Characterisation of gene expression in the airway epithelium of children with asthma

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BSc. (Hons)

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

School of Paediatrics and Child Health

2007
Declaration

This thesis has been completed during my period of candidature for the degree of Doctor of Philosophy at the University of Western Australia. I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that, to the best of my knowledge, the thesis contains no materials previously published or written by another person, except where due reference has been made. This thesis contains published work, some of which has been co-authored. The bibliographic details of the works and where they appear in the thesis are set out below.

Research article – Chapter 4
Lane: 55%, Knight: 10%, Burgess: 5%, Franklin: 5%, Horak: 5%, Legg: 5%, Moeller: 5%, Stick: 10%.

Research article - Chapter 3
Lane: 50%, Burgess: 20%, Kicic: 10%, Knight: 10%, Stick: 10%.

Review article - Chapter 7
Knight: 50%, Lane: 10%, Stick: 40%.

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Catherine Lane

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A/Prof. Stephen Stick
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Abstract

Most information regarding common childhood diseases has come from studies in adults. This is primarily due to the difficulty in obtaining target organ tissue from children. Studies in adults have established a clear role for the airway epithelium in a number of respiratory diseases, but at present there are few data regarding the paediatric epithelium. The current study has addressed this deficit by developing a method to sample the airway epithelium in an unselected population of children. The use of non-bronchoscopic brushing to safely sample useful quantities of epithelial cells for use in multiple investigative techniques was evaluated. Whilst children were anaesthetised and intubated for elective surgery, a small cytology brush was inserted directly into the endotracheal tube and brushed against the airway to sample epithelial cells. Children undergoing brushing experienced no complications. Cells sampled from the airway were identified as 95% epithelial, and were successfully used for immunocytochemistry and extraction of RNA and protein. This thesis has, for the first time, outlined a method which allows the study of multiple aspects of airway epithelial cell biology, using a single sample of epithelial cells, taken quickly and safely from children.

This method was then applied to the investigation of the epithelium in asthma. Of the children recruited for this study, 23% had atopic asthma, 25% were healthy atopic, and 52% were not atopic or asthmatic. The airway epithelium is clearly abnormal in adult asthma; however, it was unclear whether differences in gene or protein expression would be detectable in the epithelium of asthmatic children. This study aimed to determine whether non-bronchoscopic brushing could be used in children to investigate epithelial abnormalities in asthma. Three areas of fundamental importance to asthma were investigated: inflammation, damage and repair.
Firstly, inflammation was investigated by examining eosinophils in BAL fluid, exhaled nitric oxide (FeNO), and epithelial expression of the nitric oxide synthase (NOS) enzymes. Although FeNO was increased in asthmatic children, this was likely due to atopy, rather than asthmatic inflammation, as there was no difference between FeNO in healthy atopics and atopic asthmatics. Neither the number of eosinophils in BAL fluid, nor the expression of NOS in the epithelium provided any evidence of asthmatic inflammation in these children. Secondly, epithelial damage was investigated by examining epithelial cell shedding, and expression of the cellular adhesion molecule CD44. Neither of these markers was increased in the asthmatic children, suggesting that the airway epithelium was not damaged. Finally, epithelial repair was examined. EGF and EGF-R are central to the epithelial repair response, and are over-expressed in severe and adult asthma, yet expression of the genes encoding both of these molecules was significantly decreased in the epithelium of children with mild asthma. Despite this, epithelial proliferation, as indicated by immunoreactivity to PCNA, was increased in the asthmatic children.

These findings demonstrate that bronchial brushing can be used in conjunction with techniques such as real-time PCR and immunohistochemistry, to investigate airway diseases. This study provides evidence for abnormalities in epithelial function in children with asthma, and suggests that mild childhood asthma may have a different aetiology to either severe childhood asthma, or adult asthma. To provide proof of concept, the scope of this research was initially restricted to individual genes and pathways known to be important to asthma. However, in order to extend our knowledge of airway diseases, and identify novel pathways and genes of importance, the possibility of using microarray technology to investigate broader epithelial function was examined.
Gene expression was analysed in 9 asthmatic and 7 healthy children using Affymetrix U133A microarrays. Preliminary data from the microarrays showed that gene expression in epithelial cells from asthmatic subjects was significantly different to that in healthy subjects. Six databases of gene sets were analysed. Significantly enriched gene sets were found in all databases. In total, 50 gene sets were enriched in the asthmatics, and 29 gene sets were enriched in the healthy non-atopics. Based on gene expression, children with mild asthma have significant differences in their airway epithelium as compared to their healthy counterparts. This study has demonstrated that bronchial brushing can be used in conjunction with techniques such as PCR, immunohistochemistry and microarray to examine the airway epithelium in respiratory diseases. Even though the asthmatic children involved in this study had mild, asymptomatic airway disease, a number of novel, and important differences were identified. Further investigation is needed to directly compare this cohort with healthy and asthmatic adults, and also with severely asthmatic children. The microarray data have also presented further opportunity for research, and prompt detailed investigation of the pathways implicated in asthma, using cell culture, real-time PCR, and western blotting.
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<tr>
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</tr>
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<tr>
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</tr>
<tr>
<td>FEV₁</td>
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<tr>
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<td>THE INTERNATIONAL STUDY OF ASTHMA AND ALLERGIES IN CHILDHOOD</td>
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<td>LEUKOTRIENE B₄</td>
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MAP  MITOGEN-ACTIVATED PROTEIN
MCP  MONOCYTE CHEMOATTRACTANT PROTEIN
MIP  MACROPHAGE-INFLAMMATORY PROTEIN
MMP  MATRIX METALLOPROTEINASE
mRNA  MESSENGER RIBONUCLEIC ACID
NIH  NATIONAL INSTITUTE OF HEALTH
nNOS  NEURONAL NITRIC OXIDE SYNTHASE
NO  NITRIC OXIDE
NOS  NITRIC OXIDE SYNTHASE
PBS  PHOSPHATE BUFFERED SALINE
PCNA  PROLIFERATING CELL NUCLEAR ANTIGEN
PCR  POLYMERASE CHAIN REACTION
PDGF  PLATELET-DERIVED GROWTH FACTOR
PGE\textsubscript{2}  PROSTAGLANDIN E\textsubscript{2}
DPX  DIXYLOL-PHTHALATE MOUNTANT
RANTES  REGULATED UPON ACTIVATION, NORMALLY T-EXPRESSED, AND PRESUMABLY SECRETED
RAST  RADIO-ALLERGOSORBENT TEST
RNA  RIBONUCLEIC ACID
RNase  RIBONUCLEASE
RSV  RESPIRATORY SYNCITIAL VIRUS
RT  REVERSE TRANSCRIPTION
SDS  SODIUM DODECYL SULFATE
STAT  SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION
TAMRA  6-CARBOXY-TETRAMETHYL RHODAMINE
TGF  TRANSFORMING GROWTH FACTOR
TIMP  TISSUE INHIBITOR OF METALLOPROTEINASE
TNF  TUMOUR NECROSIS FACTOR
Publications and Conference Proceedings arising from this work

Publications


Conference Proceedings


*This abstract was presented in the Young Investigator Forum at the 2004 annual meeting of the Thoracic Society of Australia and New Zealand, and was awarded the Ann Woolcock Young Investigator Award.
1.0 Literature Review

1.1 Epithelium

Epithelial tissue is found in many forms throughout the body, and performs many different functions. As well as forming the skin that covers the outside of the body, the epithelium lines the inside of many body cavities, including the lung. The epithelium forms a barrier, protecting underlying nerves and smooth muscle, but is also an active participant in the airway. Epithelial cells interact with surrounding cells, and produce complex secretions that are expelled from the airway by ciliary action. The airway epithelium also participates in the inflammatory response, and has the capacity to repair itself following injury. This chapter will provide an overview of epithelial structure and function in health and disease.

1.1.1 Epithelial Structure

There are many different kinds of epithelium, each with a different structure and cellular composition suited for their specialised function. For instance, skin must provide a tough, impermeable barrier, and therefore contains a high proportion of keratinized epithelial cells. Epithelial cells lining the digestive tract have microvilli that greatly increase the surface area of the gut, increasing absorption of nutrients. Although there are at least eight morphologically distinct epithelial cell types in the respiratory epithelium, they may be grouped into three categories: ciliated, secretory and basal [1]. These vary in abundance throughout the lung: in the proximal airways, (the main bronchi) the epithelial cell layer is thicker, and composed of tall, ciliated cells, basal cells, and secretory cells known as goblet cells. As the airways branch out and become smaller, the epithelial layer becomes thinner, until at the level of the bronchioles, it is composed of a single layer of short cuboidal cells (Figure 1-1).
Figure 1-1: Structure of the epithelium throughout the airways. The proximal airways are lined with pseudostratified columnar epithelium. As the airways branch out and become narrower, the height of the epithelium decreases, until, at the level of the bronchioles, it is composed of short cuboidal cells. Adapted from Laitinen et al (1).

The majority of cells in the epithelium are ciliated; these account for over 50% of all epithelial cells (2). Ciliated epithelial cells typically possess up to 300 cilia per cell, and many mitochondria directly beneath the apical service, energising the cilia to allow directional transport of mucus from the lung to the throat (3).

Goblet cells are glandular epithelial cells characterized by membrane bound mucin granules, which are secreted to trap inhaled foreign objects (4). Mucins are produced in increased quantities in response to acute exposure to stimuli such as tobacco smoke (5). In normal human trachea, there are approximately 6800 mucus-secreting cells per mm² of surface epithelium. However, in inflammatory airway diseases such as asthma, there is an increase in the number and size of mucous cells (6).

Basal cells are found throughout the airway epithelium, although they are more numerous in the larger airways (7). In addition, there is a direct correlation between epithelial thickness and the number of basal cells. The basal cell is generally accepted to be the primary stem cell, giving rise to the mucous and ciliated epithelial cells (8). In addition, basal cells are the only epithelial cell type that is firmly attached to the
basement membrane (9). As such, they play an important role in the attachment of other cells to the basement membrane (10).

Pulmonary neuroendocrine cells (PNEC) are specialized epithelial cells found throughout the airway either alone or in clusters (2). Recent studies have shown that PNECs are more abundant than initially thought, and that they are not homogenously distributed within the epithelium: densities range from 65/mm$^2$ to patches of 250/mm$^2$ (11). These cells secrete a variety of biogenic amines and peptides, and are thought to be involved in regulating localized epithelial cell growth and regeneration (12).

In addition to the resident structural cells, a variety of cells migrate to and reside within the epithelium. Cells involved in the immune response and its reactions may migrate through the epithelial basement membrane: some of these remain within the surface epithelium, whereas others are in the process of passing through to the lumenal surface (13). Although neutrophils are present within the airway lumen of healthy individuals (14), they are greatly increased in conditions such as the late-phase of asthma (15). Monocytes and macrophages are also present within the airways under normal conditions (16). Lymphocytes are also found in healthy airways, occasionally forming aggregates sometimes referred to as bronchial-associated lymphoid tissue (16). Finally, eosinophils and mast cells can be present in increased numbers in both airway wall and lumen, most notably in asthma (17).

Epithelial cells are joined to each other by junctional complexes. These bind the cells together, permit intercellular communication, and form a barrier which regulates the passage of water and solutes from the lumen across the epithelium. Junctional complexes consist of tight junctions, adherens junctions and desmosomes (Figure 1-2).
In addition to these junctions, the gap junction forms an intercellular channel that allows the exchange of ions and small molecules between adjacent cells. Epithelial cells are not only attached to each other, but are also anchored by hemidesmosomes to the basement membrane, a thin sheet of collagen, laminin and fibronectin.

![Diagram](image)

**Figure 1-2:** Epithelial cells are connected by junctional complexes. Junctional complexes consist of tight junctions, adherens junctions and desmosomes. Gap junctions allow the exchange of ions and small molecules. Epithelial cells are anchored by hemidesmosomes to the basement membrane.

Epithelial tissues can be classified on the basis of their structure. Stratified epithelium consists of more than one layer of cells. For instance, the oesophagus is lined with stratified squamous epithelium consisting of flattened (squamous) cells on the surface overlying multiple layers of cells that are more cuboidal. On the other hand, blood vessels are lined with simple endothelium, consisting of a single layer of cells. In the proximal airways, the epithelium is pseudostratified. Although there appears to be a layer of ciliated columnar cells overlying basal cells, each cell is in fact anchored to the basement membrane.
1.1.2 Epithelial barrier function

Although the airway epithelium is an internal tissue, it is constantly exposed to particulates, viruses, bacteria, viruses, pollen, allergens, oxidants, and other potential hazards to the lung. The epithelium acts as a barrier, protecting the highly sensitive underlying smooth muscle and sensory nerves from stimulation by these agents. Overlying the epithelium is a mucus layer which traps inhaled particles (18). Epithelial cilia propel the mucus layer upwards (the mucociliary escalator) towards the throat, where it is expectorated or swallowed. The mucus layer also contains antioxidants in sufficient quantities to protect the lung from inhaled oxidants. After this first line of defence, the epithelial tight junctions, which play a major role in maintaining epithelial integrity, are also able to restrict the movement of molecules across the epithelium. Desmosomes, intermediate junctions, and gap junctions also contribute to the structural integrity of the epithelium. Finally, epithelial cells are capable of internalising particles (19) to clear them from the lung.

1.1.3 The epithelium plays an active role in the airway

Although the epithelium was traditionally thought of as a simple barrier, it is also an essential and active participant in the healthy lung. Firstly, the epithelium plays a central role in maintaining airway homeostasis. Secondly, the epithelium is in a key position to respond to inadvertently inhaled agents such as allergens and microorganisms, and intentionally inhaled agents such as inhaled therapy. These functions are accomplished through the actions of a number of mediators released by the epithelium (Table 1).
<table>
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<th>Actions</th>
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Epithelial involvement in airway homeostasis

The airway epithelium plays a central role in airway homeostasis by regulating airway tone, maintaining the epithelial barrier function, and producing airway surface liquid. Epithelial cells can directly influence underlying smooth muscle by releasing an array of bronchoconstrictors and bronchodilators. Airway epithelial cells produce prostaglandin \( \text{E}_2 \), which relaxes human airway smooth muscle (20) and nitric oxide (21), which has vasodilatory and bronchodilatory activity. Conversely, epithelial cells also release bronchoconstrictors such as endothelin (22) and arachidonic acid metabolites such as leukotriene \( \text{B}_4 \) (\( \text{LTB}_4 \)) (23) which are potent constrictors of airway smooth muscle (24). In addition to producing mediators which influence airway tone, the epithelium metabolises pre-existing modulators of airway tone. Inflammatory cells produce mediators such as kinins, endothelin and angiotensin, which are potent bronchoconstrictors (25). Epithelial enzymes such as neutral endopeptidase are responsible for the breakdown of tachykinins, bradykinin, endothelin, and angiotensins I and II (26), thereby maintaining airway homeostasis.

Epithelial involvement in the inflammatory response

The first point of contact for inhaled pathogens and microbes is the airway epithelium, therefore it is essential that the epithelium is capable of recognising pathogens, so that the immune system can respond appropriately. Epithelial cells express a variety of toll-like receptors, a family of pattern recognition receptors (27). The toll-like receptors (TLR) are key receptors of the innate immune system, and activate a protective host response to microbial pathogens (28). Toll-like receptors recognise molecules termed ‘pathogen-associated molecular patterns’ such as lipopolysaccharide, which is a ligand for TLR-4 (29). Upon binding of the specific ligands, molecules such as nuclear transcription factor kappa-B (NF-\( \kappa \)B), MyD88 and mitogen-activation protein kinases
(MAP), are activated, leading to the production of a broad spectrum of cytokines to signal cells of the immune system (30).

Airway epithelial cells can also be activated by inhaled allergens and particulates to produce pro-inflammatory mediators. The epithelium is directly activated when the inhaled substances interact with the epithelium itself. Antigens such as the house dust mite allergens Der p1 and Der p9 induce epithelial release of the proinflammatory mediators granulocyte-macrophage colony stimulating factor, interleukin (IL)-6, and IL-8 (31). Diesel exhaust particles (19), bacterial endotoxins (32) and pollutants such as NO2 (33) also activate epithelial cells and cause increased release of pro-inflammatory mediators. Alternatively, indirect activation can occur when foreign antigens cause macrophages or other inflammatory cells to release cytokines that activate the epithelium. Macrophages stimulated by allergens and viruses release cytokines such as TNF-α, IL-1β and IL-6 (34), which in turn affect the epithelium.

Stimulation of the airway epithelium can result in recruitment of inflammatory cells, through the release of chemoattractant cytokines. For example, lipopolysaccharide induces release of LTB4, a potent neutrophil chemoattractant (23). Indirect activation of epithelial cells by TNF or IL-1, causes them to release RANTES, a cytokine with potent chemotactic activity for monocytes (35) and eosinophils (36). Epithelial cells also release IL-8, a major neutrophil chemoattractant (37).

Epithelial cells can also enhance the inflammatory response. The epithelium can delay the clearance of inflammatory cells from the lung, or prevent inflammatory cell apoptosis. ICAM-1, expressed on epithelial cells, is a ligand for integrins expressed on the surface of neutrophils, and leads to their retention in the airway (38). In addition,
the epithelium is capable of prolonging the life of neutrophils in the airway by releasing G-CSF and GM-CSF, cytokines which promote neutrophil survival (39).

1.1.4 Epithelial damage

Despite the capacity of the epithelium to protect the lung from endogenous mediators and inhaled stimulants, epithelial damage still occurs. Damage to the epithelium may be caused by external factors such as particulates and viruses, or inadvertently through the response of the inflammatory system to inhaled stimulants. Seemingly inert particulate matter, such as dust and diesel exhaust particles, induces apoptosis, DNA damage and epithelial cell death (40). The mechanisms by which particulates exert damage are not clear, but it is possible that toxic agents associated with the particulate matter are the true cause of damage (41). Pollens release a variety of proteases which cause epithelial cell detachment and disrupt epithelial barrier function (42). Other allergens such as the house dust mite allergen Der p1 induce epithelial cell detachment and increase epithelial permeability (43), most likely due to their associated proteinase activity (44).

Damage to the epithelium may also be caused by factors produced by the lung. When inhaled agents are not cleared from the lung, they remain and provoke an inflammatory response. Mediators produced by the influx of inflammatory cells to the lung can then damage the epithelium. Although the epithelium produces protective anti-oxidants, reactive oxygen species produced by eosinophils, mast cells, and macrophages still cause damage (45). Eosinophils recruited to the airways release major basic protein, eosinophil cationic protein, and eosinophil peroxidase, all of which injure the epithelium (46). Neutrophil elastase also significantly disrupts the epithelium, decreasing ciliary beat frequency and causing epithelial detachment (47). Finally,
factors synthesized by the epithelium itself, such as nitric oxide, can be destructive if present in sufficiently high concentrations (48).

1.1.5 Epithelial proliferation and repair

The airway epithelium plays a central role in maintaining normal respiratory function, and must respond quickly to injury. A number of studies have investigated repair and restitution of the epithelium, using various models of epithelial damage. Experimental damage is easily induced in epithelial cultures, either mechanically by scrape wounding (49), or chemically using hydrogen peroxide (50). The selective loss of columnar epithelial cells has been investigated in isolated human and guinea pig airways using a drop of glue applied to the epithelial surface of the airways to remove columnar epithelial cells (51). Investigating epithelial damage and repair in vivo is significantly more difficult, however a method was developed by Erjefalt et al which used a steel probe to remove a strip of epithelial tissue in guinea pigs (52). Both in vivo and in vitro models have yielded valuable insights into the process of epithelial repair.

The epithelium responds rapidly to damage

The epithelium responds very quickly to damage. Following wounding, secretory and ciliated cells surrounding the wound site dedifferentiate, flatten, and migrate rapidly to cover the area (53). Within 8 to 15 hours, a new, flattened epithelium covers the wound (Figure 1-3). These cells are undifferentiated, and in comparison to normal epithelium are only loosely connected to each other. However, by 30 hours post-wounding, a tight epithelial barrier is established, and after five days the epithelium has redifferentiated to contain ciliated and secretory cells (53). The speed of epithelial repair was underestimated by studies using cultured epithelium and isolated trachea (54, 55), suggesting that the factors required for rapid changes are supplied by local tissues such as the nerves, or microcirculation (53).
Figure 1-3: The epithelial response to damage. Adapted from Holgate et al (56).

**Proliferation of epithelial cells follows migration**

Although the initial repair of the epithelium occurs through the migration of existing cells into the wound site, proliferation must occur to reconstitute the epithelial barrier. In normal, intact epithelium, proliferation occurs in only a few scattered cells. However, in damaged epithelium, proliferation increases significantly after the damaged area is covered by a new, flat and well-connected epithelium. Proliferation also increases in areas of epithelium surrounding the wound site, but to a lesser degree (53, 57). Increased proliferation is not restricted to the epithelium; mitotic activity also increases in subepithelial cells (57).

**Epithelial stem cells**

The concept of a single stem cell capable of giving rise to all cell types in all regions of the lung has not been widely accepted (58). Studies on the response of the lung to injury appear to show that the lung makes use of several different cell types for
homeostasis and repair, although the basal cell has been proposed as a pluripotent stem cell. In the distal lung, where there are no basal cells, evidence suggests that specialised Clara cells in specific micro-environments can divide and also form new ciliated and nonciliated cells (59). In the alveoli, damaged type I cells can be restored from type II cells, suggesting that type II pneumocytes are the alveolar stem cell (60).

In the proximal lung (trachea and main bronchi), undifferentiated basal cells can function as classical stem cells; they can both replicate themselves and give rise to ciliated and secretory cells. Basal cells have a histological appearance midway between ciliated and goblet cells (16), and are observed to accumulate at sites of injury (61). In juvenile rat epithelium, basal cells take up 3H-thymidine before goblet or ciliated epithelial cells (62). In vitro models of tracheal denudation in rats and rabbits have shown that the basal cells can give rise to all major tracheal cell types (63-65). In addition, an investigation of epithelial regeneration in mechanically injured rat trachea revealed that under these conditions basal cells dedifferentiate into a highly proliferative cell phenotype from which a mucociliary epithelium redifferentiates. Basal cells have also been shown to be the progenitors of primary rat tracheal (66, 67) and human bronchiolar epithelial cell cultures (68). Finally, basal cells, along with parabasal cells, account for 84% of proliferating cells in the epithelium of the conducting airways (8), underscoring the stem-cell role of these cells.

**Mediators involved in epithelial repair**

Epithelial repair is achieved by cellular migration, proliferation and differentiation, orchestrated by the expression of a number of mediators. Expression of these mediators is evidence of the altered phenotype of the repairing epithelium, and while several have been identified, the repair process remains poorly characterised. The epidermal growth factor receptor and its ligands have been identified as central to the repair process, as
epidermal growth factor (EGF) is a potent mitogen for bronchial epithelial cells (69), mechanical damage of the epithelium induces rapid phosphorylation of EGF receptor (EGF-R) (49), and epithelial wound closure is enhanced by EGF (49). The role of EGF and its receptor is covered in detail in section 5.1. Airway epithelial cells in isolation are capable of producing the autocrine mediators necessary to achieve epithelial repair (49). However, the process of epithelial repair is considerably faster in the presence of other cell types (70), emphasizing the role of paracrine signalling.

1.2 The role of the epithelium in airway disease

The airway epithelium provides an important barrier function, protecting underlying tissues from stimulation and damage from inhaled agents. The epithelium is also an active participant in the airway, as it plays an essential role in maintaining airway homeostasis, and is involved in the airway inflammatory response. Primary defects in epithelial cells, or in their response to the external environment have the potential to exacerbate or cause diseases such as cystic fibrosis, primary ciliary dyskinesia, chronic obstructive pulmonary disease, and asthma.

1.2.1 Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease, resulting from a defect in the gene encoding the cystic fibrosis transmembrane conductance regulator (71). Initially, the major clinical manifestation of CF was pancreatic failure, however pancreatic symptoms can now be controlled through the use of oral pancreatic enzyme replacement therapy, making CF primarily a respiratory disorder. Individuals with CF exhibit impaired mucociliary clearance, predisposing them to recurrent bronchial infections, which progressively destroy the lung and lead to respiratory failure.
The airway epithelium is central to the respiratory component of CF. Transepithelial electrolyte transport controls the quantity and composition of the epithelial lining fluid, and is necessary for normal mucociliary clearance. In CF, the epithelium is relatively impermeable to chloride ions, but demonstrates increased sodium ion absorption, resulting in thick, dehydrated airway secretions which predispose the lung to infection (71). In addition, epithelial expression of inducible nitric oxide synthase (72), and levels of exhaled nitric oxide (73) are decreased in cystic fibrosis. As exhaled nitric oxide is an effective bactericidal agent (72), reduced expression of this molecule may contribute to the increased susceptibility to infection seen in cystic fibrosis. Further study of the epithelium is needed to determine how a defect in epithelial chloride transport leads to the variety of airway symptoms associated with cystic fibrosis.

1.2.2 Primary Ciliary Dyskinesia

Primary ciliary dyskinesia (PCD) is a disease of the upper and lower respiratory tract resulting from abnormal structure and function of cilia (74). Symptoms can be relatively mild, although the condition has a well recognized morbidity. PCD is actually an inclusive term for a number of disorders that result from ciliary defects. Airway cilia must beat in a co-ordinated fashion in order to effect mucociliary clearance. However, in PCD, ciliary motility is impaired, resulting in delayed mucociliary clearance and therefore recurrent infections and bronchiectasis (75). To improve screening and diagnosis of PCD, additional research needs to be conducted in the epithelium. Furthermore, identification of the genes responsible for PCD would allow the development of improved treatment.
1.2.3 Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation leading to fixed narrowing of the small airways and alveolar wall destruction (76). Individuals with COPD experience gradual loss of lung function, and symptoms such as chronic cough, excess sputum production, and shortness of breath. The most important risk factor for development of COPD is smoking, and the inflammation and proteolysis in COPD appears to be an amplification of the normal inflammatory response to cigarette smoke (77).

The role of the airway epithelium in COPD is not clear, although epithelial expression of fibrogenic mediators such as TGF-β (78) and inflammatory mediators such as IL-6 and IL-8 (79) is increased in COPD. This evidence has led to the hypothesis that COPD may be associated with enhanced airway epithelial cell activation, particularly at exacerbation. However, to date the role of the epithelium in modulating inflammatory processes in COPD has not been identified, and further investigation is required to establish the contribution of the epithelium to the development and progression of COPD, and its full potential as a therapeutic target.

1.2.4 Asthma

Asthma is a chronic respiratory disease characterized by inflammation of the airways resulting in wheezing and dyspnea. Originally, the main component of asthma was thought to be bronchial hyperresponsiveness, and most therapy was directed at controlling bronchospasm. However, evidence implicating the epithelium in asthmatic disease is accumulating, and many structural and biochemical aspects of airway epithelial cell biology have been found to be abnormal. The following sections discuss asthma, and the role of the airway epithelium in asthmatic disease.
1.3 Asthma

1.3.1 Defining asthma

Although asthmatic disease was described as early as the second century AD (80), the most current definition of asthma was not developed until 1997 (81). A Greek physician first described the symptoms of an asthma attack, detailing difficulty breathing and increased symptoms at night. In the 17\textsuperscript{th} century, the difficulty in breathing was linked to reversible narrowing of the airways (82). Subsequent studies identified other features of asthma, such as mucous plugging of the airway and thickening of the airway walls (83). However, until recently, the fundamental component of asthma was considered to be bronchial hyperresponsiveness, and therefore most treatment was directed at controlling the bronchospasm that occurs during an asthma attack (84). However, classic pathological studies by Huber and Koessler (85) and Dunnill (83) defined inflammation in asthma, and introduced the concept of asthma as an inflammatory disease. Subsequent studies using bronchoalveolar lavage (86) and bronchial biopsies (87) have confirmed that inflammation is a prominent feature of the asthmatic lung. The central component of asthma is now considered to be inflammation associated with bronchospasm. In keeping with this, the National Institute of Health (NIH) issued the following definition of asthma in 1997:

“Asthma is a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils, and T lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough in early morning. These symptoms are usually associated with widespread but variable airflow limitation that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli.” (88)
1.3.2 Epidemiology

Asthma is one of the most common diseases of childhood, and affects approximately 1 in 15 children worldwide (89). There are numerous studies from various countries which have investigated the prevalence of asthma in the community, however, the ‘true’ prevalence of asthma is very difficult to determine. Asthma is not easily defined, and not all studies use the same criteria. This has resulted in a wide range of estimates of asthma prevalence. Despite this, some interesting trends in asthma prevalence have been revealed. In 1998, the International Study of Asthma and Allergy in Childhood (ISAAC) investigated 463,801 13-14 year-old children (90). One of the major findings was that asthma prevalence was highest in Western countries such as Australia, New Zealand, and the United Kingdom. This and other studies have demonstrated a clear link between Western lifestyle and asthma prevalence (91). The prevalence of asthma is high in Australia compared with other countries (92). The 2004-5 National Health Survey found that 10.3% of the Australian population had current asthma (93); although the prevalence of asthma in Western Australia was slightly higher than the national average, this difference was not statistically significant. The prevalence of asthma in children was higher: 12.3% of children aged 0-17 reported having current asthma (93).

