3.3. Environment

Whilst inherited susceptibility is important, it is also clear that the environment plays a role in the disease process. Some of the most dramatic evidence has come from epidemiological studies of asthma and atopy amongst schoolchildren at the time of German re-unification (von Mutius *et al.* 1994). Prior to reunification, bronchial hyperreactivity and sensitisation to aeroallergens were more prevalent in the west. This was considered to be responsible for the higher rates of asthma in these

schoolchildren. A similar survey three years later showed that atopic disease had increased but asthma had not. This suggested that the environmental effects that precipitate asthma occur during the preschool years, whilst those of atopy may occur at any age (von Mutius *et al.* 1998). There are many environmental factors that have been implicated in the development of asthma. Some of the main areas are discussed below and were reviewed by Strachan (Strachan 2000b).

2.3.3.1. Parental smoking

Passive smoking is associated with an increased risk for wheeze and all respiratory diseases in childhood (Cogswell *et al.* 1987; Le Souef 2000). Timing of exposure is important. A study of 159 infants showed that maternal smoking during pregnancy had a deleterious affect on both pulmonary function and lung volume, and that these changes were still present at one year of age (Tager *et al.* 1995). Maternal smoking is associated with a doubled risk of lower respiratory tract illness in the first year of life. When the mother is a smoker, the effect of passive exposure to environmental tobacco smoke after birth is difficult to separate from that of antenatal exposure to tobacco smoke *in utero*. However, a study of Turkish children (Le Souef 2000), demonstrated decreased lung function amongst households where only the father smoked, indicating that there is also an ex-utero effect of exposure to passive or environmental tobacco smoke.

2.3.3.2. Pollutants

Whilst the role of industrial pollutants and vehicle exhaust gasses are popularly associated with asthma, the evidence to implicate them in the disease process is more limited. Changes in pollutant levels are probably involved in less than half of epidemics of acute asthma. However, it is noteworthy that closure of a steel mill in a valley in Utah (USA) was associated with a dramatic reduction in preschool wheezing and wheezy bronchitis (Pope, III 1989). Also, the frequency of wheezy bronchitis was higher in the more polluted east of Germany at reunification (von Mutius *et al.* 1994), which suggests that environmental pollution may play a role in preschool wheeze.

2.3.3.3. Indoor environment

Although the outdoor environment has captured the popular imagination, it is likely that the indoor environment, where most time is spent during both waking and sleeping hours, contributes most to environmental exposure to pollution. In this respect, allergens in bedding provide the highest levels of exposure.

Sensitisation to the House Dust Mite (HDM) *Dermatophagoides* is a significant risk factor for the development of asthma although not all sensitised children have asthma (Platts-Mills *et al.* 2000; Sporik and Platts-Mills 2001). Changes in housing such as the advent of central heating may have increased exposure to indoor allergens including HDM. However, although HDM burden does not seem to be related to the development of sensitisation, there is clearly a dose response relationship between exposure and sensitisation (Platts-Mills *et al.* 2000).

Pet cats and dogs in the home expose human cohabitants to high levels of dander allergens. The role of dander exposure in the development of asthma is uncertain, although it is clear that sensitised adults have exacerbations when exposed, this is not the same as a factor that precipitates disease. In one study, exposure to cat dander early in life may even be protective against sensitisation in non-atopics (Celedon *et al.* 2002). But even in this study, cat dander was associated with increased frequency of persistent and late onset wheezing. Exposure to dog dander seems to cause fewer problems than cat dander, possibly due to the nature of the allergen or exposure to zoonotic infection (Strachan 2000a).

High levels of fungal spores in the home increase the risk of Lower Respiratory Tract Infection (LRTI) and may be the link between damp housing and chest symptoms (Stark *et al.* 2003). However fungal spores are not acting as a true allergen in this situation because the rate of sensitisation to fungal spores is very low, even amongst severe adult asthmatics.

2.3.3.4. Diet and infant feeding

Changes in infant feeding from breast milk to cow's milk based formula have been implicated in allergic disease. A careful review of the literature concluded that

breast- feeding is protective, especially amongst children born to atopic parents (van Odijk *et al.* 2003). However, this conclusion remains controversial (Zemann and Rot 2003). Unlike formula milk, breast milk contains immunoglobulins, cytokines and immune effector cells. It also provides a sample of the maternal environment. The impact of infant feeding upon the developing child may not be immediately apparent, as breastfeeding has been associated with wheezing and allergic sensitisation in mid childhood (Zemann and Rot 2003).

2.3.3.5. Iatrogenic mechanisms

There is also evidence that modern medicine may have played its part in the asthma epidemic. The use of antibiotics in infancy has a dose dependent association with the development of atopy (Mattes and Karmaus 1999). This association with antibiotics may be due to reduced exposure to micro organisms as a consequence of their prescription, or may be a surrogate marker for lower respiratory tract viral infections, where antibiotics are prescribed frequently and inappropriately. Childhood asthma has also increased further since aspirin was substituted by paracetamol for the treatment of minor febrile illnesses during the late 1980's (Varner *et al.* 1998). This followed concerns over Reye's syndrome associations with aspirin. As a lipoxygenase inhibitor, aspirin may have reduced leukotriene production modulating the immune response to viral infection (Varner *et al.* 1998).

2.4. The hygiene hypothesis

The reduced exposure to infections in infancy has been implicated in the development of atopy (Martinez 2001). It is known as the *hygiene hypothesis* (Strachan 2000a). The lifestyles of the developed world had reduced the opportunity for cross infection and that infection in early childhood had a protective effect on the development of allergy (Strachan 1989).

Atopy is inversely related to family size indicating that minor infections, passed on by contact with siblings, are protective. As such, sharing a bedroom as a child has been shown to be protective (Strachan 2000a). However, in day care environments, where preschool children are exposed to other children and their infections, results

have not been so definitive, with some studies showing no effect on the development of allergy (Kemp and Bjorksten 2003). Celedon *et al* observed that day care was protective in some but not all allergic families, and that maternal atopy in particular was associated with development of wheeze in this situation (Celedon *et al.* 2003).

The hygiene hypothesis remains contentious even though there is considerable epidemiological evidence in its favour. The most damning direct evidence against the hypothesis is that the burden of infection in early life does not relate to protection from atopy (Kemp and Bjorksten 2003). Studies looking at a range of infections including minor viral infections, measles and tuberculosis, have not shown a consistent relationship with atopy. Those that have found protection have been criticised in their methodology, casting doubt upon the validity of their conclusions. However, not all suggestive studies have such methodological issues, for instance, acute otitis media appears to be protective amongst the offspring of atopic Norwegians (Nja *et al.* 2003).

Atopy is a disease of the city and children raised on farms are protected from atopy (von Mutius 2002). This is especially true of livestock farms and it is held that exposure to endotoxin from bovine faecal flora supplies the 'infective' immune stimulus for protection. Endotoxins are lipopolysaccarides (LPS) that are part of the outer membrane of gram-negative bacteria. They are detected by the innate immune system. LPS is detected by CD14 on the cell surface and signalling is involved with the pattern recognition of Toll Like Receptor (TLR)-4 (Coutinho and Poltorack 2003). Mutations in CD14 and TLR-4 have been implicated in the development of atopy, but animal studies using various gene knockouts suggested that endotoxin signalling is complex and that the timing of LPS stimulus is important (Vercelli 2003). What is interesting is that the infection suggested by the hygiene hypothesis may be background exposure to micro-organisms and not overt infection itself (Martinez 2001).

2.5. The allergic immune response

Allergy is closely associated with persistence of wheeze through infancy. The pathogenesis of asthma in adults and school aged children centres around the

immunology of IgE (Busse and Lemanske, Jr. 2001; Djukanovic and Holgate 1999). This section considers the pulmonary immune response to allergen and its relationship to asthma.

2.5.1. Antigen processing

In order for an allergen to be recognised by the immune system it must first be processed and presented to lymphocytes. Allergens are taken up in the bronchi and alveoli by professional antigen presenting cells (APC). APC are those cells that express MHC class II on their surface and include dendritic cells, macrophages, B and T cells. Dendritic cells process allergen proteins by degradation into short peptide fragments, which are then displayed on the cell surface in conjunction with MHC class II molecules. Dendritic cells migrate to local lymphoid tissue where allergen is presented to T lymphocytes (Coutinho and Poltorack 2003; Eisenbarth *et al.* 2003; Ono *et al.* 2003).

2.5.2. Th1 / Th2 balance

Within the confines of the lymphoid tissue the specific interaction between the APC and T cell takes place. The T cell bears a T Cell Receptor (TCR) on its surface, specific for a peptide fragment of that allergen. Specific binding of the allergen epitope, in the grasp of MHC class II molecule, to the TCR leads to helper T cell (T_h) activation. This occurs with a change in T cell surface receptors and notably up regulation of the IL-2 receptor and production of cytokines.

On activation, T_h cells produce a battery of cytokines that have been divided into two categories. These are known as the T_h1 and T_h2 responses (Figure 5). In broad terms the cytokines are mutually exclusive and inhibitory, with cytokines from Th1 responses inhibiting production of T_h2 cytokines. T_h1 responses recruit cell mediated (CD8 T cell) immunity and encourage IgG synthesis by B cells. They are assumed to be an antiviral response. T_h2 cytokines cause B cell class switching to IgE and recruitment of mast cells and eosinophils. T_h2 responses have been observed in allergy, as well as parasite immunity, and are predominant in adult asthma. This

imbalance between T_h1 and T_h2 immunity has influenced an entire generation of academics and has stifled discussion outside of this paradigm for the last decade.

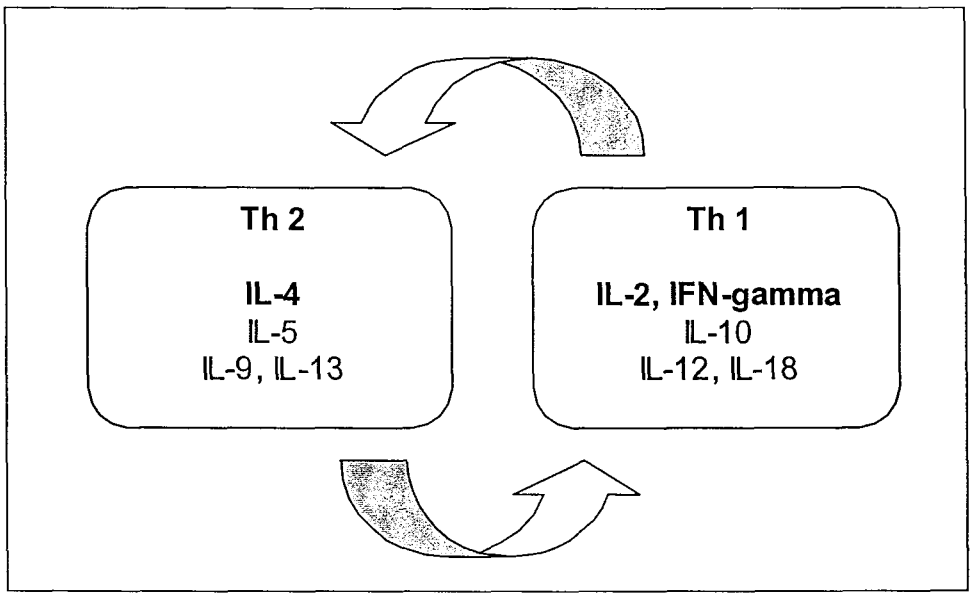


Figure 5 T cell T_h 1 and T_h 2 cytokines

Arrows indicate negative inhibition.

2.5.3. Immunoglobulin E class switching

Binding of TCR to allergen bound MHC II is just one of a series of interactions on the T_h2 cell. Co-stimulatory molecules also interact on the cell surface and modulate this recognition process and the nature of the T cell response. These co stimulatory molecules between T and antigen presenting B cells include CD40 / CD40L and CD28 / B7. This multiple binding event promotes rapid T_h2 cell secretion of IL-4, which in turn binds to IL-4 receptors on the B cell surface. Cytokine signal transduction occurs through a series of intracellular steps via Janus kinases and Signal Transducers and Activators of Transcription (STATS), known jointly as the JAK-STAT pathways (Ransohoff 1998). There are several of these factors involved in IgE class switching. These in turn bind to the epsilon (ϵ) heavy chain promoter regions of the IgE heavy chain, ensuring the alterations in the germline transcript that bring about immunoglobulin gene rearrangement and class switching from IgM to IgE.

2.5.4. Effector functions of IgE

IgE is distributed through body fluids and binds in tissues to cells bearing IgE surface receptors. These cells include mast cells, lymphocytes, eosinophils, platelets and macrophages. Binding of allergen to surface bound IgE causes cross linking of receptors that leads to activation and a variety of cellular responses. Mast cells degranulate on signalling through the high affinity IgE receptor (FcεR I), releasing a potent cocktail of inflammatory mediators including histamine, leukotrienes, chymase and tryptase enzymes. Not only do these mediators have direct toxic effects on pathogens but they also recruit other immune cells to the area. Eosinophils have a similar response to FcεR II cross-linking by antibody, releasing proteins such a major basic protein, eosinophil derived neurotoxin, and eosinophillic cationic protein from their granules.

The response of other effector cells to IgE mediated degranulation is discussed in the next section.

2.5.5. Chemokines and chemo-attraction

Leucocytes are recruited to the inflamed airway by chemo-attractants produced by the epithelium and mucosal immune system. Such molecules include complement factors C3a and C5a, leukotrienes, Platelet Activating Factor and chemokines. Chemokines are a diverse superfamily of small polypeptides (8-10 kDa) that act as chemo-attractants (Luster 1998). There are four families of chemokines, named after the arrangement of their N terminal cysteine amino acid sequence: CXC, CC, C and CX3C. Chemokines signal through specific transmembrane receptors coupled to G protein intracellular pathways. Chemokine receptor binding activates cytoskeletal rearrangement and chemotaxis, along a chemokine concentration gradient, to the site of inflammation. Eosinophils are recruited by the chemokines Eotaxin (CCL11), RANTES (CCL5), MCP-3 (CCL7) as well as by Interleukin-5 (Ono *et al.* 2003). Neutrophils are recruited predominantly by CXC chemokines enabling a differential effector cell response. Neutrophils are recruited by IL-8 (CXCL8), and also by CXCL 1, 2, 3, 5, 6, and 7.

2.5.6. Leucocyte trafficking

Leucocytes journey into the airways across the capillary vascular endothelium in response to an inflammatory event. The pathway involves specific molecular interactions between specific cellular adhesion molecules (CAM) on both leucocytes and endothelium. There are three main families of CAM, the immunoglobulin superfamily, selectins and integrins (Nourshargh 1993). Members include Intercellular Adhesion Molecule (ICAM) 1 (CD54), Vascular Cell Adhesion Molecule VCAM-1 (CD106), E and P selectins (CD62E) and (CD62P) respectively.

2.5.7. ICAM-1

ICAM-1 is a surface expressed glycoprotein with several functions. It is composed of a single 55 kDa polypeptide chain glycosylated up to 90 – 120 kDa. It is expressed on several cell types such as the vascular endothelial cells already mentioned, thymic epithelial cells and fibroblasts, and on haematopoietic cells such as macrophages, activated T-cells, germinal centre B-cells and dendritic cells in tonsils, lymph nodes and Peyer's patches.

ICAM-1 is not present on the epithelium of healthy individuals but is present in inflammatory conditions such as asthma. This indicates that it has a role in the recruitment of inflammatory cells to the airway (Manolitsas *et al.* 1994). It is expressed on vascular endothelial cells where it facilitates the adhesion of leucocytes to the endothelium, through binding to members of the β-integrin family (van der Stolpe and van der Saag 1996). ICAM-1 binds LFA-1 (Leucocyte Function Antigen 1), CD18, CD11a, Mac-1 (CD11b) and CD43 on leucocytes. It is also the endothelial receptor for rhinoviruses.

ICAM-1 is transcriptionally upregulated by the presence of pro-inflammatory cytokines such as TNF-α, IL-1, IFN-γ, and inhibited by glucocorticoids. The endothelium regulates the attachment of leucocytes according to inflammatory status of the airway.

When expressed on antigen presenting cells, ICAM-1 has a further role as a co-stimulatory molecule for T-cell help. ICAM-1 and its counter receptor LFA-1 act as accessory molecules in the activation of T lymphocytes. The binding avidity of LFA-

1 and ICAM-1 increases when other co-stimulatory molecules bind at the T-cell - APC surface without a change in the level of expression of either molecule (Collins *et al.* 1994).

ICAM-1 is shed by the endothelium by proteolytic cleavage of an 82 kDa peptide known as soluble ICAM-1 (sICAM-1). sICAM-1 is increased in many disease states including asthma, some malignancies, viral infections and autoimmune disorders (Kokuludag *et al.* 2002). The exact function of sICAM-1 remains unclear. However, what is clear is that ICAM-1 plays several roles in the immune response on different cells both in cellular attachment and intercellular signalling.

2.5.8. The importance of timing

Development of the respiratory tract and immune system continues throughout gestation into infancy. Inflammation occurring in this context may modulate (or remodel) tissue development leading to permanent changes in lung and immune architecture.

The epithelium of the lung and nose develop from different embryological sources. The lung develops from foregut endoderm with the respiratory tree budding off from the ventral pharyngeal groove (Simons 1999). Nasal mucosa is formed from the ectodermal nasal placode (Bousquet *et al.* 2004). Despite these differences in ontogeny, both sites adopt a pseudostratified ciliated epithelium, with the nose having more submucosal capillary vasculature than lung. Alveoli first appear at 32 weeks and continue to multiply after birth, with the number of alveoli increasing until at least two years old and possibly up to eight (Merkus *et al.* 1996).

In contrast, the immune system is surprisingly mature early in foetal development and is able to respond to antigen. Allergen has been detected in amniotic fluid and cord blood demonstrating that the foetus is exposed to the maternal environment (Holloway *et al.* 2000). The presentation of these allergens to the foetus by an atopic mother may be important in foetal sensitisation (Warner 2004). Studies of cord blood from pre-term infants have revealed targeted antigenic T cell responses at 22 weeks gestation (Jones *et al.* 1996). Even earlier the foetal gut forms lymphoid follicles

with antigen presenting cells as well as T and B cells all present (Warner 2004), which suggests an ability to produce targeted immunoglobulin at an early age.

Pregnancy can be considered to be a state of immunosuppression (Warner *et al.* 2000). In order to protect the foetus from adverse maternal immune responses to foeto-paternal antigen, the placenta forms an immunologically protective barrier between the infant and its mother, and maternal anticellular responses are suppressed. This has been shown by the skew towards Th2 (anti-parasite responses) in neonatal T cells. An effect which wanes over the first year of life in healthy children and might well persist in atopy (Warner *et al.* 2000)

From an understanding of the development of these organ systems, it is clear that the timing of the inflammatory event in asthma may be as important as the nature of the insult itself; events that may be of no consequence if they occurred in adulthood may have major influences on the developing infant. Such temporal programming provides a mechanism for the foetal origins of adult disease or Barker hypothesis (Barker 1992).

2.6. Airway remodelling

The macroscopic changes in the lungs of acute fatal asthma are well described (Djukanovic and Holgate 1999). The lungs are hyper inflated with mucus plugging of the airways, and the airway walls are thickened and oedematous. The mucus plugs contain inflammatory and epithelial cells that can form airway casts also known as Curschmann spirals. Microscopic examination reveals hypertrophied mucous glands with thickening of the basement membrane. It is accompanied by an inflammatory cell infiltrate that involves mast cells and eosinophils. These changes are also present in the lungs of children with asthma (Cutz *et al.* 1978). The pulmonary histopathology of infant wheeze has yet to be examined.

Airway remodelling is the term given to the histopathological changes seen in asthma. It has been demonstrated in adult asthmatics at diagnosis (Laitinen *et al.* 1993) and in older children with asthma (Cutz *et al.* 1978; Pohunek *et al.* 1997). The relationship of these changes to clinical symptoms is uncertain (Redington 2000). However, it is considered that the remodelling leads to irreversible obstructive

airways disease even in the absence of asthmatic symptoms (Boulet *et al.* 1994). In adults, the duration of remodelling is believed to lead to the observed increase in the rate of decline in airway function with age (Chiappara *et al.* 2001).

The changes of airway remodelling include epithelial shedding, increased number of mucus glands in the epithelium, sub-epithelial fibrosis, thickened reticular basement membrane and smooth muscle hypertrophy (Bousquet *et al.* 2000). These changes are discussed in more detail below and are illustrated by cartoon in Figure 6.

2.6.1. Epithelial shedding and disruption

The ciliated epithelial cells are frequently shed from the airway wall in acute asthma. These cells are often vacuolated and less viable than in the healthy airway. The reason for this phenomenon is not understood. However, epithelial cells play an active role in airway inflammation and repair and this, in part may be the cause of their demise.

2.6.2. Increased numbers of mucus secreting glands

The submucosal mucus secreting glands are more numerous and become hypertrophied. They are responsible for the mucus secretion seen in fatal asthma. These secretions occlude both large and small airways reducing the area available for gas exchange.

2.6.3. Sub-epithelial fibrosis with collagen deposition

The extra-cellular matrix (ECM) provides tissue support from a mesh of structural proteins embedded in a glycosaminoglycan (GAG) gel. There is an increase in the scar tissue collagen subtypes I and III in asthmatics, and a decrease in elastic fibres. This reduces the elasticity of the tissue.

2.6.4. Thickened reticular basement membrane

The epithelial basement membrane is composed of the true basement membrane and *lamina reticularis*. Asthmatics show a thickening of the *lamina reticularis* by deposition of fibrous reticulin and collagen subtypes I and III. The thickness of large airway basement membranes is related to changes of remodelling in smaller airways and provides a surrogate marker on endobronchial biopsy for remodelling (James *et al.* 2002).

2.6.5. Smooth muscle hypertrophy

The smooth muscle surrounding the airways becomes hypertrophied and can occupy up to four times the normal volume. Several reasons are believed to cause this thickening. Firstly increase work of the muscle secondary to bronchospasm will increase its bulk. Secondly, the inflammatory environment of the asthmatic airway may stimulate smooth muscle, causing myocyte growth and division, leading to hypertrophy. *In vitro*, airway smooth muscle cells divide in the presence of BAL from asthmatics (Naureckas *et al.* 1999), a phenomenon which was augmented by the use of BAL from asthmatics following allergen challenge.

2.7. Mechanism of airway remodelling

Acute airway inflammation on allergen challenge leads to chronic inflammation, which, in turn affects the tissue healing process leading to remodelling of the airway by repair (Bousquet *et al.* 2000). The exact relationship of remodelling to inflammation and atopy is unknown (Redington 2000). However, there is experimental evidence for this hypothesis.

Paediatric asthma is an inflammatory disease (Lemanske, Jr. 2002). Comparison of BAL in different disease states has shown increased inflammatory cells from infant wheeze and childhood asthma versus normal controls (Marguet *et al.* 1999). Results of paediatric BAL studies in asthma are discussed in detail in the discussion chapter.

Allergic challenge leads to remodelling. Palmans *et al.* (Palmans *et al.* 2002) demonstrated that exposure to ovalbumin in sensitised rats lead to signs of remodelling within two weeks of exposure. This was accompanied by an eosinophillic inflammatory response as well as a clinical correlate in bronchial hyper-reactivity on carbachol challenge.

Figure 6 Airway remodelling in asthmatic lungs

The figure on the next page shows a cartoon of the changes seen in the epithelium during airway remodelling compared to normal lung. The diagrams are based upon A (Young and Heath 2000) B (Bousquet et al. 2000).

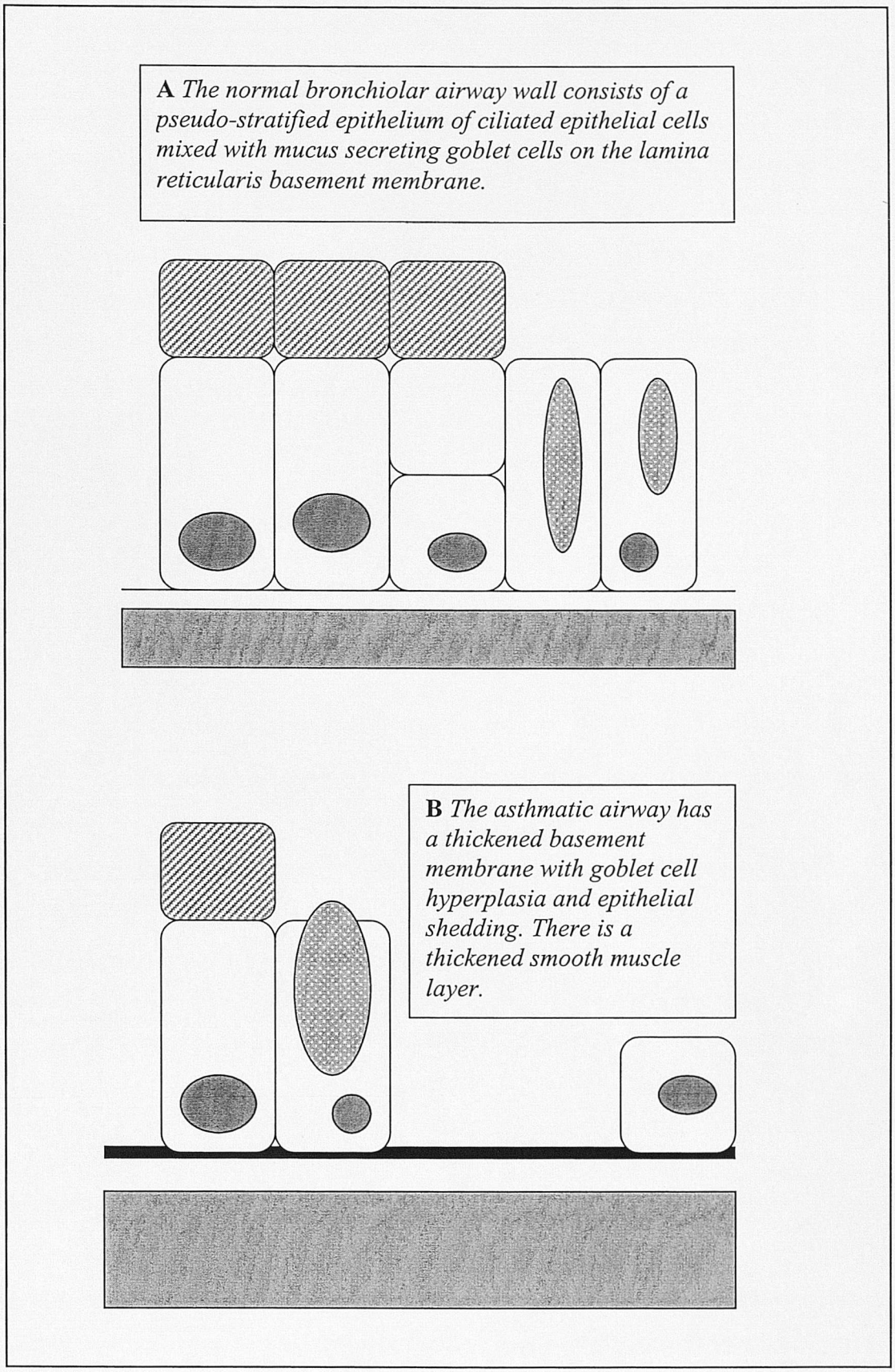


Figure 6 Airway remodelling in asthmatic lungs

2.7.1. Remodelling in preschool wheeze

There are no studies that have looked at remodelling in preschool wheezers. The persistence of wheeze amongst some infants suggests that remodelling may occur early in the disease process (Lemanske, Jr. 2002). To this end, several studies have examined pulmonary function within the first few weeks of life and its relationship to wheezing in later childhood (Murray *et al.* 2002; Turner *et al.* 2002). These experiments have shown that reduced lung function in the first few weeks of life predicts wheezing later in infancy. This suggests that airway remodelling could occur from birth in some individuals.

However, in spite of this gathering evidence, many fundamental questions about airway remodelling remain unanswered. Namely, its relationship to clinical symptoms and disease severity, the timing of onset of remodelling and the potential for reversibility in the developing infant lung remain unknown (Tiddens *et al.* 2000).

2.7.2. Inflammation and tissue repair

There is an emerging relationship between inflammation and tissue repair with fibrosis. Fundamental to these processes are the matrix metalloproteinases (MMP) and the Transforming Growth Factor TGF-β. MMP are collagenases that are released as part of the inflammatory response by myofibroblasts, neutrophils and other cells in order to digest the extracellular matrix. They are under strict physiological control and are secreted with their specific (cognate) inhibitors, the Tissue Inhibitors of Metalloproteinase (TIMP).

2.8. Matrix metalloproteinases

The matrix metalloproteinases are a family of calcium and zinc coenzyme dependent proteases, that are able to digest components of the Extracellular Matrix (ECM) (Atkinson and Senior 2003; Murphy and Docherty 1992). They are sometimes called matrixins and are part of a group of four families of metalloproteases that also include the serine proteases and the reprolysins (ADAMs) (Nagase and Woessner, Jr. 1999). MMP-9 is the predominant matrixin in pulmonary tissue (Mautino *et al.* 1999c), and has also been termed gelatinase B or Type IV neutrophil collagenase.

Matrixins are primarily involved in turnover of the ECM (Brew *et al.* 2000). This occurs in normal physiological processes such as bone remodelling, wound healing, blastocyst implantation and embryonic development. Tissue remodelling is disordered in disease states such as metastatic tumour spread, angiogenesis, atherosclerosis and tissue fibrosis. Matrixins have also been shown to be important in lung diseases such as pulmonary fibrosis and COPD. The role of matrixins and their inhibitors in asthma has recently also become apparent and will be discussed in detail. No studies have considered the role of matrixins in infant wheeze.

In lung tissue, neutrophils are the dominant source of MMP-9 (Takafuji *et al.* 2003), although bronchial epithelial cells, macrophages, B lymphocytes and eosinophils (Ohno *et al.* 1997) have also been shown to be able to produce MMP-9. Neutrophils synthesise MMP-9 during development in the bone marrow and store the latent peptide in granules (Atkinson and Senior 2003). Inflammatory stimuli cause degranulation of the neutrophil releasing proMMP-9, sometimes complexed with lipocalin (Atkinson and Senior 2003). Bacterial products such as LPS (Pugin *et al.* 1999) and *fmet-leu-phe* (FMLP) (Takafuji *et al.* 2003) have been shown to act as stimulants to neutrophil MMP release *in vitro*. Interleukin-8 is also a powerful stimulant of neutrophil MMP-9 release (Masure *et al.* 1991).

MMP-9 undergoes a two-step process to activation. The latent form of MMP-9 is a 92 kDa zymogen that is activated by the proteolytic cleavage of an N-terminal pro-peptide leaving an 84 kDa active enzyme. The first step involves limited proteolysis of the N terminal, which releases the zinc ion from a propeptide cysteinyl bond, known as the cysteinyl switch (Wart and Birkedal-Hansen 1990). This regains a degree of proteolytic function, which allows autolysis of the full pro-peptide and the completion of activation. In the lung, a variety of proteases, including opportunistic bacterial proteases may undertake the first step. MMP-3 may also be a major activator of MMP-9 *in vivo* (Goldberg *et al.* 1992).

The substrates of the matrixins demonstrate the range of functions of these proteases. MMP-9 has been shown to have several substrates including the structural ECM constituent proteins (collagen, gelatin and elastin), antiproteases (α1 anti-trypsin) and

cytokines. Proteolytic cleavage of cytokines is an immune regulatory activity (Opdenakker *et al.* 2001). IL-8 activity is increased by up to ten-fold when cleaved by MMP-9 (Van den Steen *et al.* 2000). This is in contrast to other CXC chemokines that are degraded by MMP-9 and CC chemokines such as RANTES were left unaltered by the protease. Matrixins have also shown to alter activity of latent TGF-β and TNF-α.

It is clear that matrixins require careful regulation to prevent unwanted tissue destruction. Production of matrixins is controlled at several levels. Firstly matrixins are the product of inducible genes whose transcription is regulated by growth factors, cytokines and chemical stimulants such as phorbol esters (Nagase and Woessner, Jr. 1999). MMP mRNA is transcribed into a zymogen whose latency further controls enzyme activity. Once released, the non-specific protease inhibitor α2-macroglobulin binds and inactivates the enzyme or zymogen. TIMP also provides a further level of protease control.

2.8.1. Tissue Inhibitors of Metalloproteinase

The TIMP are an ancient family of small peptides, whose structures are highly conserved amongst vertebrates (Brew *et al.* 2000). They have several known functions, and probably many more that are yet unknown. A discussion of their structure will provide an idea of their function.

There are four vertebrate TIMP. All are small 18-20 kDa polypetides with two domains (one N and one C terminal domain). The interaction between TIMP and matrixins occur at the N-terminal of the molecule. Binding between MMP and TIMP is high affinity and occurs in a 1:1 stoichiometric ratio. TIMP-1 and 2 can bind all matrixins; the other two are more selective. Inhibition occurs when the TIMP N-terminal domain inserts into the substrate-binding groove of the matrixins, obscuring their active site. X-ray crystallographic data is available only for the interactions of MMP-3/TIMP-1 and MMP-14/TIMP-2 complexes (Brew *et al.* 2000). TIMP-1 is a 28 kDa protein that binds specifically to MMP-9. The interaction between MMP-9 and TIMP-1 has not been so imaged to date.

The function of the C terminal of the TIMP family is less well understood. Although the N-terminal is primarily involved in matrixin inhibition, TIMP-1 uses the C-terminal moieties instead to inhibit MMP-9. The C terminal may be involved in the other functions of the TIMP family, which include fibroblast and epithelial cell growth, nuclear interaction within the cell cycle (perhaps the reason for their structural conservation), inhibition of cell invasion important for angiogenesis and metastasis and the induction of apoptosis in certain situations.

2.8.2. Matrixins and airway remodelling in asthma

As matrixins are capable of degradation of the extracellular matrix, including the epithelial basement membrane (EBM), their activity in asthma has been postulated to involve airway remodelling.

Murine studies have illustrated the role of MMP-9 allergic disease. Cataldo *et al* (Cataldo *et al.* 2002b) used a MMP-9 deficient knockout mouse model sensitised to ovalbumin, to demonstrate that MMP-9 was involved in allergic airway inflammation. They showed that eosinophillic airway infiltration, with associated bronchial hyper-reactivity, seen in wild type mice on aerosol challenge with ovalbumin, was not present in the MMP-9 knockout strain. Later studies showed that this was due to a failure of dendritic cell recruitment in MMP-9 deficient mice (Vermaelen *et al.* 2003). Kumagai *et al* (Kumagai *et al.* 2002) showed that basement membrane damage caused by ovalbumin challenge could be partially reversed by TIMP-2 in mice, thus implicating matrixins and their inhibitors in the remodelling process.

Human studies have considered the role of matrixins and TIMP in acute and chronic adult asthma (Kelly and Jarjour 2003). MMP-9 is the predominant matrixin in the asthmatic airway. TIMP-1 concentrations rise in order to cover the molar concentration, not just of MMP-9, but of all matrixins within the airway allowing protease – anti-protease balance at rest (Mautino *et al.* 1999c).

Human studies have shown matrixins are related to lung function in asthma. MMP-8 (Prikk *et al.* 2002) and MMP-9:TIMP-1 (Vignola *et al.* 1998) inversely correlated

with FEV_1 in adult asthma. MMP-9:TIMP-1 ratios have also been shown to correlate with FEV_1 in severe asthma and relate to steroid responsiveness in these patients (Bosse *et al.* 1999).

Immunohistochemical studies of the EBM in asthma have implicated MMP-9 in the pathogenesis of airway remodelling. MMP-9 stained the thickened (remodelled) EBM of severe asthmatics and was associated with neutrophil infiltration of the epithelium (Wenzel *et al.* 2003). This process correlated with BAL eosinophillia indicating the dual role of eosinophils and neutrophils in asthma. Hoshino *et al.* also noted a relationship between EBM thickening and MMP-9 but not TIMP-1 expression in asthmatic airway biopsies (Hoshino *et al.* 1998).

2.8.3. Matrixin – inhibitor balance

The ECM is in a constant state of turnover. Protease activity must be balanced with inactivity in order for this to occur in a controlled manner. Protease balance is one of the factors that could lead disorder in matrix turnover and may lead to tissue remodelling (Mautino *et al.* 1999a). There are two plausible mechanisms by which MMP-9 and TIMP-1 could effect a remodelling process (Figure 7): Firstly an excess of active MMP-9 over TIMP-1 would allow unregulated destruction of the ECM and basement membrane leading to inflammation and wound repair. Disorder of this process would prevent like with like replacement of the basement membrane leading to fibrosis and airway remodelling. Secondly, an excess of TIMP-1 over MMP-9 would slow the turnover of the ECM leading to build up of ECM products which may include thickening of the basement membrane, a cardinal feature of airway remodelling (Mautino *et al.* 1999c; Vignola *et al.* 1998).

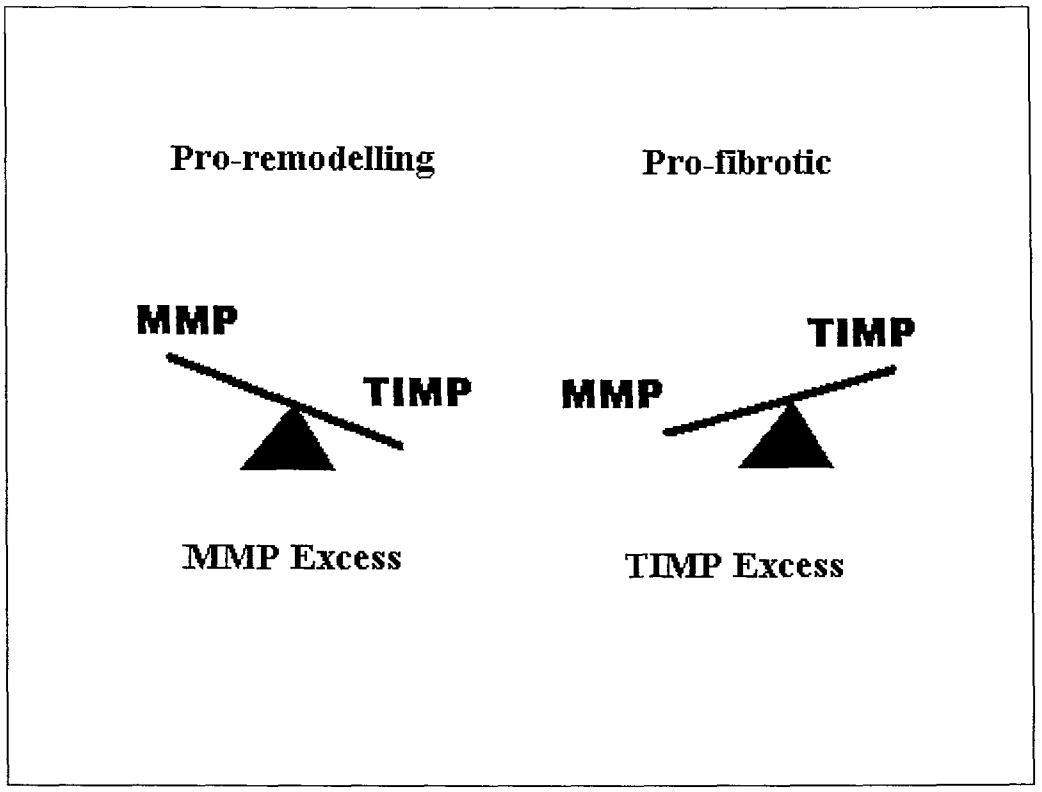


Figure 7 MMP – TIMP imbalance

The figure demonstrates the relationship between MMP and its natural inhibitor TIMP. Two mechanisms of imbalance are plausible. Firstly an imbalance of MMP over TIMP leading to unchecked proteolysis and ECM degradation, and secondly an excess of TIMP over MMP leading to decreased matrix turnover and fibrosis.

2.9. The one airway hypothesis

The nose and the lung have different functions but are all part of the respiratory tract. There is now a body of evidence to suggest that inflammation of the nasal mucosal is matched by inflammation of the lower respiratory tract, and that the two are somehow interlinked. This has been termed the one airway hypothesis (Grossman 1997), although several other terms have been coined such as the allergic inflammatory airway syndrome (Togias 2001) or allergic rhinobronchitis (Simons 1999), none of which are in widespread use. The one airway hypothesis suggests that both allergic rhinitis (AR) and asthma are atopic diseases that are part of a single continuum of disease within a common airway. Evidence for this concept is robust and comes from two main sources: that of epidemiology and more recently, from targeted allergen challenges in adult volunteers.

2.9.1. Epidemiology of allergic rhinitis and asthma

In both adults and school aged children, epidemiological studies have shown that allergic rhinitis and asthma frequently coexist (Annesi-Maesano 2001; Bousquet *et al.* 2003). Although both conditions are common, they occur together more frequently than by chance alone. Developing AR increases the chances of having asthma by up to ten fold. It is also more likely for an asthmatic with aeroallergen sensitivity to have AR than no nasal symptoms at all. The opposite situation, of seasonal bronchial hyper-reactivity without nasal symptoms is extremely rare, which suggests that the temporal process of sensitisation starts with the nose and then progresses to the lungs (Linneberg *et al.* 2002). The onset of AR in adulthood often precedes the development of asthma, normally by about two years, and is an independent risk factor for the development of asthma (Bousquet *et al.* 2003).

Adult hayfever sufferers, without any wheeze, have subtly abnormal lung physiology. Even in the absence of chest symptoms, people with AR have increased bronchial hyperreactivity to metacholine following nasal allergen exposure (Corren *et al.* 1992). However, they do not show the classical changes of obstructive airways disease as evidenced by changes in the Peak Expiratory Flow Rate (PEFR)(Kelly *et al.* 2003). Compared to asthmatics, they also have identical lower airway responses to bronchial allergen challenge, albeit at higher allergen doses than the more sensitised asthmatics (Kelly *et al.* 2003).

2.9.2. Localised allergen challenge studies

Segmental allergen challenges show that both nose and lung respond to local infiltration of either organ with allergen. Braunstahl and his colleagues from Rotterdam (Braunstahl *et al.* 2000) have performed segmental bronchial provocation via oral FOB. It is of note that they did not use the nasal route for endoscopy, which could have contaminated the nares with allergen. They challenged adults with grass pollen seasonal rhinitis (but not asthma), and healthy controls, with grass pollen extract. Adults with AR alone showed an increase in pulmonary symptoms over the next 24 hours, decrease in FEV_1, increase in blood eosinophils and increases in eosinophils in the allergen challenged biopsy specimens from the Right Lower Lobe

(RLL) as well as in specimens taken from a prior sham challenge of the Right Upper Lobe (RUL) (Braunstahl *et al.* 2001a). More importantly nasal biopsy following lung challenge showed an increase in IL-5 positive cells suggesting allergic infiltration. The number of mast cells in the nasal biopsies decreased indicating degranulation following distant challenge, although this could also indicate upper airway contamination with allergen (Braunstahl *et al.* 2001a).

The same group have also performed the reverse experiment, where nasal allergen challenge in AR produced up-regulation of adhesion molecules ICAM-1, VCAM-1 and E-selectin in bronchial biopsy specimens (Braunstahl *et al.* 2001b). This demonstrated a lung response to nasal allergen provocation, which agreed with findings from induced sputum (Beeh *et al.* 2003).

The drawback with all of these localised challenge studies is that it is impossible to isolate one part of the airway (Broide 2003). Locally deposited allergen may quickly spread to other parts of nose and lung by coughing, and assisted diffusion via the mucociliary escalator. Studies from mice have considered the issue of allergen spread following localised challenge. In mice, the distribution of nasally instilled dye particles predominantly localised to the nose (87.8% recovery) (McCusker *et al.* 2002). Following sacrifice, immediately after intranasal allergen challenge, 5.6% was recovered from the lungs and 2.8% the stomach. Both lung and stomach concentrations were not significantly different from dye recovery from the spleen, used here as a negative control. These experiments demonstrate that whilst one cannot completely isolate one part of the airway by intranasal challenge, the concentration of allergen in other areas can be assumed to be very small immediately after challenge to a non-reactive substance. However, this experimental model does not account for the spread of sensitising allergen where there is mucosal inflammation and excessive mucus production. Allergen containing mucus will be swallowed and some may enter the lower respiratory tract. Within the lung the mucociliary escalator will ensure that allergen-containing fluids ascend the respiratory tree and may be transported to distant parts of the lung by the larger airways.

Allergic responses at both lung and nose may be only part of a wider systemic response to allergen. Other tissues such as gut and skin may also be involved in a general response to local allergen challenge (Togias 2001). Anaphylaxis is the most well recognised systemic response to local allergen challenge and it seems logical that anaphylaxis may occur at a sub-clinical level following every exposure. Bone marrow has been shown to increase production of IL-5 receptor positive cells following localised allergen challenge indicating that there is a fundamental and systemic response to allergens (Denburg *et al.* 2000). Laboratory studies in mice indicated that both intra-peritoneal and nasal sensitisation to ovalbumin caused lower respiratory tract inflammation on further nasal challenge (2002). This indicates that the respiratory tract is not isolated from the rest of the immune system and that priming at one mucosal surface does not preclude response from another. In this respect there may be nothing special about the distant allergic responses in lung and nose, and that other mucosal sites would show similar responses if they had been biopsied in these experiments.

2.9.3. Airway biopsy in rhinitis

Immunological descriptions of the lower respiratory tract in allergic rhinitis are now available to add weight to the epidemiological evidence. Bronchial biopsy studies have also shown increases in eosinophils, IL-5 and lymphocytes in AR sufferers in season compared to out of season (Chakir *et al.* 2000). Recently, adults with allergic rhinitis have been shown to have signs on airway remodelling on bronchial biopsy prior to the onset of LRT symptoms (Braunstahl *et al.* 2003).

If the lung and the nose both endure a single inflammatory disease process, one would expect to remodel the nose as well as the bronchial mucosa. Although changes to the nasal mucosa have been observed in asthma they are not as striking as that of the lower airway (Bousquet *et al.* 2004). In particular the nose does not exhibit epithelial shedding, pseudo-thickening of the basement membrane and lacks substantial collagen deposition (Braunstahl *et al.* 2003; Chanez *et al.* 1999). Although AR is associated with eosinophil efflux into the mucosa (Lim *et al.* 1995), neutrophils are also present in significant numbers within the epithelium, especially in adults with seasonal symptoms. The differences between changes observed

between nasal and bronchial mucosa have been hypothesised as being due to the differing epithelial origins of the mucosae (Bousquet *et al.* 2004). The physical method of biopsy has also been implicated to play a part, with bronchial biopsy causing more epithelial disruption than nasal biopsy forceps.

2.9.4. The allergic march

The manifestation of atopic disease changes as the child gets older, a phenomenon known as the *allergic march* (Figure 8). Infants have eczema, which tends to wane in mid childhood. Asthma is common in preschool and older children. Allergic rhinitis is uncommon in infants but becomes increasingly common amongst teenagers. This development or *march* of allergic disease over time has been demonstrated by Cogswell's longitudinal study of a cohort of infants from Poole, Dorset (Sporik *et al.* 1991).

The changes seen in during allergic march are not those observed in adults developing symptomatic atopy, where hayfever occurs prior to the onset of asthma, and eczema is much less common.

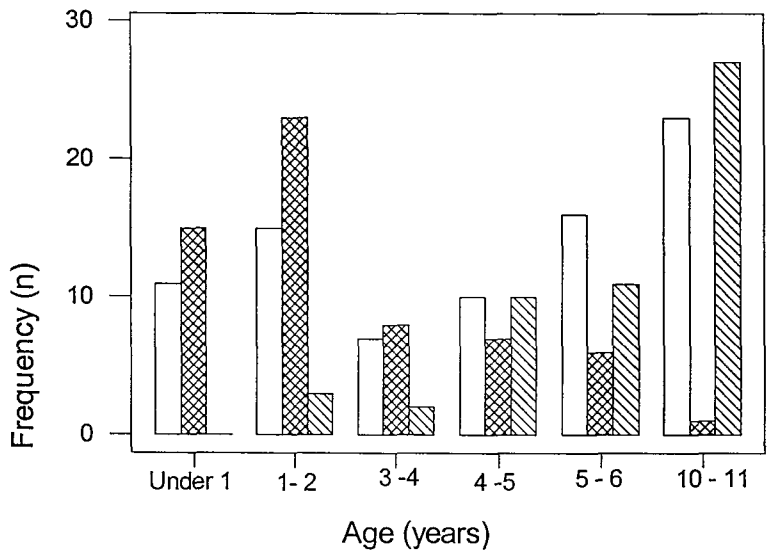


Figure 8 The allergic march.

This bar chart shows how manifestation of allergic disease changes over the first years of childhood. Wheezing is indicated by the clear bars, eczema by the cross hatched bars and allergic rhinitis by the single hatched bars. Data was taken from Sporik et al (Sporik et al. 1991).

2.9.5. Allergic rhinitis in preschool children

Allergic rhinitis in preschool children is not widely recognised. The precepts of the allergic march suggest that allergic rhinitis is the last of the atopic triad to develop, after eczema and asthma. This is clearly not the experience of adult asthma where AR precedes atopic asthma. Allergic rhinitis was found to affect nearly a quarter of 10 year olds from the Isle of Wight cohort (Arshad *et al.* 2002). AR was associated with aeroallergen sensitivity and asthma in this age group both at 10 years and at four (Kurukulaaratchy *et al.* 2003).

AR is difficult to diagnose in preschool children due to the similarity of many of the symptoms with mild viral coryza. Toddlers are also unable to report the sensation of itching that is prominent in the older age group leading to the disease being under diagnosed (Peroni *et al.* 2003).

