CILIATED EPITHELIUM
IN
RESPIRATORY DISEASES

Biju Thomas

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Abstract

Ciliated epithelium in respiratory diseases

Dr Biju Thomas

Background: The ciliated respiratory epithelium that covers the surface of human airway forms an immunologically active natural barrier to invasion and injury by inhaled noxious agents. Ciliary dysfunction and or epithelial damage compromise this innate defence mechanism.

Aim: To study the ciliary function and epithelial ultrastructure of adult patients with asthma and paediatric lung transplant recipients. To study the response of bronchial epithelial cells of patients with atopic severe asthma, to allergen and bacteria.

Methods: Digital high speed video microscopy was used to study the ciliary function on bronchoscopic bronchial epithelial brushings. Transmission electron microscopy was used to study the detailed epithelial ultrastructure. Cytokines and chemokines released by primary bronchial epithelial cells were measured using SECTOR Imager 6000 (MSD, USA).

Results: Ciliary dysfunction and ultrastructural abnormalities are closely related to asthma severity. Ciliary dysfunction is a feature of moderate to severe asthma and profound ultrastructural abnormalities are restricted to severe disease.

Primary bronchial epithelial cells of patients with atopic severe asthma and healthy controls are capable of releasing chemokines and cytokines in response to *Dermatophagoides Pteronyssinus* allergen 1 and *Streptococcus pneumoniae* in a dose and time dependent manner.

Ciliary dysfunction is a feature of native airway epithelium in paediatric Cystic Fibrosis lung transplant recipients. The allograft epithelium shows profound ultrastructural abnormalities in both Cystic Fibrosis and non-suppurative lung disease lung transplant recipients.

Summary: The phenotype of secondary ciliary dyskinesia and the differential cytokine/chemokine response of the epithelium of patients with severe asthma seen in this study extend our current paradigm of severe asthma and present a new therapeutic target. The damaged allograft epithelium seen in paediatric lung transplant recipients may increase risk of microbial colonisation of the allograft airway, which may play a role in the development of Bronchiolitis Obliterans Syndrome (BOS).

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I would like to dedicate this thesis to my beloved wife Dennis, my children Alan & Kevin and my parents.

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Chapter 1

INTRODUCTION

1.1 Overview

The ciliated respiratory epithelium that covers the surface of human airways forms an immunologically active natural barrier to invasion and injury by inhaled pathogenic organisms and particulate material. The epithelium is lined by the periciliary fluid and the mucus layer, which together constitute the airway surface liquid. The airway surface liquid provides an ideal environment in which the cilia beat at a frequency of 11-14 Hz. The mucus layer that lies above the periciliary fluid is cleared from the airway by the highly coordinated ciliary beating. This process, known as mucociliary clearance,(1) is an essential factor in pulmonary defense.(2) Effective mucociliary clearance depends on the structural and functional integrity of the cilia and quantitative and qualitative properties of the airway surface liquid. Alterations in any of these components may result in impaired mucociliary clearance that leads to mucus retention and increased susceptibility to airway infection.

The respiratory cilia are specialised membrane bound projections from apical membrane of ciliated respiratory epithelial cells. Abnormal ciliary function may result from genetic disorders that lead to a variety of ciliary ultrastructural defects that reflects the complex array of structural proteins that form the normal ciliary axoneme.(3) These genetic disorders are collectively known as primary ciliary dyskinesia (PCD). Structural and functional alterations in cilia may also result from a variety of acquired insults (such as infections and exposure to toxins or pollutants) or disorders in which there is gene-environment interaction (such as asthma).

1.2 Review of structure of ciliated respiratory epithelium and function of cilia

1.2.1 Summary

Chapter 2 focuses on the structure of human ciliated respiratory epithelium and the function of cilia. The various structural cells that form the respiratory epithelium are briefly described. This is followed by a section on periciliary fluid and airway mucus. The final section of this chapter describes the structure and function of human respiratory cilia. An overview of factors that regulate ciliary function and the role of ciliary function in determining the efficacy of mucociliary clearance is also given.

1.2.2 Structure of human respiratory epithelium

The human respiratory tract is lined by ciliated pseudo stratified columnar epithelium. The epithelial morphology changes from columnar to cuboidal at the level of terminal bronchioles. The epithelium consists of several cell types.(4) Among these, the morphologically distinct structural cell types that form the respiratory epithelium belong to one of three main categories: ciliated cells, basal cells and secretory cells (that include goblet cells and clara cells).(5) The junctional complexes that help maintain epithelial integrity include desmosomes that attach lower cell wall to adjacent basal cell or the basement membrane, tight junctions that join lateral cell walls of adjacent epithelial cells at their luminal surface and Zonula adherens that joins the cell apices together.(6-8) A brief description of the structural respiratory epithelial cells is given below.

1.2.2.1 Ciliated columnar respiratory epithelial cells

By far the commonest cell type in the human respiratory epithelium is the terminally differentiated ciliated columnar epithelial cell that accounts for >50% of all the cells.(5) They are approximately 20µm long and their breadth increases from about 2µm at their base to approximately 7µm at their apical surface.(4,9) The large number of mitochondria, located immediately beneath the apical cell surface, are involved in provision of energy for normal ciliary function. Ribosomes and secretory granules are scanty and hence the cytoplasm appears electron lucent, with the cell nucleus located towards the base of the cell and the golgi bodies placed centrally.(4,9,10)

The ciliated epithelial cell possesses approximately 200-300 cilia that project from the apical cell surface, with an increased density in the central apical cell surface.(10,11) The cilia in the proximal airways are approximately 6µm long and 0.3µm wide.(4,9,12) The cilia are surrounded by microvilli that measure approximately 1-3µm in length and 0.1-0.3µm in width (Figure 1.1).(13,14)

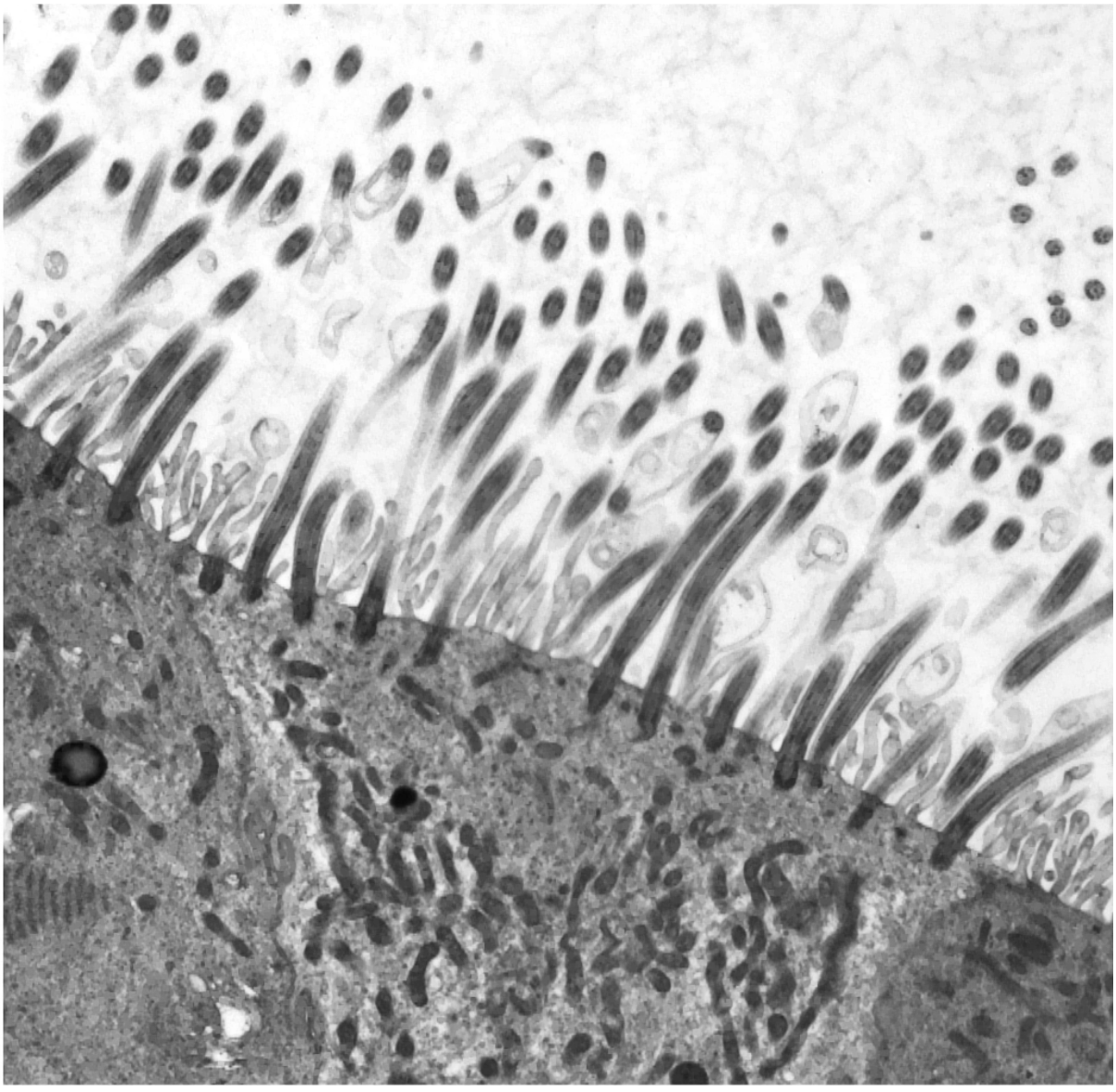

Figure 1.1: Transmission electron microscopy (TEM) image of human ciliated respiratory epithelium showing healthy ciliated epithelial cell with cilia and microvilli. This image was taken by Andrew Rutman, University of Leicester.

1.2.2.2 Basal cells

Basal cells are pyramidal in shape and are abundant in the epithelium of the conducting airways, decreasing in numbers from proximal to distal airways with decreasing airway diameter.(15) Basal cells attach themselves to the epithelial basement membrane via hemidesmosomes and to the other superficial epithelial cells via desmosomes, thus playing an important role in maintaining normal epithelial structural integrity.(7,8,16) In addition, basal cells are believed to be multipotent progenitor cells in the bronchial airways, capable of contributing to epithelial regeneration following airway injury, by developing into terminally differentiated epithelial cells.(17,18)

1.2.2.3 Goblet cells (mucus cells)

Goblet cells present in the airway epithelium, interspersed among the ciliated cells, contain mucin rich membrane bound granules from which mucus is released into the luminal surface.(4,19) Quantitative and qualitative alterations in airway mucus may play a role in the pathophysiology of diseases such as asthma and Cystic Fibrosis.

1.2.2.4 Clara cells

Clara cells are present in both proximal and distal airways. Within the bronchioles, these cells have a secretory role, contributing to production of surfactant.(20) Clara cell secretory protein (CCSP) expressing cells are also recognised to be multipotent airway progenitor cells in the bronchiolar epithelium.(21,22)

1.2.3 Periciliary fluid

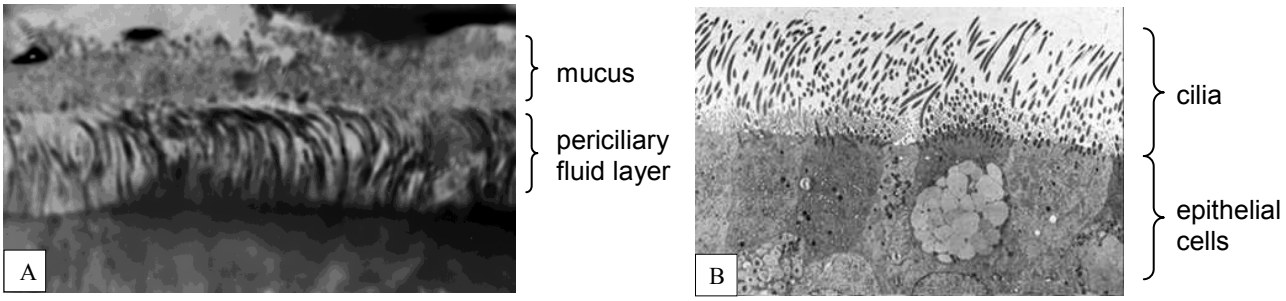

Figure 1.2: **A**: Ciliated epithelium with cilia surrounded by the periciliary fluid layer, with the mucus layer on top (X100 magnification). **B**: Transmission electron microscopy image of human ciliated respiratory epithelium showing epithelial cells and cilia. These images were taken by Andrew Rutman, University of Leicester.

The ciliated respiratory epithelium is lined by the airway surface liquid (ASL) that consists of the periciliary fluid and the mucus layers, produced by the airway epithelial cells (Figure 1.2).(1,23,24) The low viscocity periciliary fluid layer lubricates the epithelial surface and surrounds the respiratory cilia, providing an ideal environment for normal ciliary function. Using rapid fixation techniques(25,26) and confocal microscopy,(27,28) researchers have estimated the depth of periciliary fluid to be 5-10μm. A depth of 7μm has been suggested as the optimal depth for normal ciliary function. In health, the depth of periciliary fluid is tightly regulated by a number of mechanisms. Several epithelial ion channels including the epithelial sodium channel (ENaC), Ca^{2+} dependent Cl^- channel and the cystic fibrosis transmembrane conductance regulator (CFTR) are involved in the production of periciliary fluid. The expression and activity of these ion channels are regulated by a variety of stimuli in the epithelial microenvironment, including cytokines.(29-31) The mucus layer that overlies the periciliary fluid acts as a water reservoir and may therefore have an effect on the depth of periciliary fluid.(27,28) Local forces created by the ciliary beat may also have

an influence on the spread of the periciliary fluid across the airway surface and hence, maintenance of its depth.(12,24)

Studies using proteomic analysis of the airway surface liquid have identified several airway surface liquid proteins with a variety of potential functional roles.(32,33) Airway surface liquid also contains several macromolecules such as lysozyme, lactoferrin, β-defensins, immunoglobulins, glycoproteins and lipids, many of which are important in the innate defence against pathogenic microbes.(34,35) Some of the macromolecules secreted by the airway epithelial cells into the airway surface liquid may exert paracrine influences on neighbouring cells.(26)

1.2.4 Airway mucus

Mucus layer that lies above the periciliary fluid, is effectively a viscoelastic gel. Water constitutes about 95% of human airway mucus. The other components include proteins, glycoproteins called mucins and salts.(28) The mucus layer is also known to contain enzymes and immunoglobulins.(36)

Mucins are high molecular weight glycoproteins with a protein core to which carbohydrate chains are attached by O-glycoside links. The major source of mucins includes goblet cells that produce MUC5AC and submucosal glands that produce MUC5B.(2,27,36) Within the cells, mucins are synthesised in the golgi apparatus and are stored in membrane bound granules in the cell cytoplasm. The precise mechanism that leads to the release of these granules by exocytosis is largely unknown, but is believed to include parasympathetic

control.(4) Following release into the airway epithelial surface, hydration of mucins results in their expansion and conformational change. Several factors such as the acidity, osmolality and water content are known to influence this process.(1,36)

The mucus layer acts as a barrier protecting the underlying epithelium.(23,36) Inhaled pathogens and particulate matter get trapped in the mucus layer. This helps the antimicrobial macromolecules and the phagocytic cells (such as neutrophils and macrophages) to exert their effect on the pathogenic organisms. The mucus is propelled by the forces generated by the ciliary beat and is cleared from the airways. This process, known as mucociliary clearance, is an important innate defence mechanism that protects the airways.(1,2,23)

1.2.5 Structure and function of human respiratory cilia

Cilia are membrane bound projections extending from the basal body at the apical cell surface. Two main types of cilia (primary cilia and motile cilia) have been identified in mammals and they differ in their location, ultrastructure and function.(37) The usually solitary and immotile 'primary' cilia are located on non epithelial cells (including neuronal cells, Schwann cells, smooth muscle cells, fibroblasts and chondrocytes) and epithelial cells (including pancreas, thyroid, renal tubule and bile duct). The primary cilia are believed to coordinate various signal transduction pathways, thereby exerting mechanosensory, chemosensory and/or osmosensory functions in various cell types.(38) A unique type of motile primary cilium, found on the embryonic node during organ development, is believed to have an important role in determining the left-right asymmetry of the internal organs of

the body. The motile multiciliated epithelial cells are found on respiratory epithelium (Figure 1.3), ependymal cells lining the ventricles of the brain and the female oviduct.

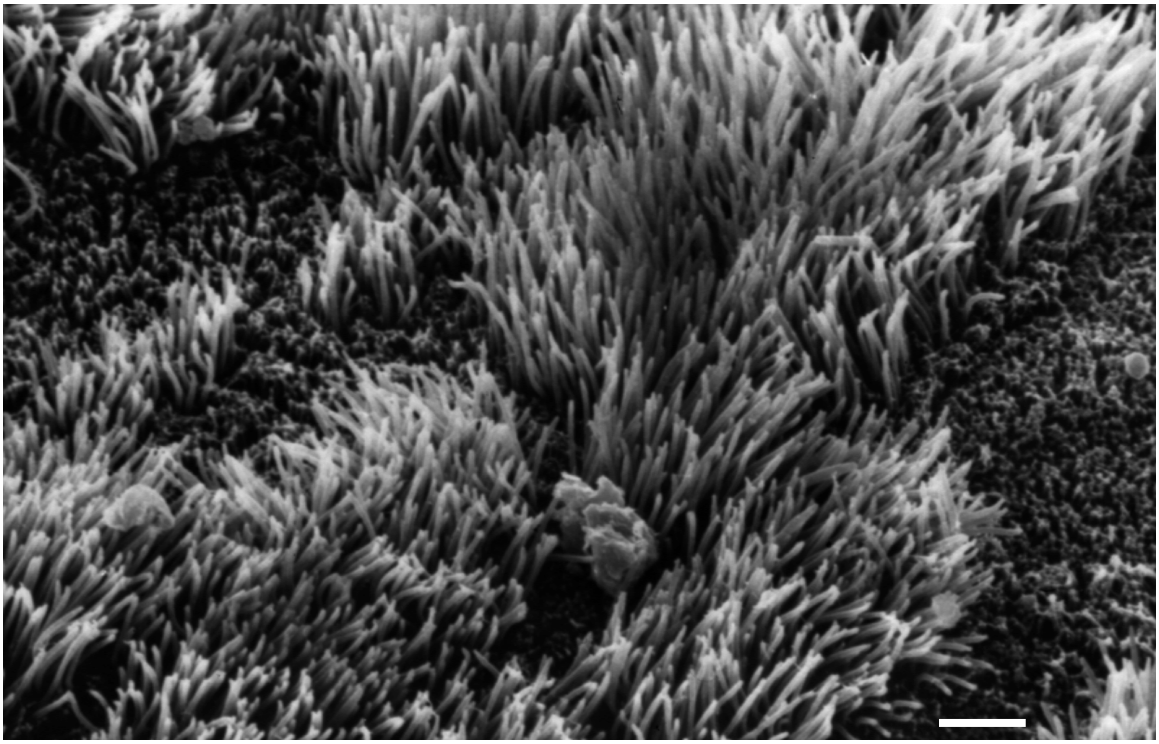

Figure 1.3: Scanning electron microscopy (SEM) image of ciliated respiratory epithelium of human nasal mucosa. Scale bar = 7μm. This image was taken by Andrew Rutman, University of Leicester.

1.2.5.1 Structure of human cilia

The membrane bound cilia, that project from the basal body at the apical cell surface of ciliated epithelial cells, have an axoneme made of microtubular cytoskeleton.(37) The axonemal structure of primary cilia differs from that of motile cilia. The 9+2 pattern, in which a central microtubular pair is surrounded by nine peripheral microtubular doublets, is

characteristic of the motile cilia (Fig 1.4). In contrast, the primary cilia have a 9+0 ciliary axoneme where the central microtubular pair is absent. Also, with the exception of the motile primary nodal cilium, the peripheral microtubular doublets of non motile primary cilia do not possess dynein arms that are responsible for ciliary motility.

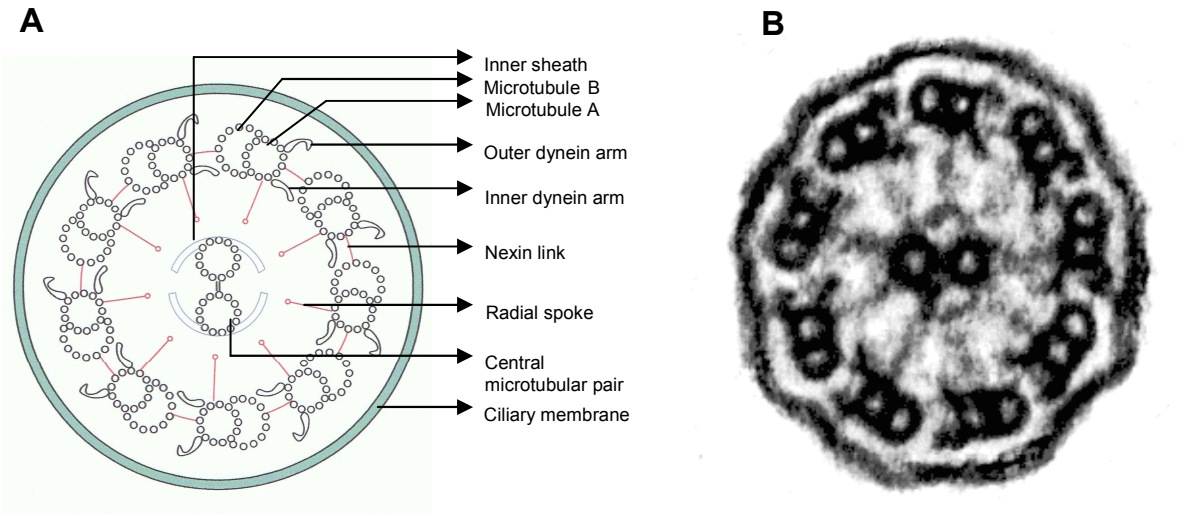

Figure 1.4: A – Schematic diagram of cross sectional image of human respiratory cilium, illustrating the classical '9+2' arrangement. (modified from PhD thesis [University of Leicester, 2010] of Dr Mina Fadaee-Shohada, with permission)**.** The central microtubular pair, enclosed in an inner sheath, is surrounded by 9 peripheral microtubular doublets. Each peripheral doublet consists of two microtubules (A and B). The inner and outer dynein arms project from microtubule A of each peripheral doublet. The peripheral microtubular doublets are connected to each other by nexin links and to the inner sheath surrounding the central microtubular pair by radial spokes. B – Cross sectional transmission electron microscopy image of human respiratory cilium. This image was taken by Andrew Rutman, University of Leicester.

1.2.5.2 Function of respiratory cilia

Cilia in the human respiratory epithelium beat in a highly coordinated fashion, with a frequency of 11-14 Hz.(1,12,39) There has been a suggestion that the respiratory cilia beat with a forward power stroke and a recovery stroke during which the cilium moves

backwards and sideways to its original position.(40) However, more recent studies using the digital high speed video imaging technique have shown that the normal beat pattern of the respiratory cilium is characterised by a forward power stroke and a backward recovery stroke within the same plane, with very little sideways movement.(41)

1.2.5.3 Factors affecting function of respiratory cilia

Ciliary beat frequency (CBF) may be affected by a number of physiological variables, pharmacological agents and inflammatory mediators.

Among the physiological variables, while there is no apparent gender difference in the ciliary beat frequency,(42,43) it is well recognised that ciliary beat frequency decreases with increasing age.(44,45) Characteristics of the periciliary environment such as temperature, pH, tonicity, viscocity, relative humidity and pressure are all known to influence ciliary beat frequency. Evidence from a number of in vitro studies suggest that the beat frequency of respiratory cilia decreases with reduction in temperature below 37^0C, while an increase in temperature above 37^0C results in an increase in ciliary beat frequency.(45-50) Ciliary beat frequency is not significantly affected if the external pH is between 7.0–10.5, but a reduction in ciliary beat frequency may be observed if the external pH falls outside this range.(51-53) Increase in viscocity of the periciliary environment causes a reduction in ciliary beat frequency.(52) Whilst the ciliary beat frequency remains stable in isotonic and hypertonic solutions, a reduction in the tonicity of periciliary fluid results in a reduction in ciliary beat frequency.(52,53) The effect of relative humidity on ciliary beat frequency is temperature dependent and a reduction in ciliary beat frequency is

observed with a reduction in relative humidity.(54,55) An enhancement in ciliary beat frequency has been observed with increase in ambient pressure, in in-vitro animal models.(56) Ciliary beat frequency also shows a diurnal variation, being slowest in the morning and fastest at mid-day.(57) Ciliary beat frequency is increased by ethanol,(58-60) while smoking has no effect(61) or only a mild inhibitory effect(62) on ciliary beat frequency. There is a suggestion that the cilia in the peripheral airways beat with a slower frequency compared to that in the upper airway and central airways,(42,63) but other authors have shown no significant difference in the ciliary beat frequency of epithelium from different levels of the tracheobronchial tree.(64)

In addition to the physiological variables, a wide variety of pharmacological agents have also been shown to exert an effect on ciliary function.(65-70) In general, the vast majority of medications have a depressive effect on ciliary beat frequency, the major exception being the short and long-acting β2-agonists that increase ciliary beat frequency.(71) The diverse effect of medications on ciliary beat frequency has been extensively reviewed by Rusznak et al.(72)

A wide variety of inflammatory mediators that may be present in the airway epithelium in disease states, are also known to affect ciliary beat frequency. For instance, histamine and acetylcholine can increase ciliary beat frequency,(73) while other mediators such as platelet-activating factor (PAF) and eosinophil major basic protein can decrease ciliary beat frequency.(74)

1.2.5.4 Regulation of ciliary beat frequency

The major intracellular second messengers that are involved in regulating the ciliary beat frequency include calcium, cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and pH.

It is well recognised that intracellular calcium (Ca^{2+}) is a key regulator of ciliary beat frequency.(75-77) Increase or decrease in intracellular calcium concentration results in an increase or decrease in ciliary beat frequency respectively.(1,78) It is believed that the effect of intracellular calcium on ciliary beat frequency is exerted via second messengers.(79) Cyclic nucleotides such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are known to be involved in regulating ciliary beat frequency.(79-82) cAMP, produced by adenylyl cyclase, activates cAMP dependent protein kinase A (PKA) localised to the ciliary axoneme, thereby leading to phosphorylation of target proteins involved in ciliary beat.(82) An increase in intracellular cAMP causes an increase in ciliary beat frequency. The precise mechanism by which cGMP regulates the ciliary beat frequency is less well understood. It is believed that the mechanism primarily involves protein kinase G (PKG).(82) It is understood that other protein kinases such as protein kinase C (PKC) causes a reduction in ciliary beat frequency.(83) Adenosine triphosphate (ATP) has been shown to induce a dose dependent increase in ciliary beat frequency. The effect is believed to be mediated through stimulation the purinergic receptor (P2Y2) and/or adenosine receptor A2B.(84,85) A rise in intracellular pH (pH_i) results in a rise in ciliary beat frequency and a fall in intracellular pH (pH_i) results in a fall in ciliary beat frequency.(86) Though acidic pH is known to inhibit protein kinase A (PKA) and activates phosphatases causing dephosphorylation of axonemal

protein targets of protein kinase A (PKA),(87) it is believed that the changes in intracellular pH (pH_i) affects ciliary beat frequency by a direct effect on ciliary outer dynein arm activity.(88) Another important regulator of the ciliary beat frequency is Nitric Oxide (NO), synthesised in the airway epithelium by nitric oxide synthase (NOS) which is present in two different isoforms (inducible [iNOS] and endothelial [eNOS] nitric oxide synthase). Nitric Oxide regulates ciliary beat frequency via soluble guanylate cyclase (sGC) and cGMP-dependent protein kinase G (PKG).(89,90)

Ciliary beat has been shown to be mechanosensitive. It has been suggested that local shear forces generated by the motion of adjacent cilia may have a role in regulating the ciliary beat. The mucus overlying the respiratory cilia exerts mechanical stimulation by activating a mechanosensitive receptor.(91,92) The resulting downward signaling cascade involves production of phospholipase C and formation of inositol 1,4,5 – triphosphate (IP3), ultimately leading to a rise in intracellular calcium.(93) Ciliated cells possess both muscarinic and adrenergic receptors.(94) Studies done in both in vivo and in vitro models suggest that both sympathomimetic and parasympathomimetic agents enhance ciliary beat frequency.(95,96)

Given the complex array of signaling pathways involved in the regulation of ciliary beat frequency, it is imperative that a degree of cross talk between these mechanisms occur, in response to physical and biochemical stimuli.