Another trend observed by epidemiological studies is the increase in asthma prevalence over time. Again, this is the subject of much debate, as it is unclear whether the trend represents a real increase in the prevalence of asthma, or simply an increase in diagnosis of asthma. However, recent studies, using a standardised approach to asthma diagnosis, confirm that the prevalence of asthma is increasing. In Australia alone, the prevalence of paediatric asthma increased by 141% between 1964 and 1990 (94).
At present, the factors driving these trends have not been determined. However, the reasons for increasing asthma prevalence may lie in the causes of asthma. Several theories relating to increased exposure to sensitizing allergens, and reduced stimulation of the immune system during critical periods of development, have been proposed (95). Childhood is a crucial period of development for both the immune and respiratory systems. The initial programming of the adaptive immune system occurs in early childhood (96), alongside intensive growth and remodelling of the lung and airways. Further investigation of paediatric asthma is urgently required to define the factors which initiate the disease.

### 1.3.3 Pathogenesis of asthma

Asthma is a complex and multifactorial disease, involving interaction between genetic factors and environmental stimuli. The majority of researchers have focused on the development of atopic asthma to identify factors involved in asthma pathogenesis, as asthma is strongly influenced by atopic status. However, not all asthma is atopic, and characteristic asthmatic disease also occurs through non-atopic inflammation. Genetics, *in utero* environment, maternal and infant diet, respiratory infections and occupational and environmental exposures have all been implicated as contributors to the development of asthma (97).

Several risk factors for the development of asthma have been identified. *In utero* exposure to agents such as antigenic proteins (98) and components of tobacco smoke (99, 100) in maternal circulation significantly increases the risk of the child developing asthma. Although asthma is not heritable, genetics are clearly involved in the development of asthma (101). Whilst major susceptibility genes for asthma have not yet been identified (102), a number of genetic loci and candidate genes are associated with asthma phenotypes. However, interactions of particular genes with the
environment are likely to be more important still in determining asthma initiation. Viral infections during childhood, especially RSV (103), increase the risk of developing asthma. However the influence of respiratory infections on the development of asthma is complex, as some infections are associated with a decreased risk of atopy and asthma (104, 105), and because children with viral-associated asthma tend to outgrow their symptoms (95). The myriad risk factors for asthma seem to suggest that any process resulting in airway inflammation during critical stages of lung development has the potential to result in persistent asthma. Therefore, investigation of the changes that occur in the asthmatic airway during childhood is also required.

1.3.4 The asthmatic airway

The asthmatic airway is characterized by a number of cellular and structural changes (106). Initially, these changes were noted in the lungs of patients who had died of asthmatic disease (107), but subsequent studies using biopsy and bronchoalveolar lavage noted significant abnormalities in the lungs of patients with mild and severe asthma (108, 109). Inhaled allergens that are not expelled through mucociliary clearance are taken up by antigen presenting cells which migrate to the draining lymph nodes and present the processed allergen to allergen-specific T and B cells (110). Activation of T-cells leads to the production of cytokines that induce B cells to produce immunoglobulin (Ig)E (111). These IgE antibodies then circulate in the blood before binding to high-affinity Fc receptors on mast cells. Re-exposure to the allergen then triggers the mast cells to release histamine and synthesise prostaglandins, leukotrienes and cytokines (112).

These mediators cause the early-phase asthmatic reaction, characterised by airway smooth muscle contraction, vascular leakage, mucus secretion, enhanced airway
hyperresponsiveness (AHR) and recruitment of inflammatory cells (113). Onset of the early-phase reaction is immediate, and lasts 30-60 minutes. Approximately 6 to 9 hours following allergen provocation, the late-phase reaction occurs. This involves the recruitment and activation of eosinophils (114), CD4+ T-cells (115), basophils (116), neutrophils (117) and macrophages (118). There is selective retention of airway T cells, expression of cellular adhesion molecules, and the release of proinflammatory mediators and cytokines involved in inflammatory cell recruitment and activation (119). The late-phase is characterized by excessive inflammation of the airways, resulting in structural changes known as airway remodelling (120).

Chronic asthma is also characterized by several cellular and structural changes to the airway. Inflammatory cells such as degranulated eosinophils, neutrophils, lymphocytes, activated macrophages, and partly degranulated mast cells are found in increased numbers (87). Structural changes are present in the airways of both mild and severe asthmatics. The asthmatic airway wall appears thickened (120). The airway wall is composed of many interrelated structural components such as connective tissue, vessels, muscle, mucus-secreting glands and epithelium, which are altered in asthma. The asthmatic reticular basement membrane is thickened (121). This was previously thought to be due to the increased deposition of type I, II and V collagen (122), but recent data suggests that reticular basement membrane thickening is due to exaggerated production of normal material, rather than deposition of interstitial collagen associated with fibrosis (123). Dilation of airway wall blood vessels, and vascular congestion contribute to asthmatic disease by increasing the stiffness of the airway (124). There is an increase in smooth muscle mass in the asthmatic airway, which is attributed to increased numbers of fibroblasts and myofibroblasts (125). Mucous secreting glands are increased in size (126). All these features lead to a thickened airway wall, and
markedly reduced airway calibre. Finally, biopsy studies of the airway of asthmatics have discovered widespread shedding of epithelial cells (127), and those cells that remain appear swollen, vacuolated, and there is often loss of cilia (128).

1.3.5 Irreversible airway obstruction

Although the importance of bronchospasm and inflammation in asthma has long been recognized, these processes do not fully explain all aspects of the disease. There are many asthmatics with symptoms that do not respond to traditional anti-inflammatory therapy (129). In addition, asthmatics generally experience an accelerated decline in respiratory function (130, 131), as demonstrated by a study which investigated 92 asthmatics and 186 healthy controls over a period of 18 years. Asthmatic patients in this cohort experienced a greater decline in airway function (FEV1) than did control subjects (50 mL per year vs. 35 mL per year) (131). These studies suggest that inflammation alone does not account for all features of asthmatic disease.

In addition to the reversible and inflammatory changes seen in the airways of asthmatics, there are irreversible structural changes termed remodelling. These structural changes involve many cells and tissues in the lung including the airway wall, the lamina reticularis, smooth muscle, myofibroblasts, and mucus producing cells. All components of the airway wall are thickened in asthma (132). Several factors contribute to airway thickening; these include an increase in airway smooth muscle, airway edema, inflammatory cell infiltration, increase in the size of airway glands, and connective tissue deposition. In combination, these components increase airway narrowing caused by smooth muscle stimulation, and therefore promote bronchospasm and hyperresponsiveness.
Initial studies of the asthmatic airway detected what was believed to be a thickening of the basement membrane (133). However, subsequent studies determined that the true basement membrane (the lamina densa and rara) is normal in asthmatics, and that the observed thickening was the result of a dense fibrotic response occurring in the lamina reticularis (134). This subepithelial fibrosis is accompanied by an increase in the accumulation of fibronectin and type I, III, and V collagen. Subepithelial fibrosis is most marked in severe asthma, and is associated with decline in FEV₁ (135). However, evidence of subepithelial fibrosis has also been found in mild asthma (122).

Increased myocyte muscle mass is another characteristic of asthmatic airway remodelling. Various studies have investigated airway smooth muscle, and implicated either hyperplasia alone, or both hyperplasia and hypertrophy of myocytes in airway remodelling (120). Regardless, this process certainly contributes to airway hyperresponsiveness and bronchospasm in asthma (136). Myofibroblasts are also involved in airway remodelling. These cells are present in increased numbers in the asthmatic airway (137), and are major producers of collagenous and non-collagenous matrix molecules which also contribute to airway remodelling. Finally, mucous hypersecretion, mucous metaplasia, and airway obstruction due to bronchial mucous plugging are well documented aspects of chronic asthma and status asthmaticus (6).

Airway remodelling was first described in fatal asthma (85) by Huber and Koessler, but has since been noted in both severe and mild asthma (119). Although the extent of airway remodelling appears to be related to asthma severity, it is not dependent on degree of atopy, or length of asthmatic history (135). The importance of this finding is emphasized by investigations of the airway of asthmatic children, which have provided evidence of airway remodelling in the early stages of mild asthma (138), and tissue restructuring up to four years before the onset of asthma symptoms (139). Although
reversible airway obstruction can develop independently of reticular basement membrane thickening (140), early remodelling of the airway in asthma suggests that it may occur concurrently with inflammation (141).

1.3.6 Causes of airway remodelling

The processes leading to airway remodelling are poorly defined, due to various difficulties facing researchers in this area. Obtaining tissue for study from human subjects is ethically and practically challenging. To study the onset and progression of airway remodelling, it would be ideal to obtain repeated tissue samples over the course of the disease, but this is even more complicated. Any study of asthma is further hampered by the difficulty in determining which cytokines and mediators are specific to the chronic, rather than the acute response to asthma. Finally, the relationship between inflammation and remodelling appears to be highly complex: chronic inflammation occurs in a number of respiratory diseases, but does not always result in airway remodelling as seen in asthma.

Generally, investigations of airway remodelling have focused on the process of subepithelial fibrosis, and the identification of causes. Using this approach, a number of factors of potential importance in the remodelling process, such as TGF-β, GM-CSF and EGF (142-144) have been identified. Mediators that regulate fibroblast proliferation and matrix production are also excellent candidates for involvement in the remodelling process. In addition, the matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) have received much attention, as the balance between MMPs and TIMPs is thought to play a critical role in the synthesis or degradation of the airway extracellular matrix (145, 146). Several cytokines and growth factors, known to be increased in the asthmatic lung, are capable of influencing fibrosis
and matrix production. These include PDGF, IL-1, TNF, IL-5, IGF-1, endothelin-1, tryptases, and leukotrienes (120). Investigation of such Th-2 type cytokines as IL-13, IL-9, IL-4, and IL-5 has demonstrated their ability to induce eosinophilia, mucus metaplasia, subepithelial fibrosis, airway obstruction and airway hyperresponsiveness. These studies suggest that the remodelling response in asthma is likely caused by the combined actions of multiple mediators (120).

1.4 The asthmatic epithelium

Many aspects of the epithelium appear to be abnormal in asthma. The asthmatic epithelium exhibits increased levels of stress, inflammation and damage. This state is marked by the altered expression of a number of mediators produced by the epithelium (table 2).
Table 2: Mediators released by the asthmatic epithelium in altered quantities

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Modification in asthma</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>Increased</td>
<td>(147)</td>
</tr>
<tr>
<td>Activator protein-1</td>
<td>Increased</td>
<td>(148)</td>
</tr>
<tr>
<td>STAT-1</td>
<td>Increased</td>
<td>(149)</td>
</tr>
<tr>
<td>STAT-6</td>
<td>Increased</td>
<td>(150)</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat shock protein 70</td>
<td>Increased</td>
<td>(151)</td>
</tr>
<tr>
<td>p21</td>
<td>Increased</td>
<td>(69)</td>
</tr>
<tr>
<td><strong>Adhesion molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>Increased</td>
<td>(152)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Increased</td>
<td>(153)</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Increased</td>
<td>(154)</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Increased</td>
<td>(155)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Increased</td>
<td>(156)</td>
</tr>
<tr>
<td>IL-11</td>
<td>Increased</td>
<td>(157)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Increased</td>
<td>(158)</td>
</tr>
<tr>
<td>IL-16</td>
<td>Increased</td>
<td>(159)</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>Increased</td>
<td>(160)</td>
</tr>
<tr>
<td>RANTES</td>
<td>Increased</td>
<td>(161)</td>
</tr>
<tr>
<td>IL-8</td>
<td>Increased</td>
<td>(162)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Increased</td>
<td>(163)</td>
</tr>
<tr>
<td>MCP</td>
<td>Increased</td>
<td>(163)</td>
</tr>
<tr>
<td><strong>Growth Factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>Increased</td>
<td>(164)</td>
</tr>
<tr>
<td>EGF, EGF-R</td>
<td>Increased</td>
<td>(49)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Increased</td>
<td>(165)</td>
</tr>
<tr>
<td><strong>Reactive Oxygen Species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peroxynitrate</td>
<td>Increased</td>
<td>(166)</td>
</tr>
<tr>
<td><strong>Gases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Increased</td>
<td>(167, 168)</td>
</tr>
<tr>
<td><strong>Peptide mediators</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelin</td>
<td>Increased</td>
<td>(169)</td>
</tr>
<tr>
<td><strong>ECM proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Increased</td>
<td>(170)</td>
</tr>
<tr>
<td>mmp-9, TIMP-1</td>
<td>Increased</td>
<td>(145)</td>
</tr>
</tbody>
</table>
1.4.1 The asthmatic epithelium is stressed

Asthmatic epithelial cells not only appear more ‘stressed’, they are more susceptible to oxidant-induced stress and apoptosis (171). Epithelial stress is manifest as increased expression and activation of various proteins. Transcription factors regulate the expression of a wide range of genes involved in immune and inflammatory responses, and can be activated by mechanical (172) or oxidative (173) stress. In asthmatic epithelium, there is increased activation of transcription factors such as nuclear factor-κB (147), activator protein-1 (148), signal transducer and activator of transcription (STAT)-1 (149) and STAT-6 (150). Heat shock proteins are induced under conditions of environmental stress: in asthma, epithelial expression of heat shock protein 70 is increased (151). The cyclin dependent kinase inhibitor p21 protects the lung (174), and is increased in response to oxidative stress: in the asthmatic epithelium expression of p21 is increased (69).

1.4.2 Inflammation in the asthmatic epithelium

As discussed earlier (section 1.1.3), the epithelium plays an active role in the airway, and participates in the inflammatory response. The asthmatic epithelium is clearly inflamed, and is a major source of inflammatory cytokines and mediators (175). In asthma, the epithelium releases increased amounts of proinflammatory cytokines such as IL-1β, IL-6, IL-11, GM-CSF, IL-16 and IL-18 (25). There is also increased expression of chemokines, including eotaxin (160), RANTES (161), IL-8 (162), MIP-1α and MCP (163). These play an important role in inflammation by recruiting eosinophils, neutrophils, basophils, lymphocytes, monocytes and T-cells to the airway.

The epithelium is also an important source of peptide mediators and small molecules. Endothelin-1 is a peptide mediator with potent bronchoconstrictor activity. Endothelin-
1 is increased in the asthmatic airway epithelium (169), and exerts its effects on the airway by constricting airway smooth muscle. Endothelin-1 may also affect the long term progression of asthma (176). Nitric oxide (NO) is a small molecule which exists as a gas, and is detectable in exhaled air. NO is produced by nitric oxide synthase (NOS) in the airway epithelium (168), and is found in increased concentrations in inflammatory lung diseases including asthma (167). This is most likely due to the increased expression of inducible NOS in the airway epithelium of asthmatics (177).

1.4.3 The asthmatic epithelium is damaged

The asthmatic airway epithelium is damaged, and exhibits increased susceptibility to injury compared to normal epithelium (178, 179). The structural integrity of the asthmatic epithelium has been investigated using techniques such as bronchial biopsy and BAL. In biopsies from asthmatic subjects, areas of epithelial damage are frequently observed (49), and occur at all levels of the airway (127). Although it has been proposed that the epithelial damage seen in asthma is artefactual (180), expression of markers of damage such as CD44 (152) and EGF-R (49) suggest that the observed damage is genuine.

Studies using BAL have found increased numbers of epithelial cells in BAL fluid from asthmatic patients (181, 182), either singly or in groups called creola bodies. Shedding of columnar, but not basal epithelial cells is common in asthma (152). The ciliated cells appear to be the most destroyed cell type in the asthmatic epithelium (127), and are significantly less viable than those obtained from healthy subjects (183).

The mechanisms causing epithelial cell shedding are yet to be determined (184). Agents such as allergens (44), inflammatory cell products (46), and NO (48, 185) are
involved in asthmatic disease, and have been shown to cause epithelial damage. However, it is uncertain whether they are responsible for the shedding of apparently viable cells in asthma (186). Given that shedding of epithelial cells occurs along a suprabasal plane (181), it is likely that abnormalities in epithelial cell tight junctions (187) play a role in epithelial shedding in asthma.

1.4.4 Repair of the asthmatic epithelium

It is vitally important that the epithelium repairs itself in response to injury. However, in asthma it appears that epithelial repair processes are compromised, and the epithelium is maintained in a repair phenotype. In the asthmatic epithelium, expression of the EGF-R is markedly increased, especially in areas where columnar cells have been shed (188). Furthermore, increased expression of EGF-R in asthmatics occurs even in areas of intact epithelium (49).

In normal bronchial epithelium, less than 5% of cells are proliferating at any given time. However, when the epithelium is damaged, proliferation must increase in order to achieve repair. Despite this, and despite the increased repair signals, proliferation is not increased in the asthmatic epithelium (189, 190). The expression of EGF-R does not correlate with the proliferative response of repairing cells (49). However, the expression of the cyclin-dependent kinase inhibitor p21 is increased in the asthmatic epithelium (69), which may explain the apparent discrepancy between EGF-R levels and proliferative response.

1.4.5 The asthmatic epithelium and airway remodelling

Epithelial wound repair is associated with the expression of a number of mediators that have been implicated in airway remodelling. Whilst repairing, the epithelium expresses
cytokines such as MCP, proinflammatory cytokines including IL-1β and TNFα, and anti-inflammatory cytokines like MIP. In addition, several growth factors are expressed, such as PDGF and EGF. Expression of these growth factors may directly influence the deposition of the extracellular matrix (191).

Direct evidence for a link between epithelial repair and airway remodelling has come from studies which co-culture epithelial cells and fibroblasts. Nakamura et al (192) found that fibroblast proliferation increased in the presence of either epithelial cells, or epithelial cell conditioned media. Another investigation, using co-cultures of mouse epithelial cells and fibroblasts demonstrated that increased expression of MCP-1, as found in repairing epithelium, results in expression of profibrotic mediators such as TNF-α, and suppression of anti-fibrotic mediators such as PGE₂ (193). Finally, Zhang et al (194) used co-cultures of human fibroblasts and a human bronchial epithelial cell line to investigate epithelial wounding. They found that epithelial cell damage increased collagen gene expression in fibroblasts, through the combined effects of several growth factors.

1.4.6 The epithelial-mesenchymal trophic unit

Epithelial-mesenchymal interactions are very important in lung development. This was demonstrated in a series of experiments in which distal lung mesenchyme was engrafted onto tracheal epithelial strips: the epithelium began to branch and reverted to an alveolar epithelial cell phenotype. In contrast, if lung epithelium was engrafted onto tracheal mesenchyme, the epithelium displayed a tracheal phenotype (195). Although these experiments seem to suggest uni-directional signalling, it is now known that the epithelium also produces signaling molecules such as EGF and TGFβ2, that are important for normal mesenchymal differentiation and proliferation (196).
The majority of transcription factors, growth factors and other signalling molecules involved in lung development have also been implicated in airway remodelling, leading to the theory that re-activation of the epithelial-mesenchymal trophic unit in asthma may lead to airway remodelling (197). In asthma, the airway epithelium is more susceptible to damage, and exhibits an inadequate repair response. Failure of appropriate growth and differentiation of airway epithelial cells will cause persistent mucosal injury, such that the epithelium is maintained in a state of repair. Repairing epithelial cells produce a variety of fibrogenic growth factors and peptide mediators such as EGF, insulin like growth factor, basic FGF and endothelin, which are all capable of inducing proliferation of sub-epithelial fibroblasts (198).

In asthma, the epithelium appears to be permanently expressing a repair phenotype. This results in the long-term expression of mediators and growth factors, which is inappropriate. When epithelial repair is experimentally blocked, the epithelium is stimulated to produce levels of mediators not seen in normal repair. For example, impairment of EGF-R activity may promote abnormal healing and airway remodelling. Blockade of EGF-R in vitro results in markedly increased expression of TGF-β. TGF-β stimulates production of collagen types I and III in fibroblasts, as well as extracellular matrix components such as fibronectin and tenascin. Furthermore, TGF-β inhibits the synthesis and activity of enzymes that degrade the extracellular matrix, and stimulates inhibitors of these enzymes such as TIMP-1 (199).

1.5 Techniques for study of the epithelium

1.5.1 Animal models

Animal models of asthma have been used for over 100 years (200). They are particularly useful for studies in which a mediator or process of interest can be
manipulated by antagonism, suppression or up-regulation. These studies allow investigators to examine its role and importance in processes such as inflammation, remodelling and airflow limitation. For example, mouse models of allergic airway inflammation allowed the identification of the relative importance of Th1 and Th2 cells (201). Studies in mice have also identified the importance of the cytokines IL-4 (202, 203), IL-5 (204), and IL-13 (205) in the perpetuation of allergic inflammation and the development of airway hyperresponsiveness. Animal models clearly represent a useful system for investigating asthma. However, interpreting the results from such models, and the extrapolating these results to human asthmatics, should be undertaken with caution.

The mouse is the most popular animal for modelling allergic airways disease. We have a detailed understanding of their genetics (206), it is easy to manipulate outcomes using transgenic technology (207), and mouse-specific probes are readily available. In addition, mice are easily sensitized to a number of antigens (208, 209). Sensitization and challenge with these antigens leads to a Th-2 type response in the lungs, although the level of IgE (210), eosinophilia (211) and airway responsiveness (212) varies between strains. In the case of allergen challenge, the influx of inflammatory cells is dominated by eosinophils (208), and the histological pattern of inflammation resembles allergic alveolitis more than asthma (213). In addition, the eosinophils rarely degranulate (214), whilst human eosinophils readily degranulate (215). Finally, one of the major criticisms of the mouse model is the lack of chronicity of the response to allergen exposure following sensitization (216). Despite these drawbacks, mice have proven to be a useful model of asthmatic disease, and an effective means of generating hypotheses that can then be tested in humans.
Rats have also been used to model allergic airways disease. They are cheap, and their larger size means a greater volume of serum and BAL can be obtained for measurements of inflammatory markers. Their size is also an advantage in terms of measuring physiological outcomes. There are a number of strains of rat available, with considerable variation in the response to sensitization and challenge (200, 217). Like mice, rats can be easily sensitized to a range of antigens, and antigen challenge produces a Th2 dominated response characterized by eosinophilia and antigen-specific IgE (218). However, unlike mice, rats demonstrate an acute response to allergen inhalation with both an early phase and late phase response (219). As with the mouse model, the rat also fails to develop a chronic allergic response. However, rats have also been useful in advancing our understanding of the mechanisms of asthma.

Guinea-pigs represent one of the oldest animal models of allergic airway responses (200). They appear to be useful as models of immediate hypersensitivity to irritants, and have pharmacological responses similar to humans (220). Studies using larger animals such as dogs and sheep are not as common, largely due to the expense involved. However, there are a number of dog models that display the characteristics of atopic asthma (221, 222), and dogs have a natural pre-disposition to develop allergic responses to antigens that are clinically relevant to humans (223).

Animal models of asthma have been the subject of a number of criticisms. Extrapolating results to humans is not always possible or advisable; this criticism is especially relevant in animals that have been sensitized via intraperitoneal injection. In addition, the antigen of interest is often injected in the presence of an adjuvant (224) which promotes the development of a Th2 phenotype; these adjuvants may alter the mechanisms of sensitization to the antigen. As mentioned earlier, mice, rats and guinea-
pigs fail to develop a chronic response to antigens: they respond to challenge initially, but when the protocol is repeated, the animals usually become tolerant to the antigen (213, 225, 226).

Another factor that needs to be taken into consideration when extrapolating data to humans is the anatomical differences between species of mammal: the branching pattern and morphology of the airway varies considerably between mammal species (227). In addition, the relative proportion of cell types in the airway varies considerably between species (227). It is also important to note that the distribution and class of receptors along the airways that respond to pharmacological agents differs between mammals (228). Finally, the animals used to model asthma are adults, and it is well recognized that early life events (see section 1.3.3) may result in immunological and structural changes that determine whether an individual does on to develop asthma. Currently, an established model of sensitization and challenge of neonatal animals is not available, making it difficult to examine the development and pathogenesis of asthma in an animal model.

1.5.2 Immortalized cell lines

*In vitro* systems for studying asthma make use of immortalized human airway epithelial cells, either alone, or in co-culture with other cell types. However, it is important to remember the limitations of this model when making extrapolations to the epithelium *in situ* (229). Firstly, primary epithelial cells will not reproduce themselves indefinitely, and must be transformed or derived from cancerous tissue in order to create a cell line. This process alters the proliferative capacity of the cells, and may have unknown effects on other cellular processes. All cells in an epithelial cell line are clones of a single progenitor cell. This progenitor cell is derived from a mixed population of epithelial
cells with different phenotypic characteristics, and may not be representative of the population. In addition, a culture of cloned cells will behave differently to a population of cells expressing distinct phenotypic characteristics. Although cell lines have been created which express features such as cell polarity, tight junction formation, secretion of mucins, and formation of cilia, these features are cell line-specific and are not generally expressed in every clone. In addition, immortalized cell lines can exhibit problems of stability and behaviour: the characteristics they express may be significantly different to the original tissue, or change over time in culture. Finally, many commonly used cell lines have been mis-identified or contaminated (230). Therefore, information derived from cell lines should be interpreted with caution, and primary epithelial cells should be obtained and used where possible.

1.5.3 Post-mortem samples

Samples of epithelial tissue for research purposes can be obtained post-mortem. Indeed, many of the initial advances in our understanding of asthma came from post-mortem specimens of lung tissue (85, 231). These studies describe epithelial cells mixed with mucus in the airway lumen, and marked epithelial desquamation (83). However, a more recent postmortem study of the asthmatic airway examined epithelial desquamation and found no increase in the detachment of epithelial cells (232). This highlights several issues concerning the use of post-mortem samples for study of the airway epithelium. Studies relying on post-mortem specimens tend to be based on small numbers, the patients are by definition atypical, and there is a lack of standard sampling protocols. Epithelial tissue is subject to a wide range of post-mortem changes which may occur very rapidly. In addition, it is often very difficult to control for use of medication such as inhaled corticosteroids, as the information simply may not be available (233).
1.5.4 Samples from lung resections

Lung resections are most commonly performed in patients with lung cancer, where the tumour, and a portion of surrounding healthy tissue is surgically removed. Tissues obtained in this manner have been used for investigation of the epithelium using immunohistochemistry (234) and epithelial cell culture (235, 236). The production of epithelial cell cultures from resected lung tissue demonstrates that, unlike post-morten samples, resected tissue is able to be processed quickly enough to retain a high level of viability. Unfortunately, studies using resected tissue are subject to the same limitations as studies using tissue obtained at autopsy: the numbers of patients are small due to tissue availability, and appropriate controls are not available due to underlying respiratory problems in patients undergoing resection. In addition, lung resections are most commonly performed for removal of tumours, or in bronchiectasis, emphysema, or tuberculosis. The incidence of these conditions in children is much lower than in adults, which further complicates the process of obtaining paediatric tissue.

1.5.5 Biopsy

Bronchial biopsy is performed using a bronchoscope, which is passed through the nose or mouth into the lungs, where the area to be sampled can be visualised. Flexible forceps are passed along the bronchoscope and used to take a sample of tissue. Bronchial biopsy is routinely performed for the diagnosis of various pulmonary diseases, and has also been used in asthmatic and otherwise healthy individuals purely for research purposes (108, 237, 238). This method is generally safe, and has been used for investigation of asthma in adults and in far fewer studies, children (239-241). Biopsy tissue has been used for histology (242, 243), gene expression (244) and cell culture (245). The quantity of lung tissue obtained by bronchial biopsy is much smaller than that obtained either at autopsy or resection; generally a 2mm$^3$ portion of tissue is
sampled (108). The sample consists of bronchial epithelial cells attached to a basement membrane, with some accompanying underlying tissue. Protease treatment can dissociate the cells, but then various methods must be used to isolate epithelial cells. There has been some controversy regarding the examination of bronchial biopsies; often it is unclear whether alterations in the epithelium are the result of disease processes, or if they are mechanical damage caused by biopsy forceps (246).