In adults, aeroallergen sensitivity and AR frequently coexist (Bousquet *et al.* 2001). Assuming that the same mechanism holds true for preschool children, the prevalence of aeroallergen sensitivity can be used as a surrogate marker for allergic rhinitis. Fortunately, aeroallergen sensitivity is better documented in this age group than rhinitis. Positive Skin Prick Tests (SPT) to inhalant allergens are found in a minority of infant wheezers being only 18% of one sample admitted with wheeze in infancy (Kotaniemi-Syrjanen *et al.* 2003a). Tariq *et al* (Tariq *et al.* 2000) showed that egg allergy in infancy predicted onset of aeroallergen sensitivity at four years old. Hattevig *et al* followed a small cohort of children through childhood and showed that food allergen sensitisation peaked in the first year of life and was replaced by aeroallergen sensitivity which first appears at two years old and persists (Hattevig *et al.* 1993).

Overall these results suggest that allergic rhinitis occurs amongst preschool children which is earlier than has been expected in the past. This may have a bearing on the development of asthma.

2.10. Therapeutics of infant wheeze

There are two main approaches to the treatment of wheeze in infancy. The aim of primary prevention is to prevent the development of disease, whilst secondary prevention aims to treat symptoms once they have occurred.

2.10.1. Primary prevention

The role of primary prevention is key to our understanding of asthma. It is likely that intervention in early life will have an impact on lung health for the adult (Holt and Sly 2000). Primary prevention studies have mainly focused on allergen avoidance.

The role of allergen avoidance strategies in the prevention of asthma remains controversial. In spite of the encouraging reports from some randomised controlled trials (Arshad *et al.* 2003), a systematic review of allergen avoidance studies did not support their use (Pearce *et al.* 2000). However, the role of sensitisation to allergen is

more robust, and it is this relationship that provides the link between allergen exposure and asthma. Sporik and Platts-Mills (Sporik and Platts-Mills 2001) argue that sensitisation is more difficult to determine from population studies, hence the difficulties in demonstrating the link between allergen and asthma.

Increasing exposure to environmental tobacco smoke both in utero and in infancy has been shown to be deleterious to lung growth (Le Souef 2000) and is associated with all forms of infant wheezing (Martinez *et al.* 1995).

There is only one study of the use of pharmacotherapy in the prevention of asthma (Bisgaard 2002). The Early Treatment of the Atopic Child (ETAC) study considered the long term use of cetirizine, an H_1 antihistamine, in infants at risk of the development of asthma before the onset of symptoms (ETAC Study group 1998). In a double blind randomised controlled trial, 817 children aged between 10 and 28 months, with atopic dermatitis or a family history of atopy were enrolled and randomised to receive placebo or cetirizine. Overall there was no difference in the development of asthma between groups over 18 months, but in a post hoc analysis those with sensitisation at enrolment to grass pollen and house dust mite had significantly lower incidence of wheeze. Those with cat dander sensitivity did not show such changes, which the authors considered was due to the pervasiveness of dander exposure in families who kept their pet. These findings were reduced after three years of follow up with only those allergic to grass pollens showing a significant reduction of incidence of wheeze (Warner 2001). The effect of constant exposure to house dust mite, as with cat dander, may the cause of this, whilst seasonal exposure to grass pollen was more easily controlled. ETAC also showed that in this group of high-risk infants, aeroallergen sensitivity was associated with a significantly higher relative risk of developing asthma.

2.10.2. Secondary prevention

Treatment of symptomatic wheeze in infancy is controversial due to the range of symptoms and difficulties in defining the diagnosis of infant wheeze (de Jongste *et al.* 2002). Oral corticosteroids (OCS) have been considered to be effective in the treatment of acute 'viral induced wheeze' from 2 months old and are the current

mainstay of treatment (Bisgaard 2002). However, a recent study of OCS in acute viral wheeze (Oommen *et al.* 2003a) suggested that they were not effective in reducing symptoms when started by parents. Steroids were commenced very early in the course of the exacerbation, which was probably the best time to intervene with an immunosuppressive therapy. No effect was seen even amongst those wheezers categorised as atopic by high urine EPX concentrations. However, the study excluded those with symptoms of rhinitis, which may have precluded those who would respond to corticosteroids.

Inhaled corticosteroids (ICS) are similarly controversial and were also reviewed by Bisgaard (Bisgaard 2002). Overall he concluded that the lack of evidence might be due in part to the difficulty in separating out the treatment effect of preventer therapy from natural loss of symptoms amongst transient wheezers. An interesting study on the role of nasal corticosteroids in prevention of viral associated wheeze also showed no differences in outcome between groups (Silverman *et al.* 2003). Other immunomodulatory agents could modulate the immune process, and this may be dependent upon timing of intervention in relation to lung development (Holt and Sly 2000). Montelukast is a leukotriene receptor antagonist. It has been used in a 28 day trial following acute RSV bronchiolitis in preschool wheezers admitted with bronchiolitis and was shown to reduce symptoms during the acute episodes and exacerbations of wheeze over the next few months (Bisgaard 2003). However, this study was criticised in the accompanying editorial (Szefler and Simoes 2003) for the wide age range of participants suggesting that both bronchiolitis and RSV associated wheeze were included. The data were not broken down by age thus obscuring the site of the effect of montelukast on RSV bronchiolitis or wheeze or both conditions.

The increase in prevalence of asthma amongst older children has been associated with a drop in asthma admissions, which demonstrates how improvements in treatment have reduced morbidity in this age group (Wennergren and Strannegard 2002). However, the same study showed that a similar reduction in admissions has not been seen for pre-school wheezers, highlighting the difficulties of effective treatment in this age group. The role of pharmacologic intervention will only become more clear when we have a better understanding of the underlying causes of wheezing in infancy.

Chapter 3: Subjects and Methods

The infant wheezers in this study were recruited as part of an international, multicentre double blind placebo controlled trial of the effect of cetirizine on nasal and bronchial ECP concentration at the onset of asthma in infancy. The primary aim of this trial was to evaluate the effect of a two-week course of cetirizine on the ECP concentration of nasal lavage fluid from infant wheezers. The timeline for the study is shown in Figure 9. As the property of UCB Pharma SA, the randomisation codes were not available for inclusion in this analysis and the results of the second nasal lavage are not shown.

Instead the exploratory aims and results of the study are discussed here, which included observational data from upper and lower respiratory tract lavage from preschool wheezers.

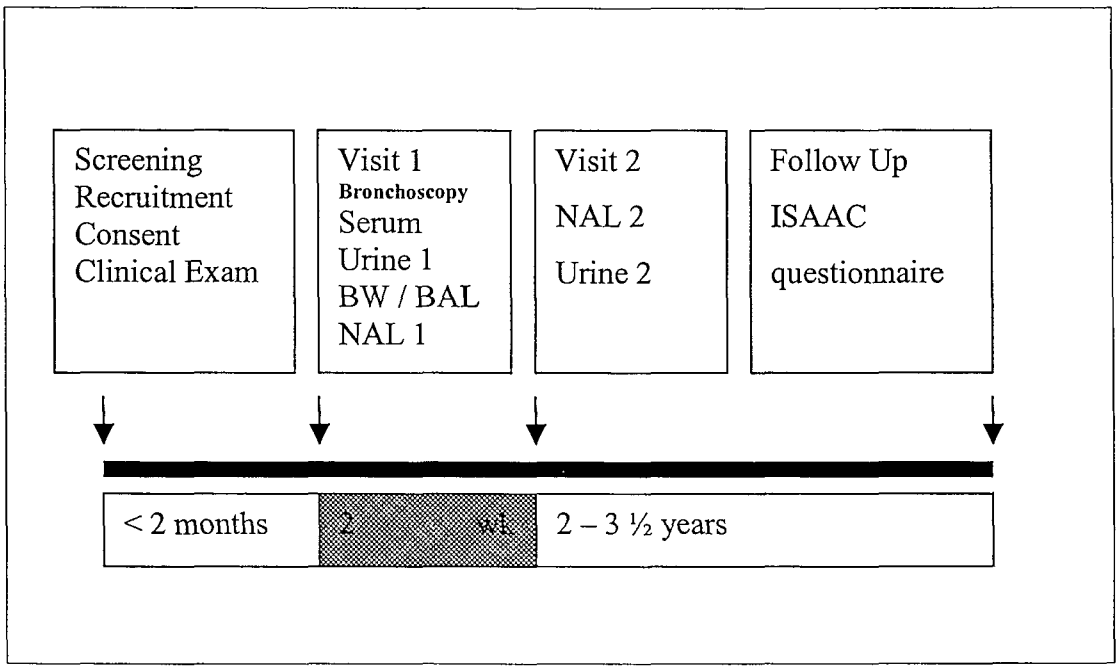


Figure 9 Timescale for the BAL study and its follow up

The time line for the study is shown in the diagram. Following recruitment and a screening visit, children underwent a clinical bronchoscopy at which samples were stored for research purposes and a concomitant nasal lavage and blood sample were taken.

Both bronchoscopy and follow up studies were fully approved by the local research ethics committees at each recruiting centre. The centres involved in the BAL study were Brussels, Paris, Prague and Southampton. Only Prague and Southampton participated in follow up. Parents or guardians provided written evidence of informed consent according to local regulations. UCB Pharma SA sponsored the trial and its exploratory study aims.

3.1. Study overview

All centres recruited preschool children to this study. All subjects had a diagnostic bronchoscopy with bronchoalveolar lavage for clinical indications of severe wheezing, with a concomitant nasal lavage. Aliquots of lavage and serum were taken for storage and later research analysis. Samples of serum and urine were also taken on the day of bronchoscopy.

Lavage cell counts were prepared and counted at each centre by local staff. Aliquots of lavage supernatant, and unstained cytocentrifuge slides were frozen and sent to the Southampton laboratory for analysis. ELISA were performed on lavage for Interleukin 8 (IL-8), soluble ICAM-1, soluble IL-2 Receptor α (sIL-2 R), Eotaxin-1, Interferon γ (γ-IFN), MMP-9 and TIMP-1 in lavage fluid. ECP was measured by radio-immunoassay in lavage and serum. Serum was also assayed for sICAM-1 and sIL-2 R by ELISA. Cytocentrifuge slides were stained using immunohistochemistry for ECP and mast cells. Dr Sarah Dobson, Dr Puja Kochhar and Dr Jane Warner performed the ELISA and zymography for MMP-9 and TIMP-1, all other assays were performed by the author. Wheezers were followed up by parental questionnaire several years after bronchoscopy.

3.2. Inclusion criteria for wheezers

All preschool wheezers in this study had recurrent respiratory symptoms, including cough and wheeze, in whom clinical, laboratory and other data were inconclusive, so that bronchoscopy with bronchoalveolar lavage was required for clinical diagnostic purposes. They were between six months and four years old at recruitment. A parent or guardian gave informed consent at a screening visit prior to the procedure. A clinical evaluation was performed following consent. All wheezers had a blood

sample taken up to maximum of two months before the procedure to estimate total serum IgE and liver function tests.

Wheezers were excluded from the study if they had a chronic disease requiring therapy (such as epilepsy or insulin dependent diabetes mellitus), severe neurological or psychological disorder of the child or their legal representative (including a drug or alcohol disorder), known cardiac dysfunction or QT abnormality on electrocardiogram, known allergy or intolerance to cetirizine or other piperazines or parabens, a personal history of central sleep apnoea or history of apnoea in siblings (if the child was younger than two years old at inclusion), participation in another clinical trial within three months preceding inclusion, metabolic condition which may compromise the absorption or other pharmacokinetic properties of cetirizine such as severe renal impairment, treatment by immunomodulators such as cyclosporin, use of antihistamines or cough medicine with antihistamines in a defined period preceding enrolment dependent upon the half life of the medication consumed and nasal septal deviation, turbinate hypertrophy or polyps preventing safe nasal lavage. Wash out periods for medication were: antihistamines or cough medicine containing these drugs in the last week, inhaled cromoglycate or theophylline in the last two weeks, inhaled, systemic or topical steroids in the last two weeks with a maximum of three days in the last month, astemizole or ketotifen in the last six weeks and any sympathomimetics in the last 24 hours prior to bronchoscopy.

3.3. Subjects

Fifty two wheezy preschool children were recruited to the BAL study and compared to 14 retrospective controls from Southampton. Wheezers were allocated to subgroups after the study closed and centres were blind to the eventual categorisation of subjects. After closure of the study, the 52 preschool wheezers were divided into atopic, infected and idiopathic wheeze subgroups according to their concomitant symptoms as below. Idiopathic wheezers were those who were neither atopic nor infected.

3.3.1. Atopic wheezers

Atopy was defined as the presence of one or more of the following: atopic dermatitis or allergic rhinitis by definition of the reporting clinician, raised serum eosinophil count or immunoglobulin E above the normal range for the local laboratory, positive skin prick test for a range of common food and aeroallergens, defined as a wheal greater than 3 mm on a flare of greater than 5 mm diameter (the diameter of the positive control was not recorded for comparison), positive RAST for a range of common food and aeroallergens (defined as > 0.7 RU/l). Serum IgE results were missing from one child with idiopathic wheeze, who had negative skin prick tests, and one child with atopic wheeze who had eczema as their defining atopic symptom. Of the four infected wheezers with missing IgE results, three had negative skin prick tests and the fourth had atopic dermatitis but no skin prick test results to compare.

3.3.2. Infected wheezers

Infected wheezers had positive bacterial growth on routine culture of BAL. It is important to remember that these children presented with cough and wheeze and were not being investigated for recurrent pneumonias.

3.3.3. Recruitement

The breakdown of recruitment by study centre is shown in Table 5 and their clinical details in Table 6. All children were under four years old at bronchoscopy. The majority were under two (Table 7, Figure 10). The median age of the control group was 1.7 years and was not significantly different to that of the wheezers, with an overall median age of 1.1 years. There were no overall significant differences in age between groups or between centres.

Centre	Control	Idiopathic wheezers	Atopic wheezers	Infected wheezers	Total
Southampton	14	3	6	1	**24**
Brussels	-	2	3	1	**6**
Paris	-	5	5	11	**21**
Prague	-	5	8	2	**15**
	14	**15**	**22**	**15**	**66**

Table 5 Recruitment of children by study centre
Wheezers were recruited from four centres and compared to a retrospective control group.

	Control	Idiopathic wheezers	Atopic wheezers	Infected wheezers
n	14	15	22	15
Males	7 (50%)	8 (53%)	13 (59%)	10 (67%)
Missing cultures	8 (57%)	4 (27%)	5 (23%)	0
Stridor	12 (86%)	1	1	1
Congenital malformation	7 (50%)	1	1	0
Abnormal serum IgE	0	0	6 (27%)	2 (13%)
Missing IgE	0	1	1	4 (26%)
Atopic dermatitis	0	0	11 (50%)	4 (26%)
Allergic rhinitis	NA	0	7 (32%)	0
Antibiotics	NA	4 (27%)	7 (32%)	8 (53%)
Inhaled corticosteroids	NA	7 (44%)	5 (24%)	9 (60%)

Table 6 Clinical features of subjects

Numbers of subjects with percentage of group total in brackets. Allergic rhinitis (AR) children had either AR or aeroallergen sensitivity. Inhaled corticosteroids or antibiotics given in the six weeks prior to bronchoscopy are shown. Medication data for controls was available at bronchoscopy but not for the preceding period. No subjects received oral glucocorticoids within six weeks of bronchoscopy. One idiopathic wheezer received intravenous immunoglobulin within three weeks of bronchoscopy but no other immunomodulating drugs were used.
NA – data not available.

	Control	Idiopathic wheezers	Atopic wheezers	Infected wheezers	p
Age	1.7 0.8 – 2.9	1.2 0.7 – 1.8	1.2 0.8 – 1.7	0.9 0.7 – 1.3	0.5

Table 7 Age of subjects by group

Ages are shown in years as median (25^{th} – 75^{th} percentiles). Kruskal Wallis Test between group medians was not significant.

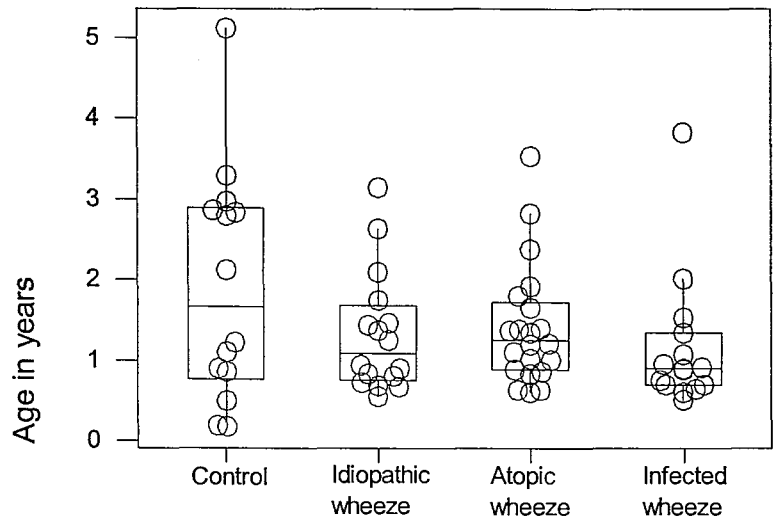


Figure 10 Boxplot of age at bronchoscopy

Boxplot showing the age of subjects at bronchoscopy compared between groups. Data points a represented as circles and the boxes show median with first and third quartile ranges. Age is given in years. There were no significant differences between groups.

3.3.4. Controls

Fourteen controls were selected retrospectively from bronchoscopies over the same time period, at our study centre in Southampton. All subjects who fitted control criteria during this time period were included as controls. They had enrolled in a separate but related ongoing study on the use of BAL in the diagnosis and treatment of lower respiratory tract disease in children.

Seven children (50%) had an upper airway diagnosis such as laryngomalacia, or a normal bronchoscopy with stridor as their sole presenting symptom. None of them had a history of wheeze. Those who presented with both cough and stridor required signs of airway malacia at bronchoscopy to warrant inclusion as controls.

Two control children did not have stridor or airway malformation. One had unexplained tachypnoea, the other a normal study following persistent radiological

changes to the right middle lobe. Both of these non-stridulous controls had negative BAL cultures, normal serum IgE and no clinical response to $\beta 2$ agonist inhalers.

3.4. Bronchoscopy

Children were admitted as day cases for the bronchoscopy. A trans-nasal flexible fibre-optic bronchoscopy was performed under continuous oximetry monitoring and clinical evaluation. A 3.5mm paediatric scope such as the Olympus BF3C20, used at the Southampton site was used for the procedure. Pre-medication consisted of midazolam, pethidine and atropine sulphate. Topical anaesthesia consisted of lignocaine 1% in the upper airways and 0.5% in the bronchial tree. Supplemental oxygen was administered as indicated by oximetry. No adverse events were associated with this procedure at the Southampton site but data were not available for the other sites.

3.4.1. Bronchoalveolar lavage method

The bronchoscope was wedged in a segmental bronchus predominantly in the right middle lobe or a site where abnormalities had been detected on chest radiograph. In order to reduce the risk of contamination from the upper airways, no suction was used until the tip of the bronchoscope had been wedged into the airway lumen. Aliquots of 10 ml of warmed 0.9% sterile saline were injected and aspirated so that the total lavage volume was between 2 and 3 ml/kg. The fluid recovered was collected into fresh sterile containers and divided into aliquots for the various investigations. A sample of the bronchial wash was sent for routine microbiological culture. Lavage was placed on ice and transferred to polypropylene tubes within an hour.

3.4.2. Bronchial washings method

The first aliquot of bronchoalveolar lavage taken by this method was called the bronchial washing. All subsequent lavages were referred to as bronchoalveolar lavage.

3.4.3. Lavage return

Although there was a protocol for bronchoalveolar lavage, instillation volumes varied between individuals but not centres. The instillation volumes for bronchial washings approached significance between centres (p = 0.066). A comparison of lavage volumes and their returns are shown in Table 8 and Table 9 respectively.

	Southampton	Brussels	Paris	Prague	p
n	9	6	21	15	
BW	10 10 -10	14 3 –38	10 6 –13	11 8 – 17	0.066
BAL	20 10 – 20	19 10 - 40	20 12 – 16	20 12 - 34	0.192

Table 8 Lavage instillation volumes

The instillation volumes of lower airway lavage (BW bronchial washing, BAL bronchoalveolar lavage) are shown between centres as median and range (ml). P values for Kruskal Wallis one-way ANOVA between groups are shown. There were no significant differences between groups although BW approached significance.

	Southampton	Brussels	Paris	Prague	p
n	9	6	21	15	
BW	35 % 20 – 50	42 % 20 – 67	37 % 14 – 57	30 % 10 – 47	0.709
BAL	50 % 20 – 85	47 % 20 - 69	42 % 15 – 100	60 % 17 – 125	0.384

Table 9 Lavage return

The lavage return volumes for lower airway lavage (BW bronchial washing, BAL bronchoalveolar lavage) are shown between centres as median and range (% of instilled volume recovered). P values for Kruskal Wallis one-way ANOVA between groups are shown. There were no significant differences between groups.

3.4.4. Nasal lavage method

Nasal lavage was performed according to a standard protocol at all centres and was similar to the methods previously used in neonates (Frischer *et al.* 2000). With the child supine a suction catheter was placed into the posterior nasopharynx and 10 ml of warmed sterile saline syringed into the nares on the contralateral side. Lavage fluid was immediately suctioned into a sterile trap and placed on ice prior to analysis.

3.4.5. Nasal lavage return

There was a significant difference between both lavage instillation and return volumes between centres (Table 10). Paris used a 5 ml NAL whilst lavage at the other centres varied around 10 ml. Brussels had the highest volume NAL with lavages of up to 20 ml.

	Southampton	Brussels	Paris	Prague	p
n	10	6	21	15	
Instilled volume	10 ml 5 -10	10 ml 6 – 20	5 ml 5 – 5	10 ml 5 – 10	< 0.001
Percentage return	57% 30 – 90	41% 10 - 100	80% 20 – 120	40% 10 - 85	0.004

Table 10 Nasal lavage volumes

The instillation volumes (ml) and lavage return (% of instillation volume) for nasal lavage are shown between centres as median and range. P values for Kruskal Wallis one-way ANOVA between groups were significantly different between centres.

Comparison of NAL between centres showed that Brussels had consistently higher ECP, IL-8 and sICAM-1 concentrations in NAL than the other centres (Table 11). Correlations between lavage volumes, lavage returns and these variables were not significant. Total nasal cells were not significantly different between centres and varied markedly between with each centre.

	Southampton	Brussels	Paris	Prague	p
TNCC x10^{5}cells/ml	1.3 0.0003 – 18.4 10	5.9 0.01 - 12.3 6	3.5 0.007 - 1165 20	9.3 0.05 – 90.2 14	0.142
ECP µg/l	11 3 –113 10	112 82 – 183 5	33 0 –162 15	13 0 –70 9	0.006
IL-8 ng/ml	64 14 – 679 9	921 297 – 1708 4	164 0 –517 15	63 0 –1127 12	0.023
sICAM-1 pg/ml	0 0 –14 9	29 14 –204 6	0 0 –25 14	0 0 –55 15	0.001

Table 11 Nasal lavage markers by centre

Total Nucleated Cell Counts (TNCC), ECP, IL-8 and sICAM-1 concentrations in nasal lavage between centres (median, range, n). P values for Kruskal Wallis one-way ANOVA between groups were significantly different between centres for all but TNCC. Cellular *differentials were not related to lavage volume and are not shown.*

3.4.6. Lavage processing

The lavage specimens were processed according to a standard method within 60 minutes of collection (Klech and Pohl 1989; Mander 1999; Postle *et al.* 1999). There was a central protocol used by each study centre. Having been placed on ice at collection, airway lavage was filtered through a 100 μm nylon cell strainer (Becton Dickinson, UK) into polypropylene tubes. Samples were then centrifuged at 400 g for 10 minutes at 4 ^{o}C to pellet the cells. The supernatant was decanted in 0.5ml aliquots and frozen at –80 ^{o}C for subsequent analysis. The pellet was then resuspended in a known volume of Hank's balanced saline solution and total, viable cell and epithelial cell count performed using the 0.4 % Trypan Blue exclusion test (Sigma-Alrdich Co Ltd, Irvine, UK). Cells were diluted to between 5 x 10^5 and 1 x 10^6 cells/ml using Hank's Buffered Salt Solution (HBSS, GibCo BRL, Life Technologies Ltd, Paisley, UK) and slides were prepared using a cytocentrifuge (Cytospin 2, Shandon, UK). The slides were stained using Haema-Gurr (BDH Laboratory Supplies Ltd, Poole, UK) and a differential cell count performed on at least 300 cells per slide.

3.5. Cell Counts

Staff at each local centre performed the cell counts. The total nucleated cell count (TNCC); viable cell count and epithelial cell count were calculated from the Trypan Blue exclusion method. Both epithelial cells and cellular viability were expressed as percentages of this TNCC. The differential leucocyte count was calculated from the cytocentrifuge differential and expressed as a percentage. Actual counts were not available from each centre, as only percentages were recorded. This had an impact on the statistical methods available for comparisons. Although a minimum of 300 cells was counted at each centre, this figure may have varied between slides.

3.5.1. Validation of cell counts

It was impossible to directly validate the live cell counts (TNCC, viability and epithelial cells) between centres because they could not be stored or independently verified. Statistical methods were employed to account for potential counting differences between centres (see 3.10).

However, cytocentrifuge slides were frozen and sent to Southampton for immunohistochemistry. Unfortunately most of the slides did not survive prolonged freezing. However, seven BAL slides could be recounted from the Paris centre. The results are shown in Table 12. Lymphocytes were significantly different between centres, suggesting that intra-observer differences were present between centres. A Bland Altman plot (Bland and Altman 1986) (Figure 11) showed that the most variation between counts occurred only for the higher lymphocyte counts demonstrating a degree of proportionality in this variation. On average central counts were 4.1 % higher than those performed locally.

Nasal lavage slides were unsuitable for recounting and so statistical compensations were made to account for potential centre differences as with BAL.

	Original	Recount	p
n	7	7	
Macrophages %	88 43 – 96	82 54 – 96	0.772
Lymphocytes %	13.0 1.0 – 22.3	14.2 4.3 – 27.2	0.041
Neutrophils %	6.3 0 – 19.6	3.7 0 – 18.7	0.091
Eosinophils %	0 0 – 27.6	0 0 – 13.0	0.225

Table 12 Comparison of cell counts at different centres

The table shows median cell counts with ranges for cell counts performed originally at the local centre and the recount performed at Southampton. Wilcoxon Signed Rank test statistic probabilities are shown.

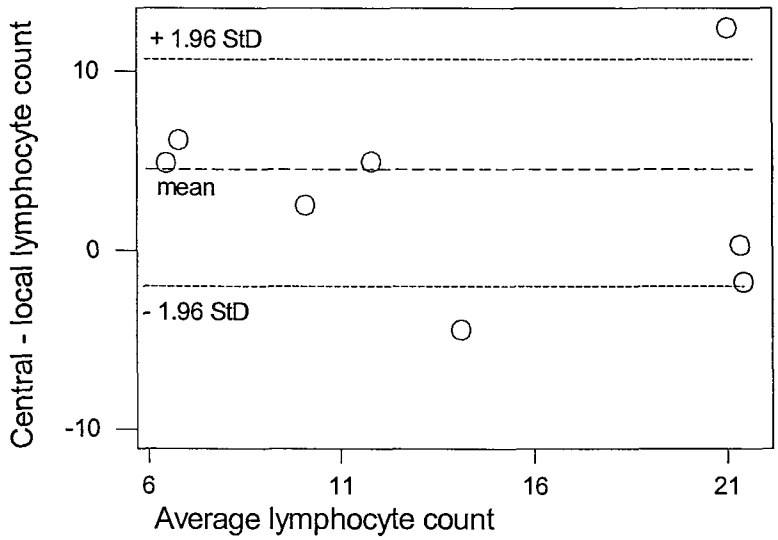


Figure 11 Bland Altman plot of lymphocyte counts

*Plot of the average lymphocyte count for the same BAL sample counted both locally and centrally compared to the absolute difference between these values. Mean of these differences and the upper and lower limit of normal as defined by + or - 1.96 * Standard deviation (StD) are shown on the plot. The graph shows that variation between centres increased as the average count increased, demonstrating a degree of proportional error between these two centres.*

3.6. Cytology varied between centres

Cell counts were seen to vary between centres. Using a two way analysis of variance (ANOVA) on transformed data, significant differences were found in counts between centres (Table 13). All cell types except for BAL neutrophils and eosinophils varied significantly between centres, which demonstrated that the cell counts were operator dependant. Further analysis of cell counts using this method would have to consider the role of centre differences in the calculations. The two way ANOVA (MIXED model method, SAS) was able to do this using either fixed or variable differences between centres. Both methods gave very similar results.

Cell count	p
TNCC	< 0.001
Cell viability %	0.004
Epithelial cells %	0.004
Macrophages %	0.002
Lymphocytes %	< 0.001
Neutrophils %	0.176
Eosinophils %	0.678

Table 13 Differences in cell counts between centres

The table shows the probability of differences between centres for each transformed BAL cell count. The probabilities were generated using a two way ANOVAwith fixed differences between centres although variable difference model was not very different from this. Only neutrophils and eosinophils did not show differences between centres.

3.7. Enzyme Linked Immunosorbent Assays (ELISA)

ELISA is a standard and commonly used method for sensitive and specific detection of immune mediators in clinical samples. It has been available since the discovery of monoclonal antibodies in the 1970s (Pathak *et al.* 1997). Several sandwich ELISA were used in this study. All of these assays work on similar principles that are described below.

3.7.1. The Sandwich ELISA

The sandwich ELISA uses two monoclonal antibodies raised to different epitopes on the target molecule. The target is thus sandwiched between them during the assay (Figure 12). The amount of secondary bound antibody can be detected and compared to known standards in order to provide a quantitative result. Most assays use standard 96 well polystyrene plates. The primary antibody is adhered to the surface of the plate by the natural affinity of hydrophobic immunoglobulin heavy chains for the plastic surface. Coating buffers or pre-activation of the polystyrene surface using UV light or glutaraldehyde ensure an even coating of antibody on the surface. The surface is then washed to remove unbound immunoglobulin and blocked with a protein coat to reduce non-specific binding sites for the target molecule.

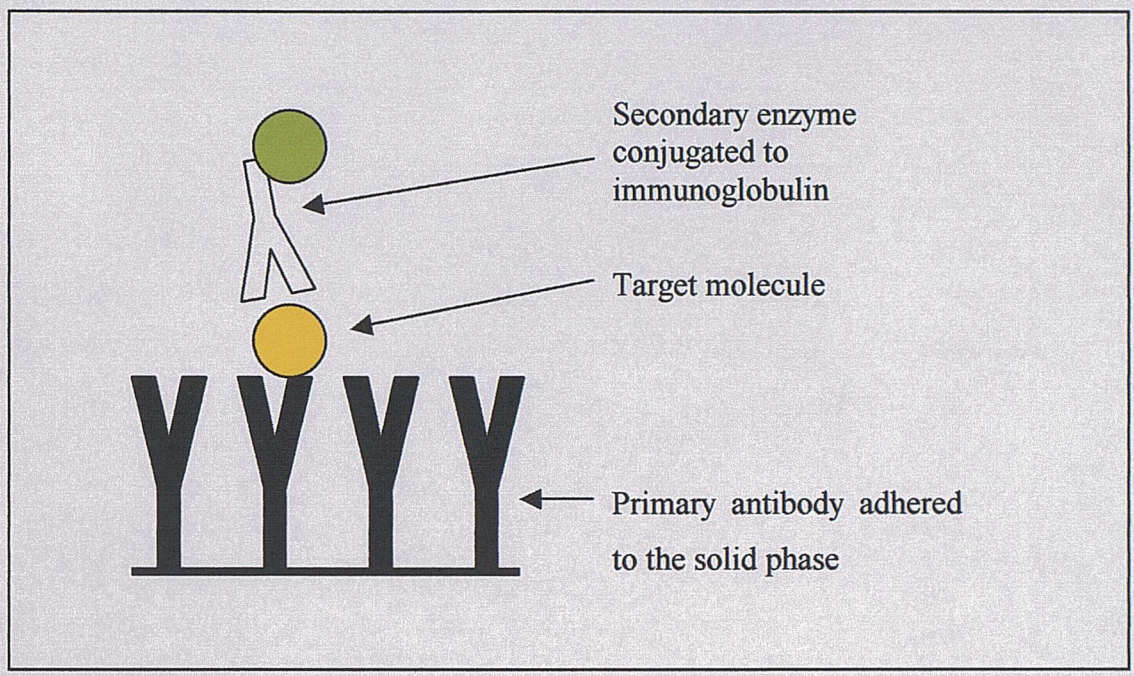


Figure 12 The sandwich ELISA method

See text for a description.

The clinical sample is then added to the pre-coated wells and the target binds to antibody attached to the solid phase surface. Following a wash cycle to remove unbound substrate the second detection antibody, which is also target specific is added. This antibody is raised against a second site on the molecule of interest so that binding is not obstructed by adhesion to the first antibody. The detection antibody has been previously conjugated to enzyme. A second wash cycle removes unbound secondary antibody and then detection can proceed.

Several methods of detection are available. They use enzymes whose products are able to produce fluorescence (fluorogenic) or a detectable colour (chromogenic). All of these methods rely on an enzyme or its substrates that are non-toxic and not present in the biological samples under investigation. Substrate is added to the wells and, following incubation, the colour or fluorescence read by fluorimetry or spectrophotometry.

The optical density of the sample by spectrophotometry is directly related to the concentration of secondary antibody present in the sample. It also relates to the

affinity of this antibody for its substrate, but, in most commercial situations, a high affinity antibody is used and binding mechanics do not factor in the assay.

3.7.2. ELISA method

The assays were performed according to the manufacturer's instructions. All samples were performed in duplicate except where repeated observation had shown that the within duplicate variation was consistently below the detection level of the assay.

Quantitative sandwich ELISA were used unless otherwise stated (Quantikine, R & D Systems Europe Ltd, Abingdon, UK). These assays used microplates pre-coated with primary antibody. In this system, horseradish peroxidase was used as the conjugate detection enzyme. It uses hydrogen peroxide to develop colour from the chromogen tetramethylbenzidine.

Plates were read at 450nm without subtraction at 540nm but using a used microplate of the same material to blank the spectrophotometer (Multiskan Plus, Titertek Ltd, UK). Direct comparison of the two reading methods showed that the single absorption at 450nm gave results that were not significantly different from the two-wavelength subtraction method.

Optical densities were converted to concentrations using a polynomial regression (4-PL) equation from the standard curve. Optical densities outside of the standard curve were repeated as dilutions until the value came within the confines of the standard curve.

3.7.3. Validation of ELISA in lavage fluid

The validity of these assays had not been proven in BAL or NAL. From the previous discussion it is clear that the characteristics of the biological sample under consideration may have a profound affect on the ELISA detection method. Validation of the ELISA for a new biological sample was performed according to the manufacturer's recommendations. Known concentrations of standard were spiked into lavage fluid throughout the range under consideration. A dilution curve was

plotted throughout the expected range with recovery ratios calculated at each point. The manufacturer's suggested acceptable recovery was between 80 – 120%. All assays fell within this range except for s-ICAM-1 where data were not available and MMP-9 where quantative zymography was used as a comparison.

Assay	Manufacturer	Lower limit of detection	Sensitivity
IL-8	R&D Systems	< 31.25 pg/ml	10pg/ml
sICAM-1	R&D Systems	< 10 ng/ml	0.35ng/ml
MMP-9	Amersham Pharmacia Biotech	< 0.125 ng/ml	0.125 ng/ml
TIMP-1	Amersham Pharmacia Biotech	< 3.13 ng/ml	1.25 ng/ml

Table 14 ELISA kit characteristics

Reproducibility of concentrations between batches was shown from manufacturer's results. The limits of detection of these assays are from the manufacturers data and are shown in the table.

3.8. Radio-Immunoassay (RIA) for ECP

The Eosinophil Cationic Protein radio-immunoassay (Pharmacia & Upjohn Diagnostics AB; Uppsala, Sweden) used in this project was the kind gift of Dr Stephan Ahlstedt of Pharmacia & Upjohn. The method was first described Peterson *et al* (Peterson *et al.* 1991).

The ECP RIA used in this study was a double antibody, competitive, immunosorbent assay (Figure 13). ^{125}I-ECP competed with ECP in the sample to bind with the primary antibody: an anti-ECP rabbit IgG. A second antibody, sheep anti-rabbit IgG, which has been coated to Sepharose beads, was added to this mixture and these complexes were then separated into a pellet by centrifugation at 1350g for 20 minutes at 18°C. The radioactivity of the pellet was measured over 60 seconds per sample in a gamma scintillation counter (Compugamma Universal Gamma Counter, LKB Wallace, Turku, Finland). The radioactivity of the pellet is inversely proportional to the amount of ECP in the original sample. The lower limit of detection was < 2.0 μg/l.

The ECP RIA was originally validated on bronchoalveolar and nasal lavage (Peterson *et al.* 1991) and so further validation was not required.

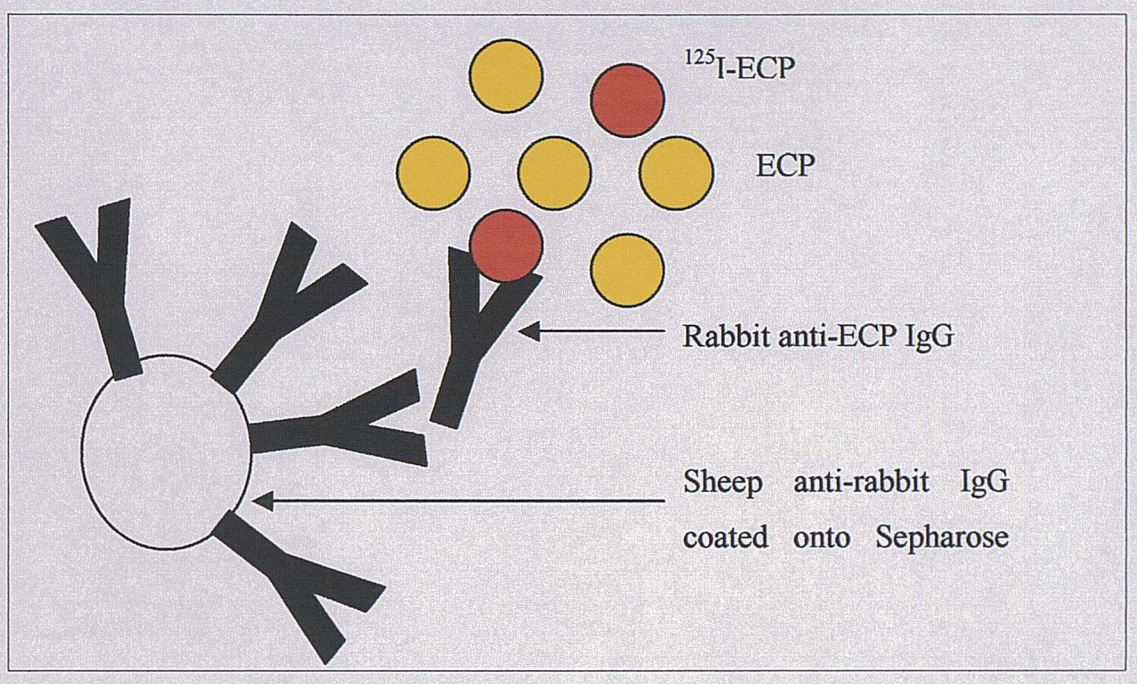


Figure 13 Competitive basis of the ECP RIA

The double antibody competitive radio immunoassay for ECP. The radioactivity of the Sepharose beads is inversely proportional to the amount of ECP in the original sample, see text for details.

3.9. Quantative zymography for MMP-9

Zymography quantifies active enzyme by capitalising on the gelatinase capabilities of MMP-9 (Kleiner and Stetler-Stevenson 1994). The biological sample is separated into component proteins by molecular weight and charge using electrophoresis. The electrophoresis is standard except that gelatin is incorporated into the gel. The gelatin can be revealed by staining with Coomassie Blue. Activated MMP-9 digests the gelatin leaving clear strips within the gel. These strips can be converted into quantified amounts of active enzyme by using image analysis software and comparing sample bands to those created by standards of known concentration.

Gelatin zymography was performed by Dr Sarah Dobson and Dr Jane Warner using the method described in Schock *et al* (Schock *et al.* 2001). To summarise, lavage

proteins were separated using SDS polyacrylamide gel electrophoresis (PAGE) incorporating 0.1% gelatin. Samples were run against a MMP-9 standard and a full range rainbow molecular weight marker. The gel was then washed twice in zymography buffer and stained with Coomassie Brilliant Blue after an overnight (18 hour) incubation. Following destaining of the gel with 7.5% acetic acid and 5% methanol, bands corresponding to the 92kDa fraction of MMP-9 were visible as clear bands on a blue background. They were analysed using an image analysis densitometer (Scan Analysis, UK).

3.9.1. Proteinase / inhibitor ratios

Molar ratios were calculated for MMP-9 (by ELISA and zymography) and TIMP-1.

Ratio = [MMP-9 (ng/ml) / 96 kDa] / [TIMP-1 (ng/ml) / 28 kDa]

The ratio is a representation of the amount of MMP that is bound to its cognate inhibitor and is the inverse of the amount of MMP free and potentially active.

3.10. Follow up study

The BAL study recruited children between January 1998 and December 2000 and questionnaire follow up occurred in November 2002, 23 months later. This means that children in the original BAL cohort were between 2 years 5 months and 6 years 11 months old at the time of the questionnaire follow up.

3.10.1. Aims

The aim of this part of the study was to examine the hypothesis that eosinophillic inflammation of the upper and lower airways will predict the persistence of wheeze. The follow up study was designed to assess the persistence of atopic symptoms and asthma amongst the BAL study cohort. This was an attempt to stratify the infant lavage specimens by the children's eventual wheezing status.

3.10.2. Exclusion criteria

Each recruiting centre carefully assessed the suitability of further contact with the family prior to sending out a questionnaire. This was to ensure that the children were still alive at follow up and that postal contact for research was appropriate. Suitability was assessed by a review of the subject's hospital notes and by contacting the subject's general practitioner, family doctor or office paediatrician by post, two weeks prior to mailing the questionnaire. Thus the only children excluded from the follow up study were those in whom it was deemed inappropriate to do so.

3.10.3. Questionnaire

The questionnaire used was the parental reporting questionnaire for 6 to 7 year olds of the International Study of Asthma and Allergies in Childhood (ISAAC)(Asher *et al.* 1995a). It assessed wheeze, wheeze severity, exercise induced symptoms and nocturnal cough as well as questions for rhinitis and atopic dermatitis. The full questionnaire and its Czech translation are shown in Appendix 1. Following assessment of exclusion a questionnaire was sent to the parents of each subject in the BAL study. A further questionnaire and follow up letter was sent two weeks later. The questionnaire contained a stamped return envelope and the introductory letter was personalised. This and the use of university headed notepaper have been shown to increase return (Edwards *et al.* 2002).

3.10.4. Recruitment centres

The BAL study was a multi-centre pan European study. Five centres actively recruited in England, France, Belgium and the Czech Republic. Questionnaires were distributed and returned locally with data the sent on to the study centre at Southampton. The patient information sheets were translated into French and Czech. The recruiting centre returned a recruitment log to ensure completeness. Questionnaires were identified with the subject's BAL study number to ensure confidentiality.

3.10.5. Validation and translation

The ISAAC questionnaire was chosen because it provided a tool for assessment of symptoms across different languages. The ISAAC questionnaire had been validated in English (Asher *et al.* 1995b) and in French (Braun-Fahrlander *et al.* 1998). The French validated questionnaire was the kind gift of Dr Isabella Annesi-Maesano but was not used in the eventual study due to the non-participation of the francophone centres.

The standard ISAAC methodology for translation and validation was used for the Czech questionnaire (Weiland *et al.* 2002). The questionnaire was translated into Czech by a UK based medical translations service and proof read by Dr Petr Pohunek, a first language Czech speaker. Several independent reviewers back translated the Czech questionnaire. The results were compared using qualitative content analysis software (NUD*IST 5, QSR Inc, Australia). No meaningful differences were found between back translations and the Czech questionnaire was used in its proof read format.

3.11. Statistical analysis

The cell and immunological markers in lavage did not follow a Normal distribution and it has been common practice in the literature to use non-parametric statistics when comparing this sort of data. Although they are biological specimens, there are several good reasons why their distribution should not follow the Gaussian curve. Firstly, lavage specimens sample a variable amount of the epithelial lining fluid and secondly, the immunological markers studies may well be the product of a cascade reaction with an exponential distribution.

Statistical analysis was performed using SPSS for Windows v11.0 (SPSS Inc., USA) and STATA v6.1 (SAS/STATA Inc, USA), graphs were plotted in Minitab (Release 13 for Windows, Minitab Inc, USA). Data were transformed and modelled for parametric analysis by Dr Linda Hunt, all other statistical calculations were performed by the author.

3.11.1. Non-parametric analysis

Groups were displayed as medians and ranges. Group comparisons were made using the Kruskal Wallis one way ANOVA. Results below the limit of detection were allocated the same lowest rank. Pair wise comparison of data were performed using a test for multiple comparisons between treatments (Siegel and Castellan 1988) according to the formula:

$$(\text{mean } Rank_1 - \text{mean } Rank_2) > or = z_{\alpha/k(k-1)} \sqrt{(N(N+1)/12 * (1/n_1 + 1/n_2))}$$

Where $\alpha = 0.05$, k the number of groups, N the total sample size and n the size of each group.

Correlation of variables was performed using Spearman's rank correlation coefficient (r_S) (Bland 2000). Markers were transformed into binary or categorical data where appropriate and comparisons between groups performed using Chi-squared tests. Two tailed Fisher's Exact tests were employed where expected numbers were smaller than five.

3.11.2. Parametric analysis

In order to compare cell counts between groups and take into account possible centre differences, a parametric analysis was performed. Variables were transformed to stabilise variances. TNCC transformed using natural logarithm whilst cellular viability, epithelial cell and the leucocyte differential percentages required arcsine transformation into radians in order to stabilise the variances of these data. Natural

logarithmic transformation could not be applied to these proportions because of the presence of data points at zero percent.

*transformed variable = arcsin * sqrt (p) where p is %/100*

Two way ANOVAs were used to compare groups taking into account centre differences using the MIXED procedure in SAS (Chapter 18 in the SAS user manual). Centre differences were considered both as a fixed and random variable. The results were very similar and only those from the fixed model are shown. T tests were performed between groups and Bonferoni's correction was applied to correct for multiple pairwise comparisons.

3.11.3. Graphs

Boxplots are used frequently in the results. They show the median and interquartile ranges as boxes. Whiskers indicate the highest or lowest value within 1.5 times the interquartile range from the first or third quartile box. Where individual values are not all shown individually, value symbols indicate any data points outside these parameters.

Chapter 4: Results from bronchoalveolar lavage

The aim of this chapter was to address the hypothesis that those infant wheezers with atopy had a different inflammatory process in the lower airways than non-atopic wheezers. The role of MMP-9 and TIMP-1 in infant wheeze is also considered here. The chapter concludes with a comparison of bronchial washings and BAL.

4.1.1. Total nucleated cell counts (TNCC)

The results for TNCC by group and centre are shown in Table 15. TNCC were significantly different between wheeze groups ($p = 0.050$). Infected wheezers had higher cell counts than idiopathic wheeze ($p = 0.019$, with Bonferoni's correction $p = 0.057$). There were no differences between atopic wheezers and the other subgroups. These differences are illustrated in Figure 14.

		Idiopathic wheezers	Atopic wheezers	Infected wheezers
Southampton	*median* *range* *n*	1.6 0.1 – 3.2 3	2.6 1.6 – 7.7 6	0.7 1
Brussels	*median* *range* *n*	2.2 1.8 – 2.6 2	1.9 0.5 – 4.0 3	15.6 1
Paris	*median* *range* *n*	1.6 0.2 – 4.0 5	1.0 0.9 – 5.0 5	3.0 0.9 – 36.0 11
Prague	*median* *range* *n*	12.6 1.6 – 30.4 5	32.7 0.8 – 184.8 8	101.9 0.3 – 200.1 2
$\log_e$ TNCC	*LS mean* *Std Dev* *n*	12.3 0.3 14	13.0 0.3 20	13.4 0.4 15
Back transformed means	*$x10^5$/ml*	2.2	4.5	6.7

Table 15 Total nucleated cell counts in BAL

Total nucleated cell counts are shown by wheeze subgroup and centre. TNCC were expressed as cells x 10^5/ml BAL. There was a significant difference in counts between centres ($p < 0.001$) with those from Prague being higher than the other counts. Accounting for centre differences, there was still an overall difference between groups ($p = 0.050$). Transformed Log_e TNCC counts are shown as least square (LS) means and standard deviations (Std Dev) for all centres, having accounted for fixed centre differences. Back transformed means for each group are also shown (x 10^5 cells/ml). Pairwise comparison showed a difference between idiopathic and infected wheezers ($p = 0.019$, with Bonferoni's correction $p = 0.057$).