1.2.5.5 Role of ciliary function in mucociliary clearance

Effective mucociliary clearance depends on normal ciliary function and optimal quality and quantity of mucus and periciliary fluid. It is difficult to determine the precise contribution of ciliary beat frequency to the overall mucus transport velocity and mucociliary clearance and previous studies have yielded conflicting results. Karnitzki et al investigated the nasal mucociliary transport time of saccharin and nasal ciliary beat frequency of twenty patients with sinusitis and twenty healthy non-smoking control subjects. They found no correlation between nasal mucociliary transport time and the ciliary beat frequency in both the groups.(97) Rutland and Cole studied ciliary beat frequency and nasal mucociliary clearance time in subjects with Cystic Fibrosis and healthy controls. They found slower nasal mocociliary clearance time in Cystic Fibrosis patients compared to healthy controls, though there was no significant difference in ciliary beat frequency between the two groups.(98) On the other hand, there also is a suggestion that increase in ciliary beat frequency may correlate with increase in mucociliary clearance. Seybold et al studied the surface liquid velocity on freshly excised sheep trachea and found that a 16% increase in ciliary beat frequency correlates with a 56% increase in tracheal surface liquid velocity.(99) Available evidence suggests that normal frequency of ciliary beat cycle is critical in maintaining optimal mucociliary clearance.(100-102). It should be noted that the studies that evaluated the correlation between ciliary function and mucociliary clearance did not assess the ciliary beat pattern and used ciliary beat frequency as the sole measure of ciliary function. More recent studies that assessed ciliary beat pattern using the digital high speed video microscopy(103) have shown that in certain conditions, cilia may beat with a normal frequency, but a markedly abnormal beat pattern. This implies that a normal ciliary beat

frequency may not be taken as equivalent to normal ciliary function. In summary, the precise effect of abnormalities of ciliary beat frequency and beat pattern on mucociliary clearance remains to be determined.

1.2.6 Common pathogens in respiratory diseases

The human respiratory tract is susceptible to infection by a variety of organisms. The common respiratory tract pathogens include viruses (such as *respiratory syncytial virus*, *rhinovirus*, *corona virus*, *adeno virus*, *influenza virus* [A&B], *parainfluenza virus* (type 1, 2 & 3) and *metapneumovirus*), bacteria (such as *Streptococcus pneumoniae*, *Haemophilus influenzae, Moraxella catarrhalis, Pseudomonas aeruginosa* and *Staphylococcus aureus*), Fungi (such as *Aspergillus fumigatus*), atypical organisms (such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*) and mycobacteria (*Mycobacterium tuberculosis* and non tuberculous mycobacteria). Impairment of the defence mechanisms may lead to increased risk of infection by any of these common respiratory pathogens.(2) However, there is predominance of certain types of pathogens in certain respiratory diseases. For example, impaired mucociliary clearance seen in patients with Primary Ciliary Dyskinesia, is associated with an increased susceptibility to infection by a variety of organisms, most commonly bacterial pathogens such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*. (2,3) Mucociliary clearance may be impaired in other respiratory diseases such as asthma, Cystic Fibrosis and following lung transplantation. This may be associated with increased susceptibility to infection in such group of patients. For instance, asthma exacerbations are known to be frequently associated with viral respiratory tract infections.(173) In addition, patients with asthma are also susceptible to infection by respiratory bacterial pathogens (such as *Streptococcus pneumoniae* and *Haemophilus influenzae*) (181) and atypical organisms (such as *Mycoplasma pneumoniae*) (184). It is well recognised that patients with Cystic Fibrosis are susceptible to respiratory infections by a variety of pathogens, but the most common bacterial pathogens include *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Respiratory infection is a common cause of morbidity and mortality following lung transplantation (142). A multitude of organisms may infect the transplanted lungs, but the common pathogens include bacteria

such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* and fungi such as *Aspergillus fumigatus*. (223,224)

1.3 Review of mucociliary clearance in asthma

1.3.1 Summary

Chapter 3 focuses on our current understanding of mucociliary clearance in asthma. The role of respiratory epithelium in the pathophysiology of asthma is briefly discussed. This is followed by a brief review of airway mucus, periciliary fluid and ciliary function in asthma.

1.3.2 Introduction

Asthma is a major public health problem, estimated to affect approximately 300 million people globally.(104) The key features of this condition include chronic airway inflammation, airway hyperresponsiveness and widespread variable airway obstruction. This results in recurrent episodes characterised by symptoms of coughing, wheezing, breathlessness and chest tightness.(104,105) The severity can vary from mild intermittent episodes at one end of the spectrum to a severe persistent phenotype at the other end, with chronic debilitating symptoms and frequent exacerbations (the phenotype of severe refractory asthma), in a minority of patients. A classification of asthma severity based on Global Initiative for Asthma (GINA) treatment steps is given in Table 1.1.(104) The American Thoracic Society criteria for refractory asthma are given in Table 1.2.(106).

Step 1	**Step 2**	**Step 3**	**Step 4**	**Step 5**
As needed rapid acting β_2 agonist	As needed rapid acting β_2 agonist			
Controller options	*Select one*	*Select one*	*To Step 3 treatment, Select one or more*	*To Step 4 treatment, add either*
	Low-dose ICS*	Low-dose ICS plus long-acting β_2 agonist	Medium-or high-dose ICS plus long-acting β_2 agonist	Oral glucocorticosteroid (lowest dose)
	Leukotriene modifier**	Medium-or high-dose ICS Low-dose ICS plus Leukotriene modifier	Leukotriene modifier Sustained release theophylline	Anti-IgE treatment
		Low-dose ICS plus sustained release theophylline		

*ICS = Inhaled glucocorticosteroids
** = Receptor antagonist or synthesis inhibitors

Table 1.1: Global Initiative for Asthma (GINA) treatment steps(104)

REFRACTORY ASTHMA: WORKSHOP CONSENSUS FOR TYPICAL CLINICAL FEATURES*,†

Major Characteristics

In order to achieve control to a level of mild–moderate persistent asthma:

1. Treatment with continuous or near continuous (≥ 50% of year) oral corticosteroids
2. Requirement for treatment with high-dose inhaled corticosteroids:

Drug	Dose (*μg/d*)	Dose (*puffs/d*)
a. Beclomethasone dipropionate	> 1,260	> 40 puffs (42 μg/inhalation > 20 puffs (84 μg/inhalation
b. Budesonide	> 1,200	> 6 puffs
c. Flunisolide	> 2,000	> 8 puffs
d. Fluticasone propionate	> 880	> 8 puffs (110 μg), > 4 puffs (220 μg)
e. Triamcinolone acetonide	> 2,000	> 20 puffs

Minor Characteristics

1. Requirement for daily treatment with a controller medication in addition to inhaled corticosteroids, e.g., long-acting β-agonist, theophylline, or leukotriene antagonist
2. Asthma symptoms requiring short-acting β-agonist use on a daily or near daily basis
3. Persistent airway obstruction (FEV_1 < 80% predicted; diurnal PEF variability > 20%)
4. One or more urgent care visits for asthma per year
5. Three or more oral steroid "bursts" per year
6. Prompt deterioration with ≤ 25% reduction in oral or inhaled corticosteroid dose
7. Near fatal asthma event in the past

* Requires that other conditions have been excluded, exacerbating factors treated, and patient felt to be generally adherent.
† Definition of refractory asthma requires one or both major criteria and two minor criteria.

Table 1.2: American Thoracic Society criteria for refractory asthma (≥1 major criteria and at least 2 minor criteria). Taken from Am.J.Respir.Crit.Care Med. 2000 Dec;162(6):2341-2351.(106)

1.3.3 Respiratory epithelium in asthma

Over the past decade, there has seen extensive research to unravel the complex pathophysiology of asthma. It is well recognised that this disorder with many phenotypes, is characterised by complex gene-environment interactions.(107) There is an overwhelming body of evidence that supports the central role that the respiratory epithelium plays in the pathogenesis of asthma, including initiation and maintenance of chronic airway inflammation and the development of airway remodeling.(108-111) The structural elements of the airways including the airway epithelium and smooth muscle constitute the epithelial mesenchymal trophic unit (EMTU) (112) which is believed to orchestrate the chronic airway inflammation and remodeling, characteristic of asthma. Aberrant epithelial homeostasis, such as abnormal epithelial injury-repair mechanism is also emerging as a key feature of asthmatic airway epithelium.(113) It is recognised that the ciliated epithelium that lines the surface of the airways is not just an innocent bystander, but provides a highly immunologically active natural barrier between the external environment and the lung parenchyma (Fig 1.5). (108-111)

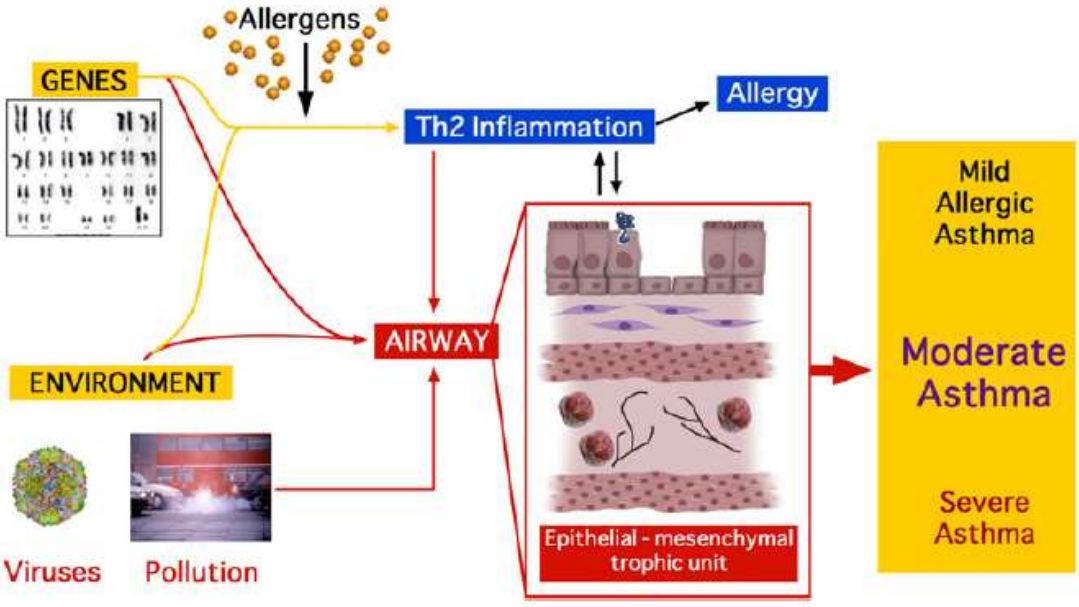

Figure 1.5: Schematic representation of the epithelial-mesenchymal trophic unit, the epithelial interactions with the external environment and the Th2 immune and inflammatory response to determine asthma phenotypes. (Taken from Holgate ST. Epithelium dysfunction in asthma. J Allergy Clin Immunol. 2007; 120(6): 1233-44)

1.3.4 Mucociliary clearance in asthma – our current understanding

Optimal mucociliary clearance depends on the structural and functional integrity of the cilia as well as the characteristics of the periciliary fluid and mucus. Though the respiratory epithelium is exposed to over 10,000 L of air per day, efficient mucociliary clearance mechanism helps avoid sustained exposure of the epithelium to inhaled noxious agents. Impaired mucociliary clearance predisposes to more persistent exposure of the airway epithelium to microbes and allergens in the inhaled air, both of which are implicated in asthma pathophysiology.(109,110)

Based on clinical observations, animal and human studies, it is recognised that there is a dysfunction of the mucociliary clearance mechanism in asthma.(73,114) Using an inhaled radio-aerosol technique, mucociliary clearance in asthma has been shown to be impaired both in the stable state and during exacerbations. Pavia et al studied eight non smoking asthma patients and eight non smoking healthy subjects. Using a radio-aerosol (5µm polystyrene particles labelled with radionuclide technetium^{99m} [^{99m}Tc]), the authors demonstrated significantly poorer mucociliary clearance in the asthma patients, compared to the healthy subjects.(115) Mezey et al measured tracheal mucus velocity radiographically, in asymptomatic asthma patients and compared that to healthy controls. The authors reported impaired baseline tracheal mucus velocity in asthma patients that decreased markedly following inhalation of an allergen, to which they are sensitised.(116) Similar finding of significantly reduced mucociliary clearance in stable asthma has been reported by other authors.(117) There is a suggestion that the degree of impairment of mucus clearance in chronic stable asthma may be related to asthma severity.(118) Mucociliary clearance has been shown to be impaired during asthma exacerbations as well. Using radiolabelled saline particles containing technetium-labelled (^{99m}TC) sulphur colloid, Messina et al studied mucus clearance in five patients during an acute asthma exacerbation. They reported significantly impaired mucus clearance during acute exacerbation that improved on recovery.(119)

1.3.5 Airway mucus in asthma

The factors that contribute to airflow obstruction in asthma include mucus hypersecretion, plasma exudation, bronchial smooth muscle constriction and airway remodelling. Mucus hypersecretion has been recognised as an important factor in the pathophysiology of asthma. A number of autopsy studies have shown widespread mucus plugging in the airways of patients who died of acute asthma exacerbation.(120,121) Submucosal gland hypertrophy and goblet cell hyperplasia are well recognised pathological features of asthma.(122) The asthmatic airway is characterised by mucus hypersecretion,(123) abnormal mucus rheology(122) and tethering of intraluminal mucins to goblet cells in the airway epithelium.(124) However, the extent to which mucus hypersecretion contributes to airflow obstruction and airway hyperresponsiveness seen in asthma, remains to be determined.(125) A significant increase in production of the gel-forming mucins, predominantly MUC5AC and to a lesser extent MUC5B, accounts for the mucus hypersecretion characteristic of asthma.(125,126) Emerging evidence suggests that a variety of inflammatory mediators implicated in asthma pathophysiology are involved in the signalling pathways leading to goblet cell hyperplasia and airway mucus hypersecretion. It is believed that the T-helper type 2 (Th2) cytokine IL-13 is a key player in the development of goblet cell hyperplasia.(127,128) Other important mediators involved in this process include tumour necrosis factor (TNF) alpha, cyclooxygenase (COX)-2 and the T-helper type 2 (Th2) cytokines IL-1β, IL-9 and IL-18.(129,130) Many of these signalling pathways appear to converge onto epidermal growth factor receptor (EGFR) activation which appears to be central in the signal transduction.(131,132) With advances in our understanding of the

role of mucus hypersecretion in airway diseases and the molecular mechanisms underlying this process, researchers are beginning to focus on blockade of mucus hypersecretion as a potential therapeutic target in the management of airway diseases including asthma.(133)

1.3.6 Periciliary fluid in asthma

Evidence suggests that the ion transport properties of the airway epithelium may be altered in response to the chronic inflammation seen in asthma, thereby altering the physicochemical characteristics of the 'sol' layer, constituted by the periciliary fluid. Inflammatory mediators present in the microenvironment of asthmatic airway epithelium may have an effect on the secretory function of airway epithelial cells.(134) For instance, in in-vitro studies using proteomic analysis, researchers have shown that the T-helper type 2 (Th2) cytokine IL-4 upregulates gelsolin mRNA expression in airway epithelial cells resulting in increased gelsolin concentration in the airway surface liquid.(33) IL-4 has also been shown in in-vitro studies, to inhibit the amiloride-sensitive Na^+ channel and activate the Ca^{2+} dependent Cl^- channel on bronchial epithelium, thereby decreasing Na^+ absorption and increasing Cl^- secretion.(31) Other proinflammatory cytokines such as IL-13, which is known to play a role in the pathophysiology of asthma, has also been shown to have similar effect. Using Ussing chamber studies Danahay et al showed that IL-13 induces a reduction in amiloride sensitive Na^+ channel activity while enhancing apical membrane anion conductance.(29) IL-1β and IL-17A have been shown to enhance HCO_3^- concentration in airway surface liquid,(135,136) which may affect the pH of the

airway surface liquid. The Th1 cytokine interferon-γ (IFN-γ) may enhance epithelial anion secretion while reducing Na^+ absorption.(30) Another proinflammatory cytokine TNFα has been shown to influence the epithelial Na^+ transport.(137) In a mouse model, allergic inflammation has been shown to enhance Ca^{2+} dependent Cl^- secretion and inhibit ENaC-mediated Na^+ absorption thereby enhancing the secretory capability of airway epithelium.(138) Allergic inflammation and respiratory viral infections have been shown to induce pendrin, which is an epithelial anion transporter that is involved in regulating the airway surface liquid thickness.(139) A variety of inflammatory mediators have been shown to alter the secretory phenotype of the epithelium, causing alterations in the protein composition of the airway surface liquid.(32,140) In summary, the myriad of inflammatory mediators present in asthmatic airways are capable of altering the airway epithelial ion transport and protein secretion and this may have an effect on the quantitative and qualitative properties of the airway surface liquid.

1.3.7 Ciliary function in asthma

While evidence that supports abnormalities of mucus and periciliary fluid provides a plausible mechanistic explanation for the reduced mucociliary clearance observed in asthma, to date no studies have assessed ciliary function (in terms of ciliary beat frequency and beat pattern) directly, in patients with asthma or considered its relationship with disease severity.

1.4 Review of mucociliary clearance following lung transplantation

1.4.1 Summary

Chapter 4 focuses on our current understanding of mucociliary clearance following lung transplantation. A brief overview of literature that examines ciliary function, airway mucus and respiratory epithelial ultrastructure following lung transplantation is also included.

1.4.2 Introduction

Lung transplantation is an accepted treatment option for patients with end stage lung disease. Despite many advances in the pre-operative, peri-operative and post transplant management, the overall survival following lung transplantation remains poor.(141) Recent reports(142) suggest that almost half the deaths within the first year post lung transplantation are attributable to infection. Emerging evidence points towards a potential role of infections (bacterial, viral or fungal) in the pathogenesis of bronchiolitis obliterans (BO), which is the major cause of death by five years post lung transplant.(143-145) The lung transplant recipients' increased susceptibility to respiratory infections may be multifactorial in causation. There is a suggestion that mucociliary clearance (MCC) is impaired following lung transplantation. Studies on mucociliary clearance in lung transplant recipients are limited and these are summarised in the following section.

1.4.3 Mucociliary clearance (MCC) in lung transplant recipients

Animal studies have shown that experimental allotransplantation decreases the bronchial mucociliary clearance in the early post operative period.(146,147) Paul et al measured the proximal airway mucociliary clearance before and after lung transplantation, in a canine lung autotransplant model. They deposited a spot of carbon particles suspended in saline in the airway mucosa through a bronchoscope, while the animals were breathing spontaneously under sedation. The authors observed the movement of the leading edge of the spot of carbon particles over a 15 minute period to estimate the proximal airway

clearance rate in millimetres per minute. They found absence of clearance of carbon particles in the transplanted bronchus (1-2 cm distal to the airway anastomosis) 3 weeks after transplantation, with partial recovery of clearance compared to pre transplant clearance rate, by 12 weeks post transplantation. (148,149) Paul et al also used a canine left upper sleeve lobectomy model and studied the preoperative and postoperative mucociliary clearance rate in the same manner. In the sleeve resected lungs, the peribronchial tissue was preserved, thereby minimising the effects of denervation and devascularisation. Interestingly, they found no significant difference in the pre and postoperative clearance rates in the sleeve resected model, suggesting that bronchial denervation and devascularisation might alter mucus rheology and epithelial integrity, thereby contributing to the impairment of mucociliary clearance following lung transplantation. Rivero and colleagues studied in situ bronchial mucociliary transport proximal and distal to the bronchial anastomosis in a rat model. The rats underwent bronchial transection and reanastomosis of the left main stem bronchus. The animals were subsequently killed and the in situ mucus clearance velocity was measured by direct observation under a video microscope, of the leading edge of a charcoal drop placed on the exposed membranous fraction of the bronchus. They observed significantly reduced mucociliary transport distal to the anastomosis, in the left main stem bronchus.(150)

Human studies also provide evidence for impairment of mucociliary clearance in lung transplant recipients. Herve et al(151) investigated bronchial mucociliary clearance in long term survivors of double lung (DLT) and heart lung transplants (HLT), using a non invasive radioisotope technique. They observed reduced bronchial mucociliary clearance

in lung transplant recipients compared to that of normal controls. Earlier, in a similar study, Dolovich et al had shown a more pronounced impairment of bronchial mucociliary clearance in patients following single lung or heart lung transplantation.(152) In another study, Shankar et al used a radio aerosol method (Tc^{99m} bound to macro aggregated albumin) to assess mucociliary clearance in 13 heart lung transplant (HLT) recipients, 12 single lung transplant (SLT) recipients and 8 healthy volunteers and demonstrated significant impairment of mucociliary clearance in the transplant group compared to healthy controls.(153)

While there is some evidence in the literature to support impairment of mucociliary clearance in lung transplant recipients, particularly in the early post transplant period, the reasons for this impairment are far from being completely understood. Several potential factors have been implicated as contributing to impaired mucociliary clearance. For example, bronchial denervation and the consequent impairment or loss of cough reflex could adversely affect clearance of secretions. Evidence from animal studies(146-148,150,154) suggests that the anastomotic site acts as a barrier to mucociliary clearance until epithelial healing is complete. Kinking or ridging at the anastomotic site might potentiate this adverse effect on mucociliary clearance. In theory, post transplant immunosuppression could adversely affect epithelial healing at the anastomotic site. The transplanted airways themselves may have inherent abnormalities. Non specific abnormalities of the airway epithelium due to a variety of factors such as bronchial denervation, devascularisation, lymphatic interruption, inadequate preservation during organ harvesting and previous episodes of lung infection or rejection also could

potentially contribute to alterations in airway epithelium and hence impairment of mucociliary clearance. More importantly, qualitative or quantitative alterations in mucus or periciliary fluid and abnormalities of ciliary function are factors that may have a more pronounced and direct effect on bronchial mucociliary clearance. There is a paucity of studies in the published literature examining the above factors with relevance to the impaired mucociliary clearance in lung transplant recipients. Few researchers have investigated ciliary function (section 4.4), viscoelastic properties of airway mucus (section 4.5) and airway epithelial ultrastructure (section 4.6) in lung transplant recipients. These are summarised in the following sections.

1.4.4 Ciliary function in lung transplant recipients

Studies that examined ciliary function in lung transplant recipients have revealed findings which are inconclusive. To investigate whether airway epithelium, ciliary structure and function are normal following lung transplantation, in an adult study, Read et al(155) obtained bronchial mucosal brush biopsy samples from 9 heart lung transplant recipients, 5 single lung transplant recipients and seven controls and studied ciliary beat frequency and epithelial ultrastructure. Although the mean ciliary beat frequency was significantly lower in transplant recipients compared to that of the control group, there was no significant difference in ciliary beat frequency between epithelial samples obtained from airways 3-5 cm proximal and 3-5 cm distal to the anastomosis. 9 of 14 patients in the Read et al study had Cystic Fibrosis or bronchiectasis and the samples were taken at varying intervals post transplant. Hence the effect of persistent infection on ciliary beat

frequency could not be excluded. Also of note is that the authors used atropine (as a pre medication), which either directly or by affecting mucus secretion, might modify the ciliary beat frequency. In another study involving 25 single lung transplant recipients, Norgaard(156) and colleagues found no difference in ciliary beat frequency in the transplanted lungs compared to the native lungs. Dolovich et al(152) studied five patients who had single lung transplant for pulmonary fibrosis and three patients who had heart lung transplant for primary pulmonary hypertension or end stage bronchiectasis and found normal ciliary beat frequency in samples taken within the first year post transplant, from all patients studied.

These findings could not be reproduced in a study be Veale et al.(157) They studied a more homogenous group of six adults who underwent single left lung transplantation for end stage cryptogenic fibrosing alveolitis, to determine whether the impaired mucociliary clearance in lung transplant recipients is accompanied by a reduction in ciliary beat frequency. They found significantly reduced ciliary beat frequency in mucosal samples from transplanted bronchi compared to that from the native bronchi.

1.4.5 Airway mucus in lung transplant recipients

Quantitative or qualitative alterations in airway mucus or periciliary fluid could result in impairment of mucociliary clearance. There is some evidence in the literature that suggests altered mucus rheology in lung transplant recipients. In a canine model of single left lung auto transplantation, Tomkiewicz and colleagues(154) studied the viscoelastic

properties of mucus obtained from main stem bronchi using magnetic microrheometry. They found that at two months post lung transplantation, mucus viscoelasticity, as indicated by the rigidity index was significantly decreased in the sample obtained from the transplanted lung, compared to that from the native lung. This difference was not seen when similar samples were compared at four months post transplant. The canine single lung autotransplantation model eliminates the problems of immunosuppression and rejection. Hence these results may not reflect what happens in patients, in whom, clinical or subclinical infection, rejection and immunosuppression play a role. Paul et al(148) examined viscoelastic properties of mucus (by microrheometry) in samples collected from a canine model of autotransplanted, allotransplanted and sleeve resected lungs. At three weeks post transplant, they found significantly increased mucus rigidity in samples obtained from autotransplanted and allotransplanted lungs compared to that from native lung. However, in the sleeve resected lung, where the peribronchial tissue was preserved, the viscoelastic properties of mucus was not significantly different from that of the native lung. The authors speculated that bronchial denervation and devascularisation may alter mucus rheology in the early post operative period in lung transplant recipients.

1.4.6 Airway epithelium in lung transplant recipients

Non specific abnormalities of the airway epithelium have been observed in studies that explored mucociliary function in lung transplant recipients. Read et al(155) studied the structure of airway epithelium in mucosal brushings obtained proximal and distal to airway anastomosis in three heart lung transplant recipients, one single lung transplant

recipient and seven controls. Compared to controls, airway epithelium of transplant recipients showed a significant reduction in the proportion of ciliated cells and an increase in proportion of dead cells and cells with mitochondrial abnormalities (including swelling and loss of internal structure), both proximal and distal to the airway anastomosis. A significantly higher frequency of ciliated cells displaying ciliary depletion was noted in the proximal epithelium. Although not statistically significant, they observed a higher frequency of projecting cells and cytoplasmic blebbing in airway epithelium proximal to the anastomosis compared to the distal.

These non-specific airway epithelial alterations may be a factor potentially contributing to the impairment of mucociliary clearance in lung transplant recipients. Although not studied in detail, denervation, devascularisation, lymphatic interruption, ischaemia during organ harvesting, infection and rejection are factors that have been implicated as contributory to airway epithelial alterations. The effects of denervation and devascularisation on airway epithelium and mucociliary clearance are unclear. Nerve regeneration is not well defined and is thought to begin sometime between 3-6 months post lung transplantation.(158) Alton and colleagues reported the lack of effect of lung denervation on the transepithelial potential difference (PD) measurements in the lower airways after human single lung transplantation.(159) Nasal denervation has been shown not to affect nasal mucociliary clearance.(160) Also, it has been shown that mucociliary clearance is not reduced in patients with chronic autonomic failure.(161) Norgaard et al(156) investigated whether bronchial artery revascularisation would contribute to preserved or even improved mucociliary clearance, measured indirectly by ciliary beat

frequency. They studied 25 single lung transplant recipients, eight of whom had complete bronchial artery revascularisation achieved. The authors concluded that bronchial artery revascularisation did not have any demonstrable influence on ciliary beat frequency. The authors also performed histological examination to compare the proportion of ciliated cells in the transplanted bronchus of 5 single lung transplant recipients with complete bronchial artery revascularisation and 9 single lung transplant recipients without bronchial artery revascularisation and found no significant difference between the two groups. However, of interest, they found abundance of metaplastic and/or squamous epithelial cells in those without bronchial artery revascularisation while none of the samples from those with complete bronchial artery revascularisation had these abnormal cell types in the epithelium of the transplanted bronchus.

1.4.7 Longitudinal changes in ciliary function, airway epithelium and mucociliary clearance in lung transplant recipients: How soon does the impaired mucociliary clearance recover?