1.5.6 Bronchoscopic brushing

Bronchoscopic brushing is performed in a similar manner to bronchial biopsy, except that a small cytology brush is inserted through the bronchoscope instead of forceps. The brush is rubbed against the surface of the airway to sample epithelial cells. This technique has proven to be a well tolerated and safe method for obtaining airway epithelial cells (247, 248). Bronchoscopic brushing has been used in association with techniques such as immunocytochemistry (153), quantification of gene expression (249) and cell culture (171) to study epithelial cells (170, 183, 250) and inflammatory cells infiltrating the epithelium in asthma (251). The cells sampled consist of greater than 90% epithelial cells, with macrophages and occasional lymphocytes (183).

Bronchoscopic brushing provides the opportunity to study the epithelium in isolation. Caution must be exercised when using epithelial samples to investigate disease, as the influence of other cell types on the epithelium, and the role of circulating factors must be considered. However, the study of isolated epithelial cells can also be beneficial, as the effect of particular endocrine or paracrine factors can be investigated. In addition, researchers have utilised co-cultures of epithelial cells and other cells types such as fibroblasts (198) to investigate specific interactions.
Whilst this technique appears to be a safe and effective method of sampling epithelial cells, it still requires the use of a bronchoscope, which necessitates the application of at least a local anaesthetic spray such as lignocaine. Unfortunately this has adverse effects on epithelial cell viability (247). Finally, bronchoscopy in children is performed under general anaesthetic, therefore elective bronchoscopy in children simply for research purposes is not ethically justifiable. However, bronchoscopic brushing could potentially be performed in children who were anaesthetised for minor surgery or investigations of a non-respiratory nature.

1.6 Scope of the thesis

The epithelium plays a vital role in maintaining respiratory homeostasis, and is central to the pathology of many airway diseases. However, most information regarding the involvement of the epithelium in common childhood diseases such as asthma has come from studies in adults, due to the lack of an appropriate method for study of the asthmatic epithelium (252). Investigation of epithelial cell biology has the potential to yield valuable information not only about asthma, but about several other respiratory diseases as well. Therefore, this study aimed to develop a model for sampling epithelial cells from healthy children for use in cellular, molecular and histochemical investigation. The safety and utility of non-bronchoscopic brushing was examined, before the model was applied to the investigation of asthma in an unselected population of children.

1.6.1 Hypotheses

This thesis initially investigates the hypothesis that epithelial cells can be sampled quickly and safely from children using non-bronchoscopic brushing, permitting study of multiple aspects of airway epithelial cell biology. Secondly, the hypothesis that the
airway epithelium in children with asthma is intrinsically abnormal was investigated by examining three areas of key importance to asthmatic disease: inflammation, damage, and repair.

1.6.2 Aims of the thesis

1. Develop and evaluate a non-bronchoscopic brushing method for sampling airway epithelial cells from a paediatric population.

2. Investigate markers of inflammation in the epithelium of children with asthma, in particular, epithelial nitric oxide synthase and the contribution it makes to NO in exhaled breath.

3. Investigate the degree of epithelial damage in asthmatic children, and levels of proliferation and repair signals in the epithelium.

4. Determine the potential of non-bronchoscopic brushing for investigation of broader epithelial function using microarrays.
2.0 Materials and Methods

2.1 Materials

Unless stated otherwise, all chemicals used were of analytical grade.

2.2 Patient recruitment

The study was approved by the Princess Margaret Hospital for Children Ethics Committee, and written informed consent was obtained from the parents of the children prior to sampling. Participants were recruited from children admitted to Princess Margaret Hospital for the purposes of elective surgery for non-respiratory complaints. Children with previous respiratory symptoms other than those that could be explained by asthma and children with evidence of reflux oesophagitis on biopsy were excluded from the analysis. Children were free of respiratory symptoms at the time of study. Children were usually having minor gastrointestinal or ENT surgery whilst under an intravenously induced anaesthetic. During anaesthesia, blood was taken for allergen-specific IgE testing (RAST). RAST analysis was performed by the pathology department at Princess Margaret Hospital, and was used in conjunction with a modified ISAAC questionnaire (See Appendix) (253) to assess the atopic and asthmatic backgrounds of study participants. Subjects were considered atopic if they had one or more response to the RAST test; asthmatic status was determined based on a doctor diagnosis of asthma, and wheeze in the past 12 months associated with atopy.

2.3 Epithelial cell sample collection

Immediately prior to surgery, a cytology brush (BC 25105, Olympus, Australia) was inserted directly through the endotracheal tube, advanced until resistance was felt, and rubbed against the epithelial surface to sample cells. The brush was then withdrawn and the tip cut off into 5ml of culture media (RPMI-1640 containing 10% (v/v) heat
inactivated foetal calf serum), and the process repeated at least once more. Typically, cells were collected from three passes with the brush; fewer passes were used if blood was apparent, more passes were used if cells were not visually apparent in the collection media. The cell suspension was immediately put on ice.

### 2.4 Bronchoalveolar lavage

Non-bronchosopic lavage was performed in a subset (n = 96) of the children. The tip of a 10-french catheter was cut off, and the catheter introduced through the endotracheal tube until resistance confirmed a wedge position. BAL was performed by instilling two 15ml aliquots of sterile saline into the airway, and immediately collecting the lavage fluid with suction. Cells in the fluid were pelleted by centrifugation, and resuspended in 300µl of phosphate buffered saline (PBS). A cell count was obtained by diluting a 20µl of the cell suspension 1:2 with trypan blue and assessing cell number using a Neubauer hemocytometer. Cytospins slides for immunocytochemistry were prepared as detailed in section 2.11.

### 2.5 Sample Processing

Samples obtained from brushings (section 2.3, 2.4) were processed immediately upon return to the laboratory. Cell number and viability were assessed via trypan blue exclusion, within 15 minutes of collection. A 20µl aliquot of the cell suspension was diluted 1:2 with trypan blue, and cell number and viability determined using a Neubauer hemocytometer. Cell number was reported as cells/mL. Each sample of cells obtained was then processed to allow for use in multiple techniques. Of the 5ml cell suspension, 2ml was used for cell culture or protein extraction, 1ml to produce cytospin slides for immunocytochemical studies, and RNA was extracted from the remaining 2ml.
2.6 Epithelial enrichment

Before the cell suspension was used for RNA and protein extraction, and to produce cytospin slides, the macrophages were removed by positive selection. The cell suspension was added to a culture dish that had been previously coated with CD68 antibody. The plate was incubated for 20 minutes (37°C, 5% CO₂) to allow the macrophages to adhere. The suspended epithelial cells were aspirated from the plate, and the macrophages removed using trypsin (0.25%) for subsequent analysis.

2.7 Protein extraction

Protein was extracted from the epithelial cells by lysing the cells in 200µl of SDS extraction buffer. As cell extracts contain a number of endogenous proteases and phosphatases, the lysis was carried out in the presence of a commercially available protease inhibitor cocktail containing a mixture of protease inhibitors with a broad specificity for the inhibition of serine, cysteine, aspartic, and aminopeptidases (Sigma-Aldrich, NSW, Australia). A commercial assay, the Micro BCA Protein Assay (Pierce Biotechnology, IL, USA), was used to determine the concentration of total protein in the cell lysate. Incubation of total protein with bicinchoninic acid (BCA) reagent results in the formation of a coloured product. Aliquots of protein samples (150µl) were mixed with 150µl of BCA reagent in a 96-well plate, and incubated at 37°C for 2 hours. Absorbance (550nm) was read in a plate reader (ELx-800UV, Biotek, VT, USA) and compared to an 8-point standard curve (0.5 – 200 µg/ml BSA).

2.8 RNA extraction

To extract RNA, pelleted epithelial cells were washed once in PBS, and then lysed in 350µl of RLT (Qiagen, Victoria) containing β-Mercaptoethanol (Sigma-Aldrich, NSW, Australia). Lysates were stored at −80°C and extracted in batches. Thawed lysates were homogenised using QIAshredder columns according to the manufacturer’s instructions.
Total RNA was then extracted using the QIAGEN RNeasy kit as specified. RNA quantity and quality was assessed using the Agilent Bioanalyser (Vic, Australia). The Agilent Bioanalyser is a microfluidics platform: a sample of RNA is electrophoretically driven through an interconnected set of microchannels, and RNA fragments are separated based on their size. Dye in the microchannels intercalates directly with the RNA and allows detection of bands of RNA molecules of differing sizes. 1µl aliquots of RNA were loaded onto the Agilent Bioanalyser to determine RNA quality and quantity.

2.9 Reverse transcription

cDNA was synthesized from up to 0.5µg of total RNA, in a volume of 25µl consisting of 1 x RT buffer, 10mM deoxyribonucleotide triphosphates, 25mM MgCl₂, 50µM random hexamers, 20U/µl RNase inhibitor, and 50U/µl Multiscribe reverse transcriptase (all from Applied Biosystems, NSW, Australia). Reactions were incubated at 25°C for 10 minutes, 48°C for 45 minutes, 95°C for 5 minutes, and then cooled to 4°C before storage at –80°C.

2.10 Taqman PCR

Gene expression was quantified relative to the expression of the housekeeping gene β-actin, using Taqman real-time PCR. Briefly, this assay makes use of primers specific for the cDNA of interest, and a ‘TaqMan’ probe which binds between the forward and reverse primers. The rate of accumulation of PCR products can be detected by an increase in fluorescence due to the probe. Relative quantitation of gene expression can be determined by analysing the rates of accumulation of PCR products in different samples.
2.11 Immunocytochemistry

Indirect immunocytochemistry was used to detect cells and proteins of interest. Briefly, a primary antibody, raised against the antigen of interest, is applied to the cells, and binds to the target antigen in the specimen. A secondary antibody is then applied that binds to the primary antibody. The secondary antibody, conjugated to a fluorescent molecule, can be excited by certain wavelengths and viewed under a fluorescence microscope.

Cytospin slides were prepared by centrifuging cells onto a glass slide in a cytocentrifuge (Hettich, Germany). To ensure an appropriate density of cells, 90,000 cells were added to each slide. Slides were air dried for 10 minutes, fixed in 4% paraformaldehyde for 10 minutes, and then stored dessicated at –20°C until required. For immunocytochemical staining, cytospins were rehydrated in PBS before cells were permeabilised with 0.1% saponin. Cells only remain permeable while saponin is present, so saponin was added at all incubation steps. Non-specific binding sites were blocked with 10% fetal calf serum (FCS). Cells were incubated with the primary antibodies at room temperature for 1 hour (Table 3). Antibody binding was detected by incubation with a fluorescent antibody conjugate (Table 4). Stained cytospins were mounted in immunomount
(DAKO, NSW, Australia). Immunofluorescence was observed using a Zeiss Axioskop 2 microscope with an AxioCam, and Axiovision software (version 3.1.1.1).

**Table 3: Specification of lectin and primary antibodies**

<table>
<thead>
<tr>
<th>Lectin/Primary Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Incubation</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated Isolectin BS-1</td>
<td><em>Bandeiraea simplicifolia</em></td>
<td>1:50</td>
<td>1 hour</td>
<td>Axxora, CA, USA</td>
</tr>
<tr>
<td>EGF-R</td>
<td>Mouse</td>
<td>1:50</td>
<td>1 hour</td>
<td>Biosource, CA, USA</td>
</tr>
<tr>
<td>PCNA</td>
<td>Mouse</td>
<td>1:50</td>
<td>1 hour</td>
<td>Santa Cruz, CA, USA</td>
</tr>
</tbody>
</table>

**Table 4: Specification of secondary antibodies**

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Antigen</th>
<th>Dilution</th>
<th>Incubation</th>
<th>Emission colour</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin-Alexa Fluor 546 conjugate</td>
<td>Biotin</td>
<td>1:200</td>
<td>30 min</td>
<td>Red</td>
<td>Molecular Probes, Vic, Australia</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>Mouse IgG</td>
<td>1:50</td>
<td>30 min</td>
<td>Green</td>
<td>Molecular Probes, Vic, Australia</td>
</tr>
</tbody>
</table>
2.12 Statistical Methods

Data are reported as mean (SE) and are analysed by independent samples t-test or two-way analysis of variance (ANOVA) to compare the difference between the three phenotype groups. Statistical analysis was performed using SPSS version 10 (IL, USA). Significance was taken as p<0.05. The power for each t-test resulting in a statistically significant result is presented in Table 5.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Section</th>
<th>Phenotypes</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeNO</td>
<td>4.3.3, p82</td>
<td>Healthy vs Healthy Atopic</td>
<td>69.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy vs Atopic Asthmatic</td>
<td>62.9%</td>
</tr>
<tr>
<td>NOS2</td>
<td>4.3.4, p83</td>
<td>Healthy vs Healthy Atopic</td>
<td>78.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy vs Atopic Asthmatic</td>
<td>59.3%</td>
</tr>
<tr>
<td>NOS3</td>
<td>4.3.4, p83</td>
<td>Healthy vs Healthy Atopic</td>
<td>86.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy vs Atopic Asthmatic</td>
<td>100.0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy Atopic vs Atopic Asthmatic</td>
<td>100.0%</td>
</tr>
<tr>
<td>EGF</td>
<td>5.3.4, p109</td>
<td>Healthy vs Atopic Asthmatic</td>
<td>95.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy Atopic vs Atopic Asthmatic</td>
<td>70.6%</td>
</tr>
<tr>
<td>EGF-R</td>
<td>5.3.4, p109</td>
<td>Healthy vs Atopic Asthmatic</td>
<td>99.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy Atopic vs Atopic Asthmatic</td>
<td>95.0%</td>
</tr>
<tr>
<td>PCNA</td>
<td>0, p113</td>
<td>Healthy vs Atopic Asthmatic</td>
<td>80.1%</td>
</tr>
</tbody>
</table>
3.0 Characterisation of the epithelial brushing model

3.1 Introduction

A number of techniques are available for assessment of airway inflammation in diseases such as asthma. Various cellular, molecular, immunological and structural markers of inflammation can be studied by direct or indirect sampling procedures in the airways. Non-invasive methods can be used to measure inflammatory markers in peripheral blood, urine, and exhaled air, however their indirect nature is a major limitation (254). BAL, airway biopsy and bronchial brushing allow direct investigation of the airway, with the drawback that they are invasive.

Depending on the technique used, different compartments of the lung may be assessed for inflammation. For example, different expiratory manoeuvres can be employed to sample exhaled nitric oxide derived from different regions of the respiratory tract (255). BAL samples inflammatory cells and epithelial lining fluid from the lumen of the distal airways, whereas bronchial biopsy produces a sample of airway mucosa from the large airways.

Airway biopsies can be obtained using flexible forceps passed through a bronchoscope that take a small sample (2mm$^3$) of airway wall. This method is generally safe, and has been used for investigation of asthma in adults and in far fewer studies, children (239, 240). Biopsy tissue has been used for histology (86), gene expression (244) and cell culture (245), but samples a heterogeneous population of cells, thereby making it difficult to study individual cell types. Epithelial cells can be detached from the surrounding tissue by protease digestion or dissection, but this decreases their viability significantly.
Bronchial brushing is another technique used to study the airway. Brushing is also performed bronchoscopically, using a small cytology brush to sample cells from the surface of the airway. This technique is reportedly safe, and yields useful quantities of cells for analysis (248). The cells sampled consist of greater than 90% epithelial cells, with macrophages and occasional lymphocytes (183). Epithelial cells obtained by bronchial brushing are more viable than those recovered from biopsies. Reported levels of viability vary between different studies, however viability appears to be decreased in asthmatic subjects compared to healthy subjects (153, 183, 248). Cells recovered from bronchial brushings have been successfully used for immunocytochemistry (153), quantification of gene expression (249) and cell culture (171).

Despite the safety and ease with which these techniques can be performed, there are ethical and practical challenges in obtaining samples from children. It is difficult to justify elective bronchoscopic procedures in children for research, where there is no direct benefit to the child. This has meant that until now, information concerning common childhood diseases such as asthma has been derived mainly from studies in adults, or from post-mortem specimens. Clearly, an ethical, non-invasive method of investigating airway inflammation is needed.

Brushing of the airway without the aid of a bronchoscope has been previously performed in adults (256). Non-bronchoscopic brushing is performed by introducing a cytology brush directly into the intubated airway. This technique has been used to aid the diagnosis of ventilator-associated pneumonia, by assessing microbiological colonisation of the lung (257). A comparison of bronchoscopic and non-bronchoscopic brushing for this purpose demonstrated that the two methods were equally useful, and that non-bronchoscopic brushing took significantly less time (257).
Researchers are now investigating the possibility of using non-bronchoscopic brushing in children to safely sample epithelial cells. A recent paper (258) outlined the use of non-bronchoscopic brushing to sample airway epithelial cells for use in cell culture. Non-bronchoscopic brushing was shown to be safe and effective, and sampled sufficiently viable epithelial cells. The major limitation of this study was that the use of the epithelial cell sample was restricted to cell culture. The present study aims to use non-bronchoscopic brushing in children admitted to hospital for the purposes of elective surgery, and determine whether non-bronchoscopic brushing can safely sample useful quantities of epithelial cells to allow investigation of the epithelium using a broad range of techniques, including real-time PCR, immunocytochemistry and cell culture.

3.2 Methods

3.2.1 Patient recruitment, cell sampling

Children were recruited from Princess Margaret Hospital as detailed in section 2.2. Epithelial cells were sampled using a non-bronchoscopic brushing technique (see section 2.3). A sub-group of 25 of these children was studied to assess how well the technique was tolerated. Respiratory variables were monitored before, during, and after the brushing procedure. Symptoms following the brushing were recorded by contacting the parents within one week of the procedure. Children who underwent non-bronchoscopic brushing were compared to 24 control children who were anaesthetised and intubated only for similar procedures.

3.2.2 Assessment of cell number and viability

Cell number and viability were assessed via trypan blue exclusion, within 15 minutes of collection. A 20µl aliquot of the cell suspension obtained by non-bronchoscopic
brushing was diluted 1:2 with trypan blue, and cell number and viability determined using a Neubauer hemocytometer. Cell number was reported as cells/mL.

### 3.2.3 Immunocytochemistry to confirm epithelial phenotype

Cytospins were prepared as detailed in section 2.11. Cytospins were stained for markers of epithelial tissue, myofibroblasts and smooth muscle cells, myoepithelial cells, and mesenchymal cells to confirm the epithelial origin of the sampled cells.

### 3.2.4 Leishman’s staining

Cytospins were prepared (section 2.11) and Leishman’s staining was performed to determine the epithelial purity of the sample. Cells were incubated with 5-6 drops of Leishman’s stain (BDH, UK) for 10 min. An equal volume of tap water was added, and the slides incubated for a further 10 min. After rinsing in tap water, the slides were mounted in DPX histology mounting material. To obtain a differential cell count, cell types were recorded until at least 100 cells per sample had been counted. Differential cell counts were performed on 15 healthy, 12 healthy atopic and 11 atopic asthmatic samples.

### 3.2.5 Immunocytochemistry to enumerate basal cells

Basal cells are widely believed to be the bronchial epithelial progenitor cell, capable of proliferation and differentiation (259). To ascertain the number of basal cells, and to test whether the proportion of basal cells differed between phenotypes, cytospins were prepared (section 2.11) and stained with isolectin B4 to identify basal cells. All incubations were performed at room temperature. Cytospins were rehydrated in 1 x PBS for 5 minutes, then permeabilised in Triton X (0.2% v/v) for 3 minutes. Non-specific binding was blocked with FCS (10% v/v) for 30 minutes before a 1:50
dilution of biotinylated isolectin was added for 1 hour. The cytospins were rinsed in PBS, and subjected to two 5 minute washes in PBS before specific binding was detected by incubation with a streptavidin-alexa fluor 546 conjugate (1:200) for 30 minutes. Cytospins were again rinsed and subjected to two 5 minute washes in PBS, then left to air-dry. Stained preparations were mounted in DAKO Immunomount histology mounting material.

3.2.6 Determination of RNA quality and quantity
RNA was extracted from epithelial cells, and RNA quantity and quality was assessed using the Agilent Bioanalyser (Vic, Australia, section 2.8).

3.2.7 Evaluation of housekeeping genes
A recent paper (260) has sparked debate regarding the choice of house-keeping genes in asthma, with the finding that the expression of the common house-keeping genes β-actin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are down-regulated in biopsy tissue from steroid-naïve asthmatics. For the present study, a panel of commonly used house-keeping genes (18S, hypoxanthine guanine phosphoribosyl transferase (HPRT), and β-actin) was evaluated for their usefulness in asthmatic epithelium. Real-time PCR was performed using SYBR-green.

Primers for β-actin, HPRT and 18S were designed using the Primer Express software program (Version 1.5, Perkin-Elmer). The primers were designed to be intron spanning, and a BLASTn search (National Center for Biotechnology Information, Bethesda, MD) was performed to confirm the total gene specificity of the sequences. Primer and probe sequences are given in Table 6. Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer, CA, USA). cDNA was incubated in a
25µl reaction volume containing 1x Taqman Universal PCR Master Mix (Applied Biosystems, NSW), forward primer (10µM) and reverse primer (10µM). The cycles used were: 95˚C for 10 min, followed by 40 cycles of 15s at 94˚C, 30s at 55˚C, and 30s at 72˚C. Signals were analysed by the ABI Prism Sequence Detection System software version 1.9.

**Table 6: Sequences of the primers used for real-time PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S forward</td>
<td>TAACCGTTGAACCCCATTC</td>
</tr>
<tr>
<td>18S reverse</td>
<td>TCCAATCGGTAGTAGCGACG</td>
</tr>
<tr>
<td>HPRT forward</td>
<td>GGTGGAGATGATCTCTCAAC</td>
</tr>
<tr>
<td>HPRT reverse</td>
<td>GCGACCTTGACCATCTTTTG</td>
</tr>
<tr>
<td>ß-actin forward</td>
<td>TCTTCCAGCCCTTCTCTCTG</td>
</tr>
<tr>
<td>ß-actin reverse</td>
<td>AGCAGTGTTGGCGGTACAG</td>
</tr>
</tbody>
</table>

### 3.2.8 Determination of protein quantity

Protein was extracted from the epithelial cells as detailed in section 2.7. A commercial assay (Pierce Biotechnology, IL, USA, (261)) was used to determine the concentration of total protein in the cell lysate (section 2.7).

### 3.2.9 Statistical Methods

The Kolmogorov-Smirnov test was used to determine whether variables were normally distributed. Where the variables were normally distributed (viability, percent basal cells, RNA quality), comparison across phenotypes was made using one-way ANOVA. Comparison between two phenotypes was made using the student’s T-test. Comparison between phenotypes was made using the Kruskal-Wallis test where the variables were not normally distributed (cell yield, RNA quantity).
3.3 Results

3.3.1 Patient Demographics

Of the 157 patients recruited for this study, 36 were atopic asthmatics, 39 were healthy atopics, and 82 were healthy (Table 7). None of the asthmatics were using inhaled corticosteroids at the time of study, and all were considered to have mild asthma. Age and gender did not differ significantly between phenotypes. None of the participants experienced any complications as a result of the non-bronchoscopic brushing. Blood staining of the cytology brush was observed in some cases, but was minor. In the subset of 25 children who were studied to assess how well the procedure was tolerated, the only symptom reported was cough (Table 8) and this was restricted to the children in whom BAL was also performed. Parentally reported cough was higher, with 40% of parents reporting cough. Cough persisting longer than 4 hours was recorded in 32% of patients, and 28% had cough persisting to the following day. Despite this, 100% of parents said that if asked by a friend “Should my child participate in this study?” they would answer “yes”. The number of children in whom cough was reported was not significantly different depending on atopic or asthmatic status. Children who underwent non-bronchoscopic brushing were, on average, ventilated for a slightly longer period than the control group, but this was not statistically significant. There was no difference in any other clinical variables recorded, including the lowest level of oxygen saturation, and the time spent in recovery.
### Table 7: Demographic details of children undergoing brushing

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Healthy atopic</th>
<th>Atopic Asthmatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>82</td>
<td>39</td>
<td>36</td>
</tr>
<tr>
<td>Age (mean, range)</td>
<td>10.83 (2.40-17.37)</td>
<td>9.80 (2.23-16.52)</td>
<td>8.14 (2.32-16.93)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>40/42</td>
<td>21/18</td>
<td>18/18</td>
</tr>
</tbody>
</table>

### Table 8: Symptoms following non-bronchoscopic brushing, and observations of respiratory parameters before, during, and after brushing. Cough was the only reported symptom, and all parents said they would recommend the study to a friend.

<table>
<thead>
<tr>
<th></th>
<th>Controls mean (SE)</th>
<th>Sampled mean (SE)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>24</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>Cough recorded</td>
<td>0%</td>
<td>20% (8%)</td>
<td>0.022</td>
</tr>
<tr>
<td>Parental reported cough</td>
<td>-</td>
<td>40% (10%)</td>
<td>-</td>
</tr>
<tr>
<td>Cough &gt; 4h</td>
<td>-</td>
<td>32% (9%)</td>
<td>-</td>
</tr>
<tr>
<td>Cough following day</td>
<td>-</td>
<td>28% (46%)</td>
<td>-</td>
</tr>
<tr>
<td>Recommend to friend?</td>
<td>-</td>
<td>100% (0%)</td>
<td>-</td>
</tr>
<tr>
<td>Length of Ventilation (mins)</td>
<td>22.3 (1.85)</td>
<td>27 (1.68)</td>
<td>0.066</td>
</tr>
<tr>
<td>Lowest oxygen saturation</td>
<td>98.6 (0.12)</td>
<td>96.8 (1.02)</td>
<td>0.083</td>
</tr>
<tr>
<td>Supplemental oxygen</td>
<td>79% (8%)</td>
<td>80% (8%)</td>
<td>0.944</td>
</tr>
<tr>
<td>Highest respiratory rate</td>
<td>22.7 (0.77)</td>
<td>22.2 (0.58)</td>
<td>0.607</td>
</tr>
<tr>
<td>Time in recovery (mins)</td>
<td>21.5 (2.22)</td>
<td>18.4 (1.40)</td>
<td>0.246</td>
</tr>
</tbody>
</table>
3.3.2 Yield, viability

Epithelial cells were brushed from the airway using a cytology brush inserted directly into the ET tube, without the benefit of direct vision. Although it was not possible to determine the region sampled, the length of the cytology brush inserted into the airway suggested that cells were sampled from the main bronchus, just beyond the carina. The quantity and viability of cells obtained in brushings was assessed with trypan blue exclusion. Each sample of epithelial cells obtained by non-bronchoscopic brushing contained, on average, 2.7 million cells (Figure 3-1A), and this did not differ significantly between phenotype groups (Figure 3-1B, p=0.484). The mean viability of the cells sampled was 17.3% (Figure 3-2A), and did not vary significantly depending on phenotype (p=0.236, Figure 3-2B).
Figure 3-1 A: Histogram of the number of cells sampled in 151 non-bronchoscopic brushings of the paediatric airway. The mean number of cells retrieved was 2.7 million. B: Number of cells obtained in brushings from healthy, healthy atopic, and atopic asthmatic subjects. There was no significant difference in the number of cells retrieved between phenotypes (p=0.484). The median is the line in each box; box limits represent 25th and 75th percentiles; the whiskers extend to the minimum and maximum values, except where extreme cases are represented by dots.
Figure 3-2 A: Histogram of the percentage of viable cells in 43 non-bronchoscopic brushings of the airway. The mean viability was 17.3% B: Percentage of viable cells obtained in brushings from healthy, healthy atopic, and atopic asthmatic subjects. There was no significant difference in viability between phenotypes (p=0.236). The median is the line in each box; box limits represent 25th and 75th percentiles; the whiskers extend to the minimum and maximum values, except where extreme cases are represented by dots.
3.3.3 Cell types sampled

Immunocytochemical staining of the cytospins was used to confirm the purity of the epithelial sample. Antibodies to pan-cytokeratin (a marker for tissue of epithelial origin), α-smooth muscle actin (a marker of myofibroblasts and smooth muscle cells), smooth muscle myosin (a marker of myocytes), and vimentin (a marker of mesenchymal cells) were used to confirm epithelial cell phenotype. Positive staining was only observed for pan-cytokeratin, indicating that the cells were epithelial in origin (Figure 3-3).