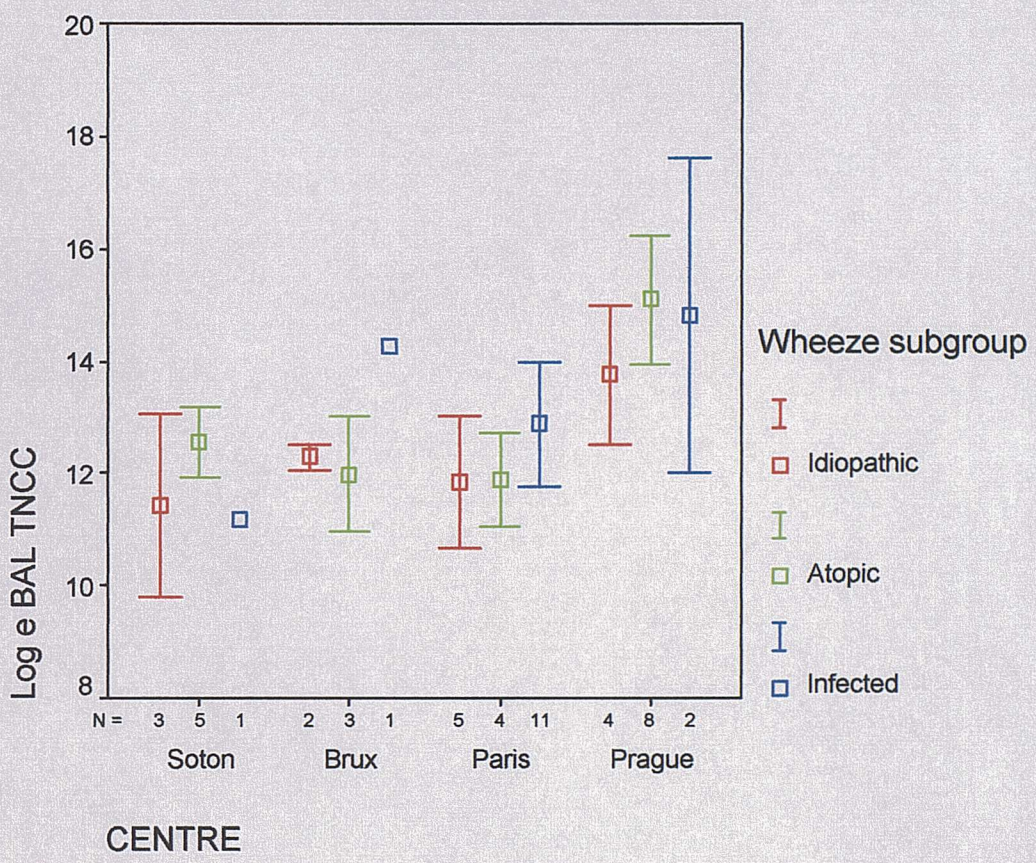


Figure 14 Total nucleated cell counts in BAL

The error bar chart shows log_e total nucleated cell counts from BAL by centre and divided by group. The marker of central tendency is the mean and error bars represent one standard deviation. Single data points are shown without error bars. The graph shows the significant differences in cytology between centres. When these differences were taken into account there were still overall differences between groups with TNCC being raised in infected wheezers compared to controls. Centre abbreviations: Soton - Southampton, Brux – Brussels.

4.1.2. Cellular viability and epithelial cells in BAL

Cell viability and epithelial cells were not significantly different between groups (p = 0.455 and 0.764 respectively. The data are shown in Table 16 and Table 17. There were no significant pairwise comparisons between groups.

		Idiopathic wheezers	Atopic wheezers	Infected wheezers
Southampton	*median*	73%	64%	61%
	range	58 – 86	51 –94	
	n	3	5	1
Brussels	*median*	68%	66%	82%
	range	65 – 71	52 –100	
	n	2	3	1
Paris	*median*	75%	64%	67%
	range	69 – 88	38 – 78	35 – 76
	n	4	4	11
Prague	*median*	92%	83%	90%
	range	63 –100	68 – 94	81 –99
	n	4	8	2
Overall mean	*LS Mean*	**80.1%**	**73.7%**	**74.2%**
Overall count (n)	*count*	**13**	**20**	**15**

Table 16 BAL cellular viability by group and centre

BAL cellular viability percentages are shown as median with ranges and counts between groups and centres. Cell viability showed a significant difference in counts between centres (p = 0.004) but no difference between groups when centre differences were accounted for (p = 0.4). Overall data was amalgamated following arcsin transformation and a comparison made using a two way ANOVAwith fixed differences between centres. The backtransoformed least square (LS) means are shown at the bottom of the table with overall counts.

		Idiopathic wheezers	Atopic wheezers	Infected wheezers
Southampton	*median*	11.0%	1.1%	2.6%
	range	0.8 – 18.8	0.8 – 6.7	
	n	3	5	1
Brussels	*median*	15.5%	12.0%	4.1%
	range	14.9 – 16.0	7.7 – 18.0	
	n	2	3	1
Paris	*median*	1.0%	1.5%	1.0%
	range	0 – 2.0	0 – 5.0	0 – 6.0
	n	4	4	10
Prague	*median*	6.9%	3.6%	8.0%
	range	0 – 2.0	1.7 – 23.8	0.4 – 15.7
	n	4	7	2
Overall mean	*LS mean*	**5.6%**	**5.0%**	**4.0%**
Overall count		**13**	**19**	**14**

Table 17 BAL epithelial cells by group and centre

BAL epithelial cell percentages are shown as median with ranges and counts between groups and centres. Epithelial cells showed a significant difference in counts between centres (p = 0.004) but no difference between groups when centre differences were accounted for (p = 0.764). Overall data was amalgamated following arcsin transformation and a comparison made using a two way ANOVA with fixed differences between centres. The backtransformed least square (LS) means are shown at the bottom of the table with overall counts.

4.1.3. Differential leucocyte counts

Differential leucocyte counts were compared between groups having taken centre differences into account. There were no overall differences between groups (Table 18). However, using pairwise comparisons, macrophages were significantly reduced (p = 0.003) in infected wheezers compared to idiopathics (median percentage 70.8% versus 86.0%). This remained significant taking Bonferoni's correction for pairwise comparisons into account (p = 0.009). Neutrophils were raised in infected wheezers compared to idiopathics (median percentage 13.3% versus 3.7%; p = 0.047). This was not significant when taking Bonferoni's correction into account (p = 0.141).

The presence of eosinophils was considered as a dichotomous variable in BAL. Eosinophils were present in BAL of 35% of controls, 30% of idiopathics, 42% of atopics and 20% of infected wheezers. Fisher's Exact test was not significant (p =

0.5). There were no pairwise significant differences between atopics and the other subgroups.

LS Mean % *LSM radians,(std)*	**Idiopathic wheezers**	**Atopic wheezers**	**Infected wheezers**	**P**
n	**14**	**20**	**15**	
Macrophages	86.0% 1.2 rad (0.06)	78.2% 1.1 rad (0.05)	70.8% 1.0 rad (0.07)	0.089
Lymphocytes	7.8% 0.28 rad (0.03)	9.8% 0.32 rad (0.02)	8.7% 0.30 rad (0.03)	0.634
Neutrophils	3.7% 0.19 rad (0.06)	6.5% 0.26, 0.05	13.3% 0.37, 0.07	0.135
Eosinophils	0.10% 0.03 rad (0.02)	0.50% 0.07 rad (0.02)	0.06% 0.02 rad (0.03)	0.330

Table 18 BAL cellular differentials

BAL cellular differential counts are shown as back transformed least squares means (LSM) in percentages. The least squares means from arcsin transformed data are shown in radians (rad) with standard deviations (in parentheses) to demonstrate spread of data. P values are for group differences using fixed effects for centres in a two way ANOVA. There was a significant difference in proportions of macrophages ($p = 0.018$) and lymphocytes ($p < 0.001$) between centres, but not for neutrophils or eosinophils. Pairwise comparison showed differences in macrophages between idiopathic and infected wheezers ($p = 0.003$, with Bonferoni's correction $p = 0.009$). Neutrophils also showed a difference between infected and idiopathic wheezers ($p = 0.047$ with Bonferoni's correction $p = 0.141$). Lymphocytes and eosinophils did not show significant differences between groups.

4.1.4. Control and wheezer cytology comparisons

Control cytology was available for 14 children from Southampton. Live cell count data was compared for the 9 Southampton wheezers and these controls. The results are shown in Table 19. Wheezers had a reduced cellular viability compared to controls (Mann Whitney U Test, $p = 0.041$). However, this was not seen when the wheezers were split into their subgroups, probably due to the small numbers of cases. Pairwise, there were no differences between atopics and controls.

median *range* *n*	**Control**	**Idiopathic wheezers**	**Atopic wheezers**	**Infected wheezers**
TNCC x 10^5 cells/ml	3.7 0.4 – 44.0 14	1.6 0.1 – 3.2 3	2.6 1.6 – 7.7 5	0.7 1
Cell viability %	81% 61 – 95 12	73% 58 – 86 3	64% 51 – 94 5	61% 1
Epithelial cells %	2.1% 0 – 4.7 12	11.0% 0.8 –18.8 3	1.1% 0.8 – 6.7 5	2.1% 1

Table 19 Southampton's live cell counts

The table compares live cell counts from the Southampton centre. Data is shown as median with ranges and cases (n). Total Nucleated Cell Count (TNCC). There were no differences between groups.

Section 3.5.1 compared slides that had been counted by the author to the original counts performed locally. This analysis only used slides from Paris, however, another four slides had survived freezing from the other centres and were stained and counted at Southampton. This provided 20 differential leucocyte counts from wheezy infants, all counted by the same observer, compared to 14 controls. The results are shown inTable 20. Comparing wheezers as a whole group to controls, lymphocytes were higher amongst wheezers (Mann Whitney U test $p = 0.025$). Macrophages were reduced amongst wheezers ($p = 0.056$). There were no significant differences overall or between groups. Eosinophils were highest amongst atopics and neutrophils highest amongst those with infection.

	Control	All wheezers	Idiopathic wheezers	Atopic wheezers	Infected wheezers
n	14	20	6	11	3
Macrophages %	90% 74 – 98	82% 54 – 96	84% 68 – 93	83% 66 – 96	79% 54 - 96
Lymphocytes %	6.7% 0.8 – 21.7	11.6% 3.8 – 27.2	12.1% 6.4 – 21.4	11.4% 3.8 – 22.9	12.1% 4.3 – 27.2
Neutrophils %	2.7% 0 – 13.9	2.4% 0 –18.7	3.8% 1.0 – 13.0	1.0% 0 – 17.3	8.6% 0 – 18.7
Eosinophils %	0% 0 – 0.7	0% 0 –13.0	0% 0 – 0.6	0% 0 – 13.0	0% 0 - 0

Table 20 Wheezers differential leucocyte counts versus controls

The table compares differential leucocyte counts, all performed by one observer, from wheezers and controls. Data is shown as median with ranges and counts. Total Nucleated Cell Count (TNCC). There were no differences between groups, however wheezers as a whole showed reduced macrophages ($p = 0.056$) and increased lymphocytes ($p = 0.025$) compared to controls.

4.2. Inflammatory markers in BAL

Bronchoalveolar lavage supernatant was analysed centrally for concentrations of IL-8, ECP and sICAM-1. Comparisons between controls and wheeze subgroups were performed using the ranks based Kruskal Wallis Test one-way ANOVA. All results below the limit of detection of the assay were assigned the lowest rank. Pairwise comparisons followed the method of Siegel & Castellan (Siegel and Castellan 1988). The results are shown in Table 21. Soluble ICAM-1 was not significantly different between groups and the results are not discussed further.

4.2.1. IL-8 in BAL

Looking at the data in Table 21 it is clear that BAL Interleukin-8 (IL-8) was higher amongst those wheezers with infection (96.8 ng/ml) than any other group. Compared to idiopathic wheezers (27.6 ng/ml), atopic wheezers (52.1 ng/ml) and controls (46.2 ng/ml) infected wheezers IL-8 concentrations appeared elevated. However, infected wheezers only had significantly higher IL-8 concentrations than idiopathic wheezers ($p < 0.05$) and even this difference was not significant when adjustments for multiple pairwise comparisons were taken into account ($p < 0.25$). There were no significant difference between controls and infected wheezers. There were also no overall

differences between groups. Dividing subjects by those with IL-8 above the limit of detection, a Fisher's Exact test was performed between groups. This was also not significant.

BAL		Control	Idiopathic Wheeze	Atopic Wheeze	Infected Wheeze	p
IL-8 pg/ml	*median*	46	28	52	97	0.549
	range	< 31 – 234	< 31- 175	< 31 – 4085	< 31 – 848	
	n	7	15	18	10	
	% LOD	57%	50%	58%	80%	
ECP µg/l	*median*	2.2	2.0	2.6	2.9	0.037
	range	< 2.0 – 5.4	< 2.0 – 12.0	< 2.0 – 90.3	2.0 – 151.8	
	n	8	16	18	11	
	% LOD	62%	47%	58%	100%	
soluble ICAM-1 ng/ml	*median*	80	78	76	77	0.846
	range	42 – 186	24 – 206	27 – 230	39 – 160	
	n	12	16	18	11	
	%LOD	100%	100%	100%	100%	

Table 21 Inflammatory markers in BAL fluid in infant wheeze

Concentrations of IL-8, ECP and sICAM-1 in BAL fluid supernatants are shown as medians with ranges and counts. The percentage of supernatants with values above the limits of detection (LOD) are shown. All lavage samples had detectable sICAM-1. P values are from Kruskal Wallis tests between all four groups. Less than symbols indicate that the value was below the cut off for the assay.

4.2.2. ECP in BAL

ECP was significantly different between groups (Kruskal Wallis Test $p = 0.037$). Pairwise comparisons between controls and infected wheezers ($p < 0.05$) and also between idiopathic and infected wheezers ($p < 0.001$) showed a significant difference, demonstrating that ECP was raised in infected children. Although ECP was raised amongst some children with atopy this did not reach significance compared to the other groups. When the presence of ECP was considered as a dichotomous variable, either below or above the limit of detection in BAL, ECP was

present in all infected wheezers and 58% of atopics. Fisher's Exact test for the presence of BAL ECP between groups was significantly different (p = 0.021).

4.3. Matrixins and TIMP in BAL

MMP-9 concentrations and its molar ratio to TIMP-1 were the only inflammatory markers that achieved significance against control lavage for wheezers as an entire group (p = 0.001 and < 0.001 respectively). However, each wheeze subgroup showed a different pattern of MMP:TIMP imbalance (as shown in the Table 22).

	Control	Idiopathic Wheeze	Atopic Wheeze	Infected Wheeze	p
N	11	12	14	10	
MMP-9 ng/ml	0.8 < 0.1 – 4.7	3.3 0.8 – 15.0	1.9 < 0.1– 30.6	4.8 1.0 – 55.7	0.005
TIMP-1 ng/ml	27 14 - 87	20 11 – 108	29 8 - 206	9 1 – 72	0.030
MMP/TIMP ratio	0.006 0 – 0.018	0.031 0.01 – 0.10	0.022 0 – 0.12	0.115 0.02 – 2.01	< 0.001

Table 22 MMP-9 and TIMP-1 in BAL fluid in infant wheeze

The table shows concentrations of metalloproteinases, TIMP and molar ratios in BAL. Data is shown as medians with ranges. P values are from Kruskal Wallis tests between all groups.

4.3.1. MMP-9 concentrations in BAL

MMP-9 was significantly different between groups (p = 0.005, see Table 22 and Figure 15). Infected wheezers had significantly elevated levels of MMP-9 in BAL compared to controls (p < 0.05). There were no other differences seen between idiopathics and controls or atopics and controls. However, if no account was made for multiple pairwise comparisons, MMP-9 concentrations in all wheeze subgroups were significantly different from controls. Idiopathic wheezers (p < 0.02) and atopics (p < 0.05) were significantly different from controls in these circumstances.

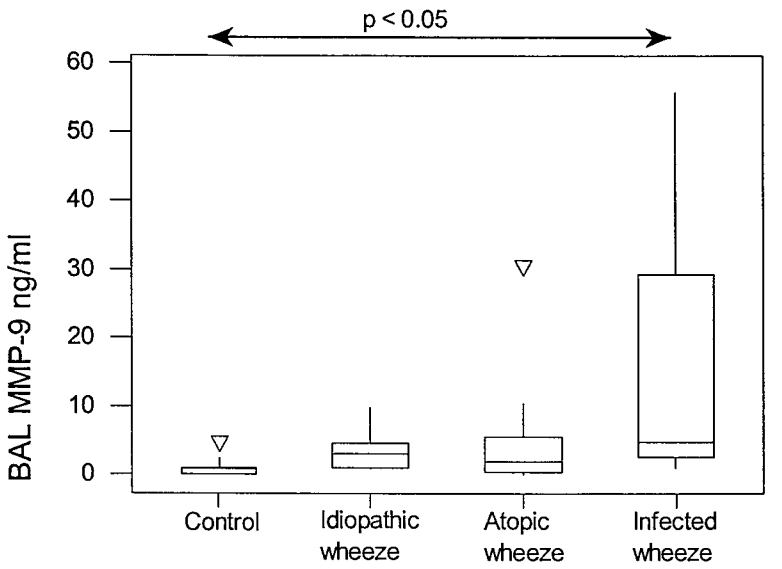


Figure 15 MMP-9 in BAL

The boxes show median and interquartile ranges, whiskers indicate the highest or lowest value within 1.5 times the interquartile range from the first or third quartile, other values are shown as inverted triangles indicating outlying data points. A Kruskal Wallis one-way ANOVA showed significant differences between groups (p= 0.005) with further pairwise difference between control and infected wheezers (p <0.05).

4.3.2. TIMP-1 concentrations in BAL

TIMP-1 concentrations were significantly different between all groups ($p = 0.030$, Figure 16). Children with atopy had the highest values for TIMP-1 in BAL and those with infected wheeze the lowest values. TIMP-1 was significantly different between atopics and infected children demonstrating this relationship ($p < 0.05$, Figure 16). However, the data suggested that infected wheezers had lower TIMP-1 concentrations than controls, but this reached significance only when data was not adjusted for multiple comparisons ($p < 0.02$). Similarly, atopic wheezers TIMP-1 concentrations approached significance compared to controls only when no adjustment was made for the effect of multiple comparisons ($p < 0.1$).

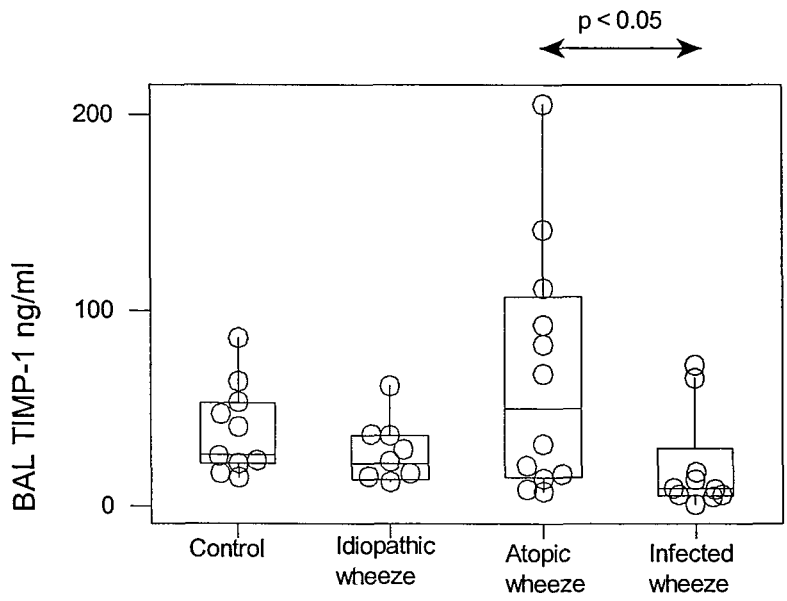


Figure 16 TIMP-1 in BAL

The boxes show median and interquartile ranges. A Kruskal Wallis test was significantly different between groups ($p = 0.030$) with further pairwise difference between atopic and infected wheezers ($p < 0.05$).

4.3.3. Molar ratio of MMP-9 to TIMP-1

The molar ratio of MMP-9 to TIMP-1 was significantly different between groups ($p < 0.001$; Figure 17). The balance between MMP-9 and TIMP-1 was tightly controlled. The maximum control value was 0.018 and all but four children maintained a ratio of below 0.15. Four wheezers with infection had grossly elevated molar ratios with the highest ratio peaking at 2.01.

Molar ratios were increased in idiopathic wheezers compared to controls ($p < 0.05$) and also between infected wheezers and controls ($p < 0.05$). Only atopic wheezers did not show a significant difference with controls. Infected wheezers also had significantly higher ratios than atopics ($p < 0.05$), which suggests that MMP-9 TIMP-1 ratios are the same as controls in atopic wheeze.

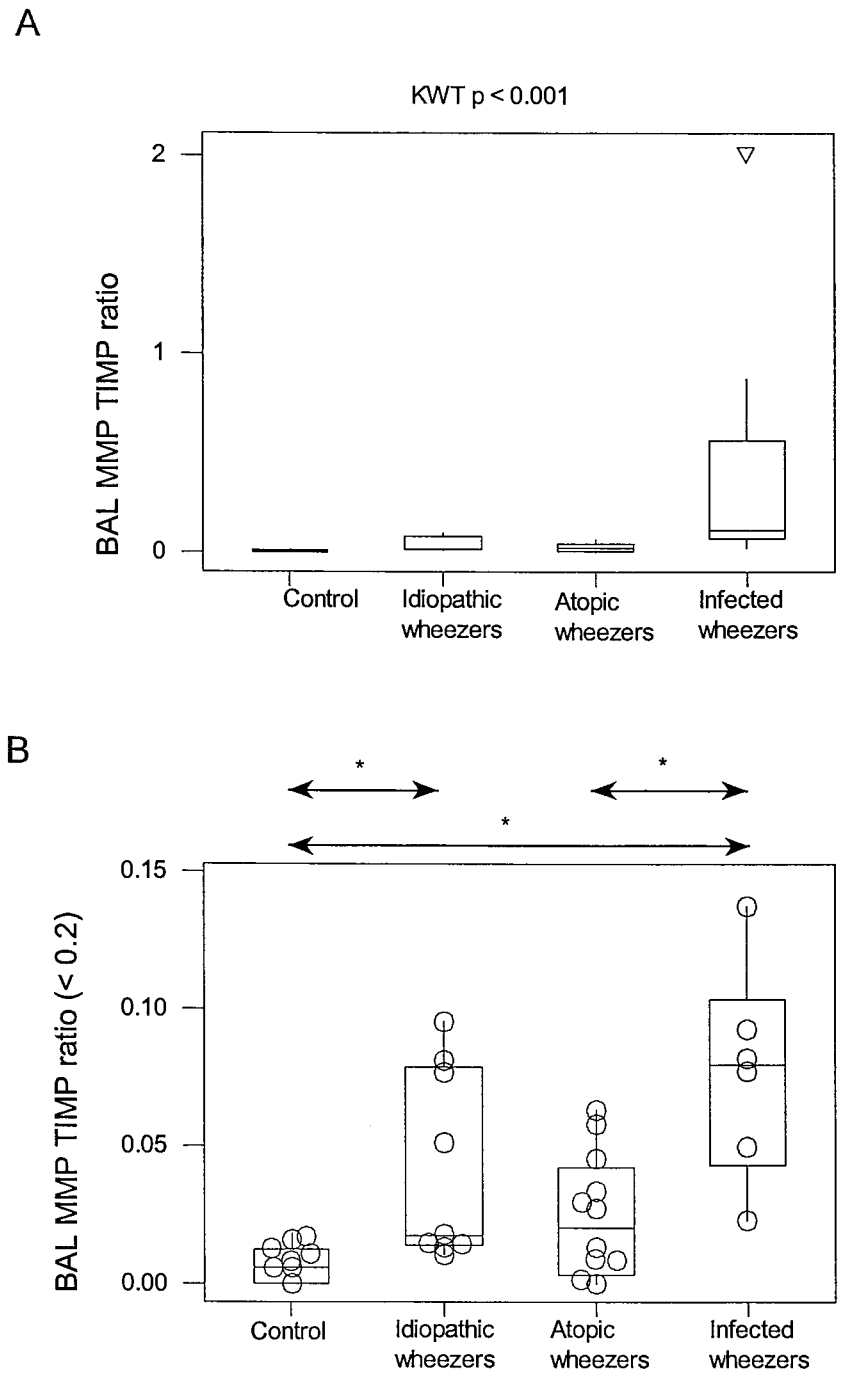


Figure 17 Molar ratio of MMP-9 to TIMP-1 in BAL

A: *The boxes show median and interquartile ranges with inverted triangles indicating outlying data points. A Kruskal Wallis test (KWT) showed significant differences between groups ($p < 0.001$).* **B:** *Values above 20% (found only amongst those with infection) were excluded to aid visual comparison. Pairwise differences were present between controls and infected wheezers, controls and idiopathic wheezers, and atopic and infected wheezers ($p < 0.05$, indicated by *).*

4.3.4. Other markers

Gamma IFN, sIL2-R and eotaxin were not detectable in BAL from any of the subjects in this study. BAL markers were measured in successive aliquots of BAL starting with ICAM-1, ECP and IL-8 and progressing through to these other markers. There were some technical problems with refrigeration before these other markers were analysed and samples had to be moved between freezers on several occasions. Defrosting may have occurred during this period. The absence of these proteins from lavage should be considered with caution in these circumstances. Lavage aliquots for MMP-9 and TIMP-1 had been stored in a separate freezer before this occurred although they had lower priority than the other markers.

4.4. Correlations between cells and markers

Spearman's rank correlation coefficients (r_S) were used to assess the relationships between cell counts and markers within BAL fluid. Wheezers were considered as a whole in the first instance and then correlations were sought within each of the three subgroups.

4.4.1. Alveolar macrophages are a marker for inflammation

The resting BAL differential count is almost entirely composed of alveolar macrophages and lymphocytes, so any cellular infiltration will reduce their relative frequency in BAL. Inverse correlations with macrophages are therefore a sensitive marker for the presence of airway inflammation. Table 23 and Figure 18 show how lymphocytes were associated with reduced macrophages across all groups. In comparison neutrophils were associated with reduced macrophages only in infant wheeze. Eosinophils did not correlate with reduced macrophages amongst any group.

		All wheezers	Controls	Idiopathic wheeze	Atopic wheeze	Infected wheeze
Lymphocytes	r_S	**- 0.661**	**- 0.851**	**- 0.922**	**- 0.675**	**- 0.543**
	p	**< 0.001**	**< 0.001**	**< 0.001**	**0.001**	**0.036**
	n	**49**	**14**	**13**	**21**	**15**
Neutrophils	r_S	**- 0.643**	- 0.459	**- 0.603**	**- 0.677**	**- 0.737**
	p	**< 0.001**	0.098	**0.029**	**0.001**	**0.002**
	n	**49**	14	**13**	**21**	**15**

Table 23 Correlation of BAL macrophages

The table shows Spearman rank correlation coefficients between BAL macrophages and other BAL cell counts. Significant relationships are in bold.

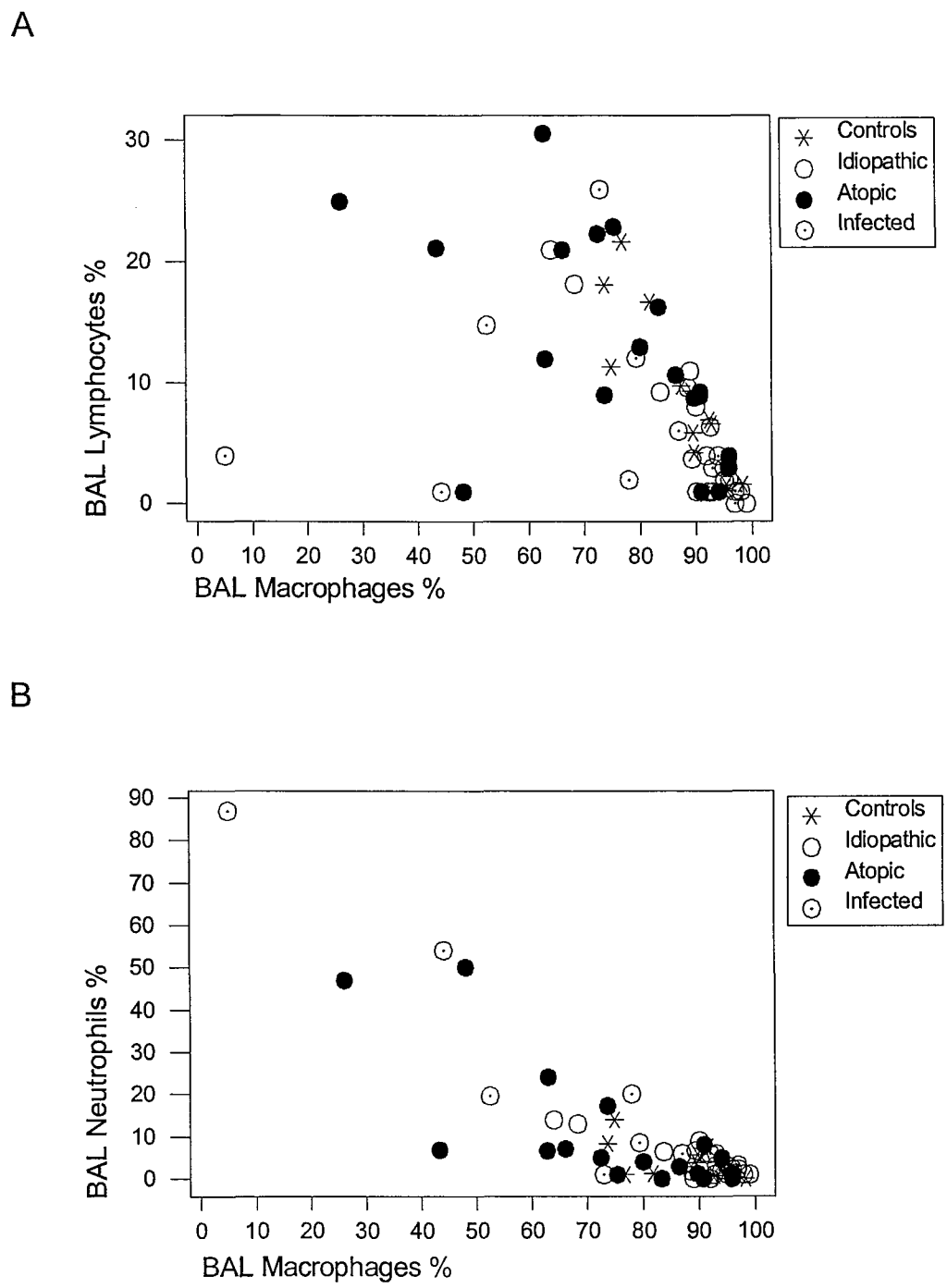


Figure 18 Cellular correlations with BAL macrophages

The scatter plots show correlations between BAL macrophages and lymphocytes (A) and neutrophils (B). The different groups are denoted by the symbols. Lymphocytes correlated with macrophages across all groups whilst neutrophils correlated only amongst wheezers.

4.4.2. Epithelial cells shed during inflammation

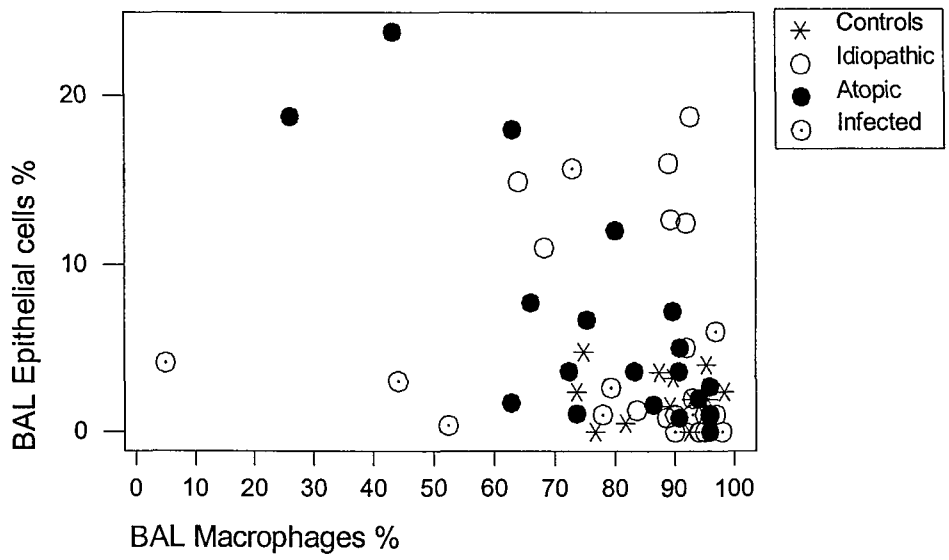
Epithelial cells are a marker for airway damage. Correlations between inflammatory and epithelial cells indicate that inflammation is involved, either directly or indirectly, with airway damage. Table 24 shows that epithelial cell shedding was correlated with reduced macrophages, and increased lymphocytes and neutrophils only amongst those with atopic wheeze. Epithelial cells did not correlate with eosinophils. Epithelial cells also correlated with neutrophils amongst controls, which suggested that the epithelial cell shedding seen in atopics was the normal response of an inflamed airway.

		All wheezers	Controls	Idiopathic wheeze	Atopic wheeze	Infected wheeze
Macrophages	r_S	**- 0.466**	0.000	- 0.182	**- 0.662**	- 0.314
	p	**0.001**	1.000	0.593	**0.001**	0.274
	n	**45**	12	11	**20**	14
Lymphocytes	r_S	**0.440**	- 0.255	0.264	**0.521**	0.018
	p	**0.002**	0.423	0.463	**0.019**	0.950
	n	**45**	12	11	**20**	14
Neutrophils	r_S	0.149	**0.619**	- 0.023	**0.464**	0.118
	p	0.329	**0.032**	0.947	**0.039**	0.688
	n	45	**12**	11	**20**	14
Eosinophils	r_S	0.247	- 0.138	- 0.347	0.420	0.360
	p	0.103	0.668	0.295	0.065	0.206
	n	45	12	11	20	14

Table 24 Correlation of BAL epithelial cells

The table shows Spearman rank correlation coefficients (rS) with probabilities (p) and counts (n) between BAL epithelial cells and other BAL cell counts. Significant relationships are in bold.

A



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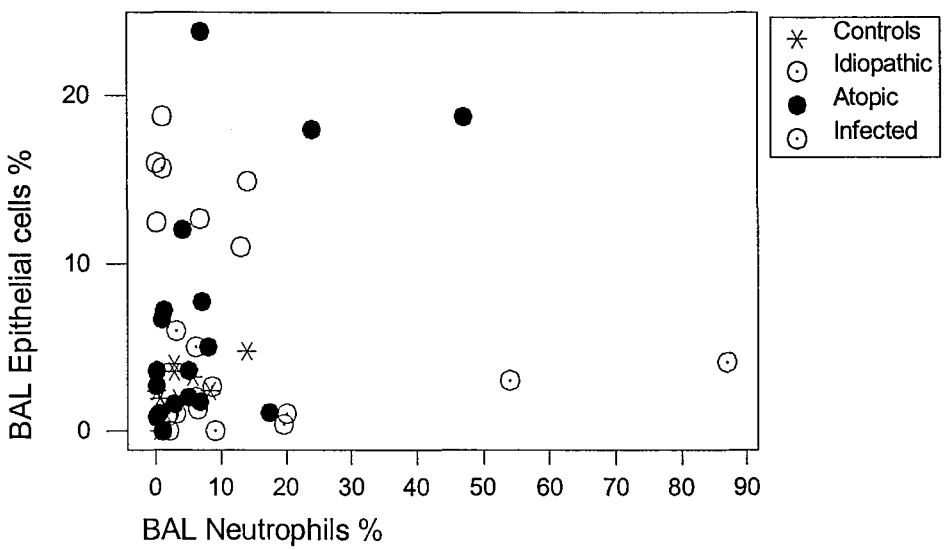


Figure 19 Correlations with BAL epithelial cells

The scatter plots show correlations between BAL epithelial cells and macrophages (A) and neutrophils (B). The different groups are denoted by the symbols. Macrophages correlated with epithelial cells only amongst atopics whilst neutrophils correlated with epithelial cells amongst atopics and controls.

4.4.3. Correlations with BAL ECP

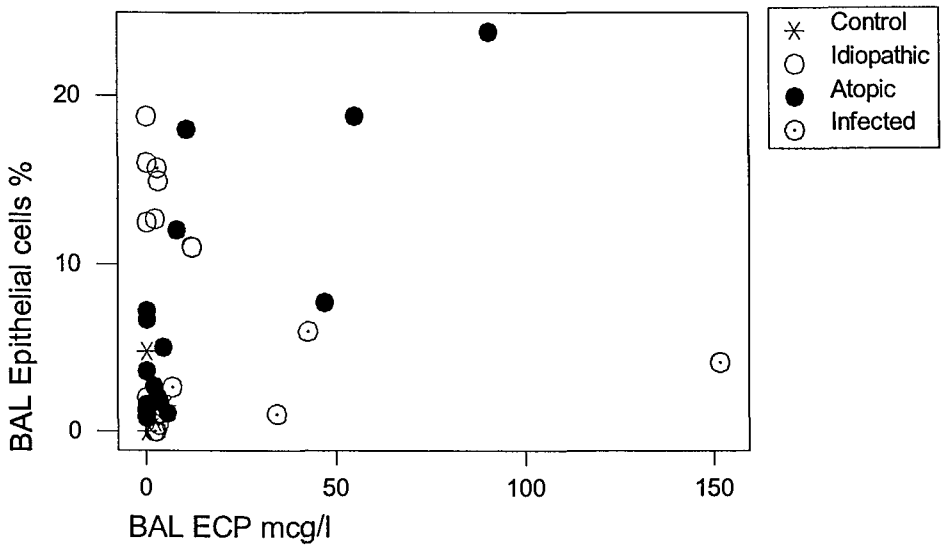
BAL ECP correlated to neutrophils but not eosinophils (Table 25). Significant correlations were seen mainly amongst the atopic groups suggesting a more prominent role in inflammation. Amongst atopic wheezers, the presence of ECP correlated to epithelial cells, reduced macrophages and neutrophils. Neutrophils also correlated with ECP amongst idiopathic wheezers, whilst ECP also correlated with epithelial cells amongst infected wheezers.

		All wheezers	Controls	Idiopathic wheeze	Atopic wheeze	Infected wheeze
Epithelial cells	r_S	0.230	0.088	- 0.165	**0.557**	**0.677**
	p	0.148	0.868	0.609	**0.013**	**0.032**
	n	41	8	12	**19**	**10**
Macrophages	r_S	**- 0.428**	- 0.268	- 0.299	**- 0.547**	- 0.410
	p	**0.004**	0.520	0.322	**0.015**	0.210
	n	**43**	8	13	**19**	11
Neutrophils	r_S	**0.698**	- 0.293	**0.555**	**0.814**	0.329
	p	**< 0.001**	0.482	**0.049**	**< 0.001**	0.324
	n	**43**	8	**13**	**19**	11
Eosinophils	r_S	0.117	0.016	0.117	0.376	- 0.300
	p	0.456	0.970	0.704	0.112	0.370
	n	43	8	13	19	11

Table 25 Correlations of BAL ECP

The table shows Spearman rank correlation coefficients between BAL ECP and BAL cell counts. Significant relationships are in bold. BAL ECP did not correlate with lymphocytes.

A



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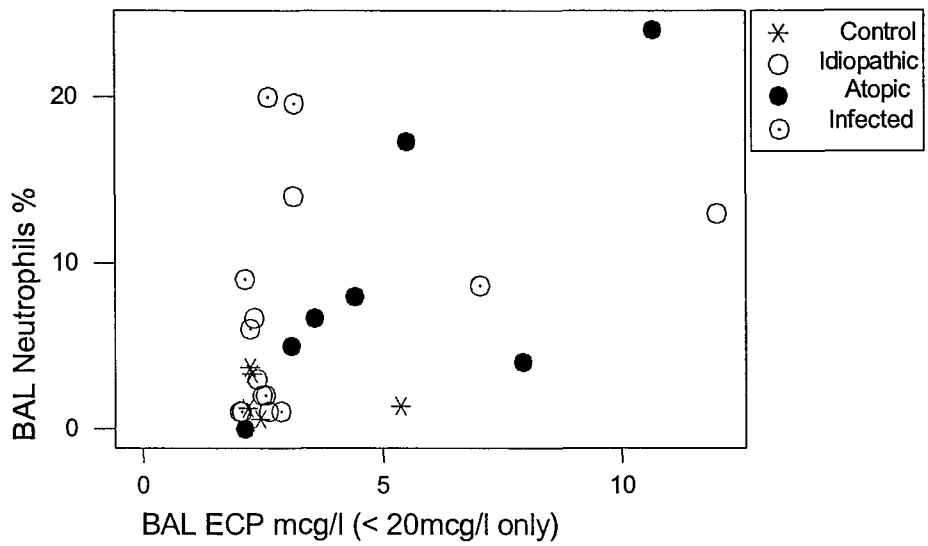


Figure 20 Correlations with BAL ECP

The scatter plots show correlations between BAL ECP and epithelial cells (A) and neutrophils (B). Plot B shows only values for ECP under 20 µg/l to aid visual comparison. The different groups are denoted by the symbols.

4.4.4. Correlation of MMP-9 and TIMP-1 in BAL

MMP-9 correlated with neutrophils. This was true for wheezers overall but also specifically for idiopathic and atopic wheezers, but not infected children (Table 26). BAL MMP-9 correlated with IL-8 and ECP concentrations. BAL IL-8 correlated with MMP-9 amongst all wheezers but not controls. MMP-9 correlated with ECP only amongst atopic wheezers.

The relationships of MMP-9 in BAL may be entirely due to the correlation of MMP-9 with BAL neutrophils, as neutrophils also strongly correlated with ECP (Table 25), BAL IL-8, MMP-9 and its molar ratio with TIMP-1 (Table 27).

		All wheezers	Controls	Idiopathic wheeze	Atopic wheeze	Infected wheeze
Neutrophils %	r_S	**0.728**	0.303	**0.745**	**0.937**	0.396
	p	**< 0.001**	0.365	**0.013**	**< 0.001**	0.257
	n	**34**	11	**10**	**14**	10
IL-8	r_S	**0.854**	0.359	**0.800**	**0.717**	**0.921**
	p	**< 0.001**	0.484	**0.002**	**0.004**	**< 0.001**
	n	**36**	6	**12**	**14**	**10**
ECP	r_S	**0.713**	0.434	0.469	**0.802**	0.410
	p	**< 0.001**	0.331	0.124	**0.001**	0.273
	n	**35**	7	12	**14**	9
TIMP-1	r_S	0.271	0.471	0.421	0.513	0.462
	p	0.110	0.144	0.173	0.061	0.179
	n	36	11	12	14	10
MMP-9:TIMP-1	r_S	**0.611**	**0.840**	0.341	0.486	0.584
	p	**< 0.001**	**0.001**	0.278	0.078	0.077
	n	**36**	**11**	12	14	10

Table 26 Correlations of BAL MMP-9

The table shows Spearman rank correlation coefficients for BAL MMP-9. Significant relationships are in bold.

		All wheezers	Controls	Idiopathic wheeze	Atopic wheeze	Infected wheeze
BAL IL-8	r_S	**0.693**	**0.778**	**0.702**	**0.779**	0.338
	p	**< 0.001**	**0.039**	**0.011**	**< 0.001**	0.339
	n	**41**	**7**	**12**	**19**	10
TIMP-1	r_S	0.072	**0.655**	0.505	**0.567**	- 0.395
	p	0.685	**0.029**	0.137	**0.034**	0.258
	n	34	**11**	10	**14**	10
MMP:TIMP	r_S	**0.570**	0.112	0.411	**0.539**	**0.644**
	p	**< 0.001**	0.744	0.238	**0.047**	**0.044**
	n	**34**	11	10	**14**	**10**

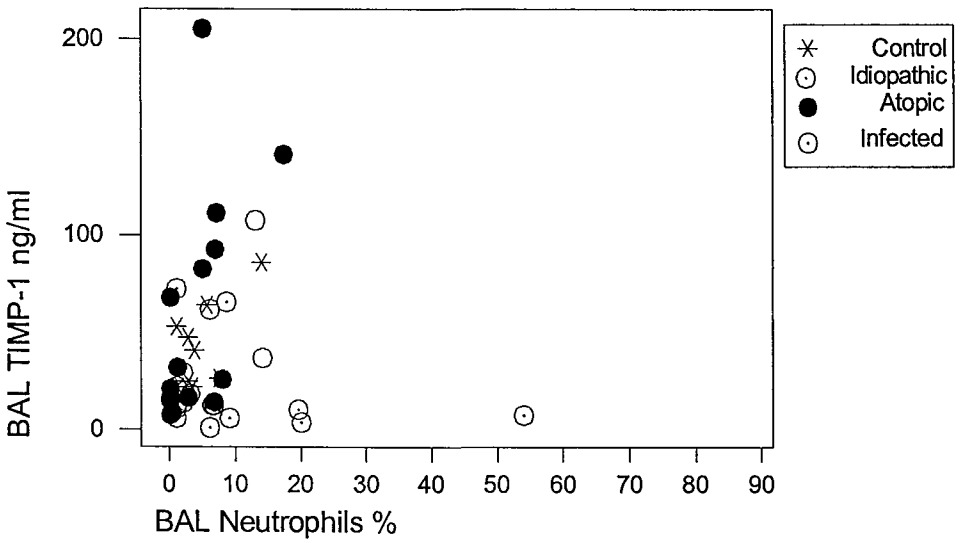
Table 27 Correlations of BAL neutrophils

The table shows Spearman rank correlation coefficients for BAL neutrophils %. Significant relationships are emboldened.

TIMP-1 did not correlate with MMP-9 amongst wheezers or controls. TIMP-1 correlated to neutrophils amongst controls (r_S = 0.655 p = 0.029) and atopic wheezers (r_S = 0.567 p = 0.034), which mirrored the pattern seen between neutrophils and epithelial cells in controls and atopic wheezers (Table 24). This tripartite relationship is shown for atopic wheezers in the 3D plot (Figure 21).

The MMP:TIMP ratio correlated neutrophils with atopic and infected wheezers and overall but not amongst controls or idiopathic wheezers.

A



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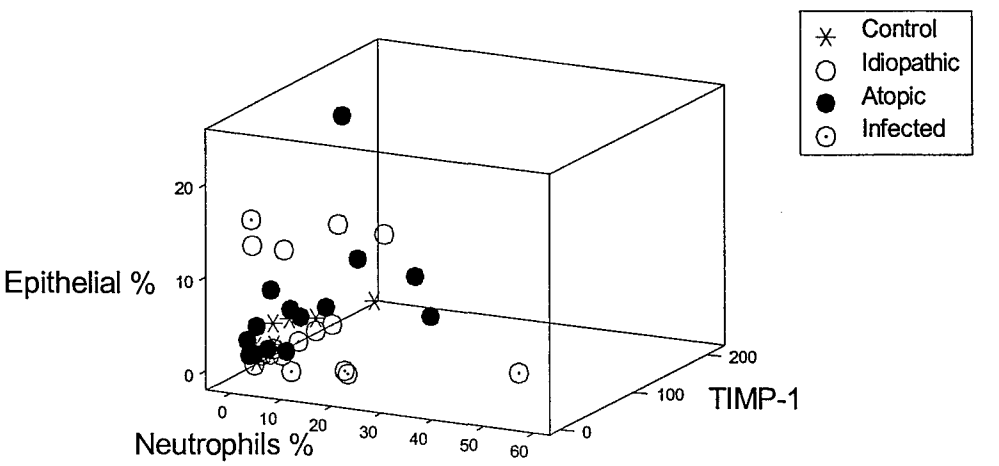


Figure 21 Correlation of TIMP-1 with neutrophils

A: Scatterplot of neutrophils to TIMP-1, significant for atopics and controls. B: 3D plot of the relationship between neutrophils, epithelial cells and TIMP-1.

4.5. Relationship between bronchial washings and BAL

Paired bronchial washings (BW: the first lavage aliquot, see section 1.4.1 and 3.4.2) were compared with BAL (second and subsequent aliquots) amongst wheezers, as BW was not available for controls. This allowed a direct comparison to be made between these lavages, which could be useful when interpreting data from other studies that have used different lavage techniques.

Correlations for each cell type showed strong positive relationships ($r_S > 0.5$) between bronchial washings and BAL that were highly significant ($p < 0.01$) which demonstrated that BW is a good surrogate for BAL. This was also true for the inflammatory markers ECP and IL-8 but not sICAM-1 for which no relationship was found. MMP-9 and TIMP-1 were not measured in BW.

Although the trends were the same, absolute values between BW and BAL differed. The Wilcoxon Signed Rank test was used to compare BAL and BW values within each individual. Epithelial cells (Figure 22) were higher in BW (median 11.4%, 3.9 – 16.2) than BAL (2.6%, 1.0 – 8.5; $p = 0.001$). Cell viability (Figure 22) was reduced in BW (60%, 46 – 83) to BAL (71%, 63 – 83; $p < 0.001$). Neutrophils (Figure 23) were lower in BAL than BW (3.0% versus 6.2 %, $p < 0.001$) and lymphocytes (Figure 23) were raised (12.5 % (6.0 –22.8) versus 9.4 % (5.0 –21.2), $p = 0.052$). Soluble ICAM-1 (Figure 24) was also reduced in BW with a median of 27pg/ml (11 – 55) compared to 77pg/ml (60 –117) in BAL ($p < 0.001$). However, ECP (Figure 24) and IL-8 (Figure 25) concentrations were raised in BW compared to BAL ($p = 0.027$ and 0.003 respectively).