Longitudinal changes in ciliary function, airway epithelium and mucociliary clearance in lung transplant recipients have not been studied in detail. It is unclear as to how soon the impaired mucociliary clearance recovers. In a canine model of left lung autotransplantation, Marelli and colleagues(149) observed partial recovery of proximal airway clearance, twelve weeks post transplant. Tomkiewicz et al(154) studied airway epithelial function in a canine single lung autotransplant model. They measured transepithelial potential difference (PD) as a measure of epithelial integrity. The

significant fall in transepithelial potential difference observed in transplanted lung at one and two months post transplantation returned to levels comparable to that of native lung, by four months post transplant. The authors also demonstrated that the decreased mucus viscoelasticity seen at two months post transplant, returned to baseline values at four months.

1.4.8 Conclusion

Available evidence suggests that there is an impairment of mucociliary clearance in lung transplant recipients, particularly in the early post transplant period. Alterations in ciliary function, airway epithelium and/or mucus rheology could contribute to the impairment of mucociliary clearance. Few studies that examined ciliary function, airway epithelium and mucociliary clearance following lung transplantation have been done in animal models or adult patients. They are mostly cross sectional studies and the findings have been inconclusive. There have been no studies to date that assessed ciliary function or airway epithelial ultrastructure in paediatric lung transplant recipients. From the limited number of human studies, all done in adult lung transplant recipients, it is unclear whether or not the ciliary function (in terms of ciliary beat pattern and beat frequency) in the native bronchi and the transplanted lungs is normal. Although few adult studies attempted to evaluate the airway epithelium following lung transplantation, detailed analysis of epithelial ultrastructure above and below the airway anastomosis has not been done yet. Also, to date no studies have assessed the ciliary function and epithelial ultrastructure in the peripheral airways of the transplanted lungs following lung transplantation. Further studies addressing these issues are needed to advance our knowledge in this area.

1.5 Aims and Hypotheses

1.5.1 Aims

The aims of this thesis were as follows:

1) To study the ciliary function (in terms of ciliary beat frequency and beat pattern) and epithelial ultrastructure of adult patients with mild, moderate and severe asthma and compare that to healthy controls.
2) To study the effect (in terms of cytokine and chemokine release) of *Streptococcus pneumoniae* on primary bronchial epithelial cells of a group of patients with atopic severe asthma phenotype and compare that to healthy controls.
3) To study the effect (in terms of cytokine and chemokine release) of *Dermatophagoides pteronyssinus* allergen 1 (Der p 1) on primary bronchial epithelial cells of a group of patients with atopic severe asthma phenotype and compare that to healthy controls.
4) To study the ciliary function (in terms of ciliary beat frequency and beat pattern) and epithelial ultrastructure of paediatric Cystic Fibrosis lung transplant recipients.
5) To study the ciliary function (in terms of ciliary beat frequency and beat pattern) and epithelial ultrastructure of paediatric non suppurative lung disease lung transplant recipients.

1.5.2 Hypotheses

Hypotheses tested in this thesis include

a). There is no difference in the ciliary function (in terms of ciliary beat frequency and beat pattern) and epithelial ultrastructure of the lower airways in patients with asthma compared to that of healthy controls.

b). There is no difference in the ciliary function (in terms of ciliary beat frequency and beat pattern) and epithelial ultrastructure of the lower airways in patients with varying grades of asthma severity.

c). There is no difference in the response (in terms of cytokine and chemokine release) to *Dermatophagoides Pteronyssinus allergen 1,* between primary bronchial epithelial cells of patients with atopic severe asthma and healthy controls.

d). There is no difference in the response (in terms of cytokine and chemokine release) to *Streptococcus pneumoniae,* between primary bronchial epithelial cells of patients with atopic severe asthma and healthy controls.

e). There is no difference in the ciliary function (in terms of ciliary beat frequency and beat pattern) and epithelial ultrastructure between the epithelium above and below the airway anastomosis and that of the peripheral airway, in paediatric Cystic Fibrosis lung transplant recipients.

f). There is no difference in the ciliary function (in terms of ciliary beat frequency and beat pattern) and epithelial ultrastructure between the epithelium above and below the airway anastomosis and that of the peripheral airway, in paediatric non suppurative lung disease lung transplant recipients.

g). There is no difference in the length and beat frequency between the cilia in the central and peripheral airways in paediatric lung transplant recipients.

CHAPTER 2

Laboratory Methods

2 Laboratory methods

2.1 Summary

Chapter 2 describes in detail, the laboratory methods used in the studies included in this thesis. The methodology used for measurement of ciliary beat frequency and assessment of ciliary beat pattern using digital high speed video imaging is described in detail. This is followed by the description of analysis of epithelial ultrastructure using transmission electron microscopy. Methodology used for culturing primary bronchial epithelial cells from bronchial epithelial brushing is included. The final sections describe the preparation of *Dermatophagoides pteronyssinus* allergen 1 and *Streptococcus pneumoniae* suspension for use in the cell culture studies, the methodology used for cytokine and chemokine assay, and the statistical software used for data analysis.

2.2 Measurement of ciliary beat frequency and assessment of ciliary beat pattern

The sample used was bronchial epithelial brushing, obtained by flexible bronchoscopy conducted according to the British Thoracic Society (BTS) guidelines,(162) The methodology used for measurement of ciliary beat frequency and assessment of ciliary beat pattern of human bronchial epithelial brush biopsy is given below.

Typically, a strip of bronchial epithelium obtained by brushing the bronchus contains a row of adjacent bronchial epithelial cells. Ciliary beat frequency was measured and beat pattern assessed on strips of bronchial epithelium using a digital high-speed video system (Fig 2.1), as described previously with nasal epithelial brushings, within six hours of sample collection.(41,163)

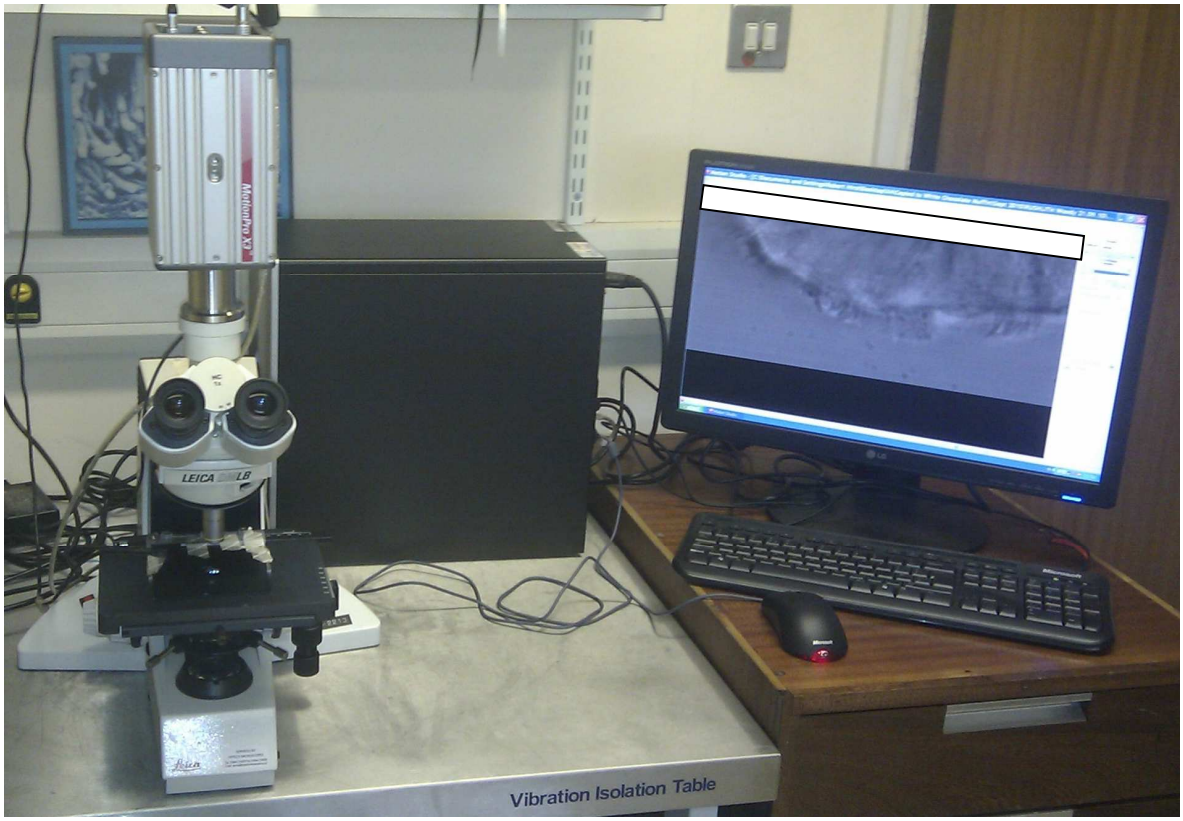

Fig 2.1: Digital high speed video microscopy imaging system.

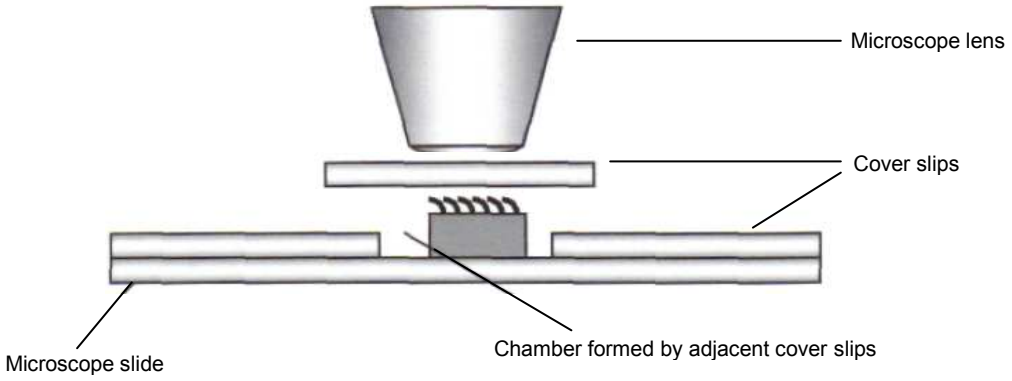

Figure 2.2: Schematic diagram showing mounting of sample on the microscope for digital high speed video microscopy imaging. Sample suspended in Medium 199 is placed in a chamber created by the separation of a cover slip and a glass slide by two adjacent cover slips. (Adapted from 'Human Respiratory Cilia', MD Thesis of Dr Mark Chilvers 2003, University of Leicester).

The sample was placed in Medium 199 (25 mM hydroxyethyl piperazine ethane sulphonic acid, Earles salt and L-glutamine [pH 7.3]; Gibco, Leicester, UK) that contained antibiotic solution (streptomycin 50 µg/mL and penicillin 50 µg/mL) and analysed within six hours after collection. The sample was suspended in a chamber created by the separation of a cover slip and a glass slide by two adjacent cover slips (Fig 2.2). The slide was placed on a heated stage (37°C) of a Leitz Diaplan microscope mounted on an anti-vibration table (Wentworth Laboratories Ltd, Sandy, UK). Ciliated epithelial strips greater than 50µm in length and devoid of mucus were observed at 37°C using a x100 interference contrast lens. They were recorded using a digital high speed video camera (Kodak Ektapro Motion Analyzer; Eastman Kodak, San Diego, CA, USA) at a frame rate of 400 frames per second. Video sequences could be recorded and played back at reduced frame rates or frame by frame.

2.2.1 Measurement of ciliary beat frequency

Ciliary beat frequency was determined directly from ciliated epithelial strips viewed in a sideways profile. Groups of beating cilia were identified and the number of frames required to complete 10 beat cycles was recorded. This was converted to ciliary beat frequency by a simple calculation (ciliary beat frequency = (400/number frames for 10 beats) x10).(41) The ciliated epithelial strip (≥50 μm) projected onto a high resolution monitor, was divided into 10 adjacent areas measuring 5μm each. One reading of ciliary beat frequency was taken from each area to obtain a total of ten measurements of ciliary beat frequency along each ciliated epithelial strip. At least 7 epithelial strips, up to a maximum of 10 strips were analysed per subject. A micrometer was used to calibrate and measure the length of cilia from digital sideways images of ciliated epithelial edges projected onto a computer screen.

2.2.2 Assessment of ciliary beat pattern

The experimental system allowed the ciliary beat pattern to be evaluated in 3 different planes: a sideways profile, beating directly toward the observer and from directly above.(41,163) The path taken by a cilium during the beat cycle was analysed frame by frame. This was characterised and compared with the normal beat pattern(41) seen on digital high speed video analysis. Dyskinesia was defined as an abnormal beat pattern that included reduced beat amplitude, stiff beat pattern, failure to bend along the length of the ciliary shaft, flickering or a twitching motion and static cilia (Fig 2.3). The ciliated epithelial strip (≥50 μm) projected onto a high-resolution monitor, was divided into 10 adjacent areas measuring 5μm each and the beat pattern of each 5μm area was assessed to

obtain a total of ten measurements of beat pattern per epithelial strip. Dyskinesia index was calculated as the percentage of dyskinetic cilia within the sample (number of dyskinetic readings/total number of readings for sample ×100). The immotility index(164) was calculated as the percentage of immotile cilia within the sample (number of immotile readings/total number of readings for sample ×100).

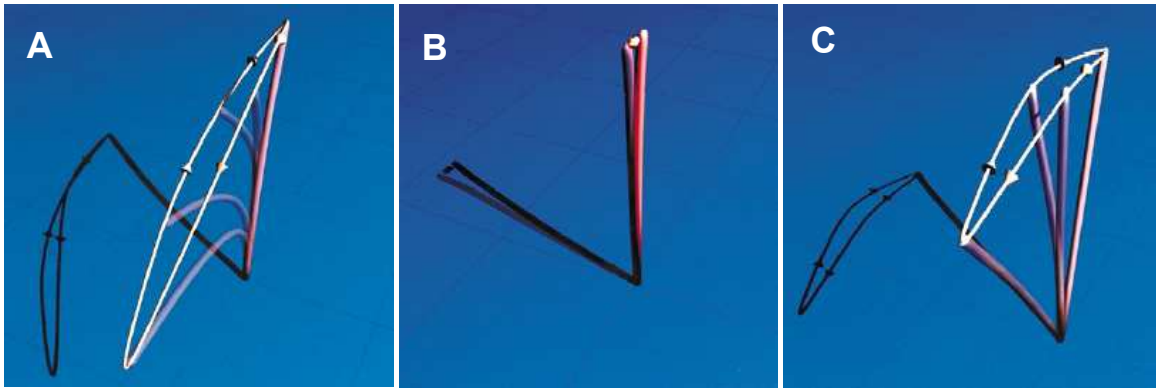

Figure 2.3: Schematic diagram of normal and abnormal ciliary beat patterns. **A** - Normal ciliary beat pattern characterised by movement of cilia in a planar motion with a forward power stroke and a backward recovery stroke, with very little sideways motion. **B** - Virtually immotile cilia, with the occasional slow, low-amplitude, stiff flickering or twitching motion. **C** - Cilia with an abnormal stiff beat pattern with markedly reduced ciliary beat amplitude. (Taken from Chilvers et al. 2003)(163)

The high speed video images were assigned a unique sample identifying number and analysed subsequently by observer 1 (B. Thomas) in a blinded fashion. The images were re-analysed by a second observer (A. Rutman) and blinded on a second occasion by the original observer (B. Thomas). This allowed estimation of agreement between the two observers (B Thomas and A Rutman) and repeatability (agreement within observer [B. Thomas]).

2.3 Assessment of ultrastructure of ciliated epithelium using transmission electron microscopy

Transmission electron microscopy was performed as described before with nasal epithelial brushings.(103) Briefly, on the day of collection, bronchial epithelial samples were fixed in 2.5% gluteraldehyde in Sorenson's phosphate buffer. After 48 h, the sample was post fixed in 1% osmium tetroxide. After rinsing in distilled water, the cells were embedded in a drop of 2% liquid agar at 45°C and allowed to solidify. This bound the cells together during dehydration and ensured that all the strips of epithelium and cilia were randomly oriented. This was processed through to resin by standard transmission electron microscopy techniques. Ultra thin sections were cut at 70 nm. These were collected on 200 mesh thin-bar copper grids and stained in 1% uranyl acetate and counter stained in Reynolds lead phosphate. The sections were then examined by transmission electron microscopy. The grids were analysed by a grid square search pattern so that all the cells in the sample were analysed, but seen only once. All the cilia that were, by random chance, captured in cross section adequate for dynein arms and microtubules to be visualised, were assessed.

The ciliated epithelium was assessed, in a blind fashion, for both epithelial and ciliary ultrastructural changes. Epithelial integrity was assessed firstly by assessing the cell type. The number of ciliated cells, unciliated cells, mucus cells, and dead cells were expressed as a percentage of all cells examined. Disruption and damage to the tissue was quantified using the criteria for epithelial integrity described by Tsang et al.(165) Percentages of ciliated cells with loss of cilia, cellular projections (extrusion of cells from the epithelial

edge), cytoplasmic blebbing and mitochondrial damage (manifested as swelling and disruption of mitochondrial cristae) among all cells examined, were also calculated. Damage to individual cilia was evaluated by examining ciliary ultrastructure for microtubular and dynein arm defects and the percentage of cilia with microtubular or dynein arm defects was calculated. Intracellular ciliary orientation, defined as the standard deviation of the angles of lines through the central pair of microtubules of cilia originating from a single ciliated cell, was determined as described previously.(166)

2.4 Culture of primary bronchial epithelial cells

The details of the reagents used for the respiratory cell cultures are given in Table 5.1

2.4.1 Collagen coating

PureCol solution of collagen was prepared as a 1% w/v solution in phosphate buffered saline (500µl in 50ml phosphate buffered saline). A sufficient volume of PureCol solution was added to completely cover the surface of plates, flasks, glass-slides and wells used in bronchial epithelial cell cultures and experiments involving primary bronchial epithelial cells. After incubating for 5 hours at room temperature, they were washed with nano pure water, left to air dry and then stored in a sealed bag at room temperature until used.

2.4.2 Bronchial brushing

Flexible bronchoscopy was conducted according to the British Thoracic Society guidelines.(162) A 2mm nylon cytology brush was passed through the channel in the bronchoscope and epithelial brushings were taken under direct vision by brushing the bronchial epithelium in a forward and backward direction. Typically, a good biopsy yielded about 1mg of ciliated tissue. Each tissue strip contained rows of 10-50 ciliated cells. The epithelial strips obtained were then dislodged by agitating in 2ml of 20mM Hepes buffered medium 199 (pH 7.4), containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml), and kept in the fridge overnight. This allowed time for the antibiotics and fungizone to work and clear any potential deep cellular infections.

2.4.3 Primary bronchial epithelial cell culture

An adaptation of a previously described method was used to grow primary bronchial epithelial cells.(167) The brush biopsy contained a heterogeneous population of both differentiated and undifferentiated bronchial epithelial cells. The contents of the brush biopsy (unknown number of cells) was placed in a collagen-coated well from a 12 well plate in 1ml of bronchial epithelial growth medium (BEGM) (BEBM + SingleQuots see Table 5.1), containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml), at 37ºC. The bronchial epithelial cells were fed every other day by removing the medium and replacing it with 1ml BEGM. When the cells were 90 to 100% confluent (after 7-10 days) the entire medium was removed and the cells were washed with 0.3ml

Trypsin/EDTA solution (0.5g porcine trypsin and 0.2g EDTA per liter of Hanks' Balanced Salt Solution with phenol red) and left at room temperature for about 2-3 minutes, occasionally agitating the tray. As soon as the cells began to detach, they were suspended by pipetting the Trypsin/EDTA solution over the cell surface. Cells were then placed in a 15ml tube containing 1ml BEGM to inactivate the trypsin. The well surface was then washed with 3ml of BEGM (to recover all the cells) and this also was placed in the 15ml centrifuge tube. The cells were then centrifuged (2,000 X *g* for 10 min) and the supernatant was removed. The pellet was re-suspended in 1ml BEGM, making sure that there were no clumps, by vigorous pipetting. The cell suspension was then added to a T80 ($80cm^2$) collagen-coated flask (Fig 2.4) containing 14ml BEGM. When the cells in the T80 flask were 90 to 100% confluent (up to 4-7 days), the entire medium was removed and the cells were washed with 1.4ml Trypsin/EDTA solution and left at room temperature for about 2-3 minutes, agitating the flask occasionally. As soon as the cells detached, 8ml BEGM was added and the cells were suspended by pipetting the medium over the cell surface. The cell suspension was pipetted into a 15ml tube and the cells were centrifuged for 5 minutes at 4,000g. The supernatant was removed and the pellet re-suspended in 1.8ml BEGM. This cell suspension was used for bronchial epithelial cell culture studies. 60 µl of the cell suspension was added to each well of 8-well collagen coated glass chamber slides. The bronchial epithelial cells in the 8-well glass chamber slides were fed with fresh BEGM (400µl) every other day, until confluent monolayers were obtained. Confluent monolayer in the glass chamber slides contained ~ 10^5 cells.

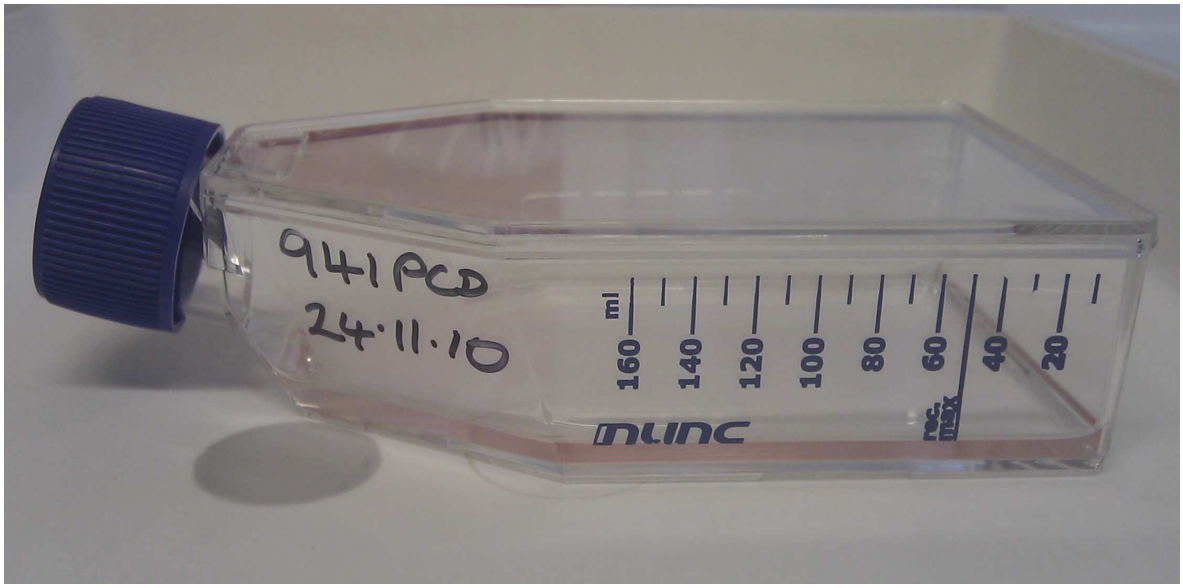

Figure 2.4: Bronchial epithelial cell suspension in a T80 (80cm^2) collagen-coated flask containing 14ml bronchial epithelial growth medium (BEGM)

2.4.4 Immunohistochemical characterisation of basal cells

This was done by a fellow researcher Dr Mina Fadaee-Shohada. Confluent basal cells, grown in glass chamber slides, as described in 5.3.3, were fixed with 4% w/v paraformaldehyde in phosphate buffered saline for 10 minutes at room temperature. The cells were then washed with 200µl phosphate buffered saline for 20 minutes with three buffer changes. The last wash was replaced with 1ml 3% w/v BSA (Bovine serum albumin) in phosphate buffered saline and left for 10 minutes at room temperature and then washed three times with phosphate buffered saline. Cells were stained for 2 hours with 200µl mouse anti-cytokeratin peptide 14 (CK14) monoclonal antibody (Sigma, UK.C8791), at a dilution of 1:200 in 1% w/v BSA in PBS at 37°C.(168) After three washes in phosphate buffered saline, FITC-Goat anti-mouse IgG, A, M (Zymed

laboratories, 65-6411) was diluted 1:50 in 1% w/v BSA in phosphate buffered saline and was added for 2 hours at 37°C. During the final 10 minutes, 1:1000 Hoechst stain (Sigma Aldrich, UK, H6024) was added to stain the nuclei. After 3 washes with PBS, the chamber was removed and a few drops of mountant (80% v/v glycerol, 3% w/v n-propyl gallate in phosphate buffered saline) was placed onto the slide, covered with a size 1.5 coverslip and sealed with nail varnish. The cells were then visualised using a Nikon eclipse TE2000-U microscope.

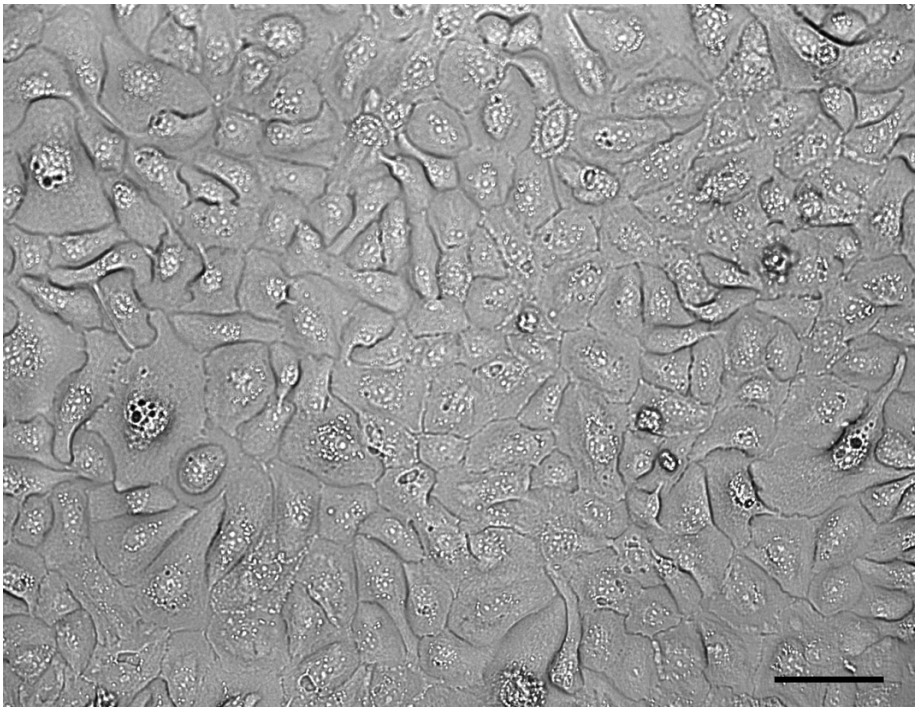

Figure 2.5: Light microscopic image of human bronchial epithelial cells in culture. Scale bar represents 20µm. (taken from PhD thesis 'The Interaction of gram positive bacteria with ciliated cells' [University of Leicester, 2010] of Dr Mina Fadaee-Shohada, with permission)**.**

Table 2.1: List of reagents and media used for the bronchial epithelial cell culture. (Adapted from the PhD thesis 'The Interaction of gram positive bacteria with ciliated cells' [University of Leicester, 2010] of Dr Mina Fadaee-Shohada, with permission)**.**

Reagents	**Source/Catalogue number/info.**
Hepes buffered medium 199	Invitrogen, UK, 21180-021
PureCol solution (collagen)	Nunclon, Holland, 5409
Bronchial epithelial cell base medium (BEBM)	Lonza, Switzerland, CC-3171
Trypsin/EDTA	Sigma, UK, T3924
(BEGM SingleQuots) contains:	Lonza, Switzerland,T3924
Bovine pituitary extract (BPE)	CC-4009
Insulin, bovine	CC-4021
Hydrocortisone (HC);	CC-4031
Gentamicin Sulfate and Amphotericin-B (GA-1000)	CC-4081
Retinoic Acid	CC-4085
Transferrin	CC-4205
Tri I odothyronine (T3)	CC-4211
Epinephrine	CC-4221
Epidermal growth factor, human recombinant (hEGF)	CC-4230
Nunc 8-well tissue culture chambers	Fisher, UK, 177402
T80 Flasks	Sigma, UK, 156499
12-well plates	Sigma, UK, 150628

2.5 Preparation of *D pteronyssinus* allergen 1

LoTox^{TM} Natural Der p 1 (Indoor Biotechnologies Ltd, Witshire, United Kingdom) with cysteine protease activity >200 RFU at 25 μg/ml concentration and an endotoxin content of ≤ 0.01 EU/μg was used. LoTox^{TM} Natural Der p 1 at concentrations of 1 μg/ml and 5 μg/ml were prepared and incubated with the reducing agent Dithiothreitol 1mM to make it enzymatically active, before addition to the bronchial epithelial cell monolayer.