Leishman’s staining of cytospins revealed that non-bronchoscopic brushings sampled a mixed population of cells consisting of epithelial cells, macrophages, neutrophils, eosinophils, and lymphocytes (Figure 3-4A). Macrophages were removed from the brushing by plating the sample on a tissue culture plate coated with CD68 antibodies (Figure 3-4B). Differential counts performed on samples that had not been subjected to macrophage removal determined that 94.16% (SE=0.84%) of cells were epithelial, with 3.36% macrophages (Figure 3-5A). Once the macrophages were removed, the remaining cell suspension consisted of 96.63% epithelial cells (Figure 3-5B). The proportion of cells that exhibited a basal phenotype was assessed by isolectin B4 positivity. The mean percentage of isolectin B4 positive basal cells was 10.04% (Figure 3-6), and did not differ significantly between phenotype groups (p=0.772).
Figure 3-3: Immunocytochemical confirmation of epithelial cell phenotype. A: Pan-cytokeratin, B: α-smooth muscle actin, C: Smooth muscle myosin, D: Vimentin
Figure 3-4: Leishman’s staining of cytospins revealed that non-bronchoscopic brushing sampled a population of cells containing 94.2% epithelial cells with 3.4% macrophages. A: group of ciliated epithelial cells, B: epithelial cells and macrophage (indicated by arrow)
Figure 3-5: Population of cells sampled by non-bronchoscopic brushing of the airway. A: Population of cells in samples not subjected to macrophage removal, B: Population of cells remaining after removal of macrophages by positive selection.
Figure 3-6 A: Histogram of the percentage of basal cells in non-bronchoscopic brushings. On average, 10.04% of epithelial cells were basal cells. B: Percentage of basal cells obtained in brushings from healthy, healthy atopic and atopic asthmatic subjects. There was no significant difference between phenotypes (p=0.772). The median is the line in each box; box limits represent 25th and 75th percentiles; the whiskers extend to the minimum and maximum values.
3.3.4 RNA yield

The quality and quantity of RNA extracted from the epithelial cells was assayed in samples from asthmatic and healthy children using the Agilent Bioanalyser system. Samples from atopics were not included in this analysis, as cells from asthmatic and healthy children were being assessed to determine whether RNA samples would be suitable for microarray analysis. This initial study was restricted to asthmatic and healthy subjects, due to the expense involved in conducting microarray experiments, and because it was unclear whether differences in gene expression would be detectable.

Results from the Bioanalyser are generated in the form of an electropherogram, with peaks corresponding to (from left to right) the marker, the 18S and 28S rRNA subunits (Figure 3-7). The ratio between 28S and 18S rRNA is used as a measure of RNA quality. Three electropherograms are presented in Figure 3-7, representing the range of quality of RNA extracted from epithelial cells. The area under the curve is used to determine the amount of RNA. The mean quantity of RNA extracted from 58 samples was 2.9µg (Figure 3-8). The quantity of RNA extracted did not vary between phenotypes (p=0.428). The mean quality of the total RNA, as determined by the ratio of 28S to 18S RNA, was 1.52 (Figure 3-9). RNA quality did not differ significantly depending on asthmatic or atopic phenotype (p= 0.173).

Sufficient RNA was extracted from all samples to allow real-time PCR to be used to evaluate gene expression. Three potential housekeeping genes were evaluated in the setting of asthma. Figure 3-10 shows the expression of the three genes in healthy and atopic asthmatic subjects. No genes expression was correlated with phenotype (β-actin: p=0.717, HPRT: p=0.311, 18S: p=0.912). HPRT was expressed at very low levels, and 18S at very high levels, making them less desirable as housekeeping genes. In light of these findings, β-actin was chosen as a housekeeping gene, as its expression did not vary depending asthmatic status, and it was expressed at appropriate levels.
Figure 3-7: Electropherogram of total RNA extracted from epithelial cells.  A: High quality RNA, with very little evidence of degradation. (rRNA ratio = 1.95).  B: Moderately degraded RNA, as evidenced by the noise between the 18S and 28S peaks (rRNA ratio = 1.34).  C: Highly degraded RNA with no clear 18S or 28S peaks (rRNA ratio = 0).
Figure 3-8 A: Histogram of the amount of RNA extracted from epithelial cells obtained from the paediatric airway. The mean amount of RNA was 2.9µg. B: Amount of RNA extracted from epithelial cells from healthy and atopic asthmatic subjects. There was no significant difference between phenotypes (p=0.428). The median is the line in each box; box limits represent 25th and 75th percentiles; the whiskers extend to the minimum and maximum values.
Figure 3-9 A: Histogram of the quality of RNA extracted from epithelial cells obtained from the paediatric airway. The mean quality of RNA was 1.52. B: Quality of RNA extracted from epithelial cells from healthy and atopic asthmatic subjects. There was no significant difference between phenotypes (p=0.173). The median is the line in each box; box limits represent 25th and 75th percentiles; the whiskers extend to the minimum and maximum values, except where extreme cases are represented by dots.
3.3.5 Protein yield

Protein was extracted from the epithelial cells (9 healthy, 3 healthy atopic, 5 atopic asthmatic) and quantified using a colorimetric method. The mean amount of protein extracted from 18 samples was 74µg (Figure 3-11). Comparison of the amount of protein extracted from different phenotypes revealed that there was a statistically significant difference (p=0.031). However, as the numbers in each phenotype were low, it is unlikely that this is a biologically meaningful difference.
Figure 3-11 A: Histogram of the amount of protein extracted from epithelial cells obtained by non-bronchoscopic brushing. The mean amount of protein extracted was 74µg. B: Amount of protein extracted from epithelial cells from healthy, healthy atopic and atopic asthmatic subjects. There was a statistically significant difference between phenotypes (p=0.031). The median is the line in each box; box limits represent 25th and 75th percentiles; the whiskers extend to the minimum and maximum values.
3.4 Discussion

The airway epithelium clearly plays a central, active role in the airway; however there are very little data about the involvement of the epithelium in common childhood diseases. This reflects the relative difficulty of obtaining tissue from children. This study outlines a method for safely obtaining useful quantities of epithelial cells from children, allowing, for the first time, detailed study of the involvement of the epithelium in airway diseases such as asthma. The quantity and quality of cells sampled was examined, and the potential for the cells to be used in multiple investigative techniques was evaluated.

3.4.1 Safety

None of the children involved in the study experienced any significant complications resulting from non-bronchoscopic brushing. The only notable symptom reported by nursing staff and parents was cough, and this was most likely attributable to the BAL procedure rather than the brushing procedure, as the patients undergoing brushing alone did not experience cough. Pneumothorax has been reported in adults following bronchoscopic brushing (262), however there were no incidences of pneumothorax in our study population. A study by Doherty et al (258) reported similar findings, including minor blood staining of the cytology brush, and no significant complications. Cough and participants’ perceptions of the study were not mentioned. The results presented here demonstrate that the non-bronchoscopic brushing technique is a safe and well accepted method for obtaining epithelial cells from children.

3.4.2 Yield, Viability

The non-bronchoscopic brushing technique was able to harvest useful quantities of epithelial cells, with a mean viability of 17.3%. Studies in adult asthmatics, harvesting
cells under direct vision, report average viability ranging from 25-30\% (153, 183, 248). The only other study to use non-bronchoscopic brushing in children (258), reported an average cell viability of 65\%, which is considerably higher than the viability of cells sampled for the present study. However, the present study may have underestimated the epithelial cell viability by using trypan blue exclusion to assess cell viability. The accuracy of this method for assessing epithelial cell viability has been questioned, due to the tendency of viable epithelial cells to take up the dye (263).

In this study, trypan blue exclusion was used in conjunction with light microscopy to assess cell viability, however a number of other methods for assessing viability are available. Flow cytometry can be used in conjunction with fluorescent dyes such as propium iodide and 7-AAD to identify non-viable cells. Alternatively, dyes such as fluorescein diacetate or rhodamine 123 can be used to identify viable cells. These dyes can also be used in conjunction with fluorescence microscopy to assess viability. Finally, dyes such as trypan blue (264), nigrosin (265), and erythrosin B (266) may be used to identify non-viable cells. Light microscopy was the method of choice for this study, as flow cytometry or fluorescence microscopy were not readily available. Trypan blue exclusion was chosen as it is the most widely used objective method of determining viable cell count.

The viability of cells retrieved in this study was not different depending on asthmatic or atopic phenotype. In adults, the viability of asthmatic epithelial cells has been shown to be significantly lower than cells from healthy adults (153, 183, 248). There are no data comparing viability of cells retrieved from the airways of children, as Doherty et al (258) do not compare levels of viability between healthy and asthmatic children. In the present study, epithelial cell viability was not significantly different in the asthmatics.
This may reflect the mild nature of asthma experienced by these children, as previous studies have reported the level of viability is correlated with the severity of the disease (153).

Asthmatic epithelium is reported to be more fragile and friable than healthy epithelium (267), so it seems reasonable to suppose that a cytology brush would detach epithelial cells more easily from an asthmatic airway, resulting in a higher cell count. In this study the number of epithelial cells retrieved was not significantly different between phenotypes. Epithelial damage appears to be relative to the degree of airway reactivity (108), however there are conflicting reports about the degree of epithelial damage in mild asthmatics. Biopsy studies comparing healthy adults, and adults with mild, asymptomatic asthma have found no difference in loss of cilia, or columnar cell shedding (108, 238), whereas other investigators have found the number of epithelial cells shed into BAL fluid to be increased in asthma. In the present study, it is likely that any damage or fragility of the asthmatic epithelium is not pronounced enough to result in a higher number of cells being sampled by non-bronchoscopic brushing. These results confirm that it is possible to make comparisons between asthmatic and healthy epithelial cells without accounting for phenotypic differences in cell number or viability.

3.4.3 Cell types sampled

Immunostaining with various cellular markers revealed that the cells obtained were of epithelial origin, and that they constituted 94.2% of the total cell sample, with 3.4% macrophages. The proportion of epithelial cells sampled in this study is similar to published data from studies using bronchoscopic brushing in adult airways (153, 183), which report ‘greater than 90%’ and 85% (248) epithelial cells. On occasion, the
cytology brush appeared to be tinged with blood, and red blood cells were apparent in the cell pellet. However, the other cell types collected (macrophages, lymphocytes and eosinophils) were present in similar proportions to those seen in the aforementioned published studies (153, 183), and once the macrophages were removed, the remaining cell suspension consisted of 96.6% epithelial cells. It is possible that positive selection of the macrophages using CD68 antibody may have altered the properties of the remaining epithelial cells, but to study epithelial cells in isolation, macrophage depletion was necessary. In addition, all samples were treated identically, thereby minimising interference when comparisons between phenotypes were made.

The proportion of basal epithelial cells did not differ between phenotypes. The proportion of basal cells was examined as the fragile nature of the asthmatic epithelium may have introduced a bias in the proportion of basal cells sampled, considering that columnar cells are selectively shed from the asthmatic epithelium (181). However, the proportion of basal cells did not differ between phenotypes, validating non-bronchoscopic brushing as a method for comparing the phenotypic differences.

3.4.4 Potential for investigation of epithelial function

On average, 2.9µg of RNA was extracted from the epithelial cells. For preparation of cRNA for microarray analysis, 1µg of RNA is required. Reverse transcription to prepare cDNA for real-time PCR requires 0.2µg of RNA. Therefore, sufficient RNA was extracted from the epithelial cells to allow analysis of gene expression using microarray and real-time PCR. It is possible that the low viability (17.3%) of the cells could have altered gene expression. Unfortunately, this was unavoidable, although a number of measures were taken to minimise changes in gene expression and mRNA degradation. Sampled cells were immediately placed on ice, and RNA was extracted
and frozen as quickly as possible. Collecting the cells directly into a lysis agent such as Trizol was considered as an alternative to collecting into culture media, however this would have precluded the assessment of cell type and viability, the removal of macrophages, the use of cells for culture, and the preparation of cytospin slides.

Epithelial cells yielded an average of 74µg of protein. Western blotting requires 10 - 30µg of protein for detection of a single protein of interest. The amount of protein extracted from the epithelial cells is sufficient to allow western blotting. In addition, cytospin slides were produced that permit not only the confirmation of epithelial cell phenotype, but investigation of the cellular location and expression of various proteins of interest.

3.4.5 Conclusions

Non-bronchoscopic brushing is a safe and effective technique for obtaining epithelial cells from children. Non-bronchoscopic brushing in children is safe and easy to perform, and is not associated with any complications. Adequate numbers of epithelial cells can be retrieved to allow western blotting, real time PCR, and microarray analysis. These techniques provide the basis for a program of study to investigate aspects of epithelial behaviour in paediatric airway diseases. As the children recruited for this study consisted of atopic asthmatics, healthy atopics and healthy children, the non-bronchoscopic brushing model was applied to the investigation of asthma and atopy. Inflammation, damage and repair are central to the pathology of asthma, therefore the following chapters outline the investigation of these areas as a validation of the bronchial brushing model.
4.0 Inflammation

4.1 Introduction

In 1992, Science selected NO as the molecule of the year, after the discovery in 1987 (268) that it plays a vital role in the regulation of many physiological functions and pathophysiological processes. Since then, there has been an explosion of research into NO in a range of organs in the digestive system, the vascular system, and the respiratory system. NO is produced in large quantities by the airways, and can be measured in exhaled breath (FeNO) (269). The levels of FeNO are increased in a number of inflammatory disorders such as asthma (270), tuberculosis (271) and COPD (272), leading to the idea that measurement of FeNO could be a valuable non-invasive method of measuring inflammation in the airways.

NO is synthesized as a product of the oxidation of L-arginine to L-citrulline. This reaction is catalysed by a family of NO synthase (NOS) enzymes (Figure 4-1), of which three isoforms have been identified. Each has a different requirement for activity, and is involved in different physiological processes. The three isoforms, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) are encoded by the genes NOS1, NOS2, and NOS3 respectively. The enzymes were named after the cell type from which they were first isolated, but all have since been found in other cell types. nNOS and eNOS are constitutively expressed enzymes, and have an absolute requirement for intracellular calcium/calmodulin for activity. nNOS was first isolated from rat neuronal tissue (273), although the predominant sites of expression in the lung are non-neuronal (274). nNOS expression has been detected in skeletal muscle (275) and capillary endothelial cells in the lung (274). Its major roles are central and peripheral neurotransmission (276). eNOS was originally isolated in endothelial cells.
and is involved in vasorelaxation. In contrast to eNOS and nNOS, iNOS is an inducible isoform of NOS and is expressed in response to exposure to agents such as cytokines and lipopolysaccharide. iNOS is regulated at the transcriptional level and produces relatively large amounts of NO that may be involved in host defense, and inflammatory processes.

Figure 4-1: Synthesis of NO from L-arginine by nitric oxide synthase. FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide) and BH₄ (tetrahydrobiopterin) are cofactors necessary for enzyme activity.

As one of the primary features of asthma is inflammation, there is great interest in monitoring inflammation in order to predict exacerbations and prescribe an appropriate level of treatment. Procedures such as bronchoalveolar lavage and bronchial biopsy are considered to be the gold standard for assessing inflammation, but the cost and invasiveness makes their routine use impractical and unethical. Recently, exhaled breath components such as NO have been investigated to evaluate their use as non-invasive markers of inflammation.

In 1991, Gustafsson et al (269) discovered that NO generated in the lungs could be measured in exhaled breath. Subsequent investigators noted that levels of exhaled NO
were increased in asthma (282), and that levels decreased after steroid use (270), leading to intense investigation of exhaled NO in asthma and other lung diseases. A variety of studies suggest that it can be used to monitor asthma control (283) and adherence to anti-inflammatory medication (284). Recent studies have shown that there is moderate agreement between FeNO and more invasive measures of inflammation such as BAL (285), and that measurements of FeNO are likely to prove useful to guide therapy in individual children (286).

FeNO can be easily measured, and many commercially produced detection systems are available. Despite the simplicity of measuring FeNO, a number of questions concerning the biology of NO remain unanswered. Exhaled NO is not increased in respiratory diseases such as cystic fibrosis (73), or primary ciliary dyskinesia (287), even though considerable inflammation is present. In atopic subjects, with no clinically apparent inflammation, FeNO is increased (288). There is evidence to suggest that iNOS plays an important role in producing the NO in exhaled breath (289), but the cellular source of NO is yet to be determined.

Assessment of airway inflammation in asthma is important to both researchers and clinicians. Asthmatic airways are characterised by the infiltration and activation of many inflammatory cells, which in turn release inflammatory mediators (25). The asthmatic epithelium is inflamed, and also plays an active role in recruiting inflammatory cells to the airway, and prolonging their life in the airway (175). These processes have been investigated in the epithelium of adults and children with severe asthma (108, 138) using bronchial biopsy, which is considered the ‘gold standard’ for assessing airway inflammation (290). However, there are very few studies which
investigate the level of inflammation in the epithelium of children, due to the paucity of tissue for study.

4.1.1 Aim
The aims of this study were two-fold: Firstly, to determine if inflammatory changes are detectable in the airway epithelium using non-bronchoscopic brushing in association with investigative techniques such as PCR and immunohistochemistry; secondly, to determine the contribution of the airway epithelium to FeNO.

4.2 Methods

4.2.1 Patient recruitment, cell sampling
Children were recruited from Princess Margaret Hospital as detailed in section 2.2. Atopic and asthmatic phenotype was determined using RAST testing and a validated asthma and allergy questionnaire (section 2.2). Epithelial cells were sampled using a non-bronchoscopic brushing technique (see section 2.3).

4.2.2 Assessment of eosinophilia
Non-bronchoscopic BAL was performed as detailed in section 2.4. Cytospin slides were prepared (section 2.11) and stained for eosinophils using a Carbol Chromotrope stain (291). Slides were stained with haematoxylin for 1 minute, rinsed in distilled water, and then stained with carbol chromotrope solution for 30 minutes. Slides were then washed in water, dried and mounted in DPX histology mounting material. To obtain a differential cell count, cell types were recorded until at least 100 cells per sample had been counted.
4.2.3 Measurement of exhaled nitric oxide

Exhaled NO was measured using a fast response (0.02 seconds) chemiluminescence analyser (NOA 280, Seivers Instruments Inc., Boulder, CO). The sensitivity of the analyser for measurement of gas phase NO is less than 1 ppb by volume. The sampling flow into the analyser was 200 ml.min\(^{-1}\). Before each test the analyser was calibrated according to the manufacturer’s instructions. This was a two point calibration using a zero (< 1 ppb) NO gas mixture (BOC Gases Australia) and a high NO gas mixture (between 10 and 100 ppm) (BOC Gases Australia). A standard single breath technique (292) was used at a flow rate of 35ml/s. This expiratory flow rate was established for related studies in this laboratory before the publication of recommendations for the measurements of FeNO in children (293). Although the 35ml/s expiratory flow rate is slightly lower than that suggested in these guidelines, it was considered appropriate for this study, as a subsequent publication reported that flow rates between 30 and 50ml/s are suitable for children.

Exhaled NO was measured while the child was waiting to go to surgery, where the epithelial cells were sampled. Each child was seated and asked to breathe through a mouthpiece containing a one-way valve. After inhalation to total lung capacity, the child exhaled into the mouthpiece at a constant flow rate, aided by a computer prompt displaying exhalation pressure. Two sampling ports near the mouthpiece fed directly into the analyser for measurement of NO and exhalation pressure. Three measurements that varied by less than 10% were averaged to obtain FeNO levels for each child.

4.2.4 Primers and Probes for Taqman PCR

RNA was extracted from the epithelial cells (section 2.8) and reverse transcribed to cDNA (section 2.9). Taqman PCR was performed using the general conditions detailed
in section 2.10, and specific primer and probe sets listed here. The β-actin, arginase I and arginase II primers and probes used for this study were obtained from Applied Biosystems (CA, USA), and as such the sequence data are proprietary. NOS2 primers and probe were designed using the Primer Express software program (Version 1.5, Perkin-Elmer). The primers were designed to be intron spanning, and a BLASTn search (National Center for Biotechnology Information, Bethesda, MD) was performed to confirm the total gene specificity of the sequences. Primer and probe sequences for NOS1, NOS2 and NOS3 are given in Table 6.

**Table 9: Sequences of the Taqman primers and probes used. All of the probes carried a 5’ FAM (6-carboxy-fluorescein) reporter dye and a 3’ TAMRA (6-carboxy-tetramethyl rhodamine) quencher dye.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS1 forward</td>
<td>CAGTGGTCCAAGCTGCAGGTA</td>
<td>(294)</td>
</tr>
<tr>
<td>NOS1 reverse</td>
<td>GGTGGCATACATTTGACATGGTTACA</td>
<td></td>
</tr>
<tr>
<td>NOS1 probe</td>
<td>TCGATGCCCGTGACTGCACCAC</td>
<td></td>
</tr>
<tr>
<td>NOS2 forward</td>
<td>ACCCTGAGCTCTTGGAATTC</td>
<td>Designed using Primer Express</td>
</tr>
<tr>
<td>NOS2 reverse</td>
<td>TTAGCTCCAGTTCGGAAACC</td>
<td></td>
</tr>
<tr>
<td>NOS2 probe</td>
<td>TGGCCATGGGAACATCCAAATACGAG</td>
<td></td>
</tr>
<tr>
<td>NOS3 forward</td>
<td>GTGCGTGTCTGCAATGGACC</td>
<td>(295)</td>
</tr>
<tr>
<td>NOS3 reverse</td>
<td>CCACGATGGTGACTTGCTGCT</td>
<td></td>
</tr>
<tr>
<td>NOS3 probe</td>
<td>AGTGGAAATCAACGTGGCCGTGCT</td>
<td></td>
</tr>
</tbody>
</table>

### 4.2.5 Statistical methods

The Kolmogorov-Smirnov test was used to determine whether variables were normally distributed. FeNO levels and real-time PCR values were log-normally transformed to achieve a normal distribution. Where the variables were normally distributed (lnFeNO, lnNOS1, lnNOS2, lnNOS3, lnArginase I, lnArginase II), comparison across phenotypes was made using one-way ANOVA. Comparison between two phenotypes was made using the student’s T-test. Comparison between phenotypes for eosinophils in BAL fluid was made using the Kruskal-Wallis test, as this variable was not normally distributed.
4.3 Results

4.3.1 Patient demographics

Epithelial inflammation was assessed in 107 subjects (53 healthy, 26 healthy atopic, 28 atopic asthmatic). A subset of samples from the entire cohort was used to investigate each parameter; demographic information, and details of subjects used for each investigation is provided in Table 10.

Table 10: Demographics of subjects investigated for epithelial inflammation. Numbers, age and gender are given for each of the inflammatory parameters investigated in this study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy n</th>
<th>Healthy Atopic n</th>
<th>Atopic asthmatic n</th>
<th>Mean age</th>
<th>Gender (m/f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophilia</td>
<td>27</td>
<td>8</td>
<td>10</td>
<td>9.17</td>
<td>28/17</td>
</tr>
<tr>
<td>FeNO</td>
<td>33</td>
<td>16</td>
<td>15</td>
<td>11.09</td>
<td>35/32</td>
</tr>
<tr>
<td>NOS1</td>
<td>23</td>
<td>9</td>
<td>7</td>
<td>11.01</td>
<td>20/19</td>
</tr>
<tr>
<td>NOS2</td>
<td>34</td>
<td>11</td>
<td>16</td>
<td>9.08</td>
<td>33/28</td>
</tr>
<tr>
<td>NOS3</td>
<td>23</td>
<td>9</td>
<td>7</td>
<td>11.01</td>
<td>20/19</td>
</tr>
<tr>
<td>Arginase I</td>
<td>19</td>
<td>10</td>
<td>10</td>
<td>9.07</td>
<td>19/20</td>
</tr>
<tr>
<td>Arginase II</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>7.98</td>
<td>25/15</td>
</tr>
<tr>
<td>Both FeNO and NOS2</td>
<td>24</td>
<td>10</td>
<td>9</td>
<td>11.07</td>
<td>22/21</td>
</tr>
</tbody>
</table>

4.3.2 Eosinophilia

To investigate the degree of eosinophilic inflammation present in the asthmatics, the percentage of eosinophils present in BAL fluid was quantified. The percentage of eosinophils found in BAL from healthy, healthy atopic, and atopic asthmatic children is shown in Figure 4-2. The majority of samples (19 of 27 healthy, 5 of 8 atopic and 6 of 10 atopic asthmatic) contained less than 1% eosinophils. One-way ANOVA indicated that there were no significant differences in the percentage of eosinophils in the BAL depending on phenotype (p=0.985). These results suggest that there was little, if any, eosinophilic inflammation in this group of asthmatics.
Figure 4-2: Eosinophils in BAL fluid, expressed as a percentage of total number of cells. The median percentage of eosinophils for each phenotype was 0. There were no significant differences according to phenotype (p=0.985)
4.3.3 Exhaled Nitric Oxide

To evaluate airway inflammation, $\text{FeNO}$ was measured in healthy, healthy atopic, and atopic asthmatic children. $\text{FeNO}$ for each phenotype is shown in Figure 4-3. The median $\text{FeNO}$ for healthy subjects was 10.20ppb, for healthy atopics was 19.15ppb and for atopic asthmatics was 13.00ppb. $\text{FeNO}$ was significantly raised in the healthy atopics ($p=0.036$), and in the atopic asthmatics ($p=0.014$) compared to the healthy subjects. However, $\text{FeNO}$ in the atopic asthmatics was not significantly different to that in the healthy atopics ($p=0.708$).

![Graph showing levels of NO in exhaled breath](image)

Figure 4-3: Levels of NO in exhaled breath, measured in healthy, healthy atopic and atopic asthmatic children. The dots represent mean values; the whiskers represent standard deviation. Median levels of $\text{FeNO}$ were: healthy: 10.20ppb, healthy atopic: 19.15ppb and atopic asthmatic: 13.00ppb.
4.3.4 Expression of Nitric Oxide Synthase

The expression of the three isoforms of nitric oxide synthase was examined in the respiratory epithelium using real-time PCR. The relative expression of the three NOS isoforms is shown in Figure 4-4. The data shown represents levels of expression from all patients, including healthy, healthy atopics, and atopics. NOS2 was expressed at the highest levels, and was detectable in all samples tested. NOS1 was expressed at much lower levels, and was detectable in only 14 of 39 (36%) samples. NOS3 was more abundantly expressed than NOS1, and was detectable in 31 of 39 (79%) of samples tested.

![Figure 4-4: Expression of the three isoforms of nitric oxide synthase in the airway epithelium. Gene expression is calculated as a ratio to the housekeeping gene β-actin, and is ln-transformed for comparison. The dots represent mean values; the whiskers represent standard deviation. Median expression: NOS1 = 0.01, NOS2 = 1.33, NOS3 = 0.08.](image)
The levels of each enzyme were compared in the epithelium of healthy, healthy atopic and atopic asthmatic subjects. Epithelial NOS1 expression in each phenotype is shown in Figure 4-5: NOS1 mRNA was detectable in the epithelium of 10 of 23 (43%) healthy subjects, 3 of 9 (33%) healthy atopics, and 1 of 7 (14%) atopic asthmatics. There was no significant difference in epithelial NOS1 expression between the phenotypes (p=0.117).

Figure 4-5: Expression of NOS1 mRNA in the airway epithelium of healthy, healthy atopic and atopic asthmatic subjects. Median expression was: healthy 0, healthy atopic 0, and atopic asthmatic 0.
NOS2 mRNA was expressed at detectable levels in all samples tested (Figure 4-6). There were significant differences in NOS2 expression depending on phenotype (p=0.023). Expression of NOS2 in airway epithelium was significantly higher in the healthy atopics (p=0.014) but not the atopic asthmatics (p=0.065) when compared to the healthy subjects. This may be due to the sample size not being large enough to detect a small change in expression, as the expression of NOS2 in the atopic asthmatics was not significantly different to that of the healthy atopics (p=0.416).

