Thesis

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Cilia, nitric oxide and non-typeable *Haemophilus influenzae* biofilm infection

by

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ABSTRACT

Introduction

Patients with Primary Ciliary Dyskinesia (PCD) have abnormal ciliary function leading to impaired mucociliary clearance. They suffer from chronic and recurrent bacterial sinopulmonary infections. Notably patients with PCD, almost universally, have low levels of nasal nitric oxide (NO). Bacterial biofilms are increasingly recognised as being important in chronic respiratory tract infections.

Hypothesis

Impaired ciliary function predisposes PCD patients to biofilm-associated infections and this may be exacerbated by the low NO levels seen.

Overview of methods

An air-liquid interface (ALI) cultured respiratory epithelial cell/*Haemophilus influenzae* co-culture model using primary epithelial cells from patients with PCD and those without PCD was developed and characterized. These two groups of patients were used in order to study respiratory epithelial cell layers with impaired and functioning cilia respectively. The cell layers were co-cultured with non-typeable *Haemophilus influenzae* (NTHi) over a time course (three days) and at a multiplicity of infection (100) that allowed biofilm development whilst maintaining cell viability, so that the role of ciliary function on NTHi biofilm development could be investigated.

In addition, the ability of epithelial cells from patients with and without PCD to biosynthesize NO was compared in order to investigate firstly its potential role as a confounding variable in the ALI/bacterial co-culture cell model; and secondly to investigate the mechanism underlying the extremely low levels of NO observed in, and used in the diagnosis of, PCD patients. Finally this project investigated the effect of exogenous NO on NTHI biofilm development.

Main results

Prior to co-culture with NTHi, the only significant difference between the PCD and non-PCD ALI cultured epithelial cell layers was in ciliary beat frequency, where all but one of the PCD group had static cilia. Levels of the cationic antimicrobial peptide, LL-37, in the apical supernatant over the PCD cell layers were lower than the non-PCD group but not significantly so. Apart from this cell layers from both groups were found to have similar surface coverage of cilia, trans-epithelial electrical resistance, numbers of

mucus-producing cells, basolateral supernatant cytokine concentrations and, in particular, apical NO concentrations.

Following co-culture with NTHi, biofilm development was found to be significantly greater on PCD as compared to non-PCD epithelial cell layers. This was evidenced by twice the bacterial biofilm colony forming units (CFU) (mean (±SEM), $3.8x10^6$ CFUs ($\pm 7.5x10^5$) vs. $2.0x10^6$ CFUs ($\pm 4.8x10^5$), $P<0.05$) and over four times the volume of NTHi as seen by fluorescence *in situ* hybridization and confocal laser scanning microscopy (712 (±152) μm^3 vs. 159 (±19) μm^3, $P<0.001$) and were corroborated by SEM images.

In vivo NO concentrations, as measured by a chemiluminescent NO analyser, were found to be reduced throughout the respiratory tract in PCD patients as compared to healthy controls. However *in vitro*, constitutive NO biosynthesis from PCD and non-PCD primary ALI cultured respiratory epithelial cell layers was similar and, furthermore, following stimulation both groups demonstrated similar 2-3 fold increases.

Finally the exogenous addition of NO was found to reduce NTHi biofilms and to act synergistically with cefotaxime to allow concentrations of antibiotic that alone had little anti-biofilm affect, to significantly reduce bacterial biofilm CFUs.

Conclusion

These data support the study hypothesis that PCD patients are at increased risk of developing biofilm-associated infections due to impaired ciliary function. Furthermore, despite the low levels of NO observed in patents with PCD, the airway epithelial cells in PCD appear to synthesize NO normally and the mechanism for this phenomenon therefore remains elusive.

There is an unmet need for novel anti-biofilm therapies. These findings suggest a therapeutic rationale for developing NO-based adjunctive anti-biofilm therapies for PCD and other chronic suppurative respiratory conditions that may be associated with bacterial biofilm infections.

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DECLARATION OF AUTHORSHIP

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Walker WT, Liew A, Harris A, Cole J, Lucas JS. Upper and lower airway nitric oxide levels in primary ciliary dyskinesia, cystic fibrosis and asthma. *Respiratory Medicine* January 2013; 107(3):380-386 DOI:10.1016/j.rmed.2012.11.021

Walker WT, Jackson CL, Lackie PM, Hogg C, Lucas JS. Nitric Oxide in Primary Ciliary Dyskinesia. *European Respiratory Journal* March 2012; 40(4):1024-32

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I dedicate this thesis to them.

Abbreviations

ALI	Air liquid interface
ASL	Air surface liquid
BEGM	Bronchial epithelial growth medium
BHI	Brain heart infusion
β-NAD	β-nicotinamide adenine dinucleotide
BSA	Bovine serum albumin
$Calv_{NO}$	Alveolar nitric oxide concentration
CAMPs	Cationic antimicrobial peptides
CBF	Ciliary beat frequency
CBP	Ciliary beat pattern
CDC	Center for Disease Control and Prevention
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CLSM	Confocal laser-scanning microscope
CO_2	Carbon dioxide
CV	Crystal violet
DAF-fm Diacetate	4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
EPS	Extracellular polymeric substance
FeNO	Fractional exhaled nitric oxide
FEV_1	Forced expiratory volume in 1 second
FFT	Fast Fourier transform
FGFb	Fibroblast growth factor basic
FISH	Fluorescence *in situ* hybridisation
FoV	Field of view
fps	Frames per second
G-CSF	Granulocyte colony stimulating factor
GMA	Glycol methacrylate acrylic
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Guanosine monophosphate
16HBE	Adenovirus SV40 transformed epithelial cell line derived from human bronchial epithelial cells
hEGF	human epidermal growth factor

Hib	*Haemophilus influenzae* type b
HSVM	High speed video microscopy
IDA	Inner dynein arm
IFN-γ	Interferon gamma
IL	Interleukin
IL-1Ra	Interleukin 1 receptor antagonist
iNOS	Inducible nitric oxide synthase
$J'aw_{NO}$	Bronchial nitric oxide flux
LCC	Liquid covered culture
LOS	Lipooligosaccharides
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein 1
MEM	Minimum essential medium
MOI	Multiplicity of infection
MIC	Minimum inhibitor concentration
MIP	Macrophage inflammatory protein
NADP	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NLR	NOD like receptor
nNO	Nasal nitric oxide
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NTHi	Non-typeable *Haemophilus influenzae*
OD	Optical density
ODA	Outer dynein arm
PAMPs	Protein associated molecular patterns
PAS	Periodic acid-Schiff
PBB	Protracted bacterial bronchitis
PBS	Phosphate buffer solution
PCD	Primary ciliary dyskinesia
PCF	Periciliary fluid
PCR	Polymerase chain reaction
PI	Propidium iodide
REC	Regional ethics committee
rcf	Relative centrifugal force
sBHI	Supplemented brain heart infusion
SCD	Secondary ciliary defect
SDS	Sodium dodecyl sulfate

SEM	Scanning electron microscopy
sMEM	Supplemented minimum essential medium
SNP	Sodium nitroprusside
TBS	Tris buffered saline
TEM	Transmission electron microscopy
TER	Trans-epithelial electrical resistance
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
UHS	University Hospital Southampton NHS Foundation Trust
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Outline

First, before focusing on PCD, an overview of the innate immunity of the respiratory tract was considered, since impairment of ciliary function forms a vital part of the innate host defence system. Second, the literature on bacterial biofilms was reviewed, with a particular focus on non-typeable *Haemophilus influenzae* (NTHi) and its role in respiratory tract infections. Third, different models of the host respiratory tract were reviewed. Fourth, the role of NO within the respiratory tract, with particular focus on why NO levels might be extremely low in PCD, was discussed. Finally the project and the study objectives are discussed with special emphasis on the wider clinical relevance of this work.

1.2 Innate immunity of the respiratory tract

1.2.1 Introduction

We inhale thousands of litres of air a day and, in doing so, expose our lungs to a significant number of pathogens, allergens and other particulate matter (Wanner, Salathe et al. 1996). Despite this lower respiratory tract infections are relatively rare in healthy individuals, which is a testament to the pulmonary host defences, particularly given the fact that the adult lungs have a surface area of approximately 70m^2 (Notter 2000).

The initial defence of the respiratory tract against infection is through the anatomical barriers, mucosal cells and effector cells of the innate immune system. The first line is comprised of the anatomical barriers of the upper airway, such as the nasal turbinates and nasal hairs, which filter out most particulate matter larger than 5μm. Small particles and organisms that breach these defences and enter the lower respiratory tract will generally land on the respiratory epithelia of the conducting airways.

1.2.2 Respiratory epithelia

The respiratory epithelia had long been considered to be a passive, physical barrier that acted to protect the underlying tissue. However, in addition to the physical barrier it provides, it is now clear that epithelial cells play a more active role in providing immunological protection to the airway.

1.2.2.1 Physical barrier

The respiratory epithelium is a highly organized polarized, endoderm-derived cell layer that protects and moistens the airways. The focus here will be on the ciliated, pseudostratified columnar epithelial cells that line the large airways, however more distally the epithelium becomes cuboidal in the smaller branching airways before becoming squamous at the alveoli (Diamond, Legarda et al. 2000).

1.2.2.1.1 Ciliated pseudostratified columnar epithelia

There are three cell types present in the epithelial layer in the larger airways; columnar cells that are often ciliated and mucus producing cells, both of which have an apical surface in contact with the airway lumen, and basal cells, which do not have contact with the airway and have the ability to differentiate. These form a single layer of cells, with all cells having contact with the basement membrane. However, the cell nuclei are not aligned in the same plane and appear as several layers of cells, hence the term pseudostratified (Figure 1). The cells are polarized with specific apical and basolateral surfaces embedded with different receptors and ion channels. Mucus is produced by sub-mucosal glands in the large, cartilaginous airways and by columnar secretory Clara cells in the smaller, distal airways. In the trachea there are approximately 6500 mucus cells/mm^2 of surface area (Ellefsen and Tos 1972).

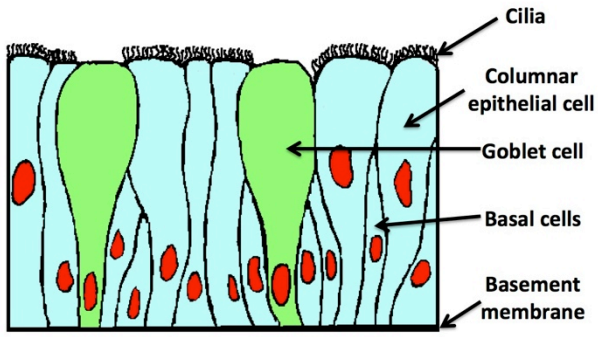

Figure 1 *– Diagram of the respiratory epithelia showing pseudostratified columnar, ciliated epithelium with mucous secreting goblet cells*

1.2.2.1.2 Intercellular junctions

Key structures that maintain the physical barrier function of the respiratory epithelia are the intercellular junctions; these include among others tight junctions, adherens junctions and desmosomes (Roche, Montefort et al. 1993). Most important of these are tight junctions, found near the apical surface of the cells where they form a continuous band, adhering neighbouring cells together. In doing so they inhibit the passage of water, molecules and ions through the paracellular space facilitating cellular control over their movement through the epithelial cells. They also have an important role in maintaining the apical/basolateral polarity of the cell surface by preventing movement of membrane proteins between the two (Aijaz, Balda et al. 2006). In addition, they hinder access of pathogens to basolateral epithelial membrane receptors (Bergelson 2009).

Tight junctions are composed of branching networks of sealing strands that are embedded in the plasma membranes of both adjoining cells joined together via extracellular domains (Aijaz, Balda et al. 2006). While there are over 40 proteins identified within tight junctions the most important of these are the integral membrane proteins claudins and occludins (Yamazaki, Okawa et al. 2008; Kojima, Go et al. 2013), which interact intracellularly with cytoplasmic proteins including zonula occludens proteins. These in turn connect to the actin cytoskeleton within the cell (Furuse 2010). Trans-epithelial resistance (TER) is a widely utilised measurement of tight junction function. This is a measure of ionic permeability of the epithelial layer.

Tight junction function has been shown to vary with respiratory pathologies, for example in severe asthma it has been demonstrated that tight junction protein expression and TER are significantly reduced, which may allow the passage of allergens and pathogens into the airway tissues (Xiao, Puddicombe et al. 2011). Wide ranges of exposures affect the function of tight junctions (Meyle, Gultig et al. 1999; Kim, Sajjan et al. 2005). For example viruses have been demonstrated to both increase, in the case of respiratory syncytial virus (Masaki, Kojima et al. 2011), and decrease, in the case of rhinovirus (Yeo and Jang 2010), expression of tight junction molecules. The antimicrobial azithromycin has been shown to have a positive effect on tight junction protein expression and TER (Asgrimsson, Gudjonsson et al. 2006)as has NTHi endotoxin in the initial 24 hours following infection (Khair, Devalia et al. 1994).

1.2.2.2 Immunological components of respiratory epithelia defence

There are a number of immunological components that are produced and modulated by the respiratory epithelia and act to offer additional protection to the airway. Bacteria, or bacterial components, that overcome the anatomical defences of the upper airway (1.2.1) and the mucociliary escalator (1.2.3), can interact with the epithelial cell apical surface. Through an array of apical receptors epithelial cells can recognise and distinguish pathogenic molecular determinants, by their surface expression of protein associated molecular patterns (PAMPs). This leads to stimulation of appropriate inflammatory responses via cytokine and chemokine pathways. In addition, epithelial cells synthesise a number of molecules, including cationic antimicrobial peptides, to further protect the airway.

1.2.2.2.1 Epithelia cell receptors

The immune response is dependent on being able to distinguish between self and non-self. The main receptors of the innate immune system on the epithelia cell surface that are tasked with this are toll-like receptors (TLR) and 10 human TLRs have now been reported (Akira and Takeda 2004). They each recognize particular pathogens, groups of pathogens and microbial components, although there is some overlap between them. For example for TLR2 this includes lipoproteins, such as lipoteicoic acid from Gram-positive bacteria, but also modulins from *Pseudomonas aeruginosa* flagella, a Gram-negative bacteria (Adamo, Sokol et al. 2004; Akira and Takeda 2004). TLR4 recognises Gram-negative bacteria components such as LPS and has been highlighted for its important role in recognising and initiating the innate immune response to *Haemophilus influenzae* (Wang, Moser et al. 2002), however it also recognises *P. aeruginosa*. In addition to these cell surface receptors there are also intracellular nucleotide-binding oligomerisation domain receptors (NOD-like receptors, NLRs), which recognise pathogens that have breached the cell surface, initiating appropriate inflammatory signalling pathways (Inohara, Chamaillard et al. 2005).

1.2.2.2.2 Cytokines

Cytokines are soluble polypeptides and glycoproteins, secreted by cells, which act as signalling molecules. They are generally present in low levels in the circulation but can be dramatically up-regulated in disease conditions. They can be highly potent, due to cascades creating amplification loops, and bind to specific receptors (Stadnyk 1994). They have a wide range of roles including cell growth and differentiation, stimulation,

propagation and inhibition of immune responses and chemo-attraction of leucocytes (Stadnyk 1994; Gomez and Prince 2008)

Cytokine biosynthesis by epithelial cells is up-regulated both by other cytokines and by pathogens. Recognition of the bacterial components by the epithelial receptors leads to the up-regulation of an array of pro-inflammatory cytokines including IL-8, IL-6, IL-1β, GM-CSF, granulocyte colony stimulating factor (G-CSF) and transforming growth factor α and β (Gomez and Prince 2008). These pro-inflammatory cytokines, via intermediaries, ultimately lead to gene induction mediated by rapid acting primary transcription factors such as nuclear factor-κB (NF-κB) which in turn leads to further propagation of the immune and inflammatory cellular response (Hayden and Ghosh 2008). While the pro-inflammatory response of respiratory epithelial cells to pathogenic microorganisms is essential to ensure their clearance from the airway, its persistence, if left unchecked, can be significantly detrimental. There are a number of important anti-inflammatory cytokines, such as IL-10, which act to inhibit production of pro-inflammatory cytokines and antigen presentation while increasing production of IL-1 receptor antagonist (IL-1Ra), which competes with IL-1 for receptor binding (Mosser and Zhang 2008). The balance between efficient clearance of pathogens and maintaining the key role of the lungs, gas exchange, is critical.

As our understanding of the role of cytokines in the airway has expanded it has led to the development of cytokine-specific therapeutic agents for use in inflammatory disease, in particular asthma, such as mepolizumab (anti IL-5 antibody) and tralokinumab (anti IL-13 antibody) (Flood-Page, Swenson et al. 2007; Pavord, Korn et al. 2012; Piper, Brightling et al. 2013). However there are little data available on the cytokine response of PCD epithelial cells to pathogens to potentially guide development of specific therapeutics for the condition. Furthermore, it remains unclear whether a number of cytokines, known to be produced by immune cells, are also synthesised by epithelial cells.

1.2.2.2.3 Cationic antimicrobial peptides

Cationic antimicrobial peptides (CAMPS) are ancient evolutionary defences that have allowed organisms, such as insects and plants, to flourishes in the face of microbes in the absence of adaptive immune effector cells. Over 2300 CAMPS have been identified to date, in nearly all species (Wang, Li et al. 2009). They are produced by airway epithelial cells and neutrophils and not only have microbial killing effects but also in recruiting cells of the innate immune system (Zasloff 2002).

CAMPs target one of the fundamental differences between the cell membranes of microbes and multicellular organisms. The outermost part of bacterial cell membranes are mainly populated by lipids with negatively charged phospholipid head-groups, as oppose to in multicellular organisms, where this layer has no net charge (Matsuzaki 1999). While there is significant diversity between CAMPs, all are net positively charged and have about 50% hydrophobic residues which allows them to fold into an amphiphilic conformation upon interaction with the bacterial membrane lipid bilayer. There are many hypotheses as to how this affects bacterial viability but, in essence, they disrupt membrane integrity by creating physical holes that allow leakage of cellular content and fatal depolarization of the bacterial cell (Westerhoff, Juretic et al. 1989; Yang, Weiss et al. 2000).

One of the most studied human CAMPs is the cathelicidin LL-37. LL-37 is a 37-residue peptide found at the C-terminus of the human cationic antimicrobial protein 18 (hCAP-18) (Larrick, Hirata et al. 1995; Gudmundsson, Agerberth et al. 1996). It is widely express at mucosal surfaces including the epithelial surface of the conducting airways (Bals, Wang et al. 1998). In addition to its antimicrobial properties, it has a number of immunomodulatory effects, such as chemotactic activation of neutrophils, and has been shown to decrease trans-epithelial permeability and prevent epithelial cell invasion by *P. aeruginosa* (Byfield, Kowalski et al. 2011). Furthermore, LL-37 has been demonstrated to potently inhibit *P. aeruginosa* biofilm formation (Overhage, Campisano et al. 2008).

There is variability in CAMP activity demonstrated in different respiratory pathologies, for example, it has been shown that the periciliary fluid (PCF, 1.2.3.3) of patients with CF has elevated salt concentrations that could inhibit the action of CAMPs (Smith, Travis et al. 1996). Furthermore the high protease concentrations, particular of neutrophil elastase, seen in the CF lung may degrade these anti-microbial peptides (Forde, Humphreys et al. 2014). The reality of the pathogenesis of CF is however clearly much more complex, with subsequent work highlighting the importance of periciliary liquid depletion as oppose to abnormal ion composition (Matsui, Grubb et al. 1998). Interestingly, more recent work by Dif *et al.* demonstrated that CF mice had increased mucus production and MUC5AC expression modulated by cytosolic phospholipase A2a interacting with CFTR independently of chloride channel function (Dif, Wu et al. 2010). While both CF and PCD are thought to be small airway diseases, differences are seen in the distribution of bronchiectasis within the lung between the two conditions. CF patients predominantly are found to have upper lobe bronchiectasis whereas in PCD the middle and lower lobes are more commonly affected. To date, there is no literature on LL-37 in PCD or its effect on NTHi.

1.2.3 Mucociliary clearance

Effective mucociliary clearance is a key component of innate immunity in the respiratory tract. The mucociliary escalator acts to clear mucus, and the pathogens trapped within it, up into the oropharynx from where it is swallowed or expectorated (Figure 2). Estimates of the velocity at which the mucociliary escalator moves mucus through the trachea range between 4-20 mm/min, depending on the technique used to assess it (Sackner, Rosen et al. 1973; Yeates, Aspin et al. 1975).

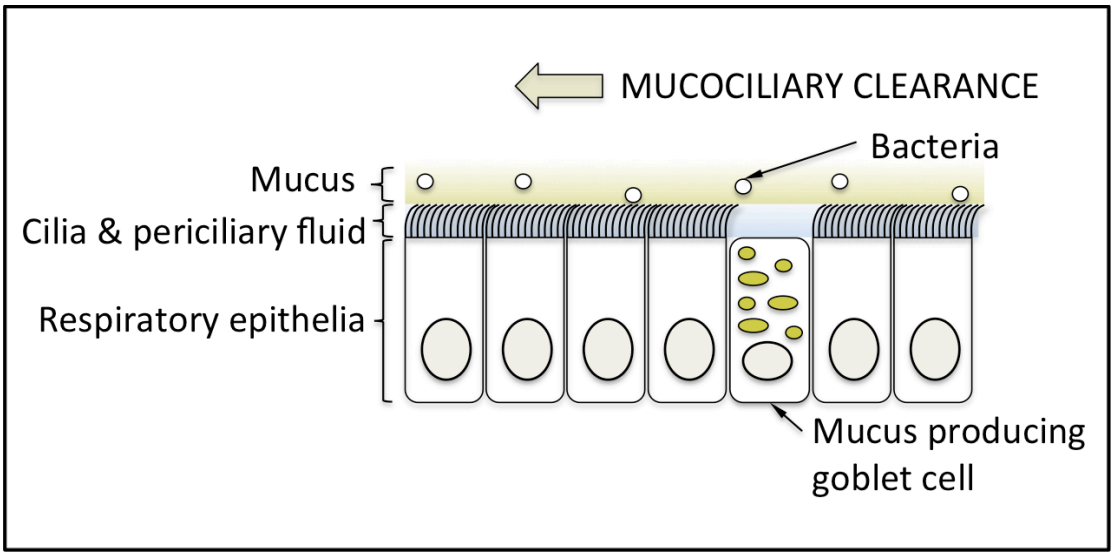

Figure 2 *- Schematic image of mucociliary clearance in the lung. The cilia, which are bathed in periciliary fluid, make coordinated sweeps into the overlaying mucus, directing the mucus and associated particles towards the oropharynx.*

The fundamental constituents of the mucociliary escalator are mucus, cilia, and the PCF layer.

1.2.3.1 Mucus

A layer of mucus, produced by airway secretory cells, covers the respiratory epithelium. This acts both to maintain epithelial moisture and as a further physical and chemical barrier, protecting the epithelial cell layer by trapping inhaled pathogens, allergen and particulates; then transporting them out of the lung via the mucociliary escalator before they are in contact with the epithelial cells. It is an extracellular gel and its main constituents are water and mucins, alongside other glycoproteins, proteoglycans and antimicrobial peptides, covered further detail in 1.2.2.2.3 (Kaliner, Shelhamer et al. 1986).

Mucins are large, highly anionic glycoproteins and the two most strongly expressed in the airway are MUC5AC and MUC5B (Thornton, Rousseau et al. 2008). They are structured in long single chains and form a gel by entanglement in a mesh by cross-linking with adjacent polymers (Thornton, Rousseau et al. 2008). The glycan side chains enable mucins to bind large amounts of water allowing mucus to act as a both a lubricant and liquid reservoir (Knowles and Boucher 2002). Bacterial components, such as flagellin, bind to mucins to facilitate clearance from the lungs by the mucociliary escalator (Arora, Ritchings et al. 1998). Interleukin (IL)-13 is key to MUC5AC expression but it is also known to be up-regulated by lipopolysaccharide (LPS), bacteria, viruses and other pro-inflammatory cytokines, such as IL-1β and TNF-α (Dohrman, Miyata et al. 1998; Kuperman, Huang et al. 2002; Strieter, Belperio et al. 2002; Zhen, Park et al. 2007). Mucins are stored intracellularly in a dehydrated state in granules. When released into the airway they can deplete the PCF and, in doing so, swell significantly, which can lead to airway obstruction (Lai, Wang et al. 2009). In health, mucus is 97% water however this can decrease to 85% in disease leading to viscous mucus that adheres to the airway wall impeding mucociliary clearance (Smart 2005).

One paper has considered the properties of sputum in children with PCD, comparing it to children with cystic fibrosis (CF) (Bush, Payne et al. 2006). CF sputa is generally considered to have increased viscosity due to dehydration secondary to defective cystic fibrosis transmembrane conductance regulator (CFTR) function. However no differences were seen in the biophysical and transport properties of the sputum between the two groups and, interestingly, they found IL-8 levels, a key neutrophil chemokine, to be three times higher in the PCD group (Bush, Payne et al. 2006). Whilst the numbers in the study were small (19 PCD patients & 30 CF patients) and the viscosity of sputa will depend on the clinical picture of the patient at the time of collection, whether stable or during an exacerbation, it suggests that mucus viscosity in PCD may be similar to that of patients with CF.

1.2.3.2 Cilia

From the upper airway to the respiratory bronchioles, the apical surface of the pseudostratified columnar epithelial cells are covered in cilia. In the trachea approximately half the epithelial cells are ciliated and this decreases to about 15% in the fifth generation bronchioles (Serafini and Michaelson 1977). The cilia beat in a coordinated pattern at a frequency of 11 to 20 Hertz (Hz).

1.2.3.2.1 Ciliary ultrastructure

Cilia are hair-like structures, typically 6 μm in length and 250 nm in diameter. Most motile cilia have a "9+2" arrangement with nine peripheral microtubule doublets surrounding a central pair of singlet microtubules (Figure 3). There are typically 200 cilia per cell and they beat in a coordinated planar motion, acting to move mucus and particles across the surface of the epithelial cell layer (Sleigh, Blake et al. 1988; Roomans, Ivanovs et al. 2006).

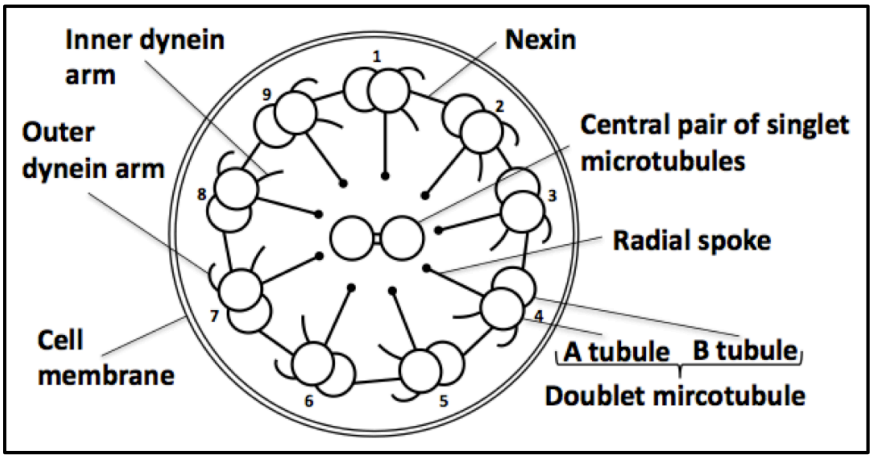

Figure 3 *- Schematic image of transverse section of a respiratory cilial axoneme.*

Microtubules consists of heterodimers of α- and β-tubulin which are assembled into 13 protofilaments, apart from B microtubules that have 11 protofilaments and share two with the A microtubules in the peripheral doublets. At the base of the cilia these microtubules are anchored to the cytoskeleton of the cell by the basal bodies and the feet of the basal bodies directs the ciliary stroke (Boisvieux-Ulrich, Laine et al. 1985). Within a cell all the basal body feet point in nearly the same direction leading to a coordinated ciliary waveform of adjacent motile cilia generating effective mucociliary clearance (Sanderson and Sleigh 1981; Porter and Sale 2000).

Dynein is a mechano-chemical ATPase and forms complexes that make up the inner and outer arms of the axoneme (Figure 3) (Kamiya 1995). They have different roles in the generation of a ciliary beat with the outer arm affecting ciliary beat frequency (CBF) and the inner arm, ciliary beat pattern (CBP) (Satir 1980). The ATPase activity of the dynein arms slides the A microtubules relative to the B microtubules, generating the force required for ciliary beating and bending, hence dynein arm mutations render ciliary movement ineffective (Satir 1980).

1.2.3.3 Periciliary fluid layer

In healthy individuals, the cilia are bathed in a layer of PCF. Traditionally this layer had been thought to be liquid, facilitating cilial beating, however it has recently been proposed that it is more structured, containing membrane spanning mucins, and thereby providing a further physical and osmotic layer of protection (Houtmeyers, Gosselink et al. 1999; Button, Cai et al. 2012). The depth of this PCF layer is important, too shallow and the overlying mucus layer impedes cilia beating, too deep and the tips of the cilia beat below the mucus layer; both inhibit effective mucociliary clearance.

1.3. Primary ciliary dyskinesia

1.3.1 Introduction

Primary ciliary dyskinesia (PCD) is an autosomal recessive condition caused by a range of ciliary ultrastructural defects. The resultant ciliary dyskinesia (Stannard, Chilvers et al. 2010) leads to recurrent sinopulmonary infection, bronchiectasis and recurrent sinusitis, due to the loss of function of the mucociliary escalator (Bush, Chodhari et al. 2007; Walker, Young et al. 2014). The estimated prevalence of PCD is generally reported to be between 1 in 15,000 – 30,000 live births (Barbato, Frischer et al. 2009) however this is significantly higher in certain populations. For example in a British Asian population with a high rate of consanguinity there is a prevalence of 1 in 2,300 (O'Callaghan, Chetcuti et al. 2010).

1.3.2 Clinical features

Clinical history and examination are central to the diagnosis and conventional diagnostic clues include abnormal *situs*, neonatal respiratory distress in term infants, persistent rhinorrhoea from the newborn period, recurrent “wet” cough, otitis media with effusion and male infertility (Munro, Currie et al. 1994; Coren, Meeks et al. 2002). There is an association with congenital heart disease and, more rarely, with hydrocephalus, polycystic kidney disease and retinitis pigmentosa (Lucas, Walker et al. 2011). Of these, the most universal aspect of their disease is their recurrent sinopulmonary infections.

1.3.3 Screening

Only a small percentage of patients presenting with chronic upper and lower respiratory tract infections have PCD and the diagnostic investigations require specialist skills, are time consuming, costly and only available in a small number of specialist centres (O'Callaghan, Chilvers et al. 2007). A reliable screening test was therefore desirable. NO detection in human exhaled breath was first described in 1991 (Gustafsson, Leone et al. 1991). The majority of NO originates from the paranasal sinuses, with lower concentrations found in exhaled breath (Lundberg, Rinder et al. 1994; Lundberg, Farkas-Szallasi et al. 1995). PCD patients have low nasal NO (nNO) and fractional exhaled NO (FeNO) compared to healthy controls (Lundberg, Rinder et al. 1994; Karadag, James et al. 1999; Narang, Ersu et al. 2002). However nNO is sufficiently low in PCD to be used as a screening test for the condition (Lundberg, Rinder et al. 1994; Karadag, James et al. 1999; Horvath, Loukides et al. 2003; Wodehouse, Kharitonov et al. 2003; Corbelli, Bringolf-Isler et al. 2004; Pifferi, Caramella et al. 2007) as recommended by the ERS task force on the approach to the diagnosis of PCD (Barbato, Frischer et al. 2009).

University Hospital Southampton (UHS) has one of three national PCD diagnostic centres in the UK. Paired nasal NO (nNO) and diagnoses data are collected for patients referred to the PCD diagnostic service. Over the 6 years up to December 2013 these paired data were collected for 301 patients, 34 of who were found to have PCD (Figure 4). There was a significant difference in the concentration of nNO between PCD and non-PCD patients (median 38.5 ppb vs. 570 ppb, $P<0.0001$) with most PCD patients having nNO concentrations less than 100 ppb. However, consistent with the literature, there were a few outliers in the PCD group in addition to some overlap of patients without PCD who had low levels (Marthin and Nielsen 2010) therefore diagnostic tests continue to be required.

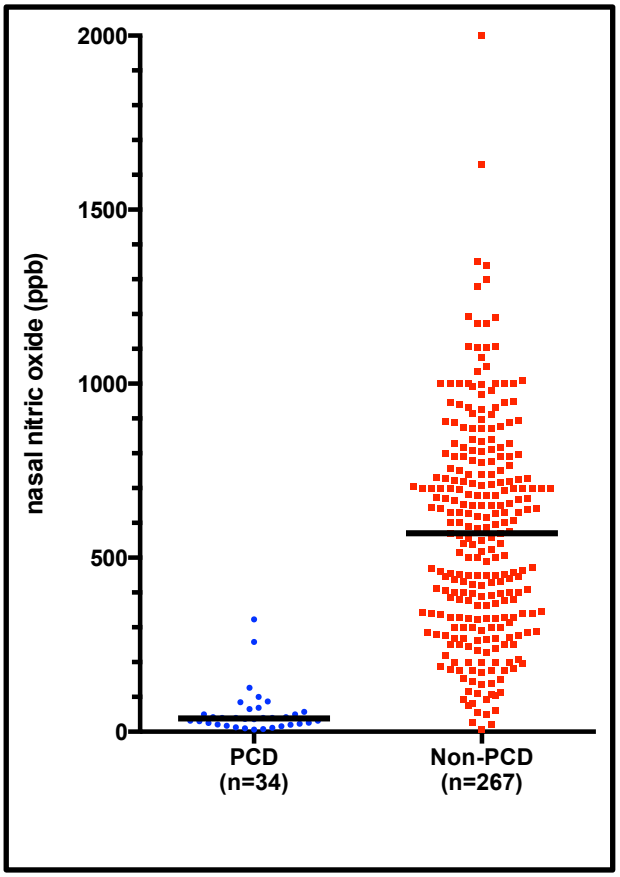

Figure 4 *- Nasal NO concentrations (ppb) in PCD (n=34) and non-PCD (n=267) patients referred to the Southampton PCD diagnostic service over the last 5 years. (Median 38.5 ppb vs. 570 ppb, P < 0.0001).*

1.3.4 Diagnosis

Diagnosis of PCD can be confirmed by analysis of ciliated nasal, or bronchial, epithelia obtained by brushing with a cytology brush. There is no 'gold standard' test that will diagnose all PCD phenotypes, and hence a diagnostic workup requires a consistent clinical history, the rigorous assessment of CBF and CBP by high-resolution, high-speed video microscopy (HSVM) and of ciliary ultrastructure by transmission electron microscopy (TEM) (Stannard, Chilvers et al. 2010). For difficult diagnostic cases the re-differentiation of basal epithelial cells at an air-liquid interface (ALI) in cell culture allows reassessment of ciliary function and ultrastructure (O'Callaghan, Chilvers et al. 2007; Barbato, Frischer et al. 2009; Hirst, Rutman et al. 2010).

1.3.4.1 Ciliary beat frequency and pattern analysis

In non-PCD patients referred to the national PCD diagnostic centre in UHS, the CBF is between 11-20 Hz (at 37˚C). However in patients with PCD this is typically markedly

reduced, approximately 3 Hz, static or, more rarely, hyper-frequent (Chilvers, Rutman et al. 2003; Leigh, Pittman et al. 2009). In patients with outer dynein arm (ODA) or inner dynein arm (IDA) defects, the majority of cilia are static, 55% and 80% respectively, with a mean CBF of 2.3 Hz and 0.8 Hz (Chilvers, Rutman et al. 2003). In patients with isolated IDA or microtubule disorganisation, cilia have a reduced amplitude and do not bend fully however only 10% and 30% respectively are static with mean CBF of 9.3 Hz and 6.0 Hz (Chilvers, Rutman et al. 2003). Ciliary transposition defects lead to a rotational beat pattern and a mean CBF of 10.7 Hz (Chilvers, Rutman et al. 2003). There are also a small group of PCD patients with the *DNAH11* gene mutation who have hyperkinetic beat frequency, with a severely altered CBP (Schwabe, Hoffmann et al. 2008).

1.3.4.2 Transmission electron microscopy

Transmission electron microscopy (TEM) allows visualization of the ciliary ultrastructure and, until recently, was considered the gold standard for PCD diagnosis. However it has now been shown that 10-15% of PCD patients have normal ultrastructure as seen at the resolution of electron microscopy (Schwabe, Hoffmann et al. 2008; Papon, Coste et al. 2010; Lucas, Walker et al. 2011). Of those with ultrastructural defects detectable by TEM, 70-80% of PCD patients have ODA defects, with or without inner dynein arm involvement (Plesec, Ruiz et al. 2008; Papon, Coste et al. 2010). Defects to the radial spokes and transposition defects, where a peripheral doublet of mircotubules is found in the centre of the axoneme, are rarer occurring in 18-25% of patients (Figure 5) (Plesec, Ruiz et al. 2008; Papon, Coste et al. 2010). In studies of normal individuals defects were seen in 3-5% of cilia and secondary ciliary defects (SCD), due to viral & bacterial infection, pollutants and inflammation, have been shown to lead to abnormalities in up to 10% of cilia. Both however are distinguishable from PCD by the lack of universality of the defect (Wisseman, Simel et al. 1981; Papon, Coste et al. 2010).

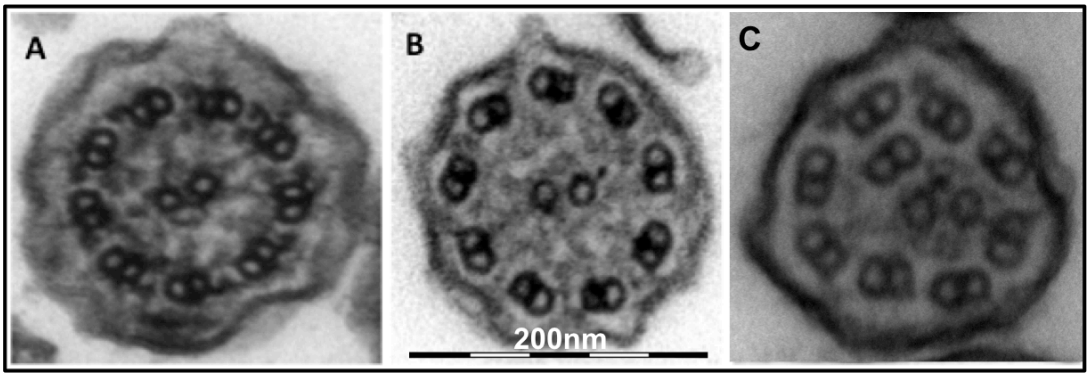

Figure 5 *- Transmission electron micrographs of cilial ultrastructure showing, A – normal ultrastructure, B - inner and outer dynein arm defects in PCD positive patient and C – microtubule disorganisation with inner dynein arm defect in PCD positive patient (images obtained using FEI Tecnai 12 TEM @ 80kV.)*

1.3.4.3 Air-liquid interface cell culture

Cell culture techniques have been used to aid the diagnosis of PCD for over a decade and presently in the UK the re-differentiation of basal epithelial cells to ciliate is achieved using an air-liquid interface (ALI) culture technique (de Jong, van Sterkenburg et al. 1994; Hirst, Rutman et al. 2010). This allows reassessment of ciliary function and ultrastructure that may differentiate primary from secondary ciliary dyskinesia and hence might stop the need for a repeat nasal brushing (O'Callaghan, Chilvers et al. 2007; Barbato, Frischer et al. 2009; Hirst, Rutman et al. 2010).