2.6 Preparation of suspensions of *S pneumoniae*

Streptococcus pneumoniae (D39), a wild type laboratory strain (serotype 2) from National Collection Type, Culture 7466, London, UK was used. *Streptococcus pneumoniae* was grown on blood agar plates in a CO_2 jar (BBL GasPak system, USA). A candle was lit and placed inside the jar, the lid tightly closed and incubated at 37°C overnight. The candle was placed to create anaerobic conditions by eliminating any oxygen present. Colonies from the blood agar plate were used to inoculate 100ml brain heart infusion broth (BHI) which was then incubated overnight at 37ºC. The next day the OD_{500} was adjusted by the adding BHI broth until it was between 0.8-1.0 (equivalent to mid exponential phase). The culture was then separated into Eppendorf tubes (1ml per tube) containing 10% v/v glycerol and frozen at -70°C until required. Numbers of viable bacteria in the stock were determined by colony counting on blood agar plates. Viable counts were calculated as average colony forming units (cfu) formed from duplicate 50 μl volumes plated onto an appropriate agar plate,(169) following ten-fold serial dilutions in sterile phosphate buffered saline. The cfu/ml was determined by the following equation;

cfu/ml = y x 10^d x 20, where y is the average colony count in 50µl and d is the dilution factor.

Before use, frozen stocks were thawed at room temperature and the bacteria were then sedimented (4,000 X g for 10 min) and re-suspended in 1ml bronchial epithelial basal medium (BEBM, Lonza, UK) and diluted to the required concentrations (10^6 cfu/ml and 10^7 cfu/ml).

2.7 Cytokine and chemokine assay

Lists of cytokines and chemokines measured in studies included in this thesis are given in Tables 2.2 and 2.3. The principal biologic functions of these cytokines and chemokines are also included in these tables.

Chemokines and cytokines were measured using a 96-well multi spot assay (Meso Scale Discovery [MSD], Maryland, USA) according to the manufacturer's instructions. Briefly, the assay employs a sandwich immunoassay format where capture antibodies are coated in a single spot, or in a patterned array, on the bottom of the wells of a multi-spot plate (Figure 2.6). Samples or standards are incubated in the multi-spot plate, and each cytokine binds to its corresponding antibody spot. Following this, when a potential is applied to the electrode, bound labelled sulpho tag produces light and this is proportional to the amount of inflammatory proteins in the sample. Unknown samples are calculated by comparing their light emitted to that of a known amount of the protein on the standard curve.

Cytokines were measured using a human Th_1/Th_2 standard 10 spot plate (Catalog number N01010A-1) and human chemokines were measured using a high band MS6000 10 spot plate (Catalog number N01001B-1), using SECTOR Imager 6000 (MSD, Maryland, USA). The lower limit of detection was 1 pg/ml. Further details of the methodology may be obtained from the following link.

http://www.meso-scale.com/CatalogSystemWeb/Documents/Human_96_well_Base.pdf

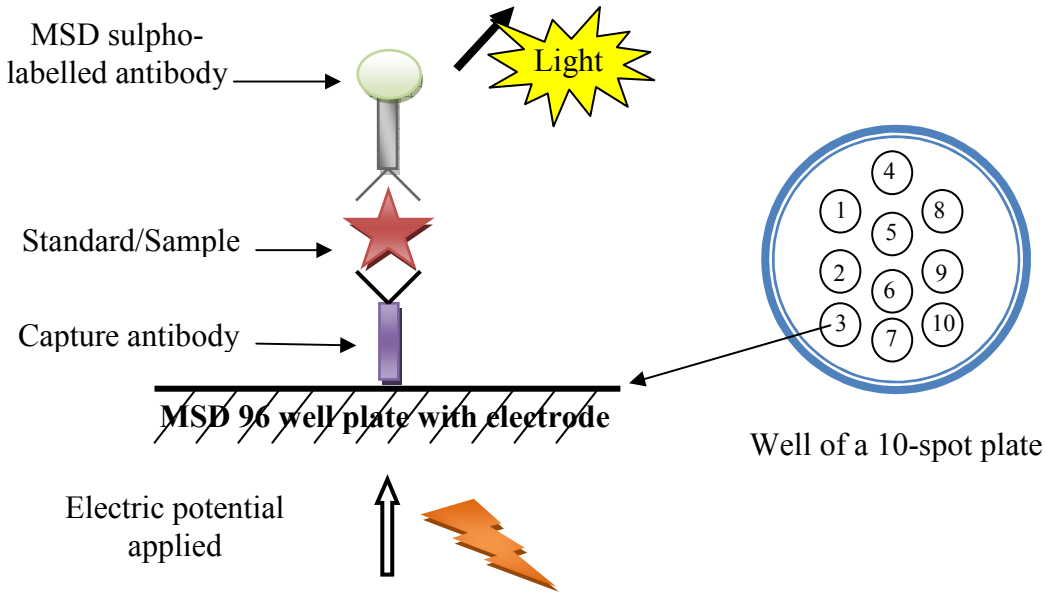

Figure 2.6: Schematic diagram showing the antibody sandwich system in Meso Scale Discovery assays. Cytokine capture antibody is pre-coated on specific spots of a multi-spot plate. Sample or standards are incubated in the multi-spot plate, and each cytokine binds to its corresponding antibody spot. When a potential is applied to the electrode, bound labelled sulpho tag emits light that is proportional to the amount of cytokine/chemokine of interest in the sample. Unknown samples are calculated by comparing the light emitted by them to that of a known amount of the protein on the standard curve. (taken from PhD thesis 'The Interaction of gram positive bacteria with ciliated cells' [University of Leicester, 2010] of Dr Mina Fadaee-Shohada, with permission)**.**

Cytokines of innate immunity		
Cytokine	**Principal cell source**	**Principal cellular targets and biologic effects**
Tumor necrosis factor (TNF)	Macrophages, T cells	Neutrophil and endothelial cell activation; Cell apoptosis Hypothalamus: fever; Liver: synthesis of acute phase proteins
Interleukin-1 (IL-1)	Macrophages, Endothelial cells, Some epithelial cells	Endothelial cell activation Hypothalamus: fever; Liver: synthesis of acute phase proteins
Interleukin-12 (IL-12)	Macrophages, dendritic cells	T cells: Th1 differentiation NK cells and T cells: IFN-γ synthesis
Interleukin-10 (IL-10)	Macrophages, T cells	Macrophages, dendritic cells: Inhibition of IL-12 production and expression of class II MHC molecules
Interleukin-6 (IL-6)	Macrophages, T cells, Endothelial cells	B cells: proliferation of antibody producing cells Liver: synthesis of acute phase proteins
Cytokines of adaptive immunity		
Cytokine	**Principal cell source**	**Principal cellular targets and biologic effects**
Interleukin-2 (IL-2)	T cells	T cells: proliferation, increased cytokine synthesis B cells and NK cells: proliferation
Interleukin-4 (IL-4)	$CD4^+$ T cells (Th2), mast cells	B cells: isotope switching to IgE; Mast cells: proliferation T cells: Th2 differentiation, proliferation
Interleukin-5 (IL-5)	$CD4^+$ T cells (Th2)	Eosinophil: activation and proliferation B cells: proliferation and IgA production
Interleukin-13 (IL-13)	$CD4^+$ T cells (Th2), NK cells, mast cells	B cells: isotope switching to IgE Epithelial cells: Increased mucus production Fibroblasts and macrophages: increased collagen synthesis
Interferon-γ (IFN-γ)	T cells (Th1, $CD8^+$ T cells), NK cells	Macrophage: activation; T cells: Th1 differentiation B cells: Isotope switching to IgG subclasses

Table 2.2: List of cytokines, their principal cell source and functions. Adapted from 'Cellular and Molecular Immunology', 6th Edition, Eds. A Abbas, A Lichtman and S Pillai, Elsevier Saunders, ISBN: 978-1-4160-3122-2)

Chemokine	Original name	Chemokine receptor	Major function
CXCL8	IL-8	CXCR1, CXCR2	Neutrophil recruitment
CXCL10	IP-10	CXCR3, CXCR3B	Effector T cell recruitment
CCL2	MCP-1	CCR2	Mixed leukocyte recruitment
CCL4	MIP-1β	CCR5	T cell, dendritic cell, monocyte and NK cell recruitment
CCL5	RANTES	CCR1, CCR3, CCR5	Mixed leukocyte recruitment
CCL11	Eotaxin	CCR3	Eosinophil, basophil and Th2 recruitment
CCL13	MCP-4	CCR2, CCR3	Mixed leukocyte recruitment
CCL17	TARC	CCR4	T cell and basophil recruitment
CCL22	MDC	CCR4	T cell and basophil recruitment
CCL26	Eotaxin-3	CCR3	Eosinophil, basophil and Th2 recruitment

Table 2.3: List of chemokines, their receptor types and major functions. Adapted from 'Cellular and Molecular Immunology', 6th Edition, Eds. A Abbas, A Lichtman and S Pillai, Elsevier Saunders, ISBN: 978-1-4160-3122-2)

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad, San Diego, CA, USA) and SAS/STAT® software (SAS Institute Inc, NC, USA). Advice on the use of statistical analyses was given by Dr John Bankart (Statistician, Department of Health Sciences, University of Leicester, UK). Details of specific statistical analysis used are included in relevant chapters, as appropriate.

CHAPTER 3

Study of ciliated respiratory epithelium of adult patients with mild, moderate and severe asthma and comparison to healthy controls.

3 Study of ciliated respiratory epithelium of adult patients with mild, moderate and severe asthma and comparison to healthy controls

3.1 Summary

Background

Epithelial dysfunction has been implicated in asthma pathophysiology, but no studies have directly assessed ciliary function in asthma.

Objective

To study the ciliary function (in terms of ciliary beat frequency and beat patten) and epithelial ultrastructure of patients with asthma and healthy controls.

Methods

The study involved assessment of ciliary beat frequency and beat pattern using digital high speed video microscopic imaging; and ultrastructure by transmission electron microscopy; of bronchial epithelial strips from 7 subjects with mild, 7 with moderate, and 19 with severe asthma, and 9 healthy controls.

Results

The median [IQR] ciliary beat frequency was decreased in moderate (6.5 [4.4-8.5] Hz) and severe asthma (6.7 [6.1-7.6] Hz) compared to controls (10.5 [9.7-11.8] Hz, ($p<0.01$). Dyskinesia and immotility indices were higher in severe asthma (65 [43-75] %; 6.3 [1-9.5] %) compared to controls (4 [0-6.7] %; 0%, $p<0.01$). These abnormalities were related to

disease severity [ciliary beat frequency r_s=-0.68; dyskinesia index r_s=0.86; and immotility index r_s=0.65; $p<0.0001$]. The ultrastructure of the epithelium was abnormal in severe asthma with a reduction in ciliated cells, an increase in dead cells, and ciliary disorientation compared to all other groups ($p<0.05$). Compared to mild asthmatic and healthy controls, severe asthmatics showed increased ciliary depletion, microtubular defects, mitochondrial damage and cytoplasmic blebbing ($p<0.01$). All of these changes were related to disease severity.

Conclusion

Ciliary dysfunction and ultrastructural abnormalities are closely related to asthma severity. Ciliary dysfunction is a feature of moderate to severe asthma and profound ultrastructural abnormalities are restricted to severe disease. Whether these changes contribute to the development of severe asthma phenotype remains to be determined.

3.2 Background to study

Asthma affects approximately 300 million people globally.(170) Though the majority of patients are well controlled, there remains a subgroup of asthmatic adults that accounts for about 10% of asthmatic patients, who continue to have debilitating chronic and persistent symptoms despite optimal standard asthma treatment.(171) This group with severe refractory asthma(106) represents those with a high risk of severe exacerbations and asthma related mortality and accounts for >50% of asthma related health care costs.(172,173) There has been extensive research to unravel the complex pathophysiology of severe asthma, but it remains uncertain what the immunopathological hallmarks of severe asthma are.(171,173) In this respect, the role of the dysfunctional respiratory epithelium in severe asthma has been of much recent interest.(109,110)

The structure of ciliated respiratory epithelium and function of cilia have been reviewed in Chapter 1.2. A review of mucociliary clearance in asthma is given in Chapter 1.3.

Available evidence suggests that there is an impairment of mucociliary clearance mechanism in asthma.(117,119) Optimal mucociliary clearance depends on the structural and functional integrity of the cilia as well as the characteristics of the airway surface liquid and mucus. Submucosal gland hypertrophy and goblet cell hyperplasia are well recognised pathological features of asthma.(122) The asthmatic airway is also characterised by mucus hypersecretion,(123) abnormal mucus rheology(122) and tethering of intraluminal mucins to goblet cells in the airway epithelium.(124) While evidence that supports abnormalities of mucus(122-124) and airway surface liquid(29) provides a plausible mechanistic explanation for the reduced mucociliary clearance observed in asthma, to date no studies have assessed

ciliary function directly in asthma or considered its relationship with disease severity. Indeed impaired ciliary function would have a pronounced effect on mucociliary clearance.

In this context, the aim of this observational study was to characterise the ciliated respiratory epithelium from the lower airways of adults with mild, moderate and severe asthma compared to healthy controls, by assessing the function of cilia (in terms of ciliary beat frequency and beat pattern) and detailed ultrastructure of the ciliated epithelium.

3.3 Methods

3.3.1 Subjects

Patients with mild (n=7), moderate (n=7) and severe (n=19) asthma were recruited from clinics at Glenfield Hospital, Leicester, United Kingdom over a two year period (2007-2009). Healthy control subjects (n=9) were recruited from hospital staff and by local advertising. Asthma was diagnosed based on presence of clinical features consistent with asthma and objective measures of airway hyperresponsiveness and variable airflow obstruction [the concentration of methacholine required to provoke a 20 percent decrease (PC_{20}) in the forced expiratory volume in one second (FEV_1) of less than 8 mg per millilitre, increase in the FEV_1 by at least 15 percent after the inhalation of 200 μg of salbutamol, or the variation in peak flow, expressed as a percentage of the mean, exceeding 20 percent over a period of 14 days]. Asthma severity was classified as mild, moderate and severe, using the current Global Initiate for Asthma (GINA) guidelines, based upon the GINA treatment steps(104) (mild asthma = GINA treatment steps 1/2, moderate asthma = GINA treatment step 3 and severe asthma = GINA treatment steps 4/5). Patients with

severe asthma also met the American Thoracic Society (ATS) criteria for refractory asthma.(106) At the time of collection of bronchial epithelial samples, patients with asthma had been free from intercurrent respiratory infections and asthma exacerbations requiring antibiotics and/or rescue use of systemic corticosteroids for at least 6 weeks. Normal subjects had no history of respiratory disease, normal lung function and normal PC_{20}. All subjects were current non smokers and those who did smoke in the past had a smoking history of less than 10 pack years. The study protocol was approved by the Leicestershire and Rutland regional ethics committee and written informed consent was obtained from all subjects.

3.3.2 Measurements

Demographic and medical details including age, sex and current medications were recorded on all subjects. FEV_1 (forced expiratory volume in one second), FVC (forced vital capacity) and FEV_1/FVC ratio were measured on all subjects, with the use of a rolling-seal spirometer (Vitalograph Ltd, Buckingham, United Kingdom). The PC_{20} was computed from the methacholine dose–response curve (the change in FEV_1 in relation to the methacholine concentration) by linear interpolation on a log scale, using standard techniques.(174) In patients with asthma, presence of atopy was determined by allergen skin prick tests for common aeroallergens including *Dermatophagoides pteronyssinus*, dog, cat and grass pollen. Plasma IgE was measured on patients with moderate and severe asthma. Patients with asthma had single flow exhaled nitric oxide concentration measured at a rate of 50 ml per second as previously described.(175) Induced sputum was obtained from patients with asthma and sputum samples were processed as previously described.(176) All subjects underwent flexible bronchoscopy conducted according to the

British Thoracic Society (BTS) guidelines,(162) to obtain strips of bronchial epithelium by brushing the bronchus intermedius.

3.3.3 Ciliary beat frequency and beat pattern

Methodology used for assessment of ciliary beat frequency and beat pattern is given in Chapter 2 (section 2.2).

Dyskinesia index was calculated as the percentage of dyskinetic cilia within the sample (number of dyskinetic readings/total number of readings for sample ×100). The immotility index(164) was calculated as the percentage of immotile cilia within the sample (number of immotile readings/total number of readings for sample ×100).

3.3.4 Transmission electron microscopy

Methodology used for detailed assessment of ultrastructure of the ciliated respiratory epithelium is given in Chapter 2 (section 2.3).

3.3.5 Statistical analysis

Sample size was calculated based on ciliary beat frequency as the primary outcome measure. Seybold et al studied the surface liquid velocity on freshly excised sheep trachea and found that a 16% increase in ciliary beat frequency correlates with a 56% increase in tracheal surface liquid velocity.(99) Hence, we assumed that an absolute mean difference in ciliary beat frequency of 2 Hz has potential biological significance. It was estimated that, to detect a mean difference in ciliary beat frequency of 2 Hz (with a standard deviation of 1

Hz) between two groups, with a confidence interval of 95% and a power of 80%, a sample size of six (n=6) in each group would be required. Statistical analysis was performed using GraphPad Prism 5 and SAS/STAT® software. Non-parametric data were described as median (IQR). Groups were initially compared using the Kruskal-Wallis test and post-hoc analysis was performed using Dunn's method. Spearman's correlation was used to assess the univariable relationship between disease severity, and abnormalities of ciliary function and epithelial ultrastructure. A p value of <0·05 was taken as the threshold for statistical significance in each case.

3.4 Results

The baseline characteristics of the subjects are given in Table 3.1. Agreement between the two observers (B Thomas and A Rutman) was excellent for measurement of ciliary beat frequency (interclass correlation 0.94) as well as dyskinesia index (interclass correlation 0.93). Repeatability (agreement within observer) was also excellent (interclass correlation was 0.94 for ciliary beat frequency and 0.99 for dyskinesia index). Ciliary beat frequency was decreased in moderate to severe asthma. The median [IQR] ciliary beat frequency was decreased in moderate (6.5 [4.4-8.5] Hz) and severe asthma (6.7 [6.1-7.6] Hz) compared to controls (10.5 [9.7-11.8] Hz) (Kruskal-Wallis $p<0.01$; $p<0.001$ between groups, Table 3.2 and Figure 3.1A). Analysis of ciliary beat pattern showed a higher proportion of dyskinetic and immotile cilia in moderate to severe asthma. The median [IQR] dyskinesia index was increased in moderate (42 [35-56.1] %) and severe asthma (65 [43.1-74.6] %) compared to controls (4 [0-6.7] %) (Kruskal-Wallis $p<0.05$; $p<0.001$ between groups, Table 3.2 and Figure 3.1B); and was increased in severe versus mild asthma (13 [9-22] %) (Kruskal-Wallis $p<0.01$, Table 3.2 and Figure 3.1B). The median [IQR] immotility index was

increased in severe asthma (6.3 [1.0-9.5] %) compared to mild asthma (0 %) and controls (0 %) (Kruskal-Wallis $p<0.05$; $p<0.01$ between groups, Table 3.2 and Figure 3.1C). All of these abnormalities were related to disease severity (Table 3.2). The median (IQR) length of cilia was 6 (6-6)µm for healthy subjects, 6 (5.5-6.0)µm for patients with mild asthma, 6 (6-6.25)µm for patients with moderate asthma and 6 (4.5-6.25)µm for patients with severe asthma and the difference between the groups was not significant ($p=0.89$). Examples of digital high speed video recordings of ciliated epithelial edges of patients with severe refractory asthma and healthy controls are provided in a compact disc (attached to the last page of this thesis) and the video legends are given in Appendix 1.

Ten of 19 patients with severe asthma had eosinophilic asthma (sputum eosinophils >3%). In the severe asthma group, the median (IQR) ciliary beat frequencies of those with eosinophilic asthma and non-eosinophilic asthma were 7.3 (6.1-8.1) Hz and 6.3 (5.5-6.8) Hz respectively and the difference was not statistically significant ($p=0.12$). Similarly, there was no significant correlation between sputum eosinophilia and ciliary beat frequency (r_s = 0.38, $p=0.11$), dyskinesia index (r_s = -0.13, $p=0.59$) or immotility index (r_s = 0.17, $p=0.48$), in the severe asthma group. Correlation between ciliary functional characteristics and duration of asthma, FEV_1, FVC, eNO, IgE, PC_{20} and proportion of sputum neutrophils was also examined. The results showed that FEV_1 and PC_{20} correlate significantly with ciliary beat frequency (r_s = 0.32, $p<0.05$; r_s = 0.47, $p<0.01$ respectively) and the dyskinesia index (r_s = -0.42, $p<0.01$; r_s = -0.50, $p<0.01$ respectively).

Bronchial epithelium obtained from two control subjects, one patient with mild asthma, one patient with moderate asthma and seven patients with severe asthma were insufficient for

assessment by transmission electron microscopy. Therefore transmission electron microscopy was done on samples obtained from seven control subjects, six subjects with mild asthma, six subjects with moderate asthma and 12 subjects with severe asthma. The median (IQR) no of epithelial cells per sample studied was 203.5 (162.8 – 263.3). Results are summarised in Table 3.3 and example electron micrographs are shown in Figures 3.2 and 3.3. The ultrastructure of the ciliated epithelium was abnormal in severe asthma with a reduction in the proportion of ciliated cells, an increase in the proportion of dead cells, and ciliary disorientation compared to all of the other groups ($p<0.05$); with ciliary depletion, microtubular defects, mitochondrial damage, cytoplasmic blebbing, and loss of epithelial integrity compared to mild asthmatics and healthy controls alone ($p<0.01$); and with an increase in mucus cells compared to healthy controls only ($p<0.01$). There were no significant differences across groups for the proportion of unciliated cells or dynein arm defects. All of the ultrastructural changes, except the proportion of unciliated cells, were related to disease severity (Table 3.3).

Table 3.1: Baseline characteristics of the subjects

Characteristic	Control (n=9)	Mild asthma (n=7)	Moderate asthma (n=7)	Severe asthma (n=19)
Age (yr)[†]	35 [22.5-52.5]	47 [39-60]	45 [42-56]	42 [38-48]
Gender (n) Male/ Female	4/ 5	2/ 5	3/ 4	8/ 11
Age at onset of symptoms(yr)[†]	-	39 [20-49]	36 [21-47]	12 [3-28]
Atopy (n)	-	4	4	11
Plasma IgE (IU/ml)[†]	-	-	71.1 (34.7-406.5)	131 (73.4-356)
FEV_1 [†] Litres	3.7 (3.2-4.1)	2.2 (2.2-2.7)	2.3 (2.0-3.1)	2·4 (1·8-2·9)
Percentage of predicted value	102 (97-109)	80 (70-92)	77 (71-107)	76 (64-92)**
FEV_1: FVC ratio (%)[†]	91 (85-95)	72 (68-77)	71 (69-78)	72 (65-78)**
Exhaled Nitric oxide (ppb)[†]	-	0.9 (0-4.9)	14 (6.6-25.7)	46·8 (15·9-99)[¶]
Sputum measurements[†]				
Eosinophils (%)	-	0.5 (0-1.1)	1.8 (1-3.5)	4·0 (0·7-32)
Neutrophils (%)	-	32 (13-58)	60 (50-68)	59 (43-86)
PC_{20} (mg of methacholine/ml)[†]	>16	0.6 (0.2-5.8)	0.2 (0.2-0.9)	0·3 (0·06-1.2)
Inhaled steroid dose in µg (BDP equivalent)[†]	0	400 (400-800)	800 (800-1000)	1600(1600-2000)[#$]
Number of subjects on continuous oral steroids	0	0	0	7
Number of subjects on long acting bronchodilator	0	0	7	19

† Data expressed as median (IQR).
** p<0·01 compared to control group
¶ p<0.01 compared to mild asthma group, # p<0.001 compared to mild asthma group and $ p<0.01 compared to moderate asthma group
BDP = Beclomethasone dipropionate

Table 3.2: Ciliary beat frequency and beat pattern†

	Control n=9	**Mild asthma n=7**	**Moderate asthma n=7**	**Severe asthma n=19**	**Spearman's r (between asthma severity and ciliary function) and p value**
Ciliary beat frequency (Hz)	10.5 (9.7-11.8)	8.4 (7.5-8.5)	6.5 (4.4-8.5)**	6.7 (6.1-7.6)***	-0.68 (-0.85, -0.44) p<0.0001
Dyskinesia Index (%)	4 (0-6.7)	13 (9-22)	42 (35-56.1)*	65 (43.1-74.6)***¶	0.86 (0.76, 0.99) p<0.0001
Immotility Index (%)	0	0	3 (0-4.9)	6.3 (1-9.5)**‡	0.65 (0.59, 0.98) p<0.0001

† Data expressed as median (IQR).
*** p<0.001, ** p<0.01 and * p <0.05 compared to control group
¶ p<0.01 and ‡ p<0.05 compared to mild asthma group

Dyskinesia index = percentage of dyskinetically beating cilia among all cilia examined.
Immotility index = percentage of immotile cilia among all cilia examined.

Table 3.3: Analysis of epithelial ultrastructure by transmission electron microscopy†

	Control (n=7)	Mild (n=6)	Moderate (n=6)	Severe (n=12)	Spearman's r (between asthma severity and ultrastructural features) and p value
Ciliated cells	83.6 (80.6-87.7)*	85.8 (81.4-87.7)*	86.9 (72.7-90.1)*	70.6(61.3-71.8)	-0.62, p=0.0002
Unciliated cells	11.4 (9.3-15.7)	10.6 (7.7-14.2)	9.1 (7.3-13.1)	14.2 (12.7-15.2)	0.23, p=0.21
Mucus cells	3.4 (2.9-3.8)**	4.4 (3.7-5.2)	4 (2.6-7.2)	7.9 (5.3-10.2)	0.66, p<0.0001
Dead cells	0 (0-1)**	0 (0-0.3)**	0 (0-5.7)*	8.3 (5.8-13.5)	0.71, p<0.0001
Dynein arm defects	0.9 (0.6-1.9)	1.6 (1.2-2.8)	2.9 (2.8-3.6)	3.1 (1.3-6.5)	0.45, p=0.012
Microtubular defects	1.6 (1.2-2.0)**	1.4 (1.2-2.1)**	2.7 (1.6-6.2)	5.7 (2.5-11)	0.69, p<0.0001
Ciliary orientation (degrees)	13.5 (12.7-14.1)***	14 (12.7-14.9)*	14 (12.9-15.9)*	20.7 (17.5-23.2)	0.79, p<0.0001
Ciliated cells with loss of cilia	13.5 (9.7-14.9)**	13.9 (10.3-16.9)**	17.5 (11.5-21.8)	39 (29.3-51.5)	0.76, p<0.0001
Cells extruding from the surface	12.9 (8.8-18.4)***	17.7 (13-23.8)**	24.4 (19.1-26.9)	44.7 (36.6-54.8)	0.89, p<0.0001
Cells with cytoplasmic blebbing	13.7 (7.7-14.9)**	10.6 (8.9-14.4)***	19.3 (15.2-25.5)	43.6 (27.3-49.3)	0.77, p<0.0001
Cells with mitochondrial damage	8.7 (3.7-11.4)**	9.8 (5.6-13)**	11.7 (9.1-24.0)	35 (31.5-46.4)	0.76, p<0.0001

† Data expressed as median percentage (IQR). Ciliary orientation is expressed as median degrees (IQR). *** $p<0.001$, ** $p<0.01$ and * $p<0.05$ compared to severe asthma group

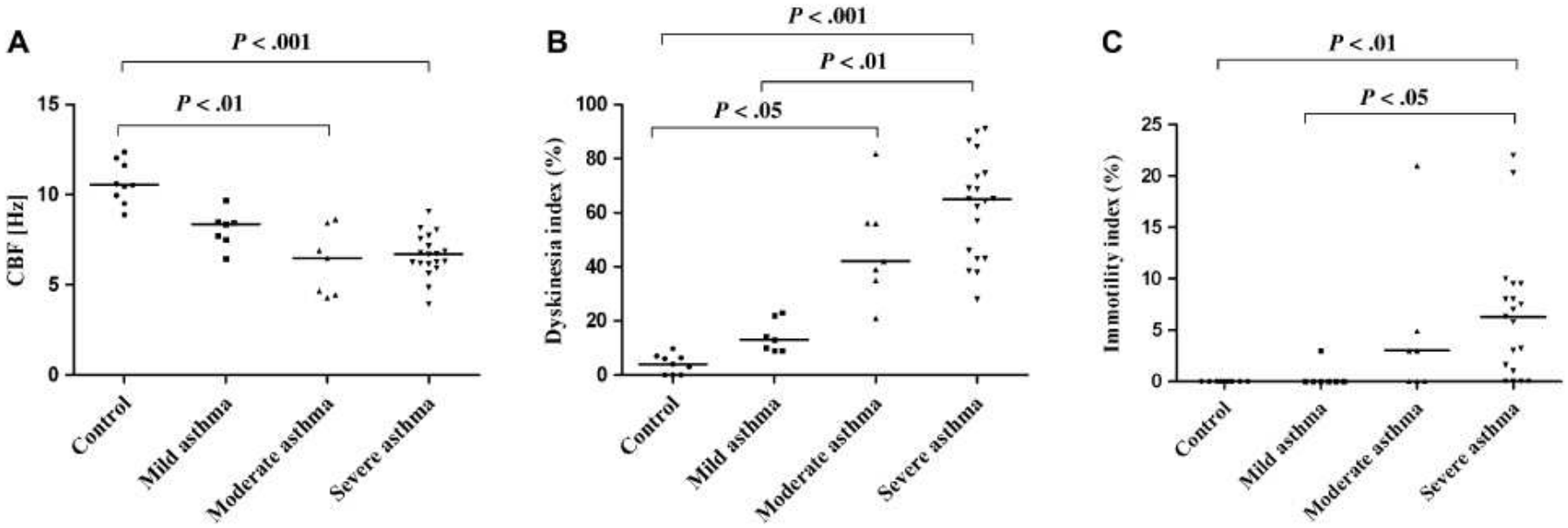

Figure 3.1: Ciliary beat frequency (A), dyskinesia index (B) and immotility index (C) of healthy control subjects and patients with mild, moderate and severe asthma.
Dyskinesia index = percentage of dyskinetically beating cilia among all cilia examined.
Immotility index = percentage of immotile cilia among all cilia examined.