Figure 4-6: Expression of NOS2 mRNA in healthy, healthy atopic and atopic asthmatic subjects. The dots represent mean values; the whiskers represent standard deviation. Median expression was: healthy: 1.22, healthy atopic: 2.51, and atopic asthmatic: 1.65.
NOS3 mRNA was expressed at detectable levels in the epithelium of 18 of 23 (78%) healthy subjects, 8 of 9 (89%) healthy atotics, and 5 of 7 (71%) atopic asthmatics; levels of expression are shown in Figure 4-7. Epithelial NOS3 expression was significantly increased in the healthy atotics compared to the healthy subjects (p=0.004), and significantly decreased in the atopic asthmatics compared to the healthy subjects (p=0.001). The expression of NOS3 was also significantly lower in the epithelium of atopic asthmatics in comparison to the epithelium of healthy atotics (p<0.001).

Figure 4-7: Expression of NOS3 mRNA in the epithelium of healthy, healthy atopic and atopic asthmatic subjects. The dots represent mean values; the whiskers represent standard deviation. Median expression was: healthy: 0.05, healthy atopic: 0.28, and atopic asthmatic: 0.01.
4.3.5 Expression of Arginase

Arginine is a substrate for both NOS and arginase, and has recently been implicated in asthma pathogenesis (296). The expression of arginase I and II mRNA was examined in airway epithelium to determine the involvement of arginase in asthma. Epithelial arginase I and II mRNAs were expressed at comparable levels between all subject phenotypes. Arginase I was detectable in 22 of 39 (59%) of samples. Arginase II was detectable in all samples (n=40).

Arginase I expression was compared in the epithelium of healthy, healthy atopic, and atopic asthmatic subjects. Arginase I was expressed in the epithelium of 10 of 19 (53%) healthy subjects, 5 of 10 (50%) healthy atopics, and 7 of 10 (70%) atopic asthmatics. Epithelial arginase I expression for each phenotype is shown in Figure 4-8. One-way ANOVA demonstrated no significant difference in arginase I expression depending on phenotype (p=0.675). Arginase II expression was also compared in the epithelium of the three phenotypes (Figure 4-8). One-way ANOVA again demonstrated no significant phenotypic differences in arginase II expression (p = 0.525).
Figure 4-8: Expression of Arginase mRNA in the epithelium of healthy, healthy atopic and atopic asthmatic subjects. The dots represent mean values; the whiskers represent standard deviation. Median expression of arginase I was: healthy 0.37, healthy atopic 0.16, and atopic asthmatic 0.59. Median expression of arginase II was: healthy 1.40, healthy atopic 1.18, and atopic asthmatic 1.32.

4.3.6 Contribution of epithelial NOS to FeNO

The epithelial contribution to FeNO was determined by examining the correlation between FeNO and the expression of each of the NOS isoforms in the airway epithelium. There was no significant correlation between FeNO and epithelial expression of either NOS1 or NOS3. As shown in Figure 4-9, FeNO was significantly and strongly correlated with epithelial NOS2 expression. The correlation coefficient was 0.672 (p<0.001), indicating that NOS within the epithelium contributes the majority of NO in exhaled breath. When the relationship between FeNO and epithelial NOS2 expression
was considered in the separate phenotype groups, the correlation was strongest in the atopic asthmatics ($R^2=0.686$), supporting the idea that the increased levels of Fe$_{NO}$ seen in asthma are due to the activity of NOS2 (Figure 4-10).

Figure 4-9: Correlation between expression of NOS2 mRNA and Fe$_{NO}$ in healthy, healthy atopic and atopic asthmatic subjects. NOS2 expression was significantly ($p<0.001$) and strongly ($R=0.672$) associated with Fe$_{NO}$. 
Figure 4-10: Correlation between NOS2 expression and FeNO for each phenotype. The correlation was strongest in the asthmatic subjects (n= 9), compared to the healthy atopics (n=10) and the healthy subjects (n=23).
4.4 Discussion

In this study, non-bronchoscopic brushing was investigated to determine its potential for detecting inflammatory changes in the airway epithelium when used in association with investigative techniques such as PCR and immunohistochemistry. In addition, the expression of nitric oxide synthase in the epithelium was examined to determine the contribution of the epithelium to Fe\textsubscript{NO}.

4.4.1 Markers of inflammation

The percentage of eosinophils in BAL fluid was not significantly increased in the asthmatics. Previous researchers have found levels of eosinophils to be increased in asthma, in bronchial biopsies (138, 297, 298) and BAL fluid (299-301). Levels of 1.5 and 2.5% eosinophils in BAL fluid have been reported in asthmatic children (300, 301), and 0.2% in healthy children (300). As eosinophil levels are related to asthma severity (86), it is not unexpected that there were no apparent differences between phenotypes in this study, as the asthmatics all experienced mild, asymptomatic asthma.

Fe\textsubscript{NO} was significantly increased in the atopic asthmatics, compared to the healthy children. A number of other authors have also found Fe\textsubscript{NO} to be increased in children with mild asthma (300, 302-304). In the healthy atopic subjects, Fe\textsubscript{NO} levels were also significantly raised. The effect of atopy on Fe\textsubscript{NO} is unclear. The majority of studies have found Fe\textsubscript{NO} to be increased in healthy atopic subjects (288, 303-308), however there are a number of studies which fail to find any significant difference in Fe\textsubscript{NO} between atopic and non-atopic subjects (300, 309-311). A recent study by Franklin \textit{et al} (303) has shown that Fe\textsubscript{NO} was associated with airway responsiveness, but only in atopic children, suggesting that Fe\textsubscript{NO} is a marker of allergic airway inflammation, rather than a simple marker of inflammation.
Zimmerman et al (296) have implicated arginase in NO production in the setting of asthma. Arginase metabolises L-arginine, and may compete for this substrate with the nitric oxide synthases, which require L-arginine to produce NO. If arginase expression was increased in the epithelium of the asthmatic children in this study, this may have affected NO production. Expression of the two isoforms of arginase (arginase I and arginase II) was examined using real-time PCR. The expression of either isoform was not different depending on phenotype. This suggests that arginase is not involved in asthma pathogenesis in this cohort of children. Levels of arginase protein were not investigated, however it is highly likely that they would mirror the levels of mRNA, as the expression and activity of arginase is primarily regulated by a transcriptional mechanism (312).

The two markers of inflammation investigated in this study (eosinophils in BAL fluid, and FeNO) seem to suggest that inflammation is present only at low levels in these children. This is not unexpected, as the asthmatics had a very mild form of asthma, were not using corticosteroids, and were asymptomatic at the time of study.

4.4.2 Expression of Nitric Oxide Synthases

In order to determine the contribution epithelial NOS makes to epithelial production of NO, the expression of each of the three NOS isoforms was measured using real-time PCR. The present study investigates levels of NOS mRNA, and not functional protein, however all three isoforms of NOS have been shown to be regulated at a transcriptional level. Transcriptional regulation of NOS2 has been established for over 20 years (313), and more recently certain compounds and conditions have been shown to regulate NOS1 and NOS3 expression (314). Therefore, although the results presented here are
based on expression of NOS mRNA, this is a valid approach for investigation of epithelial NOS enzymes.

NOS1 was expressed at very low levels, and this is not surprising, as immunohistochemical studies have not detected NOS1 in human epithelium (274, 315). There were statistically significant differences in expression of NOS1 between phenotypes, however it is unlikely that these differences represent any physiological significance, as the magnitude of change was very small. In addition, NOS1 was expressed at the limit of detection of the real-time PCR assay, and would therefore be more prone to variability.

NOS3 was also expressed at low levels, and was detectable in 31 of 39 samples tested. Although the major in vivo site of expression of NOS3 is endothelial tissue, it has previously been detected in pulmonary (278) and nasal epithelium (316). There are conflicting reports about the expression of NOS3 in cultured epithelial cells: Asano et al were unable to detect NOS3 mRNA in cultured epithelial cells, despite cytokine stimulation (279), whereas Shaul et al (317) detected NOS3 activity, protein and mRNA. This evidence suggests that NOS3 mRNA is expressed at low levels in airway epithelium, and may not always be detectable with real-time PCR.

NOS3 is considered a constitutive enzyme; however there was considerable variation in NOS3 expression between phenotypes. Compared to epithelial cells from healthy children, NOS3 levels were significantly increased in healthy atotics, and significantly decreased in asthmatic atotics. Basal expression of NOS3 is considered to be largely tissue dependent (318), but can be influenced by a number of extracellular factors. TNF-α has been shown to down-regulate NOS3 (319), and although this study did not
assess expression of TNF-α, previous studies have shown TNF-α to be increased in asthma (320). NO has also been shown to decrease NOS3 expression (321). FeNO was increased in this cohort of asthmatics, and this may have decreased expression of NOS3 in the epithelium. Although the differences in NOS3 expression are statistically significant, the magnitude of the changes suggests it is unlikely that they are biologically significant.

NOS2 is an inducible enzyme, and its expression is controlled at the level of transcription (313). Therefore, levels of mRNA can be expected to reflect levels of functional protein, and the use of real-time PCR was appropriate in this context. In this study, NOS2 was the most abundantly expressed isoform, and was detectable in all samples tested. Previous researchers, using immunohistochemistry, have found the epithelium to be the main site of NOS2 expression within the lung (21, 315, 322-324). NOS2 expression was significantly increased in healthy atopics, but not atopic asthmatics. This was unexpected, as immunostaining studies have found increased staining for iNOS in the epithelium of asthmatic subjects (323, 324). Although NOS2 expression was not significantly raised in atopic asthmatics, expression was not significantly different between healthy atopic and atopic asthmatic children. These results suggest that levels of inflammation were very low in all the children involved in this study.

4.4.3 Contribution of epithelial NOS to FeNO

The correlation between each isoform of NOS and levels of FeNO was determined. NOS2 was the only enzyme found to have a significant correlation with FeNO. The strength of the correlation between FeNO and NOS2 mRNA indicates that a large proportion of NO in exhaled breath is produced by NOS2 in the respiratory epithelium,
as the majority of variability in Fe\textsubscript{NO} is explained by NOS2 expression. The correlation between Fe\textsubscript{NO} and NOS2 was strongest in the asthmatics, but was still significant in the healthy subjects. This suggests that NOS2 expression is responsible for elevated Fe\textsubscript{NO}, and significantly contributes to baseline Fe\textsubscript{NO}. The constitutive isoforms of NOS may play a more important role in baseline Fe\textsubscript{NO} than in elevated Fe\textsubscript{NO}, however the study was not designed to test this hypothesis. The majority of variability in Fe\textsubscript{NO} can be explained by differences in epithelial NOS2 expression regardless of phenotype.

NOS2 has previously been linked with Fe\textsubscript{NO} in human airways (289), and the airway epithelium identified as the major site of NOS2 expression (21, 315, 322-324). However, this is the first study to demonstrate that epithelial NOS2 is responsible for producing the majority of NO in exhaled breath.

4.4.4 Conclusions

Non-bronchoscopic brushing was investigated to determine its potential for detecting inflammatory changes in the airway epithelium when used in association with investigative techniques such as BAL, analysis of exhaled NO, PCR and immunohistochemistry. Whilst Fe\textsubscript{NO} was increased in atopic asthmatics compared to healthy children, the number of eosinophils in BAL fluid was unchanged. This suggests there is a degree of airway inflammation in this group of mild asthmatics, but not to the extent that differences in inflammatory cell populations can be seen. Phenotypic differences were evident in the expression of the NOS isoforms NOS2 and NOS3. There was a strong correlation between epithelial NOS2 expression and Fe\textsubscript{NO}. Further evidence for this relationship could be gained by conducting an intervention study with a NOS inhibitor, however the current data indicate that epithelial NOS2 is responsible for the majority of NO in exhaled breath.
5.0 Damage and Repair

5.1 Introduction

5.1.1 The asthmatic epithelium is damaged

Whilst inflammation is clearly a cornerstone of asthma, it does not fully explain all aspects of the disease. Some asthma is refractory to corticosteroid therapy, and these asthmatics experience an accelerated decline in lung function compared to that seen in the healthy population. Huber and Koessler (85) were the first to note in 1922 that patients who had died of status asthmaticus were characterised by damage and structural changes to their lungs. Subsequent investigators found that damage to the lungs is not restricted to fatal asthma, but also occurs in mild asthma (127). Although the extent of airway remodelling appears to be related to asthma severity, it is not dependent on degree of atopy, or length of asthmatic history (135). The importance of this finding is further emphasized by investigations which have shown evidence of airway remodelling in the early stages of asthma in mildly asthmatic children (138), and tissue restructuring in children up to four years before the onset of asthma symptoms (139). A better appreciation of the factors leading to damage, and the repair processes involved in asthma is needed in order to fully understand the disease, and develop new treatment strategies.

The majority of the evidence for epithelial damage has come from adult asthmatics, due to the difficulty in obtaining target tissue from children. In specimens from patients dying in status asthmaticus, it has been difficult to find areas of normal bronchial epithelium (325). Autopsy specimens have shown marked airway edema with the shedding of airway columnar epithelial cells that leaves a layer of basal cells on the basal lamina (181). The use of endoscopic bronchial biopsy techniques has led to
recognition of structural changes in the airway epithelium of living patients with asthma (108, 127, 326, 327). Biopsy samples of asthmatic airways show sloughing of the surface epithelium (Figure 5-1) (119), and it appears that columnar cells are selectively shed, leaving the basal cells attached (181). Epithelial cells, either separately or in groups called creola bodies, are found in increased numbers in BAL sampled from asthmatics (181, 182). These cells are often viable, and it appears that they are shed because of weak attachment to the basement membrane, not simply due to cell death. The level of epithelial cell shedding is correlated with asthma severity (87, 108), and epithelial destruction occurs at all levels of the airways (127).

![Figure 5-1: Biopsy of airway wall demonstrating the altered epithelium in asthma. A: Normal airway, showing intact pseudostratified columnar epithelium. A: Fatal asthma, with sloughing of the surface epithelium (119).](image)

Although there are a number of studies that demonstrate epithelial disruption, it has been proposed that this is artefactual. However, biopsy studies have demonstrated that the expression of markers of damage is increased in the asthmatic epithelium. For example, expression of the EGF receptor is increased (49), and there is also increased
expression of the epithelial isoform of CD44 (152), indicating that epithelial damage has occurred *in vivo*. Further support for the authenticity of epithelial damage is found in the increased expression of integrins (328), and autacoid mediators (329) as well as the altered expression of adhesion molecules such as e-cadherin (154), ICAM (153) and CD44 (152). In asthmatics, expression of EGF-R and CD44 increases in proportion to disease severity, and upregulation of these two markers is found throughout the epithelium, suggesting that the damage is widespread (49, 152). EGF-R expression is not decreased by inhaled corticosteroids, and is positively correlated with the thickness of the basement membrane (49), suggesting that epithelial injury may be linked to airway remodelling. In addition, EGF-R expression is particularly upregulated in regions where basal cells have lost their columnar cell attachments. Finally, the epithelial shedding characteristic of asthma is not seen in other inflammatory airway diseases such as chronic obstructive pulmonary disease.

In the asthmatic airway, intercellular spaces become progressively wider at the base of the epithelium, whilst the tight junctions at the luminal side appear intact. The epithelium appears more fragile and friable. In places, edema fluid starts to separate the basal and luminal portions of the epithelium, and more severe damage is seen where columnar cells detach, leaving only basal cells attached to the basement membrane (1, 127). Occasionally, squamous metaplasia and ciliogenesis may be seen, providing evidence of epithelial repair (330). Damage of the airway epithelium in asthma has been reported to directly correlate with hyperresponsiveness (108). Animal models of asthma have shown that the tight junctions at the apex of the epithelial cells apparently remain intact, and that the passage of fluid through the epithelium into the lumen occurs through tri-cellular corner structures (331). It has been suggested that this may result in solutes being retained in the epithelium, leading to osmotic changes and contraction of
epithelial cells, which results in the epithelial cells being shed (331, 332). In addition, ciliated epithelial cells are prone to damage and loss of cilia (127), and are the most affected epithelial cell in asthma (17, 133, 333). The cytoplasm of these cells appears vacuolized, due to intracellular edema and cell destruction, and the number of ciliated cells appears to be inversely related to inflammatory events in the epithelium (334).

Damage to the airways can be caused by a number of factors, both from the external environment, and the milieu of the lung. External factors include allergens such as house dust mite (HDM) and pollen. HDM has been shown to cause damage to the epithelium: exposure to the major HDM allergen Der p1 induces an increase in bronchial permeability, most likely mediated by epithelial cell detachment (43). Exposure to Der p1 can also cleave intercellular junction proteins, and HDM proteinases have been shown to induce cell death (44). Pollen, another common allergen, releases proteases that cause epithelial cell detachment (42). Respiratory viruses are another significant causative agent (335). Mediators released by cells involved in the inflammatory response can also cause damage: major basic protein (336) and proteolytic enzymes (337) derived from eosinophils and mast cell derived tryptase and chymase (338) all cause epithelial damage.

5.1.2 Epithelial repair

The epithelium responds very quickly to damage. Where damage has completely destroyed the epithelium, cells surrounding the wound site rapidly dedifferentiate and migrate into the damaged area to form a simple squamous cell barrier (52). In cases of less severe damage, where only the columnar cells are lost, the remaining basal cells promptly flatten out to cover the basement membrane, at the cost of neighbouring columnar cells, which are shed (51). In both cases, the first response of the epithelium
is to reconstitute the barrier that protects the basement membrane. The speed with which the epithelium responds to damage emphasizes the importance of its barrier function. Once an interim barrier has been formed, the cells proliferate and differentiate to reconstitute the epithelium (Figure 5-2).

![Diagram](image)

**Figure 5-2: Repair of the airway epithelium in vivo.** Initially, cells dedifferentiate and migrate into the wound to form a simple squamous barrier. This is followed by proliferation and differentiation of the cells to reconstitute an intact epithelium.

The repair response is co-ordinated by an intracellular network of cytokines, growth factors and extracellular matrix proteins. Perhaps the best studied is the EGF family and its receptors (EGF-R). There is abundant evidence to support a role for EGF and EGF-R in epithelial repair: EGF-R expression and activation is increased around wound sites (49), and in response to damaging agents such as endotoxin (339), bleomycin (340), naphthalene (341) and ozone (342). In both *in vitro* (343) and *in vivo* (344, 345) systems, EGF has been shown to accelerate epithelial repair. Furthermore, blockade of the EGF-R inhibits repair (49). The EGF-R is clearly central to the repair response.
5.1.3 Epidermal Growth Factor Receptor

The EGF-R family elicits a variety of responses within the cell, ranging from growth promotion, growth inhibition, protection against apoptosis, induction of differentiation, reorganisation of the cytoskeleton, and cell migration (346). EGF-R exists as a transmembrane glycoprotein, with an extracellular ligand binding domain, and a single, helical transmembrane domain. The intracellular domain consists three parts: a site for feedback attenuation by protein kinase C and mitogen-activated protein (MAP) kinases, a tyrosine kinase domain, and a carboxy-terminal tail containing five autophosphorylation motifs which link to proteins containing SH2 or phosphotyrosine binding domains (347).

Ligands to these receptors are produced as transmembrane precursors which are processed and released by proteolysis. EGF, amphiregulin and TGF-α bind to the ErbB1 receptor. Betacellulin, HB-EGF and epiregulin can bind to either the ErbB1 or B4 receptors. Neuroregulin-1 and 2 bind either ErbB3 or B4, whereas neuroregulin-3 and 4 bind only ErbB4. No direct ligand has been discovered for ErbB2, but there is increasing evidence to suggest that its primary function is as a coreceptor. ErbB ligands usually act over short distances, in an autocrine or paracrine fashion, with the exception of EGF, which is found in many bodily fluids (348).

The EGF-R exists as a single, inactive unit. Ligand binding to the receptor drives dimerisation – either with another EGF receptor (homodimerization) or with different member of the ErbB family to form a heterodimer. The ability to form a number of different heterodimers contributes to the signalling diversity of this family of receptors. Ligand binding and dimerisation of the receptor activates the intrinsic tyrosine kinase, which phosphorylates specific carboxy-terminal tyrosine residues. This provides
docking sites for proteins containing Src homology 2 or phosphotyrosine binding domains. These proteins, recruited from the cytoplasm by the receptor, form a linked complex, the interactions between proteins in the complex triggering the activation of various intracellular pathways. For example, activation of Ras (an intracellular GTPase switch protein) initiates a cascade of phosphorylations, activating MAP kinase. MAP kinase takes the signal through the cytoplasm to the nucleus where it triggers accumulation of cyclin D. Cyclins are fundamentally involved in cell proliferation, and in association with cyclin dependent kinases, activate the cell division process (Figure 5-3).

**Figure 5-3: EGF-R signalling.** Binding of ligand to the EGF-R induces receptor dimerisation, and activates the intrinsic tyrosine kinase. This provides docking sites for Src proteins. MAP kinase takes the signal to the nucleus and triggers accumulation of cyclin D. Cyclin D, in association with cyclin dependent kinases (CDK), activates the cell division process.
5.1.4 Defects in epithelial repair may influence airway remodelling

The airway epithelium has the capacity to produce and respond to a number of mediators and receptors which are associated with airway remodelling. For example, epithelial cells can release extra-cellular matrix proteins (183), including fibronectin (349). They produce cytokines responsible for recruiting and activating inflammatory cells, and prolong their survival in the airways (350). The epithelium releases fibrogenic growth factors such as insulin growth factor (351) and TGF-β (352), and as such is able to regulate fibroblast function (192).

Altered expression of repair mediators is appropriate for short periods resulting in repair. However, in asthma, the epithelium is thought to be permanently expressing an abnormal repair phenotype. Expression of EGF-R is increased even in areas of intact epithelium (49). Although the epithelial cells must proliferate to reconstitute the epithelium, the expression of the proliferating cell nuclear antigen (PCNA) is not increased in asthmatic epithelium (190), despite increased expression of EGF-R. In addition, expression of the cyclin-dependent kinase inhibitor, p21, is increased in asthmatic epithelium (69).

5.1.5 Aims

Epithelial damage is well documented in asthma. In addition, epithelial repair appears to be abnormal: markers of epithelial repair are increased in areas of damage, but also in intact epithelium. This study aims to use epithelial cells sampled using non-bronchoscopic brushing to detect differences in markers of damage and repair depending on asthmatic or atopic phenotype. The level of epithelial damage in children is evaluated, and the expression of epithelial EGF and EGF-R is quantified.
5.2 Methods

5.2.1 Patient recruitment, cell sampling

Children were recruited from Princess Margaret Hospital as detailed in section 2.2. Atopic and asthmatic phenotype was determined using RAST testing and a validated asthma and allergy questionnaire (section 2.2). Epithelial cells were sampled using a non-bronchoscopic brushing technique (see section 2.3).

5.2.2 Immunocytochemistry

Enumeration of epithelial cells in BAL fluid

Cytospins were prepared from BAL fluid (section 2.11) and Leishman’s staining was performed to determine the epithelial purity of the sample. Cells were incubated with Leishman’s stain (BDH, UK) for 10 min. The stain was then diluted 1:1 with tap water and incubated for a further 10 min. After rinsing in tap water, the slides were mounted in DPX histology mounting material. To obtain a differential cell count, cell types were recorded until at least 100 cells per sample had been counted.

Immunostaining of EGF-R and isolectin positive cells in epithelial brushings

Cytospins were prepared (section 2.11) and double-stained with isolectin B4 to identify basal cells, and also with anti-EGF-R antibody to determine EGF-R expression. Cytospins were rehydrated in PBS for 5 minutes, then permabilised in Triton X (0.2% v/v) for 3 minutes. Non-specific binding was blocked with 10% FCS for 30 minutes. Slides were incubated with a 1:50 dilution of monoclonal anti-EGF-R antibody for 1 hour, subjected to two 5-minute washes in PBS, and then incubated with Alexa fluor 488-labelledd antibody (1:50) for 30 minutes. Slides were again washed before a 1:50 dilution of biotinylated isolectin was added for 1 hour. The cytospins were rinsed in
PBS, and subjected to two 5 minute washes in PBS before isolectin binding was detected by incubation with a streptavidin-alexa fluor 546 conjugate (1:200) for 30 minutes. Cytospins were rinsed and washed again, before being mounted in DAKO immunomount.

** Enumeration of PCNA expressing cells in brushings **

Cytospins were prepared (section 2.11) and stained for PCNA to identify proliferating cells. Cytospins were rehydrated in PBS for 5 minutes, then permeabilised in Triton X (0.2% v/v) for 3 minutes. Non-specific binding was blocked with 10% FCS for 30 minutes before a 1:50 dilution of anti-PCNA antibody was added for 1 hour. The cytospins were rinsed in PBS, and subjected to two 5 minutes washes before PCNA binding was detected following a 30 min incubation with Alexa fluor 488 labelled secondary antibody (1:200). Cytospins were rinsed and washed again, before being mounted in DAKO immunomount.

** 5.2.3 Primers and probes for Taqman PCR **

RNA was extracted from the epithelial cells (section 2.8) and reverse transcribed to cDNA (section 2.9). Taqman PCR was performed using the general conditions detailed in section 2.10, and specific primer and probe sets listed here. The ß-actin, CD44 and p21 primers and probes used for this study were obtained from Applied Biosystems (CA, USA), and as such the sequence data are proprietary. EGF-R primers and probe were synthesized according to the sequence published in Brabender et al (353). EGF primers and probe were designed using the Primer Express software program (Version 1.5, Perkin-Elmer). The primers were designed to be intron spanning, and a BLASTn search (National Center for Biotechnology Information, Bethesda, MD) was performed
to confirm the total gene specificity of the sequences. Primer and probe sequences for EGF and EGF-R are given in Table 11.

Table 11: Sequences of the Taqman primers and probes used. All of the probes carried a 5’ FAM (6-carboxy-fluorescein) reporter dye and a 3’ TAMRA (6-carboxy-tetramethyl rhodamine) quencher dye.

<table>
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<th>Gene</th>
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<th>Reference</th>
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<tbody>
<tr>
<td>EGF-R forward</td>
<td>TGCGTCTCTTGGCCGGAAT</td>
<td>(353)</td>
</tr>
<tr>
<td>EGF-R reverse</td>
<td>GGCTCACCTTCCAGAAGCTT</td>
<td></td>
</tr>
<tr>
<td>EGF-R probe</td>
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<tr>
<td>EGF forward</td>
<td>TGCAAGGGATACGCCCTAA</td>
<td>Designed using Primer Express</td>
</tr>
<tr>
<td>EGF reverse</td>
<td>CCAGAGTACAGGCATGATTTCAAA</td>
<td></td>
</tr>
<tr>
<td>EGF probe</td>
<td>TCATTAACATCTTCAGTGACTCCGGTCTCGA</td>
<td></td>
</tr>
</tbody>
</table>

5.3 Results

5.3.1 Patient demographics

Epithelial damage and repair was assessed in 82 subjects (42 healthy, 18 healthy atopic, 22 atopic asthmatic). Demographic information, and details of subjects used for each investigation is provided in Table 12.

Table 12: Demographics of subjects investigated for epithelial damage and repair. Numbers, age and gender are given for each of the inflammatory parameters investigated in this study.

<table>
<thead>
<tr>
<th></th>
<th>Healthy n</th>
<th>Healthy Atopic n</th>
<th>Atopic asthmatic n</th>
<th>Mean age</th>
<th>Gender (m/f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial shedding</td>
<td>10</td>
<td>7</td>
<td>6</td>
<td>9.5</td>
<td>12/11</td>
</tr>
<tr>
<td>CD44</td>
<td>19</td>
<td>10</td>
<td>10</td>
<td>8.7</td>
<td>22/17</td>
</tr>
<tr>
<td>EGF</td>
<td>25</td>
<td>8</td>
<td>10</td>
<td>10.5</td>
<td>22/21</td>
</tr>
<tr>
<td>EGF-R</td>
<td>19</td>
<td>9</td>
<td>10</td>
<td>9.8</td>
<td>19/19</td>
</tr>
<tr>
<td>PCNA</td>
<td>12</td>
<td>-</td>
<td>12</td>
<td>9.2</td>
<td>11/13</td>
</tr>
<tr>
<td>p21</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>8.1</td>
<td>19/21</td>
</tr>
</tbody>
</table>
5.3.2 Epithelial cells in BAL fluid

Epithelial cells collected in BAL were enumerated to assess the level of epithelial shedding. Epithelial cell numbers were expressed as a percentage of the total nucleated cell count. The mean (SE) percentage of epithelial cells was 38.9% (9.1%) in healthy patients, 37.8% (5.9%) in healthy atotics, and 49% (11.8%) in atopic asthmatics (Figure 5-4), which was not significantly different depending on phenotype. This suggests that the level of epithelial shedding is not increased in this cohort of asthmatics.