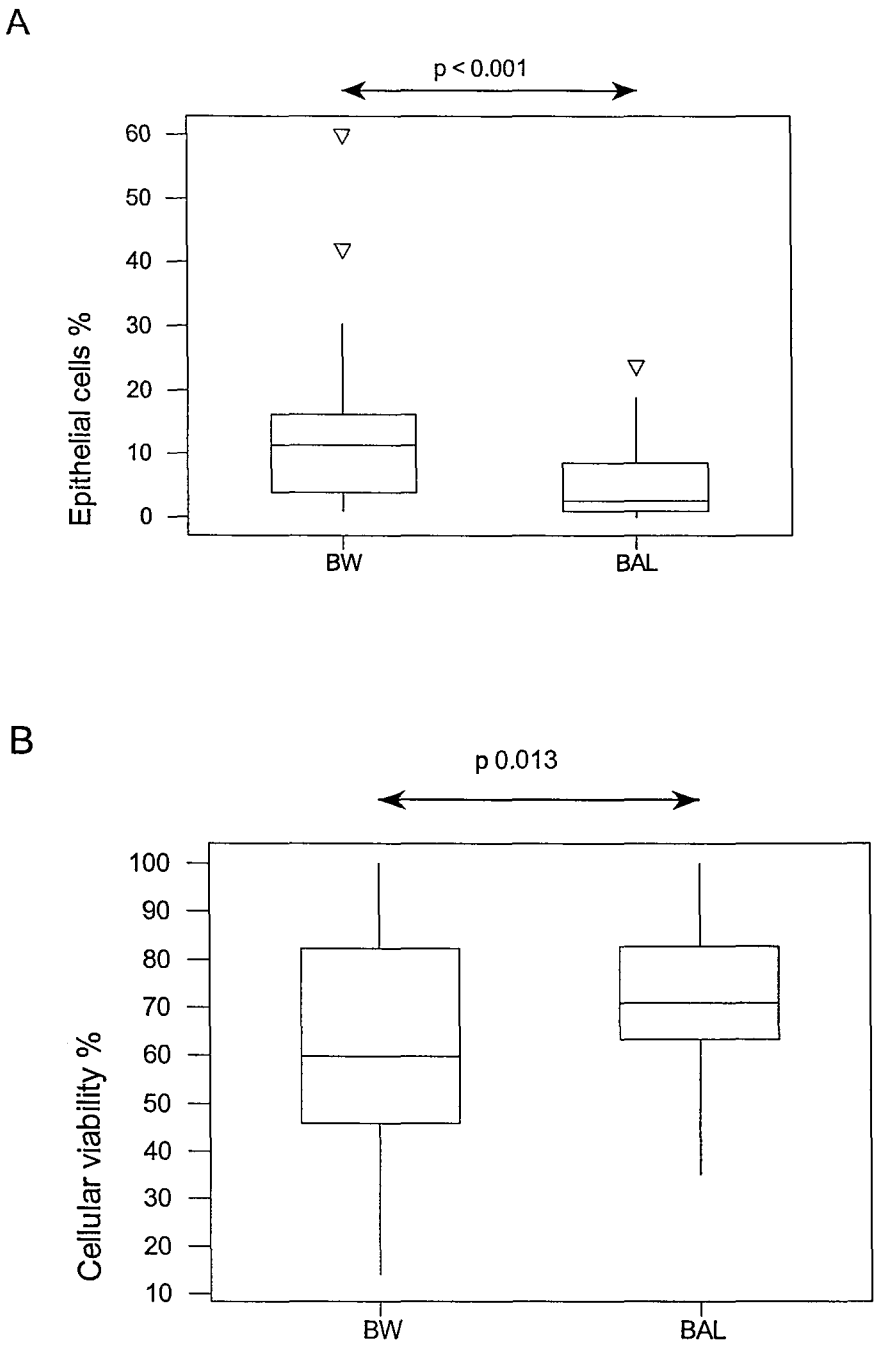


Figure 22 Live cell counts in BW and BAL

The boxes show median and interquartile ranges, whiskers indicate the highest or lowest value within 1.5 times the interquartile range from the first or third quartile, values outlying these ranges are shown as inverted triangles. Boxplots showing the relationship between A: epithelial cell counts in BW and BAL ($p < 0.001$) and B: cellular viability in BW and BAL ($p = 0.013$).

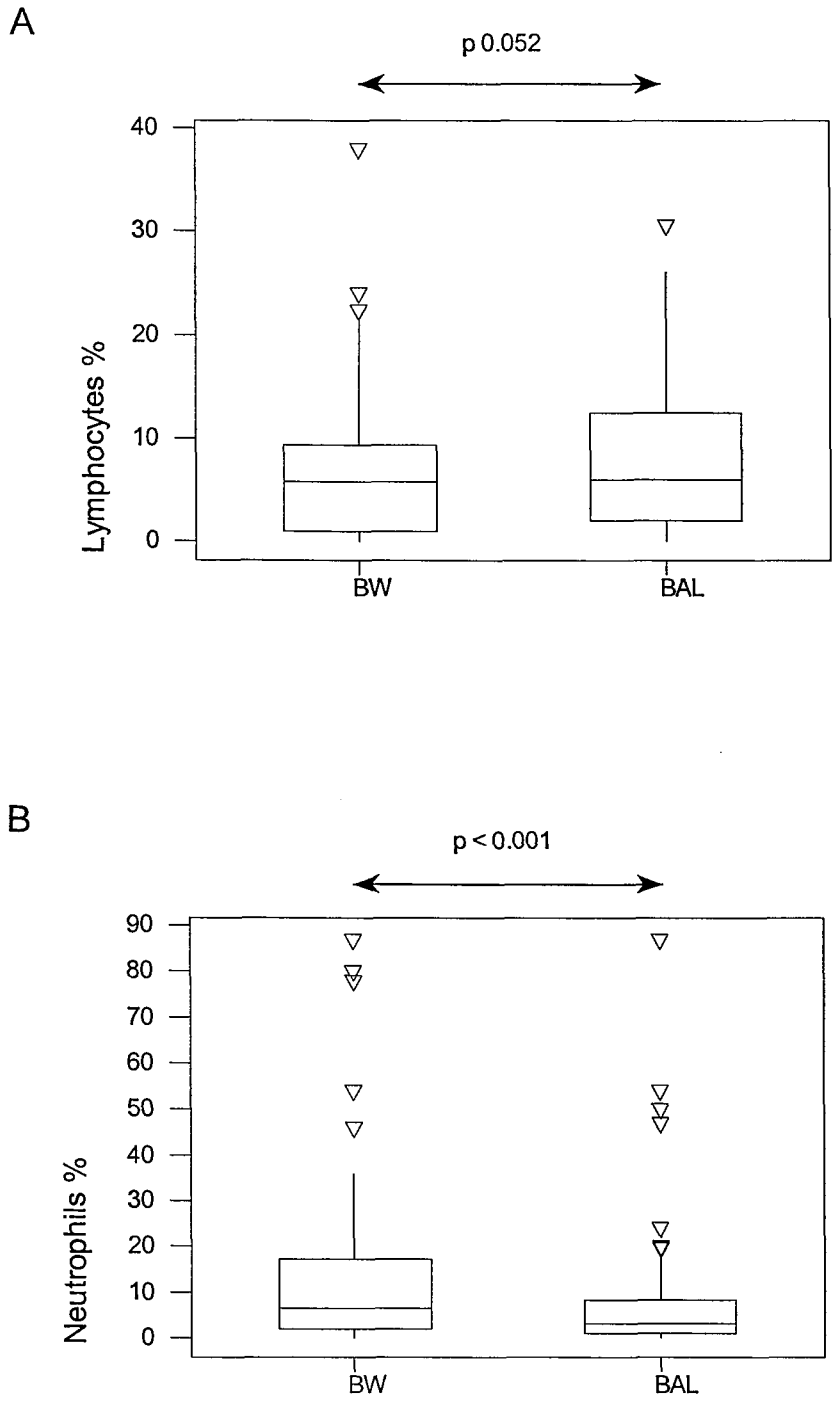


Figure 23 Leucocytes in BW and BAL

The boxes show median and interquartile ranges, whiskers indicate the highest or lowest value within 1.5 times the interquartile range from the first or third quartile, values outlying these ranges are shown as inverted triangles. Boxplots showing relationship between A: lymphocytes in BW and BAL ($p < 0.052$) B: neutrophils in BW and BAL ($p < 0.001$).

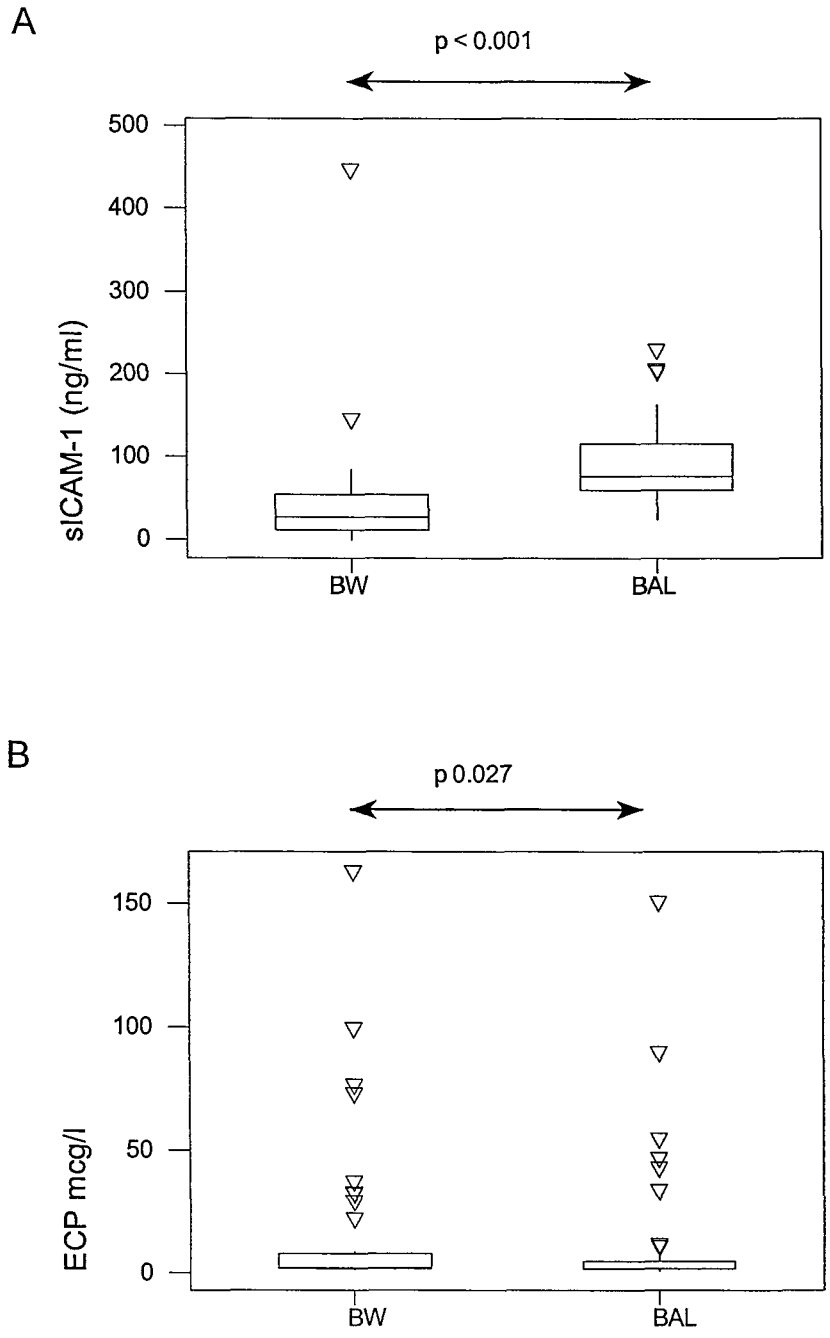


Figure 24 Markers in BW and BAL

The boxes show median and interquartile ranges, whiskers indicate the highest or lowest value within 1.5 times the interquartile range from the first or third quartile, values outlying these ranges are shown as inverted triangles. Boxplots showing relationship between A: sICAM-1 (ng/ml) in BW and BAL ($p < 0.001$)B: ECP (µg/l) in BW and BAL ($p = 0.027$).

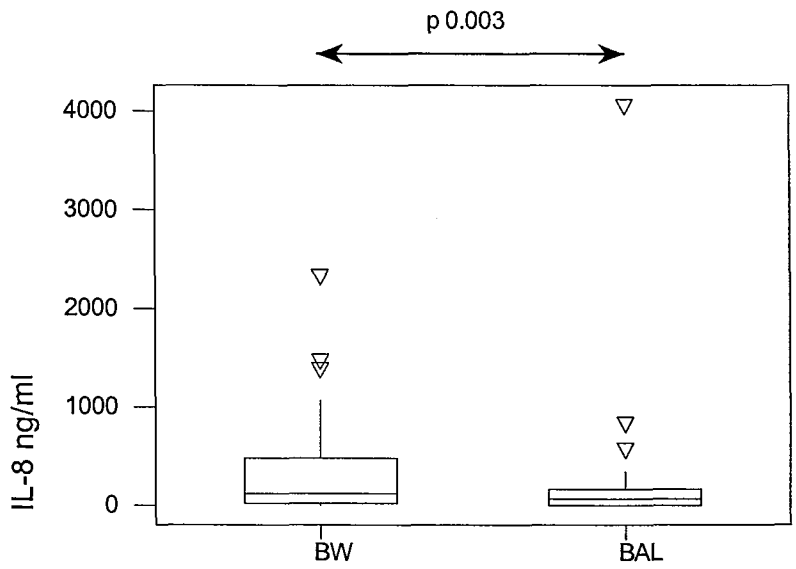


Figure 25 IL-8 in BW and BAL

Boxplot showing relationship between IL-8 (ng/ml) in BW and BAL (p = 0.003) The boxes show median and interquartile ranges, whiskers indicate the highest or lowest value within 1.5 times the interquartile range from the first or third quartile, values outlying these ranges are shown as inverted triangles.

4.6. Chapter summary

Differences between centres in cell counts made comparison between groups difficult. TNCC was different between groups and was raised amongst infected wheezers compared to idiopathic wheezers. Infected wheezers also showed higher proportions of neutrophils and fewer lymphocytes than idiopathic wheezers. Compared to controls, no significant differences were seen. However atopic wheezers had higher eosinophils and infected wheezers higher neutrophil counts.

IL-8 was raised between idiopathic and infected wheezers but not controls. ECP was raised between controls and infected but not atopic wheezers. No differences were seen in sICAM-1 concentrations between groups.

MMP-9 was significantly different across all groups being raised in infected wheezers compared to controls. TIMP-1 was reduced in infected wheezers but not significantly different from controls in other wheezers. There was a trend toward raised TIMP-1 in atopic children but this did not reach significance. MMP/TIMP ratios were significantly different from controls in idiopathic and infected wheezers but not amongst atopics.

Correlations between cells and markers showed that epithelial cells increased with inflammation particularly amongst atopic wheezers but not infected children. In atopic children, epithelial shedding was related to neutrophils, IL-8, ECP and TIMP-1. This relationship between neutrophils, epithelial cells and TIMP-1 was seen amongst controls and atopics but not infected or idiopathic wheezers.

ECP did not correlate to eosinophils but instead correlated with neutrophils even in atopics. ECP correlated to more markers of inflammation in atopic wheezers than the other groups, suggesting a more important role for this protein in atopic wheeze.

MMP-9 correlated with neutrophils and also with most other markers of inflammation. These correlations may be due to the central role of neutrophils in inflammatory process in all forms of infant wheeze.

The chapter finished by considering the role of bronchial wash compared to BAL. It is clear that BW can provide a surrogate for BAL cell counts as most cells type correlated between lavages. However actual values differ significantly for both cells and markers in this respect the two are not comparable.

Chapter 5: Nasal lavage findings in infant wheeze

5.1. Introduction

This chapter considers the hypotheses that nasal lavage findings in atopic wheezers mirror changes in the lower airway, and that NAL may provide a surrogate for BAL. The wheezers in this study had nasal lavage and serum samples taken at time of bronchoalveolar lavage. Nasal lavage was not available for control subjects.

5.2. Nasal cytology

As in Chapter Four, nasal lavage was compared between the three subgroups of wheezers: idiopathic, atopic and infected. A two way ANOVA was used to account for fixed differences between centres. A random effect model gave very similar results. The results are shown in Table 28. Centre differences were seen for macrophages, neutrophils and lymphocyte proportions but not for nasal TNCC, epithelial cells, cell viability and eosinophils. Nasal neutrophils were higher in infected wheezers compared to idiopathic and this approached significance (0.083). Neutrophil counts for the different wheeze phenotypes are shown by centre in Figure 26. Infected wheezers also showed reduced proportion of lymphocytes (8.8 %; p = 0.047) when compared to idiopathics (21.3 %). Lymphocyte counts for the different wheeze phenotypes are shown by centre in Figure 27. There were no overall differences in the other cell counts between groups.

BTLSM *LSM, SE* *n*	**Idiopathic wheezers**	**Atopic wheezers**	**Infected wheezers**	**p**
TNCC	3.0 12.6, 0.6 14	3.0 12.5, 0.5 21	3.7 12.8, 0.6 15	0.924
Cellular viability	71.4 1.01, 0.07 13	76.6 1.07, 0.06 20	69.4 0.98, 0.08 14	0.646
Epithelial cells	15.2 0.40, 0.09 13	17.2 0.42, 0.08 18	9.4 0.31, 0.11 12	0.729
Macrophages	14.8 0.39, 0.09 14	24.5 0.52, 0.08 18	11.9 0.36, 0.11 12	0.411
Neutrophils	41.2 0.70, 0.09 14	51.8 0.80, 0.08 18	66.3 0.95, 0.11 12	0.216
Lymphocytes	21.3 0.48, 0.06 14	14.0 0.38, 0.05 18	8.8 0.30, 0.07 12	0.114
Eosinophils	0.5 0.021, 0.017 14	0.3 0.056, 0.014 18	0.3 0.055, 0.021 12	0.238

Table 28 Cell counts in nasal lavage

The table shows back transformed least squares means (BTLSM) with original transformed least squared means (LSM) with standard errors (SE) and counts (n). Total Nucleated Cell Counts (TNCC) are shown as cells x10^5/ml and other counts as percentages when back transformed.

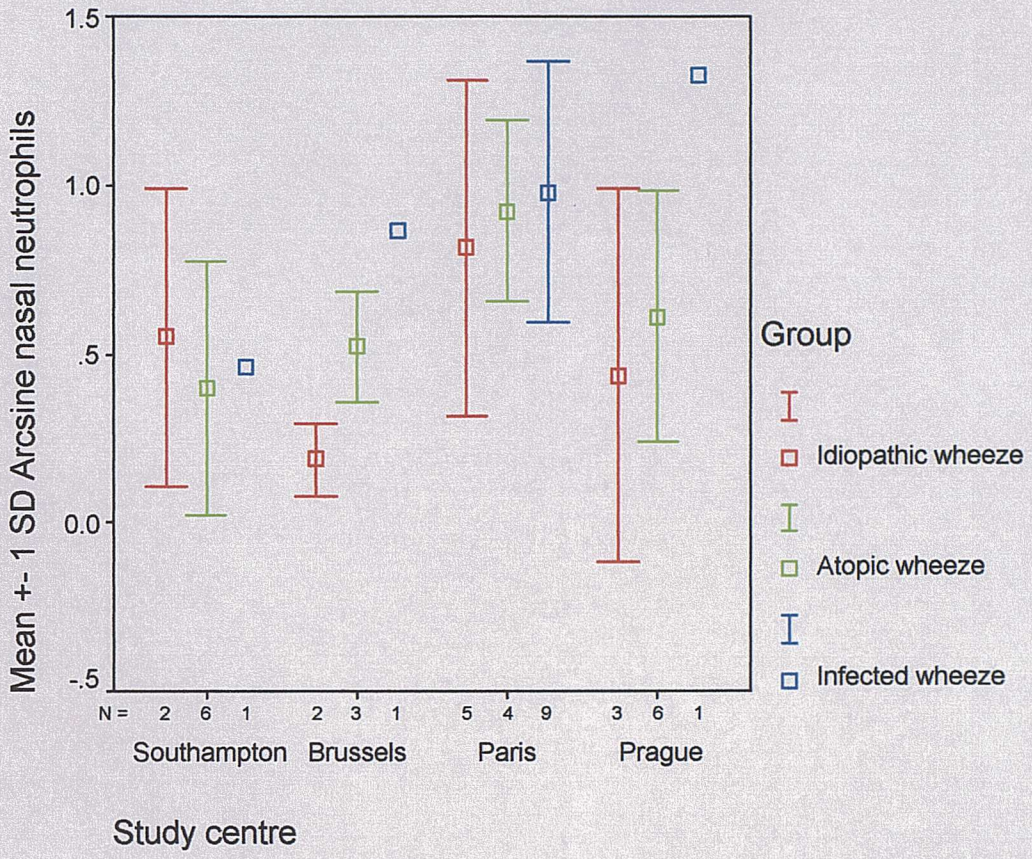


Figure 26 NAL neutrophils in infant wheeze

Error bar plot of mean and standard deviations of transformed neutrophil proportions in nasal lavage by centre and group. Trends show an increase in nasal neutrophils in the infected wheezers.

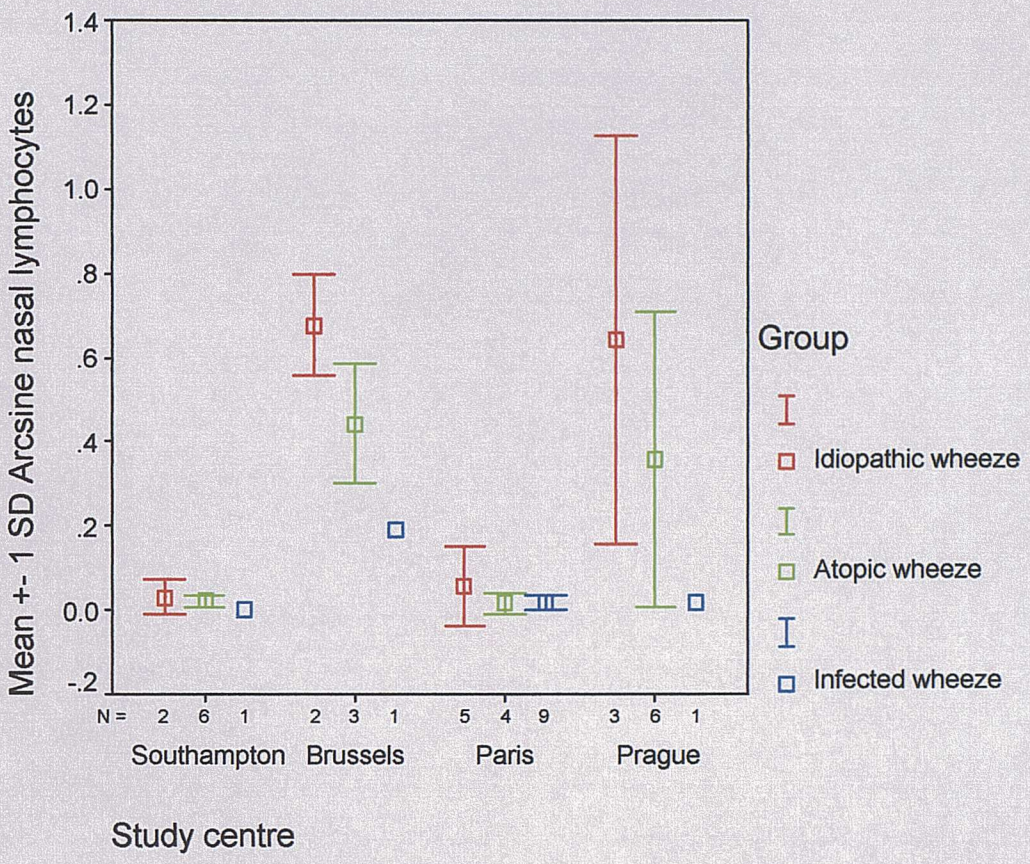


Figure 27 NAL lymphocytes in wheeze

Error bar plot of mean and standard deviations of transformed lymphocyte proportions in nasal lavage by centre and group. Trends show a decrease in nasal lymphocytes in the infected wheezers.

5.3. Nasal inflammatory markers

Nasal ECP was not significantly different between groups. Idiopathic wheezers had 14 μg/l (range 4 –93) ECP in NAL, infected 34 μg/l (8 – 92) and atopics 49 μg/l (10 –108). Nasal IL-8 and sICAM-1, were also not significantly different between wheeze subgroups. Nasal lavage was available for measurement of MMP-9 and TIMP-1 in 11 children, but numbers were too small to make a meaningful comparison between groups.

5.4. NAL as a surrogate for BAL

Correlations were performed between paired NAL and BAL. Nasal lymphocytes correlated with BAL lymphocytes (r_S 0.414, p = 0.006). They were the only nasal cell type to correlate with their lower respiratory tract counterpart (Figure 28).

There was evidence that atopic nasal inflammation was associated with bronchoalveolar inflammation. Nasal ECP correlated with BAL ECP (r_S 0.567, p = 0.001; Figure 29), but nasal eosinophils did not correlate with BAL eosinophils. Nasal eosinophils also did not correlate with either NAL or BAL ECP.

In the absence of a direct correlation for nasal and bronchial eosinophils, dichotomous variables were created using the presence of absence of eosinophils in BAL, and high or low counts for eosinophils in NAL in order to consider the co-segregation of atopic inflammation in both upper and lower respiratory tract. Eosinophils were present in both compartments in only 14% of wheezers and this result was not significant using Chi squared analysis.

Amongst wheezers as a whole group, atopic nasal inflammation was associated with bronchial epithelial shedding. Nasal lymphocytes, NAL ECP and NAL sICAM-1 correlated with BAL epithelial cells (r_S 0.437, p = 0.005; r_S 0.432, p = 0.009; and r_S 0.496, p = 0.001 respectively; Figure 30).

NAL IL-8 correlated to BAL ECP (r_S 0.512, p = 0.001) demonstrating the interaction of neutrophils and eosinophils in wheezy inflammation (Figure 31).

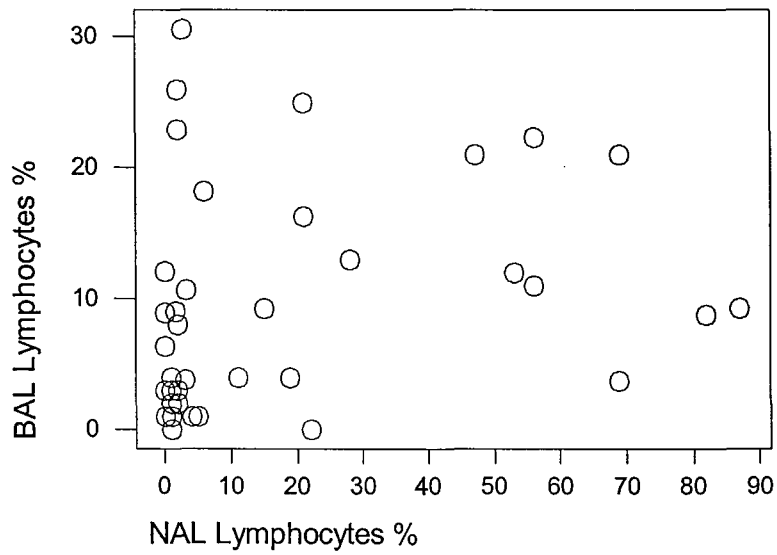


Figure 28 Correlation between NAL and BAL lymphocytes

Scatterplot showing the relationship between nasal and bronchoalveolar lymphocytes (r_S 0.414, $p = 0.006$).

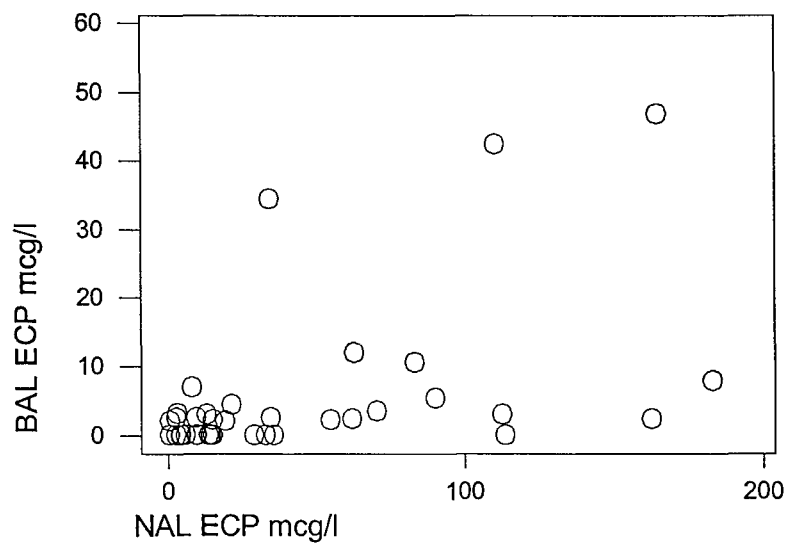
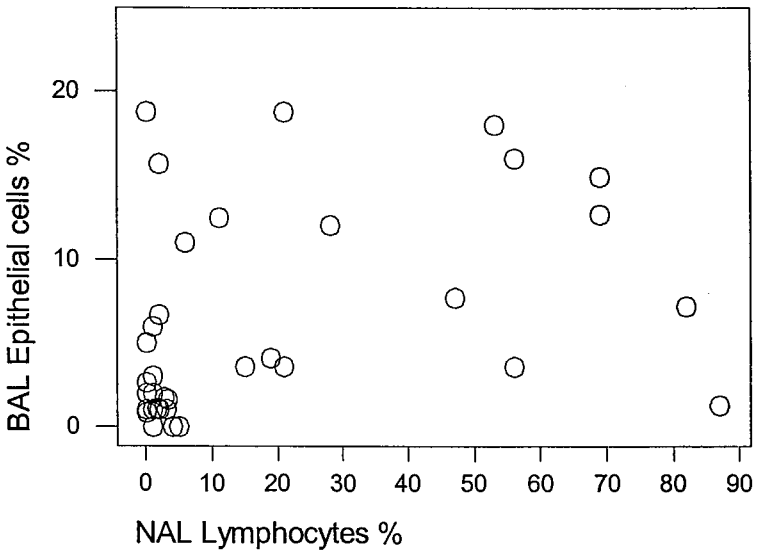


Figure 29 ECP correlation between nose and lung lavages

Scatterplot showing the relationship between nasal and bronchoalveolar ECP concentrations (r_S 0.567, $p = 0.001$). One outlier of BAL ECP 150 µg/l is excluded to aid visual comparison.

A



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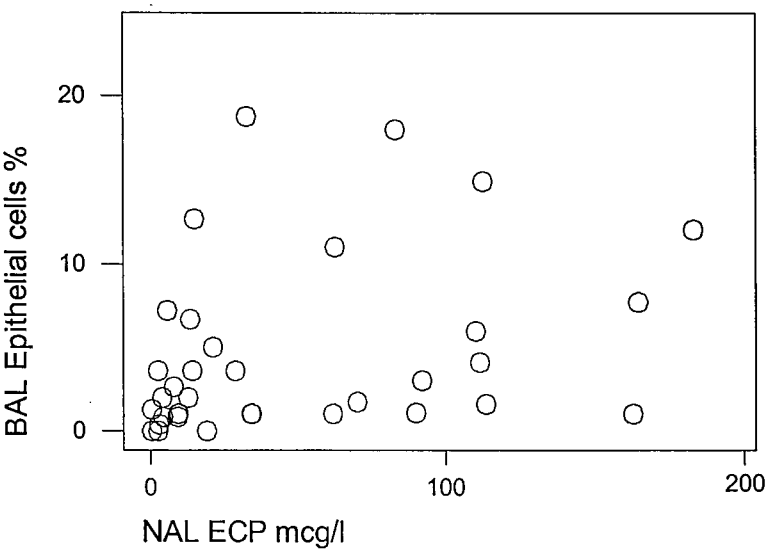

Figure 30 Nasal correlates with BAL epithelial shedding

A: Scatterplot showing the relationship between nasal and bronchoalveolar lymphocytes (r_S 0.437, $p = 0.005$). B: Scatterplot showing the relationship between nasal ECP and bronchoalveolar epithelial cells (r_S 0.432, $p = 0.009$).

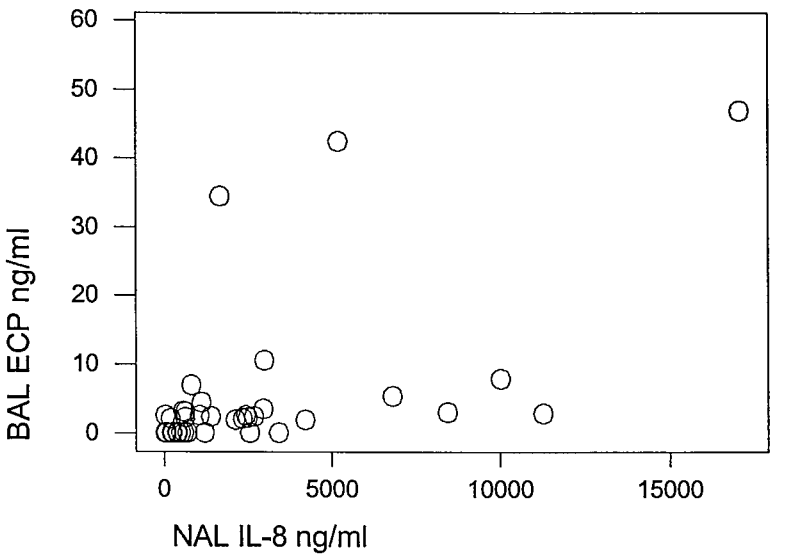

Figure 31 Nasal IL-8 correlates to BAL ECP

Scatterplot showing the relationship between nasal IL-8 and bronchoalveolar ECP (r_S 0.512, $p = 0.001$).

5.5. Serum findings in infant wheeze

Serum ECP and soluble ICAM-1 were measured from serum in infant wheeze. The results are shown in Table 29. There were no significant differences either overall or between groups. There were no significant correlations between serum markers and BAL cytology for wheezers overall or for atopic subjects in particular. Equally, nasal lavage cytology and markers did not correlate with serum ECP or sICAM-1.

median *range* *n*	**Idiopathic wheeze**	**Atopic wheeze**	**Infected wheeze**	**P**
Serum ECP µg/l	17.6 2.7 – 71.9 15	28.5 3.1 – 125.0 22	34.9 32.1 – 40.7 15	NS
Serum sICAM-1 ng/ml	335 243 – 755 15	409 307 - 589 22	334 268 – 636 15	NS

Table 29 Serum markers in infant wheeze

The table shows median with range and counts for ECP and sICAM-1 in serum. There were no significant differences between results. P values are from Kruskal Wallis one way ANOVA tests between groups.

5.6. Allergic rhinitis and infant wheeze

The bronchoalveolar lavage of infant wheezers was examined in the light of allergic nasal disease, in order to test the hypothesis that allergic upper airways disease has an impact on lower respiratory tract disease. Wheezers were divided into two groups based on the presence or absence of allergic rhinitis (AR). AR was defined as a clinical diagnosis of allergic rhinitis or aeroallergen sensitivity. By definition all these children were also atopic wheezers. As bacterial infection has been shown to influence atopic markers, such as ECP, these children were excluded from the analysis. AR data were not available for two children who were also excluded, however, wheezers with missing cultures were included in this analysis.

Twelve infants had either allergic rhinitis or sensitivity to an aeroallergen by skin prick test or RAST, compared to 24 wheezers without nasal disease. There was no difference in ages between the two groups of wheezers (Mann Whitney U test, p = 0.7). Wheezers with AR had a median age of 1.1 years (range 0.6 – 3.5) whilst those without were slightly older with a median age of 1.3 (range 0.5 – 3.1).

5.6.1. NAL and serum in allergic rhinitis

Nasal lavage showed significantly more eosinophils in subjects with AR than those without (Mann Whitney U test, p = 0.044). Children with AR had a median proportion of 1.0% (range 0 – 27.6) compared to 0 (0 – 1.2) in those without AR. NAL eosinophil counts had been previously shown not to vary according to centre and no adjustment for centres differences were made in this calculation. Another way of looking at this difference was to categorise subjects into those with high or low nasal eosinophils by rank ordering the data (Table 30). Chi squared analysis was significant (p = 0.039). However, NAL ECP was not significantly different between groups (14 μg/l, range 0 –163 versus 76 μg/l, 5 –183 in AR).

Nasal cell viability, the counts for which also did not vary significantly between centres, was lower amongst those with AR (66%) compared to those without (81%). This approached but did not reach significance (Mann Whitney U test, p = 0.091).

NAL Eosinophils	Allergic Rhinitis		
	No	Yes	
Low	16	4	20
High	4	6	10
	20	10	30

Table 30 Nasal eosinophils in AR

The table shows the number of children with allergic rhinitis who also had high proportion of eosinophils in NAL (Chi Squared p = 0.039). Whilst a higher proportion of children with AR had high nasal eosinophils they did not account for all children in this group.

Serum ECP was elevated in those with AR (p = 0.002). The median serum concentration was 63 μg/l in AR (range 16 – 124) compared to 13 in those without nasal disease (range 2.7 – 72). The relationship is shown in Figure 32.

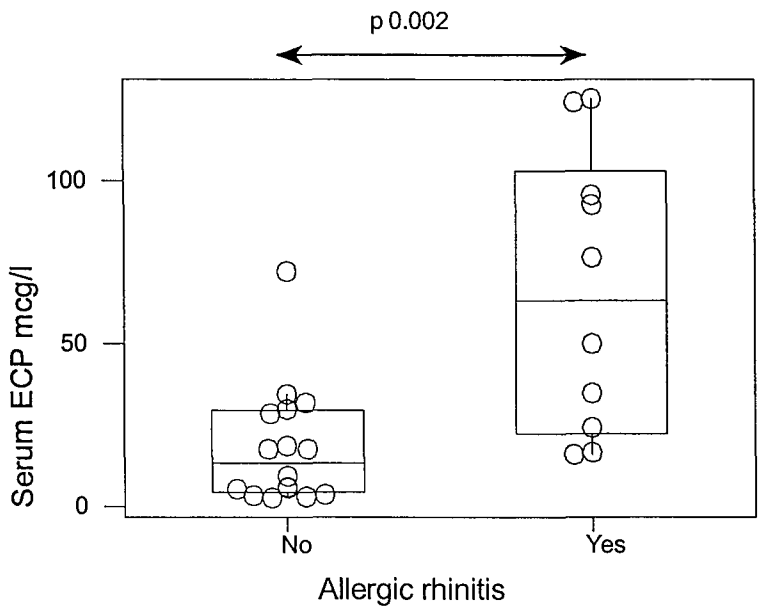

Figure 32 Serum ECP in allergic rhinitis

Serum ECP was significantly higher amongst wheezers with allergic rhinitis (p = 0.002.)

5.6.2. BAL in allergic rhinitis

The presence of allergic upper respiratory tract disease as defined by allergic rhinitis had an impact on the composition of bronchoalveolar lavage. BAL eosinophils were raised amongst subjects with AR (p = 0.05). Those without AR had few if any eosinophils in BAL (median 0, range 0 – 2.0%) whilst those with AR had a median of 1% (0 – 27.6%). Dividing subjects into those with or without eosinophils in BAL confirmed this finding (Table 31). However, the presence of high nasal eosinophils did not have a significant relationship with the presence or absence of eosinophils in BAL (data not shown). Like NAL ECP concentrations in AR, BAL ECP concentrations were not significantly different between groups. There was no difference in IL-8, sICAM-1, MMP-9 or TIMP-1 concentrations in BAL between groups in AR.

BAL Eosinophils	**Allergic Rhinitis**		
	No	**Yes**	
Absent	17	3	20
Present	4	9	13
	21	12	33

Table 31 BAL eosinophils in AR

The table shows the number of children with allergic rhinitis who also had eosinophils in BAL (Fisher's Exact test (two sided) p = 0.003).

5.6.3. Correlations in allergic rhinitis

Correlations were performed between nasal lavage cell counts and BAL cells and markers amongst wheezers with allergic rhinitis. Trends seen in the correlations within BAL fluid for atopic wheezers were more obvious amongst those with allergic rhinitis. For instance, BAL epithelial cells were related to a reduction in macrophages ($r_S = -0.820$, $p = 0.01$, $n = 12$), lymphocytes ($r_S = -0.600$, $p = 0.030$) and neutrophils ($r_S = -0.687$, $p = 0.010$) but not eosinophils. Epithelial cells were also related to ECP ($r_S = 0.791$, $p = 0.004$, $n = 11$, Figure 33) and TIMP ($r_S = 0.857$, $p = 0.014$, $n = 7$, Figure 34) but again not eosinophils.

Considering correlations between nasal and bronchoalveolar lavage, nasal neutrophils correlated with BAL neutrophils ($r_S = 0.618$, $p = 0.043$). Nasal lymphocytes also correlated with BAL epithelial cell shedding ($r_S = 0.624$, $p = 0.054$, $n = 10$, Figure 35). In wheezers with allergic rhinitis, nasal eosinophillia correlated to BAL TIMP concentrations ($r_S = 0.880$, $p = 0.021$, $n = 6$), but did not correlate to any other marker. The TIMP data did not produce a convincing graphical relationship and with such small numbers the results must be treated with caution.

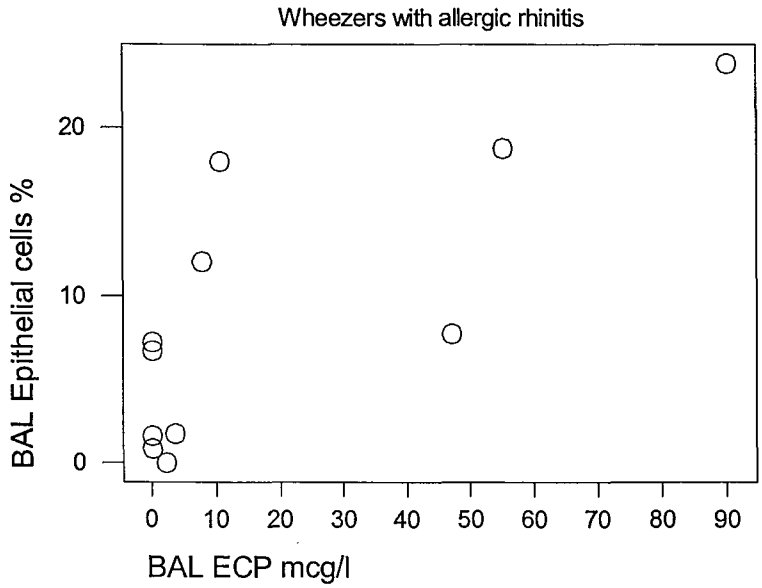

Figure 33 BAL Epithelial cells and ECP in AR

The scatter plot shows the correlation between BAL epithelial cell and ECP amongst wheezers with allergic rhinitis at bronchoscopy ($r_S = 0.791$, $p = 0.004$).

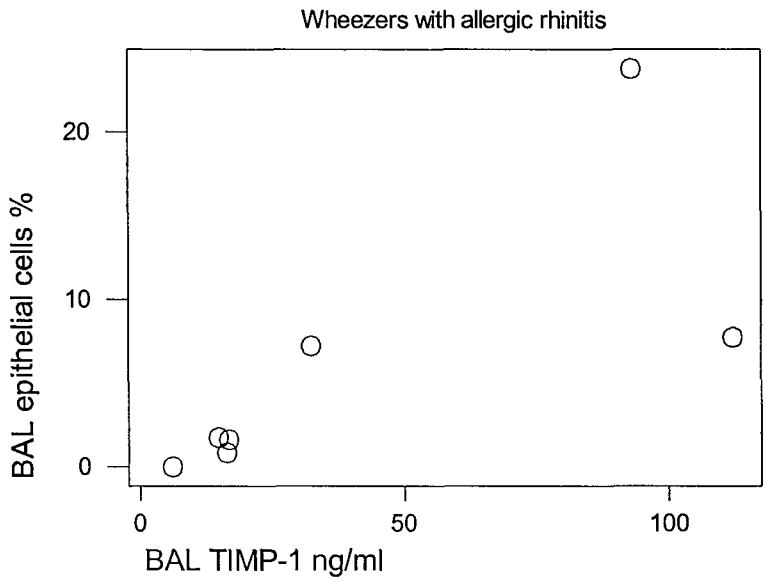

Figure 34 BAL epithelial cells and TIMP-1 in AR

The scatter plot shows the correlation between BAL epithelial cells and TIMP-1 amongst wheezers with allergic rhinitis at bronchoscopy ($r_S = 0.857$, $p = 0.014$).

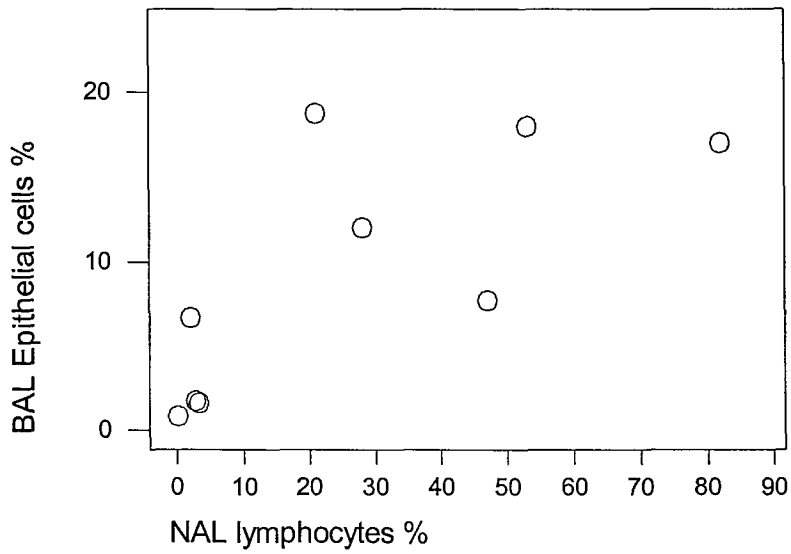

Figure 35 BAL epithelial cells and NAL lymphocytes in AR

The scatter plot shows the correlation between BAL epithelial cells and NAL lymphocytes amongst wheezers with allergic rhinitis at bronchoscopy (r_S = 0.624, p = 0.054).

5.7. Chapter summary

This chapter sought to examine the hypothesis that changes in nasal lavage mirror those found in the lower airway. The role of NAL and serum as surrogate markers for BAL were also considered.

Nasal neutrophils were raised in infected wheezers whilst nasal lymphocytes were reduced in this group. There were no differences between nasal eosinophils between groups. Similarly nasal IL-8, sICAM-1 and ECP did not vary significantly between groups.

Nasal ECP correlated with BAL ECP suggesting atopic inflammation in both compartments. However, nasal eosinophils did not correlate with BAL eosinophils. Also eosinophils did not co-segregate in both compartments. However, more subtle signs of nasal inflammation such as nasal ECP and sICAM-1 concentrations and the presence of nasal lymphocytes, correlated with lower respiratory tract inflammation as evidenced by epithelial shedding.

Separating wheezers with allergic rhinitis into a separate group showed some interesting results. As might be expected, nasal eosinophils were higher in this group, but interestingly not nasal ECP. Serum ECP was raised amongst those with AR. Lower respiratory tract changes were seen in wheezers with AR including raised eosinophils but again not ECP. Correlations within those with AR reinforced the pattern seen in atopic wheezers, where LRT inflammation was associated with epithelial cell numbers. It also included a relationship between TIMP-1 and epithelial cell shedding, but none for MMP-9.

Chapter 6: Questionnaire Follow Up Study

6.1. Introduction

The results of the follow up questionnaire for the infant wheezers are discussed in this chapter. The ISAAC questionnaire was divided into three modules (wheezing, rhinitis and eczema) and each section is discussed in turn. Current symptoms of wheeze and rhinitis were then used to compare serum, upper and lower respiratory tract lavage findings at initial bronchoscopy in an attempt to define the immunological markers, early in the disease process, that were associated with persistent symptoms.

6.2. Response rate

All four of the BAL study centres were invited to participate in the follow up study but only Prague and Southampton agreed. Questionnaires were sent to parents of all the children, from these two centres, who had been previously enrolled in the BAL study. No children were excluded from the study at these centres. This was nearly half (48%, n = 25) of the original 52 BAL study subjects. Subjects from the other two centres were excluded from further analysis. Following two rounds of postal questionnaire, 20 parents had replied giving an overall response rate of 80% (20/25). Southampton had a 60% response (6/10) and Prague 93% (14/15).

6.3. Characteristics of respondents

Characteristics of the respondents are shown in Table 32. Of the 20 who responded, 12 were atopic wheezers reflecting the excess of these children amongst the participating centres. Three were infected wheezers and five idiopathic. There were no differences in clinical characteristics in those who did not respond to the survey.

The average age of the children at follow up was 4.0 years (range 2.7-6.9). This compared to 1.4 years (0.6 – 3.8) at bronchoscopy. The average length of time between bronchoscopy and follow up was 2.6 years (2.0 – 3.6).

	Responders	Non-responders	Total/ p value
Wheeze subgroups			
Idiopathic wheezers	5	3	8
Atopic wheezers	12	2	14
Infected wheezers	3	0	3
Total (n)	**20**	**5**	**25**
Age			
Age at follow up (years)	3.7 (2.7 – 6.9)	3.8 (3.3 – 4.3)	NS
Interval since bronchoscopy (years)	2.6 (2.0 –3.6)	2.6 (2.0 –2.7)	NS
Age at bronchoscopy (years)	1.3 (0.6 – 3.8)	1.4 (0.7 – 1.8)	NS
Clinical characteristics			
Males	13	1	NS
Rhinitis and aero-allergy	6	1	NS
Atopic dermatitis	7	1	NS
Family history of atopy	8	3	NS
Family history of asthma	8	3	NS
Abnormal serum IgE	4	0	NS

Table 32 Characteristics of follow up study respondents

The table shows the number of children in the follow up study categorised by their original wheeze subgroup at bronchoscopy. Age is shown in years (median, range) with the binary variables given as number of subjects who had these findings at bronchoscopy. There were no significant differences between groups using Mann Whitney U tests.

6.4. Wheezing module

Twenty respondents answered the respiratory module, and the results of the respiratory follow up questions are shown in Table 33.

n = 20	Response (%)
Wheezed at anytime in the past	19 (95)
Diagnosis of asthma ever	14 (70)
Wheezed in the last 12 months	15 (75)
Attacks of wheezing in the last 12 months	
None	[5 (25)]
1 to 3	7 (35)
4 to 12	8 (40)
Sleep disturbance due to wheezing	
Never woken	8 (40)
Less than once per week	4 (20)
Once or more nights per week	3 (15)
Speech limiting severity in last 12 months	2 (10)
Exercise induced symptoms in last 12 months	10 (50)
Nocturnal cough in last 12 months	10 (50)

Table 33 Wheezing module responses

Results of the wheezing module of the ISAAC questionnaire shown as respondents (percentage). The square brackets indicate calculated response to this question based upon those who had indicated previously that their child was free of symptoms.