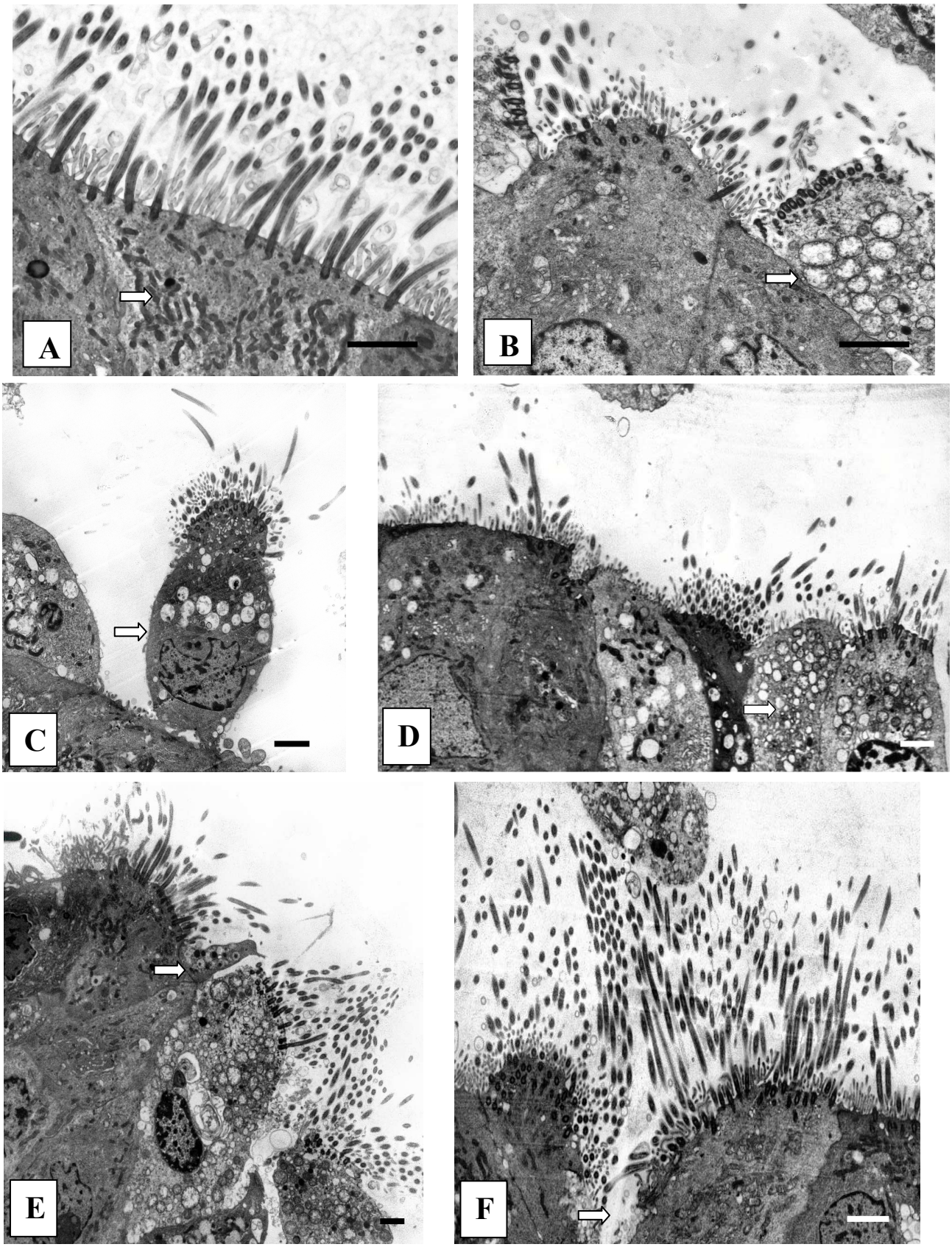

Figure 3.2: (legend on page 86)

Figure 3.2: Transmission electron micrographs of ciliated respiratory epithelium. **A**). Ciliated respiratory epithelium of a healthy control showing smooth epithelial surface, ciliated cell with cilia, microvilli and normal mitochondria (arrow). **B-F**: Ciliated respiratory epithelium of a patient with severe asthma showing, **B** – irregular epithelial surface with cellular projection, ciliary depletion and abnormal mitochondria (arrow), **C**-cell almost completely extruded from cell surface (arrow), **D** – dead cell (arrow), with ciliary depletion and abnormal mitochondria, **E** – cytoplasmic bleb (arrow) and **F** - disruption of tight junction with separation of cells (arrow). Internal scale bar = 2μm.

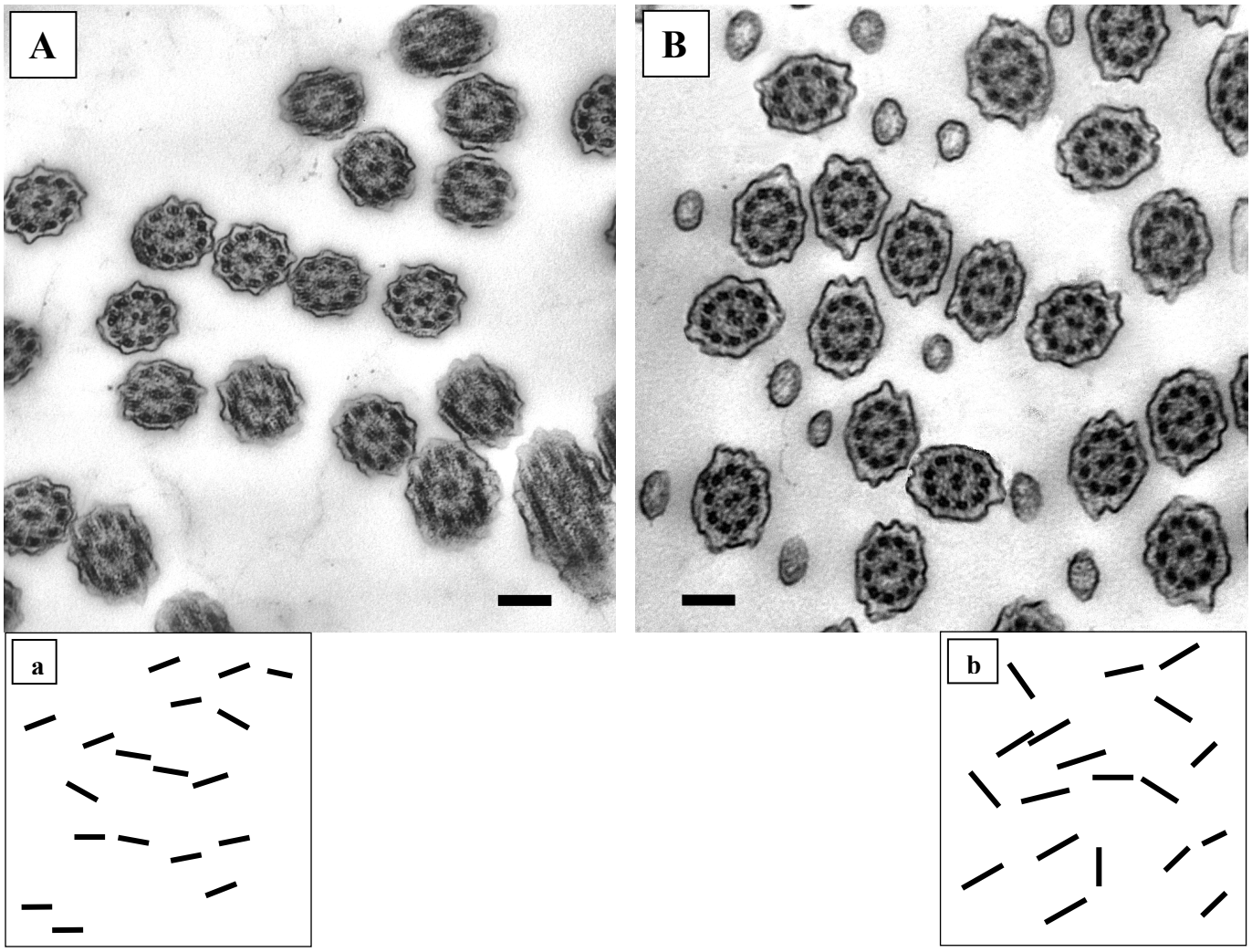

Figure 3.3: Transmission electron micrograph of ciliated cells. Cross sectional image of cilia originating from a single ciliated cell of a healthy control (**A**) showing normal ciliary orientation [11.67 degrees in this example] (**a**–distribution of lines drawn parallel to the central pair of microtubules of cilia originating from a single ciliated cell) and a patient with severe asthma (**B**) showing ciliary disorientation [21.05 degrees in this example] (**b**-distribution of lines drawn parallel to the central pair of microtubules of cilia originating from a single ciliated cell). Internal scale bar = 100nm

3.5 Discussion

These data provide evidence that ciliary dysfunction and ultrastructural abnormalities are closely related to asthma severity with dysfunction a feature of moderate to severe asthma and profound ultrastructural abnormalities restricted to severe disease. These striking changes, particularly in those subjects with severe asthma, constitute a phenotype of profound secondary ciliary dysfunction and are likely to have important functional consequences.

This is the first study that has assessed ciliary function in terms of ciliary beat frequency and beat pattern in a well characterised group of patients with asthma of varying severity. I used bronchial epithelial brushings rather than biopsies, because previous studies have shown that ciliary function (ciliary beat frequency and beat pattern) studies are technically much easier to perform on epithelial brushings and this is an accepted method for studying ciliary function.(166) This study is unique in that, in addition to showing a markedly reduced ciliary beat frequency, I determined ciliary beat pattern, which was also found to be significantly abnormal in patients with moderate and severe asthma. This was made possible by the recent advent of high-resolution digital high-speed video imaging(41) that allowed me to assess the precise beat pattern of cilia, by viewing the ciliary beat cycle frame by frame in different planes. Using this method, it has previously been shown that cilia in certain conditions may have a normal beat frequency despite markedly abnormal beat pattern(103) and normal ciliary beat frequency does not necessarily equate to normal ciliary function. The marked reduction in ciliary beat frequency and abnormal beat pattern seen in asthmatic airway epithelium, particularly in those with severe disease, is likely to be multifactorial in causation. Evidence from previous studies(177,178) suggests that sputum

from asthmatic patients and products of inflammatory cells such as eosinophil major basic protein possess ciliostatic activity. Environmental factors such as exposure to irritants or chronic infection may cause secondary ultrastructural defects of the cilia that may affect the ciliary beat frequency, as indicated in a number of reports.(179) The complex array of inflammatory mediators present in the asthmatic epithelium possesses diverse and opposing effects on ciliary beat frequency.(74)In addition, medications may influence ciliary beat frequency. Indeed both short and long-acting β2-agonists increase ciliary beat frequency.(71) Importantly, in this study, there was no significant correlation between sputum eosinophilia and ciliary beat frequency in subjects with severe asthma and a significantly reduced ciliary beat frequency was observed in patients with moderate and severe asthma, despite being on treatment with long-acting bronchodilators. Therefore, the cause of the dysfunctional ciliated epithelium observed in the severe asthma group in this study, remains to be determined. Of particular interest, significantly increased intracellular ciliary disorientation in the respiratory epithelium of patients with severe asthma, compared to healthy controls and patients with mild and moderate asthma was observed in this study. The magnitude of ciliary disorientation seen in patients with severe asthma is comparable to that previously described as a possible variant of primary ciliary dyskinesia (PCD).(166,180) In primary ciliary dyskinesia (PCD), the movement of mucus by cilia is negligible. Though mucociliary clearance per se was not studied in my study subjects and despite the fact that factors such as quantitative and qualitative properties of mucus and airway surface liquid also are known to affect mucociliary clearance, based on available evidence,(99,100) the degree of ciliary dysfunction that was demonstrated in this study is very much likely to correlate with the degree of impairment in mucociliary clearance. If the ciliary abnormalities seen in the severe asthma patients in this study were to be present

throughout the airways, these patients would have to rely on cough as their main mechanism of mucus clearance. Markedly reduced mucociliary clearance will predispose these patients to secondary bacterial infection. Indeed, asthma has been shown to be an independent risk factor for invasive pneumococcal disease.(181) The combination of impaired mucociliary clearance and infection may lead to the development of bronchiectasis. Though asthma and bronchiectasis are two diseases with distinct pathophysiologic processes, their coexistence has been reported in a number of patients, especially in those with severe persistent asthma.(182) Despite several caveats, a number of retrospective and cross sectional cohort studies suggest that asthma may a predisposing factor in the development of bronchiectasis.(182,183) In this study, all patients with moderate and severe asthma had high resolution computed tomography of chest performed and four (21%) patients with severe asthma had evidence of bronchiectasis.

The ciliary disorientation seen in patients with severe asthma is likely to be a consequence of the chronic inflammation characteristic of asthma or chronic infection(184) that may occur in some asthmatics. Indeed, there is a suggestion that inflammatory mediators such as leukotriene D4 (LTD4) may impair ciliary orientation.(185) In addition, studies done on quail oviduct and Xenopus larval skin suggest that ciliary polarity and orientation are influenced by the normal development of the apical cytoskeleton and normal tissue patterning.(186,187) Thus recent evidence of aberrant epithelial repair seen in asthmatic epithelium(109,110) could also provide a plausible mechanism contributing to ciliary disorientation seen in asthma.

This study also quantified the ultrastructural abnormalities of the respiratory epithelium. The findings in this study of loss of epithelial integrity and ultrastructural abnormalities are consistent with a number of previous studies that showed that asthmatic airway epithelium is structurally abnormal. Evidence supporting epithelial structural damage in asthmatic airways comes largely from earlier studies showing exfoliated epithelial cells in bronchoalveolar lavage (BAL) samples and marked desquamation, epithelial shedding and loss of integrity in histopathologic studies on autopsy or endobronchial biopsy specimens.(188,189) Despite the controversy(190) regarding the epithelial abnormalities seen in bronchial biopsy studies, accumulating evidence reviewed recently, strongly suggests structural abnormalities of the asthmatic epithelium.(109,110) Emerging evidence also points towards disruption of epithelial tight junctions and other junctional adhesion proteins in asthmatic airways that are responsible for maintaining the normal epithelial structural integrity.(109,110)

One limitation of this study is its cross-sectional design and therefore the within subject repeatability of these changes, the response to interventions and exacerbations is uncertain and needs to be further studied. Furthermore this study was restricted to epithelium derived from central airways and whether the findings of this study extend into the small airways is unknown. It will be of great interest to investigate the ciliary function and epithelial structure in the peripheral airways of patients with asthma. Whether these findings are specific to asthma or indeed a feature of other airways diseases such as chronic obstructive pulmonary disease or chronic cough needs to be investigated. In spite of these limitations, the measures of ciliary function and epithelial cell morphology are highly repeatable and therefore the magnitude of ciliary dysfunction in patients with moderate and severe asthma

compared to that of controls is striking, and is very likely to be clinically important. A further challenge for future studies is to determine whether these findings reflect intrinsic abnormalities of the asthmatic airway epithelium, effects of chronic inflammation, chronic infection, aberrant repair mechanisms, effect of medications or a combination of these factors.

3.6 Conclusion

In summary, this study provides evidence for profound ciliary dysfunction in adults with moderate asthma and severe refractory asthma in stable state, in addition to marked epithelial damage restricted to those with severe disease. The potential direct consequence of this phenotype of secondary ciliary dyskinesia is reduced mucociliary clearance and therefore increased susceptibility to infection, potentially more prolonged exposure to inhaled particulate pollutants and aeroallergens, all of which have been implicated in the pathophysiology of asthma.

CHAPTER 4

Chemokine and cytokine release in response to Dermatophagoides pteronyssinus allergen 1 (Der p 1) and Streptococcus pneumoniae by primary bronchial epithelial cells of patients with severe atopic asthma and healthy controls

4 Chemokine and cytokine release in response to *Dermatophagoides pteronyssinus* allergen 1 (Der p 1) and *Streptococcus pneumoniae* by primary bronchial epithelial cells of patients with severe atopic asthma and healthy controls.

4.1 Summary

Background

Injury and disruption of airway epithelium that may occur in patients with asthma potentially exposes bronchial epithelial cells to inhaled allergens and pathogenic bacteria. Sensitisation to the house dust mite *Dermatophagoides pteronyssinus* is common among patients with asthma. Infections also have been implicated in the pathophysiology of asthma.

Objective

To determine the cytokine and chemokine release by primary bronchial epithelial cells of patients with atopic severe asthma and healthy controls, in response to *Dermatophagoides pteronyssinus* allergen 1 and *Streptococcus pneumoniae*.

Methods: 8 adult patients with severe asthma and 6 healthy controls were studied. From bronchoscopic bronchial brushing, bronchial epithelial cells were cultured to confluency in collagen coated glass chamber slides, in bronchial epithelial growth medium. The cells were exposed to wild type (D39) *Streptococcus pneumoniae* at concentrations of 10^6 cfu/ml and 10^7 cfu/ml and supernatants harvested at 1 hour and 4 hours post exposure. The cells were also exposed to LoTox Der p 1 (Indoor Biotechnologies, UK) at concentrations of 1

μg/ml and 5 μg/ml in the presence of dithiothreitol and supernatants harvested at 8 hours and 24 hours following exposure. Cytokines and chemokines in the supernatant were measured using a multiplex ELISA-based protein array (SECTOR Imager 6000, Meso Scale Discovery, USA).

Results: Chemokine release by primary bronchial epithelial cells of patients with severe asthma and healthy controls in response to *Streptococcus pneumoniae* and *Dermatophagoides pteronyssinus* allergen 1 was time and dose dependent. Magnitude of release of CXCL8 (IL-8), CCL11 (Eotaxin) and CCL26 (Eotaxin-3), in response to *Streptococcus pneumoniae* by cells from healthy controls, was significantly higher compared to that from severe asthma patients. The magnitude of release of chemokines CXCL8 (IL-8), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (Eotaxin), CCL13 (MCP-4), CCL17 (TARC), CCL22 (MDC) and CCL26 (Eotaxin-3) in response to *Dermatophagoides pteronyssinus* allergen 1 by bronchial epithelial cells from patients with severe asthma, was significantly higher compared with that from healthy controls.

Conclusions: Primary bronchial epithelial cells of patients with atopic severe asthma and healthy controls are capable of releasing chemokines and cytokines in response to *Dermatophagoides pteronyssinus* allergen 1 and *Streptococcus pneumoniae* in a dose and time dependent manner. The differential response of the asthmatic epithelium seen in this study may be of significance in the context of developing novel immunomodulatory therapeutic strategies in the treatment of allergic airway inflammation.

4.2 Background to study

Unraveling the complex pathophysiology of severe asthma has proven to be a major research challenge.(106,172) There is growing interest in the role of dysfunctional and ultrastructurally abnormal airway epithelium and its interactions with inhaled aeroallergens and pathogens, in the pathogenesis of asthma in general and severe asthma in particular.(109,110,171)

The data presented in Chapter 3 suggest that ciliary dysfunction and ultrastructural abnormalities are closely related to asthma severity, with ciliary dysfunction a feature of moderate to severe asthma and profound ultrastructural abnormalities restricted to severe disease. One potential consequence of these abnormalities is prolonged and more intense exposure of the epithelium to inhaled aeroallergens and pathogens. Moreover, given the marked epithelial disintegrity seen in patients with severe asthma and the ability of the proteolytically active substances such as the *Dermatophagoides pteronyssinus* allergens to cause disruption of intercellular tight junctions, with the resulting increase in transepithelial permeability,(191) the bronchial epithelial cells could also be exposed to inhaled allergens and pathogens. Emerging evidence suggests that in addition to the adaptive immune system, the non-antigen dependent innate immune system may also play a crucial role in the pathogenesis of asthma.(192) Human bronchial epithelial cells express MHC Class II antigens,(193) CD40, ICAM-1(194) and Toll-like receptors (TLRs)(195) and share some important characteristics of antigen presenting cells such as the dendritic cells. It therefore follows that these cells can interact with microbial antigens and other inhaled allergens and this interaction is of potential significance in asthma pathogenesis.(196-198) The effect of a variety of immunomodulatory agents on allergic airway inflammation has been investigated

in recent years. Two recent reports and reviews presented data pointing towards a potential beneficial effect of immunoregulatory therapy with *S pneumoniae* on allergic airway inflammation.(199-202) In a mouse model, Preston et al(199) investigated the effect of killed *Streptococcus pneumoniae* administered before, during and after ovalbumin sensitisation on the development of allergic airways disease. They found that *Streptococcus pneumoniae* immunomodulatory therapy attenuated T cell cytokine production, goblet cell hyperplasia, airways hyperresponsiveness, and eosinophil numbers in the blood, bronchoalveolar lavage fluid and peribronchial tissue. Thornton et al(202) studied the effect of pneumococcal conjugate and polysaccharide vaccines in a mice allergic airway disease model and found that pneumococcal conjugate vaccine suppresses the hallmark features of allergic airway disease through the induction of regulatory T (T reg) cells.

In this regard, we aimed to study the effect (in terms of cytokine and chemokine release) of *Streptococcus pneumoniae* on primary bronchial epithelial cells of a group of patients with atopic severe asthma phenotype and compare that to the response of healthy controls. As a positive control, the cytokine and chemokine release in response to *D pteronyssinus* allergen 1 (Der p 1) by primary bronchial epithelial cells of patients with atopic severe asthma and healthy controls was also studied.

4.3 Methods

4.3.1 Subjects

8 subjects with severe asthma were recruited from Glenfield Hospital, Leicester, United Kingdom. 6 healthy controls were recruited from staff and by local advertising. Asthma was diagnosed based on presence of typical clinical features and objective measures of airway hyperresponsiveness and variable airflow obstruction as described previously.(176) Subjects with severe asthma met the American Thoracic Society criteria for refractory asthma(106) and belonged to the Global Initiative for Asthma (GINA) treatment steps IV-V).(104) Healthy controls were non smokers, had no history of respiratory disease and had normal lung function and PC_{20}. Subjects with severe asthma were current non smokers or had a smoking history of less than 10 pack years and had been free from infections and asthma exacerbations requiring antibiotics and/or systemic corticosteroids for at least 6 weeks at the time of collection of bronchial epithelial samples. The study protocol was approved by the Leicestershire and Rutland Regional Ethics Committee and written informed consent was obtained from all subjects.

4.3.2 Measurements

Demographics, including age, gender and current medications were recorded. All subjects had their plasma IgE measured and all underwent spirometry and methacholine challenge using the tidal breathing method.(174) Subjects with asthma underwent allergen skin prick tests for common aeroallergens, measurement of exhaled nitric oxide concentration (flow rate 50ml/s)(175) and sputum induction.(176) All subjects underwent flexible

bronchoscopy conducted according to the British Thoracic Society guidelines(162) and epithelial brushings were taken from the bronchus intermedius.

4.3.3 Primary bronchial epithelial cell culture

Methodology used for culturing primary bronchial epithelial cells from bronchial epithelial brushings is given in Chapter 2 (Section 2.4).

4.3.4 Preparation of suspensions of *Streptococcus pneumoniae* and *D pteronyssinus* allergen 1

Methodology used for preparation of suspensions of *Streptococcus pneumoniae* and *D pteronyssinus* allergen 1 is given in Chapter 2 (Sections 2.5 and 2.6).

4.3.5 Exposure of primary primary bronchial epithelial cell culture to *D pteronyssinus* allergen 1 and *Streptococcus pneumoniae*

Confluent monolayers of primary bronchial epithelial cells, on glass chamber slides, were washed twice with bronchial epithelial basal medium (BEBM) and then incubated with wild type *Streptococcus pneumoniae* (strain D39) at concentrations of 10^6 cfu/ml and 10^7 cfu/ml for up to 4 hours at 37°C. For the control, primary bronchial epithelial cells were incubated with 400µl BEBM. The supernatants were harvested after one hour and four hours after incubation and stored at -70°C. These time points were chosen based on previous (unpublished) observations in the laboratory that *Streptococcus pneumoniae*, when incubated with primary respiratory basal cell monolayer for up to 4 hours, induces release of cytokines and chemokines without causing significant cell death. Similarly,

confluent monolayers of basal cells were incubated with LoTox^{TM} Natural Der p 1 (pre-treated with dithiothreitol) at concentrations of 1 μg/ml and 5 μg/ml for up to 24 hours. The supernatants were harvested at eight hours and 24 hours after incubation (based on previous studies that showed that *Dermatophagoides pteronyssinus* allergens are capable of inducing cytokine and chemokine release from bronchial epithelial cells at these time points)(203-205) and stored at -70°C.

4.3.6 Chemokine and cytokine analysis

Methodology used for Chemokine and cytokine analysis is given in Chapter 2 (section 2.7).

4.3.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad, San Diego, CA, USA). Nonparametric data were described as median (IQR). Within group comparisons of the magnitude of chemokine/cytokine release were done using ANOVA (Kruskal–Wallis test) and Dunn's method for post-hoc analysis. Between groups comparisons were performed using the Mann–Whitney U-test. A p-value of <0.05 was taken as the threshold for statistical significance.

4.4 Results

The baseline characteristics of the subjects are given in Table 4.1. The median age was not significantly different between the two groups. Asthma patients had significantly higher IgE and significantly lower FEV_1 compared to healthy controls, consistent with airflow obstruction. The data for chemokine release in response to *S pneumoniae* and Der p 1 are

given in Tables 4.2 & 4.3 respectively. The data for cytokine release in response to *S pneumoniae* and Der p 1 are given tables 4.4 & 4.5 respectively.