![Epithelial cells sampled in BAL fluid](image)

Figure 5-4: Epithelial cells sampled in BAL fluid were counted and expressed as a percentage of the total nucleated cell count. There was no significant difference in the percentage of epithelial cells retrieved in BAL from healthy, healthy atopic, and atopic asthmatic patients.
5.3.3 Expression of CD44 mRNA

CD44 is a cellular adhesion molecule that has been shown to exhibit increased expression in areas of damaged epithelium (152). Figure 5-5 shows the expression of CD44 mRNA in epithelial cells from healthy, healthy atopic, and atopic asthmatic children. The expression of CD44 did not differ between phenotypes (p=0.210). This suggests that there is little epithelial damage in this cohort of asthmatic children.

Figure 5-5: Expression of CD44 mRNA in epithelial cells from healthy, atopic, and atopic asthmatic children. There were no significant differences in expression between phenotypes. The dots represent mean values; the whiskers represent standard deviation.
5.3.4 Expression of EGF and EGF-R mRNA

The expression of EGF mRNA was determined using real-time PCR. Figure 5-6 shows levels of EGF mRNA in epithelial cells from healthy, healthy atopic and atopic asthmatic children. The expression of EGF was significantly down-regulated in atopic asthmatics compared to healthy children (p=0.006). The expression of EGF was also down-regulated in asthmatics as compared to healthy atopics (p=0.038). Expression of EGF did not differ between healthy and healthy atopic subjects (p=0.824). This indicates that the expression of EGF is down-regulated in the setting of atopic asthma, but not atopy alone.

![Diagram showing expression of EGF mRNA levels]

Figure 5-6: Expression of EGF mRNA in epithelial cells from healthy, healthy atopic and atopic asthmatic children. EGF mRNA levels were significantly lower in atopic asthmatics compared to both healthy (p=0.006) and healthy atopic children (p=0.038). The dots represent mean values; the whiskers represent standard deviation.
Figure 5-7 shows the expression of EGF-R in healthy, healthy atopic, and atopic asthmatic children. The expression of EGF-R was significantly down-regulated in the epithelium of atopic asthmatic children compared to both healthy (p<0.001) and healthy atopic (p=0.005) children. EGF-R expression was not significantly reduced in the healthy atopic children compared to the healthy children (p=0.098). This suggests that reduced EGF-R expression is attributable to atopic asthma, but not atopy alone.

Figure 5-7: Expression of EGF-R mRNA in the epithelium of healthy, healthy atopic and atopic asthmatic children. EGF-R was significantly down-regulated in atopic asthmatics compared to both healthy (p<0.001) and healthy atopic children (p=0.005). The dots represent mean values; the whiskers represent standard deviation.
5.3.5 Immunoreactivity for EGF-R and isolectin

On average, 15.4% of cells exhibited EGF-R staining. There was no significant difference in the number of EGF-R positive cells between phenotypes (p=0.322). However, there was considerable variability in the intensity of EGF-R staining. The differences in staining intensity were not quantifiable, as it was unclear whether the variability was due to uneven density of cells in the cytospin slides, or differences in EGF-R expression. The majority of EGF-R staining was found on basal cells, indeed 72.1% of EGF-R staining was found on the isolectin B4 positive basal cells. Figure 5-8 shows co-localisation of EGF-R and isolectin staining on basal cells. The percentage of EGF-R positive basal cells was not dependent on phenotype (p=0.669).
Figure 5-8 A: Fluorescent staining for EGF-R.  B: Fluorescent staining for basal cells (isolectin) C: double staining for EGF-R and basal cells showing co-expression of EGF-R and the basal cell marker.
5.3.6 Expression of PCNA

Expression of EGF-R mRNA was significantly decreased in the atopic asthmatic, but not healthy atopic subjects in comparison to healthy controls. The decrease in EGF-R expression seen in this cohort of asthmatics may have affected proliferation levels, as EGF-R plays an important role in epithelial proliferation. Therefore, the expression of the proliferation marker PCNA in atopic asthmatic and healthy subjects was quantified in cytospin preparations. The percentage of epithelial cells staining positive for PCNA is shown in Figure 5-9. Epithelial PCNA expression was significantly higher in the asthmatic children (p=0.047): 1.72% of cells from asthmatics exhibited staining for PCNA, compared to 0.45% of cells from healthy children. These results demonstrate that epithelial proliferation is increased in children with asthma, despite markedly decreased EGF-R expression.
Figure 5-9: Percentage of cells expressing the proliferation marker PCNA in cytospin preparations of epithelial cells. PCNA was expressed in a higher proportion of cells from atopic asthmatics (p=0.047).
5.3.7 Expression of p21

In adult asthmatics, epithelial expression of the cyclin dependent kinase inhibitor p21 is increased (69); aberrant p21 expression is thought to be involved in the dysregulated repair response seen in the asthmatic epithelium (see section 5.1.4). Figure 5-10 shows the expression of p21 mRNA in the epithelium of children depending on atopic and asthmatic status. Expression of p21 was not significantly different in the asthmatics or healthy atropics compared to the healthy children (p=0.130). These results do not support a role for p21 in the regulation of epithelial proliferation in in this cohort of asthmatic children.

![Figure 5-10: Expression of p21 mRNA in epithelial cells from healthy, healthy atopic and atopic asthmatic children. The dots represent mean values; the whiskers represent standard deviation.](image)
5.4 Discussion

The epithelium in adult asthmatics has been extensively studied, and is undoubtedly damaged. The widespread shedding of epithelial cells should prompt a repair response. However, repair appears to be dysregulated, as there is no increase in proliferation, despite an increase in expression of proliferative signals such as EGF-R. The asthmatic epithelium appears to be permanently expressing a repair phenotype, and releases growth factors and mediators which may promote airway remodelling. These abnormalities are represented by a multitude of changes in the expression of various mediators, growth factors and structural proteins. In order to demonstrate that the bronchial brushing model is capable of detecting such changes, the processes of epithelial damage and repair were investigated in healthy and asthmatic children.

5.4.1 Epithelial damage is not increased in mild childhood asthma

Two indicators of epithelial damage were examined: the number of epithelial cells shed into BAL fluid, and expression of the cellular adhesion molecule CD44. The number of epithelial cells shed into BAL fluid did not differ significantly between healthy and asthmatic children. This was unexpected, as previous researchers have shown epithelial shedding to be increased in asthma (182). However, a number of investigators have established a link between epithelial shedding, asthma severity and airway hyperresponsiveness (87, 108). The children involved in this study had very mild asthma, and were asymptomatic at the time of study, which may have precluded changes in injury and repair being detected. However, it is also reasonable to suggest that there was very little, if any, damage to the airway epithelium. This finding is not unprecedented: Lozewicz et al (238) investigated the morphological integrity of the epithelium in adults with mild asthma, and found no difference to healthy subjects in terms of epithelial cell loss, widening of intercellular spaces, and loss of cilia.
Unfortunately it is not possible to examine the relationship between airway hyperresponsiveness and epithelial shedding, as airway hyperresponsiveness was not measured in this cohort.

The expression of mRNA encoding the adhesion molecule CD44 was not increased in the epithelium of children with asthma. This is consistent with the previous results, and provides more evidence to suggest that the children involved in this study sustained little epithelial damage. In contrast with the results presented here, increased expression of CD44 has previously been detected in adult asthmatic epithelium (152). It is unclear whether this discrepancy is due to the difference in the age of the subjects (adults vs. children) or the difference in disease severity. However, both epithelial CD44 expression (354) and epithelial damage (87) are related to asthma severity, and as the asthmatic children in the present study experienced only mild asthma, it is not unexpected that it was not possible to detect evidence of increased damage, or increased CD44 expression.

The epithelial damage occurring in asthma has two components – cell damage, and cell shedding. These two processes seem to occur independently of each other (128). The results presented here provide information about both aspects of epithelial damage: cellular damage was investigated by quantifying CD44 expression in the epithelium, whereas epithelial cell shedding was investigated by enumerating epithelial cells in BAL fluid. Epithelial cell damage is associated with levels of eosinophils in the airway, as eosinophils release compounds such as major basic protein (336), and metalloproteinase 9 (337). In the present study, the percentage of eosinophils in BAL fluid was not increased in the asthmatics (section 4.3.1), a finding which is again consistent with the lack of damage seen in the asthmatics.
When epithelial cells are shed in asthma, it is not only the damaged cells that are lost, but also groups of live cells called ‘creola bodies’. These groups of cells are often found in BAL fluid, with cilia still beating (183). Basal cells are not often found in creola bodies, suggesting that shedding occurs a suprabasal plane, which might be more vulnerable to various insults (181). In addition, the integrity of the tight junctions appears to be abnormal in the asthmatic airway epithelium (187). In the present study, we have shown that epithelial shedding alone is not increased in mild asthmatics. This result, together with the finding of increased FeNO (section 4.3.3), suggests that despite airway inflammation, the epithelium of this cohort of asthmatic children displays little, if any, damage.

5.4.2 EGF and EGF-R are down-regulated in mild childhood asthma

Asthma is associated with structural changes to the airway, and damage to the airway epithelium. In healthy lungs, the epithelium will respond to damage by transiently expressing growth factors necessary for repair. However, in asthma there is evidence to support abnormal expression of growth factors (355). Inappropriate expression of growth factors in the asthmatic airway epithelium has been implicated in airway remodelling. The epithelial expression of EGF and its receptor were investigated in children in the setting of asthmatic and atopic disease.

The expression of mRNA for EGF and its receptor was examined using real-time PCR. Both EGF and EGF-R expression were down-regulated in atopic asthmatics compared to healthy children. EGF expression was not down-regulated in healthy atopics compared to healthy children, suggesting that the down-regulation of this growth factor is associated with asthma, but not atopy. EGF-R expression was down-regulated in
both atopics and atopic asthmatics compared to healthy children. However, the effect was more pronounced in the atopic asthmatics; EGF-R expression was significantly lower in the atopic asthmatics in comparison to the healthy atopics.

This is one of the first studies to examine the expression of EGF-R in the epithelium of asthmatic children. The only other study of EGF-R in the paediatric asthmatic epithelium reported increased immunoreactivity for EGF-R in children with moderate to severe asthma (241). Comparisons between this study and the present investigation are complicated by the difference in methods used: expression of EGF-R mRNA levels are reported here, whereas Federov et al examined expression of EGF-R protein. Expression of EGF-R protein in asthmatic epithelium was not assessed in this study, as attempts to quantitate EGF-R immunoreactivity in cytospin slides were unsuccessful. Immunostaining revealed that the majority of EGF-R immunoreactivity was located on basal epithelial cells, but did not detect any differences between phenotypes in the total number of cells expressing EGF-R, or the number of basal cells expressing EGF-R. Whilst the number of cells expressing EGF-R may not have varied, the level of EGF-R expression may have varied. Cytospins are not appropriate for determining levels of expression, as variability in the intensity of staining is caused both by expression levels, and the density of cells on the slide. Detection of EGF-R with western blotting may yield superior results, but was not conducted in this study due to a lack of available samples of epithelial tissue.

Studies in adult asthmatics have clearly shown that both EGF and EGF-R are expressed in increased levels in asthmatic epithelium (188, 272, 356), not only in areas of damage, but also in intact epithelium (49). In both adult and paediatric asthma, epithelial expression of EGF-R in the epithelium appears to be related to disease severity (241,
Federov et al (241) demonstrated increased EGF-R in moderate asthmatics, and a further increase in EGF-R expression in severe asthmatics. All the asthmatics in this study experienced mild, asymptomatic asthma, which may also explain why the results presented here contrast those reported in children with moderate and severe asthma.

The reason for decreased EGF-R expression in this cohort of asthmatic children is unclear. However, the down-regulation of EGF-R demonstrates that the proposed model of non-bronchoscopic brushing is capable of detecting epithelial defects, even in mild, asymptomatic asthma. In addition, the finding provides evidence of a defect in the epithelium of children with mild asthma. Further investigation is needed to determine whether this effect is mediated by an in vivo factor, or if it is intrinsic to asthmatic epithelial cells. This could be accomplished by investigating levels of EGF-R mRNA in cultured epithelial cells. If decreased expression of EGF-R persisted in vitro, this would provide further evidence for an epithelial defect in mild asthma.

5.4.3 Proliferation is increased

The EGF-R plays a central role in maintenance and repair of epithelial tissue (358). EGF accelerates wound healing both in vivo (344) and in vitro (49, 345). Therefore, it was highly probable that the altered expression of EGF and EGF-R could have affected epithelial proliferation. As EGF and its receptor were down-regulated in the epithelium of this cohort of atopic asthmatics, we would expect epithelial proliferation to be similarly decreased. To investigate this hypothesis, epithelial proliferation was examined by quantifying the percentage of cells expressing the proliferation marker PCNA.
Expression of PCNA was increased in the atopic asthmatics, despite down-regulation of the EGF-R. The increase in PCNA expression was not only unexpected based on the observed levels of EGF-R in this cohort, but also based on the majority of published observations of epithelial proliferation in the asthmatic epithelium. In adult asthmatics, proliferation of asthmatic epithelial cells, as assessed by immunoreactivity to PCNA, is not increased despite increased EGF-R (69, 189, 190). However, proliferation of asthmatic epithelial cells was shown to be increased when epithelial cells were co-cultured with cells obtained from BAL (359).

PCNA is widely used to identify proliferating cells, as there is an increase in PCNA expression during the G1-phase, which peaks at the S-phase, and declines during G2/M-phases of the cell cycle (360). There have been conflicting reports on the correlation of PCNA staining with other proliferation indices (361). However, the reliability of PCNA as a proliferative marker has been questioned because detectable levels of PCNA can vary significantly among different cell types (362), and because PCNA is also involved in DNA repair (363). In this study, PCNA staining was only examined in a single cell type: epithelial cells. In addition, cells were only considered to be positively stained for PCNA if the staining was intense, and nuclear. Potential bias was minimised by blinding the observer to the asthmatic status of the sample being counted. In this manner, the utility of PCNA as a marker of proliferation was maximised.

The present study found very low levels of immunoreactivity to PCNA antigen. Indeed, only 0.45% of cells from asthmatics and 1.72% of cells from asthmatics exhibited staining for PCNA. However, these results are not unprecedented: Puddicombe et al investigated PCNA immunoreactivity in mucosal biopsies from adult non-asthmatics, and subjects with mild or severe asthma (69). They found the percentage of epithelial
cells staining positive for PCNA to be 0.4% in healthy subjects, 0.7% in subjects with mild asthma, and 0.8% in subjects with severe asthma. In addition, a number of other researchers have commented on the lack of PCNA immunoreactivity in the airway epithelium (189, 190).

Previous studies have shown that EGF-R activity is coupled to expression of the cyclin dependent kinase inhibitor p21. In adult asthmatics, increased epithelial expression of p21\textsuperscript{waf1} (69) may explain the lack of proliferation in the asthmatic epithelium. To investigate the role of p21 in the present cohort of asthmatics, p21 expression was assessed in epithelial cells from healthy, atopic and asthmatic children. Epithelial expression of mRNA encoding p21 was not different in atopic asthmatics compared to healthy atopics, or healthy children. This suggests that differences in p21 expression do not account for the exaggerated PCNA expression in this cohort. It is unclear why these results do not agree with those reported by Puddicombe \textit{et al} (69), however, it is unlikely that the discrepancy is due to differences in asthma severity: although Puddicombe \textit{et al} (69) found that p21 expression tended to be higher in severe asthma, this relationship was not statistically significant.

\textbf{5.4.4 Conclusions}

The processes of damage and repair have been extensively studied in the epithelium of adult asthmatics; therefore epithelial damage, repair signals, and proliferation were investigated in the airway epithelium of children with asthma in order to validate the proposed model of non-bronchoscopic brushing. Evaluation of epithelial cells shed into BAL fluid, and expression of the cellular adhesion molecule, CD44, revealed no evidence of epithelial damage. In contrast, expression of EGF and its receptor was significantly decreased in the epithelium of children with mild asthma. Despite this,
proliferation, as indicated by immunoreactivity for PCNA, was increased. In adults this discrepancy appears to be mediated by p21; however, this mechanism does not appear to be important in children with mild asthma. Considered together, these results demonstrate that epithelial defects are detectable in asthma through the use of non-bronchoscopic brushing. In addition, this study provides evidence for an imbalance in the relationship between proliferative signals, and proliferation.
6.0 Microarray

6.1 Introduction

6.1.1 Microarray technology

Until recently, researchers have been confined to investigating one of the many thousands of genes at a time. However, the advent of microarray technology means that it is now possible to obtain information about thousands of genes in a single experiment. This technology not only allows a much higher throughput, but is also capable of monitoring the entire genome on a single array, and permits investigation of the interactions among thousands of genes simultaneously.

A microarray consists of a glass support similar to a microscope slide, to which probes for thousands of different genes are attached at fixed locations. A labelled sample of RNA or DNA is applied to the array, allowing complementary sequences of nucleic acids in the sample to hybridise to the probes on the chip. The results are then read by creating a digital image of the array. Each location on the array corresponds to a particular sequence or gene, and the intensity of fluorescence at a particular location indicates the amount of that particular sequence in the sample. In this way, information on each sequence present on the array is compiled for a particular sample. By comparing samples of different origin (eg: healthy vs diseased) genes of importance can be identified.

6.1.2 Microarrays using DNA

Genetic material for microarray analysis can be either DNA or RNA, depending on the type of experiment to be performed. Comparative Genomic Hybridisation uses large pieces of genomic DNA to detect genomic gains and losses, or changes in the number of
copies of a particular gene involved in a disease state. This technique is particularly useful in clinical genetics, where it is used to diagnose unbalanced chromosomal rearrangements (364). Mutation microarray analysis also uses DNA, but to detect much smaller scale changes. This technique can detect DNA alterations such as heterozygous base-pair polymorphisms or mutations and single base-pair insertions or deletions. Mutation microarray analysis is widely used in pharmacogenetic studies, to investigate the genetic basis for individual variations in response to therapeutics (365).

### 6.1.3 Microarrays using RNA

Microarrays can also be used for detecting changes in gene expression levels, effectively taking a snapshot of the genes a cell is expressing at a particular point in time, and the level of expression of each of these genes. For this application, samples of RNA are prepared and hybridised to microarrays, such that differences the in expression of thousands of genes can be compared between samples. There are two types of arrays that can be used for gene expression analysis: cDNA (spotted) arrays, and oligonucleotide arrays. A cDNA array is constructed with PCR products generated from cDNA libraries or clone collections. These are printed onto glass slides as spots at defined locations. The spots are 100-300µm in size, and are roughly the same distance apart, in a grid formation. These arrays contain around 30,000 different cDNA sequences. The process of constructing a grid of spots is not accurate enough to allow comparison between arrays. Therefore, two samples for comparison are hybridised to the same array, but are labelled with different coloured dyes (Cy3 or Cy5). The slide is scanned for two separate wavelengths so the intensity of signal for each dye can be determined for each spot on the array (366).

The second type of expression array is the oligonucleotide array. Oligonucleotides (20-25 nucleotides long) are synthesized \textit{in situ} by light directed chemistry (Affymetrix
chips), or alternatively, pre-synthesized oligonucleotides are printed onto glass slides. In vitro transcription is used to prepare a sample of RNA for hybridisation to the array: RNA is reverse transcribed to cDNA, before a biotin label is incorporated in an in vitro transcription step that results in labelled cRNA. Each sample is hybridised to a separate array, and the intensity of signal compared between arrays to compare the expression between samples (Figure 6-1). Expression arrays are widely used to investigate disease states, and have been applied to investigate muscular dystrophy (367), Alzheimers disease (368), schizophrenia (369), and HIV infection (370).
6.1.4 Analysis of data

Analysis of gene expression using microarrays produces raw data in the form of signal intensity for a particular location on the array. Each signal intensity is then matched to the sequence of the probe, which corresponds to a particular gene. The challenge to the investigator lies in obtaining meaningful biological data from a list of gene expression containing several thousand genes. Several approaches to analysing microarray data have been developed, but most rely on investigating individual genes which are highly up or down-regulated. These approaches have several drawbacks. Firstly, creating a
list of statistically significant genes without any unifying biological theme means that interpretation of meaning is very dependent on the investigator’s area of expertise. In addition, these approaches examine only those genes which are highly up or down-regulated; a 20% increase in gene expression may be biologically important, but could be overlooked as statistically insignificant. Finally, analysis of individual genes may cause important effects on biological pathways to be missed. Gene Set Enrichment Analysis (GSEA) overcomes these difficulties by examining gene expression data to identify pathways of importance (371).

**Gene Set Enrichment Analysis**

GSEA represents a knowledge-based approach for interpreting genome-wide expression profiles, and allows the discovery of statistically significant pathways in expression profiling studies. Once signal intensity data have been obtained from the microarray, a signal-to-noise ratio is calculated for all genes. A ranked list is then created by listing all genes in order of their signal-to-noise ratio. Next, gene sets are obtained from sources such as the Gene Ontology project (372). Gene sets group certain genes together based on characteristics such as molecular function, or the biological process in which they are involved. For example, the gene set “integrin binding” contains 25 genes encoding proteins which interact selectively with an integrin. For each gene set, an enrichment score is calculated which reflects the degree to which the set is overrepresented at the extremes of the ranked list. A gene set with the majority of genes located at the top of the list will have a high enrichment score. Finally, a p-value is calculated to determine the statistical significance of the enrichment score, which can be adjusted for multiple hypotheses testing to obtain a q-value. Once statistically significant pathways have been identified, the investigator can use complementary
techniques such as PCR, western blotting, and cell culture to further investigate pathways of interest.

6.1.5 The present study

This investigation has demonstrated that bronchial brushing can be used in conjunction with techniques such as real-time PCR and immunohistochemistry to investigate airway diseases. This technique is sensitive enough to detect significant differences in epithelial gene and protein expression even in asymptomatic asthma. Initially, the scope of the research was restricted to individual genes and pathways known to be important to asthma. However, in order to extend our knowledge of airway diseases, and identify novel pathways and genes of importance, the possibility of using microarray technology to investigate global gene expression in the epithelium was examined. Therefore, this study aims to use Affymetrix oligonucleotide arrays to detect differences in gene expression in the epithelium of children due to asthmatic disease.

6.2 Methods

6.2.1 Preparation of cRNA

First and Second strand cDNA synthesis

RNA was extracted from the epithelial cells (section 2.8) and used to prepare cRNA according to a modified version of the protocol of Baugh et al (373). The first strand of cDNA was synthesised from 1µg of RNA in a volume of 20µl consisting of 1 x first strand buffer, 5mM DTT, 0.5mM dNTPs, 0.24mg/ml T4gp32, 40U RNase inhibitor, and 200U Superscript II. Reactions were incubated at 42°C for 1 hour and then heat inactivated at 65°C for 15 minutes. The 20µl reaction was then incubated with 1 x second strand buffer, 0.2mM dNTPs, 40U DNA polymerase I, 10U E.Coli DNA ligase,
and 2U RNase H in a final volume of 150µL, for 2 hours at 16°C. 20U of T4 DNA polymerase was added, and the reaction incubated at 16°C for a further 15 min. The reaction was then heat inactivated at 70°C for 10 min.

**Purification of double-stranded cDNA**

The cDNA was purified by phenol-chloroform extraction, and unincorporated nucleotides were removed using Bio-Spin Chromatography columns (Bio-Rad, NSW, Australia). The reaction was diluted with an equal volume of phenol/chloroform/isoamyl alcohol, and vortexed. The mixture was then added to a Phase Lock Gel tube (Eppendorf, NSW, Australia) and centrifuged at maximum speed for 5 minutes. The upper phase was applied to a Bio-Spin column and centrifuged at 3500rpm for 4 minutes. Double stranded cDNA was precipitated by incubating the eluate with 2.5 volumes of 100% ethanol, and 20mg/ml of glycogen at –80°C for 1 hour. The resulting precipitate was pelleted by centrifugation and washed with 70% ethanol then resuspended in 22µl of RNAse free water.

**In vitro transcription**

cDNA was transcribed to cRNA using the BioArray HighYield RNA Transcript Labeling Kit (Affymetrix, CA, USA). Reaction buffer, Biotin-Labelled Ribonucleotides, DTT, RNase Inhibitor Mix and T7 RNA polymerase were added to 22µl of cDNA according to the manufacturers instructions, to a final volume of 40µl. The reaction was incubated for 9 hours at 37°C. The cRNA was purified using RNeasy RNA extraction columns (Qiagen, Victoria, Australia): 160µl of RNase-free water, 500µl of 100% ethanol, and 700µl of RLT buffer was added to each reaction and mixed, then added to two separate RNeasy columns, which were centrifuged at maximum
speed for 30 sec. The columns were then washed twice with RPE buffer, and the cRNA eluted in 50µl of RNase-free water. The eluate was re-applied to the column to maximise the amount of cRNA retrieved. Samples which yielded more than 7.5µg of cRNA were accepted for fragmentation.

**Fragmentation of cRNA**

The amount of cRNA synthesised was determined using the Agilent Bioanalyser (see section 3.2.6), and samples containing approximately 10µg cRNA were selected for microarray analysis. In preparation for hybrisisation to the microarrays the cRNA was fragmented. The volume of the sample was adjusted by dessication or dilution to obtain a concentration of 1.25µg/µl cRNA. Fragmentation buffer (5x) was added to a final concentration of 1x, and the sample heated at 95°C for 30 minutes. The samples were then assayed with the Agilent Bioanalyser to ensure that fragmentation was complete. Samples which were completely fragmented were accepted for hybridisation to microarrays.

**6.2.2 Hybridisation to Microarrays**

Samples were analyzed with the use of Affymetrix U133A GeneChips. Each gene on this chip is represented by 10 to 20 oligonucleotides, termed a "probe set". The intensity of hybridization of labelled messenger RNA (mRNA) to these sets reflects the level of expression of a particular gene. The U133A GeneChip contains 22,283 probe sets, representing approximately 13,000 genes. Microarray hybridization was performed by the Arthritis and Inflammation Research Program, Garvan Institute for Medical Research, Darlinghurst, Australia.
6.2.3 Analysis of Microarray data

Normalization, transformation and data selection procedures were performed by the Division of Pulmonary & Critical Care, University of Washington, Seattle, USA. Gene expression data were analysed using GSEA Software version 1.0 (MA, USA). Statistical comparisons across groups were also performed using GSEA software, as detailed in Subramanian et al (374). Six databases of gene sets were used in the analysis (Table 13): the C2 (functional sets) (371), C3 (regulatory-motif sets) (375), and C4 (correlational sets) (376), and databases derived from the Gene Ontology project based on biological process, cellular component, and molecular function. Data provided for each gene set included the name and source of the gene set, the number of genes in the set, the percentage of active genes, the normalised enrichment score, and the nominal p-value. Gene sets were considered to be significantly enriched if they had a nominal p-value of 0.05 or less.
Table 13: Description and details for each of the six catalogs of gene sets used in the microarray analysis

<table>
<thead>
<tr>
<th>Catalog</th>
<th>Number of sets</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td>522</td>
<td>This catalog includes 472 sets containing genes whose products are involved in specific metabolic and signaling pathways, as reported in eight publicly available, manually curated databases, and 50 sets containing genes coregulated in response to genetic and chemical perturbations, as reported in various experimental papers.</td>
<td>Subramanian et al (391)</td>
</tr>
<tr>
<td>C3</td>
<td>57</td>
<td>Gene sets that contain genes that share a cis-regulatory motif that is conserved across the human, mouse, rat and dog genomes. Their motifs represent unknown or likely regulatory elements in promoters and 3'UTRs. These gene sets make it possible to link changes in a microarray experiment to a conserved, putative cis-regulatory element.</td>
<td>Xie et al (392)</td>
</tr>
<tr>
<td>C4</td>
<td>427</td>
<td>These sets are defined by expression neighborhoods centered on cancer-related genes. This database provides an initial collection of gene sets for use with GSEA and illustrates the types of gene sets that can be defined, including those based on prior knowledge or derived computationally.</td>
<td>Brentani et al (393)</td>
</tr>
<tr>
<td>BP</td>
<td>373</td>
<td>This collection of gene sets encompasses those processes specifically pertinent to the functioning of integrated living units: cells, tissues, organs, and organisms, a process being a collection of molecular events with a defined beginning and end.</td>
<td>Gene Ontology project</td>
</tr>
<tr>
<td>CC</td>
<td>101</td>
<td>Contains sets of genes encoding the part of a cell or its extracellular environment in which the gene product is located. A gene product may be located in one or more parts of a cell and its location may be as specific as a particular macromolecular complex, that is, a stable, persistent association of macromolecules that function together.</td>
<td>Gene Ontology project</td>
</tr>
<tr>
<td>MF</td>
<td>186</td>
<td>Sets of genes whose products are involved in elemental activities, such as catalysis or binding at a molecular level. A given gene product may exhibit one or more molecular functions.</td>
<td>Gene Ontology project</td>
</tr>
</tbody>
</table>
6.3 Results

6.3.1 Quantity of cRNA

RNA extracted from epithelial cells was used as a template to synthesize cRNA for microarray analysis. Even though 1μg of RNA was used as starting material for each sample, the amount of cRNA synthesized was variable. Sufficient cRNA for microarray analysis was obtained from 16 RNA samples (Table 14). There was no significant difference between the yield of cRNA from healthy or atopic asthmatic subjects (p=0.610). As shown in Figure 6-2 there was no significant correlation between the yield of cRNA and either the concentration of template RNA (R=0.024, p=0.872) or the quality of template RNA (R= -0.028, p=0.848).