6.4.1. Persistence and severity of respiratory symptoms

The majority of children continued to wheeze. Three quarters of parents (15/20) reported that their child had wheezed in the last year. All of the children in this study had been bronchoscoped for wheeze and cough in infancy. However, of the 20 parents who replied, one reported that their child had never been wheezy.

Seventy percent of the infant wheezers had reported a diagnosis of asthma by the time of follow up (70%, 14/20). A diagnosis of asthma was not related to the age of the child at follow up. As might be expected, the majority (85%, 11/14) of these *asthmatics* had continued to wheeze in the last year (Table 34). An asthma diagnosis was not related to an allergic phenotype in infancy or a family history of asthma or atopy. Most (8/10) of the children with exercise induced symptoms had a diagnosis of asthma at follow up. These symptoms are more in keeping with childhood asthma than the viral induced pattern of wheezing in infancy.

		Asthma diagnosis		
Wheezing in last 12 months		**No**	**Yes**	Total
	No	2	3	5
	Yes	4	11	15
	Total	6	14	20

Table 34 Asthma and current wheezing

Approximately one third of the children (7/20, 35%) had their sleep disturbed by wheezing. Half (10/20) reported a dry nocturnal cough not associated with a cold or chest infection. Two of these children had not wheezed in the last year. This may represent the persistence of airway reactivity, albeit at a reduced level of severity than in infancy. The two children concerned were both above 4.5 years at follow up but had been bronchoscoped at 11 months and 2.6 years. The data support a fading of symptoms with age.

Two questions addressed severity and frequency of symptoms. Half of the persistent wheezers (7/15, 47%) had one to three episodes of wheeze per year and half (8/15, 53%) between four and twelve episodes. Only two children (both of whom had frequent exacerbations) had wheezing of a speech limiting severity. That only two continued to have severe disease is somewhat surprising considering that all of these children required bronchoscopy as infants; such was the severity of their symptoms at that time.

6.5. Rhinitis module

All of the 20 parents who answered the questionnaire completed at least some questions on nasal symptoms. The results are shown in Table 35.

n = 20	Response (%)
Sneezed without a cold or flu ever	12 (60)
A diagnosis of hay fever ever	3 (15)
Sneezed without a cold in the last 12 months	9 (45)
Itchy watery eyes in last 12 months	6 (30)
Interference with daily life in the last 12 months	
Not at all	3 (15)
A little	6 (30)
A moderate amount	0
Season of symptoms in last 12 months	
Perennial	1 (5)
Spring	6 (30)
Summer	3 (15)
Autumn	3 (15)

Table 35 Responses to rhinitis module
Results of the rhinitis module of the ISAAC questionnaire shown as respondents (percentage).

Overall the symptoms of allergic rhinitis had increased since bronchoscopy. Whereas only one child was originally recorded as having rhinitis as an infant, 12 (60%, 12/20) reported having sneezed without a cold at some time (Table 36). Nine (45%) had current symptoms within the last year and three no symptoms for twelve months. Of these nine current sneezers, six also had itchy or watery eyes associated with their sneezing suggestive of an allergic aetiology. However, in spite of the increase in nasal symptoms, only one third of currently symptomatic children (3/9) had a diagnosis of hayfever. This is probably due to an under diagnosis of rhinitis in this sample.

The increasing frequency of rhinitis symptoms was not matched by an increase in the severity of disease. All parents reported that nasal and ocular symptoms did not interfere significantly with the child's daily activities.

		Sneezing ever		
Current sneezing in last year		**No**	**Yes**	Total
	No	8	3	11
	Yes	0	9	9
	Total	8	12	20

Table 36 Current sneezing symptoms

The symptoms reported are those occurring without a cold.

There was no overall pattern to the seasonality of rhinitis. Nasal symptoms were more prominent in the spring and summer, again in keeping with allergic rhinitis, although autumn affected two children. Both of these children were allergic to HDM or cat dander at bronchoscopy, and the increase in their nasal symptoms may be related to spending more time indoors as the winter months approached.

Only one child had perennial symptoms, he was allergic only to egg at bronchoscopy (but not to aeroallergens), and had an interval of 2.0 years from bronchoscopy to follow up (the minimum recorded interval). Egg allergy in infancy has been associated with the development of aeroallergen sensitivity which may be the case for this child (Tariq *et al.* 2000).

6.6. Eczema module

More children had symptoms of atopic dermatitis at follow up than at bronchoscopy. All of the 20 parents who replied answered at least some of the third module relating to eczema. Nearly half of all children (45%, 9/20) had an itchy rash coming and going for at least six months at some stage in their life. The results of the eczema module are shown in Table 37.

Parents of the symptomatic children were asked further questions regarding their children's skin. The majority (8 of 9) still had active disease and had suffered these symptoms in the last year. In total 60% (12/20) had been given a diagnosis of eczema with an average age of these responders being 3.7 years (range 2.8 –5.7

years). This was an increase from 35% (7/20) at bronchoscopy where the average age for these children was 1.4 years.

Of the eight with active eczema at follow up, five had symptoms at bronchoscopy, one developed eczema before two years old and the other two children developed a rash after they were two. Eczema began in infancy for most children in this study.

n = 20	**Response n (%)**
Itchy rash coming and going over six months ever	9 (45)
A diagnosis of eczema ever	12 (60)
Itchy rash in the last 12 months	8 (40)
Rash affected typical areas	6 (30)
Rash cleared completely in last 12 months	5 (25)
Rash started	
Under 2 years	6 (30)
2 to 4 years	2 (10)
5 or more years	0
On average sleep disturbed	
Never in last year	5 (25)
Less than one night per week	2 (10)
One or more nights per week	1 (5)

Table 37 Responses to the eczema module

Results of the eczema module of the ISAAC questionnaire shown as respondents (percentage).

6.6.1. Changes in atopic symptoms at follow up

In the interval between bronchoscopy and follow up, the frequency of atopic symptoms changed pattern in this small sample. Overall the changes are in keeping with the allergic march. Allergic rhinitis became an increasingly common diagnosis. Eczema had also increased and some wheezers had become asymptomatic during this period. The results are shown graphically in Figure 36.

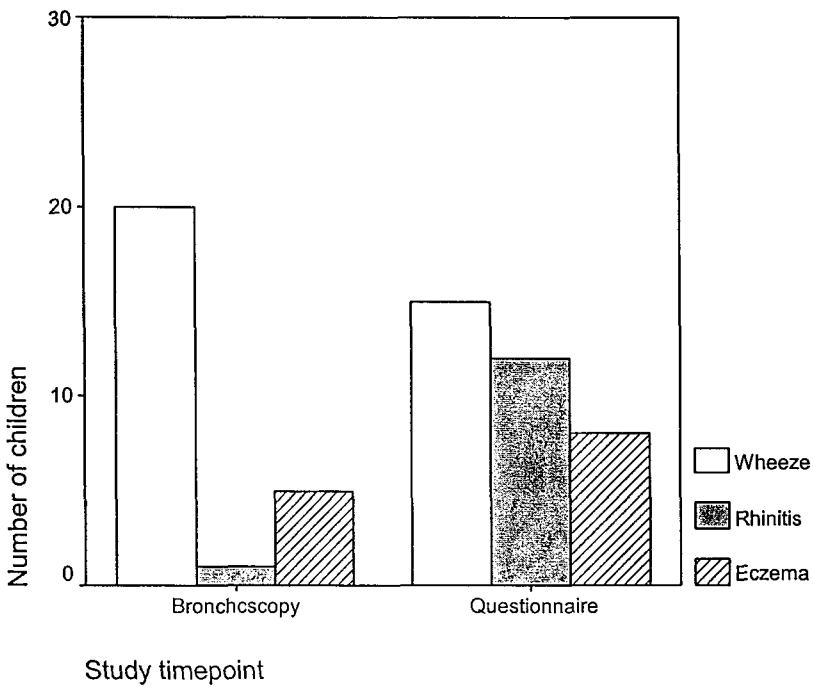

Figure 36 Frequency of atopic disease over time

The bar chart shows the differing frequencies of atopic disease between bronchoscopy at an average age of 1.4 years and the follow up questionnaire at a mean of 4.0 years.

6.7. Persistence of wheeze

The majority of children continued to wheeze at follow up, but these were not evenly distributed amongst wheeze subgroups. All but one child in the atopic group at bronchoscopy continued to wheeze at follow up (Table 38). This child was the one who had never wheezed according to his parents. In comparison 2 of 5 idiopathic wheezers and 2 of 3 infected wheezers had outgrown their symptoms. Excluding the child who never wheezed, the results were significant using Fisher's Exact test (p = 0.020).

		Wheezing in last 12 months		
Wheeze subgroup at bronchoscopy		**No**	**Yes**	Total
	Idiopathic	2	3	5
	Atopic	1	11	12
	Infected	2	1	3
	Total	5	15	20

Table 38 Persistence of symptoms by wheeze subgroup
Table showing the respiratory fate of the wheeze subgroups at bronchoscopy. Fisher's Exact test was not significantly different for this table (p = 0.060).

6.7.1. Age of persistent wheezers

At follow up, persistent wheezers were significantly younger than those who had outgrown their symptoms. Persistent wheezers had a mean age of 3.7 years (range 2.6 – 6.7 years) compared to 5.2 years in the asymptomatic group (range 4.2 – 6.9 years; t-test, p = 0.013). The time interval from bronchoscopy to follow up was also related to persistence (t-test, p = 0.001). Those without symptoms for the last year had waited longer for follow up (mean 3.2 years, range 3.0 –3.5 years) than those who still wheezed (2.4 years, range 2.0 – 3.6 years). This is represented graphically in the scatter plots in Figure 37. However, there was no difference between the ages of children at follow up when compared by wheeze subgroup (p = 0.3), suggesting that persistence of wheezing is more than just an age affect (Figure 38).

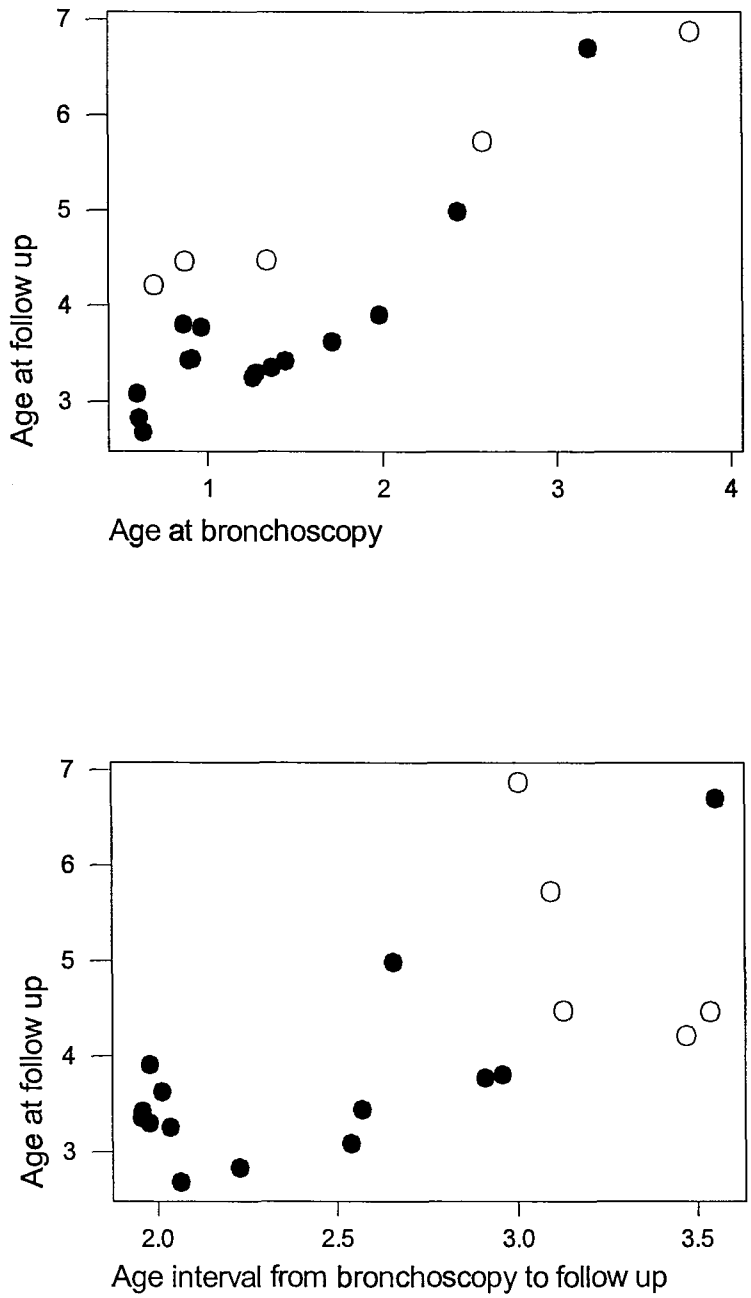

Figure 37 Age of child at follow up and persistence of wheeze

The scatter plots relate the age of the child at follow up and the time interval from bronchoscopy to follow up with persistence of wheeze. The shaded circles (•) represent persistent wheezers whereas the open circles (○) indicate that the child had not wheezed in the last year.

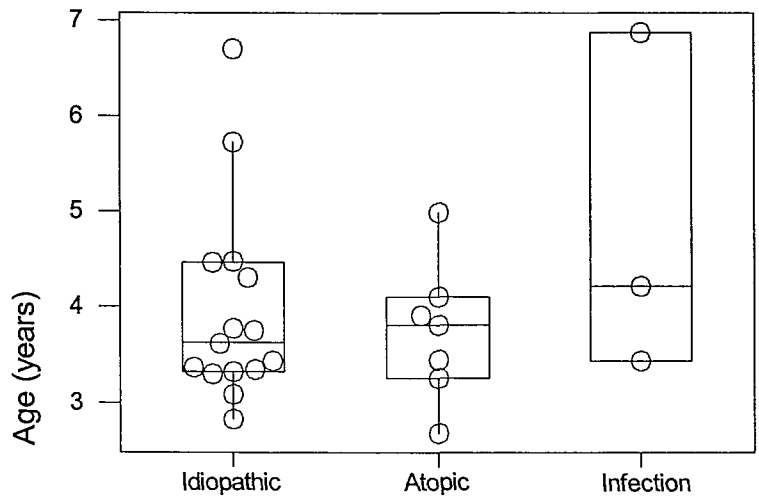

Figure 38 Age at follow up by wheeze subgroup

Age at follow up was compared according to the wheeze phenotype of respondents at bronchoscopy. There was no difference between groups (p = 0.3).

6.7.2. Current rhinitis was associated with persistent wheeze

The acquisition of allergic rhinitis by the time of follow up was associated with persistent wheezing (Table 39). Unlike persistent wheezing, current sneezing symptoms were not related to the age of the child at follow up (p = 0.3). All (45%, 9/20) of the children who had sneezing episodes unrelated to colds in the last twelve months also continued to wheeze (Fisher's Exact test, p = 0.038). Similarly, all children with itchy watery eyes at follow up still wheezed.

		Sneezing in last 12 months		
Wheezing in last 12 months		No	Yes	Total
	No	5	0	5
	Yes	6	9	15
	Total	11	9	20

Table 39 Current wheeze and current nasal symptoms

Although more atopic wheezers had symptoms of rhinitis at follow up (Table 40), not all of them had developed rhinitis. In particular those with aeroallergen sensitivity or allergic rhinitis at bronchoscopy did not necessarily have current symptoms of rhinitis at follow up. Only 4 of 6 children with upper airway symptoms at bronchoscopy had ever sneezed without a cold, and only 3 of these had done so in the last year. All of them continued to wheeze. One idiopathic wheezer and one infected wheezer had developed current sneezing symptoms and itchy watery eyes suggestive of AR. The infected wheezer had no signs of atopy at bronchoscopy. This suggests that they developed atopic sensitisation between the time of bronchoscopy and follow up.

		Sneezing in last 12 months		
Wheeze subgroup at bronchoscopy		**No**	**Yes**	Total
	Idiopathic	4	1	5
	Atopic	5	7	12
	Infected	2	1	3
	Total	11	9	20

Table 40 Current nasal symptoms by wheeze subgroup

Table showing the nasal symptoms at follow up by wheeze subgroups at bronchoscopy. Fisher's Exact test was not significantly different.

6.7.3. Active eczema was associated with persistent wheezing

All children with active eczema at follow up remained wheezy (Table 41). Most (6/8) also had symptoms of rhinitis. The two children who developed eczema late (after two years old) were both atopic in infancy and had more severe active wheeze, as evidenced by frequent exacerbations (4-12 per year) at follow up. Both also had allergic rhinitis but only one showed any tendency to nasal disease, or atopy, at bronchoscopy.

		Current rash		
Current wheeze		**No**	**Yes**	Total
	No	0	0	0
	Yes	2	8	10
	Total	2	10	10

Table 41 Relationship between current skin and wheeze symptoms

6.8. Can BAL findings in infancy predict persistence of wheeze?

Our original hypothesis stated that persistent of symptoms would be related to the changes seen in atopic wheeze. The aim of this analysis was to distinguish those cellular and inflammatory markers in infant lavage fluid and serum that could predict the persistence of chest and nasal symptoms at follow up. The age of the child at bronchoscopy was not related to persistence of wheeze, which indicates that the use of bronchoscopy data may not be skewed by age.

The results of the comparison of BAL cellularity and inflammatory markers by persistence of wheeze are given in Table 42 and Table 43. Cellular viability and TIMP-1 concentrations are the only markers significantly associated with current wheeze; each is discussed in more detail in the next sections. Sample size did not permit logistic regression of these results with other potential confounding variables.

Responses to other questions in the respiratory module were used to assess the predictive value of lavage markers, including: nocturnal symptoms of cough, waking with wheeze at night, exercise induced symptoms, and the child having been given a diagnosis of asthma. Markers of severity within the persistent wheezers were also considered using frequency of exacerbation and speech limiting severity as indicators. Subjects available for these sub-analyses were often very small, and no relationships were found between these phenotypes and lavage markers. In particular, no relationship was found between the presence of eosinophils or ECP in BAL or NAL and the child's wheezing status at follow up, both by using continuous data and by dividing the variables into two and four rank based groups.

BAL cell counts	Wheezing in last 12 months		p
	No	Yes	
TNCC	12.6 1.6 –200.1	11.2 0.7 – 184.8	0.6
Viability %	98.3 81.0-100	74.4 61.0 - 94.0	0.026
Epithelial cells %	2.7 0 –16.0	3.6 1.0 – 24.0	0.4
Macrophages %	82.5 52.4 - 96.0	83.4 26.0 – 95.9	0.7
Neutrophils %	0.5 0 – 20.0	1.2 0 – 47.0	0.5
Lymphocytes %	9.4 4.0 – 26.0	12.1 3.7 – 30.6	0.8
Eosinophils %	0 0 - 2.0	0 0 – 27.6	0.9

Table 42 BAL cytology of persistent wheezers

Table showing differences between cells counts in BAL fluid in infancy by current wheezing status at follow up (wheezing in last 12 months). Data are shown as median and range with non-parametric Mann Whitney U tests probabilities. TNCC is shown as cells x 10^5 /ml BAL.

BAL	Wheezing in last 12 months		p
	No	Yes	
sICAM-1 ng/ml	65 39 - 128	77 27 - 239	0.8
ECP ng/ml	2.1 < 2.0 – 3.2	< 2.0 < 2.0 - 90	0.3
IL-8 pg/ml	0 0 - 98	27 0 – 4098	0.3
MMP-9 ng/ml	0.9 0.9 – 2.8	9.6 0 – 21.0	0.5
TIMP-1 ng/ml	10.6 6.1 - 18.6	32.3 9.1 – 83.9	0.027
MMP-9/TIMP-1 molar ratio	0.033 0.014 – 0.077	0.013 0 – 0.092	0.3

Table 43 Lavage markers in BAL related to persistence of wheeze

Table showing differences between lavage markers in BAL fluid and current wheezing status at follow up (wheezing in last 12 months). Data are shown as median and range with non-parametric Mann Whitney U tests probabilities.

6.8.1. BAL cellular viability was decreased in persistent wheeze

Cellular viability of BAL in infancy was related to current wheezing at follow up (Figure 39). Viability was reduced from a median of 98.3 % in the asymptomatic group to 74.4 % amongst persistent wheezers (p = 0.026). A relationship was not demonstrated using bronchial wash cellular viability specimens from these subjects (p = 0.6).

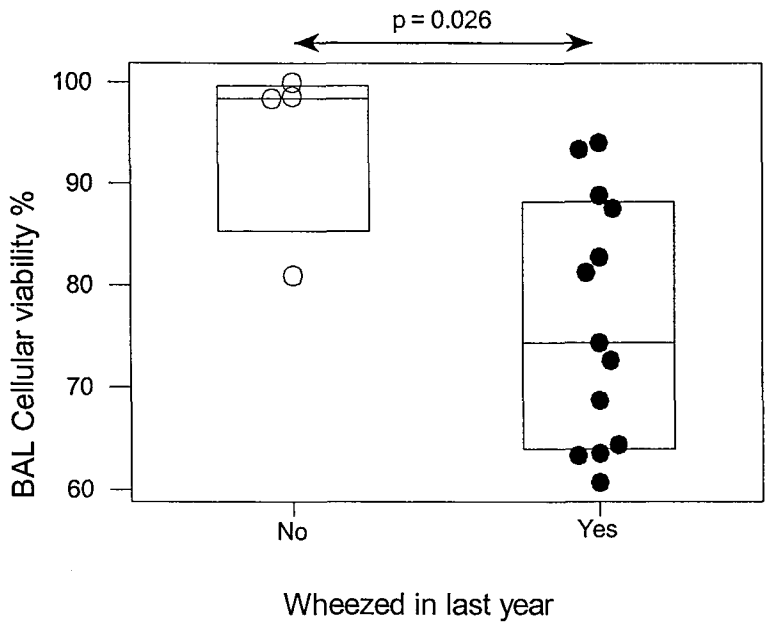

Figure 39 BAL cellular viability in persistent wheezers

Boxplot showing BAL cellular viability % at bronchoscopy versus current wheezing at follow up. Boxes indicate medians with interquartile ranges. A Mann Whitney U tests was significantly different between groups (p = 0.026). The shaded circles (•) represent persistent wheezers whereas the open circles (○) indicate that the child had not wheezed in the last year.

6.8.2. BAL TIMP-1 levels were raised amongst persistent wheezers

The concentration of TIMP-1 in BAL was raised amongst those who continued to wheeze at follow up ($p = 0.027$, Figure 40). The median concentration in BAL from the symptomatic group was 32.3 ng/ml compared to 10.6 ng/ml in the asymptomatic children.

MMP-9 and the molar ratio of MMP-9/TIMP-1 did not show significant differences between groups (Table 43). Age at follow up ($p = 0.046$) and the interval between bronchoscopy and follow up ($p = 0.003$) were also significant for these children.

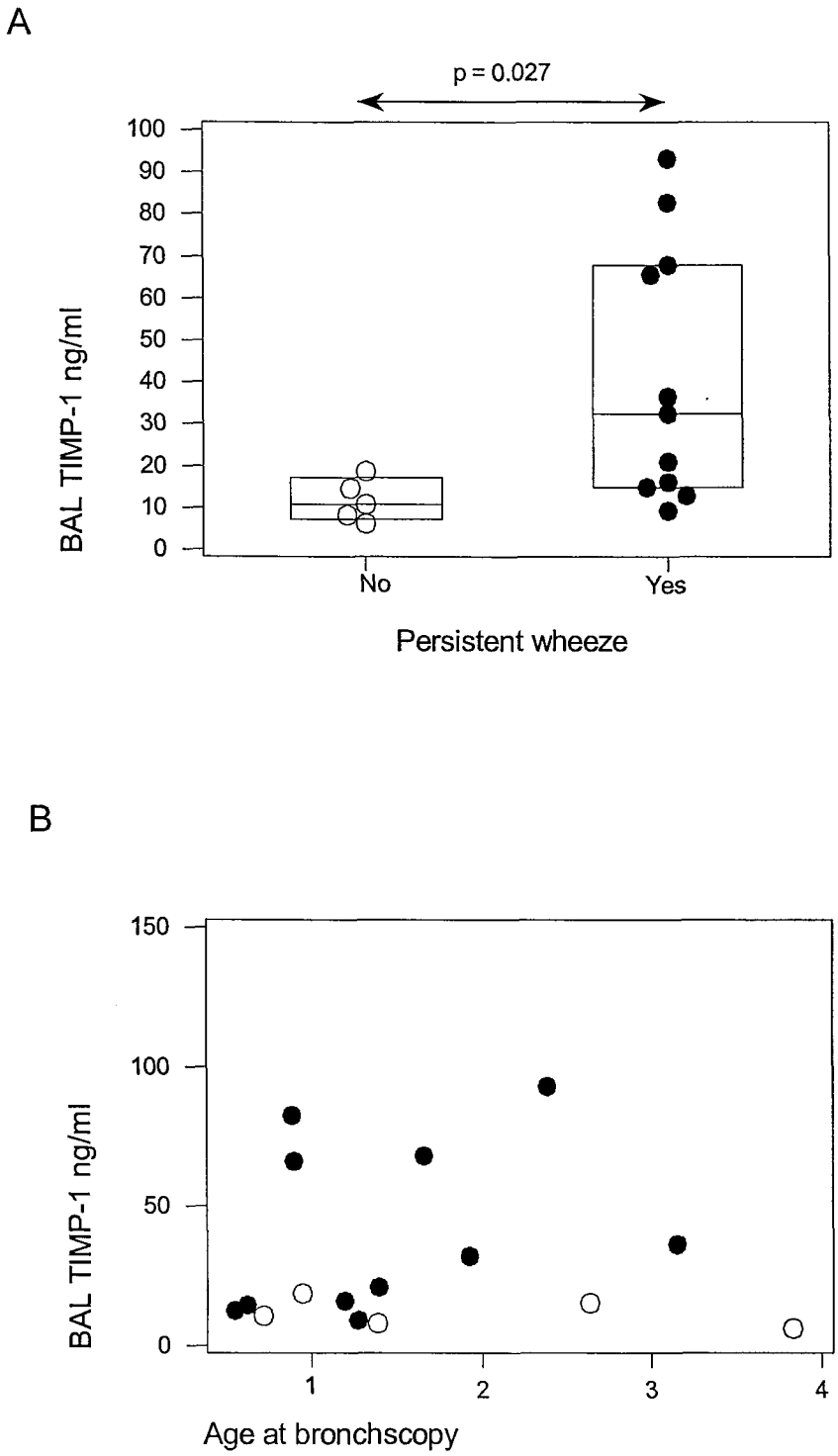

Figure 40 BAL TIMP-1 in persistent wheezing

A: The Boxplot shows TIMP-1 ng/ml concentrations in infant BAL against their symptoms at follow up (MWU test 0.027). B: Scatterplot of TIMP-1 and age at bronchoscopy demonstrating that TIMP-1 levels were not associated only with older children at bronchoscopy. The shaded circles (•) represent persistent wheezers whereas the open circles (○) indicate that the child had not wheezed in the last year.

6.8.3. Serum sICAM-1 and persistent wheezing

Serum s-ICAM-1 was related to persistence of wheezing. The median value for persistent wheezers was 394 ng/ml compared to 269 ng/ml for those who had outgrown their symptoms (p = 0.027, Figure 41 A). Age was associated with the disappearance of symptoms from these subjects (t-test, p = 0.013). However, age at bronchoscopy was not related (p = 0.218, Figure 41 B).

Serum	Wheezing in last 12 months		p
	No	Yes	
sICAM-1 ng/ml	270 251 – 337	394 243 - 589	0.015
ECP ng/ml	34.4 4.1 – 40.1	16.5 2.7 – 124.9	0.687

Table 44 Serum markers and persistence of wheeze

Table showing differences between serum markers at bronchoscopy and current wheezing status at follow up (wheezing in last 12 months). Data are shown as median and ranges with non-parametric Mann Whitney U tests probabilities.

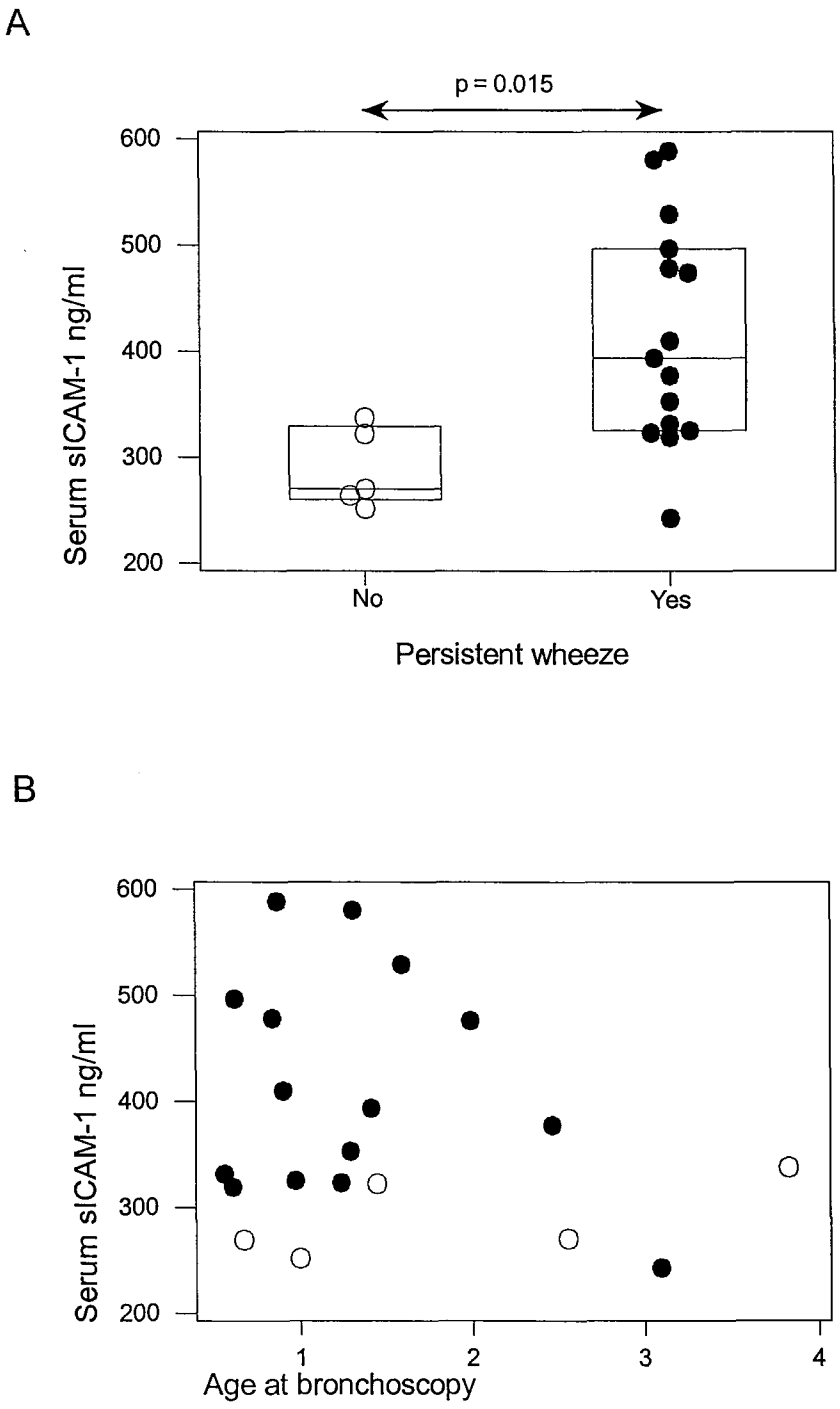

Figure 41 Serum sICAM-1 amongst persistent wheezers

A: The Boxplot shows s-ICAM-1 ng/ml concentrations from BAL in infancy compared between those children who continued to wheeze at follow up (p = 0.015). B: The Scatterplot shows the relationship between age at sampling and serum ICAM-1. Shaded circles (●) represent persistent wheezers whereas the open circles (○) indicate that the child had not wheezed in the last year.

6.9. Rhinitis and the persistence of wheeze

Current symptoms of allergic rhinitis have already been shown to be associated with persistence of wheeze. All children with aeroallergen sensitisation and allergic rhinitis at bronchoscopy also continued to wheeze but the findings in nasal lavage amongst these infants was not so well defined. Nasal lavage findings at bronchoscopy were analysed according to the presence of wheeze at follow up. Cellular and lavage constituents in nasal lavage are shown in Table 45.

High neutrophil counts in NAL (median 92.5% versus 42.4%; $p = 0.038$) and a low macrophage count (0.5% versus 21.8%; $p = 0.019$) were related to the disappearance of wheeze in this sample. However there were only two data points in the no symptoms groups, which makes further interpretation unreliable.

median *range* *n*	**Wheezing in last 12 months**		p
	No	**Yes**	
Total cell count (cells x 10^5 /ml)	0.32 0.05 - 90.2 5	0.65 0.02 – 42.0 13	0.8
Viability %	69.2 25.0 - 98.5 5	78.1 37.1 – 92.5 13	0.5
Epithelial cells %	20.0 0.5 - 75 5	5.9 2.8 - 100 14	0.9
Macrophages %	0.5 0 –1.0 2	21.8 1.6 - 100 13	0.019
Neutrophils %	92.5 88 - 97 2	42.4 0 - 93 13	0.038
Lymphocytes %	6.5 2.0 –11.0 2	3.0 0 – 8.2 13	0.8
Eosinophils %	0.5 0 –1.0 2	0 0 –11.0 13	0.8
sICAM-1 ng/ml	15 0 -18 5	5 0 -55 14	0.6
ECP ng/ml	2.4 NA 1	13.0 2.1 – 69.7 13	0.2
IL-8 ng/ml	2.11 0.52 – 11.27 3	0.65 0.53 – 2.9 14	0.4

Table 45 NAL findings in infancy amongst persistent wheezers

Table showing differences between cells counts and markers in NAL fluid taken in infancy and their current wheezing status at follow up (wheezing in last 12 months). Data are shown as median (IQR, n) with non-parametric Mann Whitney U tests probabilities. Note IL-8 is given in ng/ml not pg/ml as for BAL fluid.

6.10. Chapter summary

The follow up study was performed a mean interval of 2.6 years after bronchoscopy. It was limited by the small number of children in the sample (n = 20) and the fact that the majority of children continued to wheeze, which reduced the ability to distinguish between groups even further. Of 20 respondents, only 5 had outgrown their symptoms.

On the whole, those who continued to wheeze had been atopic at bronchoscopy or had developed symptoms of allergic rhinitis since infancy. All children who had AR or aeroallergen sensitivity at bronchoscopy continued to wheeze. A history of sneezing with colds or active eczema at follow up was associated with a persistence of disease.

Those who had outgrown their symptoms were significantly older (5.2 versus 3.7 years) than those who continued to wheeze. Persistence of symptoms was not related to the presence of eosinophils or ECP in BAL, NAL or serum. Instead, BAL cellular viability and TIMP-1 concentrations were increased in BAL from persistent wheezers. Persistence was also associated with raised serum sICAM-1 at the time of bronchoscopy.

Chapter 7: Discussion

This chapter draws together the results from the last three chapters to formulate an opinion on wheezing in infancy, and address the main hypotheses. A critique of methodology precedes a discussion of the principle findings of this work, which are then addressed in the light of published work. Finally the potential mechanisms underlying the results are considered, as are the possibilities for future research. This study was the first to measure MMP-9 and TIMP-1 in BAL from preschool wheezers. Although the cells and markers such as ECP and IL-8 have been published previously, the separation of those with bacterial infection into a distinct group, brings new insights to BAL in infant wheeze. Also this study was the first to use concomitant nasal and bronchoalveolar lavage. The use of structured follow up also sheds light upon the results.

7.1. A critique of study methods

This was a small observational study and was not powered to detect differences in any of the markers considered, so lack of significant findings does not preclude relationships between them. The invasive investigation of children is practically difficult, and research involving such procedures is ethically fraught. Studies using BAL from children cannot recruit large numbers due to the relatively small numbers of children who require these procedures. The use of a multi-centre study design can address this issue but has problems of its own (section 7.1.4.1.).

7.1.1. Selection of wheezers

The infant wheezers recruited to this study were a heterogeneous group of children whose indications for bronchoscopy included severe or atypical symptoms and failure of standard therapies. The severity of their symptoms makes extrapolation of the results to the general population of infant wheezers difficult. However, there are several factors that suggest that these results can be extrapolated to wheezers in general including the relative absence of congenital structural abnormalities found at bronchoscopy.

The range of clinical opinion contributing to the study provided a rounded sample of severe preschool wheezing. Although the inclusion of wheezers was based upon subjective clinical findings of a severe and atypical presentation, the children were recruited from four centres, over four European countries, and had at least five tertiary paediatricians contributing patients to the bronchoscopy lists. This range of clinical opinion ameliorated any potential skew of the sample towards one particular severe wheezing phenotype. The bronchscopists were also blind to the eventual categorisation of wheezers at the time of inclusion, which is reflected in the differing profiles of the three wheeze subgroups between the recruitment centres.

Skilled paediatric bronchoscopists recruited the infant wheezers in this study and because of this the severity of the study wheezers may be more comparable to the wider population as a whole. Bronchoscopy is a safe and increasingly popular tool for the investigation of infant wheeze (Schellhase *et al.* 1998; Strunk 2002) and the threshold for further investigation will be lower at centres where bronchoscopy is performed frequently and routinely (Nicolai 2001).

However, it is ironic that it is only by recording data from the exceptional few, that knowledge can be gathered that may affect the future understanding and management of all wheezy children. Studies using non-bronchoscopic lavage methods have an advantage of being able to sample from the broader population of infant wheezers who do not require investigation for chest disease (Stevenson *et al.* 1997).

7.1.2. Selection of controls

The majority of the control group was selected retrospectively from children undergoing bronchoscopy for the investigation of stridor. The careful selection of controls defined a group of individuals who, on balance, had symptoms likely to be due to a structural airway problem and not airway inflammation. None of them had recorded wheezing, symptoms of atopy or a family history of asthma. Those who coughed were only included if they also had airway malacia at bronchoscopy (which demonstrated an anatomical reason for their symptoms).

In defence of this selection policy, the generation of widely accepted normal data has been taken from bronchoscopy findings in such children (Midulla *et al.* 1995; Ratjen *et al.* 1994; Tessier *et al.* 1996). Other bronchoscopy studies on infant wheezing have also used children with airway malformation or stridor to act as controls (Marguet *et al.* 2000).

Not all studies considering BAL in infant wheeze have used such strict criteria for their controls. Several studies used a diverse array of diagnoses such as cough, recurrent infection and stridor with or without positive cultures to act as controls (Azevedo *et al.* 2001; Barbato *et al.* 2003; Le Bourgeois *et al.* 2002). Such control children included those with primary tuberculosis, suspected inhaled foreign body and even recurrent bronchitis, albeit without wheezing. At least some of the control cases will have had lower respiratory tract inflammation or infection, and some may even have had asthma. In many instances they would fit our study's criteria for inclusion as a wheezer.

Finding appropriate controls for research requiring invasive procedures in children is extremely difficult. The gold standard would be the bronchoscopic lavage of healthy infants and children. Kraweic *et al* (Krawiec *et al.* 2001) performed such a study of bronchoscopy on children attending for elective surgery without a history of acute or chronic respiratory problems. Bronchoscopic BAL has also been performed on a range of healthy children over three years old undergoing elective surgery (Ratjen *et al.* 1994) and amongst infants (Midulla *et al.* 1995).

There are ethical issues surrounding the performance of an invasive procedure, such as bronchoscopy, on children who do not require it on clinical grounds alone, and for this reason we felt that healthy children without lower airways disease could not be recruited directly. The control cases in this study are believed to represent as near to normal physiology as is possible within ethical and practical constraints.

7.1.2.1. Missing cultures

As bacterial infection played an important part in this study it is unfortunate that culture results were not available for all subjects. Missing cultures were due to

routine laboratory error or lost data and are indicative of the low priority that infection has played in asthma and wheeze. However, missing cultures occurred at random so all the available data were included. Exclusion of subjects with missing culture results made little difference to the analysis, as did assuming that all missing cultures were positive, placing cases in the infected wheezers group.

Chronic viral infection may also play a role in infant wheeze. Schellhase *et al* (Schellhase *et al.* 1998) recorded a high rate of viral infected BAL in infant wheeze. However, a later study did not find any positive viral cultures amongst their larger group of wheezers, suggesting that viral infection does not play as important a role in infantile wheeze as might be suspected (Najafi *et al.* 2003). In spite of this, the lack of virology results in our subjects remains a limitation of our study, and future studies of BAL and more importantly NAL in infancy should have full bacterial and viral cultures performed.

Some controls cases also had missing cultures. Culture results were negative for all controls with symptoms of cough and stridor but were missing for some subjects with stridor alone. In children with diagnosed laryngomalacia, lavage was performed purely for research purposes and it is likely that they would have negative cultures, having never wheezed or shown signs of LRT infection.

7.1.2.2. Clinical data collection

There were several aspects of data collection that may have been improved. Firstly, although antihistamines were discontinued and washed out prior to study entry, children were still on a range of other medications, including in some cases inhaled corticosteroids (ICS) and antibiotics. ICS have been shown to affect lavage cytology in infant wheeze (Just *et al.* 2002), where the use of this medication reduced lymphocyte counts. In our sample of severe infant wheezers discontinuation of medication may have been associated with an exacerbation of symptoms and increases in bronchial hyperreactivity making bronchoscopy hazardous.

No account was made of exposure to tobacco smoke in this study. In the original protocol urine was to be taken on the day of bronchoscopy and analysed for cotinine as a direct estimate of exposure to tobacco smoke (Rylander *et al.* 1995). However,

infants who have been fasted prior to an anaesthetic have a reduced urine output and coupled with the difficulties of collecting urine samples in this age group, and the focus of this complicated and invasive study on the respiratory tract, only a handful of samples were received and were not analysed. Questionnaire follow up also provided an opportunity to collect retrospective data on this variable. Environmental tobacco smoke played a role in all forms of infant wheeze (Martinez *et al.* 1995), and our inability to record and account for these data is a shortcoming of this study.

There was no definition for the diagnosis of allergic rhinitis or eczema at bronchoscopy and children were allocated these terms at the discretion of the recruiting physician. For this reason some children classified as idiopathic wheezers in this study may not have had these diagnoses fully excluded.

7.1.3. BAL methods

This study had the advantage of concomitant sampling of bronchial washings, BAL and nasal lavage allowing direct comparison between lavages. Inflammatory markers were analysed by subject, thus further reducing variability between compartments.

The multicentre study design increased the number of cases recruited over the study period and also reduced skew towards a particular clinical presentation of wheeze. However, several factors in the processing and counting of lavage specimens could have affected the results and many of these issues involve recruitment of subjects from several centres.

7.1.3.1. Dilutional corrections for BAL

Bronchoalveolar lavage takes a variable sample of the ELF. Several methods have been employed to try to quantify the concentration of ELF in BAL. The dilution of ELF in BAL is related to

- The volume of lavage infused,
- The amount of epithelial lining fluid diluted by this lavage,
- The amount successfully aspirated.

This is related to:

- The size of the airways, and thus the age and size of the child (Najafi *et al.* 2003),
- The quality of the bronchoscopic wedge and therefore the bronchscopist's technique.

It is impossible to standardise many of these factors, and the variation that they impose on the data is part of the difficulty in interpreting findings of bronchoalveolar lavage. Urea and albumin have both been used to assess the amount of ELF in lavage, but unfortunately both these methods have their limitations. ELF urea is considered to be in equilibrium with that of serum and as such, BAL urea concentrations have been compared to serum urea as a measure of the ELF return from lavage (Heaney *et al.* 1996). However, this method relies upon the premise that urea does not diffuse into BAL during the lavage process. Unfortunately, urea diffuses from serum into BAL and is thus increased as the dwell time increases (Marcy *et al.* 1987). For quick lavages such as those performed by non-bronchoscopic BAL or infant NAL there is little time for equilibration of serum urea to BAL. However, bronchoscopic lavage is more prolonged and urea concentrations may be falsely elevated by diffusion from serum into the BAL fluid itself, leading to an overestimation of the ELF fraction in returns. Urea concentrations in BAL may also be affected by increased alveolar vascularity and capillary permeability in certain disease states. This is also the case when considering albumin as a marker. Albumin concentrations in BAL are altered by the disease states themselves and are therefore unreliable (de Blic *et al.* 2000).

The current recommendations for reporting BAL fluid findings in children state that '*normalisation for ELF should not routinely be performed due to lack of a reliable method for its quantification*' (de Blic *et al.* 2000) pp220. In keeping with these guidelines no account was made for such sources of variation in our data.

The use of ratios of BAL components to each other negates any need to consider dilution of the ELF as this dilution is cancelled in the ratio equation, providing an internal control within the individual lavage.

i.e. (MMP-9 / BAL protein) / (TIMP-1 / BAL protein) = MMP-9 / TIMP-1

ELF sampling can be performed without recourse to lavage. Yamazaki *et al* (Yamazaki *et al.* 2003) used bronchoscopic micro-sampling with fine (1.9 mm) fibre rod probes. Such probes take up ELF by capillary attraction when the tip is placed against the airway under direct vision during bronchoscopy. This method negates the need for airway lavage, but is only able to sample a small volume of the ELF. It has not been used for the detection of inflammatory markers and ELF cellularity.

7.1.3.2. Processing of lavage

There are various techniques for the subsequent processing of BAL. The processing of BAL may affect the cytology (Baughman *et al.* 1986; Mordelet-Dambrine *et al.* 1984). However, for the majority of these processing steps, their effect on cellular constituents of infant wheeze has not been established, and they are in routine use by most research laboratories. In this respect, our study had the advantage of a single processing protocol to ensure that lavage was dealt with in the same way.

7.1.3.3. Filtering

Lavage specimens are often filtered in order to remove mucus and particulate debris, which can often make the sample unsuitable for analysis (Stevenson *et al.* 1997). Unfortunately, filtering can also selectively remove cellular components of BAL (Klech and Pohl 1989). Epithelial cells may be particularly susceptible to removal by a cotton gauze filter (Lam *et al.* 1985) but the nylon filters used in this study may remove less cellular material than the cotton gauze method (Klech and Pohl 1989). Our technique of nylon filtering lavage has been shown to increase cell yield by 17%, when compared to unfiltered lavage, although it potentially reduced the proportion of epithelial cells by 2.8% (Mander 1999).

Nasal lavage is particularly difficult to process if there is mucus in the sample. In this situation, filtering allows more samples to be successfully processed. At present the balance of expert opinion, cited in current European guidelines, suggests that filtering does not have a significant impact on results, and the recommendations are that lavage be filtered through a single layer of gauze (de Blic *et al.* 2000).

7.1.3.4. Cytology

The most direct method for performing cell counts from BAL involves placing a drop of unfiltered lavage onto a glass slide for staining and counting (Heaney *et al.* 1996; Laviolette *et al.* 1988). The advantages of this method are clear, in that less processing provides a more representative count. However, the use of neat lavage in this way reduces the volume of BAL available for further analysis. If the lavage is dilute, and cells are scattered over several microscope fields, the several hundred cells required for standard counts, can be difficult to visualise by this method. For this reason BAL is often centrifuged and cells resuspended in buffer to concentrate cells, and also to harvest the supernatant for analysis. Cytocentrifuge preparations, as used in this study, are one way to concentrate cells from dilute BAL. However, each step away from neat BAL adds the possibility of artefact into the results. For the majority of these steps the effect on the cell counts has not been quantified, but Saltini *et al* (Saltini *et al.* 1984) suggested that cytospin preparations underestimate lymphocyte counts in BAL.

To summarise, the lavage processing was standardised between centres by a single protocol. Differences between cytologists were accounted for in the statistical methods employed. No dilutional marker was used in keeping with current guidelines (de Blic *et al.* 2000).

7.1.3.5. Differences between cytologists

There was a defined protocol for cytology, which ensured that similar counting methods were used throughout. However, no validation exercise was built into the protocol. Validation was particularly important for the live cell counts, as they could not be stored for central analysis. Methods to standardise cell counts might have

included the use of sample photomicrographs and training packages, to provide a reproducible standards. Photomicrographs of the live cell preparations could also have been used to verify counts between cytologists.

The situation was less critical for the differential cell counts as cytocentrifuge slides were stained and recounted centrally. It is unfortunate that many had undergone prolonged frozen storage and were unsuitable for analysis. Prolonged storage is even more important for studies using immunohistochemistry, where six weeks is considered the maximum duration of frozen storage for cytospins (Dr S Wilson, personal communication). This probably accounts for the overall disappointing results for the immunohistochemistry performed on these slides (data not shown).

In spite of storage difficulties, we were able to compare the original counts from one centre with recounts from frozen slides at the central laboratory in Southampton. Only lymphocytes showed a significant difference between cytologists. This difference was proportional to the size of the lymphocyte count, with higher counts producing more variation between cytologists. Such a proportional effect was also seen in the eosinophil counts, where one count from the original centre was an outlier for the entire dataset. This subject had a local eosinophil percentage of 28%, which compared to the central recount value of 13%. Such proportional variation between cytologists indicates counts were subtly different between centres and this effect was amplified as the cell counts increased. Indirectly this also suggests that counting methods were standardized for each cytologist in order for such proportional differences to occur. Warke *et al* (2001) considered the role of within and between observer variability in paediatric non-bronchscopiuc BAL. They found that only lymphocytes, but not other cell types including eosinophils, varied between two observers, which in interesting in that they agree wtih our findings. Why lymphocytes should produce more variability between observers is not clear. These discrepancies in cell counts emphasize the importance of inter-rater validation in studies using different centres, or where more than one cytologist has been involved. Other studies of lavage in infancy have not considered this issue and have not routinely stated validation for cell count data, even when such data were collected over several years (Just *et al.* 2002; Le Bourgeois *et al.* 2002).

For these reasons it was not possible to directly compare cell counts between clinical groups when different centres had contributed cases. Instead, the statistical analysis of this cellular data accounted for differences between centres. Although this negated inter-centre effects, it also further reduced power to detect differences between clinical groups in an already small study. In this respect, cell count data was recorded in each centre as proportions and not the raw cell count data. This hampered statistical analysis of variability between centres, by demanding techniques that used proportions, and future studies should consider collection of absolute counts as well as proportional data.