There were no significant differences in the spontaneous release of chemokines and cytokines from bronchial epithelial cells derived from asthmatic versus healthy subjects (Table 4.2-4.5) prior to exposure to *S pneumoniae* and Der p 1. The concentration of all of the chemokines and cytokines released by asthmatic and healthy bronchial epithelial cells increased significantly following 4 hour incubation with *S pneumoniae* (10^7cfu/ml) except for IL-4, which was below the limit of detection (Table 4.2 & 4.4). Similarly, following stimulation with Der p 1 (5μg/ml) for 24 hours, the concentration of all the chemokines except CCL5 (RANTES) (Table 4.3) in the bronchial epithelial cell supernatants, and the cytokines (Table 4.5) that were above the limit of detection (except TNFα), were increased in health and disease. The release of chemokines by bronchial epithelial cells of patients with severe asthma and healthy controls in response to *S pneumoniae* (Table 4.2) and Der p 1 (Table 4.3) was time and dose dependent.

The magnitude of release of chemokines CXCL8 (IL-8) [Fig 4.1 A & B], CCL11 (Eotaxin) [Fig 4.1 C & D] and CCL26 (Eotaxin-3) [Fig 4.1 E & F] in response to *S pneumoniae* by bronchial epithelial cells from healthy controls, was significantly higher ($p<0.05$), compared to that from severe asthma patients. In contrast, the magnitude of release of chemokines CXCL8 (IL-8) [Fig 4.2 A & B], CCL11 (Eotaxin) [Fig 4.2 C & D], CCL26 (Eotaxin-3) [Fig 4.2 E & F], as well as CCL4 (MIP-1β), CCL5 (RANTES), CCL13 (MCP-4), CCL17 (TARC) and CCL22 (MDC) in response to Der p 1 by bronchial epithelial cells from patients with severe asthma, was significantly higher ($p<0.05$) compared to that from

healthy controls. The release of the other chemokines by the bronchial epithelial cells in response to stimulation by *S pneumoniae* or Der p 1 were not significantly different between subjects with severe asthma and healthy controls.

Among the ten cytokines measured, only IL-1β and IL-13 were detectable from bronchial epithelial cells from healthy controls, in response to Der p 1 (7.5). Bronchial epithelial cells from severe asthma patients released significantly higher ($p<0.05$) levels of cytokines (IL-6, IL-1β, IL-10, IL-12p70, IL-13, IL-2, and TNFα) at 24 hours post exposure to Der p 1, compared to that from healthy controls. Similar to the trend seen with chemokine release in response to *S pneumoniae*, bronchial epithelial cells from healthy controls released significantly higher ($p<0.05$) levels of cytokines IL-6 and IL-1β four hours post exposure to *S pneumoniae*, compared to that from patients with severe asthma (Table 4.4).

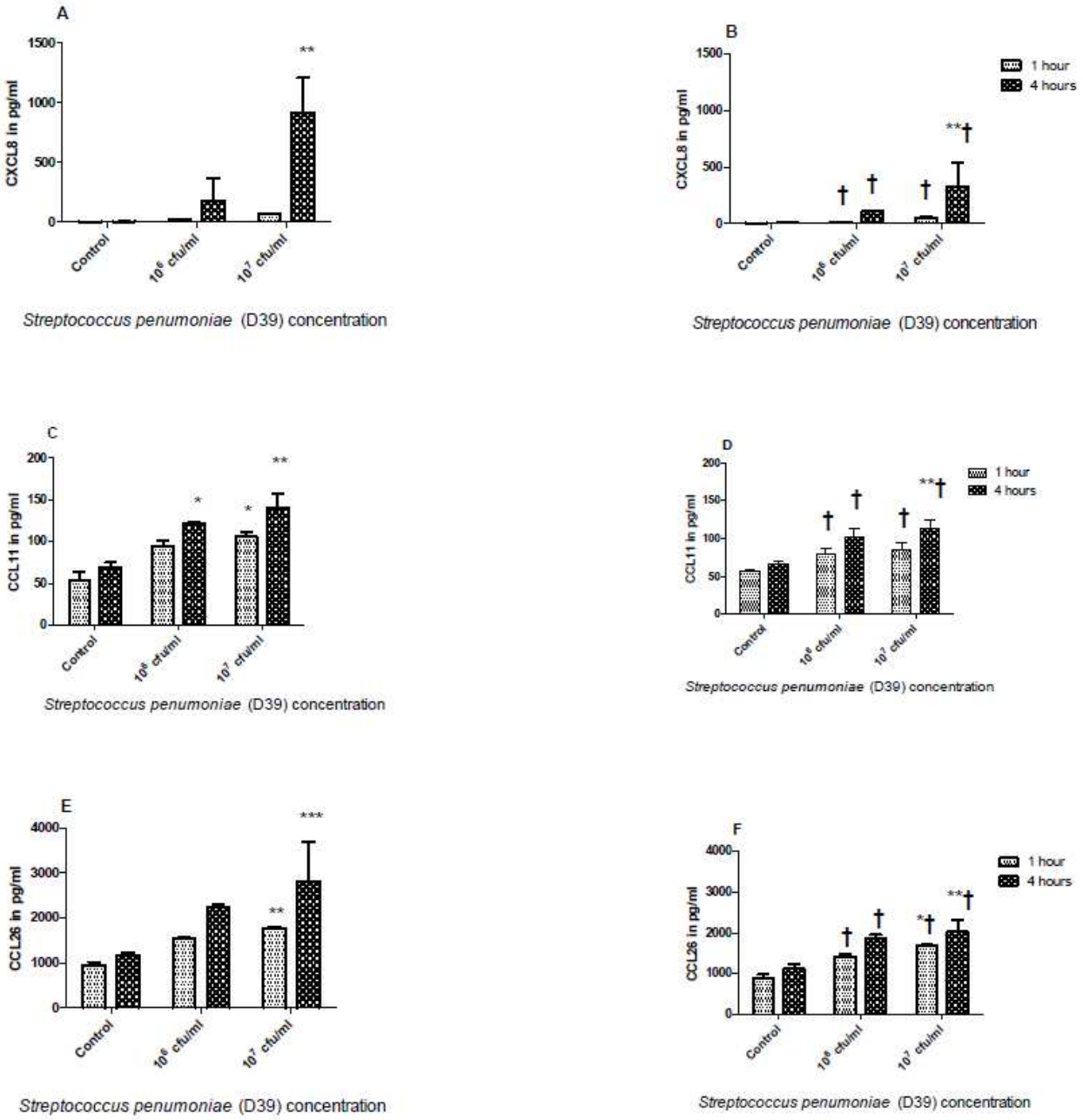

Fig 4.1: Release of CXCL8 [IL-8] (Fig 1 A & B), CCL11 [Eotaxin] (Fig 1 C & D) and CCL26 [Eotaxin-3] (Fig 1 E & F) by primary bronchial epithelial cells in response to *Streptococcus pneumoniae*. A, C & E- response of bronchial epithelial cells of healthy controls and B, D & F- patients with severe asthma. Data expressed as median (IQR).

*p<0.05, **p<0.01 and ***p<0.001 compared to control at same time point.
† p<0.01 compared to corresponding values for healthy controls.

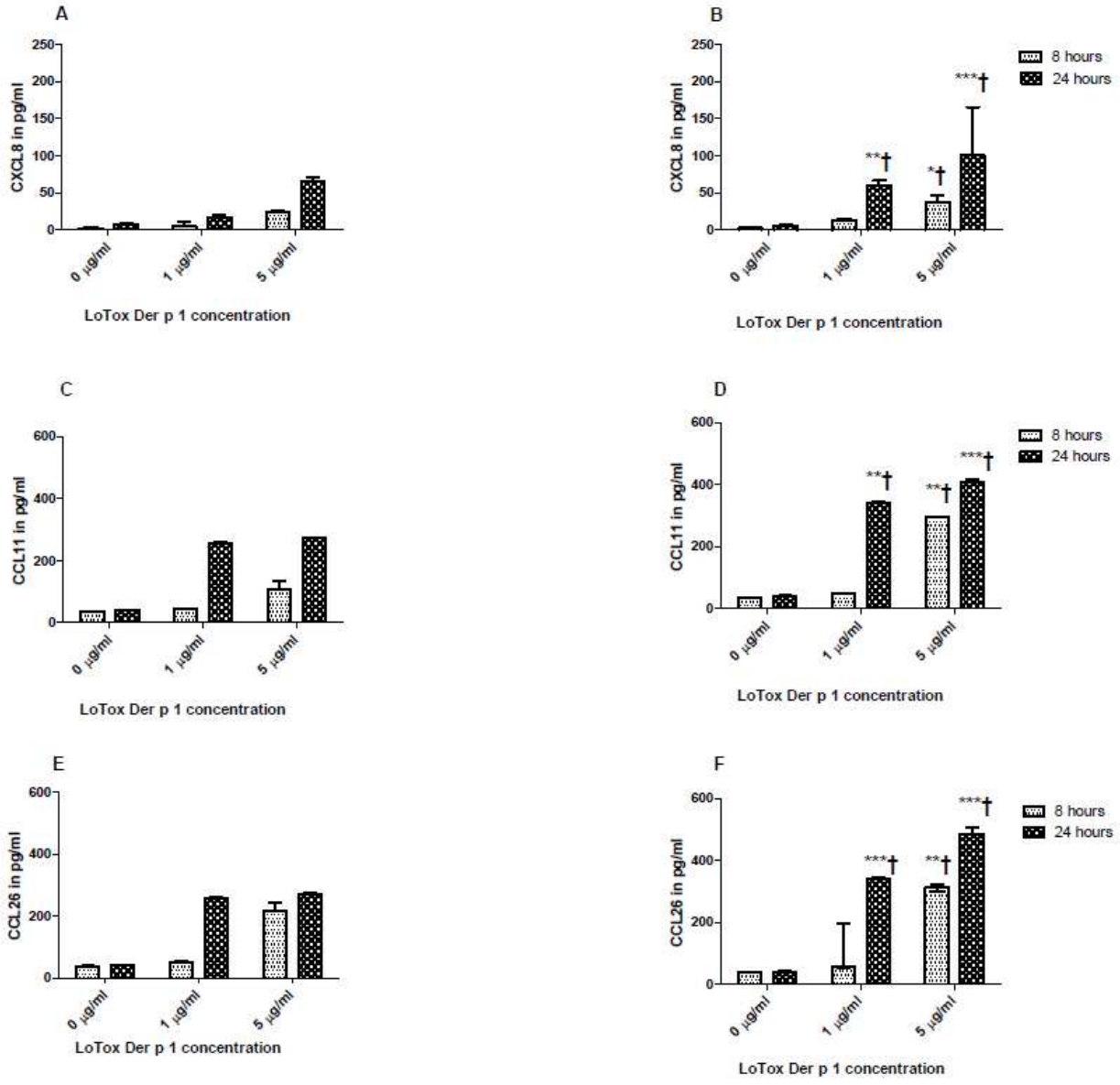

Fig 4.2: Release of CXCL8 [IL-8] (Fig 2 A & B), CCL11 [Eotaxin] (Fig 2 C & D) and CCL26 [Eotaxin-3] (Fig 2 E & F) by primary bronchial epithelial cells in response to Der p 1. A, C & E- response of bronchial epithelial cells of healthy controls and B, D & F- patients with severe asthma. Data expressed as median (IQR).

*p<0.05, **p<0.01 and ***p<0.001 compared to control at same time point.
† p<0.01 compared to corresponding values for healthy controls.

Table 4.1: Baseline characteristics of the subjects

Characteristic	**Control (n=6)**	**Severe asthma (n=8)**
Age (yr)†	29.5 [23-53]	44 [31.5-52]
Sex (no. of subjects)		
Male	2	3
Female	4	5
Age at onset of symptoms(yr)†	-	4.5 2.2-30
Atopy	-	Yes = 8
Plasma IgE (IU/ml)†	62.7 (43.4-120.4)	317 (105-564.8)*
FEV_1 †		
Litres	3.7 (2.9-4.1)	2·4 (1·5-2.8)**
Percentage of predicted value	105 (93.5-110.3)	71.5 (64-94)**
FEV_1: FVC ratio (%)†	92 (86.5-94.5)	76 (68-90)*
Exhaled Nitric oxide (ppb)†	-	58.4 (18.2-102)
Sputum measurements†		
Eosinophils (%)	-	2.6 (0·2-21.3)
Neutrophils (%)	-	69.6 (43.6-89.3)
PC_{20} (mg of methacholine/ml)†	>16	0·1 (0·06-0.31)
Inhaled steroid dose in µg (BDP equivalent)†	0	1800 (1600-2000)

† median (IQR).

**p<0.01 and *p<0.05 compared to control group

BDP = Beclomethasone dipropionate

Table 4.2: Chemokine release by basal cells from healthy controls and patients with severe asthma, in response to *Streptococcus pneumoniae*

	Healthy Controls						Patients with severe asthma					
	1 hour			4 hours			1 hour			4 hours		
Chemokine	***Control***	***10^6 cfu/ml***	***10^7 cfu/ml***	***Control***	***10^6 cfu/ml***	***10^7 cfu/ml***	***Control***	***10^6 cfu/ml***	***10^7 cfu/ml***	***Control***	***10^6 cfu/ml***	***10^7 cfu/ml***
CXCL8 [IL-8]	2.3 [1.7-2.9]	21.4 [19.6-26.8]	71.7 [69-77.4]	4.4 [4.0-7.5]	177 [158.8-364.5]	915.8 [805.3-1208]**	2.7 [1.6-2.9]	15.2 [13.4-16.8]	50.5 [35.1-58.6]	5.1 [3.5-7.3]	108.9 [91.6-116.9]	321.3 [266.6-543.3]**
CXCL10 [IP-10]	308 [282.3-318.8]	405.3 [398-409.9]	439.9 [430-455.3]	349.7 [340-361]	506.1 [486.9-523.5]	572.3 [545.8-682.8]**	311.3 [307.2-321.8]	417.6 [411.6-421.7]	456.7 [449.6-470.8]	354.2 [346.7-364.9]	528.1 [494-532.6]*	642.4 [596.4-664.2]***
CCL2 [MCP-1]	2.9 [0.1-3.8]	5.8 [5.4-6.3]	7.1 [7-7.5]	4.7 [4.5-4.9]	8.7 [8-9.5]	10.9 [10.2-13.3]*	3.1 [0.4-3.8]	6.4 [5.7-6.6]	7.4 [6.9-7.5]*	4.7 [4.2-4.9]	9.1 [8.4-9.3]*	12.3 [11.3-13]***
CCL4 [MIP-1β]	0	1.7 [0.8-2.4]	2.4 [2-4.1]	0.2 [0-0.4]	5.7 [3.9-7.2]*	7.8 [4.9-11.3]**	0	1.7 [1-1.9]	2.9 [2.3-3.3]	0.2 [0-.6]	5.8 [3.5-6.5]**	8.3 [4.6-9.1]***
CCL5 [RANTES]	0.1 [0-0.2]	1.3 [1.2-1.7]	1.9 [1.6-2.1]	1.1 [0.7-1.3]	2.8 [2.6-3.1]	4.5 [3.7-6.7]*	0.1 [0-0.3]	1.4 [1.1-1.7]	2.3 [1.9-2.4]	0.6 [0.1-1.1]	2.8 [2.6-2.9]*	4.2 [3.3-6.1]***
CCL11 [Eotaxin]	53.7 [52.3-63.7]	94.3 [91.7-100.3]	105.8 [105.3-111.3]*	69.7 [63.6-75.1]	122.2 [116.7-123.4]*	140.9 [134.5-158.2]**	57.5 [54.8-59.7]	80.4 [77 - 87.1]	85.1 [81.5-95.1]	67.3 [61.5-69.8]	102.7 [97.1-113.8]	113.1 [111.1-124.5]**
CCL13 [MCP-4]	58.9 [56.8-60.9]	92.6 [86.7-103.6]	106.7 [104.3-110.7]	77.9 [70.8-79.4]	127.2 [121.3-134.5]	144.5 [134.5-183.5]*	57.1 [42.8-65.4]	99.6 [93.6-104.2]	110.2 [101.2-113]*	72.3 [68.9-78.9]	128.7 [122.8-135.9]*	155.5 [150.3-180]***
CCL17 [TARC]	31.4 [27.4-39.5]	68.4 [64.2-71.2]	80.3 [74.9-85.9]	52.2 [40.4-56.1]	91.1 [87.5-109.2]	118.2 [100.9-144.2]**	32.4 [27.9-39.1]	67.7 [61.1-71.9]	78.5 [74.4-82.4]	51.2 [44.9-58.9]	90.9 [89.5-92.9]*	124.4 [116.1-136.2]***
CCL22 [MDC]	279.2 [259.3-285.5]	376.7 [365.1-385.8]	402.8 [374-416]	308.5 [299.4-326.6]	464.7 [447.9-507]	522.1 [476.5-607.2]*	278.9 [270.4-289.1]	376.9 [354.6-389.5]	405.3 [399.7-416.2]	305.6 [304-322]	480 [449.1-504.1]*	562.3 [518.1-584.9]***
CCL26 [Eotaxin-3]	949.3 [688.9-1021]	1560 [1536-1579]	1775 [1759-1802]	1177 [880.4-1232]	2231 [2197-2298]**	2815 [2626-3695]***	902.9 [772.8-976.7]	1422 [1361-1468]	1685 [1647-1720]	1126 [1077-1230]	1866 [1824-1968]*	2031 [1954-2307]**

Data as Median (IQR) pg/ml. *p<0.05, **p<0.01, ***p<0.001 compared to control

Table 4.3: Chemokine release by basal cells from healthy controls and patients with severe asthma, in response to Der p 1

	Healthy Controls						Patients with severe asthma					
	8 hours			24 hours			8 hours			24 hours		
Chemokine	***Control***	***Der p 1 1 µg/ml***	***Der p 1 5 µg/ml***	***Control***	***Der p 1 1 µg/ml***	***Der p 1 5 µg/ml***	***Control***	***Der p 1 1 µg/ml***	***Der p 1 5 µg/ml***	***Control***	***Der p 1 1 µg/ml***	***Der p 1 5 µg/ml***
CXCL8 [IL-8]	2.1 (1.7-3.4)	5.3 (4.9-11.3)*	25.0 (23.5-26.4) *†	7.8 (7.1-8.5)	16.9 (15.1-20.3) *‡	64.7 (51.3-71.7) *†‡	3.4 (2.5-4.1)	13.9 (11.7-14.9)*	38.3 (36.6-46.6) *†	6.3 (5.4-8.4)	60.6 (53.2-67.1) *‡	100.7 (82.2-165.8) *†‡
CXCL10 [IP-10]	54.5 (51.1-62.6)	71.5 (67.7-72.4)*	77.4 (75.2-79.4) *†	67.0 (66.3-68.7)	74.1 (67.9-74.4)	89.9 (86.4-98.6) *†‡	61.1 (58.5-64.3)	71.1 (69.0-71.8)*	78.1 (73.7-79.5) *†	67.8 (63.7-68.5)	68.5 (67.2-73.7)	88.6 (85-97.2) *†‡
CCL2 [MCP-1]	5.0 (4.6-6.8)	4.9 (4.3-5.3)	6.1 (5.6-6.7)	6 (5.2-6.3)	5.1 (4.7-5.7)	6.5 (5.7-7.7) *†‡	5.3 (4.6-5.8)	4.8 (3.8-5.7)	5.3 (5.1-5.5) †	5.5 (5.1-6.1)	5.8 (4.9-6.2)	7.4 (6.4-8.3) †
CCL4 [MIP-1β]	1.9 (1.0-2.2)	3.1 (3.0-3.2)*	3.2 (3.0-3.2)*	2.6 (2.6-2.7)	3.4 (3.1-3.4)*	3.6 (2.9-3.7)*	2.5 (2.3-2.6)	3.5 (3.1-3.6)*	3.9 (3.8-4.1) *†	2.8 (2.7-3.0)	4.8 (4.4-5.1) *‡	6.4 (5.4-7.1) *†‡
CCL5 [RANTES]	0 (0-0)	0.2 (0-0.3)	0.5 (0.4-0.6) †	0 (0-0)	0.7 (0.6-0.8) ‡	1.7 (0.9-2.2) †‡	0 (0-0)	1.0 (0.9-1.1)	1.5 (1.1-2.3) †	0 (0-0)	2.9 (2.7-3.3) ‡	4.5 (4-5.3) †‡
CCL11 [Eotaxin]	34.8 (33.2-36.7)	45.3 (44.6-46.1)*	67.8 (60.4-189.9) *†	41.1 (39.9-42.3)	255.8 (246.5-264) *‡	271.8 (269-275.6) *†‡	35.7 (33.6-36.9)	46.6 (45.6-47.7)*	289.9 (285.4-305.2)*†	42.3 (40.2-42.8)	339.1 (327-350.9) *‡	412.6 (398.9-428.9) *†‡
CCL13 [MCP-4]	37.3 (35.9-38.5)	47.1 (43.6-47.5)*	48.3 (48-49.4) *†	43.2 (42-44.8)	50.7 (49.3-51.5)*	52.7 (51.3-55.3) *†‡	39.6 (35.9-39.9)	56.1 (54.9-58.1)*	60.9 (59.7-63.6)*	44.5 (42.8-46.2)	57.8 (55.5-62.0) *‡	73.5 (69.5-86.1) *†‡
CCL17 [TARC]	28.7 (23.2-30.4)	34.3 (33.8-35.6)*	38.2 (37.6-38.3) *†	33.8 (32.1-34.6)	39.2 (38.9-39.9) *‡	40.9 (39.6-42.9) *†‡	30.4 (28.7-32.6)	36.8 (35.9-37.3)*	42.1 (39.9-42.9) *†	34.6 (32.8-34.9)	47.7 (44.7-49.3) *‡	55.6 (54.5-63.1) *†‡
CCL22 [MDC]	90.6 (83.1-92.6)	102.2 (102.1-103.4)*	105.4 (103.8-106.5) *†	98.7 (97.9-99.2)	107 (104.7-108.8) *‡	114.2 (109.1-118.5) *†‡	90 (85.5-93.6)	104.9 (103.6-105.9)*	116.5 (111.7-118.6) *†	100.1 (98.7-100.7)	127.8 (122.6-129.7) *‡	148.2 (141.4-155.3) *†‡
CCL26 [Eotaxin-3]	38.9 (38.5-39.6)	50.7 (48.6-52.9)*	218.2 (55.8-243.8) *†	41.2 (38.9-42.9)	258.5 (249.8-263.1) *‡	271.1 (268.8-275.4) *†‡	39.9 (38.7-41.3)	55.6 (53.8-195.4)*	312.9 (301.7-322.5) *†	42.1 (41.2-43.5)	342.4 (325.2-350) *‡	466.6 (455.8-551.1) *†‡

Data as Median (IQR) pg/ml. * Significantly increased compared to control

† Significantly increased compared to corresponding values for Der p 1, at concentration of 1 µg/ml

‡ Significantly increased compared to corresponding values at 8 hours

Table 4.4: Cytokine release by basal cells from healthy controls and patients with severe asthma, in response to *Streptococcus pneumoniae*

	Healthy Controls						Patients with severe asthma					
	1 hour			4 hours			1 hour			4 hours		
Cytokine	***Control***	***10^6 cfu/ml***	***10^7 cfu/ml***	***Control***	***10^6 cfu/ml***	***10^7 cfu/ml***	***Control***	***10^6 cfu/ml***	***10^7 cfu/ml***	***Control***	***10^6 cfu/ml***	***10^7 cfu/ml***
IL6	0	0	0	0	2.1 [1.7-2.9]*†	7.9 [4.9-10.9]***†	0	0	0	0	1.4 [0.8-1.6]	3.2 [2.5-4]]***†
IFN-γ	0	0	0	0	0	1.6 [1.2 -2]**	0	0	0	0	0	1.6 [1-1.9]**
IL10	0	0	0	0	1.8 [1.3-2.4]	4.5 [2.5-6.9]**	0	0	0	0	1.5 [0.8-2.7]	4.4 [2.9-6.6]***
IL12p70	0	0	0	0	1.6 [1.1-2.3]	3.1 [2.4-3.5]**	0	0	0	0	1.7 [1.1-1.8]*	3.1 [2.8-3.7]***
IL13	0	0	0	0	2.1 [1.6-3]*	5.5 [3.7-8.4]***	0	0	0	0	2.3 [1.8-2.4]**	5.1 [3.6-7.3]***
IL1b	0	2.5 [1.5-3.4]	5.2 [5-5.7]	0	10.3 [8.9-10.9]	35 [20.3-48.9]**	0	1.6 [1-3.1]	5.6 [4.1-5.7]*	0	7.1 [6.1-8.2]	15.9 [12.4-17.1]***
IL2	0	0	0	0	2.1 [1.6-3.1]	7 [6.2-8.2]**	0	0	0	0	2.3 [1.5-2.6]**	8.8 [4.4-10.1]***
IL4	0	0	0	0	0	0	0	0	0	0	0	0
IL5	0	0	0	0	0	1.4 [1.2-2.6]**	0	0	0	0	0	1.5 [0.9-2.4]***
TNFα	0	0	0	0	2.6 [1.7-5.8]	11.9 [6.9-17.2]**	0	0	0	0	4.3 [2.7-5]*	9.9 [5.9-24.2]***

Data as Median (IQR) pg/ml. *p<0.05, **p<0.01, ***p<0.001 compared to control

Table 4.5: Cytokine release by basal cells from healthy controls and patients with severe asthma, in response to *Der p 1*

	Healthy Controls						Patients with severe asthma					
	8 hours			24 hours			8 hours			24 hours		
Cytokine	***Control***	***Der p 1 1 µg/ml***	***Der p 1 5 µg/ml***	***Control***	***Der p 1 1 µg/ml***	***Der p 1 5 µg/ml***	***Control***	***Der p 1 1 µg/ml***	***Der p 1 5 µg/ml***	***Control***	***Der p 1 1 µg/ml***	***Der p 1 5 µg/ml***
IL6	0	0	0	0	0	0	0	0	0	0	0	5.1 (4.5-7.1)**
IFN-γ	0	0	0	0	0	0	0	0	0	0	1.9 (1.3-2.4)*	0
IL10	0	0	0	0	0	0	0	0	0	0	0	1.4 (1.2-2.9)**
IL12p70	0	0	0	0	0	0	0	0	0	0	0	2.1 (1.7-2.8)*
IL13	0	0	0	0	0	1.2 (1.0-1.2)*	0	0	1.3 (1.1-1.5)***	0	1.8 (1.7-1.9)**	3.7 (2.6-4.6)***
IL1b	0	0	1.1 (1-1.1)	0	1.2 (1.1-1.2)	2.1 (1.8-2.3)	0	1.5 (1.4-1.7)	3.7 (2.9-4.1)**	0	5.6 (4.8-7.5)*	24 (16.1-70.6)**
IL2	0	0	0	0	0	0	0	0	0	0	0	2.5 (1.9-3.9)***
IL4	0	0	0	0	0	0	0	0	0	0	0	0
IL5	0	0	0	0	0	0	0	0	0	0	0	0
TNFα	0	0	0	0	0	0	0	0	0	0	1.2 (0.5-1.4)	1.9 (1.8-3.6)

Data as Median (IQR) pg/ml. *p<0.05, **p<0.01, ***p<0.001 compared to control

4.5 Discussion

In this study, we have shown that primary bronchial epithelial cells are capable of releasing chemokines and cytokines in response to Der p 1 and *S pneumoniae* in a dose and time dependent manner. In response to Der p 1, the magnitude of release of chemokines from bronchial epithelial cells of severe asthma patients was significantly higher compared to that of healthy controls. In contrast to this, bronchial epithelial cells from healthy controls released significantly higher amount of chemokines in response to *S pneumoniae* compared to that from patients with severe asthma.