1.3.4.4 Genetics and novel diagnostic techniques

PCD is predominantly inherited as an autosomal recessive trait. However development of a genetic diagnosis for PCD has remained problematic due to the heterogeneity of the disease and the large number of proteins involved in the formation of cilia (probably over 400). To date there are almost 30 published PCD-causing gene mutations (Collins, Walker et al. 2014), which account for approximately two-thirds of PCD cases (Knowles and Boucher 2002; Berg, Evans et al. 2011; Merveille, Davis et al. 2011). Most of these known mutations lead to dynein arm or radial spoke defects (Figure 5) (Barbato, Frischer et al. 2009). In some countries, including the US, genetic testing is routinely used to aid diagnosis however at present in the UK it is used as a research tool only.

In addition there are a number of newer techniques, presently only used in research centres, including immunofluoresence (Omran and Loges 2009), tomography electron

microscopy (Burgoyne, Lewis et al. 2014) and pulmonary radio-aerosol mucociliary clearance (Marthin, Mortensen et al. 2007; Walker, Young et al. 2014).

1.3.5 Microbiology

There are limited available data looking at the microbiome of patients with PCD. Noone *et al.* reported that *Haemophilus influenzae*, *Staphylococcus aureus* and *Streptococcus pneumoniae* are the most prevalent pathogens in children, with *Pseudomonas aeruginosa* and non-tuberculous mycobacteria more common in adults and those with advanced lung disease (Noone, Leigh et al. 2004). Consistent with this, a clinical audit of five years of microbiology tests performed in the local paediatric PCD follow-up clinic in UHS demonstrated similar findings with *H. influenzae* the most prevalent respiratory pathogen present, accounting for almost 40% of positive cultures (Figure 6) (Walker, Jackson et al. 2012).

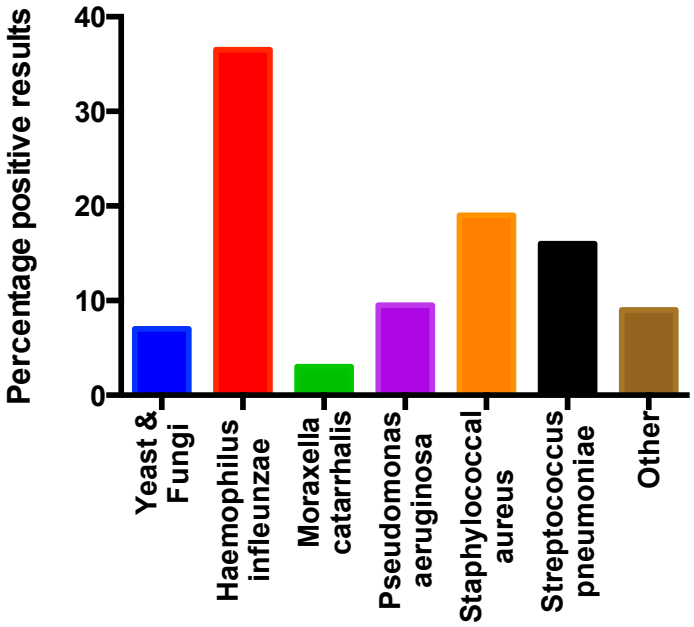

Figure 6 *- Percentage of culture positive results for the main pathogens cultured in PCD patients in University Hospitals Southampton paediatric PCD follow up clinic over the 5 years from April 2007 to April 2012*

The PCD research group at UHS has also undertaken a culture-independent assessment of the lower airway bacteriology in PCD (Rogers, Carroll et al. 2013). Thirty-three samples were collected from 24 patients aged 4 - 73 years with 16S rRNA and pyrosequencing used to determine bacterial load and community composition. In addition to finding the four respiratory pathogens commonly cultured by conventional microbiological techniques in these patients, outlined above, there was also a high

prevalence of the anaerobe *Prevotella* and the Gram-negative bacilli *Ralstonia*, the clinical significance of which is unclear. Similar to the findings by Noone *et al.*, there was a positive correlation seen between *P. aeruginosa* and age and a negative correlation between *P. aeruginosa* and a FEV_1 (Noone, Leigh et al. 2004; Rogers, Carroll et al. 2013).

In cystic fibrosis it is well established that chronic colonisation with *P. aeruginosa* increases the rate of decline in FEV_1 and is associated with an increased risk of death (Kerem, Corey et al. 1990; Kosorok, Zeng et al. 2001; Emerson, Rosenfeld et al. 2002). This has served as evidence for the aggressive use of anti-Pseudomonal treatments in CF over the last decade and during this time there have been significant improvements in the life expectancy, with infants born with CF today now expected to live into their 50's (Dodge, Lewis et al. 2007). In PCD there is little longitudinal data on the relationship between respiratory pathogens and lung function decline to evidence how aggressively clinicians should treat positive microbiology in this cohort of patients. A number of groups, including our own, have found an association between older age, *P. aeruginosa* and reduced lung function however, due to the confounding factor of age, none have derived causality (Noone, Leigh et al. 2004; Marthin, Petersen et al. 2010; Rogers, Carroll et al. 2013).

1.3.6 Management

There is limited evidence for the management of patients with PCD, much of it based on expert consensus and extrapolated from the management of CF (Barbato, Frischer et al. 2009). Ideally, patients should be managed by a multi-disciplinary team (MDT) that can coordinate the care and reduce the frequency of hospital appointments for the different systems involved.

From a respiratory point of view the mainstay of management is for the patients to perform regular airway clearance physiotherapy, as a substitute for the absent mucociliary escalator. There is limited evidence for the role of mucolytic agents, such as Dornase alpha (Pulmozyme ®, Genentech, San Francisco, US) and hypertonic (7%) saline (Forest Laboratories UK, Kent, UK). There is some case literature demonstrating that that Dornase alpha can be effective in patients with PCD (Desai, Weller et al. 1995; ten Berge, Brinkhorst et al. 1999; El-Abiad, Clifton et al. 2007) however in one large study of non-CF bronchiectasis patients it was found to led to more frequent exacerbations and a decrease in FEV_1 (O'Donnell, Barker et al. 1998). Respiratory exacerbations are treated aggressively with antibiotic therapy and, for those who

frequently exacerbate, prophylactic antibiotics are considered. The aim of management is to stabilise lung function and prevent chronic lung damage.

Recurrent otitis media with glue ear can lead to impaired hearing, which is particularly important in young children's speech development. Hearing levels can fluctuate considerably, particularly during infective exacerbations. Tympanostomy tube (grommet) insertion is generally not recommended as it can lead to persistent otorrhoea and hearing aids are the mainstay of treatment (Pruliere-Escabasse, Coste et al. 2010). These symptoms will often improve with age.

Nasal douching is used for clearing sinus mucus and congestion and, in doing so, this improves the symptoms of persistent rhinorrhoea. While the majority of males are infertile due to immotile sperm (Munro, Currie et al. 1994), *in vitro* fertilisation makes conception possible (Kay and Irvine 2000; Kaushal and Baxi 2007). Issues, such as congenital cardiac disease, are managed on a case-by-case basis by the appropriate clinicians.

1.4 Bacterial Biofilms

1.4.1 Introduction

The Centers for Disease Control and Prevention (CDC, Atlanta, U.S.) estimated that 65% of human infections are associated with bacterial biofilms (Potera 1999). The term bacterial biofilm, coined by Costerton *et al.* almost 20 years ago, is now widely found in the literature, with over 21,000 articles using the term (Ovid search, keyword "biofilm" 17/8/2014) (Costerton, Lewandowski et al. 1995).

The generally accepted definition of a bacterial biofilm is a structured community of bacterial cells enveloped in a self-produced complex of extracellular polymeric substance or exopolysaccharide (EPS) matrix growing attached to a surface (Costerton, Stewart et al. 1999). The EPS matrix is formed by a mixture of polysaccharides, extracellular DNA and proteins that contribute to the overall architecture of the biofilm (Wei and Ma 2013). Studies examining the structure of biofilms have demonstrated that the matrix-enclosed micro-colonies of bacteria within a biofilm are punctuated with open water channels (Lawrence, Korber et al. 1991; de Beer, Stoodley et al. 1994). These allow fluid to infuse through the biofilm hence facilitating nutrient and waste product exchange (Lawrence, Korber et al. 1991; de Beer, Stoodley et al. 1994).

1.4.2 Stages of biofilm development

Five distinct stages of biofilm growth have been described for *P. aeruginosa*, the best described biofilm forming bacteria, associated with changes in protein regulation affecting motility, alginate production and quorum sensing (Sauer, Camper et al. 2002; Ma, Conover et al. 2009). During the first two stages bacteria bind transiently to a surface then irreversibly with small bacterial clusters starting to form. During stage three the clusters thicken, becoming embedded in an EPS matrix and reach their maximum thickness by stage four. During stage five there is dispersal of bacteria from the biofilm that, from a proteomic perspective, more closely resemble planktonic bacteria than those in the biofilm (Sauer, Camper et al. 2002; Ma, Conover et al. 2009). The triggers of these coordinated dispersal events, where the bacteria convert back to motile planktonic bacteria (Barraud, Hassett et al. 2006), are of considerable interest from a clinical perspective. In patients with the archetypal biofilm forming disease, CF (Costerton 2001), patients suffer from recurrent infective respiratory exacerbations, which might be initiated by such dispersal events. The same may be true of other suppurative respiratory conditions associated with exacerbations, such as PCD. It is hypothesised that antibiotic therapy, initiated for a clinical exacerbation, effectively treats the planktonic bacteria, hence improving the patient's clinical picture temporarily, but is unable to eradicate the persistent biofilm bacteria from which planktonic bacteria were released. The cycle therefore continues, with the patient having further exacerbations when planktonic bacteria are subsequently dispersed from the biofilm.

1.4.3 Antibiotic recalcitrance

One of the key defining criteria of a biofilm infection is its recalcitrance to antibiotic therapy otherwise effective against the same bacteria in a planktonic culture (Parsek and Singh 2003; Hall-Stoodley and Stoodley 2009). It has been demonstrated that bacteria embedded within biofilms can be 1000-fold more resistant to antibiotic treatment (Brooun, Liu et al. 2000). There are a number of hypotheses for this and it is likely to be a complex combination of these that will vary between different bacteria and antibiotics. Firstly, the EPS may act to slow or lead to incomplete penetration of the antibiotics into the biofilm (Bolister, Basker et al. 1991; Mah and O'Toole 2001; Stewart and Costerton 2001). The EPS is not thought to directly prevent diffusion through the biofilm but antibiotics may bind to components within the biofilm matrix hence inhibiting their progress (Stewart 1998; Mah and O'Toole 2001). Secondly, most antibiotics rely, at least to some extent, on bacterial cell division to act. Due to the nutrient limitation in regions of the biofilm, bacteria can be in a near stationary,

dormant, growth phase making them tolerant to antibiotics that target cell wall or protein synthesis (Walters, Roe et al. 2003; Fux, Wilson et al. 2004). This may partially explain why it can be difficult to culture bacteria from clinical biofilms by conventional methods. One study showed only 30% of middle ear fluid in children was positive for bacteria by conventional culture compared with 100% being positive with polymerase chain reaction (PCR) and fluorescence *in situ* hybridisation (FISH) (Hall-Stoodley, Hu et al. 2006). Finally, previous antibiotic courses may lead to selection of antibiotic resistant strains of 'persister' bacteria that then survive within the biofilm (Spoering and Lewis 2001). There is also evidence that, as well as being recalcitrant to antibiotic treatment, the bacteria in biofilms are protected from the host immune system (Bjarnsholt, Jensen et al. 2005; Leid, Willson et al. 2005; Kirketerp-Moller, Jensen et al. 2008).

1.4.4 *In vitro* models of biofilm infection

A number of different *in vitro* bacterial biofilms models have been developed. While simplifying the *in vivo* situation they have nevertheless been key to investigating biofilm development, structure and physiology. They can be divided into three main groups, static or closed models, dynamic or open systems and microcosms (Lebeaux, Chauchan et al. 2013). Closed models are the most commonly utilised system due to the ease of set up and obtaining measurements, biofilms may be grown on agar or in wells in polystyrene plates, much as in tissue culture of eukaryotic cells (Christensen, Simpson et al. 1985; Stepanovic, Vukovic et al. 2007). Dynamic systems have continuous replacement of nutrients and removal of by-products. They allow control of shear forces and thereby the examination of the physical resistance of biofilms, however they require specialised equipment, the most commonly used of which are flow cells (Coenye and Nelis 2010). Microcosms are more sophisticated systems aimed to resemble the *in vivo* situation. They might include multiple bacterial species or be grown on cells as oppose to abiotic surfaces (Schaller, Schafer et al. 1998; Berry, Klumpp et al. 2009). They are more complex to set up and obtain measurements from but allow examination of more complex interactions.

Air liquid interface (ALI) epithelial cell culture models have been developed and used to provide insight into the pathophysiology of infection for a wide range of pathogens. These include *Pseudomonas aeruginosa* (Woodworth, Tamashiro et al. 2008), *Streptococcus pneumoniae* (Smith, Travis et al. 1996) and, since I have undertaken this work, Non-typeable *Haemophilus influenzae* (NTHi) (Ren, Nelson et al. 2012). Smith *et al.* co-cultured S. pneumoniae on ALI cultured primary respiratory epithelial cells for short time courses, up to 4 hours, as a model of acute infection whereas Ren *et al.* co-

cultured NTHi on commercially available cell lines, cultured at an ALI for up to ten days, assessing intracellular invasive by the pathogen (Smith, Travis et al. 1996; Ren, Nelson et al. 2012).

1.4.5 Biofilm infections in the respiratory tract

Biofilms have been demonstrated in a number of chronic infections of the upper and lower respiratory tract including chronic sinusitis, tonsillitis and bronchiectasis (Chole and Faddis 2003; Starner, Zhang et al. 2006; Psaltis, Ha et al. 2007). While *P. aeruginosa* is the best-characterised biofilm forming bacteria (Bjarnsholt, Jensen et al. 2009; Wei and Ma 2013) biofilm development occurs in a wide range of bacteria, including the common PCD pathogens *Haemophilus influenzae*, *Staphylococcus aureus and Streptococcus pneumoniae* (Noone, Leigh et al. 2004; Hall-Stoodley, Nistico et al. 2008; Arce, Carlson et al. 2009). Criteria for defining biofilm infections are outlined in Table 1 (Parsek and Singh 2003; Hall-Stoodley and Stoodley 2009; Hall-Stoodley, Stoodley et al. 2012).

Table 1 *- Proposed diagnostic criteria for biofilm infection (Parsek and Singh 2003; Hall-Stoodley and Stoodley 2009)*

- The pathogenic bacteria are surface associated
- Direct examination of infected tissue reveals aggregates of cells in clusters, encased in a matrix of bacterial or host origin
- The infection is localized with the host
- The infection is recalcitrant to antibiotic therapy despite demonstrated susceptibility of planktonic bacteria
- Negative cultures by conventional microbiological techniques despite clinically documented high suspicion of infection (as bacteria in biofilms may be missed by conventional techniques)
- Evidence of ineffective host clearance, suggested by cell clusters in discrete areas in the host tissue associated with host inflammatory cells

1.4.6 *Haemophilus influenzae*

Haemophilus influenzae, a Gram-negative coccobacilli bacterium, is a common nasopharyngeal commensal in humans, which can also lead to serious invasive infection (Satola, Schirmer et al. 2003; Shen, Antalis et al. 2005; Erwin and Smith 2007). It is known for its nutritional requirements, needing both β-nicotinamide

adenine dinucleotide (β-NAD) and haem to grow in culture (Erwin and Smith 2007). There are six antigenically distinct polysaccharide capsules (serotypes a to f) of typeable *H. influenzae*, or non-typeable (NTHi), which is unencapsulated (Gilsdorf, McCrea et al. 1997). Encapsulated serotypes are known to be more pathogenic than NTHi and *H. influenzae* type b (Hib) is recognised to be the most virulent of these, causing meningitis, pneumonia and septicaemia. However since the introduction of a Hib vaccine in 1992 invasive Hib disease has almost been eliminated in the developed world (Heath and McVernon 2002).

1.4.7 Non-typeable *Haemophilus influenzae* (NTHi)

Non-typeable *H. influenzae* colonises the majority of healthy individuals, and has led some to ignore it as a significant pathogen (Hausdorff and Dagan 2008). However it is the predominant pathogen cultured from the lower respiratory tract of children with non-CF bronchiectasis, ranging from 37% to 68% of the positive culture results (Edwards, Asher et al. 2003; Eastham, Fall et al. 2004; Li, Sonnappa et al. 2005; Banjar 2007), and has recently been shown to be the predominant pathogen in otherwise healthy children with recurrent or non-responsive pneumonia (De Schutter, De Wachter et al. 2011). It is also the commonest pathogen in recurrent otitis media (Leibovitz, Jacobs et al. 2004) and, since introduction of the 7-valent pneumococcal conjugate vaccine (Wyeth®, Philadelphia, US), the proportion of these children with NTHi has increased (Eskola, Kilpi et al. 2001; Block, Hedrick et al. 2004; Casey and Pichichero 2004).

1.4.8 Non-typeable *Haemophilus influenzae* biofilms

Non-typeable *H. influenzae* has been shown to produce biofilms both on epithelial cell lines, animal models and *in vivo* (Greiner, Watanabe et al. 2004; Jurcisek, Greiner et al. 2005; Hall-Stoodley, Hu et al. 2006; Starner, Zhang et al. 2006). Starner *et al.* co-cultured NTHi on ALI cultured cell layers, using a Calu-3 cell line (Starner, Zhang et al. 2006). They demonstrated increasing biofilm formation on the apical surface of the cell layers over the 4-day time course assessed (Starner, Zhang et al. 2006). Hall-Stoodley *et al.* examined human middle ear mucosa biopsies from children with chronic otitis media, both otitis media with effusion and recurrent otitis media, demonstrating NTHi biofilms by FISH. This study provides *ex vivo* evidence that chronic middle ear infections were biofilm related (Hall-Stoodley, Hu et al. 2006). Jurcisek *et al.* used a chinchilla model of NTHi-induced otitis media to investigate the EPS of NTHi biofilms, demonstrating the presence of sialic acid, lipooligosaccharides (LOS), double-stranded DNA and type IV pili protein (Jurcisek, Greiner et al. 2005). In addition, there

is evidence linking increased expression of phosphorylcholine and sialylation in LOS with the establishment of stable biofilm communities and a decreased host immune response (Bouchet, Hood et al. 2003; Swords, Moore et al. 2004; West-Barnette, Rockel et al. 2006; Hong, Mason et al. 2007). Nutritional limitation is a trigger for biofilm phase growth and, recent work has highlighted the importance of the availability of haem on NTHi biofilm growth, where its restriction promotes biofilm development (Szelestey, Heimlich et al. 2013). In addition NTHi biofilms development is promoted and maintained, in part, by quorum sensing. This involves the release of molecules by the bacteria in a density-dependent manner. The bacterial by-product metabolite dihydroxypentanedione (DPD) has been associated with quorum signalling for a number of bacteria (Surette, Miller et al. 1999). In NTHi both LuxS transcription and the protein RbsB have been implicated in DPD quorum signals (Armbruster, Hong et al. 2009; Armbruster, Pang et al. 2011). There are likely to be other triggers yet to be discovered.

1.4.9 Role of cilia function in biofilm formation

Bacterial respiratory tract infections are more common when the host has been co-infected with a viral infection, particularly in respiratory conditions such as CF, and add to the severity of the clinical picture (Wang, Prober et al. 1984; Armstrong, Grimwood et al. 1998; Thorburn, Harigopal et al. 2006; Wat, Gelder et al. 2008). This is particularly recognised with *H. influenzae*, where one group reported 60% of *H. influenzae* infections were associated with a viral infection (Chonmaitree, Owen et al. 1992). Traditionally the mechanism for this phenomenon had been thought to be epithelial damage caused by the initial viral infection predisposing to invasion by bacterial (Hament, Kimpen et al. 1999). However, it is also well established that viral respiratory tract infections lead to ciliary dysfunction and reduced mucociliary clearance, which may, at the very least, contribute to this association (Camner, Jarstrand et al. 1973; Tristram, Hicks et al. 1998; Pittet, Hall-Stoodley et al. 2010).

Non-typeable *H. influenzae* supernatant has itself has been shown to reduce CBF possibly via protein kinase C ε (Bailey, LeVan et al. 2012). In addition Galli *et al.*, looking at biopsy samples from patients undergoing surgery for chronic rhinosinusitis, found an absence of cilia on samples where biofilm formation was most evident, however were unable to demonstrate whether the bacteria biofilm had caused the loss of cilia or whether absent cilia had allowed bacteria biofilms development (Galli, Calo et al. 2008).

So whilst authors have questioned the role of ciliary function and mucociliary clearance in biofilm formation for over a decade there has been little published work assessing this (Miyamoto and Bakaletz 1997; Pittet, Hall-Stoodley et al. 2010).

1.5 Models of the respiratory tract

1.5.1 Introduction

The aim of any model of the human respiratory tract is to simulate the *in vivo* situation as closely as possible while controlling experimental variables. This allows for the in-depth investigation of the pathophysiology of clinical conditions, novel therapeutic targets and pre-clinical testing of new drug treatments prior to clinical trials.

1.5.2 Animal models

Animal models have been used as the pharmacological industry's standard for working up early drug discovery for the last thirty years. There are many benefits for their use in this way besides economical considerations, including the potential for genetic modification, particularly of mouse models in creating transgenic knockouts useful in studying particular pathologies. However it is now well recognised that animal models do not recapitulate the complexities of the human *in vivo* situation. This is true both because of the uniform genetic make-up of most of the animals used, being inbred strains, and the controlled environmental conditions to which they are exposed. Promising novel therapies, worked-up on such models, have not infrequently failed to show benefit in clinical trials, reflecting the genetic variation and complex environmental exposures present in the human population (Mauser, Pitman et al. 1995; Leckie, ten Brinke et al. 2000; Flood-Page, Swenson et al. 2007). For example, the use of an anti-IL-5 antibody in asthma was found to be very effective in monkeys but a number of clinical trials in humans have demonstrated only modest benefits (Mauser, Pitman et al. 1995; Leckie, ten Brinke et al. 2000; Flood-Page, Swenson et al. 2007). This has contributed to the limited number of approved novel respiratory therapies getting through phase III clinical trials over last few decades. In the case of asthma, for example, there have been only two new groups of treatments, leukotriene receptor antagonists and IgE monocloncal antibodies, licensed in the last thirty years despite vast amounts of investment. The other obvious disadvantage of animal models is the ethical issues around sacrificing them for the research.

A number of mouse models have been described with a PCD phenotype, including the *Dnahc5* mouse, the nm1054 homozygote mouse and the *Dnahc11*^{iv} mouse (Tan, Rosenthal et al. 2007; Lee, Campagna et al. 2008; Lucas, Adam et al. 2012). Mouse models offer the benefit of a complete respiratory epithelium, including immune cells and surfactant produced by the alveoli cells, as opposed to a cell culture model (1.5.4). However, while mouse models have slow or immotile ciliary function, several have additional defects that lead them to be significantly less viable and raise animal welfare issues; for example the nm1054 homozygote mouse develops hydrocephalus and severe anaemia that are usually lethal in the first week of life (Tan, Rosenthal et al. 2007; Lee, Campagna et al. 2008). The recently described *Dnahc11*^{iv} mouse does not have such viability issues and seems to closely match the phenotype of patients with PCD, having immotile tracheal cilia and suffering from rhinitis and otitis media (Lucas, Adam et al. 2012). However the *Dnahc11*^{iv} mouse has not been shown, as yet, to develop significant lung pathology (Lucas, Adam et al. 2012).

1.5.3 Explanted bronchial tissue and bronchial biopsies

Using explanted tissue, taken either at surgery or bronchial biopsy, therefore offers a valid alternative as it is human tissue and theoretically allows the architectural integrity of the whole airway wall to be preserved. In maintaining its multi-layered structure, any cell signalling between these layers might also be preserved, giving a more realistic model. However, particularly for bronchial biopsy, the architecture of the tissue is often damaged in obtaining the samples and having a completely intact epithelial cell layer is unusual. Therefore, when using this tissue in experiments there is significant risk that the sub-epithelial tissue, usually protected by this epithelial layer, would be directly exposed to any intervention being investigated, potentially increasing the risk of discrepancies between the response in the model and the *in vivo* situation.

In addition, the availability of explanted lung tissue from patients with PCD is very limited. They rarely need lung transplants and, with advances in their medical management, lobectomies are also now very uncommon. The indication for such surgeries is typically end stage bronchiectasis and as such the lung tissue recovered is almost inevitably infected by multiple respiratory pathogens. Whilst epithelial cell culture has been successful from explanted lung tissue from CF patients (Brodlie, McKean et al. 2010) tailored antibiotic regimens are required and optimisation of the technique is both resource and time consuming.

1.5.4 Respiratory epithelial cell culture

Cell culture was first conceived over a hundred years ago (Alberts 2002) and respiratory epithelial cell culture has been widely used in the study of respiratory disease for the last 30 years (Lechner, Haugen et al. 1982). Cell culture can be undertaken either with primary cells or immortalized, continuous cell lines using a various techniques.

1.5.4.1 Continuous cell lines

Primary cells can usually only divide a limited number of times before losing their ability to proliferate. By contrast cell lines, immortalised through viral or chemically induced transformation are readily available, relatively cheap and fairly simple to culture. For example, the BEAS-2B cell line was immortalized with adenovirus 12-SV40 virus hybrid Ad12SV40 (Kinnula, Yankaskas et al. 1994). Cell lines can be cultured to form cell layers but rarely differentiate fully and usually have different morphology from *in vivo* epithelial cell layers. In addition, due to the process of transformation and, potentially, the number of passages they have often undergone, cell signalling transduction networks can be deregulated (Huang, Wiszniewski et al. 2011).

1.5.4.2 Primary cell culture

Respiratory epithelial cells for primary cell culture are typically obtained either by nasal brushing or bronchial brushing at bronchoscopy. Bronchoscopies are not commonly undertaken, particular in patients with PCD, who rarely need them for clinical reasons. However the nose is readily accessible and thereby an attractive surrogate if the subsequent cultures are consistent with the lower respiratory tract epithelium.

Worldwide, in the investigation of PCD, where ciliary samples are required for diagnosis, nasal brushings are used to obtain the ciliated respiratory epithelia. (Barbato, Frischer et al. 2009) Studies have shown that the ciliary beat frequency and ultrastructure is consistent between the upper and lower respiratory tract (Rutland, Griffin et al. 1982; Verra, Fleury-Feith et al. 1993). In addition, comparisons of the morphology and function, including cell size and degree of ciliation, and constitutive and stimulated inflammatory mediator release, including IL-8, IL-6, G-CSF, MCP-1, vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) and receptor expression, of nasal versus bronchial epithelial cells have shown them to be similar (Devalia, Sapsford et al. 1990; McDougall, Blaylock et al. 2008). It is therefore

reasonable to conclude that nasal epithelial cells constitute an accessible surrogate for bronchial epithelial cells.

1.5.4.2.1 Cell culture techniques

Primary epithelial cells can be cultured as a liquid covered culture (LCC) or at an air-liquid interface (ALI), to encourage differentiation of the cell layer. ALI cultures were first developed in the late 1980's and represented a leap towards the *in vivo* epithelial biology providing an excellent model for investigating epithelial function (Whitcutt, Adler et al. 1988). Studies have shown that ALI cultures develop an epithelial cell layer that is morphologically more representative of the airway epithelium than cells cultured using liquid covered culture, with the presence of cilia, an increased depth of the cell layer and more frequent apical microvilli (Yamaya, Finkbeiner et al. 1992). Pezzulo *et al.* demonstrated that the transcriptional profile of primary ALI cultured airway epithelial cells closely recapitulates that of *in vivo* airway epithelial cells (Pezzulo, Starner et al. 2011). ALI cultured cell layers have higher TER than equivalent liquid covered cell layers, with groups reporting TER to be two to three fold greater across ALI cultured cell layers (Lee, Yoo et al. 2005; Grainger, Greenwell et al. 2006). Furthermore, ALI cultured cell layers produce secretions with a similar protein make up to that of sputum, including the mucins MUC1, MUC4, MUC5B and MUC5AC (Kesimer, Kirkham et al. 2009) Using primary epithelial cells from patient with different respiratory pathologies, as compared to healthy controls, also allows for comparison of cellular response, offering further insights into the pathophysiology of the disease processes.

1.5.5 Novel cell culture systems

Typically, when using an ALI culture system only epithelial cells are included. While this is valid for looking at the respiratory epithelial cell layer it does not fully reflect the *in vivo* airway wall, as any potential crosstalk between the different cell types present would not occur. There has therefore been increasing interest in developing complex multi-cell type culture models. In addition, culture systems are being developed incorporating microfluidics and movement, to mimic the cell stresses due to breathing, referred to as "lung-on-a-chip" systems. They enable supernatant to continuously circulate and thereby allow real time measurement of cellular responses (Nalayanda, Puleo et al. 2009; Huh, Hamilton et al. 2011). While in their infancy, reproducing the airway wall in this way should take us another leap forward in developing realistic *in vitro* systems that truly reflect the *in vivo* situation, however at presently these systems are both costly and not widely available.

1.6 Nitric Oxide

1.6.1 Introduction

Nitric oxide is a highly reactive, gaseous, and ubiquitous intracellular and intercellular signalling molecule, which governs a diverse number of dynamic physiological and pathophysiological cellular processes in humans (Ignarro, Buga et al. 1987) such as vascular homeostasis, neurotransmission, immune cell activity and tumour progression (Hong, Sun et al. 2009). Airway NO and in particular nasal NO is low in PCD (Lundberg, Rinder et al. 1994; Karadag, James et al. 1999; Horvath, Loukides et al. 2003; Wodehouse, Kharitonov et al. 2003; Corbelli, Bringolf-Isler et al. 2004; Pifferi, Caramella et al. 2007).

1.6.2 Biosynthesis and localisation

Synthesized via the oxidation of the amino acid L-arginine to L-citrulline, NO biosynthesis is catalysed by three stereo-specific isoenzymes called nitric oxide synthases (NOS) in the presence of nicotinamide adenine dinucleotide phosphate (NADP), oxygen and other cofactors (Figure 7). NO has a short half-life *in vivo*, 1 - 5 seconds, due to its molecular instability and diffuses rapidly from its point of synthesis, interacting intracellularly as well as permeating to the plasma membranes where it may be released from the cell (Liu, Miller et al. 1998; Ricciardolo 2003).

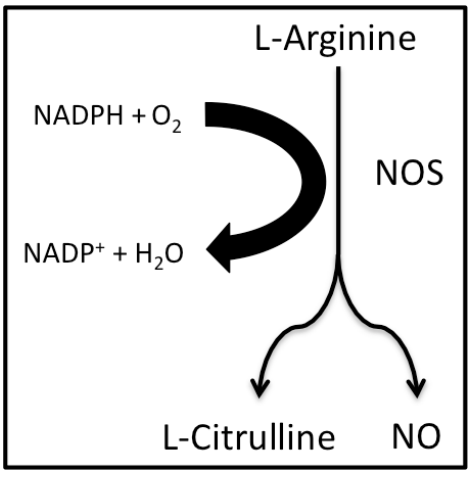

Figure 7 *- Schematic diagram to demonstrate nitric oxide (NO) biosynthesis by the conversion of L-arginine (NOS substrate) to L-citrulline via nitric oxide synthases (NOS) isoenzymes. During this process the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), is oxidized in the presence of oxygen (O_2) to form*

nicotinamide adenine dinucleotide phosphate ($NADP^+$) and water (H_2O). Other cofactors are also required for NO biosynthesis (not shown).

The concentration of NO biosynthesised is dependent on the activities of the three NOS isoenzymes, since each NOS exhibits different expressional and functional characteristics. Two of these are considered to be "constitutive", neuronal NOS (nNOS) and endothelial NOS (eNOS), the other is inducible, inducible NOS (iNOS). Expression and protein localization in the airway of the nitric oxide synthase isoenzymes are outlined in Table 2.

Table 2 *- Expression and protein localization in the airway of the nitric oxide synthase isoenzymes*

NOS isoenzyme	Protein	Gene	Chromosome	Localisation	Reference
Neuronal	nNOS	NOS1	12	Human airway neuronal cells	(Kobzik, Bredt et al. 1993)
Inducible	iNOS	NOS2	17	Human airway epithelial cells Human alveolar epithelial cells	(Warner, Paine et al. 1995; Pechkovsky, Zissel et al. 2002)
				Rat lung endothelial cells	(Ermert, Ruppert et al. 2002)
				Rat neutrophils	(Blackford, Antonini et al. 1994)
				Rat mast cells	(Gilchrist, Savoie et al. 2002)
Endothelial	eNOS	NOS3	7	Human airway epithelial cells	(Shaul, North et al. 1994)

NOS - nitric oxide synthase

Each NOS isoenzyme is structurally similar however, whilst the nNOS and eNOS are dependent on calmodulin binding and calcium activity to produce femtomole and picomole concentrations of NO (Ricciardolo, Di Stefano et al. 2006), iNOS is permanently bound to calmodulin, therefore not dependent on calcium activity, and may be transcriptionally induced to produce longer acting, nanomolar concentrations of NO (Morris and Billiar 1994; Ricciardolo, Di Stefano et al. 2006). NO biosynthesis from epithelial cells is up regulated via iNOS following stimulation with bacteria and pro-inflammatory cytokines (Warner, Paine et al. 1995).

1.6.3 Role of nitric oxide in the airway

Nitric oxide has diverse roles within the airways. Pertaining to ciliary function, *in vitro* studies of animal and human ciliated airway epithelium suggest that the induction of NOS and NO production increases CBF from baseline (Jain, Rubinstein et al. 1993; Jain, Rubinstein et al. 1995; Sisson 1995; Uzlaner and Priel 1999; Wyatt, Forget et al. 2003; Alberty, Stoll et al. 2006; Sisson, Pavlik et al. 2009). NO plays an important role in viral clearance both by direct antiviral effects and via signalling pathways (Kao, Piedra et al. 2001). Additionally in the airway, NO causes bronchodilatation by relaxation of the airway smooth muscle via activation of guanylyl cyclase (Gruetter, Childers et al. 1989) and recently there is an increasing literature on the effects of NO on bacterial growth (Barraud, Hassett et al. 2006; Major, Panmanee et al. 2010). NO has bacteriostatic and bactericidal activity; sodium nitrite can kill *Pseudomonas aeruginosa, Staphylococcus aureus* and *Burkholderia cepacia* (Major, Panmanee et al. 2010). It can also modulate biofilm formation (Barraud, Hassett et al. 2006) and cause their dispersal acting via cyclic di-GMP (Barraud, Schleheck et al. 2009), making the bacteria more susceptible to antimicrobial treatment (Barraud, Hassett et al. 2006).

1.6.4 Nitric oxide levels in respiratory disease

As outlined above, nNO levels are almost always extremely low in patients with PCD as compared to healthy controls (Walker, Jackson et al. 2012). Lower levels of nNO and FeNO are also seen, but to a lesser extent, in CF (Grasemann, Michler et al. 1997; Ho, Innes et al. 1998). In asthma, where FeNO is thought to be a marker of eosinophilic inflammation, levels can be markedly elevated, particularly in poorly controlled asthma (Silkoff, Sylvester et al. 2000). However this may be confounded by higher NO levels seen in patients with allergic rhinitis (Arnal, Didier et al. 1997).

1.6.5 Why are nitric oxide levels reduced in PCD?

While the low levels of NO seen in PCD were first described 20 years ago the underlying mechanisms have yet to be fully elucidated (Lundberg, Weitzberg et al. 1994). The potential mechanisms for this observation are both at a cellular and anatomical level; some extrapolated from the finding of reduced NO levels seen in CF (Grasemann, Tomkiewicz et al. 1997; Elphick, Demoncheaux et al. 2001; Gaston, Ratjen et al. 2002; Grasemann, Schwiertz et al. 2005). At the epithelial level it has been suggested that there is reduced biosynthesis of NO (Narang, Ersu et al. 2002; Csoma, Bush et al. 2003; Grasemann, Schwiertz et al. 2005) or increased breakdown, either within the cell or in the mucus layer (Grasemann, Tomkiewicz et al. 1997) (Elphick, Demoncheaux et al. 2001). At the anatomical level it has been suggested that NO is sequestered in the upper respiratory tract within obstructed paranasal sinuses or alternatively nasal NO biosynthesis or NO storage capacity is limited due to agenesis or opacification of the sinuses (Santamaria, De Stefano et al. 2008; Pifferi, Bush et al. 2010) (Figure 8).