7.1.4. NAL methods

There were several issues in the method of nasal lavage collection that require discussion.

7.1.4.1. Differences in lavage volumes

Firstly, the volume of nasal lavage used was different between the centres. Upper airway lavage methods influence the resulting composition of NAL (Klimek and Rasp 1999). It is also known that differences in dwell time and lavage volume to epithelial surface area will alter ELF sampling in BAL (Najafi *et al.* 2003), and are likely to influence NAL ELF concentration in a similar fashion. One centre in our study (Paris) used a 5ml lavage, whilst the other centres used 10ml. This also significantly affected the return volumes, with Paris achieving a higher return than the other centres. This does not mean that Epithelial Lining Fluid (ELF) concentrations were higher using the 5ml NAL. Although a formal dilutional marker was not measured, it seems that Brussels had a higher concentration of ELF in their lavage return evidenced by significantly higher IL-8, ECP and sICAM-1 concentrations in NAL than the other centres. Brussels median NAL volumes were equivalent to those of Southampton and Prague but Brussels had the highest range of volumes for NAL (up to 20ml) and such large volume NAL may have washed ELF from the nasal cavity more effectively than the smaller volumes, in a manner similar to the dwell time used in adult nasal lavage protocols. Bronchoalveolar lavage

volumes and returns were not significantly different between centres, neither were those of bronchial washings.

7.1.4.2. Nasal lavage methods

The nasal lavage technique used in this study differed markedly from that used in adults and older children. In this study, nasal lavage was suctioned from the nasopharynx in a manner similar to that used in studies with neonates (Frischer *et al.* 2000; Halmerbauer *et al.* 2000). In older children and adults the subject is trained to hold the lavage fluid within the nasal cavity prior to suction (Wojnarowski *et al.* 1999). Young children will not retain lavage fluid in the nose to permit a dwell time, which means that the lavage must be suctioned immediately from the nose (Frischer and Baraldi 2000). As such, the results of nasal lavage studies from adults and infants may vary markedly, and are not directly comparable as longer dwell times are associated with increases in the concentration of inflammatory and diluent markers such as urea.

7.1.4.3. Dilutional effects in NAL

The concentration of epithelial lining fluid in nasal and bronchoalveolar lavage probably account for some of the differences seen in this study. Such variation between ELF sampling in the nose and the lung would occur even if similar dwell times were being used at both sites, as other factors such as the volume of lavage and airway size would come into play. One way of overcoming ELF dilution would be to measure a standard inert marker in order estimate the concentration of ELF. Urea and albumin have been tried and discredited in BAL (de Blic *et al.* 2000), and such markers may be even more difficult to use in the upper airway, where serum proteins readily leak into nasal secretions.

Another method of ELF estimation would be to spike lavage with a dilutional marker prior to instillation. Using a lavage method similar to ours Balfour-Lynn *et al* (Balfour-Lynn *et al.* 1993) diluted saline with known concentrations of inulin prior to nasal lavage, in an attempt to estimate the amount of nasal ELF aspirated in NAL. They showed that nasal secretions accounted for a mean of 8.8% of lavage, a figure

that changed little from wheezer to non-wheezer and also by the presence or absence of infection. As our lavage volumes were greater than those used in their study (10ml compared to 2ml x 2) the sampling of nasal ELF may be different using our method. Nasal lavage using the adult dwell method is similarly reproducible in adults and older children (Nikasinovic-Fournier *et al.* 2002). However, another study showed that ECP concentrations in NAL were not reproducible amongst infants, even over a period of a few days (Frischer and Baraldi 2000), which suggests that ELF sampling by NAL may not be as constant as the previous study suggested.

7.1.4.4. Variation in NAL cytology between centres

Between centres differences were apparent for different cell types in nasal lavage. Counting differences occurred between centres for macrophages and neutrophils proportions. These cells are more difficult to distinguish in nasal lavage than BAL. This is because macrophages adopt intermediate monocyte/macrophage morphology in NAL and immature neutrophils are also seen making identification more difficult.

7.1.5. Follow up methods

Using the ISAAC questionnaire in our international multicentre study was important for several reasons. Firstly the ISAAC questionnaire was designed to enable comparisons between countries (and languages) and a set protocol was available for its translation. Secondly it was an epidemiological questionnaire designed to look at the community prevalence of symptoms, not just for those who attend for medical help. In this way the results of follow up for this sample of severe infant wheezers allowed comparison to the wider population. Thirdly, the results enable direct comparison of BAL immunology to a growing number of other ISAAC based studies and large epidemiological studies, once again contextualising these results within the wider body of evidence.

7.1.5.1. Validity of the ISAAC questionnaire

The questionnaire used in our study was a completed by parents and had been validated for children in the 6 to 7 year old age group (Asher *et al.* 1995a) but not

amongst preschool children. The average age at follow up in this study was four years and it seems unlikely that significant differences exist in the symptomatology of these children compared to six year olds. Peroni *et al.* have also successfully applied this questionnaire to the preschool age group (Peroni *et al.* 2003).

Although the ISAAC questionnaire has face validity, parents have been shown to frequently misinterpret wheezing as a non-specific term for any kind of noisy breathing (Elphick *et al.* 2001). A study using video footage of wheezing children showed that only 59% of parents were able to correctly identify wheezing (Cane *et al.* 2000; Cane and McKenzie 2001).

When considering multinational studies, the verb to wheeze does not exist in many languages outside of English (von Mutius 1998). This further impairs the specificity of other languages to define wheeze, when describing wheezing to parents. The verb to wheeze does not exist in Czech (or French), however overall it was considered that the combination of a close word for wheeze as well as a term for whistling in the chest was a suitable alternative. Similarly the Czech word for eczema is fairly non-specific and could be confused with any rash, although every care was taken to ensure accuracy and comparability of questionnaires in translation.

7.1.5.2. Size of study and length of follow up

Clearly the size of the follow up study was its biggest drawback. The numbers of children available for comparison were further reduced by the fact that the majority of children had continued to wheeze in the last year.

Children who had outgrown their symptoms were older than persistent wheezers. The small sample size meant that logistic regression analysis could not be performed to account for this fundamental confounder. However, persistent wheezers were not younger than their peers at bronchoscopy and the lavage findings remain valid. Such confounding variables are a problem in small studies and changes to the study design could have ameliorated them. An example would be to have followed up children on their fourth birthday to minimise age discrepancies.

7.2. Relationships to other studies

In this section the main findings are discussed in relation to other studies in the field.

7.2.1. Bronchoalveolar lavage findings

Total nucleated cell counts were raised in infant wheezers, especially those with infection. This is in keeping with the findings of Krawiec *et al* (Krawiec *et al.* 2001). They considered the lavage of 20 wheezing infants, who like this present study, required bronchoscopy for clinical severity of disease and compared them to a group of children attending for elective day case surgery. Like this study they also did not show differences for leucocyte differential proportions in infant wheeze.

7.2.1.1. Cellular viability in infant wheeze

The measurement of cell viability has not been widely used in paediatric lavage studies, probably because it requires a separate staining method. Our study showed that any differences between groups were largely due to counting variation between centres, and that overall cell viability was the same between the wheeze subgroups. In the only other study of infant wheeze to consider cell viability, Azvedo *et al* (Azevedo *et al.* 2001) showed that cellular viability was the same in wheezers (65%) and controls (67%).

7.2.1.2. Epithelial cells

The presence of epithelial cells in BAL is a marker of epithelial cell shedding and airway remodelling. Our study was in keeping with the findings of other studies of infant wheeze (Table 46 and Table 47) where epithelial cells are the same or reduced amongst atopic wheezers. Amongst this cohort of studies, ours was unique for its method of epithelial cell count estimations. The other studies counted these cells as part of their cellular differential whilst ours included the epithelial cell count during estimation of TNCC. Epithelial cells are more easily recognised using wet preparations as they frequently still have motile cilia, which potentially increases the yield of epithelial cells found in this study compared with the two others.

Epithelial cells may be affected by concomitant asthma medication. They were significantly reduced in children taking 450 μgof inhaled corticosteroids (ICS) per day (Krawiec *et al.* 2001), which may account for the lack of a differences in their study, especially when eosinophillic inflammation was such a prominent feature of their atopic wheezers. Other studies documented that up to half had also been pre-treated with cromoglycate or inhaled corticosteroids, which may also have affected the cell count (Stevenson *et al.* 1997). In our study no children had received oral glucocorticoids in the preceding six weeks and no children were taking ICS at the time of bronchoscopy. However, some children had received ICS in the previous six weeks and these children had significantly higher BAL ECP concentrations than their peers (data not shown). This was probably due to the fact that atopic children were more likely to receive a therapeutic trial of ICS than non-atopic wheezers, and was not an intrinsic effect of the medication.

Age may also be an important factor in the distribution of epithelial cells, as they have been shown to be higher amongst wheezy children under two years old (Najafi *et al.* 2003). This was suggested to be due to the differences in lavage sampling dynamics of young children, which produced a lavage from more proximal airways, thus making it more akin to epithelial cell rich bronchial washings. Our study did not show a correlation between age and epithelial cell percentages in BAL, BW or NAL (data not shown) although our study was not powered to detect such differences.

7.2.2. Infected wheezers are a distinct phenotype

Bacterial infection in BAL from wheezy infants is a common finding, but only a few studies have considered these wheezers as a separate group (Just *et al.* 2002; Najafi *et al.* 2003; Schellhase *et al.* 1998). The cytology of infected wheezers is shown in Table 46.

Our findings of increased cellularity with neutrophil infiltration of the airway are in keeping with the other studies. The presence of bacterial infection in BAL does alter the inflammatory milieu of the lower airway, and may well contribute to wheezing in this group of children.

	Infected wheezers	Najafi 2003	Just 2002	Schellhase 1998
n	15	16	13	12
TNCC	↑↑↑	↔	↔	↔
Macrophages %	↔	↓↓↓	↔	↓
Lymphocytes %	↔	↓↓↓	↓	↓
Neutrophils %	↑	↑↑↑	↓	↑↑↑
Eosinophils %	↔	↓	↑	↔
Epithelial cells %	↔	↔	ND	ND
Cellular viability %	↔	ND	ND	ND

Table 46 Cell counts of infected wheezers

This table shows the relative differences between cell counts from infected wheezers when compared to non-infected infant wheezers. Trends are shown as single arrows (↑ for raised, ↓ for reduced and ↔ for equivocal) whilst significant differences are shown as triple arrows (eg. ↓↓↓). (Najafi et al. 2003), (Just et al. 2002), (Schellhase et al. 1998), Infected wheezers were results from this study, ND Not done or reported, TNCC total nucleated cell count.

Not all bronchoscopy studies have accounted for infection in infant wheeze, but most have recorded positive bacterial cultures of BAL, where it was sought. Some studies did not culture BAL from infant wheezers (Azevedo *et al.* 2001; Stevenson *et al.* 1997), possibly because the prevailing belief has been that infection was not an issue in asthma. When cultures have been performed the number of infected infant wheezers varied between 10 % (Krawiec *et al.* 2001), to 55 % (Marguet *et al.* 1999; Marguet *et al.* 2001) and 60 % (Le Bourgeois *et al.* 2002) compared to 29% in our study.

The majority of cultured organisms from BAL are common upper respiratory tract flora, and their presence has been considered likely to be due to contamination (Le Bourgeois *et al.* 2002; Marguet *et al.* 1999; Marguet *et al.* 2001). It has been argued that the absence of quantative bacterial culture may make the differentiation of upper airway contamination from true infection of lavage impossible (Le Bourgeois *et al.* 2002). However, a collaborative study in association with members of our own group, has shown that using quantative culture many infant wheezers have significant bacterial loads (defined as $>10^3$ CFU/ml BALF) (Marguet *et al.* 2001).

Fayon *et al* (Fayon *et al.* 1999) showed that bacterial infection is a common finding in severe childhood asthma, and that nasal colonisation rarely predicts lower respiratory tract organisms, suggesting that bacterial isolates for the lower respiratory tract are not contaminants. The raised BAL neutrophil count and total cellularity of infected wheezers in our study also suggested that the organisms were present some time before the procedure, in order to invoke an inflammatory response.

One explanation for the frequency of bacterial infection in our study may be that it is more common amongst severe infant wheezers than the general population (Just *et al.* 2002). The clinical criteria for bronchoscopy used in this study, and others (Azevedo *et al.* 2001; Just *et al.* 2002; Le Bourgeois *et al.* 2002; Najafi *et al.* 2003), may preferentially select infected children. The majority of infected wheezers in our study were recruited from one centre suggesting a bias towards the investigation of the infected wheezer at this centre. However, this does not preclude the extrapolation of the infected phenotype to wheezers in general. Infected wheezers were found at all centres, and in all the other BAL studies of preschool wheezing, which indicate that they represent a standard presentation of severe infant wheezing. Further studies are required to consider lower respiratory tract cultures in less severe infant wheezers.

The inclusion of wheezers with bacterial infection in other bronchoscopic studies may have skewed their results. For example, Le Bourgeois *et al* (Le Bourgeois *et al.* 2002) showed that neutrophils were raised in all infant wheezers yet sixty percent of their infant wheezers, in whom cultures were performed, had documented bacterial infection in BAL. This compared favourably to their control group and was not considered significant. However, the controls also had several children with wheezy bronchitis and infective symptoms, likely to indicate LRT infection and were not a suitable comparative group. Their results showed that total cellularity was increased in wheeze, with an increase in percentage neutrophil count. This was identical to the pattern observed amongst infected wheezers in our study suggesting that their results have been materially affected. High neutrophil counts in infant wheezers have also been seen in studies where no record of infection was kept (Azevedo *et al.* 2001) suggesting that bacterial infection of these severe wheezers was underlying their findings.

7.2.3. Atopy and infant wheeze

Eosinophils were not raised amongst atopic wheezers and were only raised amongst those wheezers with allergic rhinitis. This is in contrast to the other studies that have compared atopic to non-atopic (or idiopathic) infant wheezers (Azevedo *et al.* 2001; Krawiec *et al.* 2001; Le Bourgeois *et al.* 2002; Marguet *et al.* 2001). In these studies the presence of eosinophils in atopic infant wheeze has been a consistent finding. The results of the other studies are shown in Table 47.

Atopic wheezers	Atopic Wheezers	A	B	C	D
Age (years)	1.2	2.4	ND	3.1	7.5
TNCC	↔	↓	↔	↓↓↓	↔
Macrophages %	↔	↑	↔	↑	↑
Lymphocytes %	↔	↑	↔	↓	↔
Neutrophils %	↔	↓	↓	↓	↓
Eosinophils %	↔	↑↑↑	↑↑↑	↑↑↑	↑↑↑
Epithelial cells %	↔	↑	ND	↓	↔
Cellular viability %	↔	ND	ND	ND	ND

Table 47 Comparison of cytology within atopic wheezers

This table shows the relative differences between cell counts from atopic wheezers when compared to non-infected infant wheezers. Trends are shown as single arrows (↑) whilst significant differences are shown as triple arrows (↓↓↓). A (Najafi et al. 2003), B (Just et al. 2002), C (Stevenson et al. 1997)D (Marguet et al. 2001), AW Atopic wheezers from this study, ND Not done or reported

Most of the studies considering atopy and infant wheeze have selected a sample of atopics who are older than the non-atopic infant wheezers, to which they are compared. For instance, the atopic wheezers of Najafi *et al* (Najafi *et al.* 2003) had raised eosinophils but were also older (with a mean age of 2.4 years) than their non-atopic wheezing comparison group (mean age 1.1 years). The atopic wheezers of Stevenson *et al* (Stevenson *et al.* 1997) were also older than those in our study with the viral associated wheeze group having a mean of 3.1 years (1.9 - 8.2) compared to a mean age of 1.2 years in our study. School age children with asthma are known to

have eosinophillic BAL similar to adult asthmatics (Kim *et al.* 2000; Marguet *et al.* 1999; Marguet *et al.* 2001; Stevenson *et al.* 1997). These older preschool wheezers may therefore represent the adult atopic phenotype and not that of the atopic infant airway.

7.2.4. Eosinophil Cationic Protein

BAL ECP was significantly different between controls and the three wheeze phenotypes. It was also significantly raised amongst infected wheezers when compared to idiopathic wheezers. Although BAL ECP was also raised in atopic wheezers, but not to statistical significance. Consideration of ECP as a categorical variable, as others have done (Azevedo *et al.* 2001; Marguet *et al.* 2001), also gave significant results between groups.

Azevedo *et al.* showed an increase in BAL ECP amongst there group of acute wheezers. This effect may be due to acute infection rather than their atopic status Azevedo *et al.* 2001. Marguet *et al.* showed that ECP was related to neutrophil count being raised in those with counts over 10% suggested once omor eof infection (Marguet *et al.* 2001). Other studies to consider ECP in BAL from infant wheezers have not demonstrated differences from controls (Azevedo *et al.* 2001; Ennis *et al.* 1999; Marguet *et al.* 2001). This is in contrast to older children with atopic asthma or persistent wheezing, where ECP was increased in BAL (Barbato *et al.* 2001). The differences between our study and these negative studies lies almost certainly in the definition of wheeze subgroups. The separation of infected wheezers into a separate group in our study polarised the changes in ECP between wheeze phenotypes, and was enhanced by the selection of non-inflamed controls.

Children with infection were more likely than those with atopy to have detectable ECP in BAL. ECP is increased in acute viral induced wheeze in infancy (Ingram *et al.* 1995; Oh *et al.* 2000). Urine EPX, another marker of eosinophillic inflammation, was also elevated in acute viral induced wheezers but settled on convalescence (Oommen *et al.* 2003b). Barbatao *et al* (Barbato *et al.* 2001) considered that ECP may play a role in the development of asthma, and that those with transient wheeze have lower levels of ECP in BAL. However, lower levels of ECP may be related entirely to the timing since the last exacerbation of symptoms (Shields *et al.* 1999).

Raised concentrations of ECP seen in our infected wheezers would agree with this finding.

Considering the importance of ECP in persistent inflammation, it is intriguing that ECP did not correlate to eosinophil concentrations in BAL, but correlated instead with neutrophils, epithelial cells, IL-8 and MMP-9. Ennis *et al* (Ennis *et al.* 1999) correlated ECP to eosinophils amongst a group of school age atopic asthmatics as did Marguet *et al* (Marguet *et al.* 2001). All other studies have reluctantly had to agree that in infant wheeze, ECP correlates with neutrophils and not eosinophils (Azevedo *et al.* 2001; Barbato *et al.* 2001; Marguet *et al.* 2001).

The reasons for raised levels of ECP despite the general absence of eosinophils cannot be readily explained. Degranulated eosinophils may be difficult to detect in lavage cytology under standard staining conditions, leading to an underestimation of their true number (Azevedo *et al.* 2001). As a relatively rare cell type, eosinophils can be misidentified during cytology. The use of immunohistochemistry, such as the immuno-histochemical staining methods attempted in this study, or by use of flow cytometry, may give a more accurate count. However, it is unlikely that there has been gross underestimation of eosinophils in every study on infant wheeze, so this argument can only partially explain the phenomenon. Other possibilities include the release of ECP into BAL from intraepithelial eosinophils (Azevedo *et al.* 2001). However, BAL and bronchial biopsy have shown good correlations between intraepithelial and lavage cell numbers (Foresi *et al.* 1990), which indicates that lavage from the airway lumen reflects cellular infiltration within the epithelial mucosa.

Likewise, the relationship of ECP to neutrophil infiltration of the airway is puzzling because neutrophils do not produce ECP. Neutrophils have been shown to phagocytose eosinophil granules and contain traceable quantities of ECP and other eosinophil proteins, which demonstrates that the small amounts of ECP present are imported from eosinophils (Sur *et al.* 1998). Reverse transcriptase PCR demonstrated an absence of ECP mRNA from highly purified neutrophils, which confirms that neutrophil ECP is exogenous (Bystrom *et al.* 2002). Thus, the role of

eosinoiphils in ECP production remains a mystery. It is discussed further in section 7.3.2.

7.2.5. Matrixins and TIMP in infant wheeze

This is the first study to measure the concentrations of matrixins and TIMP in infant wheeze. Although one study in cystic fibrosis used childhood asthmatics as controls (Delacourt *et al.* 1995), no other studies have considered metalloproteases in childhood asthma.

7.2.5.1. MMP-9

MMP-9 was increased in the BAL of most infant wheezers compared to controls. This was statistically significant for infected wheezers, and also for atopic and idiopathic wheezers using less stringent statistical criteria, which is in keeping with studies of adults with stable asthma who show similar increases in MMP-9 in BAL (Mautino *et al.* 1997), induced sputum (Lee *et al.* 2001; Vignola *et al.* 1998) and serum (Belleguic *et al.* 2002; Suzuki *et al.* 2001). However, in our study the only group in which MMP-9 was not statistically different from controls was the atopic wheezers, which is interesting to note because this was the group that could be considered to be most like the atopic adult asthmatics. One study of milder asthmatics did not show a difference between MMP-9 in sputum from asthmatics and controls, suggesting that the atopic infant wheezers in this study fall into this category of more mild disease (Cataldo *et al.* 2002a), despite their severe symptoms requiring bronchoscopy.

Infected wheezers had the highest concentrations of MMP-9 in BAL suggesting that they were in an acute phase of their disease. MMP-9 has been shown to be increased in poorly controlled asthma beyond levels seen in stable disease (Mattos *et al.* 2002; Mautino *et al.* 1997). *Status asthmaticus* also increased the levels of MMP-9 (Lee *et al.* 2001; Lemjabbar *et al.* 1999; Mattos *et al.* 2002; Suzuki *et al.* 2001).

MMP-9 correlated with neutrophils suggesting that neutrophils may be the predominant source of MMP-9 in infant wheeze. In adult asthmatics MMP-9 also correlates with neutrophils (Mautino *et al.* 1997; Vignola *et al.* 1998), in adults with

status asthmaticus (Lemjabbar *et al.* 1999) and following allergen challenge (Cataldo *et al.* 2002a). MMP-9 has also been directly imaged on the neutrophil surface using immunohistochemistry (Becky Kelly *et al.* 2000).

In our study, MMP-9 did not correlate with eosinophils however; two studies of asthma in adults have suggested that eosinophils may also play a role in MMP-9 production. Hoshino *et al* (Hoshino *et al.* 1998) showed that MMP-9 and TIMP-1 mRNA from bronchial biopsy specimens correlated with eosinophil infiltration of the epithelium. Wenzel *et al* (Wenzel *et al.* 2003) showed that MMP-9 staining of epithelial basement membrane increased in severe asthma. This was associated with increased in BAL neutrophils but also with an infiltration of bronchial biopsy tissue by eosinophils. This suggests that finding MMP-9 in BAL does not reflect changes to the airway wall itself, and undermines our fundamental assumption that the BAL and epithelium are synonymous.

The gelatinase activity of MMP-9 has been shown to be increased in acute exacerbations of disease (Lee *et al.* 2001; Mattos *et al.* 2002). MMP-9 is able to cleave IL-8, potentiating its action on neutrophils in preference to the eosinophil attracting CC chemokines, RANTES and eotaxin (Van den Steen *et al.* 2000). It is a drawback of our study that enzyme activity levels were not formally assessed, as they may have provided further insights into the source as well as the role of MMP-9. However, gelatin zymography (at 92 kDa) performed in tandem with ELISA showed a good correlation between methods in our study ($r_S = 0.7$, $p < 0.001$). In addition, it has been suggested that active MMP-9 may be more difficult to measure in lavage, due its preference for binding to the ECM (Shute 2002) and the neutrophil cell surface (Owen *et al.* 2003).

7.2.5.2. TIMP-1

BAL TIMP-1 concentrations were reduced in infected wheezers compared to atopics. Atopic wheezers showed a trend towards higher TIMP-1 levels compared to controls and other wheezers, but this did not reach significance. As with MMP-9,

interpretation of these findings requires a consideration of the data gleaned from the adult respiratory tract.

In our study TIMP-1 correlated with epithelial cells in BAL particularly amongst atopic wheezers, where these cells were correlated with IL-8 and ECP concentrations in BAL. It is not known whether shed epithelial cells produce TIMP or shedding is a consequence of the action of TIMP on the airway. In one study, TIMP-1 was produced by alveolar macrophages (Mautino *et al.* 1999b), but other studies of TIMP-1 in adult asthma have not been able to correlate TIMP-1 with any particular cell type. In our study TIMP-1 correlated with neutrophils and epithelial cells amongst atopic wheezers and controls, which suggests that the TIMP-1 response is that of a normal airway to an inflammatory stimulus.

The reduced concentrations of TIMP-1 seen in the infected wheezers were similar to that observed amongst adults with acute community acquired pneumonia (Hartog *et al.* 2003). It suggests that the inflammatory changes observed in infected wheeze are a response to infection, once again demonstrating the importance of bacterial infection of the lower respiratory tract in the wheezing illnesses of infancy.

Raised TIMP-1 amongst atopic wheezers was similar to that seen in adult asthma, where TIMP-1 may be raised in acute disease. However, there is some controversy over these data. Some studies of serum suggest that TIMP-1 remained the same in sera from acute asthma compared to stable disease (Belleguic *et al.* 2002; Oshita *et al.* 2003). However, induced sputum showed increased levels of TIMP-1 during spontaneous exacerbations (Mattos *et al.* 2002; Suzuki *et al.* 2001) and from BAL in adults ventilated for *status asthmaticus* (Lemjabbar *et al.* 1999). A study of blood neutrophils from asthmatics noted that they did not produce TIMP-1 on stimulation (Cataldo *et al.* 2001), unlike COPD and control subjects, which may be an explanation for the differences seen between serum and airway secretions in adults.

Further evidence from allergen challenge studies indicates that TIMP-1 is raised in acute disease. In adult asthmatics, TIMP-1 remained quiescent in sputum six hours after aerosol allergen challenge with house dust mite (Cataldo *et al.* 2002a). This is in contrast to Becky-Kelly *et al.* (Becky Kelly *et al.* 2000) where TIMP-1 was seen

to be elevated by 48 hours post challenge. The reasons for the differences in these studies are almost certainly due to the timing of post challenge sampling. It is likely that TIMP-1 is synthesised *de novo* in response to allergen whilst MMP-9 is released immediately from de-granulating neutrophils (Atkinson and Senior 2003).

7.2.6. Nasal lavage in infant wheeze

Our study was the first to compare nasal lavage directly to BAL in infant wheezers. Although other studies have considered nasal lavage (Meyer *et al.* 2003; Noah *et al.* 1995; Teran *et al.* 1999) and nasal brushings (Sale *et al.* 2003) in children with allergic rhinitis, these studies recruited schoolchildren and teenagers. The results are therefore not directly comparable to this study. Other studies have considered nasopharyngeal aspirates from acute virally infected infant wheezers (Ingram *et al.* 1995; Oh *et al.* 2000; Oh *et al.* 2002).

We showed distinct changes between neutrophils and lymphocytes in nasal lavage between wheeze subgroups. Neutrophils predominated in nasal lavage from infected wheezers whilst lymphocytes were present in higher proportions in idiopathic and atopic wheezers. Nasal eosinophils were present in children with allergic rhinitis.

7.2.6.1. Nasal neutrophillia in infant wheeze

Nasal lavage neutrophillia was associated with wheezers with bacterial lower respiratory tract infection. There are two possible explanations for this finding. The first is that the presence of upper respiratory tract inflammation caused contamination of BAL during bronchoscopy with bacteria. Although nasal endoscopy during bronchoscopy makes this a possibility it does not explain the reductions in BAL cell viability seen amongst both atopic uninfected wheezers and the infected children. Cell death would take some time to occur, much longer than the time required for the bronchoscopy procedure.

The second is that the presence of neutrophillic inflammation in the nose was associated with similar picture in the lung. Nasal lavage was not sent for bacterial or viral culture so we were unable to demonstrate the presence of URT infection in this

group. However, the implication is that infected wheezers were experiencing transient and infection induced wheezing.

Data from adult volunteers shows that viral URTI can cause lung neutrophillia (McKean *et al.* 2003). LRT differences were seen when adults who wheezed only during viral URTI were infected with rhinovirus as a model of viral induced wheeze. These participants showed a neutrophillia in induced sputum implying that LRT neutrophil driven inflammation is present during periods of viral upper respiratory tract infection.

7.2.6.2. Allergic rhinitis in infant wheeze

Although eosinophils did not dominate atopic NAL and the presence of eosinophils in NAL did not predict their presence in BAL, children with allergic rhinitis or aeroallergen sensitivity had raised NAL eosinophils suggesting the presence of allergic nasal disease. AR was associated with lower respiratory tract inflammation with evidence of epithelial shedding suggesting that a remodelling process was occurring. Soluble ICAM-1 concentrations in NAL correlated to BAL epithelial cells and are probably derived from serum exudates and reflect the significant differences seen from blood. It suggests that bronchial epithelial damage is occurring amongst atopic wheezers, more evidence that airway damage is associated with inflammation in this group.

7.2.6.3. Nasal ECP concentrations

Nasal ECP was not elevated in this study, even amongst atopic wheezers. Other studies have shown that nasal ECP was raised in acutely wheezy infants compared to those without wheeze (Ingram *et al.* 1995; Oh *et al.* 2000). In these studies NAL ECP was not associated with RAST to aeroallergens or serum IgE status suggesting that nasal ECP is a mark for acute viral infection in this age group. Neonates of atopic parents had raised nasal ECP in the first few weeks of life suggesting that atopic sensitisation may occur very early in life (Halmerbauer *et al.* 2000). Raised ECP in acute infections may then overlay the presence of atopic inflammation in infancy making ECP a less sensitive marker for allergic disease in the preschool

child. As infective exacerbations lessen with age ECP becomes specific to allergic disease once more.

7.2.7. Surrogate markers for BAL

The use of a less invasive way to study the immune response of the lower airway would provide a useful insight into childhood asthma and preschool wheezing. The use of bronchoscopy tends to be skewed towards those children with severe disease and extrapolation is required to apply these findings to the general population. A non-invasive method that correlated with lower airway disease would be extremely useful in this respect.

This study was well placed to consider the differences between lavage compartments. Lavage was taken from lung and nose on the same day, and cell counts and processing were performed simultaneously. Inflammatory markers were analysed by subject to further reduce variation between compartments.

7.2.7.1. Bronchial washings

Our study has shown that bronchial washings provide a surrogate for BAL. Correlations between lavages were strongly positive and significant for all cells and markers considered. The absolute results varied but it is already known that the first aliquot of BAL has a different composition to subsequent lavages (Pohunek *et al.* 1996). Differences found in the bronchial wash are believed to be due to the first lavage filling the larger airways, and not the alveolar spaces. In particular the cellularity and cell viability are reduced in bronchial wash, although the differential cell count remains largely unchanged (Pohunek *et al.* 1996). In keeping with our study, other sources have shown more epithelial cells, reduced macrophages and increased neutrophil counts in the bronchial wash compared to BAL (Rennard *et al.* 1998; Shields and Riedler 2000).

However, bronchial washes are just as invasive as BAL because they too require endoscopy and are not very useful as a surrogate for BAL. Non-bronchoscopic lavage (NBL) could represent a form of bronchial wash and in this respect would be less invasive than bronchoscopy (Shields and Riedler 2000). The use of a single

aliquot of lavage fluid suggests that NBL is a form of bronchial washing. This is compounded by the higher levels of epithelial cells found in NBL when compared to BAL, which suggests a lavage from the proximal airway. The proponents of NBL (Shields and Riedler 2000), argue that the smaller diameter suction catheters used in NBL provide a more distal lavage, which is therefore more akin to BAL. No studies have provided a direct comparison. In blind BAL the wedge of the catheter into a distal airway is not performed under direct vision, and may be misplaced collecting both proximal fluids from the larger airways as well as distal fluid from the alveolar space. It may also sample from the lower lobes leading to reduced lavage return (Heaney *et al.* 1996).

The cellular data from healthy infantile non-bronchoscopic lavage indicate that NBL is more likely to represent BW than BAL (Heaney *et al.* 1996). The published data on NBL is not directly comparable to our study because the cell counts include epithelial cells. However, removal of these cells from the count show macrophages to be in excess with a median value of 91.5%, neutrophils present in greater number than lymphocytes (4.2 compared to 3.9%) and eosinophils and mast cells present in low numbers (0.01 and 0.3%) respectively. This increase in neutrophils is typical of bronchial washings (Pohunek *et al.* 1996).

Our study suggests that NBL is likely to be a form of BW and as such may correlate directly to BAL. For research purposes the advantages of NBL and their use in children attending for routine surgery permits the study of a wider population of infant wheezers.

7.2.7.2. Nasal lavage

NAL cellularity was not a good predictor of BAL cellularity. This could be due to two main reasons. Firstly, that a different inflammatory process occurs in the nose to the lung. Chanez *et al* (Chanez *et al.* 1999) have compared nasal to bronchial biopsy in adults with asthma and observed similar differences. Secondly, the variation in ELF sampling between the nose and lung required a larger sample size than our study could provide to demonstrate true differences between nose and lung.

In a small study such as ours, the differential cell count proportions had the greatest opportunity of direct correlation between the nose and lungs. This is because the cells were concentrated by cytospin prior to counting and the use of cell count percentages means that the differentials were unrelated to the concentration of ELF in the lavage. As all the counts were performed at their home centres, no adjustment had to be made for differences in cytology between centres.

In spite of this, the lack of correlation between nasal and bronchoalveolar lavage indicates that nasal lavage cannot be used as a surrogate for BAL.

7.2.7.3. Serum

Our study showed that serum ECP was increased only amongst those with allergic rhinitis and that this was related to, but did not correlate with, BAL and NAL eosinophillia. Other studies have linked serum ECP to lower airway eosinophillic inflammation from BAL (Marguet *et al.* 2001; Shields *et al.* 1999), but as we have seen, this was amongst older children, not infants.

The relationship between serum ECP and infant lung function is poorly understood. In one study, serum ECP did not influence bronchial hyperreactivity in this age group (Reichenbach *et al.* 2002). However, serum ECP and atopy, but not the neutrophil associated myeloperoxidase (MPO), influenced salbutamol reversibility in infant wheezers, indicating that allergic inflammation may yet impact on infant lung function (Lodrup Carlsen *et al.* 1995). The evidence is conflicting and differences in these studies probably represent the difficulties of pulmonary function in the preschool child.

7.2.8. Follow up studies of infant wheeze

This section compares our results to those found in other similar follow up studies of wheezing in infancy. Our study showed that current symptoms of allergic rhinitis were associated with persistent wheezing at follow up. BAL TIMP-1, but not ECP, MMP-9 or its molar ratio, was raised in persistent wheezers, as was serum sICAM-1.

The role of TIMP-1 in persistent wheezing is strongly suggestive of airway remodelling and is discussed in section 7.3.4.

7.2.8.1. BAL ECP

ECP as a prominent marker for eosinophillic (and by implication therefore allergic) inflammation has received most attention as a marker for the prediction of outcome of infant wheeze. Although, eosinophillic inflammation is associated with acute viral wheeze (Ingram *et al.* 1995; Oommen *et al.* 2003b; Wojnarowski *et al.* 1999), it does not predict persistent wheezing in infancy.

Several studies have considered the role of ECP in predicting the outcome of infant wheezing. BAL ECP correlates to measures of impaired lung function in infant wheeze (Lodrup Carlsen *et al.* 1995) and acute serum ECP predicts development of recurrent wheezing (Koller *et al.* 1997; Villa *et al.* 1998). Studies in older children with asthma have shown mixed results indicating that ECP may play a more prominent role in the pathogenesis of disease in infancy than in the older child. In these studies, ECP did not correlate to either symptom severity scores or lung function in serum (Rao *et al.* 1996) or BAL (Ferguson *et al.* 1992). However, induced sputum ECP was found to correlate to lung function and bronchial hyper-responsiveness in this older age group (Wilson *et al.* 2001).

Our study has shown that BAL ECP was also raised in infected wheezers and was not particular to atopic wheeze. It is this discrepancy that may account for the inability of previous studies to demonstrate a link between ECP and persistent wheeze.

7.2.8.2. Nasal symptoms and persistence of wheeze

In our small follow up study, the persistence of preschool wheeze was strongly associated with nasal symptoms in infancy and their persistence or the development of these symptoms at follow up.

Rhinitis in infancy has been implicated in persistence of wheeze for a long time (Wright *et al.* 1994). However, there is very little work on the subject. For instance the relatively recent and comprehensive ARIA workshop on allergic rhinitis (Bousquet *et al.* 2001) considered that allergic rhinitis was rare before the third year of life, and still followed an allergic march pattern of wheeze before rhinitis. They agreed that sensitisation to aeroallergens occurs early in life but that this was not the same as rhinitis. Whereas our findings in nasal lavage would suggest that eosinophillic inflammation in the nose occurred early in those with such aero-sensitisation.

Studies on nasal ECP concentrations in neonates, born to atopic parents, would indicate that nasal sensitisation to allergen begins as early as four weeks of life (Tauber *et al.* 2000) and predicts development of wheeze and atopy (Frischer *et al.* 2000). Longer-term follow up of this cohort is required to establish the true relationship between nasal ECP and childhood asthma (Bush 2000).

7.2.8.3. Serum sICAM-1

Serum sICAM-1 was not significantly raised amongst any group in our study. The findings in infancy are in contrast to older children with asthma where serum sICAM-1 is elevated (Tang *et al.* 2002). Even the atopic wheezers in our study did not have raised serum sICAM-1. However, questionnaire follow up showed that raised serum sICAM-1 at bronchoscopy was associated with persistent wheezing. Koopman *et al* (Koopman *et al.* 2003) showed, from a random sample of 86 from a much larger birth cohort study, that serum sICAM-1 concentrations in asymptomatic one year olds were elevated amongst those who went on to develop wheezing by age two. Serum sICAM-1 was not elevated amongst one-year-old wheezers who continued to wheeze. In these children soluble IL-2 receptor was elevated compared to non-wheezers, which was in agreement with a logistic regression analysis of risk factors associated with persistent wheeze (Clough *et al.* 1999).

7.3. Possible mechanisms and implications

7.3.1. The neutrophil in infant wheeze

BAL neutrophil proportions in our study correlated with IL-8, ECP and MMP-9, suggesting an important role for the neutrophil in infant wheeze. Neutrophils have previously been shown to be important in childhood asthma (Barbato *et al.* 2001; Ennis 2003) and is in contrast to studies of older asthmatic children (Stevenson *et al.* 1997). In spite of neutrophils potential importance, neutrophil counts were only raised in infected wheezers. Neutrophils, eosinophils and their associated chemokines (IL-8 and IL-5) were increased in induced sputum from acute childhood asthma in children over 8 years old (Norzila *et al.* 2000). This study also showed that these markers decreased on resolution of the exacerbation. Infected wheezers are in an acute inflammatory phase of their disease, and the other groups are more quiescent, which may be the reason why there were not more marked differences seen in our study.

Neutrophils are being recognised as an important cell in adult asthma (Ennis 2003; O'Donnell and Frew 2002). The presence of neutrophils in asthma has been related to more severe symptoms (Jatakanon *et al.* 1999) and acute corticosteroid dependent asthma (Wenzel *et al.* 1997). Neutrophils have also been observed in response to localised bronchial allergen challenge in adults (Becky Kelly *et al.* 2000; Cataldo *et al.* 2002a).

Neutrophils seem to be important in Allergic Bronchopulmonary Aspergillosis (ABPA), which until recently was considered an eosinophil mediated disease (Gibson *et al.* 2003). In induced sputum from adults with ABPA, IL-8 correlated to neutrophil counts, MMP-9 production and severity of disease. The authors considered that IL-8 played a critical role in neutrophil inflammation leading to bronchiectasis.

7.3.2. ECP and infection

Neutrophils seem to be the dominant inflammatory cell in infant wheeze, correlating with all inflammatory markers, and yet raised ECP concentrations suggest that

eosinophils should also be present. Thus the absence of eosinophils from the wheezing airway remains a paradox and the explanations provided for this phenomenon to date remain unsatisfactory (see section 7.2.4).

It has been proposed that neutrophils may phagocytose eosinophils in the airway lumen, leading to a reduction in eosinophil cell numbers without concomitant reduction in ECP (Marguet *et al.* 2001). Why this should occur solely in infant wheeze and not be seen in asthma, where eosinophils are prominent, is not clear. Neutrophils have not been demonstrated ingesting eosinophils in lavage studies of asthma or allergen challenge. Therefore neutrophil phagocytosis cannot be the whole answer.

In our study, the presence of infection was a better stimulus to ECP production than the atopic trait, with ECP present in all children with infected wheeze compared to only 60% with atopic wheeze ($p = 0.005$). In an eloquent review, Rosenberg & Domachowske (Rosenberg and Domachowske 2001) suggest a role for eosinophils in viral infections. They consider that a primary function of ECP is as a ribonuclease against single stranded viral genomes. Mouse models of respiratory viral infections have demonstrated that eosinophils are present in the airway before the onset of symptoms; with neutrophils being almost entirely present by the time symptoms occur (Domachowske *et al.* 2000).

There is evidence that viral URTI prompt an eosinophillic response. Experimental infection with rhinovirus in adults with allergic rhinitis has shown an eosinophillic response to viral infection, with eotaxin and ECP concentrations raised in nasal lavage following infection (Greiff *et al.* 1999). Other studies have shown that both ECP (Ingram *et al.* 1995) and RANTES (Pacifico *et al.* 2000) production was increased amongst wheezers with viral URTI.

The role of eosinophils in viral infection would fit with the serum '*eosinopaenia*' that is the hallmark of transient viral induced wheezers during acute exacerbations of their disease (Garofalo *et al.* 1994; Martinez *et al.* 1998). The observed reduction in eosinophils from the circulation may be due to their recruitment to the respiratory tract early in the disease process.

7.3.3. Bacterial infection and wheeze

Our study has shown that infant wheezers with positive bacterial cultures have a distinct lavage phenotype. Infected wheezers had a different lavage cell profile from other wheezers, evidence of bronchoalveolar inflammation and metalloproteinase imbalance. Although the term *wheezy bronchitis* has gone out of fashion (Edwards *et al.* 2003), it appears to be alive and well amongst children with severe infant wheeze.

Infant wheezing has been considered to be a viral induced phenomenon. Some of the original data on viral induced wheezing in infancy came from longitudinal studies of school aged children (Horn *et al.* 1979; McIntosh *et al.* 1973; Minor *et al.* 1974). These studies showed that viruses, and in particular rhinoviruses, were isolated from the upper respiratory tracts of children with acute wheezy episodes, and agree with more recent PCR based epidemiology (Johnston *et al.* 1995)> Modern molecular biology has revealed that such exacerbations induce eosinophil chemoattractant production (Teran *et al.* 1999). All of the subjects from these studies were older children who were not in the preschool age group, and it may be specifically amongst toddlers and infants that bacterial triggers are a significant pathology. A more recent study of induced sputum from children under two with recurrent wheezing showed that 16 of 35 children had pathogenic bacteria isolated during a wheezy episode (Nagayama *et al.* 2001). Children older than two did not have a bacterial trigger for their exacerbations and the frequency of bacterial isolation was in keeping with the longitudinal studies previously described. It is interesting to note that, as in our study, bacterial infection and wheezing was associated with elevated serum ECP (Nagayama *et al.* 2001).

Even in the absence of high bacterial loads, low-grade colonisation of the lower respiratory tract may still lead to chronic airway inflammation (Simell *et al.* 2001). It is noteworthy that pneumococcal colonisation of the upper respiratory tract is highest in infancy, when it may colonise up to half of all children (Shackley *et al.* 1997). The impact of bacterial carriage on infant wheeze has not been studied.

7.3.4. Remodelling in infant wheeze

Both MMP-9 and TIMP-1 have been implicated in the process of asthmatic airway remodelling (see section 2.8.2). Ours was the first study to measure MMP-9 and TIMP-1 in lavage from wheezy infants, and as such we have an opportunity to consider the role of airway remodelling in preschool wheeze albeit by an indirect method. Our assumptions were that an imbalance of MMP-9 to TIMP-1 (section 2.8.3) would be directly related to remodelling, and that remodelling would lead in turn to persistence of wheezing at follow up. To date, persistent wheezing has been associated with atopy and our original hypothesis was that atopic wheezers would demonstrate the most pronounced matrixin imbalance. Therefore, it was surprising to find that MMP:TIMP imbalance was only seen amongst infected and idiopathic wheezers when compared to controls, and that atopic wheezers were the most balanced of our wheeze subgroups in this respect. However, BAL TIMP-1 was raised in infancy amongst those who continued to wheeze suggesting a role for TIMP-1 in airway remodelling.

The role of MMP-9, TIMP-1 and their molar ratio in prediction of airway remodelling remains uncertain. They are impossible to interpret with accuracy, as they remain unvalidated, surrogate markers of the remodelling process. Until biopsy studies are able to provide a basis for their validity, both in adults and in infancy, their significance can remain only speculative. An example of such a study was that of Wenzel *et al.* (Wenzel *et al.* 1997), who correlated MMP-9 deposition to TGF-β positive cells within the epithelium, but did not observe any co-association with basement membrane thickness, thus demonstrating that there is still considerable proof required to link the matrixins and TIMP with the remodelling process.

In cystic fibrosis it is easy to visualise airway damage from unchecked metallo-proteolytic activity, but can the low molar ratios seen in infant wheeze allow this to happen? The ratios observed in infant wheeze are miniscule compared to sputum from cystic fibrosis, where MMP-9 outweighed TIMP-1 ten fold (Delacourt *et al.* 1995), even when asthmatic children were acting as controls. The gearing of these ratios in infant wheezing was such that TIMP-1 vastly outweighed MMP-9 in BAL. Molar ratios exceeded parity in only one infected subject in our study, and apart

from four infected children, ratios of MMP-9 to TIMP-1 were consistently below 0.15 (0.15:1), indicating and excess of nearly seven times more TIMP than MMP in the airway. Mautino *et al* (Mautino *et al.* 1999c) showed that TIMP-1 concentrations in BAL were high enough to bind all matrixin species, not just the most common airway matrixin MMP-9. In healthy adults, and those with mild asthma this ratio averaged around 0.4 (Mattos *et al.* 2002) and increased to parity for severe asthmatics (Mattos *et al.* 2002). Our control data implied that molar ratios of around 0.02 are normal in infancy and that a fifty-fold excess of TIMP-1 over MMP-9 is maintained in the healthy infant lung.

The excess of TIMP on MMP may due to two possible scenarios. Firstly, the excess TIMP may be a response to its inability to completely inhibit protease activity. This would occur if MMP-9 occupied a sanctuary site. Owen *et al* demonstrated that MMP-9 was insensitive to TIMP-1 inhibition when bound to the neutrophil surface, exposing the possibility of such a sanctuary (Owen *et al.* 2003). The continued release of MMP-9 degradation products into the airway may lead to continued synthesis of TIMP-1 and TIMP excess. Secondly, active MMP-9 preferentially binds to tissue and would not be present in BAL fluid (Shute 2002). Therefore, the relatively high levels of TIMP-1 in lavage may be in order to saturate active, cell associated and EBM bound matrixins.

However, smaller differences in molar ratios may still be significant in terms of lung damage. The ratios seen in broncho-segmental allergen challenge in mild asthma were similar to those observed in our study (Becky Kelly *et al.* 2000). This would suggest that even small imbalances in molar ratios are a significant part of the atopic (and by implication remodelling) response to inflammation. In our study, no difference in molar ratio was seen between atopic wheezers and controls. A study of induced sputum in asthmatics showed that molar MMP:TIMP ratios were in fact lower in asthmatics than in controls (Vignola *et al.* 1998) and our findings in atopic wheeze may thus be more suggestive of an adult type asthmatic response.

In our study, atopic wheezers showed correlation between TIMP-1, neutrophils and epithelial cell numbers. This finding was not seen in other wheeze subgroups, but was shared by controls. This suggests two things, firstly a role for TIMP-1 in atopic

inflammatory epithelial cell shedding, which is one of the cardinal features of airway remodelling. Secondly, it also suggests that this is the response of a normal airway to an inflammatory stimulus. The abnormality may be the presence of inflammation in the lower respiratory tract and not the inflammation itself, which is a normal response to an abnormal stimulus. This idea will be expanded further when considering the nasal lavage findings.

The follow up study showed that persistent wheezers had both raised BAL TIMP-1 and serum sICAM-1 in infancy. Both of these findings may be a surrogate for the action of TGF-β which has been shown to up regulate both these markers in inflammation (Duvernelle *et al.* 2003). Thus the *see-saw* model of MMP TIMP ratios (Figure 7) tips the balance in favour of TIMP-1 as the key to airway remodelling in infancy.

These findings suggest that airway remodelling is occurring amongst some infant wheezers and that this is associated with atopy and more specifically allergic rhinitis and aeroallergen sensitivity. Remodelling occurs early in life, and that this outcome may thus be predicted from infancy. The inflammatory process that leads to remodelling may start as early as the first few weeks of life (Halmerbauer *et al.* 2000), and allergic sensitisation may occur *in utero* (Warner 2004).

7.3.5. The prognosis of transient infant wheeze

The absence of BAL epithelial shedding in infected wheezers suggests that airway remodelling is not taking place in these children. This would imply that children with this type of lower respiratory tract wheezing have transient wheezing of infancy. Colds during the first year of life are protective against the development of asthma (Illi *et al.* 2001; Johnston and Openshaw 2001), however this was not true of repeated lower respiratory tract infections.

The discussion to date has implied that the infected wheezers in this study probably represent those with transient symptoms or those who are not remodelling their airways. However, our data shows that metalloproteinase excess, with raised molar ratios, was present amongst infected wheezers. Such raised molar ratios are also seen

in infection and severe inflammation and they imply that proteolysis, with the possibility that airway damage does occur in infected wheezers.

Studies of pulmonary function have shown that if airway damage occurs during transient wheezing, it is less obvious than the clear changes to lung function observed amongst persistent wheezers. For instance seven years old transient wheezers had low to normal lung function (Gern *et al.* 2002), whereas persistent wheezing was associated with changes that persist into adulthood (Grol *et al.* 1999).