Table 14: RNA samples used to synthesize cRNA. 1μg of RNA was used as a template for cRNA synthesis.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Phenotype</th>
<th>RNA concentration (ng/ul)</th>
<th>cRNA amount (ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>Healthy</td>
<td>38.53</td>
<td>7.94</td>
</tr>
<tr>
<td>97</td>
<td>Healthy</td>
<td>41.8</td>
<td>11.88</td>
</tr>
<tr>
<td>109</td>
<td>Healthy</td>
<td>56.77</td>
<td>8.36</td>
</tr>
<tr>
<td>119</td>
<td>Healthy</td>
<td>90.24</td>
<td>11.82</td>
</tr>
<tr>
<td>122</td>
<td>Healthy</td>
<td>132.32</td>
<td>11.12</td>
</tr>
<tr>
<td>125</td>
<td>Healthy</td>
<td>59.63</td>
<td>16.00</td>
</tr>
<tr>
<td>127</td>
<td>Healthy</td>
<td>46.51</td>
<td>9.02</td>
</tr>
<tr>
<td>71</td>
<td>Atopic asthmatic</td>
<td>61.23</td>
<td>8.20</td>
</tr>
<tr>
<td>78</td>
<td>Atopic asthmatic</td>
<td>52.26</td>
<td>10.23</td>
</tr>
<tr>
<td>87</td>
<td>Atopic asthmatic</td>
<td>111.82</td>
<td>9.85</td>
</tr>
<tr>
<td>88</td>
<td>Atopic asthmatic</td>
<td>146.01</td>
<td>14.83</td>
</tr>
<tr>
<td>98</td>
<td>Atopic asthmatic</td>
<td>68.21</td>
<td>15.51</td>
</tr>
<tr>
<td>124</td>
<td>Atopic asthmatic</td>
<td>171.46</td>
<td>8.36</td>
</tr>
<tr>
<td>126</td>
<td>Atopic asthmatic</td>
<td>118.97</td>
<td>8.17</td>
</tr>
<tr>
<td>128</td>
<td>Atopic asthmatic</td>
<td>79.11</td>
<td>7.70</td>
</tr>
<tr>
<td>135</td>
<td>Atopic asthmatic</td>
<td>39</td>
<td>7.51</td>
</tr>
</tbody>
</table>
Figure 6-2: Correlation between starting RNA concentration or quality and final cRNA yield. cRNA yield was not significantly correlated with either RNA concentration (R=0.024, p=0.872) or RNA quality (R= -0.028, p=0.848).

The cRNA was fragmented to yield a sample of cRNA consisting of short oligonucleotides, ready for hybridisation to microarrays. cRNA was subjected to Agilent analysis before and after fragmentation. Figure 6-3A is a representative electropherogram showing the cRNA before fragmentation; electropherograms were used to determine the amount of cRNA present. The broad peak represents the wide range of size of RNA fragments. Figure 6-3B shows a representative sample of cRNA after fragmentation; these electropherograms were used to confirm that the fragmentation was successful. The narrow peak at 25 seconds indicates that the sample consisted of fragments of cRNA of approximately the same size.
Figure 6-3: Electropherogram of cRNA.  

A: Before fragmentation, the cRNA consists of a wide range of fragment sizes. 

B: After fragmentation, the cRNA consists of similarly sized fragments suitable for hybridisation to microarrays.
6.3.2 Summary of gene expression data

Six databases of gene sets were analysed. Significantly enriched gene sets were found in all databases. The gene expression data were summarised in six heatmaps, one for each group of gene sets investigated (Figure 6-4). These heatmaps clearly show the correlation between gene expression and phenotype. The analysis of the microarray data is too complex to present in its entirety in this thesis. Therefore, the presented data are restricted to the gene sets which were significantly enriched in the asthmatics (ie upregulated in asthmatics) and those which were significantly enriched in the healthy population (ie downregulated in asthmatics). Gene sets were considered to be significantly enriched if they had a nominal p-value of less than 0.05.

The C2 database contained 400 gene sets, 2 of which were significantly enriched in the asthmatics, and 4 of which were significantly enriched in the healthy non-atopics. In the C3 database, 10 of 144 gene sets were significantly enriched in the asthmatics, but none of the gene sets were significantly enriched in the healthy non-atopics. Of the 500 gene sets in the C4 database, 13 were significantly enriched in the asthmatics, and 5 were significantly enriched in the healthy non-atopics. 13 of the 893 gene sets in the biological process database were significantly enriched in the asthmatics, and 14 were significantly enriched in the healthy non-atopics. The cellular component database contained 190 gene sets, of which 7 were significantly enriched in the asthmatics, and 2 were significantly enriched in the healthy non-atopics. In the molecular function database, 5 of 515 gene sets were significantly enriched in the asthmatics, and 4 of the gene sets were significantly enriched in the healthy non-atopics. Gene sets which were significantly enriched in either the asthmatics or the healthy non-atopic samples are presented in table 15 and table 16.
Figure 6-4: Heatmaps of the significantly enriched gene sets. A: genes from the C2 gene sets; B: genes from the C3 gene sets; C: genes from the C4 gene sets; D: genes from the biological process gene sets; E: genes from the cellular component gene sets; F: genes from the molecular function gene set.
Table 15: Gene sets which exhibited significant enrichment in the asthmatic samples. Source: the catalogue of genes from which each gene set is derived (see section 6.2.3). Gene Set: the name of the set of genes, indicating the process or component in which they are involved. Size: the number of genes in the set. Gene %: the percentage of genes in the set which display core enrichment. NES: normalised enrichment score. NOM p-value: nominal p-value, indicating the statistical significance of the normalised enrichment score.

<table>
<thead>
<tr>
<th>Source</th>
<th>Gene Set</th>
<th>Size</th>
<th>Gene %</th>
<th>NES</th>
<th>NOM p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological Process</td>
<td>meiotic cell cycle</td>
<td>38</td>
<td>0.154</td>
<td>1.4981</td>
<td>0.002075</td>
</tr>
<tr>
<td>Biological Process</td>
<td>M phase of meiotic cell cycle</td>
<td>38</td>
<td>0.154</td>
<td>1.4981</td>
<td>0.002075</td>
</tr>
<tr>
<td>Biological Process</td>
<td>meiosis</td>
<td>38</td>
<td>0.154</td>
<td>1.4981</td>
<td>0.002075</td>
</tr>
<tr>
<td>Biological Process</td>
<td>meiosis I</td>
<td>21</td>
<td>0.128</td>
<td>1.5247</td>
<td>0.01018</td>
</tr>
<tr>
<td>Biological Process</td>
<td>DNA recombination</td>
<td>53</td>
<td>0.223</td>
<td>1.4507</td>
<td>0.01207</td>
</tr>
<tr>
<td>Biological Process</td>
<td>transmission of nerve impulse</td>
<td>196</td>
<td>0.255</td>
<td>1.4906</td>
<td>0.01285</td>
</tr>
<tr>
<td>Biological Process</td>
<td>neurotransmitter secretion</td>
<td>17</td>
<td>0.255</td>
<td>1.4894</td>
<td>0.01835</td>
</tr>
<tr>
<td>Biological Process</td>
<td>regulated secretory pathway</td>
<td>17</td>
<td>0.255</td>
<td>1.4894</td>
<td>0.01835</td>
</tr>
<tr>
<td>Biological Process</td>
<td>synaptic transmission</td>
<td>190</td>
<td>0.256</td>
<td>1.464</td>
<td>0.02336</td>
</tr>
<tr>
<td>Biological Process</td>
<td>meiotic recombination</td>
<td>16</td>
<td>0.128</td>
<td>1.4438</td>
<td>0.02459</td>
</tr>
<tr>
<td>Biological Process</td>
<td>sensory perception of taste</td>
<td>10</td>
<td>0.379</td>
<td>1.2036</td>
<td>0.02872</td>
</tr>
<tr>
<td>Biological Process</td>
<td>striated muscle contraction</td>
<td>28</td>
<td>0.208</td>
<td>1.4767</td>
<td>0.03711</td>
</tr>
<tr>
<td>Biological Process</td>
<td>gamma-aminobutyric acid signalling path</td>
<td>12</td>
<td>0.417</td>
<td>1.3112</td>
<td>0.04025</td>
</tr>
<tr>
<td>Functional Set</td>
<td>muscle myosin</td>
<td>14</td>
<td>0.142</td>
<td>1.5124</td>
<td>0.02315</td>
</tr>
<tr>
<td>Functional Set</td>
<td>SA_DAG1</td>
<td>10</td>
<td>0.0749</td>
<td>1.3423</td>
<td>0.03919</td>
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<tr>
<td>Motif based Set</td>
<td>TAAAWATAG</td>
<td>112</td>
<td>0.332</td>
<td>1.545</td>
<td>0.004167</td>
</tr>
<tr>
<td>Motif based Set</td>
<td>TGATTCRY</td>
<td>205</td>
<td>0.248</td>
<td>1.4148</td>
<td>0.004228</td>
</tr>
<tr>
<td>Motif based Set</td>
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<td>0.332</td>
<td>1.3289</td>
<td>0.003529</td>
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<tr>
<td>Motif based Set</td>
<td>RYTAAWNNNTGAY</td>
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<td>1.4143</td>
<td>0.01903</td>
</tr>
<tr>
<td>Motif based Set</td>
<td>YATTNTAC</td>
<td>249</td>
<td>0.305</td>
<td>1.3332</td>
<td>0.02743</td>
</tr>
<tr>
<td>Motif based Set</td>
<td>AAAYRNCTG</td>
<td>253</td>
<td>0.226</td>
<td>1.2785</td>
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</tr>
<tr>
<td>Motif based Set</td>
<td>CAGNMMCNNNGAC</td>
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<td>0.332</td>
<td>1.4533</td>
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</tr>
<tr>
<td>Motif based Set</td>
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<td>Motif based Set</td>
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<td>110</td>
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<tr>
<td>Motif based Set</td>
<td>RAAGNYNNCTTY</td>
<td>93</td>
<td>0.191</td>
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<td>0.04365</td>
</tr>
</tbody>
</table>
Table 16: Gene sets which exhibited significant enrichment in the healthy non-atopic samples.

<table>
<thead>
<tr>
<th>Source</th>
<th>Gene Set</th>
<th>Size</th>
<th>Gene %</th>
<th>NES</th>
<th>NOM p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological Process</td>
<td>purine ribonucleoside monophosphate metabolism</td>
<td>12</td>
<td>0.233</td>
<td>-1.5046</td>
<td>0.01163</td>
</tr>
<tr>
<td>Biological Process</td>
<td>purine ribonucleoside monophosphate biosynthesis</td>
<td>12</td>
<td>0.233</td>
<td>-1.5046</td>
<td>0.01163</td>
</tr>
<tr>
<td>Biological Process</td>
<td>purine nucleoside monophosphate metabolism</td>
<td>12</td>
<td>0.233</td>
<td>-1.5046</td>
<td>0.01163</td>
</tr>
<tr>
<td>Biological Process</td>
<td>purine nucleoside monophosphate biosynthesis</td>
<td>12</td>
<td>0.233</td>
<td>-1.5046</td>
<td>0.01163</td>
</tr>
<tr>
<td>Biological Process</td>
<td>peptidyl-amino acid modification</td>
<td>25</td>
<td>0.0806</td>
<td>-1.3692</td>
<td>0.01656</td>
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<tr>
<td>Biological Process</td>
<td>oxygen and reactive oxygen species metabolism</td>
<td>43</td>
<td>0.214</td>
<td>-1.4664</td>
<td>0.02444</td>
</tr>
<tr>
<td>Biological Process</td>
<td>retrograde vesicle-mediated transport, Golgi to ER</td>
<td>13</td>
<td>0.165</td>
<td>-1.5031</td>
<td>0.02893</td>
</tr>
<tr>
<td>Biological Process</td>
<td>energy coupled proton transport, down electrochemical gradient</td>
<td>29</td>
<td>0.294</td>
<td>-1.4771</td>
<td>0.00308</td>
</tr>
<tr>
<td>Biological Process</td>
<td>ATP synthesis coupled proton transport</td>
<td>29</td>
<td>0.294</td>
<td>-1.4771</td>
<td>0.00308</td>
</tr>
<tr>
<td>Biological Process</td>
<td>nucleoside phosphate metabolism</td>
<td>32</td>
<td>0.294</td>
<td>-1.4705</td>
<td>0.00352</td>
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<tr>
<td>Biological Process</td>
<td>ATP biosynthesis</td>
<td>32</td>
<td>0.294</td>
<td>-1.4705</td>
<td>0.00352</td>
</tr>
<tr>
<td>Biological Process</td>
<td>tRNA modification</td>
<td>30</td>
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<tr>
<td>Biological Process</td>
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<td>36</td>
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</tr>
<tr>
<td>Biological Process</td>
<td>glycerophospholipid metabolism</td>
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</tr>
<tr>
<td>Functional Set</td>
<td>MAP00052_Galactose metabolism</td>
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<td>-1.4628</td>
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<tr>
<td>Functional Set</td>
<td>MAP00510_N_Glycans_biosynthesis</td>
<td>16</td>
<td>0.081</td>
<td>-1.5402</td>
<td>0.02696</td>
</tr>
<tr>
<td>Functional Set</td>
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<td>0.164</td>
<td>-1.4537</td>
<td>0.04024</td>
</tr>
<tr>
<td>Functional Set</td>
<td>GLUT_UP</td>
<td>246</td>
<td>0.252</td>
<td>-1.313</td>
<td>0.04141</td>
</tr>
<tr>
<td>Correlational Set</td>
<td>morf1_37377_1_at_LMNA</td>
<td>91</td>
<td>0.249</td>
<td>-1.5697</td>
<td>0.005173</td>
</tr>
<tr>
<td>Correlational Set</td>
<td>gcmNML_L40391_at_TMP21</td>
<td>60</td>
<td>0.295</td>
<td>-1.5795</td>
<td>0.02650</td>
</tr>
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<td>Correlational Set</td>
<td>gcmNML_XG1190_at_ARCN1</td>
<td>95</td>
<td>0.242</td>
<td>-1.5105</td>
<td>0.03476</td>
</tr>
<tr>
<td>Correlational Set</td>
<td>morf1_37600_at_ECM1</td>
<td>61</td>
<td>0.211</td>
<td>-1.4388</td>
<td>0.04286</td>
</tr>
<tr>
<td>Correlational Set</td>
<td>gcm_M94046_at_MA1</td>
<td>66</td>
<td>0.294</td>
<td>-1.4314</td>
<td>0.04704</td>
</tr>
<tr>
<td>Cellular Component</td>
<td>proton-transporting two-sector ATPase complex</td>
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<td>-1.4776</td>
<td>0.02936</td>
</tr>
<tr>
<td>Cellular Component</td>
<td>mitochondrial outer membrane</td>
<td>22</td>
<td>0.121</td>
<td>-1.3446</td>
<td>0.04339</td>
</tr>
<tr>
<td>Molecular Function</td>
<td>hydrogen-transporting ATP synthase activity, rotational mechanism</td>
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<td>0.308</td>
<td>-1.5084</td>
<td>0.03056</td>
</tr>
<tr>
<td>Molecular Function</td>
<td>hydrogen-transporting ATP synthase activity, rotational mechanism</td>
<td>29</td>
<td>0.294</td>
<td>-1.4771</td>
<td>0.03033</td>
</tr>
<tr>
<td>Molecular Function</td>
<td>cholesterol hydrolase activity</td>
<td>40</td>
<td>0.225</td>
<td>-1.3451</td>
<td>0.03983</td>
</tr>
<tr>
<td>Molecular Function</td>
<td>GO:0016623</td>
<td>12</td>
<td>0.23</td>
<td>-1.4257</td>
<td>0.0444</td>
</tr>
</tbody>
</table>
6.3.3 Analysis: individual gene sets

The previous section presented a summary of the gene expression data, by highlighting the differences between the asthmatic and healthy atopic samples. In total, 50 gene sets were enriched in the asthmatics, and 29 gene sets were enriched in the healthy non-atopics. Of these, three will be presented here as examples of the potential of these data to identify areas of importance in asthmatic disease which require further research. For each gene set, a running enrichment plot, a volcano plot and a heat map are presented.

The running enrichment plot is presented in three sections: the top section of the plot shows the running enrichment score for the gene set as the analysis moves down the ranked list. The score at the peak of the plot is the enrichment score for the gene set. The middle portion of the plot shows where the members of the gene set appear in the ranked list of genes. The leading edge subset of a gene set is the subset of members that contribute most to the enrichment score. For a positive enrichment score (see Figure 6-5 and Figure 6-11), the leading edge subset is the set of members that appear in the ranked list prior to the peak score. For a negative enrichment score (Figure 6-8), it is the set of members that appear subsequent to the peak score. Finally, the bottom portion of the plot shows the value of the ranking metric, which measures the gene’s correlation with phenotype. A positive value indicates a correlation with asthmatic phenotype.

The volcano plot makes it possible to compare the size of the fold change to the statistical significance level. The ‘volcano plot’ arranges genes along dimensions of biological and statistical significance. Fold change is represented on the x-axis, indicating the biological impact of the change, whilst the p-value is represented on the y-axis, indicating the statistical significance of the change. Finally, the heat map
represents expression values as colours, where the range of colours from red to blue shows the range of expression values.

The “Cellular Component” list of gene sets is a database of genesets derived from information from the Gene Ontology project. The gene set “Fibrillar Collagen” was significantly enriched in the asthmatics (p=0.014). This gene set contains 10 genes encoding collagen polymers in which collagen triple helices associate to form fibrils. The major features of this gene set are summarised in Figure 6-5, 6-6 and Figure 6-7. The ranked gene list was 13,920 genes long; peak enrichment occurred at 2850 genes, and zero crossing (equal expression in the asthmatic and the healthy non-atopic samples) occurred at 8384 genes. The relationship between fold change and statistical significance for each gene in this gene set is presented in Figure 6-6 as a volcano plot: 8 genes, or 80%, exhibited core enrichment. Figure 6-7 shows the correlation between phenotype, and expression of genes encoding fibrillar collagen, as a heat map.
Figure 6-5: Running enrichment plot of the cellular component gene set “Fibrillar Collagen”. Of the ten genes in the set, eight exhibited core enrichment.
Figure 6-6: Volcano plot of the cellular component gene set “Fibrillar Collagen”. Genes exhibiting core enrichment are shown in red. Other genes in the set are shown in green.
Figure 6-7: Heat map for genes in the cellular component gene set “Fibrillar Collagen”. Eight of the ten genes in this set exhibited core enrichment.
The “Biological Process” database of gene sets is also based on information from the Gene Ontology project. The gene set “Oxygen and reactive oxygen species metabolism” was significantly enriched in the healthy non-atopics (p=0.024). This gene set contains 49 genes involved in the chemical reactions and pathways involved in metabolising oxygen, or any reactive oxygen species. The characteristics of this gene set are summarised in Figure 6-8, 6-9, and Figure 6-10. The ranked gene list was 13,920 genes long, and peak enrichment occurred at 10,942 genes (Figure 6-8). Interestingly, this gene set contained NOS2A, a gene which was investigated in section 4.3.4. In the microarray analysis, this gene was ranked 6010 of 13,920, but was not one of the genes showing core enrichment. The relationship between fold change and statistical significance for each gene in this gene set is presented in the volcano plot: 23 genes, or 46.9%, exhibited core enrichment (Figure 6-9). The correlation between phenotype and expression of genes involved in oxygen and reactive oxygen species metabolism is represented in a heat map (Figure 6-10).
Figure 6-8: Running enrichment plot of the biological process gene set “Oxygen and reactive oxygen species metabolism”. Of the 49 genes in the set, 23 exhibited core enrichment.
Figure 6-9: Volcano plot of the biological process gene set “Oxygen and reactive oxygen species metabolism”. Genes exhibiting core enrichment are shown in red. Other genes in the set are shown in green.
Figure 6-10: Heat map for genes in the biological process gene set “Oxygen and reactive oxygen species metabolism”. 23 of the 49 genes in this set exhibited core enrichment
The “C3” database of gene sets provides information on commonly conserved regulatory motifs in the promoter regions of human genes. In the current analysis, the gene set “TGATTTRY” was significantly enriched in the asthmatics (p=0.004). This gene set contains 205 genes with a 3’ untranslated region containing the motif TGATTTRY. This motif matches the annotation of Growth Factor Independence-1 (GFI1), a transcriptional repressor (375). The characteristics of this gene set are summarised in Figure 6-11, 6-12, and Figure 6-13. The ranked gene list was 13,920 genes long; peak enrichment occurred at 3455 genes (Figure 6-11), and zero crossing (equal expression in the asthmatic and the healthy non-atopic samples) occurred at 8384 genes. The volcano plot shows the relationship between fold change and statistical significance for each gene in this gene set; the 77 genes (37.6%) with core enrichment are highlighted (Figure 6-12). Finally, the heat map demonstrates the correlation between phenotype and expression of genes associated with the TGATTTRY motif (Figure 6-13).
Figure 6-11: Running enrichment plot of the C3 gene set “TGATTTRY”. Of the 205 genes in the set, 77 exhibited core enrichment.
Figure 6.12: Volcano plot of the C3 gene set “TGATTTRY”. Genes exhibiting core enrichment are shown in red. Other genes in the set are shown in green.
Figure 6-13: Heat map for genes in the C3 gene set “TGATTTRY”. 77 of the 205 genes in this set exhibited core enrichment.
Figure 6-13 continued
Figure 6-13 continued
Figure 6-13 continued
6.4 Discussion

6.4.1 The present study

Microarrays were used to compare gene expression in asthmatic and healthy children. This investigation aimed to determine whether cells obtained by non-bronchoscopic brushing were suitable for microarray analysis, and whether altered gene expression could be detected in the epithelium of children with asthma, compared to healthy children. In the epithelium of asthmatic children, 50 gene sets were enriched, and 29 gene sets were enriched in the epithelium of healthy non-atopic children, providing compelling evidence that abnormalities are detectable, even in children with asymptomatic asthma.

6.4.2 Synthesis of cRNA

RNA extracted from cells brushed from the airways of children was successfully used to synthesise cRNA for microarray analysis. As discussed in section 3.4.3, the brushing contained not only epithelial cells, but also macrophages, and a very small proportion of lymphocytes and neutrophils. After the macrophages were removed by positive selection, the remaining cells were 96.6% epithelial, raising the possibility that contaminating cells may have affected gene expression. In this case, the cRNA being used to assess epithelial gene expression may not have been entirely epithelial in origin. However, the proportion of contaminating cells was very small, and did not significantly vary between phenotypes (section 3.3.3), therefore it is unlikely that these cells affected any of the comparisons made between asthmatics and healthy children in this study.

Although the starting amount of RNA was consistent (1mg), the yield from each sample was substantially different. The reasons for the variation in the amount of cRNA
synthesised are unclear. The yield was not related to phenotype, concentration of starting RNA, or quality of starting RNA. However, the source of the samples may have played a role in the variability. Although the RNA had been extracted and purified using chromatographic columns, there may still have been some proteins present from the lung that inhibited the \textit{in vitro} transcription process. Despite this, sufficient cRNA was synthesized from epithelial cells to allow the production of high quality microarray data.

6.4.3 Gene set enrichment analysis

The analysis of microarray data has always been problematic due to the difficulties associated with assigning biological meaning to a list of differentially regulated genes. The development of GSEA has overcome a number of these problems by providing results in the form of sets of genes with similar biological function. Gene sets are considered to be enriched in a particular phenotype if they contain genes which tend to be upregulated. In the present study, the significantly enriched gene sets were involved in processes known to be important to asthma, and those for which a role in asthma has not yet been established. The three gene sets presented in section 6 are discussed to demonstrate the utility of this method for investigating the asthmatic epithelium.

The gene set “Oxygen and reactive oxygen species metabolism” was enriched in the healthy non-atopics, indicating that the genes in this set tended to be down-regulated in the asthmatic epithelium. Genes exhibiting core enrichment included glutathione peroxidises 1, 2, 3, and 7, and superoxide dismutase 2, which play a vital role in the antioxidant defenses of the lung (377). Asthmatics generate increased amounts of reactive oxygen species from peripheral blood cells and cells recovered by BAL (378), therefore the decreased levels of glutathione peroxidise and superoxide dismutase may
contribute to the pathogenesis of asthma. Previous investigators have found both systemic (379) and epithelial (380) levels of antioxidants to be decreased in asthmatics. Once again, the results from the present study emphasise the role of the epithelium in the pathogenesis of asthma, and invite further study into the mechanisms by which the lung antioxidant defenses are compromised.

The gene set ‘Fibrillar Collagen’ was also enriched in the asthmatic samples. This set contains genes encoding collagen polymers in which collagen triple helices associate to form fibrils. Among the genes showing core enrichment in this set were those encoding \(\alpha\)-subunits for collagens 1, 2, 3 and 5. Collagen production in the asthmatic airway is synonymous with remodelling and reticular basement membrane thickening (121). Whilst the asthmatic epithelium releases factors that promote collagen deposition by subepithelial fibroblasts (194), there is no evidence to suggest that the airway epithelium directly contributes to collagen deposition. It is more likely that the increase in expression of collagen genes reflects a response to damage.

The gene set TGATTTRY contains genes which have the motif TGATTTRY in their 3’ untranslated region. TGATTTRY matches the annotation for growth factor independence-1 (Gfi-1) a transcriptional repressor protein, and is therefore a potential binding site for Gfi-1 (375). In the asthmatics, there was overexpression of genes with TGATTTRY in their 3’ untranslated region. It is possible that these genes are overexpressed due to down-regulation of Gfi-1, or expression of faulty Gfi-1. This would lead to overexpression of genes in the TGATTTRY gene set, as seen in this study. The consequences of enrichment of the TGATTTRY gene set can be deduced from the genes which exhibited core enrichment, indicating biological activity. In this set, genes encoding cellular adhesion molecules, such as CD226, Claudin 8, and
Integrin β8 were biologically active. Several genes of the MAP-K signalling pathway were also upregulated, including fibroblast growth factor 7 and mitogen activated protein kinase kinase 7. In addition, IL-21, a cytokine which has potent regulatory effects on cells of the immune system, was one of the genes showing core enrichment in the asthmatics. IL-21 administration in a mouse asthma model effectively reduced IgE and IgG levels, as well as symptoms of airway hypersensitivity including eosinophil recruitment (381). Further analysis of the gene set TGATTTRY, and the expression of Gfi-1 in the asthmatic epithelium is needed.