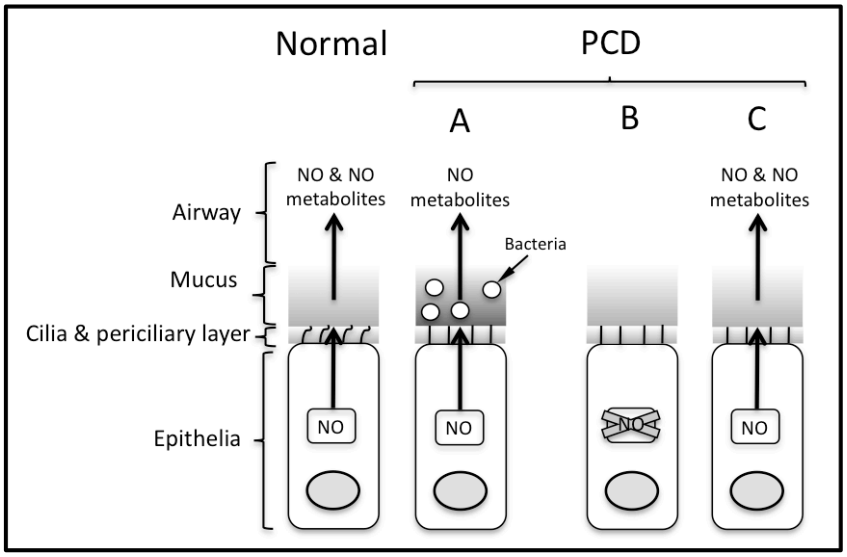

Figure 8 *- Schematic diagram to represent normal nitric oxide (NO) metabolism and release from the epithelium of healthy patients and hypotheses for the potential causes of low nasal NO concentrations in PCD, based on events at the epithelium: A) increased breakdown of highly reactive NO to NO metabolites (nitrite and nitrates); either within the epithelial cell, within extra viscous sputum or by denitrifying bacteria within the mucus, hence NO metabolites rather than NO are predominantly released into the airway; B) absence of, or reduced, NO biosynthesis within the epithelial cell; C) normal NO biosynthesis within the epithelial cell however obstruction to the osteomeatal complex inhibits NO release from the paranasal sinuses into the nasal passage, or hypoplasia, agenesis or opacification of the paranasal sinuses reduces NO storage*

capacity, hence low measurable nasal NO levels are seen (taken from Walker et al, ERJ 2012)

1.6.5.1 Increased breakdown of nitric oxide to metabolites

Nitric oxide is highly reactive and is rapidly broken down from the breath exhalate (Radi, Beckman et al. 1991; Jones, Bryan et al. 1998; Gaston 2006; Thomas, Ridnour et al. 2008). It is possible that this process occurs at a greater rate in PCD than non-PCD subjects. However, Csoma *et al*. compared NO metabolites in breath exhalate from PCD and healthy subjects and found no difference although, importantly, this work does imply that NO is biosynthesised in PCD subjects (Csoma, Bush et al. 2003).

Impaired mucociliary clearance in PCD causes mucus accumulation in the airways, potentially trapping NO, which might then be broken down into NO metabolites. In CF patients it has been suggested that the viscous mucus may trap the NO in this way (Grasemann, Tomkiewicz et al. 1997; Elphick, Demoncheaux et al. 2001) and similar biophysical properties have been identified in sputum of patients with PCD (Bush, Payne et al. 2006). This mechanism requires further consideration but the study outlined above by Csoma *et al.* (Csoma, Bush et al. 2003) would argue against it.

1.6.5.2 Reduced biosynthesis of nitric oxide

A number of reasons have been postulated to explain why NO biosynthesis may be reduced in PCD epithelia. It has been shown that iNOS is the main contributor to FeNO (Lane, Knight et al. 2004). As with CF patients (Kelley and Drumm 1998; Meng, Springall et al. 1998; Moeller, Horak et al. 2006), patients with PCD might have lower levels of iNOS expression. This was suggested by data in a recent study however significant overlap between study groups made the results difficult to interpret (Pifferi, Bush et al. 2011) Since PCD is a polygenic disorder, its genetic linkage to NOS gene polymorphisms is highly unlikely and sequencing, targeted to identify PCD causing genetic mutations, has not identified NOS genes as candidate genes for PCD (Berg, Evans et al. 2011).

Narang *et al.* proposed that normal NOS activity requires ciliary function via a mechano-chemical coupling to dynein ATPases, which would be expected to be uncoupled in patients with PCD, as seen in Duchenne muscular dystrophy (Grozdanovic, Christova et al. 1997; Gucuyener, Ergenekon et al. 2000; Narang, Ersu et al. 2002; Niebroj-Dobosz and Hausmanowa-Petrusewicz 2005). However this

explanation seems unlikely, as PCD phenotypes with hyper-frequent or motile dyskinetic cilia have similarly low NO levels to patients with static cilia.

In addition, NO synthesis occurs in patients with PCD (Csoma, Bush et al. 2003; Smith, Fadaee-Shohada et al. 2013). A recent paper by Smith *et al.* reported that constitutively, PCD epithelial cells produced similar levels of NO as controls however did not up-regulate NO biosynthesis in response to early infection with *Streptococcus pneumoniae,* observed at two hours (Smith, Fadaee-Shohada et al. 2013). Longer infective time courses, which would allow bacterial biofilm formation that, given the chronicity of infection seen in PCD patients due to the impaired ciliary function, might be more clinically relevant, have not been examined.

1.6.5.3 Nitric oxide is trapped in the obstructed paranasal sinuses

The predominant source of exhaled NO is the upper airway (Lundberg, Rinder et al. 1994) and studies using manoeuvres such as humming, which increase gas exchange across the osteomeatal complex, suggest NO is sequestered within the nasal sinuses (Weitzberg and Lundberg 2002; Lundberg, Maniscalco et al. 2003; Lundberg 2008; Santamaria, De Stefano et al. 2008). During tidal breathing it can take up to 30 minutes to completely clear sinus gases into the nasal cavity but with humming this can occur in one exhalation (Paulsson, Dolata et al. 2001; Maniscalco, Weitzberg et al. 2003). In healthy volunteers humming leads to a 15-fold peak in nasal NO, compared to quiet exhalation (Weitzberg and Lundberg 2002). This humming peak is absent in patients with chronic sinusitis, CF (Lundberg, Maniscalco et al. 2003; Struben, Sewbalak et al. 2007) and in PCD (Santamaria, De Stefano et al. 2008) the majority of whom, as outlined above, also suffer from chronic sinusitis. Interestingly a recent study reported five PCD patients with normal nNO levels noting that none suffered from chronic sinusitis (Marthin and Nielsen 2010), supporting the hypothesis that a patent osteomeatal complex permits normal nasal NO levels in PCD. There remains, however, the possibility, particularly given the vast majority of PCD patients have sinus disease and there are very few cases of PCD with normal nasal NO levels reported, that these five patients have been misdiagnosed with PCD.

It also remains unclear whether the low NO levels are a consequence of an underlying inability of the airway epithelia in PCD patients to produce NO or that the manoeuvres did not overcome the obstruction to osteomeatal complex.

1.6.5.4 Reduced nitric oxide storage capacity of the paranasal sinuses

Aplastic, hypoplastic or opacified nasal sinuses can occur in PCD and may explain why nasal NO concentrations are low and do not vary significantly with manoeuvres to improve sinus ventilation (Gomez, Perez Trullen et al. 1997; Pifferi, Bush et al. 2010). A recent study demonstrated a significant inverse correlation between the degree of aplasia/hypoplasia of each paranasal sinus and nasal NO values in PCD (Pifferi, Bush et al. 2010), supporting the hypothesis that smaller sinuses are associated with lower nasal NO levels. Although, unusually, 24% of this PCD study population had normal nasal NO and the correlation between aplasia/hypoplasia score and nasal NO level was dependent on seven such patients. There is again the possibility that they had been incorrectly diagnosed with PCD and if this were the case there would be little correlation between NO levels and aplasia/hypoplasia score.

However further evidence in support of this hypothesis is that the paranasal sinuses develop over the first 10-12 years of life and nasal NO levels are seen to increase with age over this time period (Lundberg, Farkas-Szallasi et al. 1995). In addition baboons, which do not have paranasal sinuses, have very low levels of nasal NO (Lewandowski, Busch et al. 1998). It is also noteworthy that most conditions associated with lower nasal NO levels involve disease of the paranasal sinuses, including CF (Balfour-Lynn, Laverty et al. 1996; Nishioka, Cook et al. 1996), nasal polyps (Colantonio, Brouillette et al. 2002), chronic sinusitis (Lindberg, Cervin et al. 1997) and PCD, which may lead to either obstruction of the osteomeatal complex or reduced storage capacity of the paranasal sinuses, or a combination of both.

1.6.5.5 Summary of low NO in PCD

The limited studies to date suggest that biosynthesis of NO occurs in PCD (Csoma, Bush et al. 2003; Smith, Fadaee-Shohada et al. 2013), and there is little to suggest that increased breakdown or metabolism is responsible for the low levels. However it may be that PCD patients cannot up-regulate NO biosynthesis in response to infection or the kinetics are different (Smith, Fadaee-Shohada et al. 2013; Walker, Jackson et al. 2014). If this is the case it still does not explain why constitutive NO levels are so low in patients with PCD.

There is some direct and circumstantial evidence that the reduced size of the paranasal sinuses caused by aplasia or opacification and the lack of patency of the osteomeatal complex might contribute (Lundberg, Farkas-Szallasi et al. 1995; Santamaria, De Stefano et al. 2008; Pifferi, Bush et al. 2010). However this is based

primarily on patients, diagnosed with PCD, who have normal levels of nasal NO and, given that the vast majority of PCD patients have extremely low NO levels, it raises questions over the reliability of their diagnosis.

Clarifying the mechanism for the low levels seen is increasingly important as the use of nasal NO measurement as a screening tool for the diagnosis of PCD becomes more widespread. In particular, due the difficulties in some countries accessing central facilities that can undertake the time consuming and costly PCD diagnostic investigations.

1.6.5.6 Relevance of low NO in PCD to this work

There is an increasing literature on the effects of NO on bacterial growth, at varying concentrations having bacteriostatic and bactericidal activity (Barraud, Hassett et al. 2006; Major, Panmanee et al. 2010; Regev-Shoshani, Ko et al. 2010). In addition NO can modulate *P. aeruginosa* biofilm formation (Barraud, Hassett et al. 2006; Major, Panmanee et al. 2010) and cause their dispersal acting via cyclic di-GMP (Barraud, Schleheck et al. 2009). The low nNO levels seen in PCD may, therefore, increase these patients' susceptibility to bacterial infections and, in particular, bacterial biofilm infections.

In this work ALI cultured respiratory epithelial cell layers from PCD and non-PCD patients will be compared to consider the role that ciliary function has on NTHi biofilm development. If the low NO levels seen in subjects with PCD were due to an inability of their respiratory epithelial cells to biosynthesise NO this would be a potentially important confounding variable.

1.7 Clinical importance and novel value of this work

An *in vitro* primary cell co-culture model to study the relationship between bacterial biofilms, NO and ciliary function has not been developed to date. This will be the first work assessing the role of ciliary function on NTHi biofilm development. If the study hypothesis is demonstrated to be correct it will highlight the potential importance of bacterial biofilms in PCD. This, in turn, will strengthen arguments for using anti-biofilm therapies in PCD patients as they become available.

This ALI co-culture model will also inform whether respiratory epithelial cells from patients with PCD are able to biosynthesis normal concentrations of NO. This is key to our understanding, and confidence, in using nasal NO measurements as a screen tool

in the diagnosis of PCD. This is particularly important as its use in this role is becoming more common in areas where access to full PCD diagnostic testing techniques is both difficult and costly. The model will also allow assessment of the comparative biosynthesis of CAMPs and cytokines in response to bacterial infection that may add to the fundamental understanding of the pathophysiology of the PCD.

While, due to the rarity of the condition, there are only limited numbers of patients with PCD this co-culture model is likely to be applicable more widely. For example, there is growing interest in protracted bacterial bronchitis (PBB) as a cause of chronic wet cough in children (Marchant, Masters et al. 2006; Craven and Everard 2013). The hypothesis for the development of the PBB being that a significant infection, possibly viral, leads to temporary impairment of mucociliary clearance which in turn allows bacterial biofilms to become established in the airway leading to a chronic wet cough (Everard 2012). It is further hypothesised that this, if left unchecked, may be the precursor to the development of bronchiectasis (Cole 1986; Craven and Everard 2013). If the study hypothesis were demonstrated to be correct it would lend strength to these hypotheses thereby supporting the aggressive treatment of PBB in children (Marchant, Masters et al. 2012). In addition this mechanism might also be important in the pathogenesis of other chronic respiratory conditions, such as chronic obstructive airways disease (Shaheen, Barker et al. 1994; Sethi 2000).

In the future the development of this model will allow further interactions between *H. influenzae*, and subsequently other bacteria, with primary differentiated respiratory epithelia to be studied, both using epithelia cells derived from PCD patients and from those with other respiratory conditions such as CF. In addition, this co-culture model could be used to study the interplay between respiratory viruses, bacterial biofilms and the respiratory epithelium. Finally, it will be a powerful model for developing and evaluating novel anti-biofilm therapies, for which there is an urgent and unmet need.

1.8 Summary

The normal function of cilia and the resultant mucociliary clearance plays a fundamental role in the innate immune system of the respiratory tract. Patients with PCD have ciliary ultrastructural defects that lead to recurrent sinopulmonary infection, due to the loss of function of the mucociliary escalator.

Bacterial biofilms are important in the pathophysiology of chronic suppurative respiratory diseases, due to the recalcitrance of bacteria in biofilms to antibiotics and

the host innate immune responses. Non-typeable *H. influenzae* is the commonest pathogen in non-CF bronchiectasis, chronic otitis media and in the local PCD population and, similarly to other respiratory pathogens such as *S. pneumonia* and *P. aeruginosa*, develops biofilms *in vitro* and *in vivo*.

The study hypothesis is that impaired ciliary function predisposes PCD patients to biofilm-associated infections. While authors have questioned the role of ciliary function and mucociliary clearance on biofilm development for over a decade there has been no published work considering this. A ciliated primary respiratory epithelial cell ALI co-culture model, with cells from patients with and without PCD, will therefore be developed with clinical NTHi isolates obtained from patients with PCD to investigate this.

This co-culture model would also allow assessment of the ability of respiratory epithelia of PCD patients to biosynthesis CAMPs, cytokines and, importantly NO in response to NTHi infection. Nitric oxide has diverse roles within the airways including bactericidal and biofilm dispersal activity. For reasons that have yet to be elucidated, PCD patients have extremely low levels of NO and this, alongside their impaired mucociliary clearance, may predispose them to biofilm infections. This project will help to clarify this by investigating firstly whether airway epithelial cells from PCD patients are able to biosynthesise normal levels of NO before considering its role on NHTi biofilm development.

1.9 Study hypothesis

Impaired ciliary function predisposes PCD patients to biofilm-associated infections and this may be exacerbated by the low NO levels seen.

1.10 Study Objectives

- Develop an ALI cultured primary respiratory epithelial cell co-culture model to investigate the role of impaired ciliary function in the development of NTHi bacterial biofilms.
    - Characterise and compare primary respiratory epithelial cell layers from PCD and non-PCD patients differentiated at an ALI.
    - Optimise the experimental design for an ALI cultured primary epithelial cell/NTHi co-culture model
    - Utilise the developed ALI cultured primary epithelial cell/NTHi co-culture model to investigate the study hypothesis
- Investigate NO biosynthesis by respiratory epithelia from both PCD and non-PCD patient cohorts and investigate its effect on NTHi biofilm development.

Chapter 2

Materials and Methods

2.1 Study participants

Patients with PCD were recruited from the national PCD centre in University Hospital Southampton (UHS), Southampton, UK. Samples were also obtained from healthy controls with no further characterisation or were patients seen in the PCD diagnostic service who were proven not to have the condition. Patients were diagnosed as having PCD in accordance with European consensus guidelines (Barbato, Frischer et al. 2009). This study was approved by Southampton and South West Hampshire Research Ethics Committee (A) (REC numbers: 06/Q1702/109 and 08/H0502/126). All subjects gave written informed consent. Details of PCD diagnostic investigation results for the PCD patients and the experiments that both their and the non-PCD patients samples were used for given in appendix 2.

2.2 Microbiology methods

2.2.1 *Haemophilus influenzae* confirmation

The diagnostic laboratory at the Health Protection Agency (UHS, Southampton, UK) cultured four non-typeable *H. influenzae* (NTHi) isolates from the sputum and cough swabs of four children with PCD. Isolates one and two were taken from children who either cultured NTHi for the first time or who had only had isolated it once in the previous three months as oppose to isolates three and four that were taken from children who had each recurrently isolated NTHi for four years.

2.2.1.1 Confirmation of bacterial purity

In order to check the purity of the four bacterial clinical isolates they were cultured separately in 10 ml of brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) supplemented with 10 μg/ml of haemin and β-nicotinamide-adenine dinucleotide (β-NAD) (both Sigma-Aldrich, Dorset, UK) to be referred to as supplemented BHI (sBHI). This was at 37°C with 5% carbon dioxide (CO_2), for approximately four hours, until the broth had an optical density 600 (OD_{600}) of 0.1, as measured by a using a Jenway 6300

spectrophotometer (Keison Products, Chelmsford, UK). 100 µl aliquots of each isolate were then streaked onto chocolate blood agar (CBA) plates (Oxoid, Basingstoke, UK). Chocolate agar is a non-selective growth medium where, as compared to blood agar, the red blood cells have been lysed, releasing the intracellular growth factors (haemin and NAD) required for *Haemophilus influenzae* culture. The plates were incubated inverted in a NuAire Autoflow CO_2 incubator (NuAire, Plymouth, USA) at 37°C with 5% CO_2 under sterile conditions overnight. Purity was confirmed by the consistency of the morphological appearance (shape and colour) of the colonies across the CBA plate when examined the following day.

2.2.1.2 Gram staining

This has been a standard microbiology technique to aid identification of bacterial species for over one hundred years (Gram 1884). Following purity checks (2.2.1.1), six to eight colonies from each isolate were re-suspended in sterile water then streaked onto glass slides and allowed to dry in a Holten LaminAir class II laminar flow cabinet. The samples were stained with 1% crystal violet (BDH Merck Ltd, Poole, UK) for one minute, washed, stained in iodine solution (BDH Merck Ltd, Poole, UK) for one minute, washed again then decolourised in acetone (Fisher-Scientific, Loughborough, UK), before finally washing again in water for five to ten seconds. The slides were then counter-stained in safranin red (BDH Merck Ltd, Poole UK) for one minute and washed, allowed to dry then examined by a Leica DMLB microscope (Leica Microsystems, Milton Keynes, UK).

2.2.1.3 V and X diagnostic disc testing

This is a standard method used by microbiologist to confirm that an isolate is *Haemophilus influenzae* (Jones 1982), utilising its requirement for both haemin and NAD to culture. The four NTHi clinical isolates were cultured in 10 ml sBHI until at an OD_{600} was 0.1 (2.2.1.1) and 100 µl aliquots were then streaked across a non-blood Columbia agar plate (Oxoid, Basingstoke, UK). This agar does not contain the growth factors haemin and NAD required for NTHi culture. Three diagnostic discs (disc X containing haemin, disc V containing NAD and disc X and V containing both (Oxoid, Basingstoke, UK)) were positioned equidistant from each other at 12, 4 and 8 o'clock respectively on the non-blood Columbia agar plate, which were then incubated at 37°C in 5% CO_2 overnight. NTHi colonies were confirmed by their growth in close proximity to the disc containing both factors V and X but not the discs containing factors V or X alone.

From these plates, six to eight NTHi colonies from each clinical isolate were separately cultured in 30 ml sBHI (2.2.1.1). When in exponential growth phase, determined by the optical density of the culture (2.2.2), glycerol was added to the NHTi suspension at a ratio of 1:3. This was then stored at -80°C in 1ml aliquots as the stock for each NTHi isolate to be used for subsequent experiments, each aliquot was freeze/thawed up to three times before being discarded.

Prior to any subsequent experiment, the frozen stock of the required NTHi isolate was cultured and streaked onto CBA agar and incubated overnight (2.2.1.1) to confirm bacterial colony morphology and purity, in order to control for contaminants.

2.2.1.4 Polymerase Chain Reaction (PCR)

In order to further confirm that the isolates were *Haemophilus influenzae*, PCR was undertaken on the isolates having each been cultured on polystyrene for one, three and seven days. The resultant bacterial colonies were washed, to remove non-adherent bacteria, were scraped and re-suspended in 1 ml sBHI (2.2.3.2). In addition, laboratory strains of both *Haemophilus influenzae* and *Moraxella catarrhalis* (generously gifted by Dr Stewart Clarke, HPA, Southampton, UK) were cultured for one day on polystyrene and used as positive and sham controls respectively. The purity of the adherent bacterial colonies for each isolate at each time point were confirmed by streaking 50 μl of this suspension on a CBA plate, incubating it overnight and examining it the following day for the consistency of the morphology of the colonies (2.2.1.1).

The bacterial suspension was centrifuged at 9,000 revolutions per minute (rpm) for five minutes to form a bacterial pellet and the DNA was then extracted using a QIAamp® DNA Miniprep kit (Qiagen, Crawley, UK) as per the manufacture's instructions. Briefly, bacterial pellets were re-suspended in 180 μl ATL lysis buffer containing 20 μl of proteinase K (to maintain bacterial DNA by degrading endogenous DNase and RNase enzymes) and incubated at 56°C for 15 minutes, 200 μl of AL lysis buffer was added and the mixture incubated at 70°C for a further ten minutes to cause bacterial cell lysis. 200 μl of ethanol was added in order to precipitate the DNA, which is insoluble to alcohols. After each addition the sample was vortexed. The mixture was loaded into a QIAamp mini spin column, which contained a silica membrane that absorbed the DNA binding it in the column, and centrifuged at 8,000 rpm for one minute. To purify the DNA it was rinsed in the mini spin column first by loading 500 μl of buffer AW1 and centrifuging at 8,000 rpm for one minute then loading 500 μl of buffer AW2 and centrifuging at 14,000 rpm for a further three minutes. After each spin the filtrate was discarded. Finally 200μl of buffer AE, elution buffer, was loaded to the

column to elute the purified DNA from the column. This was incubated at room temperature for one minute, centrifuged at 8000 rpm for one minute and the filtrate, containing the genomic DNA, was collected. The quality and volume of DNA was checked using a Nanodrop® ND-1000 (Thermo Scientific, Loughborough, UK) (260:280 ratios for the samples were between 1.8 and 2.2) and the DNA was then stored at -20^{0}C.

For *Haemophilus influenzae* samples, PCR reactions were carried out in a volume of 25 μl using reactions components from Bioline (London, UK). Each PCR reaction contained 2.5 μl 10x NH_4-based reaction buffer, 0.75 μl of 50 mM $MgCl_2$, 1 μl of 5 u/μl Biotaq red DNA polymerase, 2.5 μl of 10 mM deoxyribonucleotide triphosphates (dNTPs), 0.25 μl of 25 pmol *Haemophilus influenzae* forward primer (5'-GGAGTGGGTTGTACCAGAAGTAGAT-3') (Gok, Bulut et al. 2001), 0.25 μl of 25 pmol universal reverse primer (5'-AGGAGGTGATCCAACCGCA-3') (Gok, Bulut et al. 2001) (synthesized by Eurofins, Ebersberg, Germany), 15.75 μl of ultra high quality sterile water and 2 μl of DNA from each isolate. A PCR reaction containing PCR reaction components only, with no extracted DNA, and another reaction containing DNA extracted from the laboratory strain of *M. catarrhalis* as a sham template were used as negative controls. A PCR reaction containing DNA extracted from the laboratory strain of *Haemophilus influenzae* was used as a positive control. 36-cycle amplifications were run using a Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, Foster City, US) as follows; 30 seconds of denaturation at 95^{0}C, 30 seconds of annealing at 55^{0}C and 30 seconds of elongation at 72^{0}C.

A gel of 2.5% agarose was prepared in tris acetate ethylenediaminetetraacetic acid (EDTA) buffer with 10 μl ethidium bromide (all Sigma-Aldrich, Dorset, UK). Two microlitres of loading buffer (made up from 0.4 g sucrose, 0.25 mg bromo-phenol blue in 1ml ultra high quality water) was added to 10 μl of each PCR product, which were then loaded into the wells in the agarose gel (all Sigma-Aldrich, Dorset, UK). PCR fragment band sizes were compared against a DNA hyper V ladder (Bioline, London, UK) loaded into lane one of the gel. The products were then electrophoretically separated using a Bio-Rad gel electrophoresis apparatus (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) in a tris acetate-EDTA buffer (Sigma-Aldrich, Dorset, UK) at 90-95 mV for approximately one and a half hours then visualised using a M-20 UV transilluminator (UVP, Cambridge, UK).

2.2.2 Kinetics of non-typeable *H. influenzae* isolates growth in planktonic culture

Before assessing the biofilm-forming ability of the four NHTi clinical isolates their growth kinetics were compared in planktonic culture, to establish the kinetics of the exponential growth phase. Having checked the purity of the NTHi isolates (2.2.1.1) six to eight colonies of each isolate were inoculated in warm (37^{0}C) sBHI broth and incubated at 37^{0}C in 5% CO_2. Optical density at 600nm (OD_{600}) readings were taken every 30 minutes for seven hours using a Jenway 6300 spectrophotometer (Keison Products, Chelmsford, UK). This was performed in duplicate two to four times.

Regression analysis was performed to establish the OD_{600} equivalent to a number of bacterial colony forming units (CFU), as previously reported (Hall-Stoodley, Nistico et al. 2008). Following purity checks (2.2.1.1) each NTHi clinical isolate was inoculated in sBHI and incubated at 37^{o}C in 5% CO_2. The exponential growth phase was demonstrated, from the previous experiment, to start at approximately three and a half hours for each isolate. Therefore at four, five and six hours, during this phase of growth, the OD_{600} was measured and 100 μl of the broth was serial diluted to 10^{-8} for each of the isolates, in order to measure the CFU counts. Five 20 μl drops of the 10^{-4} to 10^{-8} dilutions for each isolate were pipetted onto CBA plates, allowed to dry, inverted and cultured overnight at 37^{o}C with 5% CO_2. The following day CFUs/ml of the original inoculum were calculated for the corresponding OD_{600} at the three time points. This allowed calculation of a regression curve of CFUs –v- OD_{600} values for each isolate. Each experiment was undertaken in duplicate two to three times.

2.2.3 Quantification of biofilm development on polystyrene

2.2.3.1 Crystal violet assay

Crystal violet (CV) is a cationic dye that binds to the bacterial cell surface and has been used widely in the literature to quantity biofilm formation (Hall-Stoodley, Nistico et al. 2008). In brief, 10^7 CFUs of each NTHi isolate in 100 μl of sBHI were loaded into 24 wells (six for each isolate) of two flat-bottomed 96-well plates (Nunc, Fisher-Scientific, Loughborough, UK). These were incubated for one and three days respectively at 37^{0}C with 5% CO_2, with the sBHI being replaced daily. The wells were then washed to remove non-adherent bacteria, 150 μl of 0.5% CV solution (BDH Merck Ltd, Poole, UK), was added for 15 minutes then washed six times with 200 μl of phosphate buffered solution (PBS, Gibco, Life Technologies, Paisley, UK). The CV was solubilised from the adherent NTHi bacterial colonies in ethanol, 200 μl of 100% ethanol was loaded on the

wells for five minutes, this solution was then removed and placed in a fresh 96-well plate and the OD of each well was read using a Spectra Max 340 PC plate-reading spectrophotometer (Molecular Devices, Sunnyvale, US). Six wells containing sBHI only (without bacteria) were used as negative control wells and the mean background absorbance of these wells subtracted from the test well values. The experiment was repeated on two occasions on separate days.

2.2.3.2 Biofilm colony forming units counts

This method has been widely used to quantify the number of viable bacteria in a bacterial biofilm (Hall-Stoodley, Nistico et al. 2008). In brief, approximately $2x10^8$ CFUs, corresponding to 1 ml of cultured sBHI at an OD_{600} 0.1, of each NTHi isolate were loaded into 6-well plates (Corning Life Sciences, Fisher-Scientific, Loughborough, UK) in 4 ml of sBHI, this was repeated in triplicate. The bacteria were incubated at 37°C with 5% CO_2 and adherent bacteria were quantified at one, three and seven days. Two millilitres of the sBHI was replaced daily until the endpoint was reached. To quantify CFUs each well was washed twice with 2 ml of sBHI to remove non-adherent bacteria. One millilitre of sBHI was then added and the adherent NTHi bacteria liberated from the well surface using a sterile cell scraper (Fisher-Scientific, Loughborough, UK). This suspension was vortexed three times for 30 seconds, to break up any clumps/colonies of bacteria, serially diluted, plated onto CBA plates, incubated overnight and the number of CFUs counted the following day (2.2.2). As mentioned, each isolate was cultured in triplicate for each time point and the experiment repeated on three separate occasions.

2.2.3.3 Scanning electron microscopy of bacterial biofilms

To visualise the adherent bacterial colonies and the extracellular polymeric substance matrix surrounding them, an optimised fixation method was used (Erlandsen, Kristich et al. 2004) and the samples then processed for scanning electron microscopy (SEM). 10 mm sterile coverslips were placed in the base of 6-well plates and each NTHi clinical isolate grown on top of them for up to seven days (2.2.3.2). The NTHi bacterial colonies were then washed twice with 2 ml of sBHI to remove non-adherent cells then the coverslips were removed from the 6-well plates with the adherent NTHi bacteria on their surface. The samples were fixed for 1 hour with 3% glutaraldehyde with 0.15% alcian blue in 0.1 M cacodylate buffer at pH 7.2 (all Agar Scientific, Stanstead, UK). They were then rinsed twice in a 0.1 M cacodylate buffer at pH 7.2 for ten minutes. This was the buffer used for all subsequent buffer rinses. Samples were post-fixed in 1% osmium tetroxide (Sigma-Aldrich, Dorset UK) in 0.1 M cacodylate buffer at pH 7.2

for one hour, followed by two further ten minutes buffer rinses. The samples were dehydrated by placing them through a series of ethanol solutions diluted to 30%, 50%, 70%, 95% in water and 100% for 20 minutes each. All steps were carried out at room temperature. The samples were critical point dried using a Balzers CPD 030 critical point dryer (BAL-TEC, Liechtenstein). This is a process achieved by exchanging 100% ethanol for liquid carbon dioxide then heating to 31°C under pressure at 73.9 bar (the critical point for carbon dioxide) to enable instantaneous drying and thereby minimising surface tension effects on the sample. Finally the samples were sputter coated with a gold/palladium mix, using an E5100 sputter coater (Polaron, UK). This process covers the samples in an ultra-thin coating of this conducting alloy, inhibiting charging of the specimen and increasing secondary electron emission therefore increasing the signal to noise ratio. The samples were then examined using a FEI Quanta 200 scanning electron microscope (SEM) (FEI company, Eindhoven, The Netherlands)

2.2.3.4 Fluorescence in situ hybridisation on bacterial biofilms

Fluorescence *in situ* hybridisation (FISH) was undertaken to quantify the adherent bacterial colonies further using a similar method to that previously utilised on *Streptococcus pneumoniae* (Hall-Stoodley, Nistico et al. 2008). The technique employs a fluorescent probe to detect and localise specific RNA targets which were then visualised using confocal laser scanning microscopy (CLSM), allowing depth and volume of biofilm to be analysed (Hall-Stoodley, Hu et al. 2006). Each NTHi clinical isolate was cultured on a MatTek glass bottom plate (MatTek, Ashland, UK) for three days with daily changes of sBHI (2.2.3.2). The adherent NTHi bacterial colonies were fixed in 1.5 ml 4% paraformaldehyde (Sigma-Aldrich, Dorset, UK), for one hour, washed in 2 ml PBS at 4°C for one hour then stored until processed for FISH in 2 ml 50% ethanol/PBS mix at -20°C to permeabilise the bacteria, thereby allowing target accessibility.

Four hundred microlitres of the NTHi FISH probe, diluted 1 in 10 with hybridisation buffer, was then applied to the adherent bacterial colonies in the MatTek plate and incubated for two hours at 46°C. The FISH probe was an NTHi 16S ribosomal RNA probe (16S position 185, 5'-CY5-CCGCACTTTCATCTTCCG-3', Integrated DNA Technologies, Leuven, Belgium) and its specificity had previously been validated (Hall-Stoodley, Hu et al. 2006). The hybridization buffer contained 360 μl 5M sodium chloride (NaCl), 40 μl 1M Tris-hydrochloride (Tris-HCL) pH 8.0, 400 μl of formamide (making it 20% formamide buffer) and was made up to 2 ml with milli-Q (MQ) water before addition of 2 μl of 10% sodium dodecyl sulphate (SDS) (all Sigma-Aldrich,

Dorset, UK). This step allows the CY5 tagged FISH probe to specifically bind to the NTHi 16s ribosomal RNA in the adherent bacterial colonies. One millilitre of a FISH wash buffer (made up with 1 ml 1M Tris-HCL added to 2150 μl 5M NaCl and 500 μl EDTA made up to 50 ml with MQ water before the addition of 50 μl 10% SDS, all Sigma-Aldrich, Dorset, UK) was used to remove any unhybridised NTHi FISH probe and hybridisation buffer. This was then replaced with 1 ml fresh FISH wash buffer and incubated for 15 minutes at 48°C and finally washed with MQ water. An 80% glycerol solution was applied over the adherent bacterial colonies as a mountant and they were examined using an inverted Leica SP5 CLSM (Leica Microsystems, Milton Keynes, UK).

2.2.4 Assessment of antibiotic recalcitrance

2.2.4.1 Minimum inhibitor concentrations (MIC) of bacteria in planktonic phase growth

Having checked the purity of the four NTHi clinical isolates (2.2.1.1) each was grown in sBHI broth until at an OD_{600} 0.1, corresponding to approximately $2x10^8$ CFUs/ml (2.2.2). For each isolate, 1 ml of this NTHi bacterial suspension was serially diluted and plated on CBA plates, incubated overnight and CFUs were counted the following morning (2.2.2). This was taken as the baseline CFU count for that isolate. To each isolate, increasing concentrations of cefotaxime (Bowmed Ibisqus Ltd, Wrexham, UK) (10-fold dilutions from 200 mcg/ml to 2 ng/ml, made up in sBHI, with a no antibiotic control) were added to separate 1 ml aliquots in 1.5 ml ependorfs (Fisher-Scientific, Loughborough, UK) of the NTHi bacterial suspension, which were then incubated overnight at 37°C with 5% CO_2. The following day, 20 hours later, the antibiotics were removed by twice pelleting the bacteria, centrifuging at 9000 rpm for 5 minutes, and re-suspending in 1 ml of BHI. Each bacterial suspension was then serially diluted and plated on CBA plates, incubated overnight and CFUs were counted the following morning (2.2.2). The minimum inhibitor concentration (MIC) for each isolate in planktonic phase growth was taken as the lowest antibiotic concentration that had a lower CFU count than baseline. These experiments were performed in duplicate and on two or three separate occasions dependent on isolate.

The MIC for the clinical NTHi isolates were confirmed using MIC evaluator (M.I.C.E ™) strips (0.002 mcg – 32 mcg) (Oxoid, Basingstoke, UK), a validated technique to measure the MIC (Mushtaq, Warner et al. 2010), as per the manufacturers instructions. In brief, each NTHi clinical isolate was grown to an OD_{600} 0.1 (2.2.2) then 100 μl aliquots were spread uniformly across separate CBA plates using a sterile spreader (Fisher-Scientific, Loughborough, UK). The M.I.C.E™ strips were then applied to the CBA

plates, placing them on top of the layer of bacteria. These were incubated overnight at 37°C in 5% CO_2 and read the following morning, as per the manufacturers instructions. Each was performed in duplicate on separate occasions.

2.2.4.2 Minimum inhibitor concentrations (MIC) of bacteria in biofilm phase growth

The four NTHi isolates were separately cultured in 12-well culture plates (Corning Life Sciences, Fisher-Scientific, Loughborough, UK) (2.2.3.2), with $2x10^8$ CFUs in 2 ml sBHI per well and eight wells utilised for each isolate. The plates were incubated at 37°C with 5% CO_2 for three days, with daily changes of 1 ml sBHI. On day three all NTHi bacterial colonies were washed twice with 1 ml sBHI, to remove non-adherent bacteria. A single well for each of the four isolates was then scraped, re-suspended in 1 ml BHI, vortexed, serially diluted and plated (2.2.3.2). These were incubated overnight at 37°C with 5% CO_2 and the CFUs counted the following morning to quantify the number of CFUs present for the NTHi isolate at baseline. For each isolate, increasing concentrations of cefotaxime (Bowmed Ibisqus Ltd, Wrexham, UK) (10-fold dilutions from 200 mcg/ml to 2 ng/ml, made up in 2 ml sBHI, with a no antibiotic control) were separately added to the remaining seven wells, which were then incubated overnight at 37°C with 5% CO_2. The following day, 20 hours later, the antibiotic was removed and the bacterial colonies washed twice with 2 ml BHI to remove both residual antibiotic and non-adherent bacteria. One millilitre of sBHI was then added and the adherent NTHi bacterial colonies liberated from the well surface using a sterile cell scraper (Fisher-Scientific, Loughborough, UK), vortexed, serially diluted and plated onto CBA plates (2.2.3.2). These were incubated overnight at 37°C with 5% CO_2 and the number of CFUs counted the following day. The minimum inhibitor concentration (MIC) for the adherent bacterial colonies for each isolate was taken as the lowest antibiotic concentration that had a lower CFU count than baseline. These experiments were performed in duplicate and on two separate occasions.

2.3 Cell culture and co-culture methods

2.3.1 Cell culture

2.3.1.1 Human bronchial epithelial cell line culture (16HBE14o-)

16HBE14o- cells are an adenovirus SV40 transformed epithelial cell line derived from human bronchial epithelial cells (gifted by Dr D Gruenert, University of California, San

Francisco) from now on referred to as 16HBE cells. 16HBE cells were cultured to confluence in T75 flasks (Corning Life Sciences, Fisher-Scientific, Loughborough, UK) in supplemented minimum essential medium (sMEM) (MEM with 10% fetal calf serum, 1% L-glutamine (200 mM), 1% penicillin (50 units/ml) streptomycin (50 μg/ml) (all Gibco, Life Technologies, Paisley, UK) and 0.2% nystatin (100 μg/ml) (Sigma-Aldrich, Dorset, UK). The 16HBE cells were passaged in the standard way, the sMEM was replaced with 6 ml of Hank's buffered salt solution (HBSS, Gibco, Life Technologies, Paisley, UK) and incubated for ten minutes at 37°C with 5% CO_2. This step removes any serum in the sMEM that may effect the action of the disassociation agent, trypsin. This was replaced with 3 ml of 0.25% trypsin/EDTA (Gibco, Life Technologies, Paisley, UK) and incubated for three minutes at 37°C with 5% CO_2. The flask was knocked to encourage the cells to dislodge in the flask, incubating for longer if less than 90% of the cells had detached, in which case the degree of detachment was monitored every 30 seconds. Ten millilitres of sMEM was then added and the cells pelleted, by centrifuging at 1000 relative centrifugal force (rcf) for 5 minutes. The supernatant was removed and the cell pellet re-suspended in 5 ml sMEM. Dr Claire Jackson, PCD Group Scientist, had previously established this protocol in our Department. The cells were counted using a haemocytometer chamber (Weber Scientific International, Hamilton, US).