Long term follow up of transient infant wheezers into adulthood have shown an impact on adult lung function that could not have been foretold by the childhood cohorts. Transient preschool wheezers, now in their mid-thirties, had reductions in FEV1 not seen in age matched controls (Strachan *et al.* 1996). Also there was a rapid decline in lung function of transient infant wheezers in middle age, which may herald the onset of obstructive airways disease (Edwards *et al.* 2003). Such changes are not seen in all studies (Oswald *et al.* 1997), and may be due to difficulties with initial case definitions and symptom recall.

Matrix metalloproteinase imbalance during transient preschool wheezing episodes may have an effect upon pulmonary development that is only seen much later in adulthood. Today's happy wheezer may be tomorrow's chronic bronchitic.

7.3.6. Reconsidering the allergic march

Current understanding of atopic disease in preschool children suggests that allergic rhinitis (AR) is not a major problem compared to asthma and eczema. Our study showed that, even when symptoms are present, they are light and do not interrupt the child's daily life. The paradigm of the allergic march would also suggest that for the majority of children AR occurs after the establishment of persistent wheeze. Allergic rhinitis is recognised in preschool children but little work has been performed on it as a separate entity and it remains largely under diagnosed (Peroni *et al.* 2003). This is probably because AR is a difficult diagnosis to make in a preschool child due to the frequency of intercurrent URTI. However, our follow up questionnaire showed

that parents are able to recognise symptoms of rhinitis in their children even if their physicians fail to take note.

7.3.7. A mechanism for the one airway hypothesis

The role of allergic rhinitis in persistent wheezing may provide an insight into the mechanisms of the one airway hypothesis. If the nose is the gateway to the lung then allergic rhinitis may leave the door open to the lower respiratory tract. Environmental allergens are not the only factors in the development of asthma (Pearce *et al.* 2000), and normal bronchial epithelium has been shown to have an inflammatory response to inhaled agents (Davies 2001). Access of allergen to the lower respiratory tract may be the trigger for the development of lower airway eosinophillic inflammation and persistent wheezing in predisposed individuals. Nasal symptoms and / or allergic rhinitis may be crucially involved in this process.

Several findings from our study are of relevance to the following discussion. The development of overt nasal symptoms at follow up was associated with persistent wheezing. Allergic rhinitis in infancy correlated with bronchoalveolar changes associated with epithelial shedding and airway remodelling (BAL epithelial cells, ECP and TIMP-1). Also the same pattern of correlations between BAL cells and markers in atopic wheezers were also seen in controls, which suggested that the atopic lower airway was producing a normal response to an abnormal stimulus.

Only half of those children with AR at bronchoscopy had current sneezing symptoms at follow up, but all of them continued to wheeze. The difficulties of diagnosing rhinitis in infancy have already been discussed. However, the definition of AR used in this study ensured that these children had two things in common, allergic sensitisation and or the presence of watery rhinorrhoea. Both of these may be important in the development of future asthma. The Early Treatment of the Atopic Child (ETAC) study (ETAC Study group 1998) showed that long-term administration of antihistamines to children with aeroallergen sensitivity reduced the later development of wheezy symptoms. This was the first evidence of any pharmaceutical intervention acting as primary prevention for asthma. Antihistamines such as cetirizine have been shown to reduce nasal inflammation and the recruitment

of inflammatory cells to the airway (Nelson 2003). This is probably due to the down regulation of ICAM-1 on the nasal mucosal epithelium (Fasce *et al.* 1996). The anti-inflammatory action of cetirizine on the nasal mucosa would also have reduced rhinorrhoea (Ciprandi *et al.* 1997) and it may be this effect which modulated lower respiratory tract disease.

Rhinorrhoea may be important to bronchial sensitisation because aeroallergens can dissolve in these watery nasal secretions decreasing their particle size and permitting entry to the lung. Most aeroallergens are of a particle size that prevents good deposition in the airways. An example is grass pollen whose diameter of around 10 μm does not permit access to the lower respiratory tract and the majority of particles are trapped in nasal mucus (Suphioglu *et al.* 1992). Evidence for the improved bronchial access of soluble allergen comes from a surprising source.

Epidemics of acute exacerbations of asthma are seen following thunderstorms during periods of high grass pollen counts (Knox 1993; Packe and Ayres 1985). It is often those with grass pollen allergy who are most affected. The author has personally experienced this phenomenon becoming acutely wheezy during a summer downpour in London (Celenza *et al.* 1996). He has not wheezed either before or since the event and does not have allergic rhinitis, which personally demonstrates the response of normal epithelium to such inhaled allergen.

It has been suggested that pollen particles dissolve in rainwater during these downpours, reducing their particle size dramatically, which in turn permits entry to the lower airway (Bousquet *et al.* 2003; Suphioglu *et al.* 1992). Rye grass pollen has been shown to dissolve into much smaller particles of less than five microns under certain conditions (Taylor *et al.* 2002). Each mini-particle has both starch and *Lol p1* allergen present and at this particle size could easily penetrate the lower airways.

In a similar manner aeroallergens may dissolve in the aqueous upper respiratory tract secretions of allergic rhinitis increasing their access to the lower airway, which over time may lead to bronchial sensitisation. Although this hypothesis remains purely speculative, evidence from sensitised mice suggest that nose and lung are linked in this way (Hellings *et al.* 2001).

This theory of nasal allergen solubility provides a potential mechanism for the one airway hypothesis. It also explains the results of the ETAC study (ETAC Study group 1998; Warner 2001) in that modulation of nasal symptoms, including the drying up of secretions by cetirizine reduced access of soluble allergen to the lung, preventing LRT sensitisation and slowing the onset of asthma. Several studies in adults have shown that antihistamines improve allergic asthma in adults with AR (Walsh 2002).

7.4. Unanswered questions and future research

This section considers the direction of future research based upon the findings of this study.

7.4.1. The role of bacteria in infant wheeze

The role of bacterial infection in infant wheeze clearly requires further investigation. Although most studies of severe bronchoscoped infant wheezers showed bacterial infection, it is impossible to say whether infection is implicated in less clinically severe infant wheezing. It is unfortunate that the only study to date that could have answered this question did not report the results of BAL culture (Stevenson *et al.* 1997). Further studies on children attending day case procedures are required to consider the role of infection in preschool wheeze. They may show that the anecdotal primary care treatment of wheeze with antibiotics may not be as heretical as one would at first imagine (Gaston 2002). There may yet be some mileage in the concept of bacterial bronchitis in preschool wheeze.

Although several placebo controlled trials have discredited the use of antibiotics for adult coughs (O'Brien *et al.* 1998), no such data exist for treatment of viral induced wheezers. In fact the failure of corticosteroids to abort attacks of wheezing (Oommen *et al.* 2003a), when instituted early in the disease process, is intriguing, and trials of the use of antibiotics in a similar manner (at the onset of first symptoms in acute infant wheeze) are required. Other studies using antibiotic prophylaxis may also be rewarding, it is surprising how many children with chest problems and minor immunodeficiencies improve on prophylaxis although the evidence is purely anecdotal. When considering prophylaxis against infection, the effect of pneumococcal conjugate vaccines on infant wheezing has also not been considered.

7.4.2. Bronchial lavage studies of infant wheeze

Our study has demonstrated that bronchial washings provide a cellular and inflammatory profile very similar to that of BAL. Epithelial cells were higher in BW compared to BAL that makes investigation of a disorder involving epithelial

shedding, like asthma, easier to investigate as differences between groups may be magnified. The findings in BW are similar to those reported in non-bronchoscopic lavage (NBL) (Stevenson *et al.* 1997) and our study provides further validation for this blind technique. NBL has advantages over the bronchoscopic procedure in that it is quicker and perceived as less invasive (Shields and Riedler 2000). In this respect a range of children with infant wheeze can be investigated, not just those with severe or atypical disease. Investigation still relies on the proviso that they are having a general anaesthetic for another procedure. However, this method has been used successfully by the Belfast group (Ennis *et al.* 1999; Fitch *et al.* 2000; Heaney *et al.* 1996; Shields *et al.* 1999; Stevenson *et al.* 1997), and should be recommended for further studies in infant wheeze.

7.4.3. The role of endobronchial biopsy

The true place of airway remodelling in infant wheeze will only be elucidated by studies of endobronchial biopsy (EBB) specimens. This is because airway remodelling is a histopathological diagnosis. Although other markers may also predict asthma, only studies that validate their markers against the benchmark of tissue diagnosis will provide proof of structural airway changes. This is problematic because of the relative inexperience with the use of EBB in children and the ethics of performing invasive procedures on otherwise healthy children, as well as the controversies over the storage and use of human tissue for research purposes.

Two recent studies have reported that EBB in pre-school children is safe (Saglani *et al.* 2003; Salva *et al.* 2003). Saglani *et al* compared the complication rate with a group of children undergoing bronchoscopy and BAL alone (Saglani *et al.* 2003). They found that of 66 preschool children, complication rates were the same between the two groups, although they were not randomised to these groups, which detracts to a degree from their findings. Salva *et al* (Salva *et al.* 2003) considered the safety of EBB in 170 children of whom 29 were less than five years old. In both of these studies, no child had active bleeding that required treatment with topical adrenaline, or developed a pneumothorax. The endobronchial biopsies were of sufficient quality to allow assessment of airway remodelling. These studies support the earlier report of its safety in older asthmatic children (Payne *et al.* 2001). Still more recently,

endobronchial biopsy has been used to examine the differences between persistent and intermittently symptomatic school children with steroid resistant asthma (de Blic *et al.* 2004). They have shown that eosinophillic and neutrophillic inflammation were associated with persistent symptoms amongst these children. Changes to basement membrane thickness were not seen between groups suggesting that remodelling had occurred in all children.

Coeliac disease provides a precedent for the role of biopsy specimens in the diagnosis and treatment of an organ specific inflammatory condition. The typical appearances of jejunal biopsy specimens remain the gold standard for diagnosis of this condition (Abdulkarim and Murray 2003). Biopsies are taken under sedation by Crosby capsule and radiologic screening, or more recently by endoscopy under general anaesthetic. The diagnosis of coeliac disease is important because it leads to lifelong treatment which, as well as providing symptomatic relief, reduces the incidence of gut neoplasia providing both primary and secondary prevention.

In a similar way the use of EBB in preschool wheeze should play an important part in understanding the role of airway remodelling in this condition. Until validated surrogate markers of airway remodelling are available, EBB should become a routine part of the research assessment of the infant wheezer. This would enable histological categorisation of a heterogeneous disorder and elucidation of their different responses to treatment. It may even enable secondary prevention, with disease modifying treatment given prior to the onset of airway remodelling. In order to do this, researchers will need high quality and targeted studies as well as the faith of their research ethics committees. Progress will be taken in small steps, as has been shown by the recent studies on EBB safety and their use in older asthmatic children (de Blic *et al.* 2004; Payne *et al.* 2001; Saglani *et al.* 2003; Salva *et al.* 2003).

7.4.4. Allergic rhinitis and infant wheeze

The role of allergic rhinitis in infancy is a relatively unexplored area as most physicians consider that it doesn't exist. The intriguing findings of our study, which suggest that nasal symptoms predict lower airway changes associated with remodelling in infancy, indicate that careful investigation of this area is required.

Comparisons of nasal lavage findings in infants with aeroallergen sensitivity compared to a non-sensitised but aged matched controls seems a good place to start. Consideration of the role of aqueous rhinorrhoea on allergen particle size should also be conducted, as should the role of respiratory mucins in pollen entrapment and clearance.

7.4.5. Further follow up

It seems obvious to suggest that further follow up of this sample would be useful but there are several reasons to think that this is so. Detailed information on lower respiratory tract inflammation in infants is very hard to obtain and the maximum amount of information should be collected on this precious resource. Follow up in mid-childhood would ensure that all those children with transient infant wheeze would have out grown their symptoms by this time and would give a clearer picture of the relevance of the infantile inflammatory process in this disease. The employment of follow up on the subject's birthday would dispose of the need to account for age in the analysis, which would be useful with such a small sample. A repeat age appropriate ISAAC questionnaire would also provide a direct comparison, not only with this study but also with the wider population as already mentioned. Questions on exposure to tobacco smoke in infancy and *in utero* would make up for deficiencies in this current study. Skin prick testing to common inhalant allergens and a clinical examination would also be useful in categorising children into their eventual wheeze phenotypes.

7.5. Conclusions

Wheezers with bacterial infections were a common occurrence in this small study and are probably more commonplace than has been previously recognised. They do not show signs of inflammation associated with epithelial shedding and have nasal and bronchial neutrophillia in keeping with viral associated wheeze. They also have raised levels of BAL ECP but no eosinophils, possibly associated with an inflammatory response to infection. The suggestion from this data is that infected wheezers represent transient infant wheeze phenotype that is not actively remodelling the airway. The MMP:TIMP imbalance in infected wheezers appears to be an acute response to infection. However, this may still cause damage to the developing lung.

Atopic wheezers did have a different pattern of inflammation in BAL although this was more subtle than expected and was largely inferred from correlations. The hypothesis that the presence of eosinophillic inflammation in the airway predicted the persistence of symptoms has not been confirmed in infant wheeze. However, our study suggests that eosinophils are present in those with allergic rhinitis or aeroallergen sensitivity but were not significantly different in other atopic wheezers. Amongst atopics, epithelial cell shedding was associated with inflammation. Atopic wheezers did not have a significant imbalance in MMP:TIMP. TIMP-1 was elevated amongst atopics although this was significant only in comparison with infected children. This is similar to adult atopics asthmatics and those with allergic rhinitis. TIMP-1 correlated with epithelial shedding in atopic wheezers and was also associated with persistence of wheeze at follow up. This suggests that raised BAL TIMP-1 may be important in atopic inflammation in infant wheeze and by definition, the remodelling process.

Idiopathic wheezers represent an indeterminate group. They do not have signs of inflammation in BAL. They may represent the non-atopic infected wheezers between acute infections. In this case it is interesting to note that they still had significant MMP-9:TIMP-1 imbalance compared to controls suggesting that inflammation is ongoing between acute episodes.

This is the first study to compare upper airway with lower respiratory tract lavage in preschool children. Our hypothesis was that upper respiratory tract lavage would mirror lower airway lavage. It is unfortunate that there was not a direct relationship between cells and markers in NAL and BAL, as access to the nose is much easier than the alveolar spaces. Nevertheless, changes in nasal lavage provided insight into lower respiratory tract disease. The most obvious signs of this were amongst infected children where neutrophils were present in both lavages. The presence of lymphocytes and ECP in NAL directly related to findings in BAL. Wheezers with allergic rhinitis also showed signs of atopic lower respiratory tract inflammation associated with airway damage. This links upper and lower airway inflammation in infancy. At follow up, nasal symptoms were present in persistent wheezers. Nasal disease was associated with development of persistent wheeze, as seen in adults with allergic rhinitis.

Follow up also implicated serum sICAM-1 and BAL TIMP-1 with persistence of wheezing. That any markers were related to persistence of wheezing indicates that inflammation leading to lung damage starts early in life. Raised TIMP-1 indicates that airway remodelling may be taking place, whilst raised serum sICAM-1 suggests airway inflammation.

The implications of this study are that airway inflammation leading to remodelling occurs at the onset of disease in infant wheezers and heralds the persistence of wheezing in childhood. This process is linked to allergic rhinitis and aeroallergen sensitisation and implies that the process of development of asthma in childhood may not be all that different from that observed in adults. Amongst transient infant wheezers, bacterial lower respiratory tract infections may play a greater role than has been thought and the diagnosis of wheezy bronchitis may yet see a renaissance.

MDS Erlewyn-Lajeunesse
Bristol, March 2004

Chapter 8: Appendices

8.1. ISAAC study questionnaire (English)

Questionnaire follow up of wheezing infants.

All questions require you to tick your answer in a box. If you make a mistake put a cross in the box and tick the correct answer. Tick only one option unless otherwise instructed.

8.1.1. Module 1

Has your child ever had wheezing or whistling in the chest at any time in the past?

Yes ☐ No ☐ If you answered NO please skip to question 6.

Has your child had wheezing or whistling in the chest in the last 12 months?

Yes ☐ No ☐ If you answered NO please skip to question 6.

How many attacks of wheezing has your child had in the last 12 months?

None ☐ 1 to 3 ☐ 4 to 12 ☐ More than 12 ☐

In the last 12 months, how often, on average, has your child's sleep beeen disturbed due to wheezing?

Never woken with wheezing ☐ Less than one night per week ☐ One or more nights per week ☐

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?

Yes ☐ No ☐

Has your child ever had asthma?

Yes ☐ No ☐

In the last 12 months, has your child's chest sounded wheezy during or after exercise?

Yes ☐ No ☐

In the last 12 months, has your child had a dry cough at night, part from a cough associated with a cold or chest infection?

Yes ☐ No ☐

8.1.2. Module 2

Has your 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?

Yes ☐ No ☐ If you answered NO please skip to question 14

In the past 12 months, has your 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?

Yes ☐ No ☐ If you answered NO please skip to question 14

In the past 12 months, has this nose problem been accompanied by itchy-watery eyes?

Yes ☐ No ☐

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 ☐

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 ☐

Has your child ever had hay fever?

Yes ☐ No ☐

8.1.3. Module 3

Has your child ever had an itchy rash which was coming and going for at least 6 months?

Yes ☐ No ☐ If you answered NO please skip to question 21

Has your child had this itchy rash at any time in the last 12 months?

Yes ☐ No ☐ If you answered NO please skip to question 21

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?

Yes ☐ No ☐

At what age did this itchy rash first occur?

Under 2 years ☐ Age 2 to 4 ☐ Age 5 or more ☐

Has this rash cleared completely at any time during the last 12 months?

Yes ☐ No ☐

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 ☐

Has your child ever had eczema?

Yes ☐ No ☐

Thank you for your participation.

Please return the questionnaire in the pre-paid envelope provided to:

Dr Mich Erlewyn-Lajeunesse, BAL Follow Up Study,
University Department of Child Health, Mailpoint 803,
Southampton General Hospital, Southampton SO16 6YD, United Kingdom

Version 2 dated 18th July, 2002

8.2. ISAAC study questionnaire (Czech)

Dotazníkové sledování sípajících dětí. U všech otázek je třeba, abyste svou odpověď vyznačili zaškrtnutím čtverečku. Uděláte-li chybu, přeškrtněte čtvereček křížem a vyznačte správnou odpověď. Není-li jiný pokyn, vyznačte pouze jednu z možností.

8.2.1. Modul 1

Mělo Vaše dítě vůbec někdy v minulosti sípání nebo pískání v hrudníku?

Ano ☐ Ne ☐ Pokud jste odpověděli NE, přeskočte prosím k otázce 6.

Mělo Vaše dítě za posledních 12 měsíců sípání nebo pískání v hrudníku?

Ano ☐ Ne ☐ Pokud jste odpověděli NE, přeskočte prosím k otázce 6.

Kolik návalů sípání mělo Vaše dítě za posledních 12 měsíců?

Žádný ☐ 1 až 3 ☐ 4 až 12 ☐ Více než 12 ☐

Jak často byl za posledních 12 měsíců přerušen spánek Vašeho dítěte v důsledku sípání?

Sípání ho nikdy neprobudilo ☐ Méně než jednu noc za týden ☐ Jednu nebo více nocí za týden ☐

Stalo se za posledních 12 měsíců, aby bylo sípání tak nepříjemné, až by omezilo hovor Vašeho dítěte jen na jedno slovo nebo dvě v mezidobí mezi nádechy?

Ano ☐ Ne ☐

Mělo Vaše dítě vůbec někdy astma?

Ano ☐ Ne ☐

Stalo se za posledních 12 měsíců, aby hrudník Vašeho dítěte vydával sípavé zvuky při cvičení nebo po něm?

Ano ☐ Ne ☐

Stalo se za posledních 12 měsíců, aby Vaše dítě přepadl v noci suchý kašel, kromě kašle spojeného s nachlazením nebo hrudníkovou infekcí?

Ano ☐ Ne ☐
Číslo studie BAL ☐☐☐

Dotazníkové sledování sípajících dětí.

8.2.2. Modul 2

Mělo Vaše dítě <u>vůbec někdy</u> problém s kýcháním, s tečením z nosu nebo s ucpaným nosem, když NEBYLO nachlazeno, nebo nemělo chřipku?

Ano ☐ Ne ☐ Pokud jste dopověděli NE, prosím přeskočte k otázce 14

Mělo Vaše dítě <u>za posledních 12 měsíců</u> problém s kýcháním, s tečením z nosu nebo s ucpaným nosem, když NEBYLO nachlazeno, nebo nemělo chřipku?

Ano ☐ Ne ☐ Pokud jste dopověděli NE, prosím přeskočte k otázce 14

Byl <u>za uplynulých 12 měsíců</u> tento problém s nosem doprovázen svěděním a slzením očí?

Ano ☐ Ne ☐

Ve kterém <u>z uplynulých 12 měsíců</u> se objevil tento problém s nosem?
(prosím zaškrtněte všechny, kterých se to týká)

leden ☐ únor ☐ březen ☐ duben ☐ květen ☐ červen ☐
červenec ☐ srpen ☐ září ☐ říjen ☐ listopad ☐ prosinec ☐

Jak silně překážel <u>za uplynulých 12 měsíců</u> tento problém s nosem každodenním aktivitám Vašeho dítěte?

Vůbec ne ☐ Trochu ☐ Mírně ☐ Hodně ☐

Mělo Vaše dítě <u>vůbec někdy</u> sennou rýmu?

Ano ☐ Ne ☐

Číslo studie BAL ☐☐☐

Dotazníkové sledování sípajících dětí.

8.2.3. Modul 3

Mělo Vaše dítě vůbec někdy svědivou vyrážku, která se objevovala a mizela alespoň po dobu 6 měsíců?

Ano ☐ Ne ☐ Pokud jste dopověděli NE, prosím přeskočte k otázce 211

Vyskytla se u Vašeho dítěte tato svědivá vyrážka kdykoli za posledních 12 měsíců?

Ano ☐ Ne ☐ Pokud jste dopověděli NE. prosím přeskočte k otázce 21

Napadla tato svědivá vyrážka kdykoli některé z těchto míst: loketní záhyby, podkolení, nárty, místa pod zadečkem, nebo kolem krku, uší nebo očí?

Ano ☐ Ne ☐

V jakém věku se tato svědivá vyrážka objevila poprvé?

Do 2 let ☐ Věk 2 až 4 ☐ Věk 5 a více ☐

Vymizela tato vyrážka úplně kdykoli za uplynulých 12 měsíců?

Ano ☐ Ne ☐

Jak často se za posledních 12 měsíců v průměru Vaše dítě v noci probudilo pro tuto svědivou vyrážku?

Za posledních 12 měsíců nikdy ☐ Méně než jednu noc za týden ☐ Jednu nebo více nocí za týden ☐

Mělo Vaše dítě někdy ekzém?

Ano ☐ Ne ☐

Děkuji Vám za účast.

Prosím vraťte dotazník v dodané vyplacené obálce na adresu:

Chapter 9: References

Abdulkarim, A. S. and Murray, J. A. (2003). Review article: The diagnosis of coeliac disease. *Alimentary Pharmacological Therapy*, 17: 987-995.

Annesi-Maesano, I. (2001). Rhinitis and asthma - epidemiological eidience. *Allergy and Clinical Immunology International*, 13: 147-153.

Arshad, S. H., Bateman, B. and Matthews, S. M. (2003). Primary prevention of asthma and atopy during childhood by allergen avoidance in infancy: a randomised controlled study. *Thorax*, 58: 489-493.

Arshad, S. H., Kurukulaaratchy, R. J., Fenn, M., Waterhouse, L. and Matthews, S. (2002). Rhinitis in 10-year-old children and early life risk factors for its development. *Acta Paediatrica*, 91: 1334-1338.

Asher, M. I., Keil, U., Anderson, H. R., Beasley, R., Crane, J., Martinez, F., Mitchell, E. A., Pearce, N., Sibbald, B. and Stewart, A. W. (1995). International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *European Respiratory Journal*, 8: 483-491.

Asher, M. I. and Weiland, S. K. (1998). The International Study of Asthma and Allergies in Childhood (ISAAC). ISAAC Steering Committee. *Clinical Experimental Allergy*, 28 Suppl 5: 52-66.

Atkinson, J. J. and Senior, R. M. (2003). Matrix metalloproteinase-9 in lung remodeling. *American Journal of Cellular & Molecular Biology*, 28: 12-24.

Azevedo, I., de Blic, J., Vargaftig, B. B., Bachelet, M. and Scheinmann, P. (2001). Increased eosinophil cationic protein levels in bronchoalveolar lavage from wheezy infants. *Pediatric Allergy & Immunology*, 12: 65-72.

Balfour-Lynn, I. M., Valman, B., Silverman, M. and Webster, A. D. (1993). Nasal IgA response in wheezy infants. *Archives of Disease in Childhood*, 68: 472-476.

Barbato, A., Panizzolo, C., Gheno, M., Sainati, L., Favero, E., Faggian, D., Giusti, F., Pesscolderungg, L. and La Rosa, M. (2001). Bronchoalveolar lavage in asthmatic children: evidence of neutrophil activation in mild-to-moderate persistent asthma. *Pediatric Allergy & Immunology*, 12: 73-77.

Barbato, A., Turato, G., Baraldo, S., Bazzan, E., Calabrese, F., Tura, M., Zuin, R., Beghe, B., Maestrelli, P., Fabbri, L. M. and Saetta, M. (2003). Airway Inflammation in Childhood Asthma. *American Journal of Respiratory and Critical Care Medicine*, 168: 798-803.

Barker, D. J. P. (1992) *Fetal and infant origins of adult disease.* Bristish Medical Journal Publishing Ltd., London.

Baughman, R., Strohofer, S. and Kim, C. K. (1986). Variation of differential cell counts of bronchoalveolar lavage fluid. *Archives of Pathological Laboratory Medicine*, 110: 341-343.

Becky-Kelly, E. A., Busse, W. W. and Jarjour, N. N. (2000). Increased matrix metalloproteinase-9 in the airway after allergen challenge. *American Journal of Respiratory & Critical Care Medicine*, 162: 1157-1161.

Beeh, K. M., Beier, J., Kornmann, O., Meier, C., Taeumer, T. and Buhl, R. (2003). A single nasal allergen challenge increases induced sputum inflammatory markers in non-asthmatic subjects with seasonal allergic rhinitis: correlation with plasma interleukin-5. *Clinical Experimental Allergy*, 33: 475-482.

Belleguic, C., Corbel, M., Germain, N., Lena, H., Boichot, E., Delaval, P. H. and Lagente, V. (2002). Increased release of matrix metalloproteinase-9 in the plasma of acute severe asthmatic patients. *Clinical Experimental Allergy*, 32: 217-223.

Bisgaard, H. (2002). Efficacy of steroid treatments in the asthmatic preschool child. *Allergy*, 57 Suppl 74: 32-41.

Bisgaard, H. (2003). A randomized trial of montelukast in respiratory syncytial virus postbronchiolitis. *American Journal of Respiratory & Critical Care Medicine*, 167: 379-383.

Bland, J. M. and Altman, D. G. (1986). Statistical method for assessing agreement between two methods of clinical measurement. *Lancet,* i: 307-310.

Bland, M. (2000) *An introduction to medical statistics*. Oxford University Press, Oxford.

Bont, L., Heijnen, C. J., Kavelaars, A., van Aalderen, W. M., Brus, F., Draaisma, J. T., Geelen, S. M. and Kimpen, J. L. (2000). Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one-year follow-up study. *American Journal of Respiratory & Critical Care Medicine*, 161: 1518-1523.

Bosse, M., Chakir, J., Rouabhia, M., Boulet, L. P., Audette, M. and Laviolette, M. (1999). Serum matrix metalloproteinase-9:Tissue inhibitor of metalloproteinase-1 ratio correlates with steroid responsiveness in moderate to severe asthma. *American Journal of Respiratory & Critical Care Medicine*, 159: 596-602.

Bouchier, I. A. D. and Morris, J. S. (1982) *Clinical Skills: A System of Clinical Examination*. W.B.Saunders Company Ltd., London.

Boulet, L. P., Turcotte, H. and Brochu, A. (1994). Persistence of airway obstruction and hyperresponsiveness in subjects with asthma remission. *Chest*, 105: 1024-1031.

Bousquet, J., Jacquot, W., Vignola, A. M., Bachert, C. and Van Cauwenberge, P. (2004). Allergic rhinitis: a disease remodeling the upper airways? *Journal of Allergy & Clinical Immunology*, 113: 43-49.

Bousquet, J., Jeffery, P. K., Busse, W. W., Johnson, M. and Vignola, A. M. (2000). Asthma: from bronchoconstriction to airways inflammation and remodeling. *American Journal of Respiratory & Critical Care Medicine*, 161: 1720-1745.

Bousquet, J., Van Cauwenberge, P. and Khaltaev, N. (2001). Allergic rhinitis and its impact on asthma. *Journal of Allergy & Clinical Immunology*, 108: S147-S334.

Bousquet, J., Vignola, A. M. and Demoly, P. (2003). Links between rhinitis and asthma. *Allergy*, 58: 691-706.

Braun-Fahrlander, C., Gassner, M., Grize, L., Minder, C. E., Varonier, H. S., Vuille, J. C., Wuthrich, B. and Sennhauser, F. H. (1998). Comparison of responses to an asthma symptom questionnaire (ISAAC core questions) completed by adolescents and their parents. SCARPOL-Team. Swiss Study on Childhood Allergy and Respiratory Symptoms with respect to Air Pollution. *Pediatric Pulmonology*, 25: 159-166.

Braunstahl, G. J., Fokkens, W. J., Overbeek, S. E., KleinJan, A., Hoogsteden, H. C. and Prins, J. B. (2003). Mucosal and systemic inflammatory changes in allergic rhinitis and asthma: a comparison between upper and lower airways. *Clinical Experimental Allergy*, 33: 579-587.

Braunstahl, G. J., KleinJan, A., Overbeek, S. E., Prins, J. B., Hoogsteden, H. C. and Fokkens, W. J. (2000). Segmental bronchial provocation induces nasal inflammation in allergic rhinitis patients. *American Journal of Respiratory & Critical Care Medicine*, 161: 2051-2057.

Braunstahl, G. J., Overbeek, S. E., Fokkens, W. J., KleinJan, A., McEuen, A. R., Walls, A. F., Hoogsteden, H. C. and Prins, J. B. (2001a). Segmental bronchoprovocation in allergic rhinitis patients affects mast cell and basophil numbers in nasal and bronchial mucosa. *American Journal of Respiratory & Critical Care Medicine*, 164: 858-865.

Braunstahl, G. J., Overbeek, S. E., KleinJan, A., Prins, J. B., Hoogsteden, H. C. and Fokkens, W. J. (2001b). Nasal allergen provocation induces adhesion molecule expression and tissue eosinophilia in upper and lower airways. *Journal of Allergy & Clinical Immunology*, 107: 469-476.

Brew, K., Dinakarpandian, D. and Nagase, H. (2000). Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochemical Biophysical Acta*, 1477: 267-283.

Burr, M. L., Butland, B. K., King, S. and Vaughan-Williams, E. (1989). Changes in asthma prevalence: two surveys 15 years apart. *Archives of Disease in Childhood*, 64: 1452-1456.

Bush, A. (2000). Nosing out the future asthmatic? *Allergy*, 55: 685-687.

Busse, W. W. and Lemanske, R. F., Jr. (2001). Asthma. *New England Journal of Medicine*, 344: 350-362.

Bystrom, J., Garcia, R. C., Hakansson, L., Karawajczyk, M., Moberg, L., Soukka, J. and Venge, P. (2002). Eosinophil cationic protein is stored in, but not produced by, peripheral blood neutrophils. *Clinical Experimental Allergy*, 32: 1082-1091.

Cane, R. S. and McKenzie, S. A. (2001). Parents' interpretations of children's respiratory symptoms on video. *Archives of Disease in Childhood*, 84: 31-34.

Cane, R. S., Ranganathan, S. C. and McKenzie, S. A. (2000). What do parents of wheezy children understand by "wheeze"? *Archives of Disease in Childhood*, 82: 327-332.

Carlsen, K. H. (1997). What distinguishes the asthmatic amongst the infant wheezers? *Pediatric Allergy & Immunology*, 8: 40-45.

Castro-Rodriguez, J. A., Holberg, C. J., Wright, A. L. and Martinez, F. D. (2000). A clinical index to define risk of asthma in young children with recurrent

wheezing. *American Journal of Respiratory & Critical Care Medicine*, 162: 1403-1406.

Cataldo, D., Munaut, C., Noel, A., Frankenne, F., Bartsch, P., Foidart, J. M. and Louis, R. (2001). Matrix metalloproteinases and TIMP-1 production by peripheral blood granulocytes from COPD patients and asthmatics. *Allergy*, 56: 145-151.

Cataldo, D. D., Bettiol, J., Noel, A., Bartsch, P., Foidart, J. M. and Louis, R. (2002a). Matrix metalloproteinase-9, but not tissue inhibitor of matrix metalloproteinase-1, increases in the sputum from allergic asthmatic patients after allergen challenge. *Chest*, 122: 1553-1559.

Cataldo, D. D., Tournoy, K. G., Vermaelen, K., Munaut, C., Foidart, J. M., Louis, R., Noel, A. and Pauwels, R. A. (2002b). Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *American Journal of Pathology*, 161: 491-498.

Celedon, J. C., Litonjua, A. A., Ryan, L., Platts-Mills, T., Weiss, S. T. and Gold, D. R. (2002). Exposure to cat allergen, maternal history of asthma, and wheezing in first 5 years of life. *Lancet*, 360: 781-782.

Celedon, J. C., Wright, R. J., Litonjua, A. A., Sredl, D., Ryan, L., Weiss, S. T. and Gold, D. R. (2003). Day care attendance in early life, maternal history of asthma, and asthma at the age of 6 years. *American Journal of Respiratory & Critical Care Medicine*, 167: 1239-1243.

Celenza, A., Fothergill, J., Kupek, E. and Shaw, R. J. (1996). Thunderstorm associated asthma: a detailed analysis of environmental factors. *Bristish Medical Journal*, 312: 604-607.

Chakir, J., Laviolette, M., Turcotte, H., Boutet, M. and Boulet, L. P. (2000). Cytokine expression in the lower airways of nonasthmatic subjects with

allergic rhinitis: influence of natural allergen exposure. *Journal of Allergy & Clinical Immunology*, 106: 904-910.

Chanez, P., Vignola, A. M., Vic, P., Guddo, F., Bonsignore, G., Godard, P. and Bousquet, J. (1999). Comparison between nasal and bronchial inflammation in asthmatic and control subjects. *American Journal of Respiratory & Critical Care Medicine*, 159: 588-595.

Chiappara, G., Gagliardo, R., Siena, A., Bonsignore, M. R., Bousquet, J., Bonsignore, G. and Vignola, A. M. (2001). Airway remodelling in the pathogenesis of asthma. *Current Opinion in Allergy & Clinical Immunology*, 1: 85-93.

Christie, G. L., Helms, P. J., Godden, D. J., Ross, S. J., Friend, J. A., Legge, J. S., Haites, N. E. and Douglas, J. G. (1999). Asthma, wheezy bronchitis, and atopy across two generations. *American Journal of Respiratory & Critical Care Medicine*, 159: 125-129.

Christie, G. L., Helms, P. J., Ross, S. J., Godden, D. J., Friend, J. A., Legge, J. S., Haites, N. E. and Douglas, J. G. (1997). Outcome of children of parents with atopic asthma and transient childhood wheezy bronchitis. *Thorax*, 52: 953-957.

Ciprandi, G., Tosca, M., Ricca, V., Passalacqua, G., Riccio, A. M., Bagnasco, M. and Canonica, G. W. (1997). Cetirizine treatment of rhinitis in children with pollen allergy: evidence of its antiallergic activity. *Clinical Experimental Allergy*, 27: 1160-1166.

Clement, A. Chadelat, K., Massliah, J., Housset, B., Sardet, A., Grimfeld, A., Tournier, G. (1987). A controlled study of oxygen metabolite release by alveolar macrophages from children with interstitial lung disease. *American Review of Respiratory Disease* 136: 1424 – 28.

Clough, J. B., Keeping, K. A., Edwards, L. C., Freeman, W. M., Warner, J. A. and Warner, J. O. (1999). Can we predict which wheezy infants will continue to wheeze? *American Journal of Respiratory & Critical Care Medicine*, 160: 1473-1480.

Cogswell, J. J., Mitchell, E. B. and Alexander, J. (1987). Parental smoking, breast feeding, and respiratory infection in development of allergic diseases. *Archives of Disease in Childhood*, 62: 338-344.

Collins, T. L., Kassner, P. D., Bierer, B. E. and Burakoff, S. J. (1994). Adhesion receptors in lymphocyte activation. *Curent Opinion in Immunology*, 6: 385-393.

Corren, J., Adinoff, A. D. and Irvin, C. G. (1992). Changes in bronchial responsiveness following nasal provocation with allergen. *Journal of Allergy & Clinical Immunology*, 89: 611-618.

Coutinho, A. and Poltorack, A. (2003). Innate immunity: from lymphocyte mitogens to Toll-like receptors and back. *Current Opinion in Immunology*, 15: 599-602.

Culley, F. J., Pollott, J. and Openshaw, P. J. (2002). Age at First Viral Infection Determines the Pattern of T Cell-mediated Disease during Reinfection in Adulthood. *Journal of Experimental Medicine*, 196: 1381-1386.

Cutz, E., Levison, H. and Cooper, D. M. (1978). Ultrastructure of airways in children with asthma. *Histopathology*, 2: 407-421.

Davies, D. E. (2001). The bronchial epithelium: translating gene and environment interactions in asthma. *Current Opinion in Allergy & Clinical Immunology*, 1: 67-71.

de Blic, J., Marchac, V. and Scheinmann, P. (2002). Complications of flexible bronchoscopy in children: prospective study of 1,328 procedures. *European Respiratory Journal*, 20: 1271-1276.

de Blic, J., Midulla, F., Barbato, A., Clement, A., Dab, I., Eber, E., Green, C., Grigg, J., Kotecha, S., Kurland, G., Pohunek, P., Ratjen, F. and Rossi, G. (2000). Bronchoalveolar lavage in children. ERS Task Force on bronchoalveolar lavage in children. European Respiratory Society. *European Respiratory Journal*, 15: 217-231.

de Blic, J., Tillie-Leblond, I., Tonnel, A. B., Jaubert, F., Scheinmann, P. and Gosset, P. (2004). Difficult asthma in children: an analysis of airway inflammation. *Journal of Allergy & Clinical Immunology*, 113: 94-100.

de Jongste, J. C., Janssens, H. M. and Van der, W. J. (2002). Effectiveness of pharmacotherapy in asthmatic preschool children. *Allergy*, 57 Suppl 74: 42-47.

de Vries, T. W., Wormmeester, L., Pinxteren-Nagler, E., Bakker, A. J. and Brand, P. L. (2000). [Routine laboratory tests unnecessary for children referred for recurrent wheezing and/or asthma]. *Nederlands Tijdschrift voor Geneeskunde*, 144: 2107-2111.

Delacourt, C., Benoist, M. R., Waernessyckle, S., Rufin, P., Brouard, J. J., de Blic, J. and Scheinmann, P. (2001). Relationship between bronchial responsiveness and clinical evolution in infants who wheeze: a four-year prospective study. *American Journal of Respiratory & Critical Care Medicine*, 164: 1382-1386.

Delacourt, C., Le Bourgeois, M., D'Ortho, M. P., Doit, C., Scheinmann, P., Navarro, J., Harf, A., Hartmann, D. J. and Lafuma, C. (1995). Imbalance between 95 kDa type IV collagenase and tissue inhibitor of metalloproteinases in sputum of patients with cystic fibrosis. *American Journal of Respiratory & Critical Care Medicine*, 152: 765-774.

Denburg, J., Sehmi, R., Saito, H., Pil-Seob, J., Inman, M. and O'Byrne, P. (2000). Systemic aspects of allergic disease: Bone marrow responses. *Journal of Allergy & Clinical Immunology*, 106: 242-246.

Djukanovic, R. and Holgate, S. T. (1999) Pathogenesis of asthma. In Djukanovic, R. and Holgate, S. T. (eds), *An atlas of asthma*. Parthenon Publishing Group, London.

Dold, S., Wjst, M., von Mutius, E., Reitmeir, P. and Stiepel, E. (1992). Genetic risk for asthma, allergic rhinitis, and atopic dermatitis. *Archives of Disease in Childhood*, 67: 1018-1022.

Domachowske, J. B., Bonville, C. A., Dyer, K. D., Easton, A. J. and Rosenberg, H. F. (2000). Pulmonary eosinophilia and production of MIP-1alpha are prominent responses to infection with pneumonia virus of mice. *Cellular Immunology*, 200: 98-104.

Duvernelle, C., Freund, V. and Frossard, N. (2003). Transforming growth factor-beta and its role in asthma. *Pulmonary Pharmacological Therapeutics*, 16: 181-196.

Edwards, C. A., Osman, L. M., Godden, D. J. and Douglas, J. G. (2003). Wheezy bronchitis in childhood: a distinct clinical entity with lifelong significance? *Chest*, 124: 18-24.

Edwards, P., Roberts, I., Clarke, M., DiGuiseppi, C., Pratap, S., Wentz, R. and Kwan, I. (2002). Increasing response rates to postal questionnaires: systematic review. *Bristish Medical Journal*, 324: 1183.

Eisenbarth, S. C., Piggott, D. A. and Bottomly, K. (2003). The master regulators of allergic inflammation: dendritic cells in Th2 sensitization. *Current Opinion in Immunology*, 15: 620-626.

Elphick, H. E., Sherlock, P., Foxall, G., Simpson, E. J., Shiell, N. A., Primhak, R. A. and Everard, M. L. (2001). Survey of respiratory sounds in infants. *Archives of Disease in Childhood*, 84: 35-39.

Ennis, M. (2003). Neutrophils in asthma pathophysiology. *Current Allergy & Asthma Reports*, 3: 159-165.

Ennis, M., Turner, G., Schock, B. C., Stevenson, E. C., Brown, V., Fitch, P. S., Heaney, L. G., Taylor, R. and Shields, M. D. (1999). Inflammatory mediators in bronchoalveolar lavage samples from children with and without asthma. *Clinical & Experimental Allergy*, 29: 362-366.

ETAC Study group (1998). Allergic factors associated with the development of asthma and the influence of cetirizine in a double-blind, randomised, placebo-controlled trial: first results of ETAC. Early Treatment of the Atopic Child. *Pediatric Allergy & Immunol*, 9: 116-124.

Fasce, L., Ciprandi, G., Pronzato, C., Cozzani, S., Tosca, M. A., Grimaldi, I. and Canonica, G. W. (1996). Cetirizine reduces ICAM-I on epithelial cells during nasal minimal persistent inflammation in asymptomatic children with mite-allergic asthma. *International Archives of Allergy & Immunology*, 109: 272-276.

Fayon, M., Just, J., Thien, H. V., Chiba, T., Pascual, L., Sandouk, G. and Grimfeld, A. (1999). Bacterial flora of the lower respiratory tract in children with bronchial asthma. *Acta Paediatrica*, 88: 1216-1222.

Ferguson, A. C., Whitelaw, M. and Brown, H. (1992). Correlation of bronchial eosinophil and mast cell activation with bronchial hyperresponsiveness in children with asthma. *Journal of Allergy & Clinical Immunology*, 90: 609-613.

Fielder, H. M., Lyons, R. A., Heaven, M., Morgan, H., Govier, P. and Hooper, M. (1999). Effect of environmental tobacco smoke on peak flow variability. *Archives of Disease in Childhood*, 80: 253-256.

Fitch, P. S., Brown, V., Schock, B. C., Taylor, R., Ennis, M. and Shields, M. D. (2000). Chronic cough in children: bronchoalveolar lavage findings. *European Respiratory Journal*, 16: 1109-1114.

Foresi, A., Bertorelli, G., Pesci, A., Chetta, A. and Olivieri, D. (1990). Inflammatory markers in bronchoalveolar lavage and in bronchial biopsy in asthma during remission. *Chest*, 98: 528-535.

Frey, U. (2001). Why are infants prone to wheeze? Physiological aspects of wheezing disorders in infants. *Swiss Medical Weekly*, 131: 400-406.

Frischer, T. and Baraldi, E. (2000). Upper airway sampling. *American Journal of Respiratory & Critical Care Medicine*, 162: S28-S30.

Frischer, T., Halmerbauer, G., Gartner, C., Rath, R., Tauber, E., Schierl, M., Koller, D. Y., Urbanek, R., Forster, J. and Kuhr, J. (2000). Eosinophil-derived proteins in nasal lavage fluid of neonates of allergic parents and the development of respiratory symptoms during the first 6 months of life. Collaborative SPACE team. Study on the Prevention of Allergy in Children in Europe. *Allergy*, 55: 773-777.

Garofalo, R., Dorris, A., Ahlstedt, S. and Welliver, R. C. (1994). Peripheral blood eosinophil counts and eosinophil cationic protein content of respiratory secretions in bronchiolitis: relationship to severity of disease. *Pediatric Allergy & Immunology*, 5: 111-117.

Gaston, B. (2002). Rethinking doctrine: Bronchitis, eosinophils, and bronchoscopy in pediatric asthma. *Journal of Allergy & Clinical Immunology*, 110: 24-25.

Gern, J. E., Martin, M. S., Anklam, K. A., Shen, K., Roberg, K. A., Carlson-Dakes, K. T., Adler, K., Gilbertson-White, S., Hamilton, R., Shult, P. A., Kirk, C. J., Da Silva, D. F., Sund, S. A., Kosorok, M. R. and Lemanske, R. F., Jr. (2002). Relationships among specific viral pathogens, virus-induced interleukin-8, and respiratory symptoms in infancy. *Pediatric Allergy & Immunology*, 13: 386-393.

Gibson, P. G., Wark, P. A., Simpson, J. L., Meldrum, C., Meldrum, S., Saltos, N. and Boyle, M. (2003). Induced sputum IL-8 gene expression, neutrophil

influx and MMP-9 in allergic bronchopulmonary aspergillosis. *European Respiratory Journal*, 21: 582-588.

Godfrey, S., Avital, A., Maayan, C., Rotschild, M. and Springer, C. (1997). Yield from flexible bronchoscopy in children. *Pediatric Pulmonology*, 23: 261-269.

Goldberg, G. I., Strongin, A., Collier, I. E., Genrich, L. T. and Marmer, B. L. (1992). Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *Journal of Biological Chemistry*, 267: 4583-4591.

Greiff, L., Andersson, M., Andersson, E., Linden, M., Myint, S., Svensson, C. and Persson, C. G. (1999). Experimental common cold increases mucosal output of eotaxin in atopic individuals. *Allergy*, 54: 1204-1208.

Grimfeld, A. (1997). Bronchoscopy and bronchoalveolar lavage in childhood asthma. When and what for? *Pediatric Pulmonology*, Suppl 16: 92-93.

Grol, M. H., Gerritsen, J., Vonk, J. M., Schouten, J. P., Koeter, G. H., Rijcken, B. and Postma, D. S. (1999). Risk factors for growth and decline of lung function in asthmatic individuals up to age 42 years. A 30-year follow-up study. *American Journal of Respiratory & Critical Care Medicine*, 160: 1830-1837.

Grossman, J. (1997). One airway, one disease. *Chest*, 111: 11S-16S.

Halmerbauer, G., Gartner, C., Koller, D., Schierl, M., Kuhr, J., Forster, J., Urbanek, R. and Frischer, T. (2000). Eosinophil cationic protein and eosinophil protein X in the nasal lavage of children during the first 4 weeks of life. SPACE Collaborative Study Team. *Allergy*, 55: 1121-1126.

Hartog, C. M., Wermelt, J. A., Sommerfeld, C. O., Eichler, W., Dalhoff, K. and Braun, J. (2003). Pulmonary Matrix Metalloproteinase Excess in Hospital-acquired Pneumonia. *American Journal of Respiratory & Critical Care Medicine*, 167: 593-598.

Hattevig, G., Kjellman, B. and Bjorksten, B. (1993). Appearance of IgE antibodies to ingested and inhaled allergens during the first 12 years of life in atopic and non-atopic children. *Pediatric Allergy & Immunology*, 4: 182-186.

Heaney, L. G., Stevenson, E. C., Turner, G., Cadden, I. S., Taylor, R., Shields, M. D. and Ennis, M. (1996). Investigating paediatric airways by non-bronchoscopic lavage: normal cellular data. *Clinical Experimental Allergy*, 26: 799-806.

Hellings, P. W., Hessel, E. M., Van Den Oord, J. J., Kasran, A., Van Hecke, P. and Ceuppens, J. L. (2001). Eosinophilic rhinitis accompanies the development of lower airway inflammation and hyper-reactivity in sensitized mice exposed to aerosolized allergen. *Clinical Experimental Allergy*, 31: 782-790.

Holloway, J. A., Warner, J. O., Vance, G. H., Diaper, N. D., Warner, J. A. and Jones, C. A. (2000). Detection of house-dust-mite allergen in amniotic fluid and umbilical-cord blood. *Lancet*, 356: 1900-1902.

Holloway, J. W., Beghe, B. and Holgate, S. T. (1999). The genetic basis of atopic asthma. *Clinical & Experimental Allergy*, 29: 1023-1032.

Holt, P. G. and Sly, P. D. (2000). Prevention of adult asthma by early intervention during childhood: potential value of new generation immunomodulatory drugs. *Thorax*, 55: 700-703.

Horn, M. E., Reed, S. E. and Taylor, P. (1979). Role of viruses and bacteria in acute wheezy bronchitis in childhood: a study of sputum. *Archives of Disease in Childhood*, 54: 587-592.

Hoshino, M., Nakamura, Y., Sim, J., Shimojo, J. and Isogai, S. (1998). Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in

asthmatic airway inflammation. *Journal of Allergy & Clinical Immunology*, 102: 783-788.