The house dust mite *Dermatophagoides pteronyssinus* allergens have been implicated in the pathophysiology of asthma, perennial rhinitis and atopic dermatitis. These allergens are capable of mounting both immunologic and non immunologic interactions with the airway epithelium. Of the several allergen groups identified in house dust mites, the group 1 (e.g Der p 1) allergens possess cysteine protease activity and the groups 3, 6 and 9 allergens possess serine protease activity.(203,206) Previous authors have investigated the effect of house dust mite extract,(204,205) natural Der p 1(203) and recombinant Der p 1(207) on epithelial cell lines (such as A549, BEAS-2B, 16HBE) and bronchial epithelial cells cultured from patients with asthma or explanted lungs. These studies have shown that Der p 1 induces release of cytokines (such as IL-6 and GM-CSF) and chemokines (such as CXCL8 [IL-8], CCL2 [MCP-1], CCL5 [RANTES] and CXCL10 [IP-10]) from bronchial epithelial cells. Data from this study and other reports (203-207) show that bronchial epithelial cells are capable of releasing a range of other cytokines (IL-1, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, TNF-α and IFN-γ) and chemokines (CCL4

[MIP-1β], CCL11 [Eotaxin], CCL13 [MCP-4], CCL17 [TARC], CCL22 [MDC] and CCL26 [Eotaxin-3]), in a time and dose dependent manner. (203-207)

The role pathogenic organisms in the development, maintenance and exacerbation of asthma has always been of great interest.(208) Recently, it has been suggested that newborns with hypopharyngeal bacterial colonisation are at increased risk of asthma in early life.(209) Both Gram-positive and Gram-negative bacteria have been shown to up regulate release of inflammatory mediators such as CXCL8 (IL-8) from airway epithelial cells.(209-211) Also, concurrent stimulation of epithelial surface by multiple microbes has been shown to induce a synergistic increase in CXCL8 (IL-8) release by the epithelial cells.(212)

In the context of profound ciliary dysfunction and epithelial disintegrity seen in patients with severe asthma,(213) I wanted to investigate if the response of primary bronchial epithelial cells from these patients to a common aeroallergen (Der p 1) and a common bacterial pathogen (*Streptococcus pneumoniae*) differ from that of healthy controls. The differential chemokine response of primary bronchial epithelial cells from severe asthma patients to Der p 1 and *Streptococcus pneumoniae* compared to healthy controls, that we found in this study, is of great interest due to two main reasons. Firstly, asthma and allergies have been reported as risk factors for carriage of *Haemophilus influenzae* and *Streptococcus pneumoniae.*(214) Asthma has been shown to be an independent risk factor for invasive pneumococcal disease as well.(181) It is well recognised that generation of CXCL8 (IL-8), a potent neutrophil chemo attractant, by epithelial cells is important in

clearance of pathogenic bacteria from epithelial surface.(215) It may be argued that the reduced CXCL8 (IL-8) release by asthmatic airway epithelium compared to that of healthy controls may potentially lead to a reduction in neutrophil influx and a delay in bacterial clearance. Secondly, it has been suggested that in individuals with atopic sensitisation to aeroallergens, there may be an altered mucosal immune response to bacterial antigens.(216,217) In recent studies using mouse models of allergic asthma, immunomodulatory therapy using *Streptococcus pneumoniae* has been shown to attenuate the key features of allergic airway disease such as Th2 cytokine production, peripheral blood and airway eosinophilia, goblet cell hyperplasia and airway hyperresponsiveness.(200,201) These effects are believed to be mediated by an increase in regulatory T-cells which in turn reduces T-cell proliferation and Th2 cytokine release.(202,218)

In this study we did not attempt to elucidate the mechanisms underlying the bronchial epithelial cells response to Der p 1 or *Streptococcus pneumoniae*. Previous work on airway epithelial cells has shown that the cysteine protease Der p 1 stimulates chemokine release by both protease activated receptor (PAR) dependent and independent mechanisms.(203,204) In addition, emerging data point towards a potential role of the innate immune system in airway epithelial response to house dust mite allergens.(219,220) It remains to be determined if similar mechanism(s) help explain the bronchial epithelial cell responses that we have seen in this study. Secondly, we have not investigated if the response of bronchial epithelial cells from non atopic patients with severe asthma is different to that of those with atopic asthma. Also, given that the basal

cells are important progenitor cells,(17) the effect of this aberrant chemokine mileu on epithelial injury-repair mechanism and the effect on other airway epithelial progenitor cells, need further investigation. Thirdly, it would be of interest to investigate if prior exposure of asthmatic airway epithelium to *Streptococcus pneumoniae* leads to an attenuated response to Der p 1.

4.6 Conclusions

Our study shows that primary bronchial epithelial cells of patients with atopic severe asthma and healthy controls are capable of releasing a range of chemokines and cytokines in response to Der p 1 and *Streptococcus pneumoniae* in a dose and time dependent manner. The differential response of the asthmatic epithelium seen in this study may be of significance in the context of developing novel immunomodulatory therapeutic strategies in the treatment of allergic airway inflammation.

CHAPTER 5

Ciliary function and epithelial ultrastructure in paediatric lung transplant recipients

5 Ciliary function and epithelial ultrastructure in paediatric lung transplant recipients

5.1 Summary

Background

Though mucociliary clearance is recognised to be impaired following lung transplantation, it is unclear if the ciliary function is abnormal.

Objective

Aim was to study the ciliary function and epithelial ultrastructure above and below the airway anastomosis and the peripheral airway of children following lung transplantation.

Methods

I studied the ciliary beat frequency and beat pattern using digital high speed video imaging and ultrastructure by transmission electron microscopy, of bronchial epithelium from above and below the airway anastomosis and the peripheral airway of 10 Cystic Fibrosis and 10 non-suppurative lung disease paediatric lung transplant recipients.

Results

Compared to epithelium below the anastomosis, the epithelium above the anastomosis in the Cystic Fibrosis group showed reduced ciliary beat frequency (median [IQR] ciliary beat frequency: 10.5 [9.0–11.4]Hz vs 7.4 [6.4–9.2]Hz; $p<0.01$) and increased dyskinesia

(median [IQR] dyskinesia index: 16.5 [12.9–28.2]% vs 42.2 [32.6– 56.4]%; $p<0.01$). No difference in ciliary function was observed between epithelium from the three sites in the non-suppurative lung disease group. In both Cystic Fibrosis and non-suppurative lung disease groups, compared to epithelium above the anastomosis, the epithelium below the anastomosis showed marked ultrastructural abnormalities including a significant reduction ($p<0.05$) in ciliated cells and a significant increase ($p<0.01$) in unciliated cells, dead cells, microtubular defects, ciliary depletion, mitochondrial damage, cytoplasmic blebbing, and projecting cells.

Conclusions

Ciliary dysfunction is a feature of native airway epithelium in paediatric Cystic Fibrosis lung transplant recipients. The epithelium below the airway anastomosis shows profound ultrastructural abnormalities in both Cystic Fibrosis and non-suppurative lung disease lung transplant recipients.

5.2 Background to study

Lung transplantation is an accepted treatment option for children with end stage lung disease. Compared to other solid organ transplants, the overall survival remains poor for paediatric lung transplant recipients.(142) According to recent reports,(142) almost half the deaths within the first year post lung transplant are attributable to infection. Also, available evidence suggests that among a multitude of factors, infections (viral, bacterial or fungal) may also play a role in the pathogenesis of bronchiolitis obliterans syndrome (BOS), which is the major cause of death by five year post lung transplant.(221-224) Paediatric lung transplant recipients' increased susceptibility to respiratory infections is primarily related to immunosuppression, but other factors such as impaired cough reflex and impaired mucociliary clearance may be contributory.

The structure of ciliated respiratory epithelium and function of cilia have been reviewed in Chapter 1.2. Our current understanding of ciliary function, airway epithelium and mucociliary clearance following lung transplantation is reviewed in Chapter 1.4.

In summary, though available evidence from both animal(146-148,150) and human(151-153) studies suggests that the mucociliary clearance may be impaired in lung transplant recipients, particularly in the early post transplant period, the reasons for this impairment are far from being completely understood. To date, there have been no studies that examined ciliary function in children following lung transplantation.

The aim of this study was to assess the ciliary function (in terms of ciliary beat frequency and beat pattern) and detailed ultrastructure of the ciliated respiratory epithelium in the native airway and the transplanted lungs in paediatric lung transplant recipients. Secondary aims included comparison of ciliary function and epithelial ultrastructure of the peripheral and central airways in paediatric lung transplant recipients. An additional objective was to study the ciliary function and epithelial ultrastructure of the lower respiratory tract of paediatric Cystic Fibrosis lung transplant recipients.

5.3 Methods

5.3.1 Subjects

At Great Ormond Street Hospital for Children (London, United Kingdom), surveillance bronchoscopy is performed at 1 week, 1, 3, 6 and 12 months post lung transplantation, as part of the treatment protocol. Bronchoscopy may be performed on clinical grounds both during and after the first year post transplantation. Over a two year period (2007-2009), 20 children (<18 years) who underwent flexible bronchoscopy following lung transplantation at Great Ormond Street Hospital for Children were studied. Demographic and clinical details were collected including indication for transplant, type of transplant, duration post lung transplantation and indication for bronchoscopy. All subjects underwent pH/impedance study at 3 months post lung transplantation, to look for evidence of pathological gastroesophageal reflux. Flexible bronchoscopy was conducted according to the British Thoracic Society guidelines.(162) Epithelial brushings were

taken from 2-3cm above and 2-3cm below the airway anastomosis under direct vision and from a peripheral airway under fluoroscopic screening (Fig 8.1B). Bronchoalveolar lavage was performed and the fluid was tested for various pathogens including bacteria, mycobacteria, legionella, fungi and viruses using microscopy, culture, immunofluorescence, and polymerase chain reaction (PCR), as appropriate. Bronchoalveolar lavage fluid was also processed using the Oil Red O staining technique to estimate the proportion of lipid laden macrophages and a proportion greater than 10% was considered significant. Transbronchial lung biopsy samples were obtained and histopathologic studies were performed to look for evidence of rejection. At the time of collection of bronchial epithelial samples, the subjects had been free from intercurrent respiratory infections requiring rescue use of antimicrobials for at least 6 weeks.

The study protocol was approved by the Institute of Child Health and Great Ormond Street Hospital Research Ethics Committee. Participating children provided assent and written informed consent was obtained from parents.

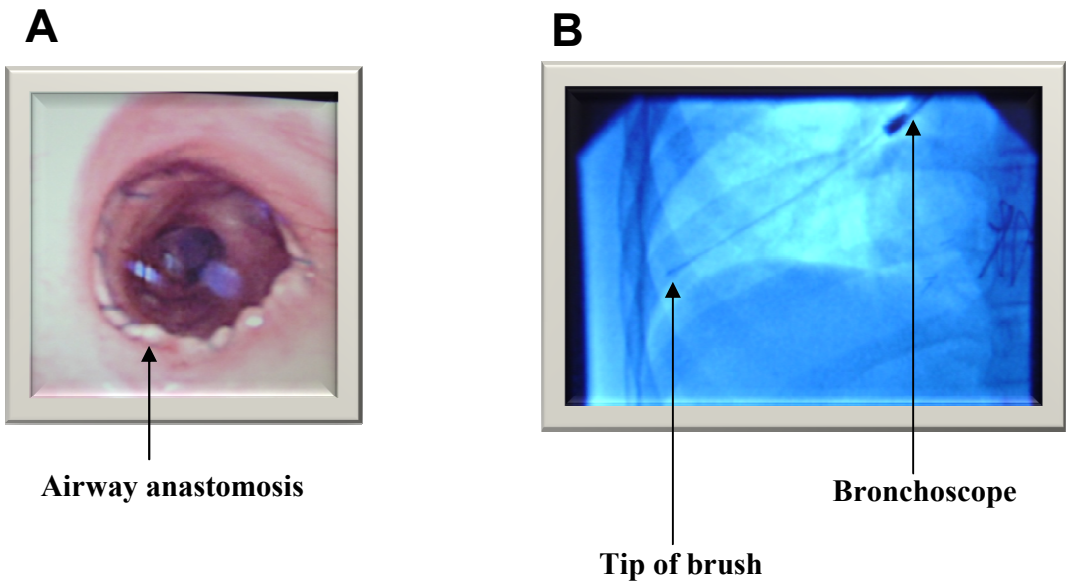

Figure 5.1: **A** - Bronchoscopic image of airway anastomosis (arrow) in a paediatric lung transplant recipient. **B** - Radiograph showing epithelial brushing from a peripheral airway under fluoroscopic control in a paediatric lung transplant recipient. Arrows point to tip of the flexible bronchoscope and tip of the brush (passed through the bronchoscopic channel) located in a peripheral airway of the right lung.

5.3.2 Ciliary beat frequency and beat pattern

Methodology used for assessment of ciliary beat frequency and beat pattern is given in Chapter 2 (section 2.2).

5.3.3 Transmission electron microscopy

Methodology used for detailed assessment of ultrastructure of the ciliated respiratory epithelium is given in Chapter 2 (section 2.3).

5.3.4 Statistical analysis

Sample size was calculated based on ciliary beat frequency as the primary outcome measure. As explained in section 6.3.5, it was estimated that, to detect a mean difference in ciliary beat frequency of 2 Hz (with a standard deviation of 1 Hz) between two groups, with a confidence interval of 95% and a power of 80%, a sample size of six (n=6) in each group would be required. Statistical analysis was performed using GraphPad Prism 5. Non-parametric data were described as median (IQR). Groups were initially compared using the Friedman test and post-hoc analysis was performed using Dunn's method. A p value of <0.05 was considered statistically significant. Agreement between the two observers (B Thomas and A Rutman) was excellent for measurement of ciliary beat frequency (interclass correlation 0.94) as well as dyskinesia index (interclass correlation 0.93). Repeatability (agreement within observer) was also excellent (interclass correlation was 0.94 for ciliary beat frequency and 0.99 for dyskinesia index).

5.4 Results

The demographic and clinical details including results of bronchoalveolar lavage and transbronchial biopsy studies are given in Table 5.1. None of the transbronchial biopsy samples studied showed evidence of acute cellular rejection. Of the 20 children studied, two children who had a pathogenic organism in the bronchoalveolar lavage, were excluded from the ciliary function studies and analysis of epithelial ultrastructure. Ciliary beat frequency and beat pattern were studied in 18 children (9 each in the Cystic Fibrosis group and the non-suppurative lung disease group) and the results are given in Figures 5.2 and 5.3.

In the Cystic Fibrosis group, the median [IQR] ciliary beat frequency was significantly decreased in the epithelium above the airway anastomosis (7.4 [6.4-9.2] Hz) compared to that below (10.5 [9.0-11.4] Hz) (Friedman $p<0.01$, Figure 5.2A). Analysis of ciliary beat pattern showed a higher proportion of dyskinetic cilia in the epithelium above the airway anastomosis (42.2 [32.6-56.4] %) compared to that below the anastomosis (16.5 [12.9-28.2] %) and that of the peripheral airway (13.9 [6.5-16.6] %) (Friedman $p<0.01$, Figure 5.2B). The epithelium above the airway anastomosis also showed significantly higher immotility index (4.1 [3-5.9] %) compared to that of the peripheral airway (0 %) (Friedman $p<0.001$, Figure 5.2C). In contrast, in the non-suppurative lung disease group, there was no significant difference in the ciliary beat frequency, dyskinesia index or immotility index between the epithelium above and below the airway anastomosis and that of the peripheral airway (Figure 5.3). The median (IQR) length of cilia in the central (above and below the airway anastomosis) and peripheral airways were 5.8 (5.4 – 6.2) μm and 6.0 (5.6 – 6.2) μm respectively and the difference was not statistically significant ($p=0.17$).

Bronchial brushing obtained from one child each in the Cystic Fibrosis group and the non-suppurative lung disease group was excluded from analysis of epithelial ultrastructure because of identification of a pathogenic organism in the bronchoalveolar lavage fluid. The samples obtained from two other children each in the Cystic Fibrosis and the non-suppurative lung disease groups were insufficient for assessment by transmission electron microscopy. Therefore transmission electron microscopy was done

on samples obtained from seven children in each group. Results are summarised in Tables 5.2 & 5.3 and example electron micrographs are shown in Figures 5.4 & 5.5. Striking ultrastructural abnormalities were observed in the ciliated epithelium below the airway anastomosis, in both the Cystic Fibrosis and non-suppurative lung disease groups. Compared to epithelium above the airway anastomosis and that of the peripheral airway, the epithelium below the anastomosis in the Cystic Fibrosis group showed a significant reduction in the proportion of ciliated cells ($p<0.05$) and a significant increase in the proportion of ciliated cells with loss of cilia ($p<0.05$) and cilia with microtubular defects ($p<0.05$). In addition, compared to the epithelium above the airway anastomosis, the epithelium below the anastomosis in the Cystic Fibrosis group showed a significant increase in the proportion of unciliated cells, dead cells, cells extruding from the cell surface, cells with cytoplasmic blebbing and cells with mitochondrial damage ($p<0.01$). Also, there was significantly higher ciliary disorientation in the epithelium below the airway anastomosis compared to that above the anastomosis, in the Cystic Fibrosis group ($p<0.05$).

In the non-suppurative lung disease group, the epithelium below the airway anastomosis showed a significant reduction in the proportion of ciliated cells ($p<0.01$) and a significant increase in the proportion of unciliated cells, dead cells, ciliated cells with loss of cilia, cells extruding from the cell surface, cells with cytoplasmic blebbing, cells with mitochondrial damage and cilia with microtubular defects ($p<0.01$) compared to epithelium above the anastomosis. A significantly higher ciliary disorientation was seen in the epithelium below the airway anastomosis and the peripheral airway, compared to

the epithelium above the airway anastomosis ($p<0.05$). The epithelium below the airway anastomosis also showed a significant increase in the proportion of mucus cells compared to that above the anastomosis and that of the peripheral airway ($p<0.05$).

Overall, there was marked epithelial ultrastructural abnormalities in the epithelium below the airway anastomosis in both the Cystic Fibrosis and non-suppurative lung disease groups. In view of possible effect of normal epithelial healing process in the immediate post operative period on epithelial ultrastructural changes, the data were re-analysed excluding the bronchial brushing samples taken within the first four months post transplantation (two patients in each group). This did not alter the finding of significant epithelial ultrastructural abnormalities in the epithelium below the airway anastomosis compared to that above the anastomosis, in both the groups (Tables 5.4 & 5.5).

Table 5.1: Demographic and clinical details

	Cystic Fibrosis (n=10)	**Non-suppurative lung disease (n=10)**
Age (yr)*	14.1 (12.8 – 15.7)	13.1 (8.6 – 15.4)
Sex M/F	2/8	7/3
Diagnosis	Cystic Fibrosis	Obliterative Bronchiolitis (n=3) Pulmonary arterial hypertension (n=4) Pulmonary venoocclusive disease (n=1) Interstitial lung disease (n=1) Eisenmenger syndrome (n=1)
Type of transplant	Bilateral lung (n=9) Heart lung (n=1)	Bilateral lung (n=7) Heart lung (n=3)
Indication for bronchoscopy	Surveillance (n=9) Suspected rejection (n=1)	Surveillance (n=9) Suspected rejection (n=1)
Duration post transplant (months)*	12 (2.9 – 15.5)	7 (2.3 – 15)
FEV_1 (% of predicted)* at time of bronchoscopy	75.5 (54.5 – 96)	74 (60.5 – 97)
Bronchoalveolar lavage	No organisms (n=6) URT commensals (n=3) Mycobacterium Abscessus (n=1)	No organisms (n=5) URT commensals (n=4) Streptococcus pneumoniae (n=1)
Transbronchial Biopsy	No acute cellular rejection (n=10) A0B0 = 8 A0BX = 2	No acute cellular rejection (n=10) A0B0 = 7 A0Bx = 3
Gastroesophageal reflux disease requiring Nissen fundoplication	9	8
BAL lipid laden macrophages >10%	3	4

* Median (IQR) URT = Upper Respiratory Tract BAL = Bronchoalveolar lavage

Table 5.2: Analysis of epithelial ultrastructure (Cystic Fibrosis group) by transmission electron microscopy*

	Above anastomosis	Below anastomosis	Peripheral airway
Ciliated cells	67.8 [64.1-73.9]†	36.9 [34.9-39.1]	67.7 [55-71.1]‡
Unciliated cells	25.5 [20.8-30.4]†	45.1 [42.9-46.1]	28.1 [19.7-35.4]
Mucus cells	5.5 [4.6-6.7]	6.4 [5.7-6.9]	6.9 [4.9-7.2]
Dead cells	0†	11.7 [10.9-13.1]	1.2 [0-2.3]
Dynein arm defects	2.9 [1.1-5.2]	3.6 [0.4-4.2]	3 [2.6-4.5]
Microtubular defects	2.2 [2-2.6]†	6.3 [5.3-8.7]	2.1 [2.1-3.9]‡
Ciliated cells with loss of cilia	22.6 [14.8-26.7]†	87.7 [78.3-92.4]	29.7 [17.8-49.4]‡
Cells extruding from the surface	20.9 [14.1-25.5]†	83.8 [77.2-89.7]	28.1 [25.5-31.3]
Cells with cytoplasmic blebbing	10.7 [8.5-12.9]†	40.7 [33.7-44.2]	17.9 [15.6-20.6]
Cells with mitochondrial damage	8.8 [7.1-11.6]†	34.3 [26.5-39.8]	16.2 [13.5-20.4]
Ciliary orientation (degrees)	12.9 [12.2-14.1]‡	19.8 [19.3-23.6]	17.5 [13.1-18.2]

* Data expressed as median percentage (IQR). Ciliary orientation is expressed as median degrees (IQR).
† $p<0.01$ and ‡ $p<0.05$ compared to epithelium below the airway anastomosis

Table 5.3: Analysis of epithelial ultrastructure (Non-suppurative lung disease group) by transmission electron microscopy*

	Above anastomosis	Below anastomosis	Peripheral airway
Ciliated cells	83.2 [79.9 - 84.9]†	40.6 [30.9 - 59.3]	59.6 [53.6 - 68.5]
Unciliated cells	12.7 [11.3 - 13.7]†	49.7 [32.8 - 59.6]	37 [27.4 - 43.3]
Mucus cells	4.7 [3.5-5.6]‡	8.5 [7.1-11.4]	3.4 [2.7 - 4.7]‡
Dead cells	0†	12.4 [9.9 - 15.9]	1.8 [0.6-9.7]
Dynein arm defects	2.3 [1.9 - 3.6]	5.4 [2.9 - 8.1]	3.3 [2.4 - 9.5]
Microtubular defects	2.4 [1.9 - 3.5]†	7.7 [6.6 - 9.1]	2.7 [1.9 - 8.6]
Ciliated cells with loss of cilia	11.2 [8.3 - 24.5]†	66.1 [55.3 - 83.5]	29.9 [16.2 - 53.6]
Cells extruding from the surface	16.3 [5.1 - 18.6]†	49.2 [37.1 - 58.4]	41 [14.9 - 81.3]
Cells with cytoplasmic blebbing	7.2 [4.5 - 10.3]†	45.3 [41.6 - 48.3]	27.9 [15.3 - 41]
Cells with mitochondrial damage	6.4 [4.3 - 9.0]†	26.6 [17.9 - 36.5]	10.2 [9.3 - 48.1]
Ciliary orientation (degrees)	11.8 [10.6 – 12.7]	19.5 [17.2 - 22.2]§	23.6 [20.3 - 26.8]§

* Data expressed as median percentage (IQR). Ciliary orientation is expressed as median degrees (IQR).
† $p<0.01$ and ‡ $p<0.05$ compared to epithelium below the airway anastomosis
§ $p<0.05$ compared to epithelium above the airway anastomosis

Table 5.4: Analysis of epithelial ultrastructure (Cystic Fibrosis group) by transmission electron microscopy* (excluding samples taken within the first 4 months of transplantation. n=5)

	Above anastomosis	Below anastomosis	Peripheral airway
Ciliated cells	66.1 [62.5 - 70.9]	38.7 [35.7 - 42.7]‡	68.2 [47.7 - 74.6]
Unciliated cells	29.3 [24.2 - 32.4]	47.2 [45.2 - 50.1]	28.3 [18.6 - 44.6]
Mucus cells	4.7 [3.9 - 6.1]	6.7 [5.9 - 8.3]	5.6 [4.2 - 8]
Dead cells	0	6.1 [4.7 - 8.2]‡	0 [0 - 1.3]
Dynein arm defects	2.4 [0.6 - 4.8]	3.7 [0.9 - 5.9]	2.8 [2.5 - 3.9]
Microtubular defects	2.1 [1.5 - 2.3]	6.3 [4.5 - 8.2]§	2.1 [1.8 - 3.7]
Ciliated cells with loss of cilia	22.7 [12.9 - 28.9]	91.6 [87.7 - 95.3]‡	28.4 [16.9 - 37.6]
Cells extruding from the surface	18.6 [12.2 - 22.2]†	86.6 [81.5 - 90.4]	28.1 [26.2 - 30.2]
Cells with cytoplasmic blebbing	9.9 [8.1 - 12.3]†	43.8 [32.7 - 53.3]	19.6 [15.8 - 24.6]
Cells with mitochondrial damage	7.3 [6.1 - 9.4]†	28.7 [24.2 - 40.8]	17.9 [15.2 - 20.8]
Ciliary orientation (degrees)	13.4 [12.6 - 14.3]†	21.5 [19.6 - 23.5]	15.0 [13.9 - 16.4]

* Data expressed as median percentage (IQR). Ciliary orientation is expressed as median degrees (IQR).
† $p<0.01$ compared to epithelium below the airway anastomosis.
‡ $p<0.05$ compared to epithelium above the anastomosis and that of peripheral airway.
§ $p<0.05$ compared to epithelium above the airway anastomosis

Table 5.5: Analysis of epithelial ultrastructure (Non-suppurative lung disease group) by transmission electron microscopy* (excluding samples taken within the first 4 months of transplantation. n=5)

	Above anastomosis	Below anastomosis	Peripheral airway
Ciliated cells	83.9 [82.5 - 84.9]†	37.8 [24.2 - 49.9]	60.8 [50.5 - 67.1]
Unciliated cells	12.4 [11.9 - 13.8]†	46.2 [39.0 - 59.9]	31.9 [25.8 - 43.7]
Mucus cells	3.6 [3 - 5.4]†	7.9 [6.4 - 9]	4.6 [3.2 - 5.8]
Dead cells	0†	5.9 [4.7 - 9.4]	2.5 [1.2 - 2.9]
Dynein arm defects	2.3 [2.1 - 2.7]‡	8 [4.5 - 11.5]	6.9 [3.7 - 10.9]
Microtubular defects	2.5 [2 - 3.3]†	8.7 [7.4 - 11.5]	4.2 [2.4 - 6.6]
Ciliated cells with loss of cilia	20.3 [9.3 - 37]†	67.7 [59.2 - 88.9]	41.7 [17.3 - 70.3]
Cells extruding from the surface	18.5 [8.7 - 22.6]†	57.4 [40.3 - 61.6]	46.4 [21.3 - 61.8]
Cells with cytoplasmic blebbing	9.7 [5.3 - 10.3]†	23.1 [22.1 - 29.2]	15.3 [11.1 - 19.3]
Cells with mitochondrial damage	6.7 [1.7 - 7.9]†	32.2 [25.0 - 40.1]	16.4 [9.3 - 36.9]
Ciliary orientation (degrees)	12.1 [11.6 - 13.5]‡	17.6 [16.8 - 23.3]	16.2 [14.2 - 19.6]

* Data expressed as median percentage (IQR). Ciliary orientation is expressed as median degrees (IQR).
† $p<0.01$ and ‡ $p<0.05$ compared to epithelium below the airway anastomosis