Transwell 12-well plate inserts (Corning Life Sciences, Fisher-Scientific, Loughborough, UK) were pre-coated with 100 μl 1:10 PureCol collagen (Nutacon, Leimuiden, The Netherlands), final concentration 0.3 mg/ml, diluted in sterile ultra high quality (UHQ) water for one hour at room temperature. The PureCol was removed and the transwell left to dry in a Holten LaminAir class II laminar flow cabinet (Fisher-Scientific, Loughborough, UK). $4x10^5$ 16HBE cells were loaded on the apical surface of the coated transwells in 500 μl sMEM with 700 μl sMEM added to baso-lateral surface. These were left until confluent, with alternate day changes of media, for one week then the apical media was removed to allow an air-liquid interface (ALI).

2.3.1.2 Primary respiratory air-liquid interface cell culture

Respiratory epithelial cells were cultured at an ALI until differentiated and ciliated as described previously (Hirst, Jackson et al. 2014) (Figure 9). Dr Claire Jackson, PCD Group Scientist, had previously established this protocol in our Department. The epithelial cells were obtained from patients by brushing the nasal epithelium once, or twice if tolerated, with a cytology brush (Olympus Keymed Ltd, 2 mm diameter, Southend, UK). This method has been reported to yield approximately $1x10^6$ cells with a median viability of 42% (Stokes, Kieninger et al. 2014). The brushes were placed in 1.5 ml bronchial epithelial growth medium (BEGM) supplemented with SingleQuots

(Clonetics™, Lonza, Castleford, UK) (bovine pituitary extract (2 ml); hydrocortisone (0.5 ml); human epidermal growth factor (hEGF) (0.5 ml); epinephrine (0.5 ml); transferrin (0.5 ml); insulin (0.5 ml); retinoic acid (0.5 ml); triiodothyronine (0.5 ml); GA-1000 (0.5 ml) per 500 ml BEGM) with 1% penicillin/streptomycin (Gibco, Life Technologies, Paisley, UK) and 0.2% nystatin (Sigma-Aldrich, Dorset, UK) (from now on termed BEGM+) in a 5 ml sample tube (BD Falcon, VWR International, Magna Park, UK) and transferred to the laboratory. The epithelial cells were removed from the cytology brush with vigorous pipetting then the suspension was centrifuged to pellet the cells. In this protocol all centrifugation was carried out at 1400 rpm for 7 minutes at 10°C. The supernatant was carefully removed, the epithelial cells re-suspended in 2 ml BEGM+ and seeded onto a single PureCol coated 12-well plate well (Corning Life Sciences, Fisher-Scientific, Loughborough, UK). The 12-well plate was incubated at 37°C in 5% CO_2 and 100% humidity and the media replaced on alternate days to allow cell expansion.

Once 80-90% confluent (usually after five to seven days) the cells were passaged. The BEGM+ was removed and 0.5 ml of trypsin/EDTA added for 1-2 minutes at 37°C in 5% CO_2. Once rounded up, the cells were pipetted off and transferred into 7 ml BEGM+ in a 15 ml centrifuge tube (Fisher-Scientific, Loughborough, UK). The cells were centrifuged, re-suspended in 1 ml BEGM+, centrifuged again, re-suspended in 5 ml BEGM+ and seeded into a PureCol coated T25 culture flask (Corning Life Sciences, Fisher-Scientific, Loughborough, UK).

The flask was incubated at 37°C in 5% CO_2 and 100% humidity until 80-90% confluent (usually a further five to seven days). The cells were then passaged, using the same technique as the first passage except that following the first centrifugation the cell pellet was re-suspended in 1 ml ALI media, centrifuged again then re-suspended in a further 1 ml ALI media. ALI media was similar to BEGM+ except that it was made up from 1:1 BEGM: Dulbecco's modified eagle medium (DMEM) (Gibco, Life Technologies, Paisley, UK) and only 1/5th of the SingleQuot hEGF concentration was used. 0.5 ml of the cell suspension was then seeded onto the apical surface of each of two PureCol coated transwells 12-well plate inserts. 1 ml ALI media was then loaded into the well under the transwell insert, hence covering the baso-lateral surface.

The 12-well plate, with transwell inserts, was incubated at 37°C in 5% CO_2 and 100% humidity until the cells were 100% confluent (usually one to two days). The apical media was then removed to allow an ALI and the baso-lateral media was replaced with 650 μl ALI media supplemented with 100 nM retinoic acid to encourage differentiation (Sigma-Aldrich, Dorset, UK) (ALI+). This basolateral ALI+ media was replaced three

times per week. Ciliation was confirmed by light microscopy (typically occurring approximately 21 days later). The ALI cultures were utilised for experiments when they were deemed to be well ciliated, evidenced by ciliary movement across the cell layer with evidence of mucociliary clearance if debris present. These cultures were found to be on average 10.1% (5.7 - 27.6) (median, min-max) ciliated once further assessment was undertaken (2.3.3.8).

Low passage numbers were used by this method to reduce the possibility of cellular changes as compared to the *in vivo* epithelial cells. However, in doing so, this limited the number of epithelial cells and hence ALI cultured epithelial cell layers produced.

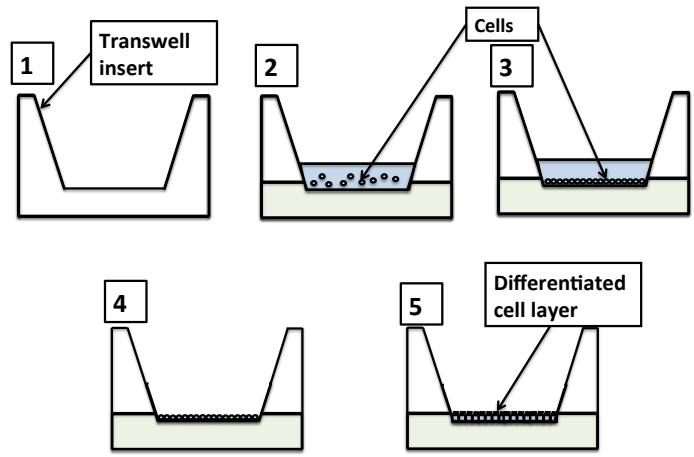

Figure 9 *- Simplified schematic outlining the method for re-differentiation of basal primary airway epithelial cells on air-liquid interface (ALI) culture. 1) 12-well plate well with Costar® transwell insert; 2) primary basal epithelial cells were applied to top of transwell in ALI media and ALI media also applied basolaterally; 3) basal cells adhere to transwell membrane and become confluent; 4) ALI media removed from apical surface of cell layer and basolateral media supplemented with retinoic acid to encourage differentiation; 5) cell layer differentiates and ciliates.*

The number of respiratory epithelial cells present on an ALI membrane when fully confluent was measured using a haemocytometer chamber. This was measured on five separate ALI cell cultures and the mean used to inform the multiplicity of infection estimations in the subsequent co-culture experiments (2.3.2).

2.3.1.2.1 Characterisation of primary air-liquid interface cell cultures

For characterisation of the primary ALI cultured ciliated epithelial cell layers, derived from both PCD and non-PCD patients, the ALI+ media was replaced on the baso-lateral surface with 900μl ALI media and 500μl ALI media was applied to the apical surface of the cells; then incubated at 37^{0}C in 5% CO_2 and 100% humidity for 24 hours. Trans-epithelial electrical resistance, ciliary beat frequency (CBF) and ciliation by fast Fourier transform (FFT) measurements were then taken (2.3.3.1, 2.3.3.7 and 2.3.3.8.2 respectively). The baso-lateral and apical supernatants were collected, aliquoted and frozen at -80°C and the baso-lateral media then replaced with 650 μl ALI+ media. These supernatants were used to measure constitutive cationic antimicrobial peptide (CAMP) and cytokine release by the ALI cultured cell layers (2.3.3.10 and 2.3.3.11 respectively). Total nitric oxide concentration was measured from the apical surface (2.4.2.1). In addition, transwell inserts from a number of PCD and non-PCD ALI culture respiratory epithelial cell layers were cut from their transwell insert membranes. Once removed these membranes were circular in shape and were then divided into sections from the centre. These sections were then processed for localisation of the three isoforms of nitric oxide synthase (NOS) (2.4.2.5.2), assessment of ciliation by b-tubulin antibody labelling (2.3.3.8.1), SEM (2.2.3.5), periodic acid-Schiff (PAS) staining to assess the amount of mucus producing cells in the cell layers (2.3.3.9) and 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-fm Diacetate, Molecular Probes®, Life Technologies, Paisley, UK) labelling to corroborate nitric oxide biosynthesis (2.4.2.2).

2.3.2 NTHi bacterial co-culture on ALI cultured ciliated respiratory epithelial cell layers

Differentiated, ciliated ALI cultured respiratory epithelial cell layers were produced (2.3.1.2). The baso-lateral media of the ALI cultured primary respiratory epithelial cell layers was replaced with 900 μl antibiotic free ALI+ media two days prior to co-culturing experiments. TER, CBF, ciliation by FFT and total NO measurements were taken at baseline for each sample (2.3.3.1, 2.3.3.7, 2.3.3.8.2 and 2.4.2.1 respectively). Baseline baso-lateral and apical supernatants, incubated with the ALI cultured cell layers for 24 hours were collected for each sample, aliquoted and frozen at -80°C, these were subsequently used to measure cytokine and CAMP concentrations at baseline respectively (2.3.3.10 and 2.3.3.11 respectively). 16 HBE cells, cultured at an ALI on transwell inserts, were used for comparison, TER and total NO measurements were taken at baseline and apical and baso-lateral supernatants collected.

NTHi isolate four, demonstrated to be the best biofilm forming isolate (4.3), was used for co-culturing experiments. The isolate was cultured in sBHI (2.2.2) until at an OD_{600} 0.1 (equivalent to $2x10^8$ CFUs/ml) then pelleted and re-suspended (dependent on the required concentration) in antibiotic free ALI media. The bacteria CFU/ml concentration was dependent on the multiplicity of infection (MOI, ratio of bacteria CFUs to epithelial cells) required for the individual experiment, which varied from 0.1 to 200. 500 μl of NTHi isolate four suspension in ALI media was place on the apical surface of the ALI cultured respiratory epithelial cells which were then incubated at 37°C in 5% CO_2 in 100% humidity for the desired time-course.

Daily TER, CBF and FFT measurements, to assess ciliation, were obtained and baso-lateral and apical supernatants collected, aliquoted, frozen at -80°C and replaced with fresh media, 900 μl in baso-lateral compartment and 500 μl on apical surface. Removing and replacing the apical surface media also served as a wash to remove non-adherent bacteria.

On the final day of a co-culturing experiment measurements were made and supernatants collect as above. In addition, the ALI cultured epithelial cell layers were washed twice with 500 μl of ALI media, to remove non-adherent bacteria, a total NO measurement taken (2.4.2.1) and the basal side of the transwell membrane was scraped, to remove any bacteria adherent to the underside of the membrane. The ALI transwell insert membranes, with the epithelial cell layer attached, were then cut out from the transwell inserts. These membranes were circular in shape and were divided into sections from the centre (2.3.1.2.1); half the membrane used to measure NTHi bacterial CFU counts of bacteria adherent to the epithelial cells (2.3.3.3), and a quarter each fixed for FISH (2.3.3.4) and SEM (2.2.3.5). The co-culture technique and experiment was developed as part of this work.

2.3.3 Characterisation of ALI cultured ciliated respiratory epithelial cell layers at baseline, during and after co-culture experiments with NTHi

The characterisation and co-culture experiments were run (as described in 2.3.1.2.1 and 2.3.2 respectively.) Specific assays and measurements were performed either at pre co-culture, daily during or at the desired end point of the experiment as described below.

2.3.3.1 Trans-epithelial electrical resistance

Trans-epithelial electrical resistance (TER) is a well-recognized surrogate measure of tight junction formation and hence cellular integrity and health (Xiao, Puddicombe et al. 2011). Corrected TER across an ALI cultured primary epithelial cell layer is reported to range from 150-1000 Ohms.cm^2 (Xiao, Puddicombe et al. 2011). TER measurements were taken at baseline, prior to co-culturing experiments, and daily during them, using EVOM 2 epithelial voltohmmeter (World Precession Instruments, Sarasota, US) as per the manufacturers instructions. The TER probe has two measuring prongs; the longer of which was placed down the side of the transwell insert, into the baso-lateral media, and the short prong into the apical media over the epithelial cell layer. This allowed measurement of the resistance across the ALI cultured epithelial cell layer. Three measurements were taken across each cell layer daily and the mean of these used, after correcting for the TER of the transwell membrane alone without a cell layer (170 Ohms) and converting into Ohms.cm^2. The correlation of variation between these three TER readings was low, varying from 0.07 to 0.009.

2.3.3.2 Propidium iodide

Propidium iodide (PI) is an intercalating agent that is fluorescent when bound to DNA. It is impermeant to viable cells and hence is often used as a marker of cell viability as will only become fluorescent when the cell membrane is compromised. PI was therefore assessed for use as a measure of the epithelial cell layer viability. However the adherent NTHi bacteria became fluorescent in the presence of PI, presumably due to the presence of extracellular DNA, known to be a constituent of the extracellular polymeric substance matrix of bacterial biofilms (Wei and Ma 2013). It was not possible to easily differentiate between extracellular DNA in the biofilm and the underlying non-viable cells and this method of assessing cell viability was therefore not taken forward.

2.3.3.3 Biofilm CFUs

Half of the transwell insert membrane from each of the epithelial cell layers of interest, cut from the transwell at the endpoint of the co-culture experiment, was used to measure the CFU counts of adherent NTHi bacteria (2.3.2). The washed sections of cell membrane, with overlying epithelial cell layer and any adherent bacterial, were individually placed in a 12-well plate in 500 μl BHI and were both vigorously scraped with a sterile cell scraper (Fisher-Scientific, Loughborough, UK) and pipetted to dislodge the cells and bacteria from the membranes. The pipetting broke up bacterial

cell clusters to form a suspension and, in order to further break up any clumps of bacteria noted on visual inspection, the suspension was transferred into 1.5 ml ependorfs (Fisher-Scientific, Loughborough, UK) and was vortexed three times for 30 seconds each time. If any clumps remained further pipetting was undertaken until these were not evident. The suspension was then serially diluted, plated onto CBA plates and incubated overnight at 37^{0}C in 5% CO_2 and CFUs were counted the following day (2.2.3.2).

2.3.3.4 Fluorescence in situ hybridisation with fluorescence immunocytochemistry labelling with β-tubulin

Fluorescence *in situ* hybridisation was performed on a washed section of the transwell insert membrane, from each cell layer of interest, cut from the transwell at the endpoint of the co-culture experiment (2.3.2). Sections of cell membrane, with overlying epithelial cell layer and any adherent bacterial, were individually placed in a 24-well plate (Corning Life Sciences, Fisher-Scientific, Loughborough, UK). The samples were fixed in 1.5 ml 4% paraformaldehyde for one hour, washed in 1.5 ml PBS for one hour (both at 4^{o}C) then stored in 1.5 ml 50% ethanol/PBS mix at -20^{0}C to permeabilise the bacteria, thereby allowing target accessibility, until processed for FISH. FISH processing was carried out as described in 2.2.3.4 except the ALI membrane sections were processed in a 24-well plate.

Once FISH processing was complete, immunohistochemistry techniques were used to label β-tubulin, a fundamental protein component of the ciliary axoneme, present on the cell layers. This processing was carried out by placing the membrane section, inverted onto 100 μl droplets of each of the solutions from the protocol in turn on a wax surface, hence exposing the cell layer to the solutions. Initially the membranes were placed on a droplet of blocking medium (80 ml DMEM (Sigma-Aldrich, Dorset, UK), 20 ml newborn bovine serum (Fisher-Scientific, Loughborough, UK) with 1 g BSA and 0.05% Triton-X) for 30 minutes, in order to reduce binding to non-specific proteins. The samples were then moved through a wash step, the primary antibody (applied for 90 minutes), a wash step then the secondary antibody (applied for a further 90 minutes) then a further wash step. The primary antibody was a mouse monoclonal antibody to β-tubulin (Clone 2-28-33, product no. T5293, Sigma-Aldrich, Dorset, UK) diluted 1:2000, the secondary antibody was an Alexa Fluor® 594 chicken anti-mouse IgG antibody (dilution 1:500) (Code A21201, Molecular Probes®, Life Technologies, Paisley, UK), both were diluted in PBS with 0.05% Triton-X and 1% BSA. Each wash step involved placing the inverted membranes on 100 μl droplets of PBS (Gibco, Life Technologies, Paisley, UK) with 0.05% Triton-X and 1% bovine serum

albumin (BSA) (both Sigma-Aldrich, Dorset, UK) three times for five minutes. The samples were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Life Technologies, Paisley, UK), a nuclear counterstain diluted 1:360, for 30 minutes before being washed and mounted on slides. All steps were carried out at room temperature.

For analysis, every field of view in a strip across the sample, from the edge to the centre, were imaged using a Leica SP5 CLSM (Leica Microsystems, Milton Keynes, UK) at x400 magnification. The volume of the adherent NTHi bacterial colonies on the epithelial cell layers in each field of view were calculated using Volocity 3D image analysis software (version 6.0.1, PerkinElmer, Coventry, UK).

2.3.3.5 Scanning electron microscopy

Once the washed ALI transwell insert membrane was removed and divided into sections (2.3.2) a section from each cell layer of interest was fixed and processed for SEM in a 24-well plate (as described in 2.2.3.3).

2.3.3.6 Transmission electron microscopy

Once the washed ALI transwell insert membrane was removed and divided into sections (2.3.2) a section from each cell layer of interest was fixed and processed for TEM. The sections were individually placed in a 24-well plate and initially fixed with 1.5 ml 3% glutaraldehyde with 0.15% alcian blue in 0.1 M cacodylate buffer pH 7.2 for 15 minutes at room temperature before a further hour at 4°C (all Agar Scientific, Stanstead, UK). The fix was discarded and the samples washed twice in 2 ml 0.1 M cacodylate buffer at pH 7.2, ten minutes for each wash. This buffer was used for subsequent buffer washes. 1.5 ml 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 post-fix was then applied for one hour, followed by two further buffer washes (all Agar Scientific, Stanstead, UK). The specimens were washed in distilled water for one minute to remove excess phosphate ions to prevent uranyl acetate precipitation. 1.5 ml 2% aqueous uranyl acetate (Agar Scientific, Stanstead, UK) was applied for 30 minutes and the samples were then dehydrated by placing them through a series of ethanol solutions diluted to 30%, 50%, 70%, 95% in water and 100% for 20 minutes each. 1.5 ml acetonitrile (BDH, Poole, UK) was applied for 30 minutes then replaced with 1.5 ml 50:50 acetonitrile:Spurr resin (Agar Scientific) mix and incubated overnight at 4°C. The following day this was replaced with Spurr resin alone for 6 hours then embedded in fresh Spurr resin and polymerised at 60°C for 17 hours. All processing was carried out at room temperature, unless otherwise stated. Specimens were cut

using a Ultracut E (Reichert-Jung, Vienna, Austria) counterstained with Reynold's lead citrate stain then and examined using a FEI Tecnai 12 TEM @ 80kV (FEI company, Eindhoven, The Netherlands)

2.3.3.7 Measurement of ciliary beat frequency

Ciliated epithelial cell layers were cultured at an ALI on transwell inserts (2.3.1.2.1 and 2.3.2). The 12-well plate, containing the transwell inserts of interest, was transferred to an environmental chamber (Solent Scientific Ltd, Segensworth, UK) surrounding an x600 inverted IX71 phase contrast microscope (Olympus, London, UK) that had been pre-warmed to 37°C. Videos of ciliary beating on each cell layer were obtained using a Fastcam MC2 high-speed video camera (Photron, West Wycombe, UK) with Photron Fastcam Viewer software (version 2.4.3.3, West Wycombe, UK). The videos were recorded at 250 frames per second (fps) then played back at 30 fps and the number of frames equivalent to ten ciliary beats recorded. The ciliary beat frequency (CBF) was then calculated by dividing this by ten (to give frames needed to achieve one beat) and multiplying by 0.004 (time in seconds for one frame) to give seconds per ciliary beat (x). This number was inverted (1/x) in order to give the CBF in Hertz (Hz). Six measurements were obtained at random across each cell layer and the mean used as the CBF for that cell layer at that time point. This has been standard used by the national PCD diagnostic for ALI cultured cell layers for many years. Previously ten CBF measurements were taken however it was demonstrated that six measurements were adequate and this has been validated across the three National PCD diagnostic centres.

2.3.3.8 Assessment of ciliation

Two methods were used to assess the surface area of the primary ALI cultured epithelial cell layers that was ciliated. The first, undertaken on fixed cells, used immunohistochemistry techniques with β-tubulin antibody labelling, a fundamental component of the ciliary axoneme. It did not require ciliary beating for assessment to be made thereby allowing comparison between PCD and non-PCD epithelial cell layers but, obviously, could not be undertaken on live cells. The alternative method used a Fast Fourier Transform (FFT) algorithm with high-speed video microscopy on live cell layers. This required ciliary beating and was therefore not possible on PCD cell layers with static cilia. This technique was used to assess the effect of co-culture with NTHi on percentage ciliation in the non-PCD epithelial cell layers.

2.3.3.8.1 Method 1 - Fluorescence immunocytochemistry labelling with β-tubulin

This technique uses a primary antibody to bind to the epitope of interest, in this case β-tubulin, a fundamental protein component of the ciliary axoneme. A secondary antibody, tagged with a specific fluorophore, was then bound to the primary antibody allowing the epitope to be visualised under a fluorescence microscope.

Once the washed ALI membrane was removed and divided into sections (2.3.1.2.1), sections from each cell layer of interest were placed in 24-well plates and fixed with 1.5 ml 100% methanol at -20°C for ten minutes then washed with 2 ml PBS for 20 minutes. Once fixed subsequent processing was carried out by placing the membrane sections inverted onto 100 μl droplets of each of the solutions from the protocol in turn on a wax surface, hence exposing the cell layer to the solutions. The protocol was exactly the same as that used on the sections of cell layers that had been processed for FISH (described in 2.3.3.4).

The samples were mounted on slides and examined using an Axioskop 2 fluorescent microscope (Carl Zeiss Ltd, Cambridge, UK) at x100 magnification. KS400 image analysis software (version 3.0, Carl Zeiss Ltd) was then used to calculate the percentage ciliation by thresholding the positively stained areas as shown in Figure 10.

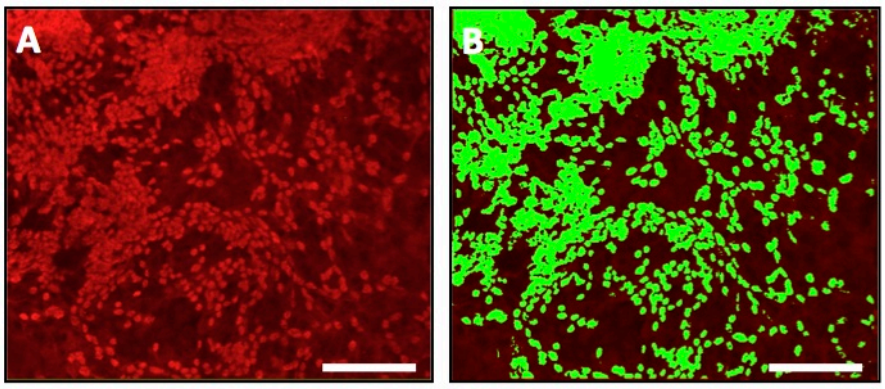

Figure 10 *- A pair of images of A) a β-tubulin labelled ALI cultured epithelial cell layer and B) the thresholding using KS400 image analysis software (version 3.0) used to calculate the percentage of ciliation. Images obtained using a Zeiss Axioskop 2 fluorescent microscope at x100 magnification. In this example the sample was well ciliated with a percentage ciliation 31.7% as calculated with KS400 image analysis software (version 3.0). Scale bars represent 200 μm.*

Ten random images from the epithelial cell layers of PCD (n=5) and non-PCD (n=5) patients were assessed and the mean for each cell layer used as the percentage ciliation for that sample.

2.3.3.8.2 Method 2 - High-speed video microscopy and fast Fourier transform

This was undertaken on live ALI cultured cell layers whilst *in situ* on the transwell inserts in 12-well plates using an Olympus IX71 phase contrast inverted microscope with a Fastcam MC2 high-speed video camera, as used for CBF measurements (2.3.3.7). It had previously been observed in our research laboratory by light microscopy inspection of ALI culture epithelial cell layers, that the edge tended to be more ciliated than the centre of the transwell insert membrane. This was evident in many cases when using this fast Fourier transform technique for measuring ciliation (data not shown). To take account of this, videos were taken at magnification of x200 at every other field of view across the whole diameter of the transwell membrane, as represented in Figure 11.

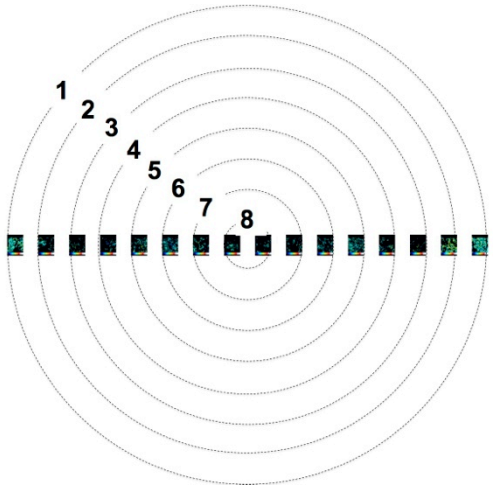

Figure 11 *- Diagrammatic representation of the ALI cultured epithelial cell layer on the transwell insert with images of fields of view taken as shown using an Olympus IX71 phase contrast inverted microscope with a Fastcam MC2 high-speed video camera. Numbering of rings utilised in table 3 below. Ring 1 represented the outer edge of the transwell insert membrane.*

FFT analysis using an 'add on' for Image J software (version 1.47a) (Rasband 1997-2012) was used to assess the percentage of a field of view that was moving and this was used as a surrogate for the percentage surface area of the cell layer that was ciliated within that field of view (Figure 12).

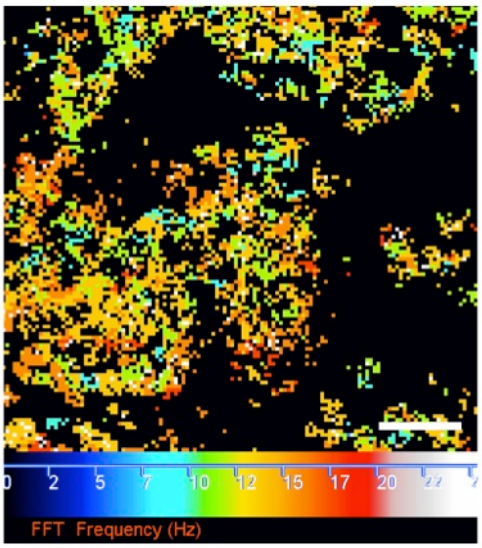

Figure 12 *- Representative image of Fast Fourier transform (FFT) analysis (using an 'add on' for Image J software (version 1.47a) (Rasband 1997-2012)) based on high speed video of ALI cultured epithelial cell layer obtained using an Olympus IX71 phase contrast inverted microscope with a Fastcam MC2 high-speed video camera at x200 magnification. Colour represents cilial movement with the increasing warmth of the colour reflecting an increasing ciliary beat frequency as shown in the FFT frequency scale below the image, black represents no movement. Scale bar represents 100 µm.*

In calculating the percentage of surface area of the cell layer that was ciliated across the whole transwell membrane, account was taken of the fact that a greater number of field of views made up the ring at the outside of the circular membrane due to the larger diameter (represented by ring 1 in Figure 11) as compared to more central rings. For each cell layer assessed 16 fields of view across the cell layer diameter were assessed, as shown in Figure 11. The percentage of the cell layer that was ciliated, as calculated by FFT method, for the two fields of view at that diameter was averaged then multiplied by number of field of view at that diameter (Table 3), to give the appropriate correction ratio for the more distal field of views in the overall percentage ciliation of the cell layer. The multiplying factor was calculated on the basis of the number of fields of view that would be present around the whole transwell at that radius and equalled the diameter of the circle at that radius divided by the width of the field of view. These multiples, at each radius, were then added together and divided by the total number of fields of view (453) to give an estimated percentage ciliation of the whole cell layer. To take an example, the average percentage ciliation of the 2 fields of view on ring 1, Figure 11, was multiplied by 101, the number of field of view at that radius (Table 3). This was repeated for each ring. These were then added together and divided by 453. An example is included in appendix 3.

Table 3 *- Weighting given to each field of view in calculating percentage ciliation of the cell layer across the whole transwell membrane by FFT technique.*

Field of view	Numbers of field of view at that diameter
1	101
2	88
3	75
4	63
5	50
6	38
7	25
8	13

The advantage of this method, using a live cell layer, was that it could be utilised daily during co-culturing experiments to make estimates of the percentage ciliation, but only on the non-PCD cell layers. The ALI culture transwell inserts were marked to ensure approximately the same diameter across the membrane was used for measurements on each day. This then allowed assessment of the affect of bacterial co-culture on percentage ciliation.

2.3.3.9 Periodic acid-Schiff staining

Periodic acid-Schiff (PAS) staining is used to detect mucosubstances, such as mucins and glycoproteins. The periodic acid oxidizes vicinal diols, breaking the bond between to monosaccharides creating a pair of aldehydes at the free tips. These aldehydes react with the Schiff reagent staining pink/magenta.

Periodic acid-Schiff staining was undertaken on un-infected cell layers. Once the ALI membrane was removed and divided into sections (2.3.1.2.1) a section from each cell layer of interest were placed in 24-well plates and fixed with 1.5 ml 100% methanol at -20°C for ten minutes then washed with 2 ml PBS for 20 minutes.

Once fixed subsequent processing was carried out (as described in 2.3.3.4) by placing the membrane sections inverted onto 100 μl droplets of each of the solutions from the protocol in turn on a wax surface, hence exposing the cell layer to the solutions.

The samples were placed on 100 μl droplets of 1% periodic acid solution (Sigma-Aldrich, Dorset, UK) for five minutes. They were then moved through a wash step, onto Schiff's reagent for 15 minutes. Schiff's reagent was made in house using 1 g pararosaniline, 2 g sodium metabisulphite, 0.2 g decolourising charcoal, and 200 ml distilled water (all Sigma-Aldrich, Dorset, UK) and 0.2 ml analar concentrated hydrochloric acid (Fisher-Scientific, Loughborough, UK). The samples were washed again then counterstained on a droplet of Mayer's haematoxylin for 90 seconds. Mayer's haematoxylin was again made in house using 1 g haematoxylin, 50 g potassium Alum, 0.2 g sodium iodate, 1 g citric acid, 50 g chloral hydrate and 1000 ml distilled water (all Fisher-Scientific, Loughborough, UK). The samples were then washed in tap water, mounted and viewed under a Zeiss Axioskop 2 microscope. All steps were carried out at room temperature and each wash step involved placing the inverted membranes on 100 μl droplets of distilled water six times for one minute unless otherwise stated. KS400 software (version 3.0, Carl Zeiss Ltd) was used with a macro to aid cell counting in order to assess the percentage of cells/field of view that were PAS stain positive. Ten random images were taken of each PCD (n=5) and non-PCD (n=9) sample at x200 magnification and the mean percentage of PAS stain positive cells for each cell layer used.

2.3.3.10 LL-37 concentrations in apical supernatant

Human cathelicidin, LL-37, is a key CAMP and therefore an important part of the innate immunity system of the lung (Larrick, Hirata et al. 1995; Gudmundsson, Agerberth et al. 1996). LL-37 concentrations were assessed at baseline and daily following co-culture in the apical supernatant of PCD and non-PCD cell layers. This assay used a Bio-Dot 96-well apparatus (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) to apply the supernatant to a membrane then used immunohistochemistry techniques to label the LL-37 that was compared to a standard curve to quantify the concentrations present (Figure 13 is an example of the standard curve). This work was carried out with the help of Dr Ben Nicholas (Clinical Experimental Sciences, University of Southampton, UK).

One hundred microlitre aliquots of supernatant, frozen at -80°C following incubation on the apical surface of the PCD (n=5), non-PCD (n=9) and 16HBE (n=6) cell layers for 24 hours at baseline and daily following co-culture (2.3.2) were thawed and vortexed. Recombinant human cathelicidin protein, LL-37 (0.5 mg/ml, AB140725, Abcam®, Cambridge, UK) was diluted in ALI media in a doubling dilution series, including an ALI only negative control, in order to produce a standard curve. A nitrocellulose membrane, cut to size for Bio-Dot 96-well apparatus, was placed in a tray and hydrated

in 50 ml towbins transfer buffer with 20% methanol (10 ml x10 Tris/glycine (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) with 70 ml distilled water and 20 ml methanol) then rinsed three times in 50 ml distilled water. The membrane was positioned in the Bio-Dot 96-well apparatus, which was attached to a vacuum pump, as per the manufacturers instructions. In turn 200 μl PBS then 100 μl PBS with 100 μl of apical supernatant or serially diluted recombinant LL-37 peptide (AB140725, Abcam®, Cambridge, UK) followed by two washes with 200 μl PBS were loaded into the wells and drawn through the Bio-Dot apparatus and across the membrane. In this way the proteins in the supernatants were captured in specific dots to the membrane. The membrane was removed from the Bio Dot apparatus, placed in a tray and incubated for five minutes in 25 ml Ponceau S solution (Sigma-Aldrich, Dorset, UK), a rapidly reversible protein stain. This allowed the sample dots to be visualised to facilitate the membrane being trimmed, which was then washed to remove the Ponceau S. The membrane was blocked with 25 ml PBS with 0.05% tween and 5% non-fat dried milk for 30 minutes, in order to reduce binding to non-specific proteins. It was then incubated overnight in 25 ml rabbit polyclonal antibody to LL-37 (AB64892, Abcam®, Cambridge, UK), diluted 1:1000 in blocking buffer, at 4^{0}C. The following morning the membrane was washed, incubate for two hours in 25 ml goat anti-rabbit horseradish peroxidase (HRP) secondary conjugate diluted 1:10000 in PBS/tween then washed again. In order to visualise the antibody labelling the membrane was incubate for five minutes in 4 ml 50:50 HRP buffer and HRP luminol enhancer (both Immuno-star™, Bio-Rad Laboratories Ltd) and imaged using a VersaDoc™ Imager (Bio-Rad Laboratories Ltd). All steps were carried out at room temperature unless otherwise stated and all washes were undertaken three times for five minutes in 50 ml PBS with 0.05% tween. The image of the membrane was analysed using Quantity One® analysis software (version 4.6.9, Bio-Rad Laboratories, Hemel Hempstead, UK). The antibody labelling for each of the samples was compared to the standard curve to allow quantification (Figure 13). The experiment was undertaken twice and the mean LL-37 concentration used. For results below the limit of detection of the assay, taken as half the value of the lowest standard dilution assessed, equivalent to an LL-37 concentration of 0.32ng/ml, an arbitrary value of 0.16 ng/ml were assigned.

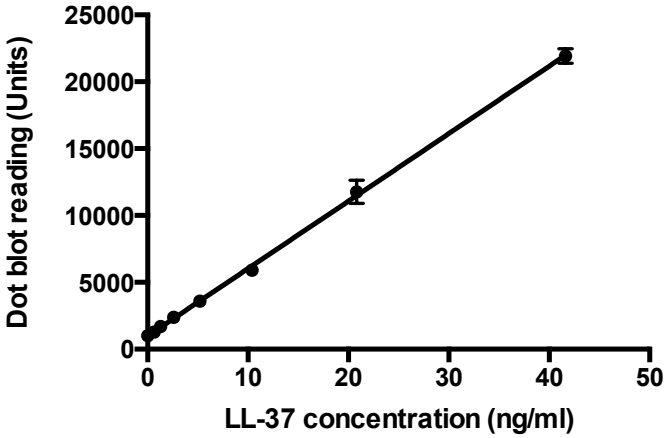

Figure 13 *– Example of the standard curve showing LL-37 concentration (ng/ml) against dot blot readout.*

2.3.3.11 Cytokines concentrations in baso-lateral supernatant

Aliquots of supernatant, frozen at -80°C following incubation on the basolateral surface of the PCD (n=5), non-PCD (n=9) and 16HBE (n=6) cell layers for 24 hours at baseline and daily following co-culture (2.3.2) were thawed and vortexed. IL-8 was not included in the multi-analyte processing as an initial experiment suggested that it would be out of the measurable range and the samples would therefore need to be diluted. It was therefore analysed separately in an individual enzyme-linked immunosorbent assay (ELISA). This work was carried out with the help of Dr Laurie Lau (Clinical and Experimental Sciences, University of Southampton, UK)

2.3.3.11.1 Multi-analyte cytokine profiling

The concentrations of 18 cytokines in the basolateral supernatant were analysed using a fluorokine® MultiAnalyte Profiling (MAP) kit (Human MAP base kit A, LUH000, with the cytokines of interest, R&D systems®, Abingdon, UK) following the manufacturers instructions. This is a Luminex bead-based multiplex assay that allows multiple cytokines concentrations to be measured whilst only requiring small amounts of sample. The assay uses colour-coded polystyrene beads coated with cytokine specific antibodies. Beads for the cytokines of interest are mixed together and incubated with the sample, capturing the cytokines of interest that are then detected using biotinylated detection antibodies and a streptavidin-phycoerythrin conjugate. The beads are read by a dual-laser flow-based detector, in this case a Bio-Plex® 200 System (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). One laser determines the bead type and the second the magnitude of the phycoerythrin-derived signal that is directly

proportional to the amount of cytokine present. The concentration is then calculated by comparison with a standard curve.

The cytokines profiled were fibroblast growth factor basic (FGFb) (LUH233), granulocyte colony stimulating factor (G-CSF) (LUH214), granulocyte macrophage colony stimulating factor (GM-CSF) (LUH215C), interferon (IFN)-γ (LUH285), interleukins (IL)-1α (LUH200B), IL-1β (LUH201), IL-1Ra (LUH280), IL-2 (LUH202), IL-4 (LUH204), IL-5 (LUH205), IL-6 (LUH206), IL-10 (LUH217), and IL-17 (LUH317), monocyte chemotactic protein 1 (MCP-1) (LUH279), macrophage inflammatory protein (MIP)-1α (LUH270) and -1β (LUH271), tumour necrosis factor (TNF)-α (LUH210) and vascular endothelial growth factor (VEGF) (LUH293). A wide spectrum of pro-inflammatory and other cytokines were included as the literature on whether many of these were produced by respiratory epithelial cells is limited and their role in infection in PCD was unknown. Most of the literature measuring cytokine profiles from ALI cultured cells utilised the basolateral supernatant, hence that was also used in this work, however there are some recent data suggesting higher levels may be seen in the apical supernatant (Khair, Devalia et al. 1994; Clemans, Bauer et al. 2000; Ren, Nelson et al. 2012).