Illi, S., von Mutius, E., Lau, S., Bergmann, R., Niggemann, B., Sommerfeld, C. and Wahn, U. (2001). Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. *Bristish Medical Journal*, 322: 390-395.

Ingram, J. M., Rakes, G. P., Hoover, G. E., Platts-Mills, T. A. and Heymann, P. W. (1995). Eosinophil cationic protein in serum and nasal washes from wheezing infants and children. *Journal of Pediatrics*, 127: 558-564.

James, A. L., Maxwell, P. S., Pearce-Pinto, G., Elliot, J. G. and Carroll, N. G. (2002). The Relationship of Reticular Basement Membrane Thickness to Airway Wall Remodeling in Asthma. *American Journal of Respiratory and Critical Care Medicine*, 166: 1590-1595.

Jartti, T., van den, H. B., Garofalo, R. P., Osterhaus, A. D. and Ruuskanen, O. (2002). Metapneumovirus and acute wheezing in children. *Lancet*, 360: 1393-1394.

Jatakanon, A., Uasuf, C., Maziak, W., Lim, S., Chung, K. F. and Barnes, P. J. (1999). Neutrophilic inflammation in severe persistent asthma. *American Journal of Respiratory & Critical Care Medicine*, 160: 1532-1539.

Johnston, S. L. and Openshaw, P. J. (2001). The protective effect of childhood infections. *Bristish Medical Journal*, 322: 376-377.

Johnston, S. L., Pattemore, P. K., Sanderson, G., Smith, S., Lampe, F., Josephs, L., Symington, P., O'Toole, S., Myint, S. H., Tyrrell, D. A. and . (1995). Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *Bristish Medical Journal*, 310: 1225-1229.

Jones, A. C., Miles, E. A., Warner, J. O., Colwell, B. M., Bryant, T. N. and Warner, J. A. (1996). Fetal peripheral blood mononuclear cell proliferative responses

to mitogenic and allergenic stimuli during gestation. *Pediatric Allergy & Immunology*, 7: 109-116.

Just, J., Fournier, L., Momas, I., Zambetti, C., Sahraoui, F. and Grimfeld, A. (2002). Clinical significance of bronchoalveolar eosinophils in childhood asthma. *Journal of Allergy & Clinical Immunology*, 110: 42-44.

Kelly, E. A. and Jarjour, N. N. (2003). Role of matrix metalloproteinases in asthma. *Current Opinion in Pulmonary Medicine*, 9: 28-33.

Kelly, E. A. B., Busse, W. W. and Jarjour, N. N. (2003). A comparison of the airway response to segmental antigen bronchoprovocation in atopic asthma and allergic rhinitis. *Journal of Allergy & Clinical Immunology*, 111: 79-86.

Kemp, A. and Bjorksten, B. (2003). Immune deviation and the hygiene hypothesis: A review of the epidemiological evidence. *Pediatric Allergy & Immunology*, 14: 74-80.

Kim, C. K., Chung, C. Y., Choi, S. J., Kim, D. K., Park, Y. and Koh, Y. Y. (2000). Bronchoalveolar lavage cellular composition in acute asthma and acute bronchiolitis. *Journal of Pediatrics*, 137: 517-522.

Klech, H. and Pohl, K. (1989). Technical recommendations and guidelines for bronchoalveolar lavage (BAL). Report of the European Society of Pneumology Task Group. *European Respiratory Journal*, 2: 561-585.

Kleiner, D. E. and Stetler-Stevenson, W. G. (1994). Quantitative zymography: detection of picogram quantities of gelatinases. *Annals of Biochemistry*, 218: 325-329.

Klimek, L. and Rasp, G. (1999). Normal values for eosinophil cationic protein in nasal secretions: influence of specimen collection. *Clinical Experimental Allergy*, 29: 367-374.

Knox, R. B. (1993). Grass pollen, thunderstorms and asthma. *Clinical Experimental Allergy*, 23: 354-359.

Kokuludag, A., Sin, A., Terzioglu, E., Saydam, G. and Sebik, F. (2002). Elevation of serum eosinophil cationic protein, soluble tumor necrosis factor receptors and soluble intercellular adhesion molecule-1 levels in acute bronchial asthma. *Journal of Investigations in Allergology & Clinical Immunology*, 12: 211-214.

Koller, D. Y., Wojnarowski, C., Herkner, K. R., Weinlander, G., Raderer, M., Eichler, I. and Frischer, T. (1997). High levels of eosinophil cationic protein in wheezing infants predict the development of asthma. *Journal of Allergy & Clinical Immunology*, 99: 752-756.

Koopman, L. P., Savelkoul, H., van Benten, I. J., Gerritsen, J., Brunekreef, B. and Neijens, J. (2003). Increased serum IL-10/IL-12 ratio in wheezing infants. *Pediatric Allergy & Immunology*, 14: 112-119.

Korppi, M. and Kotaniemi-Syrjanen, A. (2003). Infection-induced wheezing during the first year of life does not mean asthma: a 50-year-old observation. *Acta Paediatrica*, 92: 1494-1495.

Kotaniemi-Syrjanen, A., Reijonen, T. M., Korhonen, K. and Korppi, M. (2002). Wheezing requiring hospitalization in early childhood: Predictive factors for asthma in a six-year follow-up. *Pediatric Allergy & Immunology*, 13: 418-425.

Kotaniemi-Syrjanen, A., Reijonen, T. M., Romppanen, J., Korhonen, K., Savolainen, K. and Korppi, M. (2003a). Allergen-specific immunoglobulin E antibodies in wheezing infants: the risk for asthma in later childhood. *Pediatrics*, 111: E255-E261.

Kotaniemi-Syrjanen, A., Vainionpaa, R., Reijonen, T. M., Waris, M., Korhonen, K. and Korppi, M. (2003b). Rhinovirus-induced wheezing in infancy--the first

sign of childhood asthma? *Journal of Allergy & Clinical Immunology*, 111: 66-71.

Krawiec, M. E., Westcott, J. Y., Chu, H. W., Balzar, S., Trudeau, J. B., Schwartz, L. B. and Wenzel, S. E. (2001). Persistent wheezing in very young children is associated with lower respiratory inflammation. *American Journal of Respiratory & Critical Care Medicine*, 163: 1338-1343.

Krieger, B. P. (2002). When wheezing may not mean asthma. Other common and uncommon causes to consider. *Postgraduate Medicine*, 112: 101-8, 111.

Kuehni, C. E., Davis, A., Brooke, A. M. and Silverman, M. (2001). Are all wheezing disorders in very young (preschool) children increasing in prevalence? *Lancet*, 357: 1821-1825.

Kumagai, K., Ohno, I., Imai, K., Nawata, J., Hayashi, K., Okada, S., Senoo, H., Hattori, T. and Shirato, K. (2002). The involvement of matrix metalloproteinases in basement membrane injury in a murine model of acute allergic airway inflammation. *Clinical Experimental Allergy*, 32: 1527-1534.

Kurukulaaratchy, R. J., Fenn, M. H., Waterhouse, L. M., Matthews, S. M., Holgate, S. T. and Arshad, S. H. (2003). Characterization of wheezing phenotypes in the first 10 years of life. *Clinical Experimental Allergy*, 33: 573-578.

Laitinen, L. A., Laitinen, A. and Haahtela, T. (1993). Airway mucosal inflammation even in patients with newly diagnosed asthma. *American Review of Respiratory Disease*, 147: 697-704.

Lam, S., Leriche, J. C. and Kijek, K. (1985). Effect of filtration and concentration on the composition of bronchoalveolar lavage fluid. *Chest*, 87: 740-742.

Laviolette, M., Carreau, M. and Coulombe, R. (1988). Bronchoalveolar lavage cell differential on microscope glass cover. A simple and accurate technique. *American Review of Respiratory Disease*, 138: 451-457.

Le Bourgeois, M., Goncalves, M., Le Clainche, L., Benoist, M. R., Fournet, J. C., Scheinmann, P. and de Blic, J. (2002). Bronchoalveolar cells in children < 3 years old with severe recurrent wheezing. *Chest*, 122: 791-797.

Le Souef, P. N. (2000). Pediatric origins of adult lung diseases. 4. Tobacco related lung diseases begin in childhood. *Thorax*, 55: 1063-1067.

Lee, Y. C., Lee, H. B., Rhee, Y. K. and Song, C. H. (2001). The involvement of matrix metalloproteinase-9 in airway inflammation of patients with acute asthma. *Clinical Experimental Allergy*, 31: 1623-1630.

Legg, J. P., Hussain, I. R., Warner, J. A., Johnston, S. L. and Warner, J. O. (2003). Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. *American Journal of Respiratory & Critical Care Medicine*, 168: 633-639.

Lemanske, R. F., Jr. (2002). Inflammation in childhood asthma and other wheezing disorders. *Pediatrics*, 109: 368-372.

Lemjabbar, H., Gosset, P., Lamblin, C., Tillie, I., Hartmann, D., Wallaert, B., Tonnel, A. B. and Lafuma, C. (1999). Contribution of 92 kDa gelatinase/type IV collagenase in bronchial inflammation during status asthmaticus. *American Journal of Respiratory & Critical Care Medicine*, 159: 1298-1307.

Lim, M. C., Taylor, R. M. and Naclerio, R. M. (1995). The histology of allergic rhinitis and its comparison to cellular changes in nasal lavage. *American Journal of Respiratory & Critical Care Medicine*, 151: 136-144.

Linneberg, A., Henrik, N. N., Frolund, L., Madsen, F., Dirksen, A. and Jorgensen, T. (2002). The link between allergic rhinitis and allergic asthma: a prospective population-based study. The Copenhagen Allergy Study. *Allergy*, 57: 1048-1052.

Lodrup Carlsen, K. C., Halvorsen, R., Ahlstedt, S. and Carlsen, K. H. (1995). Eosinophil cationic protein and tidal flow volume loops in children 0-2 years of age. *European Respiratory Journal*, 8: 1148-1154.

Luster, A. D. (1998). Chemokines--chemotactic cytokines that mediate inflammation. *New Engand Journal of Medicine*, 338: 436-445.

Mander, A. (1999). *Pulmonary surfactant and neutrophil function in cystic fibrosis*. PhD Thesis. University of Southampton.

Manolitsas, N. D., Trigg, C. J., McAulay, A. E., Wang, J. H., Jordan, S. E., D'Ardenne, A. J. and Davies, R. J. (1994). The expression of intercellular adhesion molecule-1 and the beta 1-integrins in asthma. *European Respiratory Journal*, 7: 1439-1444.

Marcy, T. W., Merrill, W. W., Rankin, J. A. and Reynolds, H. Y. (1987). Limitations of using urea to quantify epithelial lining fluid recovered by bronchoalveolar lavage. *American Review of Respiratory Disease*, 135: 1276-1280.

Marguet, C., Dean, T. P., Basuyau, J. P. and Warner, J. O. (2001). Eosinophil cationic protein and interleukin-8 levels in bronchial lavage fluid from children with asthma and infantile wheeze. *Pediatric Allergy & Immunology*, 12: 27-33.

Marguet, C., Dean, T. P. and Warner, J. O. (2000). Soluble intercellular adhesion molecule-1 (sICAM-1) and interferon-gamma in bronchoalveolar lavage fluid from children with airway diseases. *American Journal of Respiratory & Critical Care Medicine*, 162: 1016-1022.

Marguet, C., Jouen-Boedes, F., Dean, T. P. and Warner, J. O. (1999). Bronchoalveolar cell profiles in children with asthma, infantile wheeze, chronic cough, or cystic fibrosis. *American Journal of Respiratory & Critical Care Medicine*, 159: 1533-1540.

Martinez, F. D. (2001). The coming-of-age of the hygiene hypothesis. *Respiratory Research*, 2: 129-132.

Martinez, F. D. (2002). Development of wheezing disorders and asthma in preschool children. *Pediatrics*, 109: 362-367.

Martinez, F. D., Morgan, W. J., Wright, A. L., Holberg, C. J. and Taussig, L. M. (1988). Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. *New England Journal of Medicine*, 319: 1112-1117.

Martinez, F. D., Stern, D. A., Wright, A. L., Taussig, L. M. and Halonen, M. (1998). Differential immune responses to acute lower respiratory illness in early life and subsequent development of persistent wheezing and asthma. *Journal of Allergy & Clinical Immunology*, 102: 915-920.

Martinez, F. D., Wright, A. L., Taussig, L. M., Holberg, C. J., Halonen, M. and Morgan, W. J. (1995). Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *New England Journal of Medicine*, 332: 133-138.

Masters, I. B., Chang, A. B., Patterson, L., Wainwright, C., Buntain, H., Dean, B. W. and Francis, P. W. (2002). Series of laryngomalacia, tracheomalacia, and bronchomalacia disorders and their associations with other conditions in children. *Pediatric Pulmonology*, 34: 189-195.

Masure, S., Proost, P., Van Damme, J. and Opdenakker, G. (1991). Purification and identification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8. *European Journal of Biochemistry*, 198: 391-398.

Mattes, J. and Karmaus, W. (1999). The use of antibiotics in the first year of life and development of asthma: which comes first? *Clinical Experimental Allergy*, 29: 729-732.

Mattos, W., Lim, S., Russell, R., Jatakanon, A., Chung, K. F. and Barnes, P. J. (2002). Matrix metalloproteinase-9 expression in asthma: effect of asthma severity, allergen challenge, and inhaled corticosteroids. *Chest*, 122: 1543-1552.

Mautino, G., Capony, F., Bousquet, J. and Vignola, A. M. (1999a). Balance in asthma between matrix metalloproteinases and their inhibitors. *Journal of Allergy & Clinical Immunology*, 104: 530-533.

Mautino, G., Henriquet, C., Gougat, C., Le Cam, A., Dayer, J. M., Bousquet, J. and Capony, F. (1999b). Increased expression of tissue inhibitor of metalloproteinase-1 and loss of correlation with matrix metalloproteinase-9 by macrophages in asthma. *Laboratory Investigations*, 79: 39-47.

Mautino, G., Henriquet, C., Jaffuel, D., Bousquet, J. and Capony, F. (1999c). Tissue inhibitor of metalloproteinase-1 levels in bronchoalveolar lavage fluid from asthmatic subjects. *American Journal of Respiratory & Critical Care Medicine*, 160: 324-330.

Mautino, G., Oliver, N., Chanez, P., Bousquet, J. and Capony, F. (1997). Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *American Journal of Cellular & Molecular Biology*, 17: 583-591.

McCann, D., McWhirter, J., Coleman, H., Devall, I., Calvert, M., Weare, K. and Warner, J. (2002). The prevalence and management of asthma in primary-aged schoolchildren in the south of England. *Health Education Research*, 17: 181-194.

McCusker, C., Chicoine, M., Hamid, Q. and Mazer, B. (2002). Site-specific sensitization in a murine model of allergic rhinitis: Role of the upper airway in lower airways disease. *Journal of Allergy & Clinical Immunology*, 110: 891-898.

McIntosh, K., Ellis, E. F., Hoffman, L. S., Lybass, T. G., Eller, J. J. and Fulginiti, V. A. (1973). Association of viral and bacterial respiratory infection with exacerbations of wheezing in young asthmatic children. *Chest*, 63: Suppl.

McKean, M., Hewitt, C., Lambert, P., Myint, S. and Silverman, M. (2003). An adult model of exclusive viral wheeze: inflammation in the upper and lower respiratory tracts. *Clinical Experimental Allergy*, 33: 912-920.

Merkus, P. J., Have-Opbroek, A. A. and Quanjer, P. H. (1996). Human lung growth: a review. *Pediatric Pulmonology*, 21: 383-397.

Meyer, P., Andersson, M., Persson, C. G. and Greiff, L. (2003). Steroid-sensitive indices of airway inflammation in children with seasonal allergic rhinitis. *Pediatric Allergy & Immunology*, 14: 60-65.

Midulla, F., Villani, A., Merolla, R., Bjermer, L., Sandstrom, T. and Ronchetti, R. (1995). Bronchoalveolar lavage studies in children without parenchymal lung disease: cellular constituents and protein levels. *Pediatric Pulmonology*, 20: 112-118.

Minor, T. E., Dick, E. C., DeMeo, A. N., Ouellette, J. J., Cohen, M. and Reed, C. E. (1974). Viruses as precipitants of asthmatic attacks in children. *Journal Of The American Medical Association*, 227: 292-298.

Mordelet-Dambrine, M., Arnoux, A., Stanislas-Leguern, G., Sandron, D., Chretien, J. and Huchon, G. (1984). Processing of lung lavage fluid causes variability in bronchoalveolar cell count. *American Review of Respiratory Disease*, 130: 305-306.

Murphy, G. and Docherty, A. J. (1992). The matrix metalloproteinases and their inhibitors. *American Journal of Cellular & Molecular Biology*, 7: 120-125.

Murray, C. S., Pipis, S. D., McArdle, E. C., Lowe, L. A., Custovic, A. and Woodcock, A. (2002). Lung function at one month of age as a risk factor for infant respiratory symptoms in a high risk population. *Thorax*, 57: 388-392.

Nagase, H. and Woessner, J. F., Jr. (1999). Matrix Metalloproteinases. *Journal of Biological Chemistry*, 274: 21491-21494.

Nagayama, Y., Tsubaki, T., Toba, T., Nakayama, S. and Kiyofumi, O. (2001). Analysis of sputum taken from wheezy and asthmatic infants and children, with special reference to respiratory infections. *Pediatric Allergy & Immunology*, 12: 318-326.

Najafi, N., Demanet, C., Dab, I., De Waele, M. and Malfroot, A. (2003). Differential cytology of bronchoalveolar lavage fluid in asthmatic children. *Pediatric Pulmonology*, 35: 302-308.

Naureckas, E. T., Ndukwu, I. M., Halayko, A. J., Maxwell, C., Hershenson, M. B. and Solway, J. (1999). Bronchoalveolar lavage fluid from asthmatic subjects is mitogenic for human airway smooth muscle. *American Journal of Respiratory & Critical Care Medicine*, 160: 2062-2066.

Nelson, H. S. (2003). Prospects for antihistamines in the treatment of asthma. *Journal of Allergy & Clinical Immunology*, 112: S96-100.

Ng Man, K. G., Proctor, A., Billings, C., Duggan, R., Das, C., Whyte, M. K., Powell, C. V. and Primhak, R. (2001). Increasing prevalence of asthma diagnosis and symptoms in children is confined to mild symptoms. *Thorax*, 56: 312-314.

Nicolai, T. (2001). Pediatric bronchoscopy. *Pediatric Pulmonology*, 31: 150-164.

Nikasinovic-Fournier, L., Just, J., Seta, N., Callais, F., Sahraoui, F., Grimfeld, A. and Momas, I. (2002). Nasal lavage as a tool for the assessment of upper-airway inflammation in adults and children. *Journbal of Laboratory & Clinical Medicine*, 139: 173-180.

Nja, F., Nystad, W., Hetlevik, O., Lodrup Carlsen, K. C. and Carlsen, K. H. (2003). Airway infections in infancy and the presence of allergy and asthma in school age children. *Archives of Disease in Childhood*, 88: 566-569.

Noah, T. L., Henderson, F. W., Henry, M. M., Peden, D. B. and Devlin, R. B. (1995). Nasal lavage cytokines in normal, allergic, and asthmatic school-age children. *American Journal of Respiratory & Critical Care Medicine*, 152: 1290-1296.

Norzila, M. Z., Fakes, K., Henry, R. L., Simpson, J. and Gibson, P. G. (2000). Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. *American Journal of Respiratory & Critical Care Medicine*, 161: 769-774.

Nourshargh, S. (1993). Mechanisms of neutrophil and eosinophil accumulation in vivo. *American Review of Respiratory Disease*, 148: S60-S64.

O'Brien, K. L., Dowell, S. F., Schwartz, B., Marcy, M., Phillips, W. R., and Gerber, M. A. (1998). Cough Illness/Bronchitis - Principles of Judicious use of Antimicrobial Agents. *Pediatrics,* 101: 178-181.

O'Donnell, R. A. and Frew, A. J. (2002). Is there more than one inflammatory phenotype in asthma? *Thorax*, 57: 566-568.

Oh, J. W., Lee, H. B., Park, I. K. and Kang, J. O. (2002). Interleukin-6, interleukin-8, interleukin-11, and interferon-gamma levels in nasopharyngeal aspirates from wheezing children with respiratory syncytial virus or influenza A virus infection. *Pediatric Allergy & Immunology*, 13: 350-356.

Oh, J. W., Lee, H. B., Yum, M. K., Kim, C. R., Kang, J. O. and Park, I. K. (2000). ECP level in nasopharyngeal secretions and serum from children with respiratory virus infections and asthmatic children. *Allergy & Asthma Proceedings*, 21: 97-100.

Ohno, I., Ohtani, H., Nitta, Y., Suzuki, J., Hoshi, H., Honma, M., Isoyama, S., Tanno, Y., Tamura, G., Yamauchi, K., Nagura, H. and Shirato, K. (1997). Eosinophils as a source of matrix metalloproteinase-9 in asthmatic airway

inflammation. *American Journal of Cellular & Molecular Biology*, 16: 212-219.

Ono, S. J., Nakamura, T., Miyazaki, D., Ohbayashi, M., Dawson, M. and Toda, M. (2003). Chemokines: roles in leukocyte development, trafficking, and effector function. *Journal of Allergy & Clinical Immunology*, 111: 1185-1199.

Oommen, A., Lambert, P. C. and Grigg, J. (2003a). Efficacy of a short course of parent-initiated oral prednisolone for viral wheeze in children aged 1-5 years: randomised controlled trial. *Lancet*, 362: 1433-1438.

Oommen, A., McNally, T. and Grigg, J. (2003b). Eosinophil activation and preschool viral wheeze. *Thorax*, 58: 876-879.

Opdenakker, G., Van den Steen, P. E., Dubois, B., Nelissen, I., Van Coillie, E., Masure, S., Proost, P. and Van Damme, J. (2001). Gelatinase B functions as regulator and effector in leukocyte biology. *Jopurnal of Leukocyte Biology*, 69: 851-859.

Openshaw, P. J. and Hewitt, C. (2000). Protective and harmful effects of viral infections in childhood on wheezing disorders and asthma. *American Journal of Respiratory & Critical Care Medicine*, 162: S40-S43.

Openshaw, P. J. and O'Donnell, D. R. (1994). Asthma and the common cold: can viruses imitate worms? *Thorax*, 49: 101-103.

Oshita, Y., Koga, T., Kamimura, T., Matsuo, K., Rikimaru, T. and Aizawa, H. (2003). Increased circulating 92 kDa matrix metalloproteinase (MMP-9) activity in exacerbations of asthma. *Thorax*, 58: 757-760.

Oswald, H., Phelan, P. D., Lanigan, A., Hibbert, M., Carlin, J. B., Bowes, G. and Olinsky, A. (1997). Childhood asthma and lung function in mid-adult life. *Pediatric Pulmonology*, 23: 14-20.

Owen, C. A., Hu, Z., Barrick, B. and Shapiro, S. D. (2003). Inducible Expression of Tissue Inhibitor of Metalloproteinases-Resistant Matrix Metalloproteinase-9 on the Cell Surface of Neutrophils. *American Journal of Respiratory Cell and Molecular Biology*, 29: 283-294.

Oymar, K. and Bjerknes, R. (2001). Urinary eosinophil protein X in children with asthma: influence of atopy and airway infections. *Pediatric Allergy & Immunology*, 12: 34-41.

Pacifico, L., Iacobini, M., Viola, F., Werner, B., Mancuso, G. and Chiesa, C. (2000). Chemokine concentrations in nasal washings of infants with rhinovirus illnesses. *Clinical Infectious Diseases*, 31: 834-838.

Packe, G. E. and Ayres, J. G. (1985). Asthma outbreak during a thunderstorm. *Lancet*, 2: 199-204.

Pala, P., Bjarnason, R., Sigurbergsson, F., Metcalfe, C., Sigurs, N. and Openshaw, P. J. (2002). Enhanced IL-4 responses in children with a history of respiratory syncytial virus bronchiolitis in infancy. *European Respiratory Journal*, 20: 376-382.

Palmans, E., Vanacker, N. J., Pauwels, R. A. and Kips, J. C. (2002). Effect of age on allergen-induced structural airway changes in brown Norway rats. *American Journal of Respiratory & Critical Care Medicine*, 165: 1280-1284.

Palmer, L. J., Rye, P. J., Gibson, N. A., Burton, P. R., Landau, L. I. and Lesouef, P. N. (2001). Airway responsiveness in early infancy predicts asthma, lung function, and respiratory symptoms by school age. *American Journal of Respiratory & Critical Care Medicine*, 163: 37-42.

Papadopoulos, N. G., Stanciu, L. A., Papi, A., Holgate, S. T. and Johnston, S. L. (2002). A defective type 1 response to rhinovirus in atopic asthma. *Thorax*, 57: 328-332.

Park, E. S., Golding, J., Carswell, F. and Stewart-Brown, S. (1986). Preschool wheezing and prognosis at 10. *Archives of Disease in Childhood*, 61: 642-646.

Pathak, S. S., van Oudenaren, A. and Savelkoul, H. F. J. (1997) Quantifiaction of immunoglobulin concentration by ELISA. In Lefkovits, I. (ed), *Immunology methods manual*. Academic Press Ltd., London, pp. 1056-1075.

Payne, D., McKenzie, S. A., Stacey, S., Misra, D., Haxby, E. and Bush, A. (2001). Safety and ethics of bronchoscopy and endobronchial biopsy in difficult asthma. *Archives of Disease in Childhood*, 84: 423-426.

Payne, D. N., Lincoln, C. and Bush, A. (2000). Lesson of the week: right sided aortic arch in children with persistent respiratory symptoms. *Bristish Medical Journal*, 321: 687-688.

Pearce, N., Douwes, J. and Beasley, R. (2000). Is allergen exposure the major primary cause of asthma? *Thorax*, 55: 424-431.

Peroni, D., Piacentini, G., Alfonsi, L., Zerman, L., Di Blasi, P., Visona', G., Nottegar, F. and Boner, A. (2003). Rhinitis in pre-school children: prevalence, association with allergic diseases and risk factors. *Clinical Experimental Allergy*, 33: 1349-1354.

Peterson, C. G., Enander, I., Nystrand, J., Anderson, A. S., Nilsson, L. and Venge, P. (1991). Radioimmunoassay of human eosinophil cationic protein (ECP) by an improved method. Establishment of normal levels in serum and turnover in vivo. *Clinical Experimental Allergy*, 21: 561-567.

Platts-Mills, T. A., Rakes, G. and Heymann, P. W. (2000). The relevance of allergen exposure to the development of asthma in childhood. *Journal of Allergy & Clinical Immunology*, 105: S503-S508.

Pohunek, P., Pokorna, H. and Striz, I. (1996). Comparison of cell profiles in separately evaluated fractions of bronchoalveolar lavage (BAL) fluid in children. *Thorax*, 51: 615-618.

Pohunek, P., Roche, W. R., Tuzikova, J., Kurdmann, J., and Warner, J. O. (1997). Eosinophillic inflammation in the bronchial mucosa of children with bronchial asthma. *European Respiratory Journal,* 10: 160s

Pope, C. A., III (1989). Respiratory disease associated with community air pollution and a steel mill, Utah Valley. *American Journal of Public Health*, 79: 623-628.

Postle, A. D., Mander, A., Reid, K. B., Wang, J. Y., Wright, S. M., Moustaki, M. and Warner, J. O. (1999). Deficient hydrophilic lung surfactant proteins A and D with normal surfactant phospholipid molecular species in cystic fibrosis. *American Journal of Cellular & Molecular Biology*, 20: 90-98.

Postma, D. S. and Howard, T. (2004). ADAM 33 gene: confirming a gene without linkage. *Clinical Experimental Allergy*, 34: 1-3.

Prikk, K., Maisi, P., Pirila, E., Reintam, M. A., Salo, T., Sorsa, T. and Sepper, R. (2002). Airway obstruction correlates with collagenase-2 (MMP-8) expression and activation in bronchial asthma. *Laboratory Investigations*, 82: 1535-1545.

Pugin, J., Widmer, M. C., Kossodo, S., Liang, C. M., Preas, H. L., II and Suffredini, A. F. (1999). Human Neutrophils Secrete Gelatinase B In Vitro and In Vivo in Response to Endotoxin and Proinflammatory Mediators. *American Journal of Respiratory Cell and Molecular Biology*, 20: 458-464.

Raine, J. and Warner, J. O. (1991). Fibreoptic bronchoscopy without general anaesthetic. *Archives of Disease in Childhood*, 66: 481-484.

Ransohoff, R. M. (1998). Cellular responses to interferons and other cytokines: the JAK-STAT paradigm. *New England Journal of Medicine*, 338: 616-618.

Rao, R., Frederick, J. M., Enander, I., Gregson, R. K., Warner, J. A. and Warner, J. O. (1996). Airway function correlates with circulating eosinophil, but not mast cell, markers of inflammation in childhood asthma. *Clinical Experimental Allergy*, 26: 789-793.

Ratjen, F., Bredendiek, M., Brendel, M., Meltzer, J. and Costabel, U. (1994). Differential cytology of bronchoalveolar lavage fluid in normal children. *European Respiratory Journal*, 7: 1865-1870.

Redington, A. E. (2000). Fibrosis and airway remodelling. *Clinical & Experimental Allergy*, 30 Suppl 1: 42-45.

Reichenbach, J., Jarisch, A., Khan, S., Homberg, M., Bez, C. and Zielen, S. (2002). Serum ECP levels and methacholine challenge in infants with recurrent wheezing. *Annals of Allergy & Asthma Immunology*, 89: 498-502.

Rennard, S. I., Aalbers, R., Bleecker, E., Klech, H., Rosenwasser, L., Olivieri, D. and Sibille, Y. (1998). Bronchoalveolar lavage: performance, sampling procedure, processing and assessment. *European Respiratory Journal*, 26: 13S-15S.

Rhodes, H. L., Thomas, P., Sporik, R., Holgate, S. T. and Cogswell, J. J. (2002). A birth cohort study of subjects at risk of atopy: twenty-two-year follow-up of wheeze and atopic status. *American Journal of Respiratory & Critical Care Medicine*, 165: 176-180.

Rosenberg, H. F. and Domachowske, J. B. (2001). Eosinophils, eosinophil ribonucleases, and their role in host defense against respiratory virus pathogens. *Journal of Leukocyte Biology*, 70: 691-698.

Ruiz, R. G., Kemeny, D. M. and Price, J. F. (1992). Higher risk of infantile atopic dermatitis from maternal atopy than from paternal atopy. *Clinical Experimental Allergy*, 22: 762-766.

Rylander, E., Pershagen, G., Eriksson, M. and Bermann, G. (1995). Parental smoking, urinary cotinine, and wheezing bronchitis in children. *Epidemiology*, 6: 289-293.

Saglani, S., Payne, D. N. R., Nicholson, A. G., Scallan, M., Haxby, E. and Bush, A. (2003). The safety and quality of endobronchial biopsy in children under five years old. *Thorax*, 58: 1053-1057.

Sale, R., Silvestri, M., Battistini, E., Defilippi, A. C., Sabatini, F., Pecora, S. and Rossi, G. A. (2003). Nasal inflammation and bronchial reactivity to methacholine in atopic children with respiratory symptoms. *Allergy*, 58: 1171-1175.

Saltini, C., Hance, A. J., Ferrans, V. J., Basset, F., Bitterman, P. B. and Crystal, R. G. (1984). Accurate quantification of cells recovered by bronchoalveolar lavage. *American Review of Respiratory Disease*, 130: 650-658.

Salva, P. S., Theroux, C. and Schwartz, D. (2003). Safety of endobronchial biopsy in 170 children with chronic respiratory symptoms. *Thorax*, 58: 1058-1060.

Scheinmann, P., Pedersen, S., Warner, J. O. and de Blic, J. (1998). Methods for assessment of airways inflammation: paediatrics. *European Respiratory Journal*, 26: 53S-58S.

Schellhase, D. E., Fawcett, D. D., Schutze, G. E., Lensing, S. Y. and Tryka, A. F. (1998). Clinical utility of flexible bronchoscopy and bronchoalveolar lavage in young children with recurrent wheezing. *Journal of Pediatrics*, 132: 312-318.

Schellhase, D. E., Tamez, J. R., Menendez, A. A., Morris, M. G., Fowler, G. W. and Lensing, S. Y. (1999). High fever after flexible bronchoscopy and bronchoalveolar lavage in noncritically ill immunocompetent children. *Pediatric Pulmonology*, 28: 139-144.

Schock, B. C., Sweet, D. G., Ennis, M., Warner, J. A., Young, I. S. and Halliday, H. L. (2001). Oxidative stress and increased type-IV collagenase levels in bronchoalveolar lavage fluid from newborn babies. *Pediatric Research*, 50: 29-33.

Sears, M. R., Greene, J. M., Willan, A. R., Wiecek, E. M., Taylor, D. R., Flannery, E. M., Cowan, J. O., Herbison, G. P., Silva, P. A. and Poulton, R. (2003). A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *New England Journal of Medicine*, 349: 1414-1422.

Shackley, F., Diggle, L., Heath, P., Griffiths, D., Crook, D., and Moxon, R. (1997). Streptococcus Pneumoniae: acquisition and carriage in infancy. *British Paediatric Immunology and Infectious Diseases Group Meeting 1997,*

Shamssain, M. H. and Shamsian, N. (1999). Prevalence and severity of asthma, rhinitis, and atopic eczema: the north east study. *Archives of Disease in Childhood*, 81: 313-317.

Shields, M. D., Brown, V., Stevenson, E. C., Fitch, P. S., Schock, B. C., Turner, G., Taylor, R. and Ennis, M. (1999). Serum eosinophilic cationic protein and blood eosinophil counts for the prediction of the presence of airways inflammation in children with wheezing. *Clinical & Experimental Allergy*, 29: 1382-1389.

Shields, M. D. and Riedler, J. (2000). Bronchoalveolar lavage and tracheal aspirate for assessing airway inflammation in children. *American Journal of Respiratory & Critical Care Medicine*, 162: S15-S17.

Shute, J. (2002). Matrix metalloproteinase-9: marker or mediator of tissue damage in asthma? *Clinical Experimental Allergy*, 32: 168-171.

Siegel, S. and Castellan, N. J. (1988) *Non-parametric statistics for the behavioural sciences*. McGraw-Hill Book Company, London.

Sigurs, N., Bjarnason, R., Sigurbergsson, F. and Kjellman, B. (2000). Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *American Journal of Respiratory & Critical Care Medicine*, 161: 1501-1507.

Silverman, M., Wang, M., Hunter, G. and Taub, N. (2003). Episodic viral wheeze in preschool children: effect of topical nasal corticosteroid prophylaxis. *Thorax*, 58: 431-434.

Silverman, M. and Wilson, N. (1997a). Asthma--time for a change of name? *Archives of Disease in Childhood*, 77: 62-64.

Silverman, M. and Wilson, N. (1997b). Wheezing phenotypes in childhood. *Thorax*, 52: 936-937.

Simell, B., Korkeila, M., Pursiainen, H., Kilpi, T. M. and Kayhty, H. (2001). Pneumococcal carriage and otitis media induce salivary antibodies to Pneumococcal Surface Adhesin A, Pneumolysin, and Pneumococcal Surface Protein A in children. *Journal of Infectious Diseases*, 183: 887-896.

Simons, F. E. (1999). Allergic rhinobronchitis: the asthma-allergic rhinitis link. *Journal of Allergy & Clinical Immunology*, 104: 534-540.

Sporik, R., Holgate, S. T. and Cogswell, J. J. (1991). Natural history of asthma in childhood--a birth cohort study. *Archives of Disease in Childhood*, 66: 1050-1053.

Sporik, R. and Platts-Mills, T. A. (2001). Allergen exposure and the development of asthma. *Thorax*, 56 Suppl 2: ii58-ii63.

Stark, P. C., Burge, H. A., Ryan, L. M., Milton, D. K. and Gold, D. R. (2003). Fungal levels in the home and lower respiratory tract illnesses in the first year of life. *American Journal of Respiratory & Critical Care Medicine*, 168: 232-237.

Stein, R. T., Holberg, C. J., Morgan, W. J., Wright, A. L., Lombardi, E., Taussig, L. and Martinez, F. D. (1997). Peak flow variability, methacholine responsiveness and atopy as markers for detecting different wheezing phenotypes in childhood. *Thorax*, 52: 946-952.

Stein, R. T., Sherrill, D., Morgan, W. J., Holberg, C. J., Halonen, M., Taussig, L. M., Wright, A. L. and Martinez, F. D. (1999). Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet*, 354: 541-545.

Stevens, C. A., Turner, D., Kuehni, C. E., Couriel, J. M. and Silverman, M. (2003). The economic impact of preschool asthma and wheeze. *European Respiratory Journal*, 21: 1000-1006.

Stevenson, E. C., Turner, G., Heaney, L. G., Schock, B. C., Taylor, R., Gallagher, T., Ennis, M. and Shields, M. D. (1997). Bronchoalveolar lavage findings suggest two different forms of childhood asthma. *Clinical Experimental Allergy*, 27: 1027-1035.

Stewart, B., Cochran, A., Iglesia, K., Speights, V. O. and Ruff, T. (2002). Unusual case of stridor and wheeze in an infant: Tracheal bronchogenic cyst. *Pediatric Pulmonology*, 34: 320-323.

Strachan, D. P. (1989). Hay fever, hygiene, and household size. *Bristish Medical Journal*, 299: 1259-1260.

Strachan, D. P. (2000a). Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax*, 55 Suppl 1: S2-10.

Strachan, D. P. (2000b). The role of environmental factors in asthma. *British Medical Bulletin*, 56: 865-882.

Strachan, D. P., Griffiths, J. M., Johnston, I. D. and Anderson, H. R. (1996). Ventilatory function in British adults after asthma or wheezing illness at ages 0-35. *American Journal of Respiratory & Critical Care Medicine*, 154: 1629-1635.

Strunk, R. C. (2002). Defining asthma in the preschool-aged child. *Pediatrics*, 109: 357-361.

Suphioglu, C., Singh, M. B., Taylor, P., Knox, R. B., Bellomo, R., Holmes, P. and Puy, R. (1992). Mechanism of grass-pollen-induced asthma. *The Lancet*, 339: 569-572.

Sur, S., Glitz, D. G., Kita, H., Kujawa, S. M., Peterson, E. A., Weiler, D. A., Kephart, G. M., Wagner, J. M., George, T. J., Gleich, G. J. and Leiferman, K. M. (1998). Localization of eosinophil-derived neurotoxin and eosinophil cationic protein in neutrophilic leukocytes. *J Leukoc.Biol.*, 63: 715-722.

Suzuki, R., Kato, T., Miyazaki, Y., Iwata, M., Noda, Y., Takagi, K., Nakashima, N. and Torii, K. (2001). Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in sputum from patients with bronchial asthma. *J Asthma*, 38: 477-484.

Szefler, S. J. and Simoes, E. A. (2003). Montelukast for respiratory syncytial virus bronchiolitis: significant effect or provocative findings? *American Journal of Respiratory & Critical Care Medicine*, 167: 290-291.

Tager, I. B., Ngo, L. and Hanrahan, J. P. (1995). Maternal smoking during pregnancy. Effects on lung function during the first 18 months of life. *American Journal of Respiratory & Critical Care Medicine*, 152: 977-983.

Takafuji, S., Ishida, A., Miyakuni, Y. and Nakagawa, T. (2003). Matrix metalloproteinase-9 release from human leukocytes. *J Investig.Allergol.Clin Immunol*, 13: 50-55.

Tang, R. B., Chen, S. J., Soong, W. J. and Chung, R. L. (2002). Circulating adhesion molecules in sera of asthmatic children. *Pediatric Pulmonology*, 33: 249-254.

Tariq, S. M., Matthews, S. M., Hakim, E. A. and Arshad, S. H. (2000). Egg allergy in infancy predicts respiratory allergic disease by 4 years of age. *Pediatric Allergy & Immunology*, 11: 162-167.

Tauber, E., Halmerbauer, G., Frischer, T., Gartner, C., Horak, F., Jr., Veiter, A., Koller, D. Y. and Studnicka, M. (2000). Urinary eosinophil protein X in children: the relationship to asthma and atopy and normal values. *Allergy*, 55: 647-652.

Taylor, P. E., Flagan, R. C., Valenta, R. and Glovsky, M. M. (2002). Release of allergens as respirable aerosols: A link between grass pollen and asthma. *Journal of Allergy & Clinical Immunology*, 109: 51-56.

Teran, L. M., Seminario, M. C., Shute, J. K., Papi, A., Compton, S. J., Low, J. L., Gleich, G. J. and Johnston, S. L. (1999). RANTES, macrophage-inhibitory protein 1alpha, and the eosinophil product major basic protein are released into upper respiratory secretions during virus-induced asthma exacerbations in children. *Journal of Infectious Diseases*, 179: 677-681.

Tessier, V., Chadelat, K., Baculard, A., Housset, B. and Clement, A. (1996). BAL in children: a controlled study of differential cytology and cytokine expression profiles by alveolar cells in pediatric sarcoidosis. *Chest*, 109: 1430-1438.

Tiddens, H., Silverman, M. and Bush, A. (2000). The role of inflammation in airway disease: remodeling. *American Journal of Respiratory & Critical Care Medicine*, 162: S7-S10.

Toelle, B. G., Ng, K., Belousova, E., Salome, C. M., Peat, J. K. and Marks, G. B. (2004). Prevalence of asthma and allergy in schoolchildren in Belmont, Australia: three cross sectional surveys over 20 years. *Bristish Medical Journal*, 328: 386-387.

Togias, A. (2001). Systemic cross-talk between the lung and the nose. *American Journal of Respiratory & Critical Care Medicine*, 164: 726-727.

Turner, S. W., Palmer, L. J., Rye, P. J., Gibson, N. A., Judge, P. K., Young, S., Landau, L. I. and Le Souef, P. N. (2002). Infants with flow limitation at 4 weeks: outcome at 6 and 11 years. *American Journal of Respiratory & Critical Care Medicine*, 165: 1294-1298.

Van den Steen, P. E., Proost, P., Wuyts, A., Van Damme, J. and Opdenakker, G. (2000). Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood*, 96: 2673-2681.

van der Stolpe, A. and van der Saag, P. T. (1996). Intercellular adhesion molecule-1. *Journal of Molecular Medicine*, 74: 13-33.

Van Eerdewegh, P., Little, R. D., Dupuis, J., Del Mastro, R. G., Falls, K., Simon, J., Torrey, D., Pandit, S., McKenny, J., Braunschweiger, K., Walsh, A., Liu, Z., Hayward, B., Folz, C., Manning, S. P., Bawa, A., Saracino, L., Thackston, M., Benchekroun, Y., Capparell, N., Wang, M., Adair, R., Feng, Y., Dubois, J., FitzGerald, M. G., Huang, H., Gibson, R., Allen, K. M., Pedan, A., Danzig, M. R., Umland, S. P., Egan, R. W., Cuss, F. M., Rorke, S., Clough, J. B., Holloway, J. W., Holgate, S. T. and Keith, T. P. (2002). Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature*, 418: 426-430.

van Odijk, J., Kull, I., Borres, M. P., Brandtzaeg, P., Edberg, U., Hanson, L. A., Host, A., Kuitunen, M., Olsen, S. F., Skerfving, S., Sundell, J. and Wille, S. (2003). Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966-2001) on the mode of early feeding in infancy and its impact on later atopic manifestations. *Allergy*, 58: 833-843.

Varner, A. E., Busse, W. W. and Lemanske, R. F., Jr. (1998). Hypothesis: decreased use of pediatric aspirin has contributed to the increasing prevalence of childhood asthma. *Annals of Allergy & Asthma Immunology*, 81: 347-351.

Venn, A., Lewis, S., Cooper, M., Hill, J. and Britton, J. (1998). Increasing prevalence of wheeze and asthma in Nottingham primary schoolchildren 1988-1995. *European Respiratory Journal*, 11: 1324-1328.

Vercelli, D. (2003). Learning from discrepancies: CD14 polymorphisms, atopy and the endotoxin switch. *Clinical Experimental Allergy*, 33: 153-155.

Vermaelen, K. Y., Cataldo, D., Tournoy, K., Maes, T., Dhulst, A., Louis, R., Foidart, J. M., Noel, A. and Pauwels, R. (2003). Matrix metalloproteinase-9-mediated dendritic cell recruitment into the airways is a critical step in a mouse model of asthma. *The Journal of Immunology*, 171: 1016-1022.

Vignola, A. M., Riccobono, L., Mirabella, A., Profita, M., Chanez, P., Bellia, V., Mautino, G., D'accardi, P., Bousquet, J. and Bonsignore, G. (1998). Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *American Journal of Respiratory & Critical Care Medicine*, 158: 1945-1950.

Villa, J. R., Garcia, G., Rueda, S. and Nogales, A. (1998). Serum eosinophilic cationic protein may predict clinical course of wheezing in young children. *Archives of Disease in Childhood*, 78: 448-452.

von Mutius, E. (1998). The rising trends in asthma and allergic disease. *Clinical & Experimental Allergy*, 28 Suppl 5: 45-49.

von Mutius, E. (2001). Paediatric origins of adult lung disease. *Thorax*, 56: 153-157.

von Mutius, E. (2002). Environmental factors influencing the development and progression of pediatric asthma. *Journal of Allergy & Clinical Immunology*, 109: S525-S532.

von Mutius, E., Martinez, F. D., Fritzsch, C., Nicolai, T., Roell, G. and Thiemann, H. H. (1994). Prevalence of asthma and atopy in two areas of West and East Germany. *American Journal of Respiratory & Critical Care Medicine*, 149: 358-364.

von Mutius, E., Weiland, S. K., Fritzsch, C., Duhme, H. and Keil, U. (1998). Increasing prevalence of hay fever and atopy among children in Leipzig, East Germany. *Lancet*, 351: 862-866.

Walsh, G. M. (2002). Second-generation antihistamines in asthma therapy: is there a protective effect? *Amercian Journal of Respiratory Medicine*, 1: 27-34.

Warke, T.J., Kamath, S., Fitch, P.S., Brown, V., Shields, M.D., Ennis, M. (2001). The repeatability of nonbronchopscopic bronchoalveolar lavage differential cell counts. *European Respiratory Journal* 18: 1009 – 12.

Warner, J. A., Jones, C. A., Jones, A. C. and Warner, J. O. (2000). Prenatal origins of allergic disease. *Journal of Allergy & Clinical Immunology*, 105: S493-S498.

Warner, J. O. (2001). A double-blinded, randomized, placebo-controlled trial of cetirizine in preventing the onset of asthma in children with atopic dermatitis: 18 months' treatment and 18 months' posttreatment follow-up. *Journal of Allergy & Clinical Immunology*, 108: 929-937.

Warner, J. O. (2004). The early life origins of asthma and related allergic disorders. *Archives of Disease in Childhood*, 89: 97-102.

Wart, H. E. V. and Birkedal-Hansen, H. (1990). The Cysteine Switch: A Principle of Regulation of Metalloproteinase Activity with Potential Applicability to the Entire Matrix Metalloproteinase Gene Family. *Proceedings of the National Academy of Sciences*, 87: 5578-5582.

Wennergren, G. and Strannegard, I. L. (2002). Asthma hospitalizations continue to decrease in schoolchildren but hospitalization rates for wheezing illnesses remain high in young children. *Acta Paediatrica*, 91: 1239-1245.

Wenzel, S. E., Balzar, S., Cundall, M. and Chu, H. W. (2003). Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9:

Association with asthma severity, neutrophilic inflammation, and wound repair. *Journal of Allergy & Clinical Immunology*, 111: 1345-1352.

Wenzel, S. E., Szefler, S. J., Leung, D. Y. M., Sloan, S. I., Rex, M. D. and Martin, R. J. (1997). Bronchoscopic Evaluation of Severe Asthma . Persistent Inflammation Associated with High Dose Glucocorticoids. *American Journal of Respiratory and Critical Care Medicine*, 156: 737-743.

Werner, M., Topp, R., Wimmer, K., Richter, K., Bischof, W., Wjst, M. and Heinrich, J. (2003). TLR4 gene variants modify endotoxin effects on asthma. *Journal of Allergy & Clinical Immunology*, 112: 323-330.

Wilson, N. M., James, A., Uasuf, C., Payne, D. N., Hablas, H., Agrofioti, C. and Bush, A. (2001). Asthma severity and inflammation markers in children. *Pediatric Allergy & Immunology*, 12: 125-132.

Wojnarowski, C., Roithner, B., Koller, D. Y., Halmerbauer, G., Gartner, C., Tauber, E. and Frischer, T. (1999). Lack of relationship between eosinophil cationic protein and eosinophil protein X in nasal lavage and urine and the severity of childhood asthma in a 6-month follow-up study. *Clinical Experimental Allergy*, 29: 926-932.

Wright, A. L. (2002). Epidemiology of asthma and recurrent wheeze in childhood. *Clinical Reviews in Allergy and Immunology*, 22: 33-44.

Wright, A. L., Holberg, C. J., Martinez, F. D., Halonen, M., Morgan, W. and Taussig, L. M. (1994). Epidemiology of physician-diagnosed allergic rhinitis in childhood. *Pediatrics*, 94: 895-901.

Yamazaki, K., Ogura, S., Ishizaka, A., Oh-hara, T. and Nishimura, M. (2003). Bronchoscopic Microsampling Method for Measuring Drug Concentration in Epithelial Lining Fluid. *American Journal of Respiratory and Critical Care Medicine*, 168: 1304-1307.

Young, B., Fitch, G. E., Dixon-Woods, M., Lambert, P. C. and Brooke, A. M. (2002). Parents' accounts of wheeze and asthma related symptoms: a qualitative study. *Archives of Disease in Childhood*, 87: 131-134.

Young, B. and Heath, J. W. (2000) *Wheater's Functional Histology*. Churchill Livingstone, London.

Zemann, B. and Rot, A. (2003). What dogs may teach humans about the vertical transmission of allergy predisposition. *Immunology*, 110: 427-429