6.4.4 Application of microarray analysis to the asthmatic epithelium

Microarrays have been used with some success to investigate experimentally-induced asthma in animals. These studies have been conducted in mice models of asthma. Karp et al (382) used microarray analysis of gene expression to identify genes involved in airway hyperresponsiveness in mouse strains of differing susceptibility. These investigators identified complement factor 5 as a susceptibility locus for airway hyperresponsiveness. In an experimentally induced mouse model of asthma, Zimmerman et al (296) attempted to identify asthma signature genes. Among the genes which were differentially regulated in asthma were those involved in the uptake and metabolism of amino acids. The authors focused on arginine metabolism, and were able to identify a role for arginase in asthma pathogenesis. Zou et al (383) used a monkey model of experimentally-induced asthma to identify genes involved in bronchoconstriction and allergic reaction. The study identified a number of novel genes that were differentially expressed in allergen-induced pulmonary inflammation. The authors also investigated the expression of 80 genes with real-time PCR, and found agreement between the two methods in 55% (44) of the genes.
Array analysis has also been used to investigate asthma in humans. Individual cell types investigated include airway eosinophils and peripheral blood lymphocytes. Cho et al (386) profiled gene expression in resting and activated human mast cells. However, very few studies have used microarray analysis to investigate epithelial gene expression in asthmatics. Laprise et al (387) applied microarray analysis to bronchial biopsies taken from asthmatics before and after inhaled corticosteroid therapy. This study investigated only eight subjects to identify 79 genes with significantly different expression between asthmatics and healthy controls. Interestingly, almost twice as many genes showed differential expression between pre- and post-corticosteroid therapy. Only one published study has used bronchial brushing to obtain cells for microarray analysis in asthma. Lilly et al (388) sampled airway epithelial cells from five adult asthmatics before and after a segmental airway challenge with allergen. Whilst they identified a number of genes involved in the response to allergens, they did not make any comparison to healthy controls. Guajardo et al (389) sampled nasal epithelial cells from healthy children, and those with stable and exacerbated asthma. Although this study made the assumption that gene expression in the nasal mucosa reflects gene expression in the respiratory epithelium of the lung, it identified a number of immune genes, and cilia-related genes as significantly dysregulated in the asthmatics.

Whilst microarrays have facilitated advances in our understanding of a number of diseases, they still have disadvantages. Although the cost of arrays has decreased significantly in the last few years, they remain expensive. This has meant that the number of chips per experiment is generally kept to a minimum, either by investigating fewer subjects, or by pooling RNA from many subjects. Unfortunately, this increases the risk of incorrectly identifying differentially expressed genes. Another hurdle for clinical studies is the challenge of obtaining RNA of sufficient quantity and quality.
for microarray studies. Epithelial cell cultures generally yield RNA of higher quality and have been used for array analysis of asthma (391). However, gene expression in vitro may not accurately represent in vivo expression.

Clinical studies are further complicated by the composition of tissue that can be sampled. Whole lung or biopsy samples are composed of many different cell types, of which only a few may be relevant for the disease (390). In addition, changes in asthma such as airway remodelling, epithelial shedding and inflammatory cell infiltrate can interfere with the composition of the sample (387). The present study avoids a number of these complications. Cells were sampled directly from the lung, so gene expression was representative of in vivo conditions. The samples were close to homogeneous, consisting of 96.6% epithelial cells (section 3.3.3). In addition, the population of cells sampled was not changed in asthmatics.

Other challenges faced by clinical studies include overcoming difficulties in obtaining tissue, and accounting for the effect of therapy on gene expression. It is difficult to justify elective bronchial biopsy in children, but the non-bronchoscopic brushing method presented here allows researchers to safely obtain useful quantities of epithelial cells from healthy children (chapter 0). Inhaled corticosteroid therapy is widely used in treating asthma, and is known to alter gene expression (387). However, all asthmatics involved in this study had mild asthma and did not require inhaled corticosteroids.

6.4.5 Conclusions
This study aimed to determine whether children with asthma had evidence of abnormal epithelial function based on gene expression. Significant differences in gene expression were found, indicating that the airway epithelium is abnormal, even in children with
asymptomatic asthma. These findings emphasize the role of the epithelium in asthma, and highlight its role as an active participant within the airway. The data presented here clearly demonstrate the potential of microarray analysis to investigate the airway epithelium in asthma. Future investigators should concentrate on specific genes and pathways of importance as identified by the microarray, and the investigation of their functional significance.
7.0 General Discussion

The epithelium plays a vital role in maintaining respiratory homeostasis, and is central to the pathology of many airway diseases. Epithelial cells are the first point of contact for any inhaled stimuli, and provide a barrier, protecting underlying tissues. However, the epithelium is also an essential and active participant in the healthy lung, releasing a number of mediators which are central to normal airway function (392). Disruption of normal epithelial function can have serious consequences for respiratory health (393).

Investigation of epithelial cell biology has the potential to yield valuable information about several respiratory diseases. Previous investigators have used a variety of techniques to obtain epithelial tissue from adults and children with severe airway disease; however it is difficult to justify the use of such invasive techniques in otherwise healthy children. The lack of an appropriate method for study of the epithelium has hindered the investigation of the paediatric epithelium (252). This study aimed to address this issue, by developing a model for sampling epithelial cells from healthy children for use in cellular, molecular and histochemical investigation.

7.1 Statement of principal findings

The safety and utility of non-bronchoscopic brushing was investigated in an unselected population of children. The non-bronchoscopic brushing model was then applied to the study of asthmatic and atopic disease in the epithelium. Asthma is one of the most common childhood diseases, and yet most information concerning the disease comes from studies in adults (394). Little is known about the early stages of the disease, and a better understanding of the mechanisms involved in its progression would greatly enhance our ability to treat and manage this syndrome (395). Subsequently, global gene
expression was investigated using microarray analysis, in order to identify novel pathways and genes involved in the disease, and extend our knowledge of this common childhood disease.

Non-bronchoscopic brushing was found to be a safe and effective method for obtaining cells of sufficient quality and quantity to allow western blotting, real time PCR, and microarray analysis. None of the children undergoing brushing experienced any complications. Cells sampled from the airway were identified as 94.2% epithelial with 3.4% macrophages. Once the macrophages were removed, the epithelial cells were successfully used for immunocytochemistry and extraction of RNA and protein.

Asthma is a multifactorial disease, involving many cells, cytokines, chemokines and growth factors. In order to investigate asthma in the paediatric airway epithelium, three target areas were identified: inflammation, damage and repair. These processes are central to asthma, therefore genes and cellular markers representing each of the three areas were examined to identify asthma-related differences in the epithelium of children (Figure 7-1).

![Inflammation Damage Repair](image)

**Figure 7-1:** Asthma was investigated in the paediatric epithelium by investigating the three processes central to asthmatic disease: inflammation, damage and repair.

Investigation of various markers of airway inflammation such as FeNO, iNOS and eosinophilia revealed little evidence of inflammation in the asthmatic children in this cohort. FeNO was raised in the asthmatics compared to the healthy children. However,
FeNO has previously been shown to be increased due to atopy (303), and further increased in atopic asthma (396). In addition, there was no difference in FeNO between the healthy atopic children, and the atopic asthmatic children, suggesting that the raised FeNO in the atopic asthmatics was due to their atopic status, and not the result of asthma per se. Furthermore, there was no evidence of eosinophilia in the epithelial samples, and no increase in NOS2 expression in the asthmatics, suggesting that there was little, if any inflammation in this cohort of asthmatic children. As FeNO (397) and eosinophil levels (86) are both related to asthma severity, it is not unexpected that there were no apparent differences between phenotypes in this study, as the asthmatics all experienced mild, asymptomatic asthma.

Cellular signatures of epithelial damage, repair and proliferation were also investigated. Although epithelial damage is considered to be characteristic of asthma (127), evaluation of epithelial cells shed into BAL fluid, and expression of the cellular adhesion molecule, CD44, revealed no evidence of epithelial damage. Since studies in adult asthmatic epithelium have shown repair signals such as EGF to be increased even in intact asthmatic epithelium (49), we examined expression of EGF and its receptor in our cohorts. Expression of EGF and EGF-R was found to be significantly decreased in the epithelium of the asthmatic children. Previous researchers have shown that expression of the proliferation marker PCNA is not increased in adult asthmatic epithelium (189), despite increased EGF-R expression. In contrast, this investigation revealed that epithelial immunoreactivity to PCNA was increased in asthmatics, despite decreased EGF-R levels.

To identify novel genes and pathways of importance to asthma, and thereby expand our understanding of asthmatic disease, global gene expression was investigated using
microarrays. In the past, analysis of microarray data has concentrated on the expression of single genes, causing difficulties in the extraction of biologically meaningful data. The interpretation of meaning is very dependent on the investigator’s area of expertise, and small changes in gene expression may be overlooked as statistically insignificant. Gene Set Enrichment Analysis (GSEA) overcomes these difficulties by examining gene expression data to identify pathways of importance (371). In the present study GSEA was used to interpret the microarray data, using six databases of gene sets derived from gene ontology and published information. Overall, 50 gene sets were enriched in the asthmatic epithelium, and 29 were enriched in the healthy non-atopic epithelium. Among the gene sets enriched in the asthmatic epithelium were a set of genes with a 3’ untranslated region containing the motif TGATTTRY, which matches the annotation for growth factor independence-1, and a set of genes encoding fibrillar collagens. These gene sets may be important in the abnormal repair and remodelling processes seen in asthma, and require further investigation. In the healthy non-atopic epithelium, a set of genes involved in oxygen and reactive oxygen species metabolism was significantly enriched. As these genes play a role in the response to oxidative stress, they may be important in the asthmatic epithelium. In summary, these data indicate that epithelial cells obtained from asthmatic children exhibit a different gene expression profile compared to healthy subjects.

7.2 Strengths and weaknesses of the study

This thesis presents, for the first time, a method for investigating the epithelium of otherwise healthy children, using non-bronchoscopic brushing. The value of this method for the study of airway diseases was clearly demonstrated through the investigation of aspects of epithelial cell biology known to be important in asthmatic and atopic disease. Although several of the findings presented here agree with previous
research conducted in the asthmatic airway epithelium, there were also numerous discrepancies. This is most likely due to the mild, asymptomatic asthma experienced by the children recruited for this study. Although the study of this particular phenotype of asthma has limitations, there are several advantages. Data presented here allow the generation of two hypotheses concerning the behaviour of the airway epithelium in asthma, which require further investigation:

1. Studying children with mild asthma permits investigation of the causes of asthma, rather than changes that result from asthma.
2. Investigation of children with mild asthma allows elucidation of factors that influence the persistence of asthma.

This study has provided the first systematic examination of gene expression in children, and has demonstrated striking differences to adult data. In addition to the discovery that there are distinct patterns of gene expression in mild asthma, it was revealed that the gene expression encoding for EGF-R is dramatically down-regulated. Despite this, and despite signs of minimal epithelial damage, there is increased proliferation in the epithelium of children with mild asthma, as reflected by exaggerated PCNA expression. The data from this study demonstrate that there are differences in the epithelium of children with mild asthma that are not related to current symptoms, and are not related to inhaled corticosteroid therapy. These differences may represent intrinsic abnormalities in the epithelium, present before asthmatic disease becomes clinically apparent.

7.3 **Strengths and weaknesses in relation to other studies**

In adult asthmatics, there are extensive changes to the airway (398). Some of these changes are structural, such as the thickening of the basement membrane, increases in
smooth muscle mass, and shedding of the epithelium, but there are also alterations in the production of signalling molecules: the epithelium expresses many chemokines, cytokines, peptides and growth factors in augmented quantities (354). Several changes may have been present before asthmatic disease became clinically apparent, but the majority will relate to inflammation, damage and repair, as well as corticosteroid therapy. Investigation of epithelial restitution has revealed that resolution of inflammation and repair of damage do not occur normally in asthmatics (69). Furthermore, it appears that the asthmatic lung is inappropriately maintained in a cycle of damage and repair (2). This has been proposed as a factor in the decline in lung function seen in susceptible asthmatics (130). Clearly then, any examination of gene expression in adult asthmatics is complicated by the progression of the disease, and as such it is difficult to distinguish between any causative abnormalities and changes relating to symptoms and treatment.

In children with severe asthma, there are also extensive changes to the airway. A limited number of studies have investigated airway remodelling and reticular basement membrane thickening in children with severe or difficult asthma, and shown that the degree and extent of airway remodelling in these children is very similar to that seen in adult asthmatics (399-401). Furthermore, in some cases, airway changes were observed prior to the onset of symptoms. In addition to irreversible changes, there are reversible changes associated with the presence of current symptoms, including denudation of airway epithelium, mucous hyper secretion, and infiltration by inflammatory cells (402). Cytokines released by inflammatory cells induce changes in epithelial cells. For example, IL-17 secreted by T-cells stimulates epithelial cell cytokine production (403), and IL-1β, in conjunction with TNF-α and IFN-γ, can induce expression of ICAM-1 on epithelial cells (404). In addition, inhaled allergens impacting on the epithelium cause
damage and inflammatory changes (405). Children with severe asthma also require inhaled corticosteroid therapy, which alters expression of many genes (406). Whilst inhaled corticosteroids are employed to reduce inflammation, their mechanism of action is not well defined. Furthermore, they influence different aspects of inflammation with varying efficiency. For example, inhaled corticosteroids are highly potent inhibitors of NOS (177), and reduce FeNO very rapidly (407). However, their effect on other indicators of airway inflammation, such as the presence of neutrophils, occurs to a lesser degree, over a longer time-period (408). Therefore, children with severe asthma also display extensive changes to their airways that complicate the study of intrinsic epithelial abnormalities related to asthma.

The asthmatics in the present study were asymptomatic and were not using inhaled corticosteroid therapy at the time of study, so neither treatment nor current symptoms would have influenced epithelial gene expression. Therefore, the differences in epithelial gene expression observed in this cohort are likely to represent intrinsic abnormalities that contribute to the development of asthma, not abnormalities resulting from the progression of asthma. Alternatively, it is possible that abnormalities in the epithelium of children with mild asthma represent the early stages of severe asthma, or a mild form of persistent asthma. However, this is highly unlikely, as the pattern of expression of EGF-R and PCNA seen in the epithelium of mildly asthmatic children is not only different to the pattern seen in their healthy counterparts, but also the opposite to the pattern of expression seen in adult asthma (56), and moderate to severe asthma in children (241). These findings strongly suggest that differences in the epithelium of children with mild asthma represent intrinsic abnormalities in the epithelium.
7.4 Meaning of the study

The novel findings of this investigation indicate that asthma in children is associated with abnormal epithelial function, and also suggest dysregulation of the repair process. The abnormalities seen suggest that mild childhood asthma may have a different aetiology to either severe childhood asthma, or adult asthma. Alternatively, the data presented here may reflect a different response to the same stimuli, or an early stage of asthmatic disease that is not seen in studies of adult or severe asthmatics. The findings are summarised in Figure 7-2.

Figure 7-2: The paediatric epithelium in asthma. In this cohort of children, the parameters investigated revealed that there was very little inflammation, no evidence of damage, and increased proliferation despite decreased repair signals in the epithelium of asthmatics.

Epidemiologic data suggest that children with mild asthma will tend to grow out of the disease, whereas children with severe asthma are much more likely to have asthma that persists into adulthood (409, 410). This prompts a further hypothesis: study of the epithelium of children with mild asthma allows the identification of factors influencing the resolution or persistence of asthma. The data presented here consider three aspects of asthma pathology: inflammation, damage, and repair, to support this hypothesis.
Unlike adult asthmatics and children with severe asthma, the mild asthmatics in this study had very little evidence of inflammation at a cellular level. Although inflammation is central to asthma pathophysiology, it is not intrinsically injurious. Acute inflammation is a normal and necessary physiological response that allows the body to fight off infection. Conversely, chronic inflammation, as occurs in asthma, is not appropriate, and is associated with destruction of airway epithelium, mucous hypersecretion, collagen deposition beneath the basement membrane, and infiltration of inflammatory cells which release proinflammatory cytokines such as IL-4, IL-5, and IL-13 (402). The asthmatics involved in this study had no evidence of cellular inflammation in either BAL or mucosal brushings, consistent with their asymptomatic nature. Although this is different to the scenario of children with severe asthma and adult asthmatics, it is unlikely to represent a factor affecting asthma persistence. Studies have shown that while inhaled corticosteroids attenuate asthma symptoms, they have little effect on airway remodelling, and do not result in asthma resolution (134). A study examining the effect of corticosteroids on expression of TGF-β, IL-11, IL-17 and collagens type I and III found that corticosteroids reduced the expression of IL-11 and IL-17, but not TGF-β or collagen type I or III (411). This may account for the relative resistance of airway remodelling to corticosteroid therapy. Therefore, it is unlikely that the different level of inflammation in these children represents a factor contributing to the persistence of asthma.

Although inflammation appears to have limited influence on asthma persistence, it causes epithelial damage, which may have a greater effect. In this cohort there was very little evidence of damage in terms of CD44 expression or epithelial cells shed into BAL fluid. This contrasts published reports of epithelial damage in adult asthmatics and children with severe asthma. In addition, the asthmatic children in this study exhibited a
different response to damage than that previously published in adult or severe asthmatics: EGF and EGF-R expression were decreased, whilst proliferation was increased. Epithelial damage and repair processes are likely to influence the persistence of asthma. In persistent asthma, it would appear that the repair process is somehow compromised (2), as the epithelium is fragile in appearance, and maintained in a damaged state (108). While damaged, the epithelium expresses an altered array of mediators in order to effect repair (412). Unfortunately, these mediators are also able to affect other cells, and have been linked to airway remodelling (179). In addition, when the process of epithelial repair is blocked, the production of the profibrogenic mediator TGF-β2 is enhanced (49). Published data from adults show increased repair signals (EGF and EGF-R) and decreased proliferation (PCNA), suggesting the epithelium is maintained in a damaged state. Data from the present study demonstrate decreased EGF and EGF-R, and increased proliferation as evidenced by PCNA expression. Clearly this is not normal; however, it is possible that this represents an appropriate response to damage, and one that leads to resolution of asthma.

7.5 Future Research

This research has outlined a program of study of the airway epithelium in children of varying disease states, including asthma. Preliminary analysis of the data generated has highlighted key abnormalities in the epithelium of children with asthma that warrant further investigation. In this study, comparisons were made between adult and paediatric data and between severe and mild asthma. This was only possible by comparing data from children recruited for this study, and published observations of the epithelium in adults and children with severe asthma. Clearly this is not optimal, as differences between studies in recruiting patients, sampling tissue, and obtaining data will have affected the outcomes. Tissue collected from a comparable cohort of children
with severe asthma, and healthy and asthmatic adults would allow a complete and direct comparison of gene expression data.

The preliminary analysis of the array data demonstrated that there are significant differences in the epithelium of asthmatic children. However, the data presented here have barely scratched the surface of the wealth of information provided by microarray analysis. Future investigators should focus their efforts by examining particular genes or pathways to determine their relevance to asthma. Initially, this will require validation of the array data by examining expression of individual genes with real-time PCR. While changes in gene expression provide a highly useful indicator of cellular function, they do not always represent changes in the expression of functional protein. Western blotting should be employed to validate differential expression of individual proteins. In addition, very few studies have applied proteomics to the investigation of asthma (413). Large-scale protein studies through proteomic techniques are required for the understanding of biological processes, and should be employed to further our understanding of the asthmatic epithelium. Next, functional significance should be determined using primary cultures of epithelial cells derived from bronchial brushings.

This study has shown that EGF-R expression is decreased in the epithelium of children with asthma. Further investigations using primary cultures of epithelial cells should determine whether the down-regulation of EGF-R is maintained in culture, or whether there are in vivo factors inducing receptor down-regulation. In this study, a marker of epithelial proliferation (PCNA) was used to investigate the effect of EGF-R down-regulation on epithelial proliferation. Primary cultures of epithelial cells derived from bronchial brushings should be used to investigate potential differences in proliferation in vitro, both under normal conditions, and in response to scrape wounding. Factors
known to influence epithelial proliferation should be investigated in order to determine what is causing increased proliferation in the epithelium of these asthmatic children. In conclusion, the data presented here prompt careful re-evaluation of the repair-remodelling paradigm. The role of the airway epithelium as an active participant in asthmatic disease is emphasised. In addition, the need for investigation of asthmatic disease in children is highlighted, both by the paucity of paediatric studies of asthma, and by the surprising results presented in this study.
8.0 References


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9.0 Appendix

QUESTIONNAIRE

1. Has your child ever had wheezing or whistling in the chest at any time in the past?       Yes ❑       No ❑

IF YOU HAVE ANSWERED “NO” PLEASE SKIP TO QUESTION 6

2. Has your child had wheezing or whistling in the chest in the last 12 months?      Yes ❑       No ❑

IF YOU HAVE ANSWERED “NO” PLEASE SKIP TO QUESTION 6

3. How many attacks of wheezing has your child had in the last 12 months?       None ❑       1 to 3 ❑       4 to 12 ❑       > 12 ❑

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

5. In the last 12 months, has the wheezing been severe enough to limit your child’s speech to only one or two words at a time between breaths?      Yes ❑       No ❑

6. Has your child ever had asthma?       Yes ❑       No ❑

7. In the last 12 months, has your child’s chest sounded wheezy during or after exercise?      Yes ❑       No ❑

8. In the last 12 months, has your child ever had a dry cough at night, apart from a cough associated with a cold or chest infection?      Yes ❑       No ❑

9. Has your child ever had a problem with sneezing, or a runny, or blocked nose when he/she did not have a cold or the flu?      Yes ❑       No ❑

10. Was your child treated for this with medication?       Yes ❑       No ❑

11. In the past 12 months, has your child had a problem with sneezing, or a runny, or blocked nose when he/she did not have a cold or the flu?      Yes ❑       No ❑

IF YOU HAVE ANSWERED “NO” PLEASE SKIP TO QUESTION 15

12. In the past 12 months, has this nose problem been accompanied by itchy-watery eyes?       Yes ❑       No ❑

13. In which of the past 12 months did this nose problem occur? (please tick any which apply)
1. In the past 12 months, how much did the nose problem interfere with your child’s daily activities? Not at all ☐
   A little ☐
   A moderate amount ☐
   A lot ☐

2. Has your child ever had hayfever? Yes ☐
   No ☐

3. Has you child ever had an itchy rash which was coming and going for at least six months? Yes ☐
   No ☐

IF YOU HAVE ANSWERED “NO” PLEASE SKIP TO QUESTION 22

4. Has your child had this itchy rash at any time in the last 12 months? Yes ☐
   No ☐

IF YOU HAVE ANSWERED “NO” PLEASE SKIP TO QUESTION 22

5. Has this itchy rash at any time affected any of the following places: The folds of the elbows, behind the knees, ankles, under the buttocks or around the neck, ears or eyes? Yes ☐
   No ☐

6. At what age did this rash first occur? Under 2 years ☐
   Age 2-4 years ☐
   Age >4 years ☐

7. Has the rash cleared completely at any time during the last 12 months? Yes ☐
   No ☐

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

9. Has your child ever had eczema? Yes ☐
   No ☐

10. Medication in the last 3 months:

11. Family History:
    Hay fever Mother ☐
        Father ☐
        Sibling(s) ☐
    Asthma Mother ☐
        Father ☐
        Sibling(s) ☐
    Eczema Mother ☐
        Father ☐
        Sibling(s) ☐

25. Does anyone smoke in the family? Mother Inside ☐
    Outside ☐
    Father Inside ☐
    Outside ☐
    Sibling(s) ☐
Parent Information Sheet

Does raised exhaled nitric oxide reflect unrecognised airway inflammation in healthy children?

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

GENERAL INFORMATION:
Nitric oxide (NO) is a molecule that is involved in many physiological processes in the body including inflammation. NO is detectable in exhaled breath (eNO) and is raised in asthmatics. Many studies have demonstrated that exhaled NO might be a useful marker of airway inflammation in asthma thus aiding diagnosis and monitoring of disease activity. However, children who respond to skin allergy tests (atopic) and who do not have any respiratory symptoms also have raised eNO. We do not know why eNO is raised in healthy atopic children but it may also be due to inflammation in the airways that is not presently causing respiratory problems. If we can determine what causes raised eNO in healthy atopic children we will better understand how this test will help us monitor airway disease.

"What will happen if I agree for my child to take part?"
The present study will use standard diagnostic techniques to investigate whether inflammation that might not otherwise be recognised is the cause of raised NO levels in the breath of children with atopy. In order to study this issue we will recruit children, WITH and WITHOUT ATOPY, who are at Princess Margaret Hospital for day surgery.

We will use the following strategy:
- Prior to surgery we will measure lung function, eNO and do a skin allergy test. We will also collect the water vapour from your child’s exhaled air (breath condensates) for measurements of possible markers of inflammation.
- During surgery we will obtain some samples of liquid (BAL) from the airways, swab the surface of the windpipe (brushings) and collect a small amount of blood for genetic analysis.
- These samples will be analysed in the laboratory for signs of inflammation and DNA from the blood will be analysed for genes known to be associated with asthma.
- The parents and GP's of children with abnormal lung function or signs of inflammation will be advised regarding any further investigations or possible treatment.

Measurements we will make:
1. Prior to surgery
- Lung function tests – These are routine, painless tests that give us information about how well the lungs work. Your child will be asked to take a full breath and blow out hard into a measuring device.
- Exhaled nitric oxide – To measure eNO we will ask your child to take a deep breath in and blow into a machine while trying to maintain a constant expiratory flow.
- Exhaled Breath Condensate – To collect the condensate your child will be asked to breathe normally through a mouthpiece that is attached to a cooling apparatus. Our exhaled air is saturated with water and cooling down this air results in condensation. We can collect the condensation to measure different markers of airway inflammation. It will take about 10 minutes of normal breathing to collect enough condensation for analyses.
- Skin tests - These are routine “allergy tests” and tell us if a child is allergic to common environmental allergens such as house dust mite, grass and tree pollens etc. A small amount of the allergen is placed on the skin, which is then scratched. An allergic reaction is like mosquito bite but clears up after a couple of hours.
2. During surgery

**BAL** (bronchoalveolar lavage)- after your child is asleep a fine silicon, flexible tube is passed through the vocal cords into the airways. The tube is small compared to the diameter of the airway and through it a small amount of saline (salt water) is introduced into the airway and then immediately sucked out. This irrigation allows us to collect samples of cells, liquid and chemicals produced by the lung. Over half is immediately sucked out the remainder is almost immediately cleared by the lung as part of it's normal function.

**Brushings** – To collect cells from the wall of the airways we will pass a fine brush through the larynx into the windpipe and rub along the wall of the windpipe a few centimetres below the vocal cords. If you wish, we will demonstrate the techniques and equipment used before you decide to go ahead.

**Blood sample** – Blood will be collected once your child is asleep.

**Risks:**
Both the BAL and brushings are simple tests and take less than 5 minutes to complete. In adults these tests are performed without anaesthetic. Neither test will be carried out if your child's anaesthetist or surgeon believes the test will interfere with your child's treatment. We have routinely performed hundreds of these tests without incident. A dry cough is the only adverse symptom reported, it seems to occur in approximately half of the children involved in our study.

**Benefits:**
We will be able to provide you with information regarding the allergic status and lung function of your child. Furthermore, eNO measurements and the determination of other inflammatory markers from breath condensates and samples obtained during anaesthesia will help determine whether your child might have asthma or are at increased risk of asthma.

All information that is collected about your child during the course of the research will be kept strictly confidential. Any information about your child that leaves the hospital will have your/his/her name and address removed so that you/he/she cannot be recognised from it. It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care your child receives. If you have any complaints about any aspect of the study you can contact the Executive Director Medical Services of PMH (Dr Geoff Masters) on 9340 1550

Thank you for reading this information sheet.

**If you have any further questions with regard to this study they can be discussed with**
- **Dr Stephen Stick** (Telephone 9340 8830)
- **Dr Scott Burgess** (Hospital switch board - Telephone 9340 8222 page 2025 at any time)
CONSENT FORM – INOS STUDY

PLEASE INITIAL EACH BOX

1. I confirm that I have read and understand the information sheet dated …………… for the above study and have had an opportunity to ask questions.

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

3. I understand that sections of any of my child’s medical notes may be looked at by responsible individuals where it is relevant to his/her taking part in research. I give permission for those individuals to have access to my child’s records.

4. I agree to my child taking part in the above study.

_________________  _______________  ___________________
Name of parent           Date                         Signature

_________________  _______________  ___________________
Name of researcher     Date                         Signature
I consent to the collection from my child of blood via venepuncture from which DNA will be extracted and stored. I also consent for the collection of respiratory epithelial cells via bronchial brushing, from which RNA will be extracted and stored.

I consent to my child’s DNA/RNA being used for research into asthma. I understand that the DNA/RNA will be stored in good faith but that its integrity cannot be guaranteed.

I understand that the DNA/RNA will not be used for purposes other than that specified above and will not be used for diagnostic purposes.

Name of child: ………………………………

Name of Parent / Guardian: ………………………………………

Signature: ……………………………………… Date: …………………

Witness: ……………………………………… Date: …………………