The reagents and working standards were reconstituted as per the manufacturers instructions. The filter-bottomed 96-well micro-plate was pre-wet with 100 μl wash buffer, drawn through the plate using an Aurum™ vacuum manifold (all Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), before adding 50 μl of microparticle mixture (containing capture polystyrene microparticles for each of the cytokines of interest diluted 1:100 in microparticle diluent). 50 μl of either the basolateral supernatant, standard or blank were incubated in the wells of the microplate for three hours then washed off. 50 μl diluted biotin antibody cocktail (containing 50 μl detection antibody for each of the cytokines of interest added to the biotin antibody diluent) was then incubated for one hour and the plate was washed. 50 μl diluted streptavidin-phycoerythrin conjugate was incubated on it for 30 minutes then it was washed again. The microparticles were re-suspended in 100 μl wash buffer for 2 minutes then the plate was read on a Bio-Plex® 200 System (Bio-Rad Laboratories Ltd). All incubations were undertaken on a horizontal orbital microplate shaker set at 550 rpm with the plates covered with a light impermeable adhesive strip at room temperature. All washes were undertaken using 100 μl wash buffer, using the vacuum manifold to remove the liquid through the bottom of the plate so as to avoid loss of any microparticles.

2.3.3.11.2 Interleukin-8 assessment

The concentrations of IL-8 in the basolateral supernatant, diluted 1:5 with 1% BSA/PBS, was analysed using a human IL-8 DuoSet ELISA development kit (DY208 R&D systems®) following the manufacturers instructions, except using 96-well half area microplates (Corning Life Sciences, Fisher-Scientific, Loughborough, UK). The reagents and working standards were reconstituted as per the manufacturers instructions. 50 μl capture antibody, mouse anti-human IL-8 (4.0 μg/ml), was incubated in each well of a 96-well microplate overnight at room temperature. The following morning the microplate was washed, each well was blocked with 50 μl 1% BSA/PBS for one hour then 50 μl of either diluted basolateral supernatant, standard or blank was incubated in each for two hours. The microplate was washed and 50 μl detection antibody, a biotinylated goat anti-human IL-8 (20 ng/ml), was incubated in each well for two hours then washed again. 50 μl Streptavidin-HRP was then incubated for 45 minutes in each well, washed then 50 μl substrate solution (1:1 H_2O_2:tetramethybenzidine) was added for 20 minutes before adding 50 μl of stop solution (2N H_2SO_4). The plate was then read at a wavelength of 450 nm, with correction wavelength of 595 nm, on a ThermoMax Microplate Reader (Molecular Devices, Sunnyvale, US). Each wash was undertaken using 100 μl 1% BSA/PBS three times for 1 minute. The experiment was undertaken in duplicate and the mean concentration for each sample used.

2.4 Methods for the assessment of nitric oxide biosynthesis

2.4.1 Clinical measurements of upper and lower airway nitric oxide

Measurement of nasal and lower airway nitric oxide levels was undertaken using a chemiluminescent NO analyser, NIOX® Flex (Aerocrine, Sweden) and followed American Thoracic Society/European Respiratory Society (ATS/ERS) recommendations (2005). In brief, nasal NO was measured during a breath holding manoeuvre to close the velum whilst a nasal probe sampled gas aspirated from the nostril at a rate of 5ml/sec. Patients were encouraged to hold each breath for approximately 20 seconds until the analyser recorded a plateau in nitric oxide concentrated from the aspirated gas. Three measurements were obtained from each child using the same nostril and the mean nNO reading was recorded. Fe_{NO} was measured at multiple flow-rates (50, 100, 200 and 250 ml/s) whilst maintaining a constant exhalation pressure >5cm H_20 through visual feedback. Two consistent readings, within 10%, were obtained at each flow-rate. Calculation of the alveolar NO ($Calv_{NO}$) (ppb) and bronchial NO flux ($J'aw_{NO}$) (pl/s) were

based on the mathematical model of pulmonary NO exchange dynamics proposed by Tsoukias and George (Tsoukias and George 1998): $V'_{NO} = V_E \times Calv_{NO} + J'aw_{NO}$. Where NO elimination ($V'_{NO}$) (nl/s) is the exhaled NO concentration (ppb) x flow rate (V'_E) (ml/s), $J'aw_{NO}$ (pl/s) is bronchial NO flux, and $Calv_{NO}$ (ppb) is the steady state NO concentration in alveolar air. Therefore the gradient and intercept of a regression line on a graph of NO elimination (V'_{NO}) against flow-rate (V'_E) represent $Calv_{NO}$ and $J'aw_{NO}$ respectively (Figure 14) (Tsoukias and George 1998).

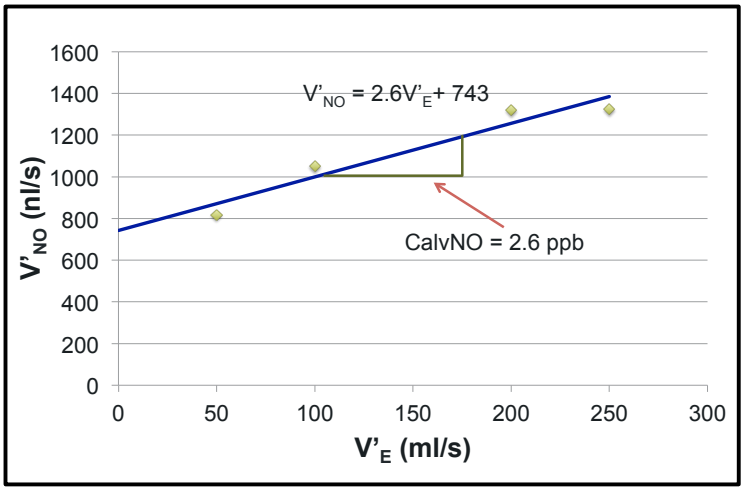

Figure 14 *- Example of the two-compartment mathematical technique used to estimate bronchial ($J'aw_{NO}$) and alveolar ($Calv_{NO}$) contributions to Fe_{NO}. Fe_{NO} was measured at multiple expiratory flow-rates (50, 100, 200 and 250 ml/s.) The gradient and intercept of a regression line between NO elimination (V'_{NO}) and flow-rate (V'_E) are recorded as $Calv_{NO}$ (ppb) and $J'aw_{NO}$ (pl/s), respectively.*

2.4.2 Assessment of NO biosynthesis in primary ciliated epithelial cells

2.4.2.1 Nitric oxide assay

Due to the transient and volatile nature of NO, which is rapidly broken down into its more stable products nitrate and nitrite, total NO biosynthesis was assess by reducing all nitrate to nitrite then measuring nitrite levels. This was undertaken using a total nitric oxide detection kit (Enzo Life Sciences, Exeter, UK). The reagents and working standards were reconstituted as per the manufacturers instructions (standard curve range 3.125-100μmol/L). 100 μl PBS was incubated on the apical surface of the ALI cultured respiratory epithelial cell layers from PCD and non-PCD subjects (prepared as described in 2.3.1.2) for 30 minutes at 37°C in 5% CO_2. The PBS was then removed from the cell culture, diluted 1:1 with reaction buffer and ultra-filtered through a 10,000 molecular weight cut-off (MWCO) filter (Millipore, Watford, UK) by centrifuging

at 13,000 rpm for 20 minutes. In a 96-well plate, 25 μl of both nitrate reductase enzyme and β-nicotinamide adenine dinucleotide (NADH) were added to 50 μl of the filtered samples, standards or blank and incubated at 37°C in 5% CO_2 for 30 minutes. 50 μl sulphanilamide and N-(1-Naphthyl) ethylenediamine (both in 2M hydrochloric acid) (Griess reagents I and II respectively) were then added and incubated for 10 minutes. The sulphanilamide forms diazonium salts with any nitrite present and when the azo dye agent, N-(1-Naphthyl) ethylenediamine, is added a pink colour develops. The plate was read at 540 nm using a Spectra Max 340 PC (Molecular Devices, Sunnyvale, US) and the nitrite concentration calculated from the standard curve. All steps were carried out at room temperature unless otherwise stated and all samples were run in duplicate with the mean concentration used.

2.4.2.2 DAF-fm Diacetate

4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-fm Diacetate, Molecular Probes®, Life Technologies, Paisley, UK) was used to further corroborate the presence of NO in ALI cultured cell layers from both PCD and non-PCD subjects. DAF-fm is essentially non-fluorescent until it reacts with NO to form a fluorescent benzotriazole. Air-liquid interface cultured epithelial cell layers derived from PCD and non-PCD patients were produced as described in 2.3.1.2. 500 μl of 10 μM DAF-fm Diacetate was incubated on the apical surface of the cell layers for 40 minutes at 37°C with 5% CO_2. This was discarded and the cell layers washed with 500 μl PBS for five minutes three times. The transwell membranes were cut from the transwell inserts, mounted on slides and examined under a Leica DMRBE fluorescent microscope (Leica Microsystems, Milton Keynes, UK).

2.4.2.3 Stimulation of nitric oxide biosynthesis by pro-inflammatory cytokines

The three pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α have been demonstrated to act synergistically in combination to increase NO biosynthesis by epithelial cells (Kwon, Newcomb et al. 2001). These were therefore used to stimulate NO biosynthesis in PCD and non-PCD ALI cultured cell layers. Air-liquid interface cultured epithelial cell layers derived from PCD and non-PCD patients were prepared as described in 2.3.1.2. In keeping with previously published work (Kwon, Newcomb et al. 2001), 10 ng/ml of each of IL-1β, IFN-γ and TNF-α (Invitrogen, Life Technologies, Paisley, UK) were added to 650 μl ALI media, replacing the basolateral media of the cell layers of interest, and was incubated at 37°C with 5% CO_2 for 18 hours. Total nitrite detection assays were carried out in duplicate on the cell layers before and after this pro-inflammatory cytokine stimulation (2.4.2.1) and the mean used.

2.4.2.4 Stimulation of nitric oxide biosynthesis by non-typeable Haemophilus influenzae infection

Total nitrite levels (2.4.2.1) were measured on ALI cultured cell layers from PCD and non-PCD subjects before and after three days of co-culture with NTHi isolate four (as described in 2.3.2). All samples were run in duplicate with the mean concentration used.

2.4.2.5 NOS isoform localisation

Two methods of NOS isoform localisation were utilised, the first on bronchial biopsy samples embedded in glycol methacrylate acrylic (GMA) resin; the second on methanol fixed sections of ALI cultured epithelial cell layers. The reason to develop the second technique was to allow comparison of NOS localisation in PCD and non-PCD derived epithelial tissue, as no bronchial biopsy samples were available from PCD subjects.

2.4.2.5.1 Respiratory epithelial biopsy samples embedded in glycol methacrylate acrylic resin

This protocol used was adapted from the protocol for staining GMA resin embedded tissue used in the Histochemistry Research Unit, University of Southampton, UK as described previously (Britten, Howarth et al. 1993). Bronchial tissue from healthy adult control patients (kindly gifted by Professor P. Howarth, University of Southampton, Southampton, UK) embedded in GMA resin was sectioned using an Ultracut E (Reichert-Jung, Vienna, Austria) and placed on glass slides. These slides were placed on racks over trays, to catch solution and buffer as it was washed off the slides. All reagent steps were carried out by placing adequate droplets of a reagent over a sectioned sample to cover it, typically 300 μl per slide. All wash steps were carried out using liberal amounts of tris-buffered saline (TBS, Gibco, Life Technologies, Paisley, UK) (at least 5 ml per slide) to wash off the reagent on the sectioned sample then leaving for 5 minutes, this was repeated three times. The steps were all carried out at room temperature unless stated otherwise.

Endogenous peroxidases from the specimens were inhibited by incubating with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide in reverse osmosis water for 30 minutes then washed. Two drops of avidin block followed by two drops of biotin block (both Vector, Burlingame, US) were separately placed on each specimen and incubated 30 minutes with wash steps between them. Blocking medium (made up of 80 mls DMEM (Sigma-Aldrich, Dorset, UK), 20 mls fetal calf serum (PharmAust Ltd,

Perth, Western Australia) with 1g BSA (Sigma-Aldrich, Dorset, UK)) was applied for 30 minutes and the specimens were incubated overnight with the primary antibodies. The primary antibodies to the three NOS isoforms were rabbit polyclonal antibodies (Calbiochem, Nottingham, UK), nNOS (1414-1434, Cat No ST1095) was diluted 1:100 and eNOS (599-613, Cat no. KP5302) and iNOS (1131-1144, Cat no KP5303) diluted 1:1000. EN4 (Code MON6002, Monosan, Uden, The Netherlands), a monoclonal antibody which specifically binds to endothelial tissue, was used as a positive control for eNOS, which is known to be localised to vessel walls in the airway epithelial, at a 1:300 dilution and β-tubulin (Clone 2-28-33, product no. T5293, Sigma-Aldrich, Dorset, UK), a monoclonal antibody that specifically binds to cilia, was used at a dilution of 1:6000. In addition, half the sections incubated with the nNOS primary antibody had an nNOS specific blocking peptide (1414-1434, Cat no KP5304, Calbiochem, Nottingham, UK, 1:4 dilution) added as a negative control. The slides were washed and a biotinylated rabbit anti-mouse secondary antibody (Dako UK Ltd, Ely, UK) at a 1:1000 dilution was applied for two hours and washed. All antibodies were diluted in TBS. Vectastain® avidin biotin complex (Vector, Burlingame, US) (made up by a 1:1:75 ratio of avidin:biotin:TBS) was then applied for two hours and washed. Liquid 3,3'-diaminobenzidine chromagen (Biogenex, San Ramon, US) was applied for ten minutes, washed in running tap water for five minutes, counter-stained with Mayer's haemotoxylin for 100 seconds then washed again in running water for a further five minutes. Finally the slides were mounted and coverslips applied then examined with Leica DMLB light microscope (Leica Microsystems, Milton Keynes, UK).

2.4.2.5.2 ALI cultured primary respiratory epithelial cell layers

Cell layers from PCD and non-PCD subjects were cultured at an ALI until differentiated and ciliated (2.3.1.2). The transwell membranes were then removed from the transwell inserts and sectioned (2.3.1.2.1). These sections were then fixed in methanol and washed in PBS. Once fixed subsequent processing was carried out (as described previously in 2.3.3.4) by placing the membrane section inverted onto 100 μl droplets of each of the solutions from the protocol in turn on a wax surface, hence exposing the cell layer to the solutions. Each wash step involved placing the inverted membranes on 100 μl droplets of PBS with 0.05% Triton-X and 1% BSA three times for five minutes.

The sections were incubated in blocking medium (80 ml DMEM (Sigma), 20 ml newborn bovine serum (Fisher Scientific, Loughborough, UK) with 1 g BSA and 0.05% Triton-X (both Sigma-Aldrich, Dorset, UK)) for 30 minutes. The samples were then taken through a wash step, primary antibodies incubation for 90 minutes, wash step then

secondary antibody incubation for a further 90 minutes before a final wash step. The same NOS isoform primary antibodies were used as for the previous section (2.4.2.5.1) however all were diluted 1:200, the isotype control was a rabbit IgG antibody (X0903, Dako, Ely, UK) diluted 1:4000, the secondary was a Alexa Fluor® 488 goat anti-rabbit IgG antibody (dilution 1:100) (Code A11034, Molecular Probes®, Life Technologies, Paisley, UK). All were diluted with PBS with 0.05% Triton-X and 1% BSA and all steps were carried out at room temperature. The samples were mounted and examined using a Leica DMRBE fluorescent microscope (Leica Microsystems, Milton Keynes, UK).

To assess co-localisation of nNOS to cilia non-PCD ALI culture cell layers were initially labelled with nNOS primary and secondary antibodies as outlined above. Once the nNOS secondary antibody had been washed off the sections were then labelled for the specific ciliary protein β-tubulin. A β-tubulin primary mouse monoclonal antibody (Clone 2-28-33, product no. T5293, Sigma-Aldrich, Dorset, UK) diluted 1:2000 was incubated with the sections for 90 minutes then washed off. A secondary antibody, an Alexa Fluor® 594 conjugate chicken anti-mouse IgG antibody (Code A21201, Molecular Probes®, Life Technologies, Paisley, UK), diluted 1:500 was then incubated with the sections for a further 90 minutes. The sections were then wash before being mounted and examined using a Leica SP5 confocal laser-scanning microscope (Leica Microsystems, Milton Keynes, UK). All dilutions were with PBS with 0.05% Triton-X and 1% BSA and all steps were carried out at room temperature.

2.4.3 Assessing the role of nitric oxide on NTHi in planktonic and biofilm culture

Nitric oxide has been demonstrated to disperse *P. aeruginosa* biofilms but its role on NTHi biofilms has not been investigated (Barraud, Hassett et al. 2006). The affected of NO on NTHi, both in planktonic culture and when adherent to a surface, was therefore assessed using the NO donor sodium nitroprusside, SNP. Dr Aneurin Young (Academic Foundation Year 2 Doctor) undertook this work under my supervision.

2.4.3.1 NO effect on NTHi in planktonic culture

Having confirmed purity (2.2.1.1), identical 15 ml broths of NTHi isolate four were made up in pre-warmed (37^{0}C) sBHI broths in 15 ml centrifuge tubes (2.2.2). To each of these a range of concentrations of the NO donor SNP were added in addition to a no SNP control that were then incubated at 37^{0}C in 5% CO_2. In different experiments the SNP concentrations assessed ranged from 10 nM to 10 mM. NTHi bacterial growth was assessed every 30 minutes for 7 hours then check again at 24 hours by measuring

optical density at 600nm (OD_{600}) (2.2.2). The experiment was undertaken in duplicate up to five times in separate experiments dependent on concentration of SNP.

2.4.3.2 NO effect on NTHi in biofilm culture

Having confirmed purity (2.2.1.1) NTHi isolate four was cultured in 24-well polystyrene plates (2.2.3.2). 0.5 ml of NTHi sBHI broth at OD_{600} 0.1 was added to 1 ml sBHI in each well. These were incubated for three days at 37^{0}C in 5% CO_2 with 1 ml sBHI being replaced daily. On day three the NTHi colonies were washed to remove non-adherent bacteria and then incubated with varying concentrations of SNP (10nM to 100μM) made up in 1.5 mls sBHI for six hours at 37^{0}C in 5% CO_2. The supernatant was collected and the NTHi colonies remaining in the well washed a further two times with 1 ml sBHI, to remove non-adherent bacteria and any residue SNP. CFU counts from both the supernatant and the adherent NTHi bacterial colonies were calculated (2.2.2 and 2.2.3.2). In further experiments 20 ng/ml cefotaxime was added either alone or in combination with 100 μM SNP alongside a no treatment control and processed in the same way to assess any synergistic effects of the combination of this antibiotic and SNP. In releasing nitric oxide SNP is broken down to sodium ferrocyanide, which in itself potential has a bactericidal/biofilm dispersal effect. Experiments were therefore controlled with 1mM potassium ferrocyanide (KFeCN) (Sigma-Aldrich, Dorset, UK). All experiments were undertaken in triplicate and repeated two or three times.

2.5 Statistical methods

For the clinical measurements of upper and lower airway nitric oxide data analysis the natural log of $J'aw_{NO}$, $Calv_{NO}$ and Fe_{NO50} were found to be normally distributed hence were used for statistical analysis. Non-parametric analysis (Mann-Whitney test) was used for nNO. Least squares regression models were used to evaluate univariate relationships between the NO parameters and independent variables: age, sex, height, weight, FEV_1, ambient NO, use of inhaled corticosteroids and antibiotics. If log transformation was necessary to achieve normal distribution, log transformed data were used in the model. Linear regression analysis and a standard backwards model selection process were used in order to assess relationships between these variables and NO parameters and to identify confounding variables. Nasal NO was compared between groups using the Kruskal-Wallis test. Confounding variables were adjusted for during the analyses. The data were evaluated using statistical analysis software SPSS version 19.0.0 (IBM, USA).

For other measurements, parametric data were compared using student t-tests and non-parametric data using Mann Whitney U tests. The Sidak-Bonferroni method was used to adapt p values where multiple comparisons using t-tests were made, for example with the cytokine analysis (5.3.3.3.1). In addition, where repeated measurements were taken, for example in the case of ciliary beat frequency comparing ALI and MEM media (4.4.2.2.3), repeated measures ANOVA was used to assess significance. These were calculated using Graphpad Prism® software (version 6.0a, 2012, Graphpad Software Inc.). This software was also used to calculate means, standard deviations, standard error of the means and correlation coefficients in addition to generating all graphs.

A statistical significance level of 0.05 was used throughout.

Chapter 3

Comparison of primary respiratory epithelial cells from PCD and non-PCD patients, differentiated at air-liquid interface

3.1 Introduction

The study hypothesis was that impaired ciliary function predisposes PCD patients to biofilm-associated infections. The aim of the work was therefore to investigate the effect of respiratory epithelial ciliary function on bacterial biofilm development by developing a co-culture model using airway epithelial cells derived from patients with and without PCD.

The first step was therefore to characterise and compare primary respiratory epithelial cell layers derived from PCD and non-PCD control patients, differentiated at an air-liquid interface (ALI). The ALI culture model was established in-house to culture basal epithelial cells from patients referred to the National PCD Diagnostic Centre at University Hospital Southampton (UHS) for cell differentiation and ciliogenesis to assess ciliary function *in vitro* (Hirst, Jackson et al. 2014). In this chapter a number of parameters were therefore assessed to compare the ALI cultured epithelial cell layers derived from PCD patients to non-PCD controls. The parameters included ciliary beat frequency (CBF), by high-speed video microscopy; percentage of ciliated surface area, quantified by two methods using either fluorescent or light microscopy and image j analysis; tight junction formation, assessed by trans-epithelial electrical resistance (TER); the number of mucus producing epithelial cells, quantified by Period acid-Schiff (PAS) staining and light microscopy; and the biosynthesis of the cationic antimicrobial peptide (CAMP) LL-37 and 19 cytokines, investigated by dot blot and multiplex ELISA methods (all described in 2.3.3). In addition, of particular interest, was the biosynthesis of nitric oxide (NO) by the epithelial cell layers given that, almost universally, patients with PCD have extremely low nasal NO levels, the mechanism for which is unknown. This was assessed by quantifying total nitrite concentration in apical surface washes of ALI cultured epithelial cells however these data are presented in Chapter 6.

3.2 Primary respiratory epithelial cell collection and culture

Primary respiratory epithelial cells were obtained by brushing the inferior turbinates of PCD and non-PCD patients with cytology brush biopsies. The basal epithelial cells then underwent two passages prior to being seeded onto collagen coated 12-well plate transwell inserts, once confluent the apical supernatant was removed to allow an ALI. Cultures were fed on alternate days and once observed by light microscopy to be ciliated, typically 21 days after ALI culture, were characterised (2.3.1.2). These cultures were on average 10.1% (5.7 - 27.6) (median, min-max) ciliated once further assessment was undertaken.

3.3 Characterisation of the ALI culture epithelial cell model

3.3.1 Ciliary beat frequency

Ciliary beat frequency measurements of the ALI cultured epithelial cell layers were obtained by high-speed video microscopy and analysis by reviewing ciliary function slowed to eight times the original speed (2.3.3.7). The mean CBF in Hertz (Hz) for each ALI culture was calculated from six separate and random fields of view.

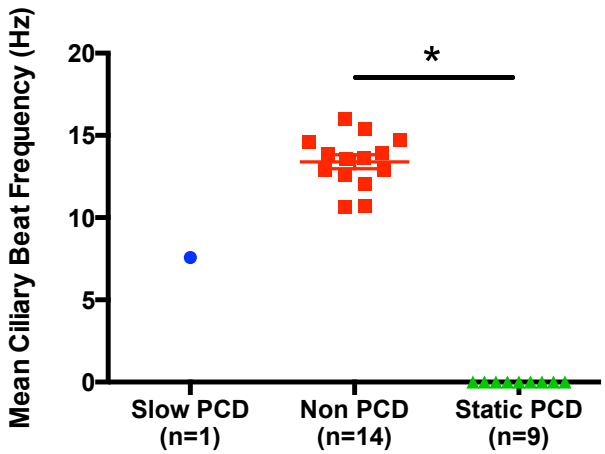

Figure 15 *– Mean ciliary beat frequency (CBF) of cilia on ALI cultured cell layers from a slow CBF PCD (n=1), non-PCD (n=14) & PCD (n=9) patients (mean and SEM, where appropriate) (* P<0.0001)*

The normal range for CBF in healthy patients in the national PCD diagnostic centre at UHS is 11-20 Hz, (Hirst, Jackson et al. 2014). The non-PCD patients all fell within this range following ALI culture ((mean±SEM) 13.4 Hz (±0.4)). One PCD positive patient had a measurable but slow CBF, consistent pre and post ALI culture (7.6 Hz and 7.3 Hz respectively) and all other PCD cell layers (n=9) had static cilia recorded as 0 Hz. There was a significant difference between the CBF of static PCD compared to non-PCD patients ($P<0.0001$) (Figure 15).

3.3.2 Assessment of ciliation

Two methods were used to assess the percentage of the surface area of the primary ALI cultured epithelial cell layer that was ciliated (2.3.3.8). The first, undertaken on fixed cells, used immunohistochemistry techniques with β-tubulin labelling, a fundamental protein component of the ciliary axoneme. This did not require the cilia to be beating for assessment to be made thereby allowing comparison between PCD and non-PCD epithelial cell layers. The alternative method, using a Fast Fourier Transform (FFT) algorithm with high-speed video microscopy on a live cell layers, required ciliary beating and was therefore not possible on PCD cultures that were, in the main, static. However this technique would subsequently be used to assess the affect of co-culture with non-typeable *Haemophilus influenzae* on ciliation on the non-PCD epithelial cell layers.

3.3.2.1 Method 1 – β-tubulin labelling on fixed epithelial cell layers

Figure 16 shows β-tubulin labelling of cilia (bright red) on ALI cultured epithelial cell layers from PCD (A and C) and non-PCD (B and D) patients at x100 magnification. A and B show examples of well ciliated fields of view (31.7% and 24.7% of the surface area respectively was ciliated as assess by KS400 (version 3.0) image analysis software) whereas C and D show examples of poorly and averagely ciliated fields of view (6.5% and 10.5% ciliated respectively)

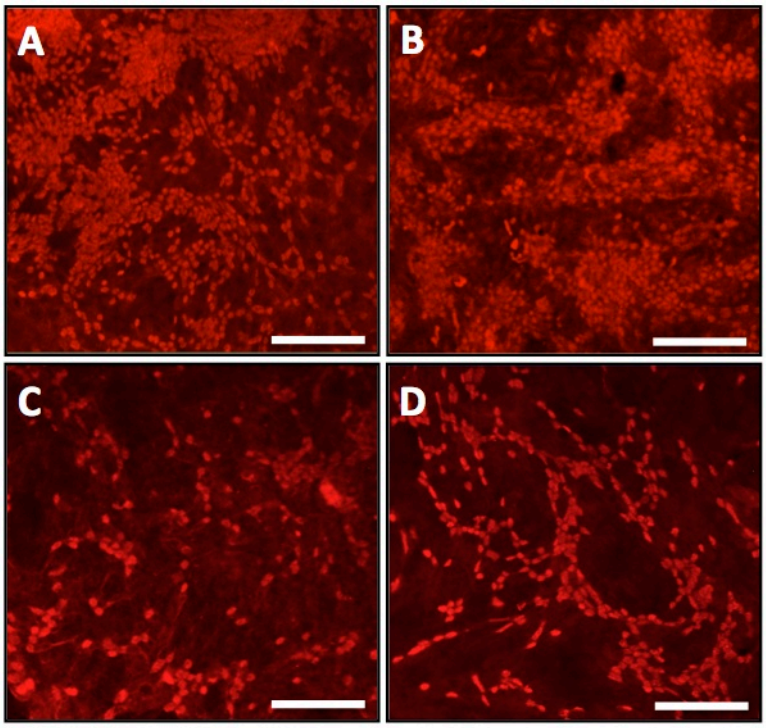

Figure 16 *- Fluorescent microscopy images showing examples of β-tubulin labelling of cilia (bright red) on ALI cultured epithelial cell layers from PCD (A and C) and non-PCD (B and D) patients. Images were taken on Zeiss Axioskop 2 fluorescent microscope at x100 magnification and analysed using KS400 image analysis software (version 3.0). A and B show examples of well ciliated fields of view (31.7% and 24.7% of the surface area respectively was ciliated) whereas C and D show examples of poorly and averagely ciliated fields of view (6.5% and 10.5% ciliated respectively). Scale bars represent 200 µm.*

Figure 17 shows the median percentage of ciliated surface area across PCD (n=5) compared with non-PCD (n=5) ALI cultured cell layers. For each cell layer, ten randomly selected fields of view were analysed and the mean used. While variation was observed between cell layers the median percentage of ciliated surface area between the PCD and non-PCD groups were similar (median (min-max) 10.7% (5.7-27.6) vs. 9.7% (6.4 - 20.6) respectively).

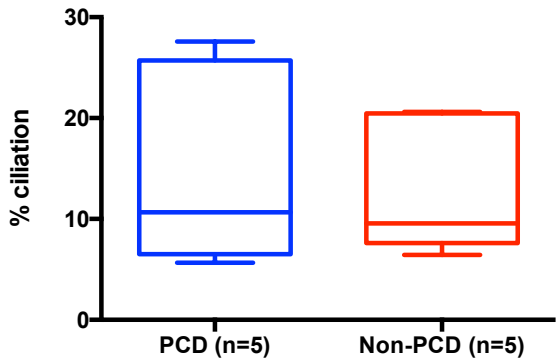

Figure 17 *– Box and whisker plot of percentage ciliation (by β-tubulin staining) of PCD (n=5) and non-PCD (n=5) ALI cultured respiratory epithelial cell layers (median, min-max). No differences were seen between the two groups.*

3.3.2.2 Method 2 – Fast Fourier transform on live epithelial cell layers

This method used an 'add on' for Image J software (version 1.47a) (Rasband 1997-2012) using an FFT algorithm to permit mapping of areas within a field of view with moving pixels. This was taken as a surrogate of the area that was ciliated but, as mentioned above, could only be used on non-PCD cell layers, with motile cilia. In calculating the mean percentage ciliation of the whole cell layer account was taken to the observation that the ALI cultured cell layers were sometimes better ciliated towards the edge of the transwell membranes as compared to the centre (2.3.3.8.2).

Figure 18 shows the mean (±SEM) percentage surface area that was ciliated across the ALI cultured cell layers from non-PCD (n=8) patients was of 10.1% (±1.0).

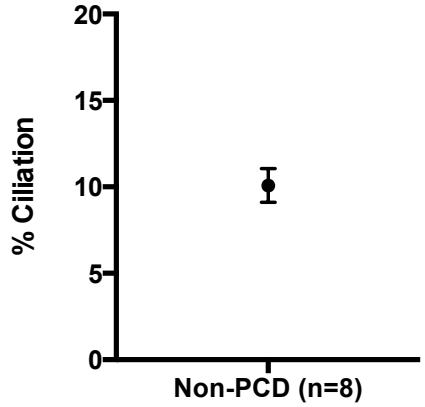

Figure 18 *– Percentage ciliation (by fast Fourier transform method) of non-PCD (n=8) ALI cultured respiratory epithelial cell layers (mean, SEM)*

3.3.3 Assessment of mucus producing cells

Periodic acid-Schiff (PAS) staining (dark purple) was used to detect secretory cells in the ALI cultured epithelial cell layers with Mayer's haematoxylin counterstaining (2.3.3.9). Figure 19 shows representative images of A) PCD and B) non-PCD.

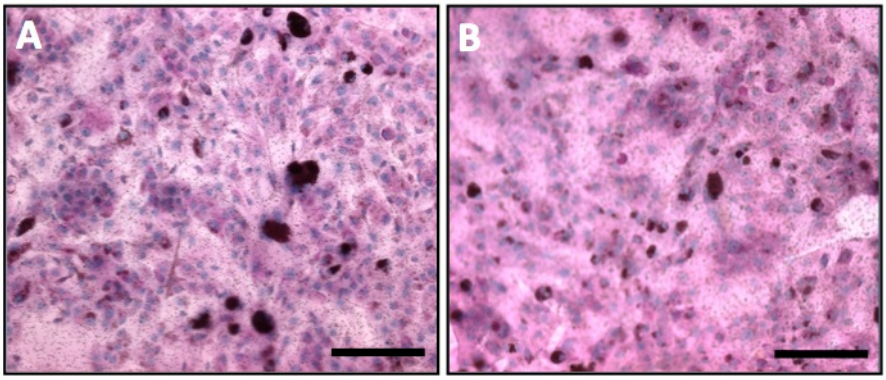

Figure 19 *- Representative images of A) PCD and B) non-PCD ALI cultured respiratory epithelial cell layers following PAS staining with Mayer's haematoxylin counterstaining. Images taken on Leica DMRBE light microscope at x200 magnification. (Positive PAS stained cells are dark purple labelling secretory cells). Scale bars represent 100 µm.*

The positively and negatively labelled cells within a field of view were counted and the percentage of cells positively labelled calculated. This was performed on ten random fields of view and the mean calculated for each cell layer (2.3.3.9). Figure 20 shows the median percentage of PAS positive labelled cells in ALI cultured epithelial cell layers from PCD (n=5) and non-PCD patients (n=9).

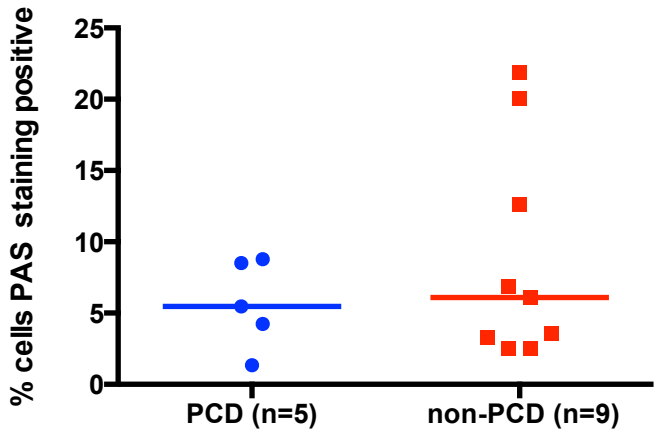

Figure 20 *- Percentage of cells with positive PAS staining on PCD (n=5) and non-PCD (n=9) ALI cultured respiratory epithelial cell layers (median). The groups overlapped.*

Despite there being two outliers with more PAS stained cells in the non-PCD group there was no difference seen in the median percentage of positively PAS stained cells between the two groups (5.5% vs. 6.1% respectively.) The clinical history of the patients from whom the two outlying ALI cultured cells layers were derived was similar to the other non-PCD patients in this experiment.

3.3.4 Trans-epithelial electrical resistance

Trans-epithelial electrical resistance is a well-recognized surrogate measure of tight junction formation and hence cellular integrity and health (Xiao, Puddicombe et al. 2011). It was measured across the ALI cultured cell layers using an EVOM 2 epithelial voltohmmeter (World precision instruments, Sarasota, US) (2.3.3.1). Figure 21 shows the mean TER, taken from three readings and corrected for the TER of the transwell membrane alone in Ohms.cm^2, for PCD (n=5) and non-PCD (n=14) cell layers. There was no difference seen between the two groups (mean (±SEM) 753 Ohms.cm^2 (±124) vs. 964 Ohms.cm^2 (±88) respectively).

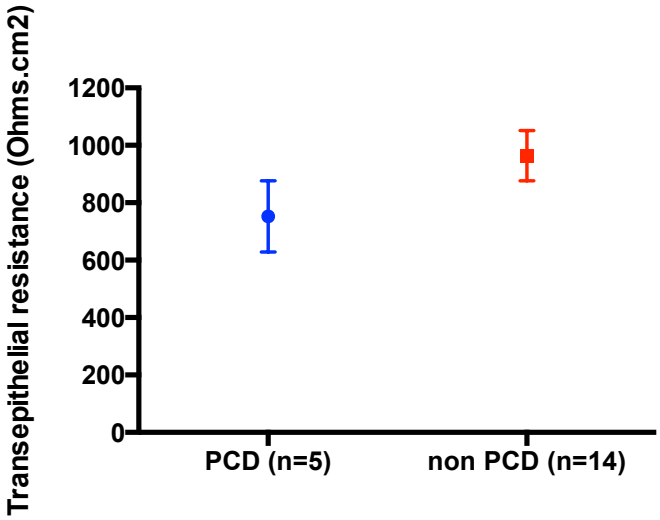

Figure 21 *- Corrected TER measurements across PCD (n=5) and non-PCD (N=14) ALI cultured epithelial cell layers (Ohms.cm^2) (mean, SEM). No differences were seen between the two groups.*

3.3.5 Cationic antimicrobial peptide – cathelicidin (LL-37)

Human cathelicidin, LL-37, is a key CAMP and therefore an important part of the respiratory innate immunity system of the lung (Larrick, Hirata et al. 1995; Gudmundsson, Agerberth et al. 1996). Constitutive LL-37 concentrations in the apical supernatant, incubated for one day on ALI cultured epithelial cell layers from PCD and non-PCD patients, were measured using a dot blot technique (2.3.3.10) (Figure 22).

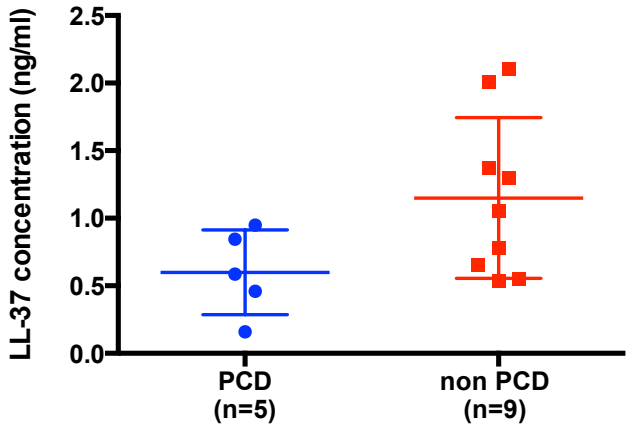

Figure 22 – Concentrations of LL-37 in the apical supernatant collected after 24 hours incubation over ALI cultured primary respiratory epithelial cell layers of PCD (n=5) and non-PCD (n=9) patients (ng/ml) (mean, SD). While mean LL-37 concentrations were lower in the PCD group the difference was not significant. Lower limit of detection of assay was 0.32ng/ml, for results below this a value of 0.16 ng/ml was used.

The constitutive mean LL-37 concentrations in the apical supernatant from PCD ALI cultured epithelial cell layers were half that of non-PCD cell layers, (mean±SD) 0.60ng/ml (±0.31) vs. 1.15ng/ml (±0.6) respectively. However the difference between the groups did not reach significance.