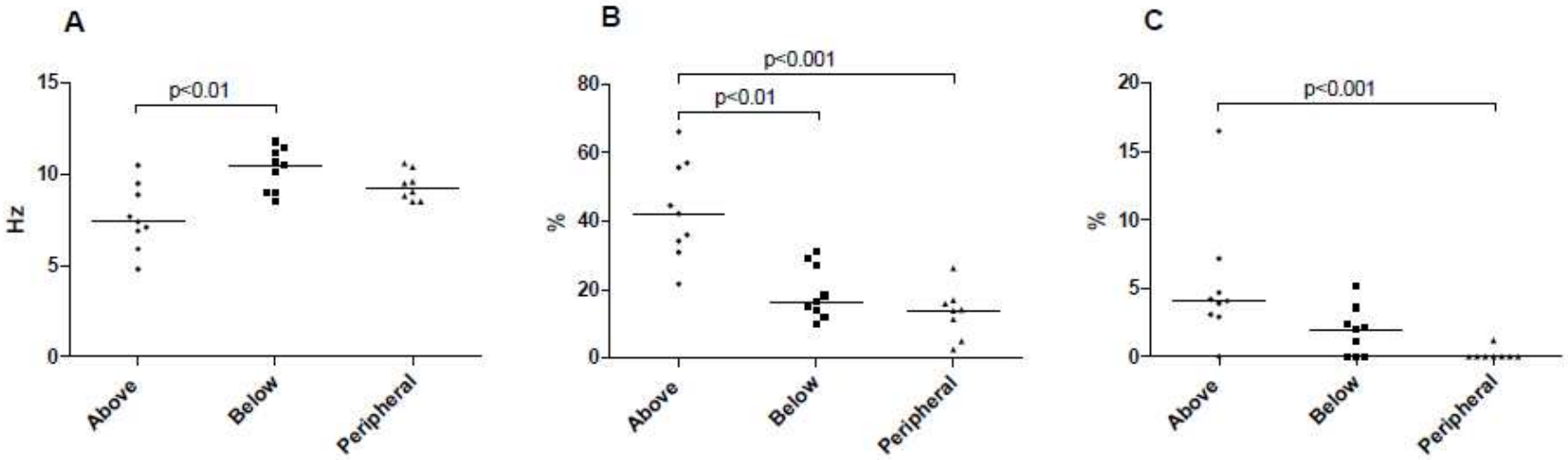

Figure 5.2: Ciliary beat frequency (A), dyskinesia index (B) and immotility index (C) of respiratory epithelium above and below the airway anastomosis and the peripheral airway of paediatric Cystic Fibrosis lung transplant recipients

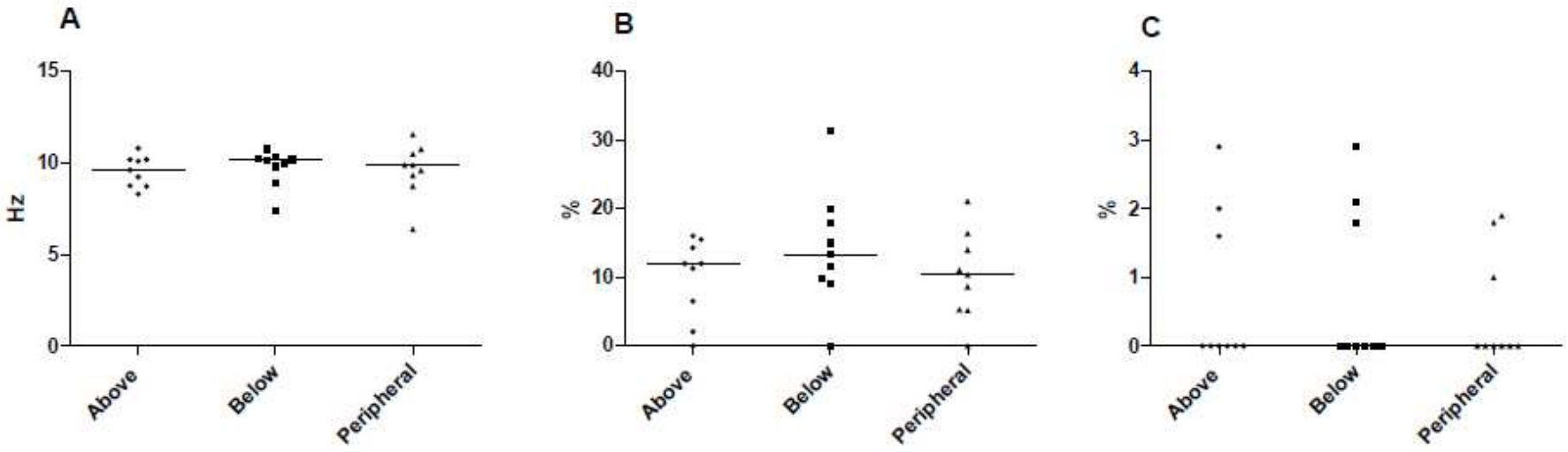

Figure 5.3: Ciliary beat frequency (A), dyskinesia index (B) and immotility index (C) of respiratory epithelium above and below the airway anastomosis and the peripheral airway of paediatric non-suppurative lung disease lung transplant recipients

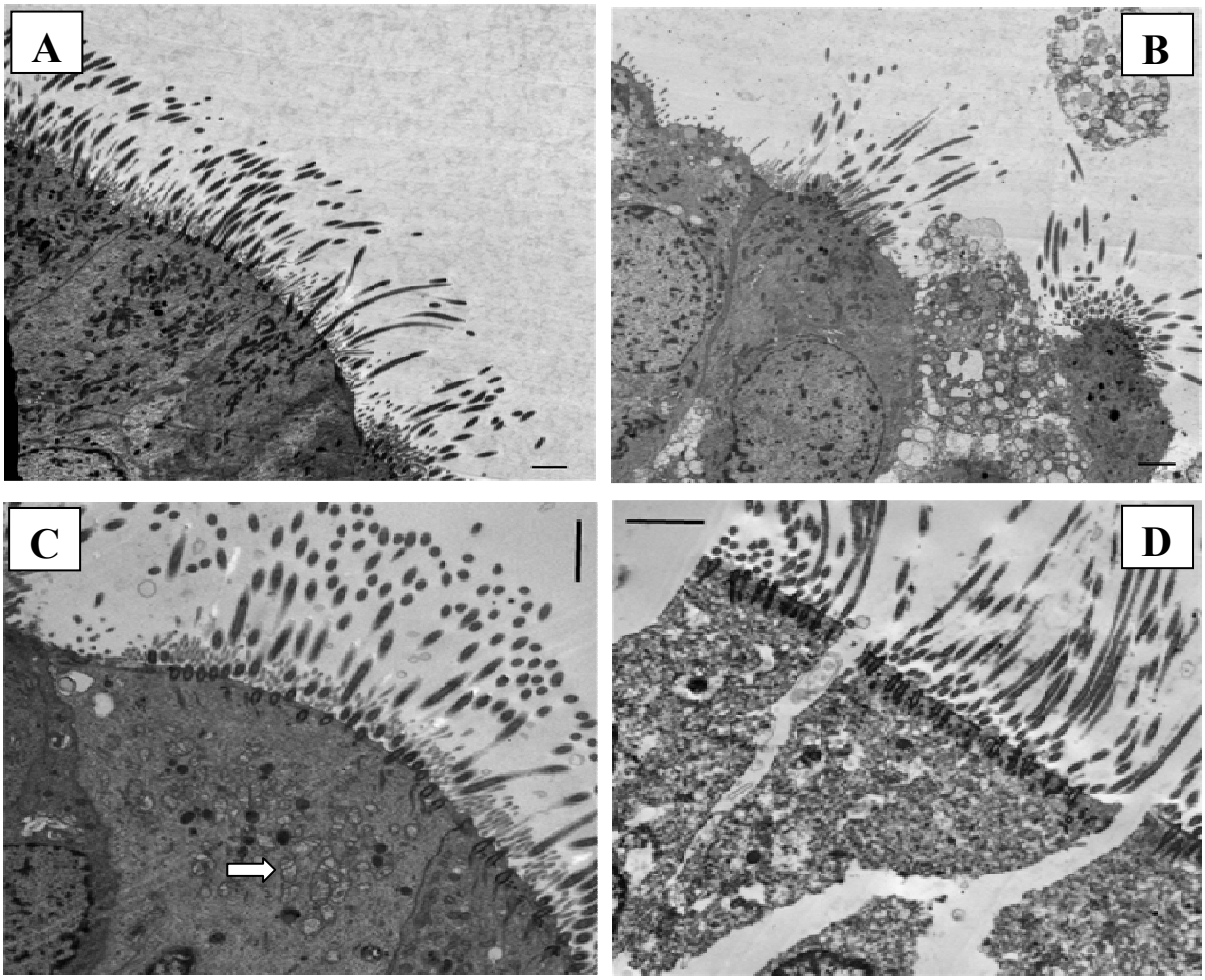

Figure 5.4: Shows representative transmission electron microscopy images of normal healthy ciliated respiratory epithelium (**A**), epithelium showing loss of cilia, projecting cell and disruption of epithelial tight junction with separation of cells (**B**), ciliated cell with disrupted and swollen mitochondria [arrow] (**C**) and dead cells (**D**). Internal scale bar = 2μm.

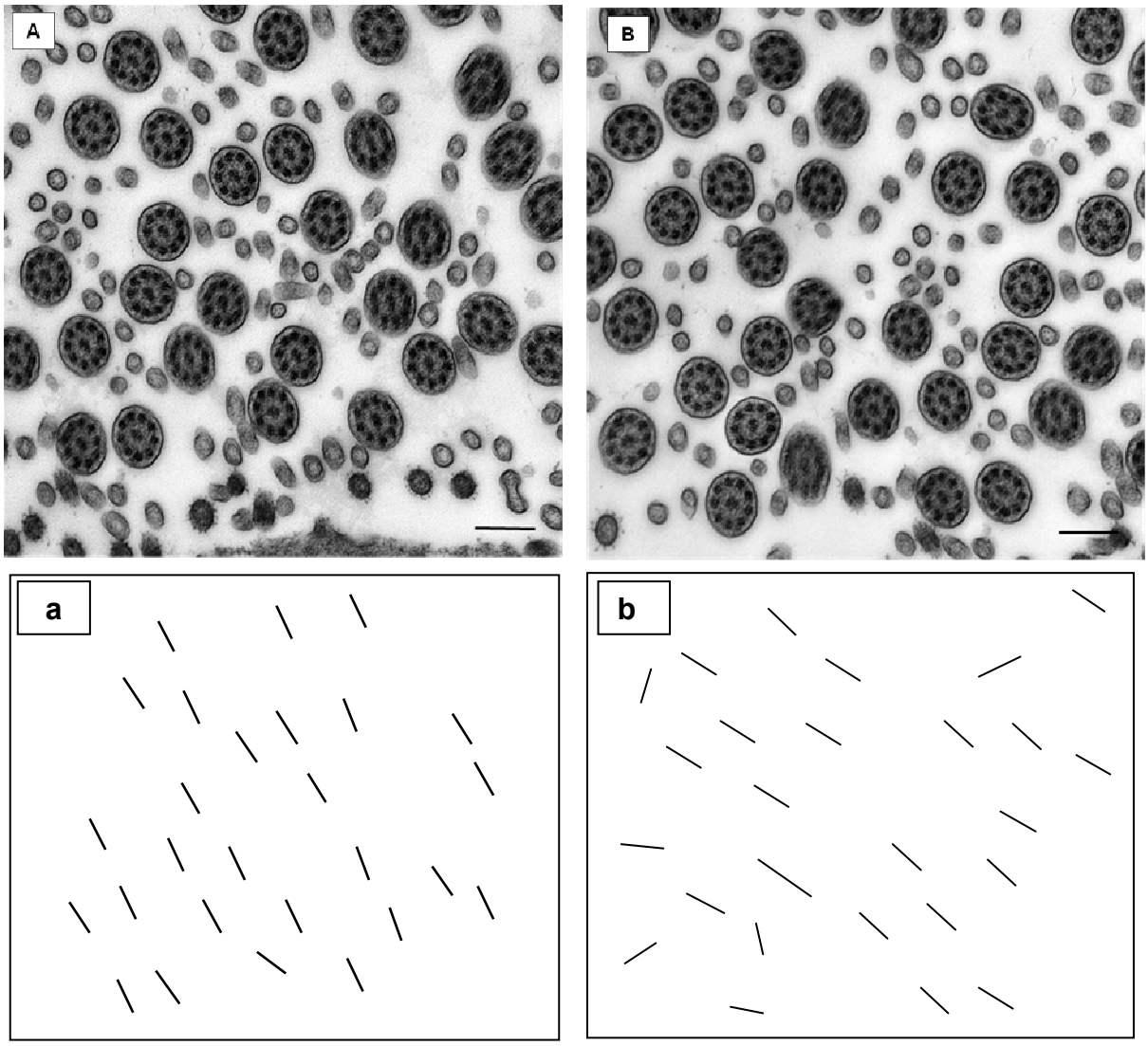

Figure 5.5: Cross sectional image of cilia originating from a normal healthy ciliated cell (**A**) showing normal ciliary orientation [9.48^0 in this example] and a ciliated cell (**B**) showing ciliary disorientation [35.12^0 in this example]. **a & b**–distribution of lines drawn parallel to the central pair of microtubules of cilia originating from a single ciliated cell. Internal scale bar = 250nm

5.5 Discussion

There are three novel findings in this study. Firstly, these data provide evidence of significant ciliary dysfunction in the lower airway of paediatric Cystic Fibrosis patients. Significant abnormalities of ciliary beat frequency and beat pattern were observed in the native airway compared to the airway of the transplanted lungs in paediatric Cystic Fibrosis lung transplant recipients. In contrast, the ciliary function of the native airway of paediatric non-suppurative lung disease lung transplant recipients was comparable to that of the transplanted lungs. Secondly, data from this study suggests that there is no difference in the length and beat frequency of cilia from peripheral airways compared to that from central airways of the transplanted lungs. Thirdly, this study provides evidence that profound epithelial ultrastructural abnormalities persist in the epithelium below the airway anastomosis, for several months post transplantation, in both Cystic Fibrosis and non-suppurative lung disease paediatric lung transplant recipients.

This is the first study that assessed ciliary function and epithelial ultrastructure in paediatric lung transplant recipients. Previous authors(152,155-157) studied ciliary beat frequency in adult lung transplant recipients and no difference in ciliary beat frequency between the native and transplanted bronchi were reported in all except one study.(157) Heterogeneity in the subject characteristics, indication for transplant, type of transplant and methodology used for sample collection and analysis of ciliary beat frequency makes direct comparison with our study results difficult. This study is unique in that, in addition to measurement of ciliary beat frequency, ciliary beat pattern was also determined as a

measure of ciliary function. This was made possible by the recent advent of high-resolution digital high-speed video imaging(41) that allowed me to assess the precise beat pattern of cilia, by viewing the ciliary beat cycle frame by frame in different planes. Using this method, it has previously been shown that cilia in certain conditions may have a normal beat frequency despite markedly abnormal beat pattern(103) and normal ciliary beat frequency does not necessarily equate to normal ciliary function.

There is a paucity of studies that assessed the function of cilia in the lower airways of Cystic Fibrosis patients. The finding in this study of significantly reduced beat frequency and increased ciliary dyskinesia in the epithelium of native airway in the Cystic Fibrosis transplant recipients is in contrast to the Read et al study(155) that showed a higher median (IQR) ciliary beat frequency of 10.8 (8.8 – 11.1) Hz in the native airway epithelium of the six adults with Cystic Fibrosis they studied. However, Read et al did not study the ciliary beat pattern and used the photometric technique rather than the high speed digital video analysis technique to assess ciliary beat frequency. Though chronic inflammation, which is often present in the native Cystic Fibrosis airway epithelium, is known to cause ciliary dysfunction and ultrastructural defects,(179) the precise mechanism(s) underlying ciliary dysfunction in the native Cystic Fibrosis epithelium remains to be determined. There has been a suggestion that compared to cilia in central airways, cilia in peripheral airways are shorter(225,226) and beat at a slower frequency.(42,63) The results of this study contradicts this suggestion and is in agreement to the study by Yager et al(64) who showed no difference in ciliary beat frequency of epithelium from different levels of the tracheobronchial tree.

This study also quantified the ultrastructural abnormalities of the respiratory epithelium of the native airway and transplanted lungs. Limited animal and human studies have examined epithelial ultrastructure following lung transplantation. In a canine autotransplant model, Marelli et al demonstrated ciliary depletion distal to the anastomosis.(149) Shankar et al studied a heterogeneous group of three heart-lung and one single-lung transplant recipients and found a higher proportion of ultrastructural abnormalities proximal to the airway anastomosis compared to distal, whilst there was no difference in the proportion of ciliated cells and dead cells between the two sites.(153) This is in contrast to the findings in this study of profound loss of epithelial integrity and striking ultrastructural abnormalities of the epithelium below the airway anastomosis, observed several months post transplantation. These abnormalities are likely to be multifactorial in causation. Injury during organ harvesting, allograft preservation, reperfusion and acute cellular rejection may cause epithelial damage. Although not studied in detail, devascularisation and lymphatic interruption due to transplantation, medications such as corticosteroids that may impair wound healing and aspiration into airways due to gastro oesophageal reflux(227) have also been implicated in epithelial damage following lung transplantation.

The marked epithelial ultrastructural abnormalities observed below the airway anastomosis in this study are very likely to have important functional consequences. Firstly, these may result in quantitative and qualitative alterations in mucus and periciliary fluid which may lead to impaired mucociliary clearance despite a normal ciliary beat frequency. More importantly, these epithelial abnormalities may increase the

risk of allograft colonisation by pathogenic organisms. Indeed, bacteria such as *Pseudomonas* have been shown to adhere preferentially to injured, disrupted and regenerating areas of airway epithelium(228,229) and colonise the allograft in both Cystic Fibrosis(223,230,231) and non Cystic Fibrosis(230,231) lung transplant recipients. Though not convincingly robust, a growing body of evidence points towards the potential role of allograft colonisation with microbes such as *Pseudomonas*(223,230,231) and *Aspergillus*(224) in the development of bronchiolitis obliterans syndrome (BOS).

5.6 Conclusion

In summary, this study provides evidence for ciliary dysfunction in the native airway of paediatric Cystic Fibrosis lung transplant recipients and marked epithelial damage below the airway anastomosis in both Cystic Fibrosis and non-suppurative lung disease paediatric lung transplant recipients.

The main limitation of this study is its cross-sectional design and therefore the within subject repeatability and longitudinal changes in ciliary function and epithelial ultrastructure need to be further studied. Furthermore there is no data on healthy control children to compare this study results. Though previous studies(149,154) suggested that the epithelial ultrastructural abnormalities following lung transplantation may recover as early as 3-4 months post transplant, this study results are not consistent with these observations and only long term prospective studies will determine the time course of allograft epithelial ultrastructural changes post transplantation. Unraveling the cause(s) of ciliary dysfunction in the native Cystic Fibrosis airway epithelium, epithelial

ultrastructural abnormalities below the airway anastomosis and its potential effects on mucus and periciliary fluid pose a further challenge for future studies. The observation in animal models that preservation of peribronchial tissue during lung transplantation might potentially reduce the abnormalities in mucus rheology and airway epithelium is of interest.(148) Future studies could also assess the effect of improved surgical techniques aimed at minimising effects of denervation, devascularisation and lymphatic obstruction, on longitudinal changes in ciliary function and epithelial ultrastructure following lung transplantation. In spite of these limitations, the measures of ciliary function and epithelial cell morphology are highly repeatable and the magnitude of epithelial ultrastructural abnormalities seen below the airway anastomosis is striking, and is very likely to be clinically important.

CHAPTER 6

General Discussion

6.1 Summary of the thesis

The aim of this thesis was to examine the ultrastructure of the respiratory epithelium and function of cilia (in terms of ciliary beat frequency and beat pattern) in two groups of patients; adults with varying grades of asthma severity and children who have had lung transplantation (for Cystic Fibrosis and non-suppurative lung disease). An additional objective was to study the response of the respiratory epithelium (in terms of cytokine and chemokine release) of patients with atopic severe asthma to a common aeroallergen (*Dermatophagoides Pteronyssinus*) and a common bacterial pathogen (*Streptococcus pneumoniae*).

The hypothesis in the study of adult patients with asthma was that there is no difference in the ciliary function (in terms of ciliary beat frequency and beat pattern) and epithelial ultrastructure of the lower airways, between patients with varying grades of asthma severity and between asthma patients and healthy subjects. The data generated from my study show that the ciliary dysfunction and ultrastructural abnormalities are closely related to asthma severity, in adult asthmatics. It has been demonstrated that, compared to healthy controls, ciliary dysfunction is a feature of moderate to severe asthma and profound ultrastructural abnormalities are restricted to patients with severe asthma. A new phenotype of marked ciliary dysfunction and striking epithelial ultrastructural abnormalities including ciliary disorientation in adult patients with severe asthma has been unraveled.

An additional hypothesis in the study of adult patients with atopic severe asthma was that there is no difference in the response (in terms of cytokine and chemokine release) to *Dermatophagoides Pteronyssinus allergen 1* and *Streptococcus pneumoniae*, between primary bronchial epithelial cells of patients with atopic severe asthma and healthy controls. My study results have shown that whilst primary bronchial epithelial cells of patients with atopic severe asthma and healthy controls are capable of releasing chemokines and cytokines in response to *Dermatophagoides pteronyssinus* allergen 1 (Der p 1) and *Streptococcus pneumoniae* in a dose and time dependent manner, there is a striking differential response of the asthmatic airway epithelium compared to that of healthy subjects. Whilst the chemokine release from primary bronchial epithelial cells of patients with atopic severe asthma in response to Der p 1 was significantly higher compared to that of healthy subjects, an exactly opposite trend was demonstrated on studying the response to *Streptococcus pneumoniae*. The magnitude of release of chemokines in response to *Streptococcus pneumoniae* by primary bronchial epithelial cells of healthy subjects was significantly higher compared to that of patients with atopic severe asthma.

The hypothesis in the study of children who have had lung transplantation (for Cystic Fibrosis and non-suppurative lung disease) was that there is no difference in the ciliary function (in terms of ciliary beat frequency and beat pattern) and epithelial ultrastructure between the epithelium above and below the airway anastomosis and that of the peripheral airway, in paediatric lung transplant recipients. An additional hypothesis was that there is no difference in the length and beat frequency between the cilia in the central

and peripheral airways in paediatric lung transplant recipients. The study results revealed significantly reduced ciliary beat frequency and increased ciliary dyskinesia in the native epithelium in the Cystic Fibrosis group. There was no difference in ciliary function between the native airway epithelium and the epithelium of the transplanted lungs in the non suppurative lung disease group. The data from my study has also shown that there is no significant difference in the length and beat frequency of cilia from peripheral airways compared to that from central airways, in paediatric lung transplant recipients. Of particular interest, this study showed striking epithelial ultrastructural abnormalities in the epithelium immediately below the airway anastomosis in the transplanted lungs, in both the Cystic Fibrosis group and the non suppurative lung disease group. These changes were shown to persist many months after lung transplantation.

6.2 Conclusions and future research directions

Though the airway epithelium has been recognised to play a central role in asthma pathophysiology, the novel findings of the phenotype of profound ciliary dysfunction and marked epithelial ultrastructural abnormalities in patients with severe asthma extend our current paradigm of severe asthma. This phenotype of secondary ciliary dyskinesia is likely to result in reduced mucociliary clearance and therefore increased susceptibility to infection. These abnormalities may contribute to the susceptibility of severe asthma patients to frequent and severe exacerbations triggered by respiratory tract infections, in particular viral infections. Asthma has been recognised as a risk factor for bacterial infections such as invasive pneumococcal disease (181) and also as a predisposing factor

in the in the development of bronchiectasis. (182,183). The findings in this thesis may help explain the increased susceptibility of patients with severe asthma to infections. Given the marked ciliary dysfunction seen in patients with severe asthma, it is highly likely that these patients have impaired mucociliary clearance and this may make the airway epithelium susceptible to prolonged and intense exposure to inhaled pathogens and aeroallergens. The data in this thesis suggest that the response of the epithelium of patients with atopic severe asthma to bacterial pathogens such as *Streptococcus pneumoniae* may be altered compared to that of healthy subjects. It may be argued that such altered response may have functional implications. For instance, the significantly reduced release of a powerful neutrophil cehmoattractant such as CXCL8 (IL-8) by asthmatic airway epithelium may contribute to delayed clearance of pathogenic organisms from the epithelium, an effect that may be additive to the impaired mucociliary clearance seen in these patients.

These findings provoke several important questions that need to be addressed in future research. The epithelial abnormalities seen in patients with asthma may be multifactorial in nature and the role of intrinsic abnormalities of the asthmatic airway epithelium, effects of chronic inflammation, chronic infection, aberrant repair mechanisms and effect of medications need to be studied in detail. It is unknown if the ciliary function and epithelial ultrastructure of small airways of patients with asthma are normal or not and this needs to be studied. Also, it remains to be determined if similar ciliary dysfunction and epithelial changes are present in other chronic respiratory conditions such as chronic obstructive pulmonary disease (COPD). It would be of great interest to see if the ciliary

dysfunction and/or epithelial ultrastructural changes such as ciliary disorientation can be reversed by potential therapeutic strategies. For instance, it has been shown in in-vitro models that macrolide antibiotics such as roxithromycin may increase ciliary beat frequency.(68,232) There is also a suggestion that prolonged treatment with certain antibiotics may reverse ciliary disorientation.(233) Such observations have important potential therapeutic implications and need to be robustly studied in patients. In the light of recent observations of immunomodulatory effect of *Streptococcus pneumoniae* in mouse models of allergic asthma (200,201), the finding of differential chemokine response of primary bronchial epithelial cells from atopic severe asthma patients to Der p 1 and *Streptococcus pneumoniae* compared to that of healthy controls, generates great interest. It would be of interest to investigate if prior exposure of the asthmatic airway epithelium to *Streptococcus pneumoniae* would lead to an attenuated response to Der p 1. If such a response is demonstrated in robust studies in animal models or humans, that may have potential clinical implications. The mechanism(s) underlying the release of cytokine and chemokine release from airway epithelial cells in response to the challenges used in my studies and the effect of an aberrant chemokine mileu on epithelial injury-repair mechanisms need further investigation. Also future studies could assess if the response of airway epithelial cells from non atopic patients with severe asthma is different to that of those with atopic asthma.

The findings in this thesis of persistent and marked epithelial ultrastrctural abnormalities in the transplanted lungs below the airway anastomosis may help explain the increased susceptibility of lung transplant recipients to microbial colonisation of allograft airway

and infections – a major cause of post lung transplant mortality and a factor that is increasingly recognised to be a key player in the development of chronic allograft rejection, a process known as the Bronchiolitis Obliterans Syndrome (BOS). Moreover, these epithelial abnormalities may have an effect on the qualitative and quantitative properties of the periciliary fluid and airway mucus that may help explain the impairment of mucociliary clearance in the transplanted lungs, demonstrated in previous studies in animal models and humans. Though there may not be a significant difference in the ciliary function between the native airway and the transplanted lungs, as I have demonstrated in paediatric non suppurative lung transplant recipients, the potential effects of the marked allograft epithelial abnormalities below the airway anastomosis on airway surface liquid properties, can not be overlooked. The data in this thesis generate several important questions that need to be addressed in future research. Unraveling the cause(s) of the epithelial ultrastructural abnormalities below the airway anastomosis and its potential effects on mucus and periciliary fluid pose a major challenge for future studies. Studies targeting the effect of medications commonly used post lung transplantation, such as immunosuppressants, on the allograft epithelial changes may provide information that may guide the optimal immunomodulatory therapy post lung transplantation with the least harmful effects on allograft epithelial healing. There is a suggestion in an animal model study that preservation of peribronchial tissue during lung transplantation might potentially reduce the abnormalities in mucus rheology and airway epithelium.(148) This is of interest and future studies could assess the effect of improved surgical techniques aimed at minimising effects of denervation, devascularisation and lymphatic obstruction, on allograft epithelial ultrastructure following lung

transplantation. Long term prospective studies may help determine the time course of allograft epithelial ultrastructural changes following lung transplantation and such information may guide therapeutic decisions such as the duration of prophylactic antimicrobials.

Appendix 1

Video legends

Video1: Sideways view of a ciliated epithelial edge from a healthy control subject showing normal ciliary beat pattern.

Video 2: Sideways view of a ciliated epithelial edge from a patient with severe asthma, showing dyskinetic beat pattern.

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Publications and presentations originating from this thesis

Published papers

Thomas B, Rutman A, Hirst RA, Haldar P, Wardlaw AJ, Bankart J, Brightling CE, O'Callaghan C. Dysfunctional cilia and ultrastructural abnormalities are features of severe asthma. Journal of Allergy and Clinical Immunology 2010; 126: 772-779.

Paper currently being peer reviewed

Thomas B, Aurora P, Spencer H, Elliott M, Rutman A, Hirst R A, O'Callaghan C. Disrupted ciliary epithelium several months after pediatric lung transplantation. (Thorax)

Published abstracts/Presentations

B Thomas, RA Hirst, G Williams, J Bankart, P Haldar, AJ Wardlaw, CE Brightling, C O'Callaghan. Chemokine release in response to *Streptococcus pneumoniae*, by primary bronchial epithelial cells of patients with severe asthma and healthy controls. Eur Respir J 2009; 34: 145s

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