3.3.6 Cytokine biosynthesis

Basolaterally applied supplemented ALI media, incubated below ALI cultured epithelial cell layers of PCD (n=5) and non-PCD (n=9), were harvested after 24 hours and frozen at -80°C. Concentrations of 19 cytokines were measured from these supernatants, 18 using a multi-analyte technique and one (IL-8) measured by a separate enzyme-linked immunosorbent assay (ELISA), as it needed to be diluted (2.3.3.11).

Ten cytokines had measurable levels, fibroblast growth factor basic (FGFb), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interleukins (IL) -6, IL-8 and IL-1 receptor antagonist (IL-1Ra), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP) -1α, tumour necrosis factor (TNF) -α and vascular endothelial growth factor VEGF (Figure 23).

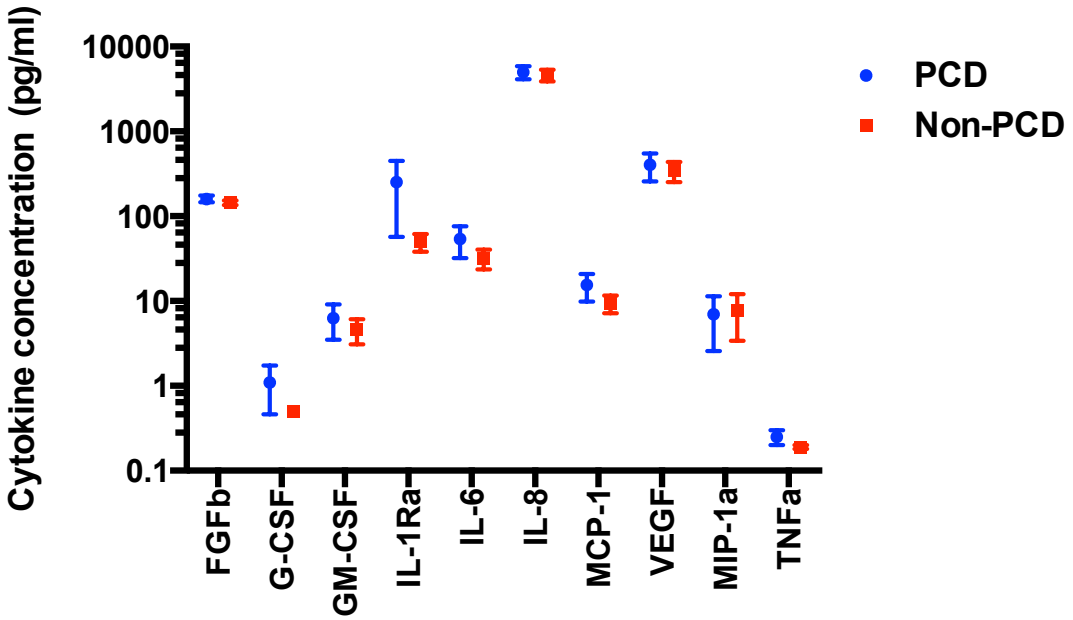

Figure 23 *– Concentrations of cytokines in the basolateral supernatant collected after 24 hours incubation at 37°C with 5% CO_2 from under ALI cultured primary respiratory epithelial cell layers of PCD (n=5) and non-PCD (n=9) patients (pg/ml) (mean, SEM). (FGFb - fibroblast growth factor basic, G-CSF - granulocyte colony stimulating factor, GM-CSF - granulocyte macrophage colony stimulating factor, IL – interleukins, MCP-1 - monocyte chemotactic protein 1, MIP-1a - macrophage inflammatory protein, TNFα - tumour necrosis factor & VEGF - vascular endothelial growth factor.) No differences were seen between the two groups for any cytokine.*

There were no differences seen in the concentrations of these 10 cytokines in the basolateral supernatant of PCD as compared with non-PCD cell layers. Interleukins (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-10 & IL-17, interferon (IFN)-γ and macrophage inflammatory protein (MIP)-1β were not detectable by the assay used.

3.3.7 Scanning electron microscopy

In addition to the parameters consider so far in this chapter, ALI cultured cell layers from PCD and non-PCD patients were fixed, processed and imaged by SEM (n=5 for

each group) (2.3.3.5). Whilst not quantified, the SEM images confirmed good cell differentiation, with these images showing excellent ciliation across the cell layer surfaces of both PCD and non-PCD patients (Figure 24 A and B).

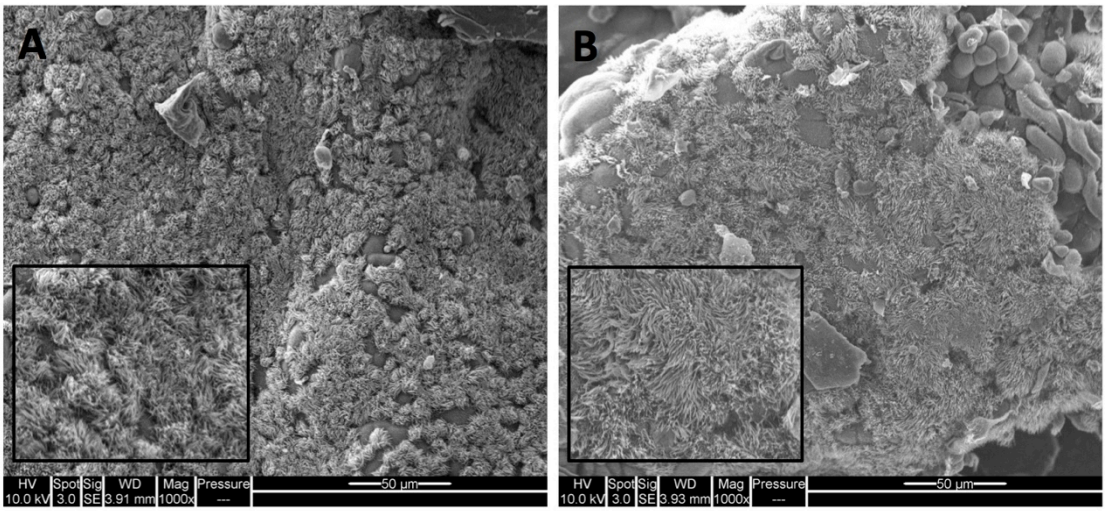

Figure 24 *- SEM images of ALI cultured A) PCD and B) non-PCD primary epithelial cell layers respectively, magnification x1000 with x2000 images inlayed. SEM images were taken on an FEI Quanta 200 SEM (FEI company, Eindhoven, The Netherlands).*

3.3.8 Nitric oxide biosynthesis

Assessment of nitric oxide (NO) biosynthesis by the ALI cultured primary ciliated respiratory epithelial cells from PCD and non-PCD patients and localisation of the three nitric oxide synthase (NOS) isoforms within these cell layers, will be consider in the Chapter 6

3.4 Discussion

The only significant difference seen between PCD and non-PCD ALI cultured epithelial cell layers, in any of the parameters considered in this chapter was in CBF, where all but one of the PCD group had static cilia. Both PCD and non-PCD cell layers were observed to have similar percentages of ciliated surface area, TER, mucus-producing cells and basolateral supernatant cytokine concentrations.

Levels of the CAMP LL-37 in the apical supernatant over PCD cell layers were lower than the non-PCD group, although not significantly so. There has been no previous literature investigating LL-37 in PCD patients. LL-37 is widely expressed at mucosal surfaces including the epithelial surface of the conducting airways (Bals, Wang et al.

1998) and, in addition to its antimicrobial effects, has been demonstrated to potently inhibit *P. aeruginosa* biofilm formation (Overhage, Campisano et al. 2008). Furthermore, LL-37 expression is upregulated following both *Mycobacterium tuberculosis* infection and stimulation with lipopolysaccharides (LPS) in a dose-dependent manner however, to our knowledge, no data are published on LL-37 expression following NHTi infection (Mendez-Samperio, Miranda et al. 2008; Rivas-Santiago, Hernandez-Pando et al. 2008). Given the lower constitutive levels seen from the PCD epithelial cell layers, their ability to upregulate LL-37 biosynthesis in response to NTHi infection is of interest and will be investigated in Chapter 5.

Several groups have assess constitutively expressed cytokines concentrations from ALI cultured epithelial cell layers (Ren, Nelson et al. 2012; Blume, Swindle et al. 2013), however there is no literature on levels produced by primary epithelial cell layers derived from PCD patients. It is interesting to observe that constitutive expression of these cytokines is similar between PCD and non-PCD cell layers. One group reported a panel of cytokines produced by ALI cultured cell layers derived from asthmatic patients, three of which also were assessed in this work, IL-8, GM-CSF and TNF-α, finding comparable levels to those seen here (Blume, Swindle et al. 2013). Another group assessed eleven cytokines in the basolateral media of a commercially available airway epithelial tissue model (EpiAirway™, MatTek, Ashland, USA), detecting measurable concentrations of IL-6, IL-8, GM-CSF, TNF-α and finding the concentrations to be approximately a fold greater than those observed here (Ren, Nelson et al. 2012). However, they incubated the basolateral media below the EpiAirway™ cell layers for two days, whereas in this work one day was used and, furthermore, the passages and processing required to produce this tissue model might have led to the different constitutive expression of these cytokines. Interestingly, consistent with this work, the group were unable to detect measurable levels of cytokines IL-2, IL-4, IL-10 & IL-17 or IFN-γ (Ren, Nelson et al. 2012).

The percentage surface area of the ALI cultured cell layers that were ciliated was lower than that seen *in vivo* in the respiratory tract, reported to be between 15-50% dependent on the site within it (Serafini and Michaelson 1977). However it was consistent with previously report ALI cultured epithelial cell layers, Ketterer *et al.* finding 10-15% of the cell layer to be ciliated (Ketterer, Shao et al. 1999). In addition, in this work, non-PCD cell layers that had this degree of ciliation were observed, by light microscopy, to clear cell debris effectively.

The mean number of secretory cells in the ALI cultured cell layers from PCD and non-PCD patients were observed to be 5.7% and 8.8% respectively. This is consistent with

that observed in differential cell counts on bronchial brushings of respiratory epithelial cells in several papers, where between 5% and 10% of cells were reported to be secretory cells (Harvey, Heguy et al. 2007; Pezzulo, Starner et al. 2011).

3.4.1 Choice of respiratory tract model

The aim of any model of the human respiratory tract is to simulate the *in vivo* situation as closely as possible while controlling experiment variables. There are a variety of different models of the respiratory tract available, from animal models through to the ALI cultured ciliated primary respiratory epithelial cell model used here (1.5).

Respiratory epithelial cell culture has been widely used in the study of respiratory disease for the last 30 years (Lechner, Haugen et al. 1982). However ALI cultured techniques, first developed in the late 1980's, represented a leap towards the *in vivo* epithelial biology, providing an excellent model for investigating epithelial function (Whitcutt, Adler et al. 1988). Studies have shown that ALI cultures develop an epithelial cell layer that is morphologically more representative of the airway epithelium than cells cultured using liquid covered culture (LCC), with the presence of cilia, an increased depth of the cell layer and more frequent apical microvilli (Yamaya, Finkbeiner et al. 1992). Pezzulo *et al.* demonstrated that the transcriptional profile of primary ALI cultured airway epithelial cells closely recapitulates that of *in vivo* airway epithelial cells (Pezzulo, Starner et al. 2011). ALI cultured cell layers have higher TER than equivalent LCC, with groups reporting TER to be two to three fold greater across ALI cultured cell layers compared with LCC (Lee, Yoo et al. 2005; Grainger, Greenwell et al. 2006). Furthermore, ALI cultured cell layers produce secretions with a similar protein make up to that of sputum, including the mucins MUC1, MUC4, MUC5B and MUC5AC (Kesimer, Kirkham et al. 2009). Using ALI cultured cell layers in this work allowed the role of the epithelial cells, and their cilia, to be investigated without interference by other cell types.

Nasal, as oppose to bronchial, epithelial cells were utilised in the ALI cultured cell model here. Studies have shown that the ciliary beat frequency and ciliary ultrastructure are consistent between the upper and lower respiratory tract (Rutland, Griffin et al. 1982; Verra, Fleury-Feith et al. 1993). In addition, comparisons of the morphology and function, including cell size and degree of ciliation, and constitutive and stimulated inflammatory mediator release including, among others IL-8, IL-6, G-CSF, MCP-1, and receptor expression of nasal versus bronchial epithelial cells have shown them to be similar (Devalia, Sapsford et al. 1990; McDougall, Blaylock et al. 2008). It is therefore reasonable to conclude that nasal epithelial cells constitute an

accessible surrogate for bronchial epithelial cells. Furthermore, there is limited opportunity to obtain bronchial brushing from PCD patients as bronchoscopies are rarely indicated. In contrast to nasal brushings, which are routinely performed on referrals to the National PCD Diagnostic Centre at UHS in order to obtain the respiratory epithelial cells required for investigation of this diagnosis.

However this primary respiratory ALI cultured epithelial cell model is not without its limitations. *In vivo* efficient ciliary beating promotes clearance of mucus and pathogens, eventually moving them from the lower respiratory tract towards the pharynx to be swallowed or expectorated. In this ALI cultured model the cell layers were cultured on transwell inserts in 12-well culture plates. Therefore, while the cilia would be able to move the bacteria away from the underlying area of epithelial cells, they would remained trapped within the transwell insert. To allow for this during the co-culture experiments undertaken in Chapters 4 and 5, the apical surface of the co-cultures would be washed daily, removing any non-adherent bacteria, debris and waste products. For future work a culture system where the apical surface fluid is continuously circulated across and off the cell layer might be considered. However the shearing forces this movement would create might, in itself, affect the ability of bacteria to adhere to the epithelial cell layer. Further work would be required to investigate the use of this and other novel culture systems, which are discussed in Chapter 7.

Alternative airway models include the use of explanted human lung tissue and mouse models. However the availability of explanted lung tissue from patients with PCD is very limited; they rarely need lung transplants and, with advances in their medical management, lobectomies are also now very uncommon. In addition, the indication for such surgeries is, typically, end stage bronchiectasis and the lung tissue recovered is almost inevitably infected by multiple respiratory pathogens. A number of mouse models have been described with a PCD phenotype including the *Dnahc5* mouse, the nm1054 homozygote mouse and the *Dnahc11*^{iv} mouse (Tan, Rosenthal et al. 2007; Lee, Campagna et al. 2008; Lucas, Adam et al. 2012). Mouse models offer the benefit of a complete respiratory epithelium, including immune cells. However, while mouse models have slow or immotile ciliary function, several have additional defects that lead them to be significantly less viable and raise animal welfare issues; for example the nm1054 homozygote mouse develops hydrocephalus and severe anaemia that are usually lethal in the first week of life (Tan, Rosenthal et al. 2007; Lee, Campagna et al. 2008). The recently described *Dnahc11*^{iv} mouse does not have such viability issues and seems to closely match the phenotype of patients with PCD, having immotile tracheal cilia and suffering from rhinitis and otitis media (Lucas, Adam et al. 2012). However

the *Dnahc11*^{iv} mouse has not been shown, as yet, to develop significant lung pathology (Lucas, Adam et al. 2012). Whilst these models might have a place in future work in this area, the use of a human primary respiratory ALI cultured epithelial cell model was felt to best recapitulate the *in vivo* situation and was therefore chosen for this work.

3.5 Conclusion

The characterisation work undertaken in this chapter has demonstrated that ALI culture produces similar ciliated epithelial cell layers from both PCD and non-PCD patients with appropriate numbers of mucus producing cells and TER that biosynthesises CAMPs and cytokines as expected. In addition, NO biosynthesis between the two was also found to be similar, however these data are presented in Chapter 6. This confirms that primary respiratory ALI cultured epithelial cell layers are a reasonable model of the *in vivo* airway epithelium.

The only difference between PCD and non-PCD ALI cultured epithelial cell layers was in ciliary beat frequency, suggesting that comparison between the two groups will be a valid way of considering the role of ciliary function on biofilm development. The next step in investigating the study hypothesis is therefore to develop a co-culture model that will allow biofilm development on ALI cultured cell layers from both groups.

Chapter 4

Development of a co-culture model of non-typeable *H. influenzae* biofilms co-cultured on air-liquid interface cultured ciliated respiratory epithelial cell layers

4.1 Introduction

A bacterial biofilm is a structured community of bacterial cells enveloped in a self-produced complex of extracellular polymeric substance or exopolysaccharide (EPS) matrix growing attached to a surface (Costerton, Stewart et al. 1999). A key, defining criterion of a bacterial biofilm infection is the recalcitrance to antibiotic therapy effective against the same bacteria in a planktonic culture (Parsek and Singh 2003; Hall-Stoodley and Stoodley 2009). It has been demonstrated that bacteria embedded within biofilms can be up to 1000-fold more resistant to antibiotic treatment (Brooun, Liu et al. 2000), however there are limited data on this for non-typeable *Haemophilus influenzae* (NTHi) (Greiner, Watanabe et al. 2004; Jurcisek, Greiner et al. 2005; Hall-Stoodley, Hu et al. 2006; Starner, Zhang et al. 2006).

Haemophilus influenzae is a Gram-negative coccobacilli bacterium (Satola, Schirmer et al. 2003; Shen, Antalis et al. 2005; Erwin and Smith 2007). Non-typeable *Haemophilus influenzae* (NTHi), which is unencapsulated (Gilsdorf, McCrea et al. 1997), colonises the majority of healthy individuals, but is also the predominant pathogen cultured from the respiratory tract of children with non-CF bronchiectasis and in otherwise healthy children with recurrent or non-responsive pneumonia (Edwards, Asher et al. 2003; Eastham, Fall et al. 2004; Li, Sonnappa et al. 2005; Banjar 2007; De Schutter, De Wachter et al. 2011). In addition, a clinical audit of five years of microbiology tests performed in the local paediatric PCD follow-up clinic at University Hospital Southampton (UHS) revealed that NTHi was the most prevalent respiratory pathogen found in these children (1.3.5) (Walker, Jackson et al. 2012).

It was hypothesised that impaired ciliary function predisposes PCD patients to biofilm-associated infections. To investigate this hypothesis, an air-liquid interface (ALI) cultured ciliated primary respiratory epithelial cell co-culture model with NTHi was

developed, to allow assessment of the role of ciliary function on bacterial biofilm development. Respiratory epithelial cells used in the co-culture model were obtained by nasal brushing from patients with and without PCD (from here on termed 'non-PCD'). In the ALI co-cultured model, epithelial cells layers derived from PCD patients had abnormal ciliary function whereas those derived from non-PCD patients had normally functioning cilia, allowing the study hypothesis to be investigated.

The ALI cultured epithelial cell layers derived from PCD and non-PCD patients were characterised and compared in Chapter 3 and potential confounding variables to using epithelial cells from these two patient groups in developing this co-culture model considered. Results indicated that there was little difference seen between ALI cultured epithelial cell layers derived from PCD and non-PCD patients except for ciliary beat frequency which, as expected, was reduced or absent in the PCD group. In addition, NO biosynthesis by the epithelial cells from the two groups was assessed in Chapter 6 and was found to be similar.

4.2 Aims

The aims of the experiments described in this chapter were to:

- Confirm that the clinical isolates obtained from children with PCD were NTHi (4.3)
- Characterise the growth kinetics of the NTHi isolates both in planktonic culture and when cultured on a surface, to assess biofilm development, and compare the antibiotic recalcitrance between the two. The methods for evaluating and quantifying the adherent NTHi bacterial colonies were also assessed for use in the subsequent ALI co-culture model (4.3).

- Optimise the experimental design, including the type of media, multiplicity of infection (MOI or number of NTHi colony forming units used to infect the cell layers) and time course for the primary respiratory ALI cultured epithelial cell layer co-culture experiments, to investigate NTHi biofilm development, whilst maintaining cell viability (4.4).

4.3 Development and characterisation of NTHi biofilm formation on abiotic surfaces

4.3.1 Confirmation of the species of clinical PCD isolates

The diagnostic laboratory at the Health Protection Agency (UHS, Southampton, UK) isolated NTHi from the sputum and cough swabs of four children with PCD (Table 4). PCD isolates one and two were taken from children who either cultured NTHi for the first time, or who had only had it isolated in the previous three months. In contrast, PCD isolates three and four were obtained from children who had repeatedly cultured NTHi for four years. The children were not siblings. Genetic testing was not undertaken on these four strains. Whilst there are similar clinical concerns of cross-infection in PCD clinics as there are in CF clinics there have been no reports of this occurring between PCD patients to date but this is not routinely screened for. However, similarly to CF patients, PCD patients are isolated from each other in both outpatient and inpatient settings.

Table 4 *- Clinical details of the children with PCD from whom the Non-typeable Haemophilus influenzae isolates were cultured*

PCD isolate	Age at isolation (years)	Time since *H. influenzae* first isolated	Ciliary ultrastructural defect	CBF (Hertz)	CBP
1	8	1St time isolated	Inner & outer dynein arm	0	Static
2	15	3 months	Inner & outer dynein arm	0	Static
3	10	4 years	Inner & outer dynein arm	≈0	Static & twitching
4	5	4 years	Outer dynein arm	0	Static

CBF – ciliary beat frequency, CBP – ciliary beat pattern

4.3.1.1 Gram staining and V and X disc testing confirmation

Gram staining has been a standard microbiology technique to aid identification of bacterial species for over one hundred years (Gram 1884) (2.3.1.2). It demonstrated that all four PCD isolates were Gram-negative bacilli, consistent with *H. influenzae*. Further confirmation was performed with V and X discs tests. The technique is based

on the requirement of NTHi for both factor V (haemin) and factor X (nicotinamide adenine dinucleotide, NAD) in order to grow in culture (2.3.1.3). Confirmation that each PCD isolate was NTHi was made when the CFUs were seen around the V and X disc and not the V only or the X only disc.

4.3.1.2 PCR confirmation

In addition to the standard tests used to confirm that these PCD isolates were NTHi, real time PCR confirmation was performed on the each isolate having been cultured in 6-well plates for one, three and seven respectively (2.3.1.4). The prolonged culture period was used as any potential contaminating bacteria would have the opportunity to proliferate and, alongside the PCR experiment, each isolate at each time-point was plated onto chocolate agar plates and incubated overnight to check the purity of the isolates. In this way, contamination would be clearly visible by morphologically distinct colonies on the plates the following day.

Gel electrophoresis clearly demonstrated that adherent bacteria from each PCD isolate at each time-point assessed contained *H. influenzae* (Figure 25). Check plates taken at the same time did not show contamination by other bacteria. Stocks were then made of these four PCD isolates, which were then used for subsequent experiments.

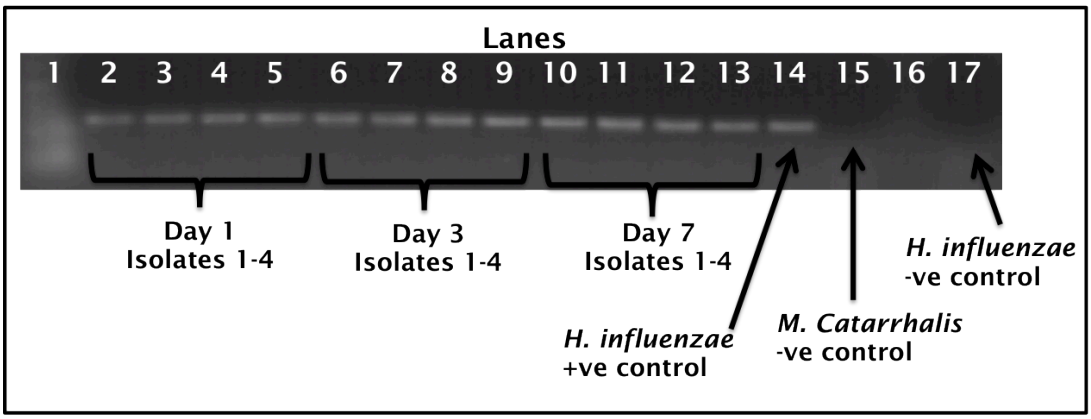

Figure 25 *- Agarose gel electrophoresis showing distinct bands for NTHi. Lane 1 – hyper V ladder (Bioline, London), lanes 2-13 – NTHi isolates 1-4 on days one, three and seven respectively, lane 14 – NTHi strain (positive control), lane 15 – M. catarrhalis strain (negative control), lane 16 – blank and lane 17 – no NTHi DNA template (negative control)*

4.3.2 Characterisation of kinetics of NTHi clinical PCD isolates in planktonic culture

Before assessing the biofilm-forming ability of the four PCD isolates their growth kinetics were assessed in planktonic culture (2.2.2). This was undertaken to determine the kinetics of exponential growth in order to standardise the number of NTHi inoculated for use in subsequent experiments.

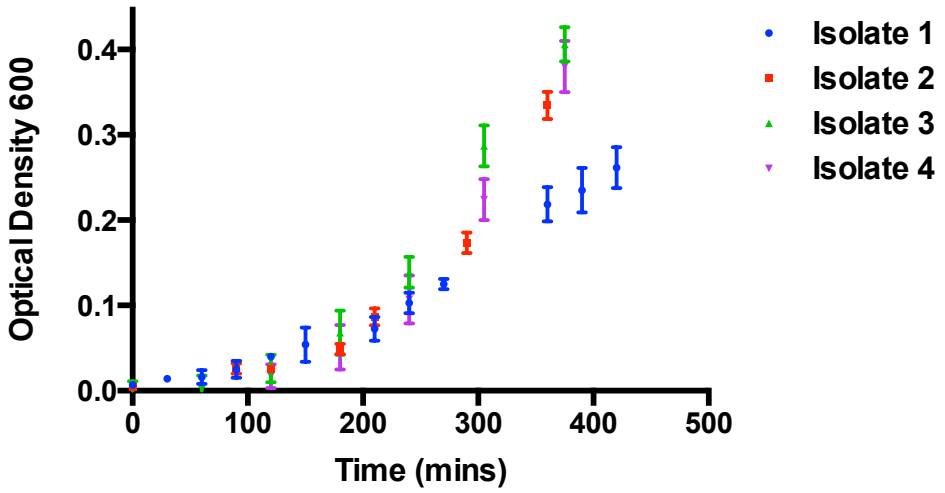

Figure 26 *- Graph of optical density$_{600}$ against time (minutes) showing the growth kinetics in planktonic culture of the four clinical PCD isolates of NTHi in supplemented BHI broth (Mean, SD) n = 4 for each isolate.*

The growth curves established the timing and OD_{600} of the lag and exponential growth phase of each PCD isolate and that the kinetics in planktonic growth of the each of the four NTHi PCD isolates were strain dependent (Figure 26). All PCD isolates had similar growth kinetics for the initial five hours. After this the growth of PCD isolate one was slower compared to the other three PCD isolates.

Having confirmed the beginning of the exponential growth for each of the PCD isolates was approximately three and a half hours; CFU counts were taken at three time points during this phase, four, five and six hours, to allow regression analysis. Regression analysis allowed the CFU counts for a specific OD_{600} to be estimated (r^2 values 0.9 - 0.99). These data were subsequently used for biofilm culture and co-culturing experiments, when the ratio of bacteria to epithelial cells, also known as the multiplicity of infection (MOI), was required. For each of the four NTHi clinical PCD isolates an OD_{600} of 0.1 was equivalent to $2x10^8$ CFUs.

4.3.3 NTHi biofilm development on abiotic surfaces

Having established the planktonic growth kinetics of the *H. influenzae* PCD isolates their ability to develop bacterial biofilms was compared. To ensure a robust assessment, multiple methods were used to quantify biofilm development, including crystal violet assays, biofilm CFU counts, scanning electron microscopy (SEM), fluorescence in situ hybridisation (FISH) and antibiotic recalcitrance.

4.3.3.1 Crystal violet assay

Crystal violet (CV) is a cationic dye that binds to the bacterial cell surface and has been used widely in the literature to quantity biofilm formation (Hall-Stoodley, Nistico et al. 2008). It was used here to estimate the biomass of adherent NTHi bacteria at one and three days. The four PCD NTHi isolates were cultured in 96-well plates, washed to remove non-adherent bacteria, stained with crystal violet that was then solubilised in ethanol and read by a plate reader (2.2.3.1). Each isolate was duplicated six times and the experiment repeated on two occasions. NTHi isolates three and four were found to have significantly more adherent biomass compared with isolates one and two both at day one and three ($P < 0.05$ for each) (Figure 27).

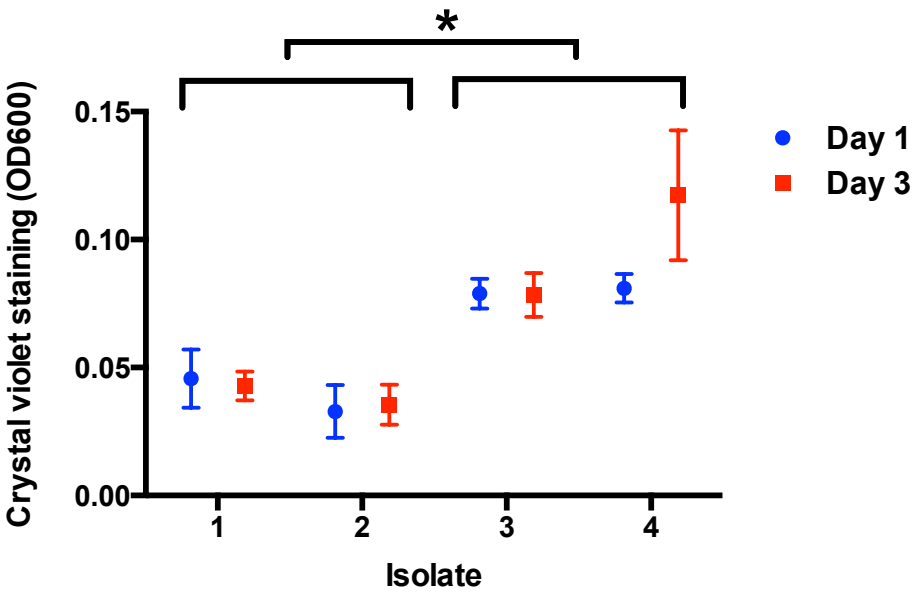

Figure 27 *- Crystal violet staining by OD_{600}, corrected for by controls, comparing the four NTHI clinical PCD isolates on polystyrene at one day (n=12) and three days (n=6). PCD isolates three and four had significantly more adherent biomass compared with isolates one and two at both day one and three (*P < 0.05 for each).*

4.3.3.2 Biofilm colony forming unit counts

The number of viable bacteria in adherent colonies at one, three and seven days was studied on polystyrene 6-well plates for each PCD isolate (2.2.3.2) (Figure 28).

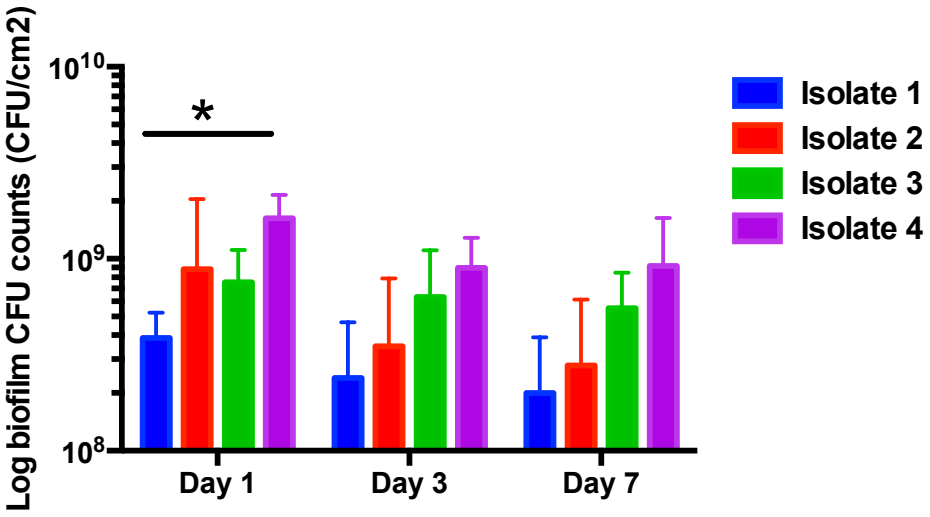

***Figure 28** – Log colony forming unit counts (CFU/cm^2) comparing adherent bacteria from the four NTHi clinical PCD isolates on polystyrene 6-well plates after one, three and seven days (mean, SEM) (n = 4 for each). (*P = 0.005)*

NTHi PCD isolate four and, at most time-points, PCD isolate three had greater biofilm CFU counts than PCD isolate one and two, however the only significant difference, as assessed by 2-way ANOVA analysis, was between isolate one and four on day one (mean (±SEM) $4.2x10^8$ CFU/cm^2 ($±8.6x10^7$) vs. $1.6x10^9$ CFU/cm^2 ($±2.7x10^8$) respectively, P = 0.005).

4.3.3.3 Scanning electron microscopy

Colony forming unit counts do not measure non-viable bacteria or the EPS matrix that makes up much of the structure of a bacteria biofilm. The PCD NTHi isolates were therefore cultured on glass cover slips in the bottom of 6-well plates, washed, then fixed and processed for scanning electron microscopy (SEM) (2.2.3.3). This allowed high-resolution visualisation of the adherent colonies of the NTHi isolates, thereby allowing assessment of the ultrastructure, EPS and the pleomorphic nature of NTHi within an adherent colony (Figure 29, 30 and 31).

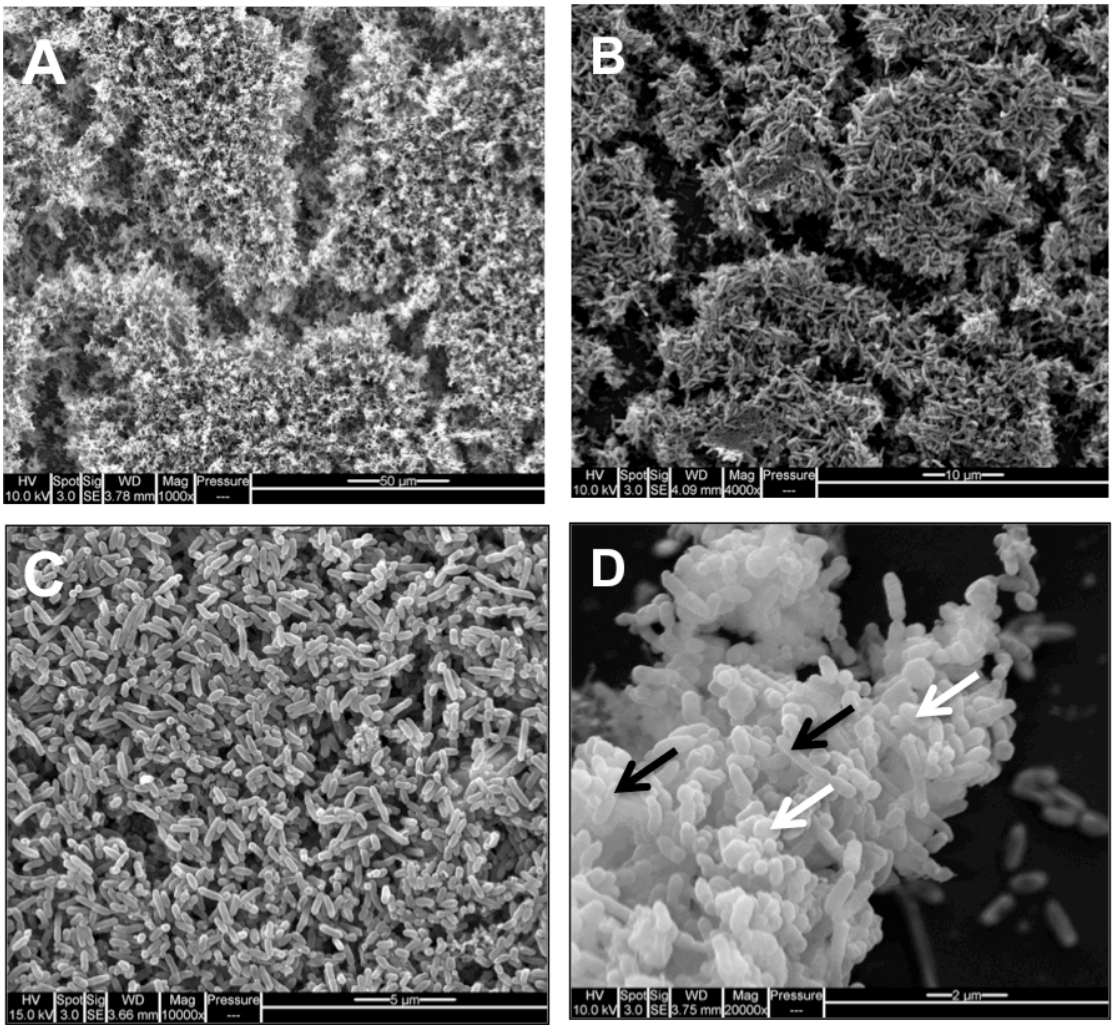

Figure 29 *- Representative scanning electron microscopy (SEM) images of adherent colonies of NTHi PCD isolate four cultured on glass cover slips in 6-well polystyrene plates at increasing magnification. A - at x1000 magnification; B - at x4000 magnification; C – at x10000 magnification and D - at x20000 magnification. Arrows point to varying morphology of bacteria within the colony (black arrows – bacilli, white arrows – coccobacilli). (Cracks in colony structure in A and B are likely to be due to dehydration artefact occurring during processing for SEM). Images taken on FEI Quanta 200 scanning electron microscope (FEI company, Eindhoven, The Netherlands)*

SEM demonstrated the self-produced EPS matrix, often seen as a mesh-like structure (Figure 30 A and B, arrows) but also as linear strands stretching between bacteria (Figure 30 C and D, arrows). In some areas the bacteria seem to aggregate along these strands (Figure 30 D, arrow) and form lines of bacteria (Figure 30 E and F, arrows) that add to the lattice-structure of the biofilm.

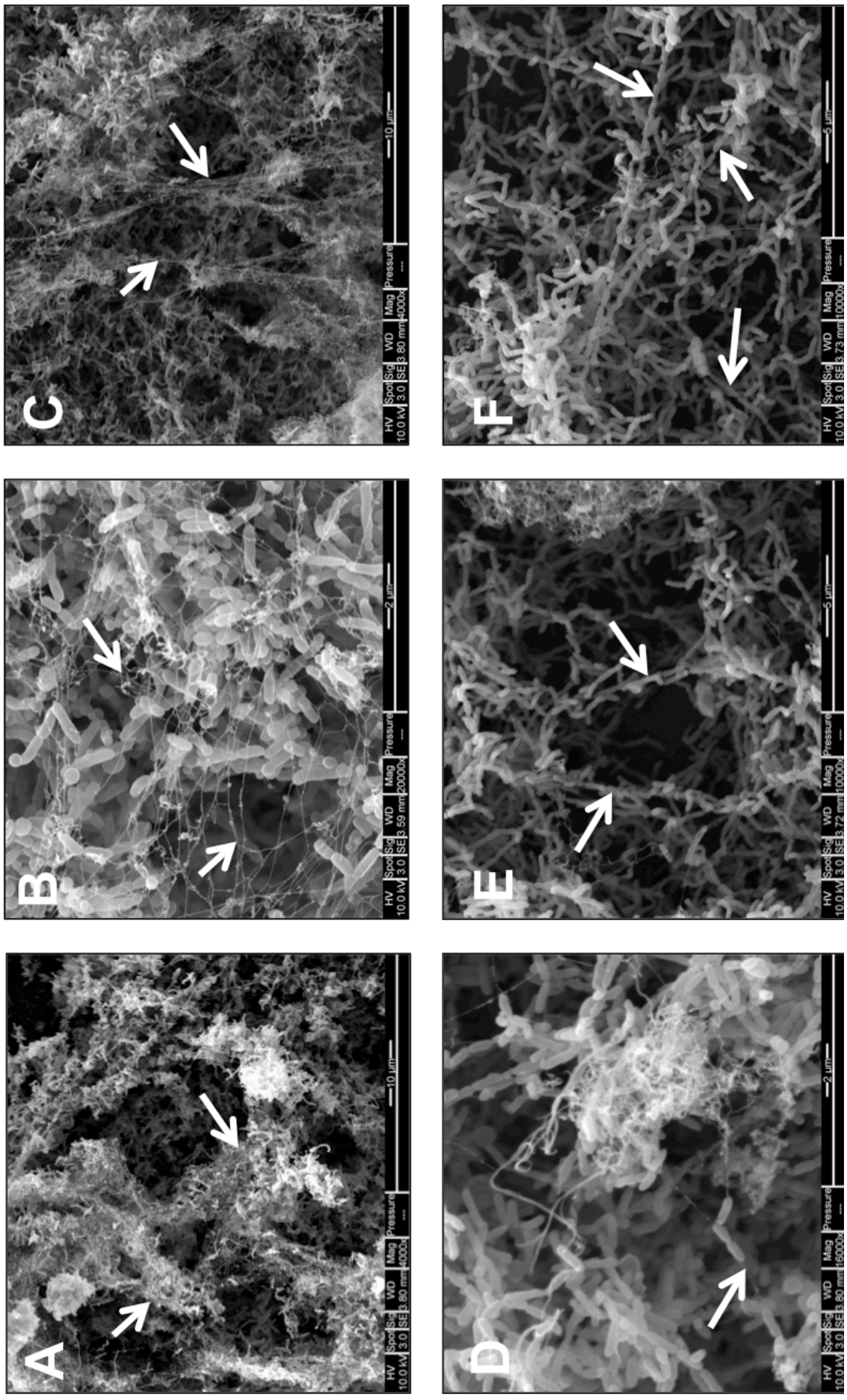
A
B
C
D
E
F

Figure 30 *- Representative scanning electron microscopy images at varying magnifications of self-produced extracellular polymeric substance (EPS) matrix by NTHi PCD isolates three and four cultured on glass cover slips in 6-well polystyrene plates at different time points. A - shows EPS mesh at low magnification (x4000) and B at higher magnification (x20,000) showing the fine strands of EPS (arrows); C and D - show linear strands of EPS between bacteria at low and high magnification (x4000 and X 16,000 respectively, arrows) D also shows bacilli beginning to aggregate along this strand (arrow); E and F - show lines of bacilli (arrows). Images taken on FEI Quanta 200 SEM.*

SEM images supported data from previous assays indicating that NTHi PCD isolate four produced more robust adherent bacterial colonies than isolate one (Figure 31). Furthermore, they allowed comparison of the EPS produced by the four isolates. PCD isolate four clearly produced more EPS than isolate one (Figure 31).

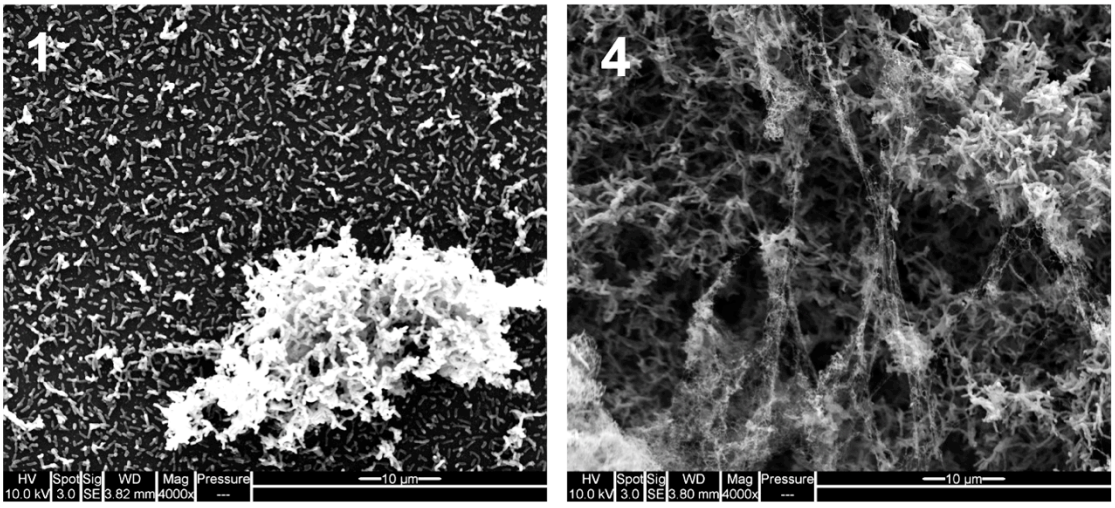

Figure 31 *- Representative scanning electron microscopy images (magnification x4000) of day seven NTHi biofilms for PCD isolates one and four cultured on glass cover slips in 6-well polystyrene plates, demonstrating PCD isolate four produced more EPS matrix and larger biofilms compared with PCD isolate one. Images taken on FEI Quanta 200 SEM.*

4.3.3.4 Fluorescence in situ hybridization

Fluorescence *in situ* hybridization allowed the depth and, in later experiments, the volume of adherent NTHi colonies to be assessed. The four NTHi PCD isolates were cultured on MatTek glass bottomed plates (MatTek, Ashland, UK) for three days then washed, fixed and processed for FISH then imaged on a Leica SP5 confocal laser scanning microscope (CLSM, Leica Microsystems, Milton Keynes, UK) (2.2.3.4).

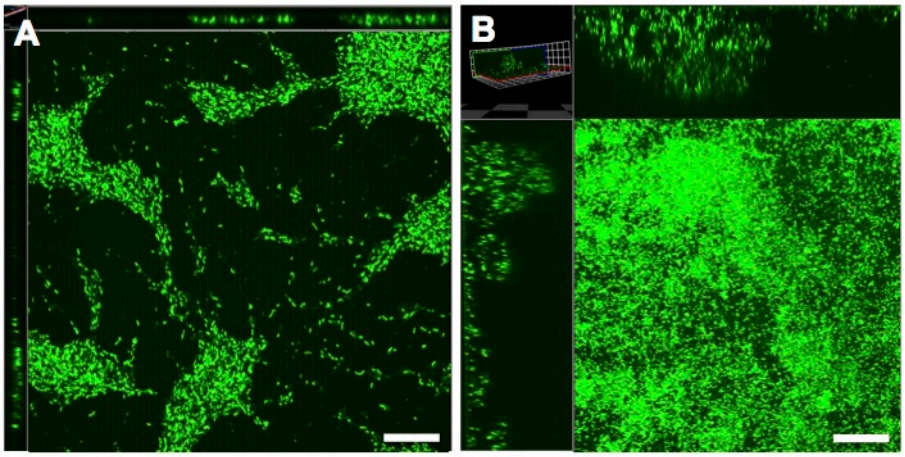

Figure 32 *– Representative confocal laser scanning microscopy merged maximal views with z planes of fluorescence in situ hybridization (FISH) of day three of PCD isolates one (A) and four (B) NTHi biofilms, using a CY3 labelled NTHi FISH probe (green). Isolates four had a adherent colony depth of up to 82μm and was much more widespread as compared to a depth of 13μm for isolate one. Images taken on a Leica SP5 confocal laser-scanning microscope (CLSM, Leica Microsystems, Milton Keynes, UK) at magnification x400. Scale bars represent 40 μm*

While limited CLSM images were taken at this stage, FISH performed on adherent NTHi colonies after three days of culture indicated, along with previous assays, that PCD isolate four formed larger adherent colonies than isolate one. The depth of adherent NTHi colonies from isolates four were six times greater in areas than isolate one (82μm vs. 13μm) and was much more widespread (Figure 32).

4.3.4 Antibiotic recalcitrance

A defining criterion of a bacterial biofilm infection is the recalcitrance of the bacteria in a biofilm to antibiotic therapy effective against the same bacteria in a planktonic culture (Parsek and Singh 2003; Hall-Stoodley and Stoodley 2009). In order to assess the antibiotic recalcitrance, both in planktonic culture and once adherent to a surface, the minimum inhibitor concentration (MIC) for NTHi for both was investigated. The MIC is the lowest concentration of a specific antibiotic that will inhibit growth of that bacterium (Andrews 2001). In the work here CFU counts were used as the measure of bacterial growth and hence the MIC was taken as the lowest concentration of antibiotic that led to a lower CFU count than at baseline.

Initial antibiotic experiments utilised amoxicillin, as this has been the standard antibiotic used in clinical practice for NTHi infections. However, we determined that

PCD isolates three and four were resistant and PCD isolate two only partially sensitive to amoxicillin therefore cefotaxime was used for subsequent experiments (2.2.4) (Figure 33).

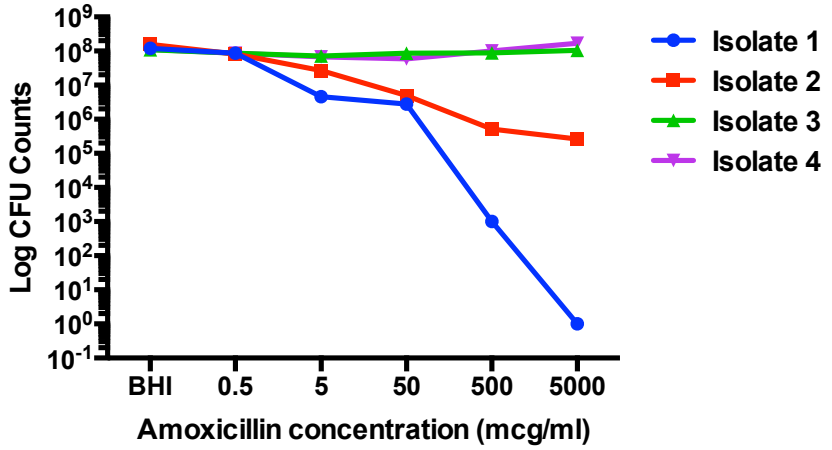

Figure 33 *– Log CFU counts for each of the four NTHi PCD isolates in planktonic culture incubated overnight with 10-fold dilutions of amoxicillin (0.5 – 5000 mcg/ml) and a no antibiotic BHI control (lines joining data points added for clarity)*

The lowest concentration of cefotaxime to inhibit the growth of PCD isolate four in planktonic culture compared to baseline (MIC) was 0.02 mcg/ml (Figure 34a). By comparison the MIC for the adherent bacteria for the same PCD isolate after a three-day culture was 0.2 mcg/ml (Figure 34b). These data demonstrate that for isolate four adherent bacteria were 10 times more recalcitrant to antibiotics than when in planktonic culture.

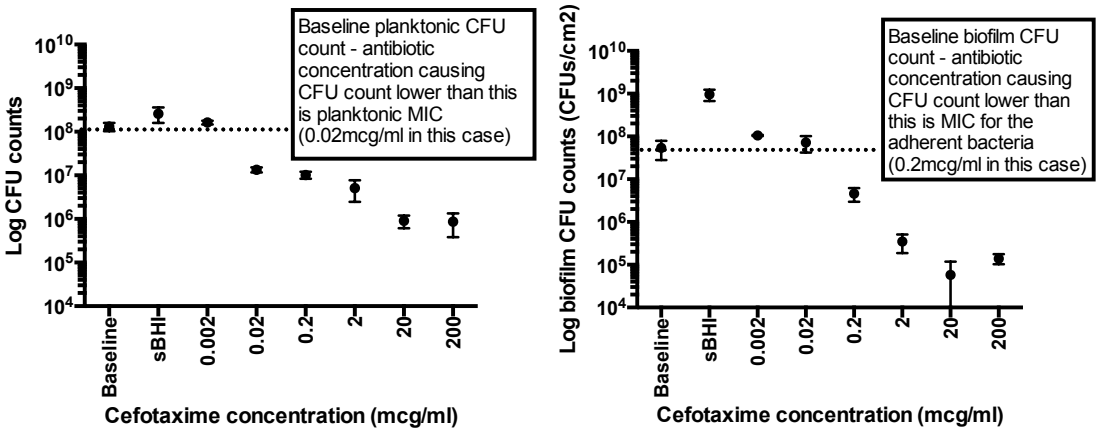

Figure 34 *– a) Log CFU counts of NTHi isolate four in planktonic culture at baseline and after overnight incubation with 10-fold dilutions of cefotaxime (0.002 – 200 mcg/ml) with no antibiotic sBHI control (n=4) (mean, SEM). Planktonic MIC is the concentration of antibiotic causing a lower CFU count than baseline, in this case*

0.02mcg/ml. b) Log CFU counts of adherent NTHi PCD isolate four bacteria cultured for three days in 12-well plates at baseline and after overnight incubation with 10-fold dilutions of cefotaxime (0.002 – 200 mcg/ml) with no antibiotic sBHI control (n=4) (mean, SEM). MIC for adherent bacteria is the concentration of antibiotics causing a lower CFU count than baseline, in this case 0.2mcg/ml.

Planktonic MIC calculated by this method was compared to MIC evaluator (M.I.C.E™) strips (Oxoid, Basingstoke, UK), a validated method to confirm MIC accuracy (Mushtaq, Warner et al. 2010), (2.2.4.1). Table 5 summarises the comparable MIC data for all the isolates.

Table 5 *– Comparing the MIC of the 4 NTHi isolates in planktonic (using both CFU and M.I.C.E™ strip method) and biofilm growth using cefotaxime (all ng/ml)*

Isolate	M.I.C.E™ strips	Planktonic CFU MIC	Adherent bacteria CFU MIC	Difference between planktonic & biofilm CFU MICs
1	15	20	20	No difference
2	30	200	200	No difference
3	12	20	200	10x increase
4	12	20	200	10x increase

There were differences seen between the two methods used to assess the isolates in planktonic growth (M.I.C.E™ strips and the CFUs) however these can be explained by the different dilutions of antibiotic that were assessed by these two methods. The M.I.C.E™ strips used a doubling dilution, with a validated method for estimating values between these concentrations, whereas the CFU method used a 10-fold dilution. Hence the M.I.C.E™ strips could give a more accurate measurement and, being the validated method widely used in microbiological centres, should be considered correct (Mushtaq, Warner et al. 2010). If isolate two was taken as an example, the M.I.C.E™ strip gave an MIC of 30 ng/ml whereas the CFU method gave an MIC of 200 ng/ml. However the next lower antibiotic concentration using the CFU method was 20 ng/ml, below the 'true' MIC as demonstrated by the M.I.C.E™ strip. Hence 200 ng/ml, the next concentration of antibiotic assessed by the CFU method, was consistent with the M.I.C.E™ strip result. This is true of the other isolates where, similarly, the MIC calculated by both methods were also consistent.

The main findings therefore were that the adherent bacteria from PCD isolates three and four exhibited an MIC 10 times greater than bacteria from the same isolates in planktonic culture. However, for PCD isolates one and two no increase in tolerance was seen.

4.3.5 Summary of the characterisation of NTHi biofilm formation on abiotic surfaces

A bacterial biofilm is a structured community of bacterial cells enveloped in a self-produced complex of extracellular polymeric substance or exopolysaccharide (EPS) matrix growing attached to a surface (Costerton, Stewart et al. 1999). However, in addition, a widely considered defining criterion of a bacterial biofilm infection is the recalcitrance of the bacteria in a biofilm to antibiotic therapy effective against the same bacteria in a planktonic culture (Parsek and Singh 2003; Hall-Stoodley and Stoodley 2009).

While it has been demonstrated that all the PCD NTHi isolates were able to form a structured community of adherent bacteria that produced EPS, only isolates three and four had increased recalcitrance to antibiotics when in adherent colonies as oppose to planktonic culture. Hence, while this confirms that PCD NTHi isolates three and four form bacterial biofilms, by the generally accepted criteria, it has also shown that isolates one and two do not. It is interesting, therefore, that the two biofilm forming NTHi isolates were both cultured from children with recurrent NTHi infections, for four years, whereas isolates one and two were obtained from children who had only recent culture this pathogen. This supports the notion that bacterial biofilms are likely to be important in chronic and recurrent infections.

The different techniques used to assess the biofilm-forming ability of the isolates demonstrated heterogeneity in NTHi biofilm ultra-structure, highlighting the importance of the EPS matrix in NTHi biofilm development.

One NTHi PCD isolate, isolate four, consistently demonstrated the most robust biofilm development. Biofilm CFU counts, FISH with CLSM and SEM were determined to be the optimal techniques for quantifying NTHi biofilm development on the co-culture model. This isolate and these techniques were therefore used in subsequent co-culturing experiments.

4.4 Development of a co-culture model of non-typeable *H. influenzae* biofilms co-cultured on air-liquid interface cultured ciliated epithelial cell layers

In this section the experimental design for the co-culture model, in particular the type of media, amount of bacteria (multiplicity of infection, MOI) and time course was optimised. Initial experiments were undertaken using an immortalised epithelial cell line, 16HBE cells, (4.4.1) before moving on to primary cell cultures (4.4.2).

4.4.1 Co-culture of NTHi on 16HBE cell layers

In order to optimise the techniques to quantify biofilm formation in the co-culture model initial experiments were carried out using 16HBE cells cultured at an ALI on transwell inserts (2.3.1.1). In addition, these experiments were used to confirm that NTHi PCD isolate four was the optimal isolate for use subsequently in the co-culture model by comparing it with PCD isolate one. 16HBE cells were used for these initial experiments due to the limited availability of primary ALI cultured respiratory epithelial cell layers. However it was recognised that immortalised cell lines might have altered cell signalling compared with primary cells and, more importantly, 16HBE cells do not ciliate (Huang, Wiszniewski et al. 2011).

In the previous experiments on abiotic surfaces (4.3) NTHi PCD isolate four consistently formed robust biofilms whereas PCD isolate one did not. To confirm this remained the case in co-culture with NTHi on epithelial cell layers, initial experiments were performed using both PCD isolates one and four. A multiplicity of infection (MOI), or ratio of bacterial colony forming units (CFU) to epithelial cells, of 1 was used. Biofilm development was assessed at one and three-day time-points by determining biofilm CFUs alongside visualising the biofilm with SEM (2.3.3) and experiments were repeated twice. In order to optimise FISH with CLSM in co-culture this technique was undertaken in one of the experiments with PCD isolate four (2.3.3).

Scanning electron microscopy images showed micro-colonies and early NTHi biofilm development on the 16HBE cell layers after 24 hours for both PCD isolates one and four (Figure 35 A and B respectively). However, at this early stage PCD isolate four demonstrated larger aggregates with more EPS matrix than isolate one. This was more evident by day three when PCD isolate four had formed a thick layer of biofilm across the surface of the 16HBE cells (seen at across the top half of Figure 35 D as highlighted), whereas isolate one exhibited small micro-colonies with less EPS matrix

evident than isolate four had demonstrated on day one (Figure 35 C and D respectively).

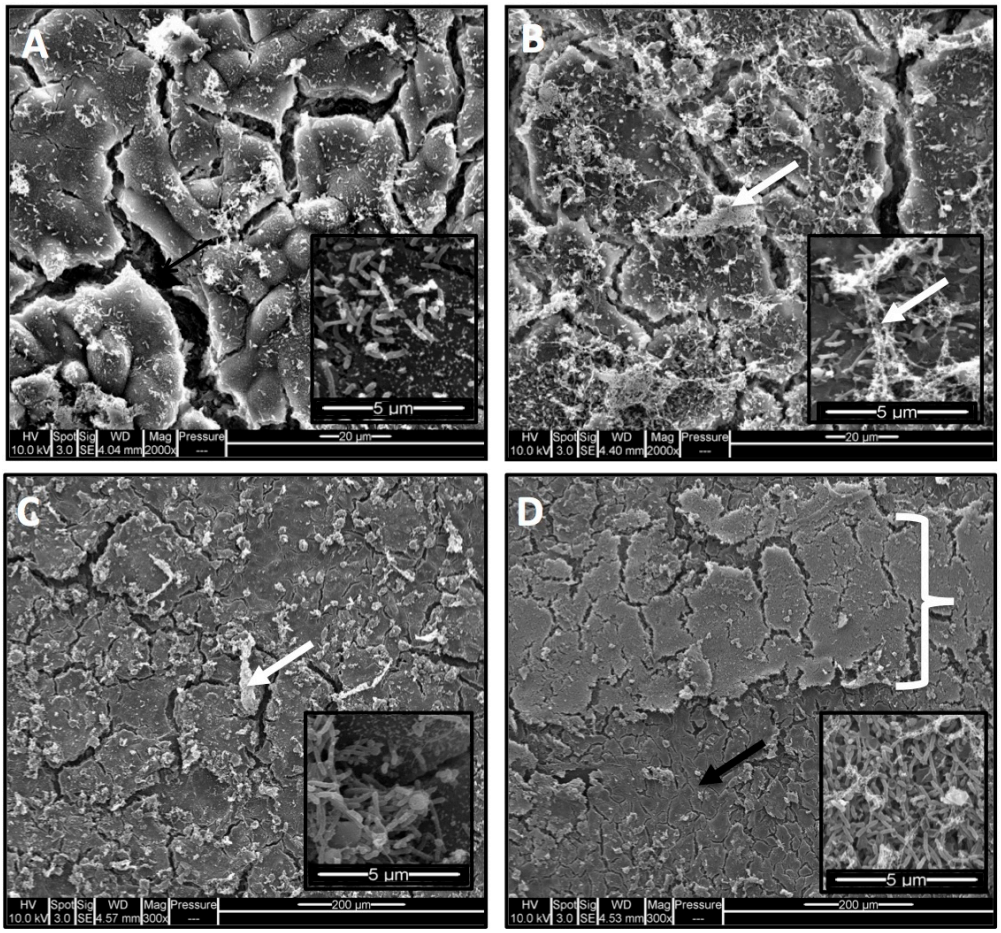

Figure 35 *- Representative scanning electron microscopy images of 16 HBE ALI cultured cell layers co-cultured with NTHi isolate one and four at a multiplicity of infection of one for one day (A and B respectively) and three days (C and D respectively) then washed leaving adherent bacteria. (Cracks in cell layer and biofilms due to dehydration artefact occurring during SEM processing). Magnification x2000 for A and B and x300 for C and D with x16000 magnification inlaid images. White annotation indicates NTHi bacterial biofilm, black arrow indicates underlying cells. Images taken on a FEI Quanta 200 SEM (FEI company, Eindhoven, The Netherlands)*

Although PCD isolate one demonstrated less EPS, as seen by SEM, both isolates had similar biofilm CFU counts at day one. By day three however PCD isolate four had significantly higher numbers of NTHi CFUs than isolate one (mean (±SD) $7.2x10^8$ CFUs ($±2x10^8$) vs. $9.0x10^7$ CFUs ($±3x10^7$) respectively, P = 0.007) (Figure 36).

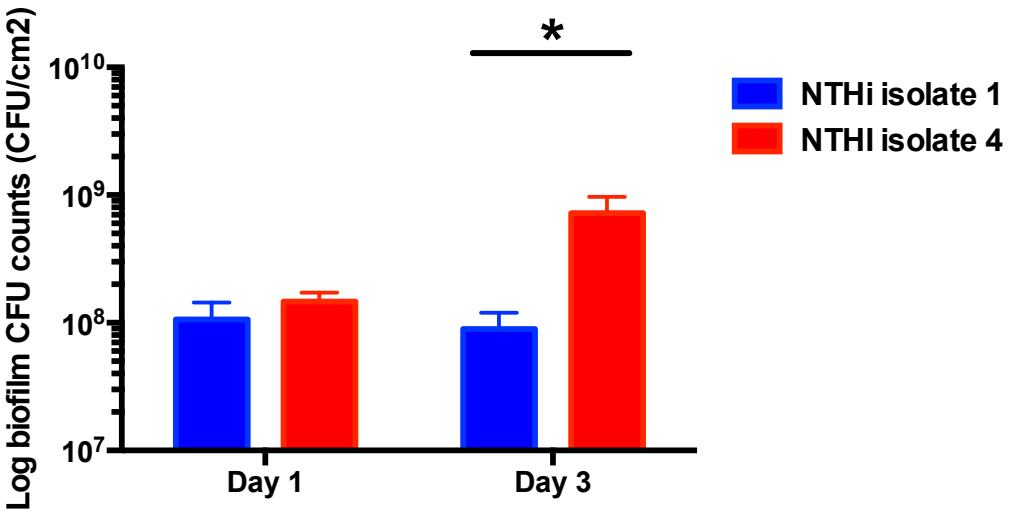

Figure 36 *– Log NTHi biofilm CFU counts on day one and three for NTHi isolates one and four co-cultured on ALI cultured epithelial cell layers of 16HBE cells (mean, SD) (* P < 0.005)*

In one of the initial experiments, PCD isolate four was used to optimise the technique of FISH with CLSM in co-culture. This corroborated day three SEM and CFU data for PCD isolate four demonstrating a thick biofilm, seen here with the NTHi bacterial biofilm in green (Figure 37).

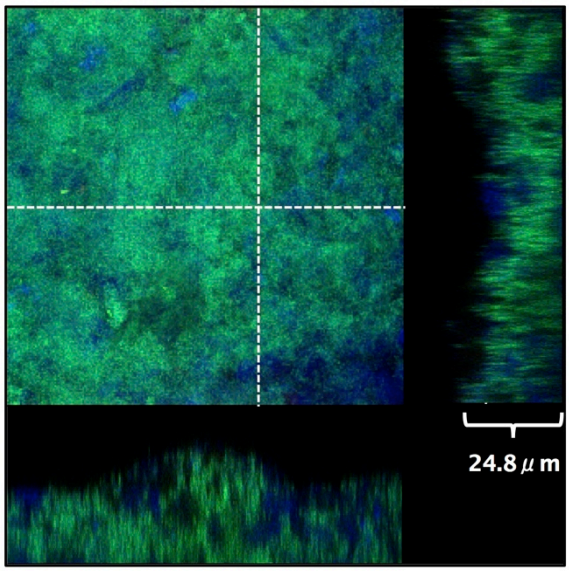

Figure 37 *- Representative CLSM image (maximal with orthogonal views) of a three day NTHi biofilm using PCD isolate four co-cultured on 16HBE cells at an MOI of 1 using a CY3 labelled NTHi FISH probe (green) and a SYTOX® blue nuclear counter-stain (blue). Images taken on Leica SP5 CLSM (Leica Microsystems, Milton Keynes, UK) at magnification x600.*

4.4.1.1 Summary

Measurement of NTHi biofilm CFUs and visualisation of the biofilm by SEM and, following FISH, by CLSM provided quantitative and qualitative data on biofilm development in this co-culture model. These experiments were consistent with NTHi biofilm development on abiotic surfaces (4.3.3) demonstrating PCD isolate four produced significantly robust biofilms whereas PCD isolate one formed smaller micro-colonies. Isolate four was therefore used for subsequent co-culturing experiments. Substantial NTHi biofilms formed by day three on 16HBE cells using an MOI of 1 and these parameters were therefore used as a basis for the initial co-culturing experiments using primary ALI cultured cell layers.

4.4.2 Co-culture of NTHi on primary ALI cultured respiratory epithelial cell layers

4.4.2.1 Initial experiment on primary epithelial cell layers

An MOI of 1 and three-day time course, informed by experiments above on 16HBEs, were used in initial co-culturing experiments of NTHi isolate four on primary epithelial cell layers (2.3.2). NTHi biofilm formation on ALI cultured primary epithelial cell layers from PCD (n=2) and non-PCD (n=1) patients were compared to 16HBE cells (n=3) in two separate experiments. The outcomes assessed were biofilm CFUs, SEM and FISH with CLSM (2.3.3).

Biofilm CFU counts were almost 3-Log fold lower for the PCD and non-PCD cell layers as compared to16HBE cells (mean (±SD where possible) $8.4x10^4$ CFUs ($\pm1.5x10^4$) and $1.0x10^5$ CFUs (N/A) vs. $4.5x10^7$ (($\pm3.0x10^7$) respectively) (Figure 38).

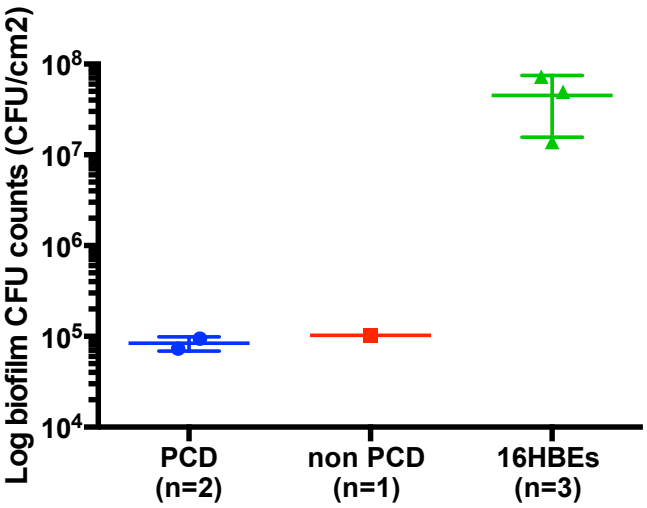

Figure 38 *- Log biofilm CFU counts of NTHi isolate four co-cultured at an MOI of 1 on ALI cultured epithelial cell layers of PCD (n=2), non-PCD (n=1) and 16HBE cells (n=3) for three days (mean, SD where applicable)*

These differences were also reflected in the SEM images where, consistent with images in 4.4.1 Figure 35, a thick biofilm was seen on the 16HBE cell layer with the cell layer itself only visible in cracks and gaps in the biofilm, as compared to only early biofilm development with small micro-colonies on the primary ALI cultured cells (Figure 39 A and B).

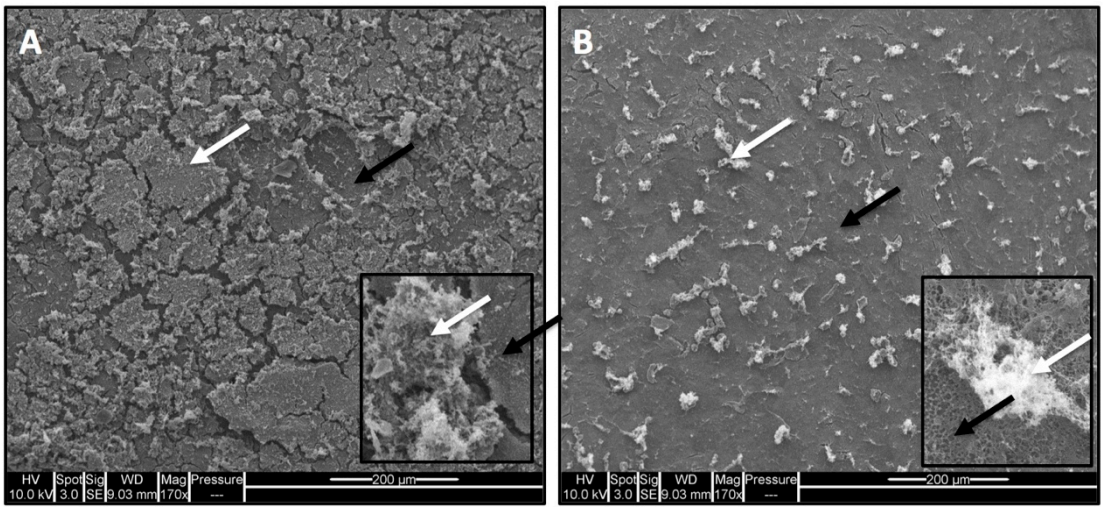

Figure 39 *- Representative SEM images of three day NTHi biofilm co-cultured at an MOI of 1 on A) ALI cultured 16HBE cell layers and B) primary ALI cultured non-PCD airway epithelial cell layers. A thick layer of PCD isolate four biofilm was seen on the 16HBE cell layer with the cell layer itself only visible in the gaps of the biofilm, see inlaid*

image, whereas on the primary cell layer only small micro-colonies with limited EPS matrix were seen. White arrows indicate bacterial biofilm, black arrows indicate underlying cells. Magnification x170 with x4000 images inlaid. Images taken on a FEI Quanta 200 SEM.

Confocal laser scanning microscopy of NTHi PCD isolate four biofilm on the co-cultures, using FISH with 16s rDNA specific probes, further corroborated the biofilm CFU and SEM data, demonstrating a thick, 33 µm, biofilm (in green) across the 16HBE cell layer (Figure 40 A). In contrast, primary cells co-culture exhibited occasional sparse NTHi biofilm clusters (Figure 40 B).

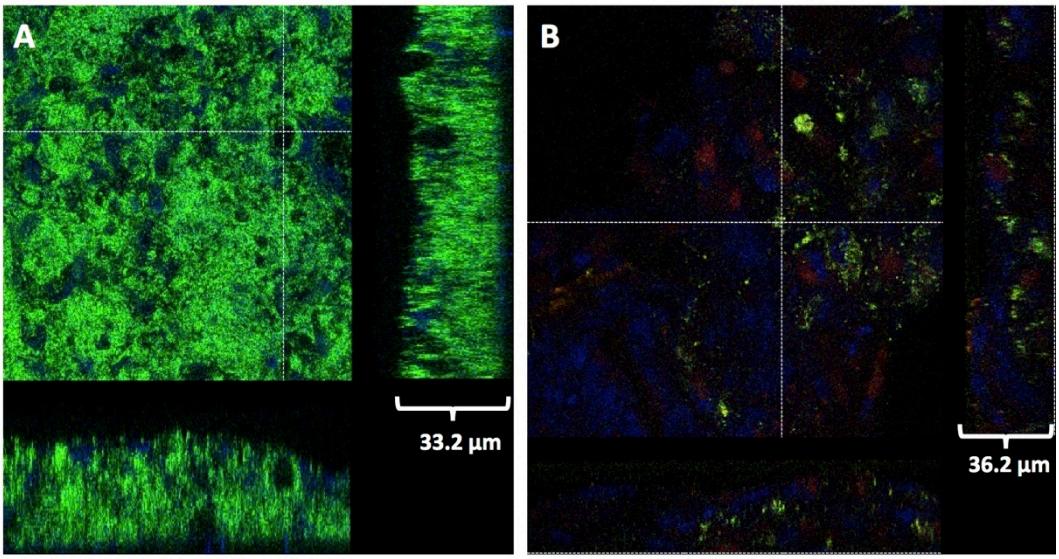

Figure 40 *- Representative CLSM images (maximal with orthogonal views) of NTHi biofilms co-cultured on A) ALI cultured 16 HBEs and B) primary ALI cultured non-PCD airway epithelial cell layer at an MOI of 1 for three days. FISH using CY3 labelled NTHi FISH probe (green), B-tubulin antibody with a 594 Alexa fluor secondary (red) and a SYTOX® blue nuclear counter-stain (blue). The images demonstrated a thick NTHi bacterial biofilm on the 16HBE cell layer whereas only occasional small micro-colonies were visible on the primary epithelial cell layer. Magnification x600. Images taken on Leica SP5 CLSM (Leica Microsystems, Milton Keynes, UK).*

4.4.2.1.1 Discussion

The most striking finding from these experiments was the clear difference in NTHi biofilm formation on immortalized 16HBEs cell layers compared with primary cell layers with a 3-Log fold increase in bacterial biofilm CFUs on the 16HBE cells, corroborated by both SEM and CLSM images of FISH.

It had been predicted that there would be a difference between the 16HBE and primary cell layers due to the presence and, in the case of the non-PCD cell layers, function of cilia, as 16HBEs cells do not ciliate. In addition, immortalised cell lines can have altered cellular responses as compared to primary cells (Huang, Wiszniewski et al. 2011). However, the difference seen was more striking than expected. There had however been a difference in the experiment conditions for the 16HBE cells as compared to the primary cells for these experiments. The primary cells were cultured in ALI media and 16HBE cells in MEM media, as these were the normal media used for culturing these cell types (2.3.1). The affect of these culture media on NTHi biofilm development therefore needed to be investigated (4.4.2.2).

4.4.2.2 Effect of media on NTHi planktonic and biofilm bacterial growth

It was hypothesised that the media used may have been responsible for the significant difference in the biofilm development seen between the primary and immortalized cell layers. If this were the case then MEM media would be found to encourage NTHi growth kinetics or ALI media to inhibit them. Experiments were therefore undertaken to investigate this in planktonic and biofilm phases of growth, both on abiotic surfaces and primary ALI cultures. In addition to MEM and ALI media, comparison was made to supplemented brain heart infusion (sBHI), which is the standard media in which NTHi is cultured.

4.4.2.2.1 Media type had minimal effect on NTHi planktonic phase growth

The planktonic growth kinetics of NTHi PCD isolate four was compared when cultured in ALI, MEM and sBHI media (2.2.2). Experiments were carried out in duplicate. Twenty-four hour bacterial growth was slightly lower for the ALI media compared to MEM and sBHI but this was not significant ($P = 0.61$) (Figure 41). The small difference demonstrated would not account for the 3-Log fold difference seen in the biofilm CFU counts on 16HBE cells as compared to primary cells. This suggests that each media contained sufficient nutrients to allow NTHi replication to occur.

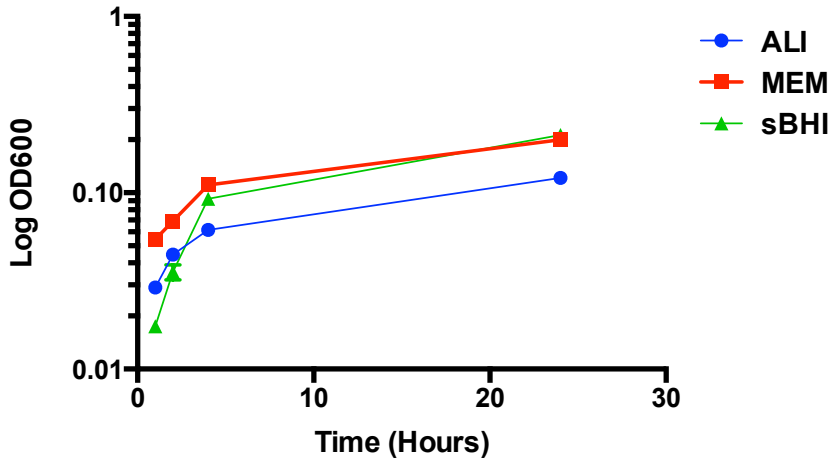

Figure 41 *- Growth kinetics (Log$_{10}$ OD$_{600}$) of NTHI isolate four in ALI, MEM or sBHI media over 24 hours (Mean, SD) (n=2 for each) (lines between data points added for clarity). No significant differences were seen between the different media.*

4.4.2.2.2 Media type had minimal effect on NTHi biofilm development on abiotic surfaces

Non-typeable *Haemophilus influenzae* isolate four biofilm development, cultured on polystyrene for two days, was then compared in the three media (2.2.3). This was measured by biofilm CFU counts and SEM (Figures 42 and 43). Experiments were carried out in duplicate.

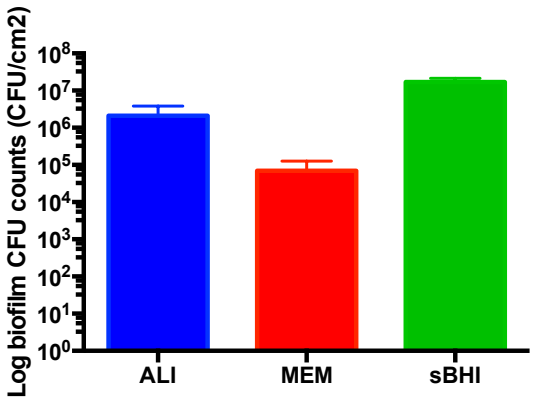

Figure 42 *- Log$_{10}$ biofilm CFU counts for NHTi isolate four grown on an abiotic surface in ALI, MEM and sBHI media for 48 hours (mean, SD) (n=2 for each)*

Biofilm formation was found to be lower in the MEM media by 1-Log fold compared with the ALI (Figure 43). These data were supported by SEM images, where less biofilm formation was seen with the MEM media than both the ALI and sBHI media (Figure 43 B, A and C respectively).

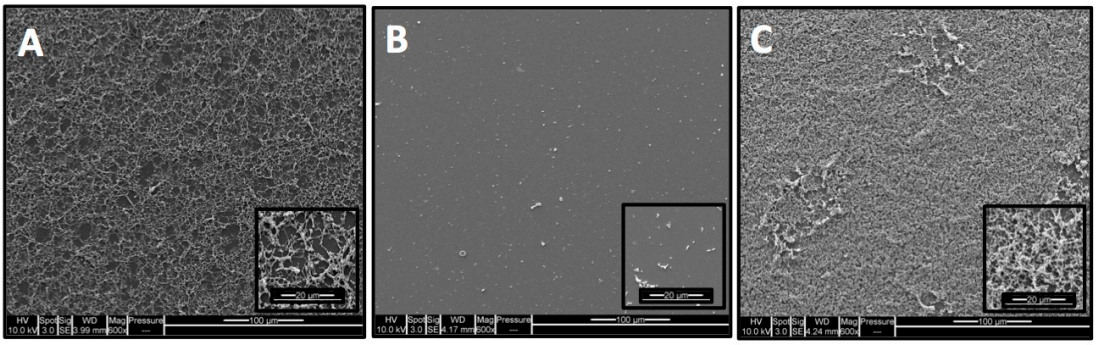

Figure 43 *– Representative SEM images of NHTi isolate four grown on polystyrene in A) ALI, B) MEM and C) sBHI media for two days. A thick biofilm was seen in sBHI media and to a slightly lesser extent in ALI media whereas only occasional micro-colonies were seen in MEM media. Magnification x600 with x2000 images inlaid. Images taken on a FEI Quanta 200 SEM.*

4.4.2.2.3 Media type had no effect on NTHi biofilm development on ALI cultured primary epithelial cell layers however MEM media was detrimental to cell viability

Finally, an experiment using MEM and ALI media on non-PCD primary epithelial cell layers was undertaken. An MOI of 1 and a three-day time course were used for the experiment. Biofilm development was measured by biofilm CFUs and cell viability by baseline and then daily measurements of ciliary beat frequency (CBF) and trans-epithelial electric resistance (TER) (2.3.3). Given the limited number of ALI cell layers available experiment was undertaken once.

Biofilm CFU counts for ALI and MEM media were similar in this co-culturing experiment on ALI cultured primary cell layers ($4x10^6$ vs. $9x10^6$ respectively) (Figure 44).

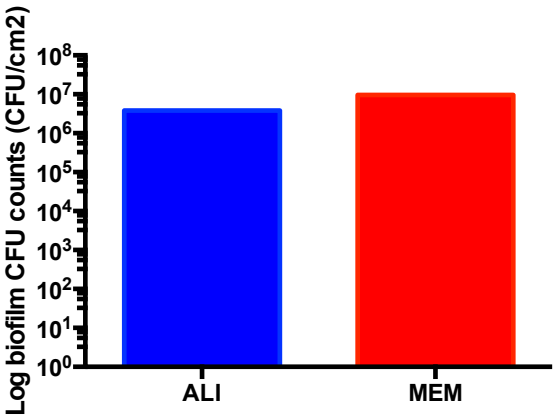

Figure 44 *– Log biofilm CFU counts on a pair of non-PCD ALI cultured epithelial cell layers three days post co-culture with NTHi isolate four at MOI 1 in either ALI or MEM media (n=1 for each)*

The primary cell layer in ALI media maintained a CBF in the normal range (11-20 Hz) throughout the co-culture experiment with NTHi (Figure 45). The CBF in ALI media was significantly higher than for the cell layer in MEM media both at baseline and daily thereafter (p=<0.0001 using repeated measures ANOVA). The CBF in MEM media dropped below the normal range by day one and remain below it throughout the experiment.

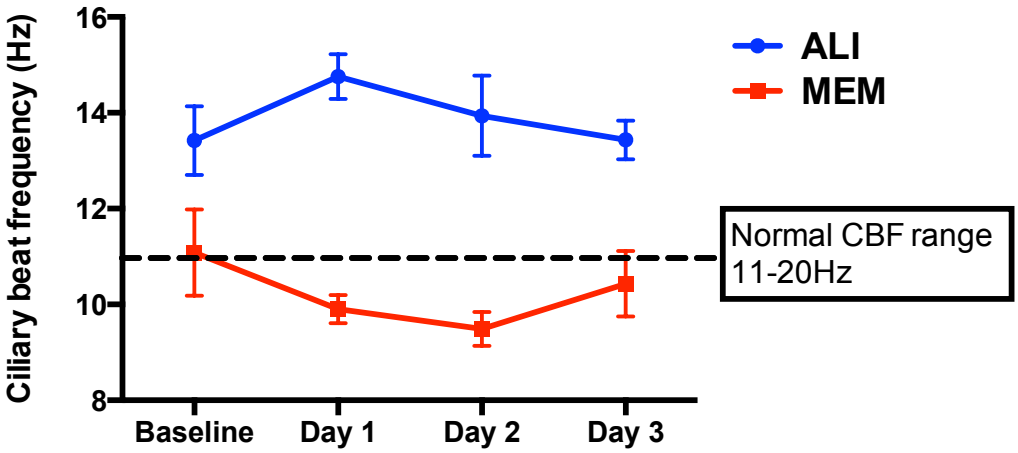

Figure 45 *– Mean ciliary beat frequency (CBF) at baseline and daily from non-PCD ALI cultured epithelial cell layers co-cultured with NTHi isolate four at an MOI of 1 for three days in either ALI or MEM media (mean, SEM) (mean of six CBF readings, lines between data points added for clarity) (P < 0.0001 using repeated measures ANOVA)*

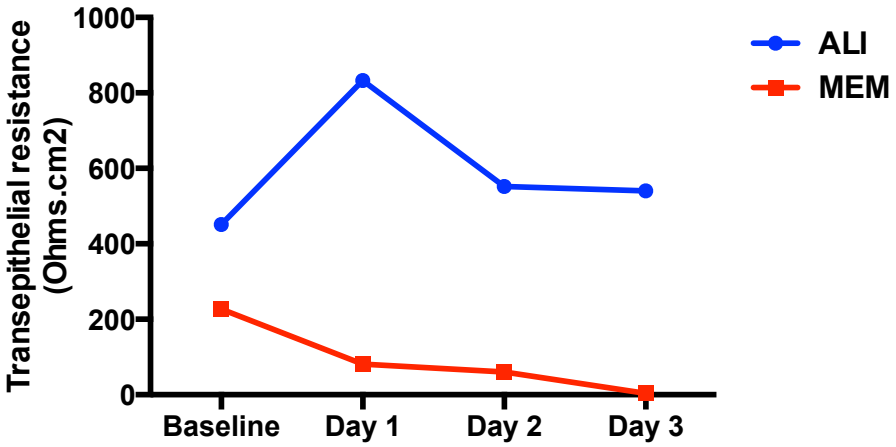

Figure 46 *- Mean corrected trans-epithelial resistance at baseline and daily across non-PCD ALI cultured epithelial cell layers co-cultured with NTHi isolate four at an MOI of 1 for three days in either ALI or MEM media (mean) (n=1 for each) (lines between data points added for clarity)*

Similarly to CBF, in ALI media TER for the cell layer remained above baseline following co-culture with NTHi, increasing on day one (Figure 46). However, in MEM media the TER for the cell layer dropped below baseline by day one and decreased to zero suggesting that tight junction formation of the primary cell layer was compromised in this media type.

4.4.2.2.4 Discussion

Experiments did not demonstrate that MEM media encouraged NTHI growth, or that ALI inhibited it. In fact, NTHi biofilm growth on an abiotic surface was lower with the MEM media than ALI. These findings therefore refute the hypothesis that the different media used in the initial primary cell co-culture experiments (4.4.2.1) accounted for the 3-Log fold difference seen in biofilm formation on 16HBEs as compared to primary cell layers, suggesting other factors were at play. These potentially included the lack of ciliation, possible differences in pro-inflammatory cellular responses or cell layer morphology. In addition, while the ALI media did not seem to be detrimental to biofilm formation the MEM media had significant adverse effects on epithelial cell viability, evidenced by reductions in both TER and CBF in primary cell layers when using this media (Figures 45 and 46).

Due to concerns over epithelial cell viability when using MEM media, future co-culturing experiments used ALI media for both primary and 16HBE cells.

4.4.2.3 Optimisation of time course

In order to optimise time course a limited number of experiments were undertaken comparing biofilm formation on days three and seven at MOIs of 1 and 50. Biofilm CFU counts were used as the outcome measure of biofilm formation (Figures 47) with TER and CBF as measurements for epithelial cell viability (Figures 48 and 49).

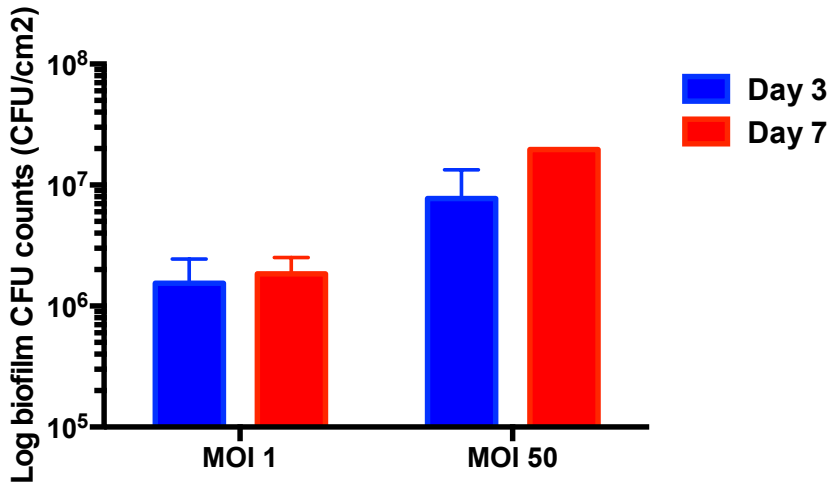

Figure 47 *– Log biofilm CFU counts of NTHi isolate four co-cultured at an MOI of either 1 or 50 on ALI cultured epithelial cell layers of non-PCD for three and seven days (mean, SEM where applicable) (n=4 for MOI 1 day three, n=3 for MOI 1 day seven, n=2 for MOI 50 day three, n=1 for MOI 50 day seven)*

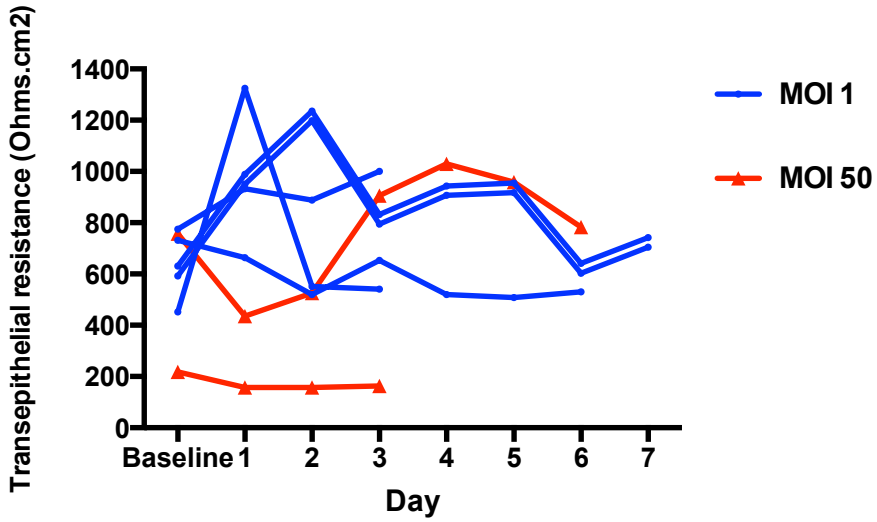

Figure 48 *– Graph showing corrected trans-epithelial resistance (Ohms.cm²) at baseline and then daily across non-PCD ALI cultured epithelial cell layers co-cultured with NTHi isolate four at MOIs of 1 or 50 for up to seven days (For MOI 1 n=5 to day three, n=2 to day six and n=1 to day seven, for MOI 50 n=2 to day three then n=1 to day six).*

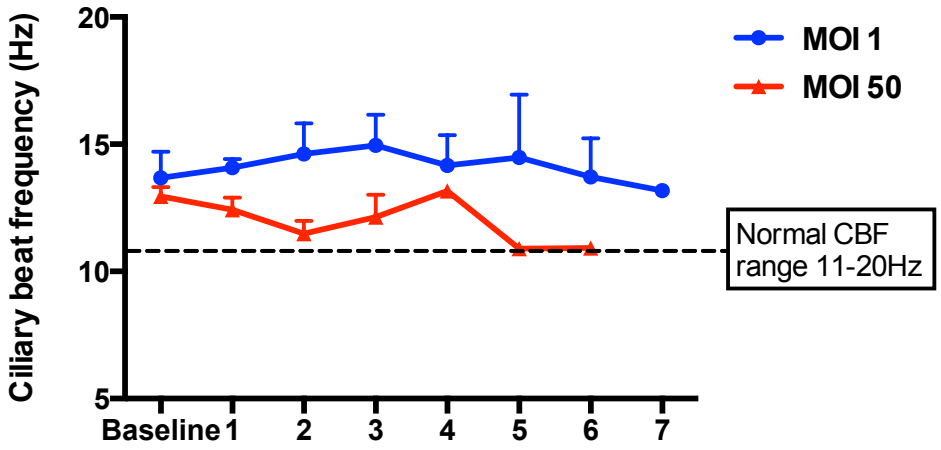

Figure 49 *– Mean ciliary beat frequency (Hz) at baseline and then daily on non-PCD ALI cultured epithelial cell layers co-cultured with NTHi isolate four at MOIs of 1 or 50 for up to seven days (mean, SEM where applicable) (For MOI 1 n=3 to day three, n=2 to day six and n=1 to day seven, for MOI 50 n=2 to day three then n=1 to day six). (For MOI 50 - data available until day six only). (Lines between data points added for clarity).*

Modest increases were seen in biofilm CFU counts on day seven compared with day three at an MOI of 50 (Figure 47). At an MOI of 1 both CBF and TER were stable throughout the seven-day time course (Figure 48 and 49). At an MOI of 50 one of the cell layers had a lower TER throughout the three-day time course of that experiment. This is likely to reflect poor health in that particular ALI cultured cell layer. While the TER across the other MOI 50 ALI cell layer dropped initially it subsequently improved back up above its baseline level. Ciliary beat frequency at MOI 50 was maintained until day five when it dropped below the normal range.

4.4.2.3.1 Summary

Little difference was observed in biofilm development between days three and seven and in the MOI of 50 experiment CBF dropped at day five. Therefore to ensure cell viability with optimal biofilm development a three-day time course was selected for the main experiment addressing the study hypothesis, undertaken in Chapter 5.

4.4.2.4 Optimisation of multiplicity of infection

Initial experiments on primary cell layers (4.4.2.1) using an MOI of 1 showed limited biofilm formation on primary ALI culture cell layers. This was therefore increased using a range of MOI to optimise NTHi infection over a three-day time course on non-PCD ALI cultured epithelial cell layers. Biofilm CFU counts were used as the outcome measure of biofilm development (Figures 50) with TER and CBF as measurements of epithelial

cell viability (Figures 51 and 52). Primary ALI cultured cell layers were a limited resource hence few repeats were possible.

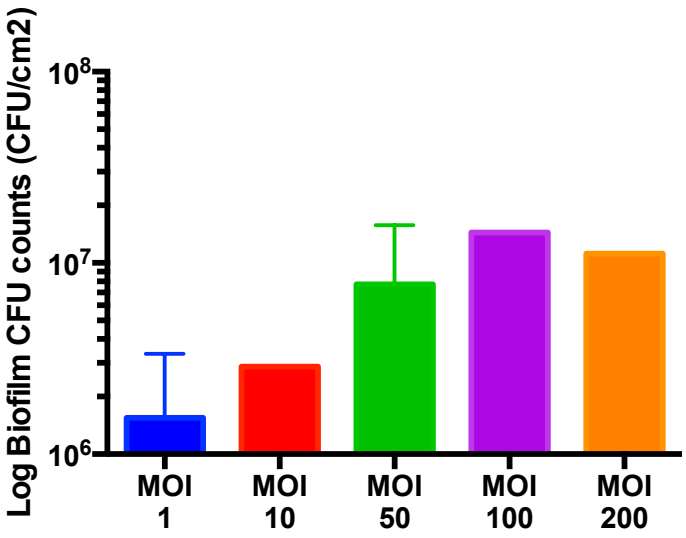

Figure 50 *– Log biofilm CFU counts of NTHi isolate four co-cultured at MOIs ranging from 1 to 200 on non-PCD ALI cultured epithelial cell layers for three days (mean, SEM where applicable) (n=4 for MOI 1, n=2 for MOI 50 and n=1 for the others)*

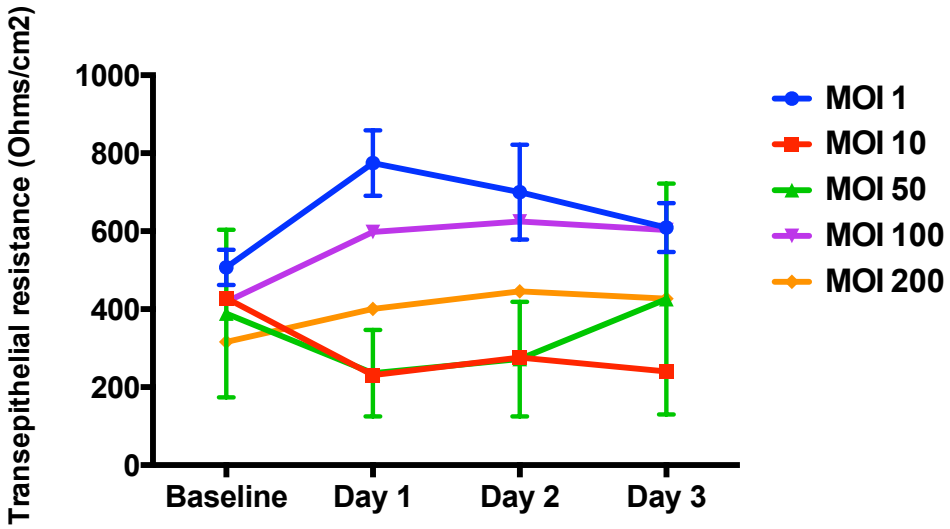

Figure 51 *– Mean corrected trans-epithelial resistance (Ohms.cm^2) at baseline and then daily across non-PCD ALI cultured epithelial cell layers co-cultured with NTHi isolate four at MOIs ranging from 1 to 200 for three days (mean, SEM where applicable) (n=5 for MOI 1, n=2 for MOI 50 and n=1 for others) (colour and lines between data points included for clarity)*

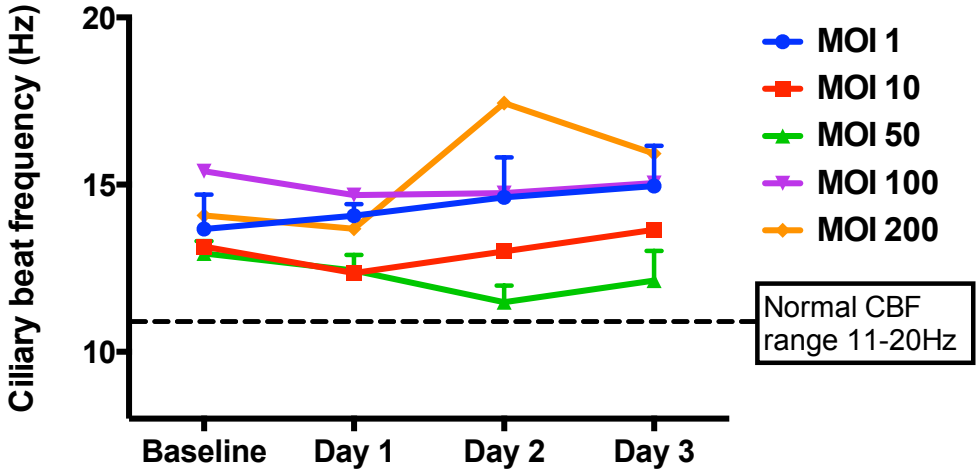

Figure 52 *– Mean ciliary beat frequency at baseline and then daily on non-PCD ALI cultured epithelial cell layers co-cultured with NTHi isolate four at MOIs ranging from 1 to 200 for three days (mean, SEM where applicable) (n=3 for MOI 1, n=2 for MOI 50 and n=1 for others) (colour and lines between data points included for clarity)*

Biofilm development, measured by biofilm CFU counts, increased with increasing MOIs, up to an MOI of 100 (Figure 50). Epithelial cell viability, as measured by TER for MOIs of 10 and 50 dropped from baseline to day one then stabilised however, despite using a higher MOI, TER for MOIs of 100 and 200 increased from baseline for the first 2 days then stabilized (Figure 51). CBF was maintained within the normal range (11-20Hz) for all MOIs however again at MOI 10 and 50 the CBF initially drop (Figure 52). However, given the limited number of ALI cell layers assessed at the various MOIs, this could reflect poor health in those particular cell layers.

4.4.2.4.1 Summary

To maximally preserve epithelial cell viability and integrity during challenge with NTHi whilst optimising biofilm development an MOI of 100 was selected and would be utilised for the main experiment addressing the study hypothesis, undertaken in Chapter 5.

4.5 Discussion

A bacterial biofilm is a structured community of bacterial cells enveloped in a self-produced complex of EPS matrix growing attached to a surface (Costerton, Stewart et al. 1999) that is recalcitrance to antibiotic therapy effective against the same bacteria in a planktonic culture (Parsek and Singh 2003; Hall-Stoodley and Stoodley 2009). It

has been demonstrated here that while all the PCD NTHi isolates were able to form a structured community of adherent bacteria that produced EPS only isolates three and four had increased recalcitrance to antibiotics. Therefore PCD NTHi isolates three and four formed bacterial biofilms whereas isolates one and two did not. PCD NTHi isolate four consistently demonstrated the best biofilm forming ability of the clinical isolates and was therefore used for subsequent co-culturing experiments. In addition the experimental design for the next experiments was optimised, which would use ALI media, an MOI of 100 and a three-day time course, to investigate the study hypothesis.

As outlined in Chapter 1, nutritional limitation is a trigger for biofilm formation and recent work has highlighted the importance of the availability of haem on NTHi biofilm growth, where its restriction promotes biofilm development (Szelestey, Heimlich et al. 2013). Jurcisek *et al.* used a chinchilla model of NTHi-induced otitis media to investigate the EPS of NTHi biofilms, demonstrating the presence of sialic acid, lipooligosaccharides (LOS), double-stranded DNA and type IV pili protein (Jurcisek, Greiner et al. 2005). There is evidence linking increased expression of phosphorylcholine and sialylation in LOS with the establishment of stable biofilm communities and a decreased host immune response (Bouchet, Hood et al. 2003; Swords, Moore et al. 2004; West-Barnette, Rockel et al. 2006; Hong, Mason et al. 2007). In addition, NTHi biofilms development is promoted and maintained, in part, by quorum sensing. This involves the release of molecules by the bacteria in a density-dependent manner. The bacterial by-product metabolite dihydroxypentanedione (DPD) has been associated with quorum signalling for a number of bacteria (Surette, Miller et al. 1999). In NTHi both LuxS transcription and the protein RbsB have been implicated in DPD quorum signals (Armbruster, Hong et al. 2009; Armbruster, Pang et al. 2011).

4.5.1 Quantification of biofilm formation

The biofilm development of the four PCD NTHi clinical isolates was assessed using multiple assays. Interestingly, despite the planktonic growth of the four NTHi isolates being broadly comparable, there was marked differences between their biofilm forming abilities. This is consistent with the literature, Starner *et al* finding that the 10 clinical NTHi isolates cultured from patients with cystic fibrosis had similar growth kinetics during planktonic growth however had varying degrees of bacterial biofilm growth (Starner, Zhang et al. 2006).

There is significant variation in the literature as to the extent to which authors try to quantify biofilm development; from solely using the CV assay (Izano, Shah et al. 2009) to using a biofilm forming index, combining CV assay, biofilm CFUs, biofilm thickness

and biofilm biomass measurements (Hall-Stoodley, Nistico et al. 2008). For this work a number of methods were utilised to ensure accurate quantification as individually each assay measures different factors and has specific issues.

While widely reported in the literature to assess biofilm formation the CV assay gives a crude assessment of adherent biomass (Hall-Stoodley, Nistico et al. 2008; Izano, Shah et al. 2009). Nevertheless, it is a suitable measure of early bacterial adhesion to an abiotic surface and is useful to compare biofilm formation by different bacterial strains. However the number of wash steps, used to remove excess stain, also removed a significant amount of NTHi biofilm. While these steps were performed similarly between isolates, allowing comparisons to be drawn, this assay alone does not reflect the full extent of the original biofilm. In addition, whilst used to assess biofilm development on an abiotic surface, the assay was not appropriate for assessing it in the co-culture model.

A limitation of biofilm CFU counts is that they do not take into account the EPS and dead bacteria that may make up a significant proportion of the biofilm biomass. However the data here, contrary to the hypotheses of previous groups (Moxon, Sweetman et al. 2008), indicate that there are a large number of live bacteria remaining in NTHi biofilms. Measurements up to day seven showed no indication the bacterial numbers were falling, if anything they were increasing. It is also notable that biofilm CFU counts may underestimate the number of viable bacteria within a biofilm, as bacteria in biofilms form aggregates and CFU counts rely on a single bacterium forming a single micro-colony on the agar plate. In order to minimise this, the samples were repeated vortexed and passed through a fine needle syringe a number of times, although it is still possible that some bacteria remained in small aggregates. Nevertheless, CFU enumeration remained an important method of biofilm quantification and was utilised going forward for epithelial cell co-culture model alongside with microscopy evaluation.

Biofilm formation is difficult to quantify by SEM however this technique offers high-resolution images of the ultrastructure of the bacterial biofilm and surrounding EPS. Interestingly, NTHi was seen to aggregate along strands of EPS that stretch between bacteria, forming a lattice-like structure. It is also notable that isolate one was only seen to form micro-colonies and produce small quantities of EPS compared with isolate four, which formed substantial biofilms and produced much more EPS. We hypothesise that there may be an important relationship between the ability of the NTHi bacterial isolate to produce EPS and its ability to develop biofilms, as seen with other bacteria such as *Streptococcus pneumoniae* (Hall-Stoodley, Nistico et al. 2008). If this were the

case it suggests that EPS would make an excellent potential target for future anti-biofilm therapeutic strategies. Further work focusing on the molecular constituents of the NTHi biofilm matrix is required. SEM was utilised in co-culture experiments for this project.

FISH is a useful and specific method for assessing biofilm development allowing *in situ* measurement of the biofilm co-localised with epithelial cells and was therefore an important method of biofilm quantification in the co-culture model.

4.5.2 Antibiotic Recalcitrance

Interestingly, the two isolates that formed the largest adherent colonies and produced the most EPS (three and four) were each 10 times more recalcitrant to cefotaxime in biofilms than when in planktonic phase growth, as compared to isolates one and two that were not. A number of hypotheses for the antibiotic recalcitrance of bacteria in biofilms have been proposed (outlined in 1.4.3) and it is likely that a combination of these is at play, varying between different bacteria and antibiotics. Firstly, the EPS may act to slow or lead to incomplete penetration of the antibiotics into the biofilm (Bolister, Basker et al. 1991; Mah and O'Toole 2001; Stewart and Costerton 2001). Secondly, as most antibiotics rely on bacterial cell division to act and as bacteria in biofilms can be in a near stationary growth phase, they can become tolerant to antibiotics that target cell wall or protein synthesis (Walters, Roe et al. 2003; Fux, Wilson et al. 2004). Finally, previous antibiotic courses may lead to selection of antibiotic resistant strains of ‘persister’ bacteria that then survive within the biofilm (Spoering and Lewis 2001). Whether the ultra structure of the biofilm, adaptions by the bacteria that are advantageous to biofilm growth or development of resistance mechanisms are responsible for this will require further work. However these findings are consistent with other bacterial species (Amorena, Gracia et al. 1999; Brooun, Liu et al. 2000) and the limited literature available on NHTi (Slinger, Chan et al. 2006).

The standard way to assess the activity of an antibiotic on bacteria in planktonic culture is to calculate the minimum inhibitory concentration (MIC). This is the lowest concentration of antibiotic that will inhibit the growth of bacteria after an overnight incubation, as seen by visual inspection of the turbidity of the bacterial broth as compared to a no antibiotic control. There are a number of problems with this technique, however, to determine the MIC of bacteria in biofilms. Biofilms are attached to a surface and removing them prior to antibiotic exposure could potentially affect their antibiotic recalcitrance. Antibiotics were therefore co-cultured with biofilms whilst attached to a surface, rinsed off, then the biofilms detached from the surface and the

MIC determined by CFUs. In addition, utilising turbidity as the marker for MIC in biofilms is problematic since the quantity of EPS matrix is responsible for much of the turbidity in a suspended biofilm once removed from its surface. Therefore less turbidity may not reflect inhibition of bacterial growth, rather less EPS. This is particularly important as each isolates produce markedly different amounts of EPS, which makes comparisons between isolates difficult. Therefore CFU counts were utilised here instead of using visual inspection of turbidity as a measure of bacterial growth (2.2.4). As this technique differed from the standard approach, previously validated MIC evaluator (M.I.C.E™) strips (Oxoid, Basingstoke, UK) (Mushtaq, Warner et al. 2010) were used were on isolates in planktonic cultures to validate this method. These were demonstrated to be consistent with the CFU technique developed here.

4.5.3 Biofilm formation on 16HBE immortalised cell layers

The striking difference in biofilm formation on 16HBE cell layers compared with primary epithelial ALI cultured cell layers, evidenced by 3-Log fold higher biofilm CFU counts, was not accounted for by the different media used (4.4.2.2). A number of factors may therefore account for this including potential differences in the cytokine or cationic anti-microbial peptide biosynthesis by 16HBE cells in response to bacterial infection or differences in the morphology of the cell layer that might have facilitate biofilm development on 16HBE cells. However importantly, given that 16HBE cells do not ciliate, it does suggest that cilia, whether motile or static, play a protective role against biofilm development. Further comparison between primary and 16HBE cell layers are made in Chapter 5, in particular considering relative cellular responses to NTHi infection. Identification of any such differences might point towards potential novel therapies for the treatment of biofilm infections.

4.5.4 Biofilm formation on primary ALI culture epithelial cell layers

Although initial experiments on primary ALI cultures (4.2.2.1) compared PCD and non-PCD cell layers and found no difference in biofilm formation between the two, only three cell layers were used (2 PCD and 1 non-PCD) with an MOI of 1. Subsequent optimisation experiments were therefore undertaken to attempt to increase the inoculating dose of NTHi whilst maintaining cell viability.

Multiplicity of infection was increased up to 200, however an MOI of 100 resulted in optimal biofilm development, while preserving epithelial cell viability and function. In the limited literature in this area the MOI is sometimes difficult to ascertain from the methods however, consistent with the work here, a range of 1 to 200 has been

reported (Starner, Zhang et al. 2006; Morey, Cano et al. 2011). In addition to increasing initial bacterial load, prolonging the time course of the experiment might have allowed for increased biofilm development, however little difference in biofilm CFU counts resulted when increasing the duration of experiments from three to seven days. This is consistent with one paper that used similar methods to those here however used a time course of up to ten days (Ren, Nelson et al. 2012). While their endpoint was intracellular bacteria CFU counts, they observed that this peaked on days four and five, depending on the epithelial cell type used for co-culture (Ren, Nelson et al. 2012).

Therefore an MOI of 100 and a three-day time course was selected for the subsequent co-culturing experiments. However it was accepted that the difference between biofilm formation on PCD and non-PCD cell layers might be small, particularly in comparison to the dramatic differences seen between primary and 16HBE cell layers.

4.5.5 Clinical context

It is interesting that isolates three and four, the to biofilm forming isolates, were obtained from children who had regularly cultured NTHi for over four years whereas isolate one was newly cultured for the first time and isolate two had only been seen in that child's respiratory culture for three months. While it cannot be proven that these two children with recurrent infection were chronically colonised with the same NTHi isolated for this whole time course it adds support the notion that bacterial biofilms are likely to be important in chronic and recurrent infections.

Non-typeable *Haemophilus influenzae* is not only the commonest pathogen seen in the local PCD population at UHS but also more generally in non-CF bronchiectasis (Edwards, Asher et al. 2003; Eastham, Fall et al. 2004; Li, Sonnappa et al. 2005; Banjar 2007) and is becoming increasingly recognised more widely, in particular as a cause chronic cough in children with persistent bacterial bronchitis (Marchant, Masters et al. 2006). Data presented in this Chapter suggest that some NTHi isolates more readily develop bacterial biofilms, particularly when taken from children with recurrent infections. These data strengthen the potential importance of the role of NTHi bacterial biofilms in these clinical settings and would advocate aggressive attempts to eradicate NTHi from patients.

4.5.6 Limitations

The main limitation of the development and optimisation work of the co-culture model undertaken here was the limited availability of ALI cultured cell layers for these experiments.

Primary ciliary dyskinesia is a rare condition and, whilst the patient cohort managed at UHS is motivated to take part in research, obtaining nasal brushing is uncomfortable. It is therefore understandable that the numbers of children willing to allow samples to be collected for research purposes alone was limited. In addition, given the rarity of this condition, there are only relatively small numbers of adults and children managed at UHS therefore limiting the availability of volunteers who might allow nasal brushing samples to be collected for research. Most of the samples were therefore collected as part of clinical investigation for referrals to the National PCD Diagnostic Service in UHS. Each brushing provided only two transwells of ALI cultured epithelial cell layers if it were cultured successfully, which was typically 40-50% of the time. The priority for these clinical samples was to confirm or exclude the diagnosis of PCD, which usually required at least one of the two available transwells. The remaining limited samples needed to be divided between the characterization and optimisation experiments here and the final comparative experiments of biofilm formation on PCD and non-PCD epithelial cell layers presented in Chapter 5. Given these factors the number of samples used for this characterisation work was limited but practical. For future work using this ALI cultured epithelial cell model, ways of successful yielding larger numbers of transwells cell layers that truly reflected the original epithelial cells are required. This all stated, in retrospect, more nasal brushings samples could have been obtained from healthy adult volunteers in order to increase the numbers of ALI cultured cell layers available for these optimisation experiments and future work will take account of this.

4.6 Conclusion

The experiments described in this chapter demonstrated that NTHi biofilm development occurs both on abiotic surfaces and on primary ALI cultured respiratory epithelial cell layers. NTHi PCD isolate four consistently demonstrated superior biofilm development, evidenced by biofilm CFU counts, FISH and SEM, and these were found to be the best techniques for quantifying biofilm development on the co-culture model. This isolate and these techniques would therefore be used in subsequent co-culturing experiments. In addition, the experimental design of using ALI media, an MOI of 100

and a three-day time course to investigate the study hypothesis was found to be optimal.

The data here strengthens evidence of the clinical importance of NTHi bacterial biofilms in PCD respiratory infections. Better understanding the adaptations that NTHi undergoes when in transition from a planktonic to an aggregate biofilm phase of growth will allow for novel therapeutic interventions that will help to reduce the risk chronic infections. The methods of biofilm quantification assessed here and the co-culture model developed will potentially be important in assessing the effectiveness of such interventions in the future.

Chapter 5

The role of ciliary function on non-typeable *Haemophilus influenzae* biofilm development

5.1 Introduction

Patients with primary ciliary dyskinesia (PCD) have impaired mucociliary clearance due to abnormal ciliary function. Bacterial biofilms are important in the pathophysiology of chronic suppurative respiratory diseases (Chole and Faddis 2003; Starner, Zhang et al. 2006; Psaltis, Ha et al. 2007). Non-typeable *Haemophilus influenzae* (NTHi) is the commonest pathogen in non-CF bronchiectasis and in the local PCD population at UHS (Edwards, Asher et al. 2003; Eastham, Fall et al. 2004; Li, Sonnappa et al. 2005; Banjar 2007; Walker, Jackson et al. 2012) and develops biofilms *in vitro* and *in vivo* (Greiner, Watanabe et al. 2004; Jurcisek, Greiner et al. 2005; Hall-Stoodley, Hu et al. 2006; Starner, Zhang et al. 2006). While authors have questioned the role of ciliary function and mucociliary clearance on biofilm formation for over a decade there has been little published work investigating this (Miyamoto and Bakaletz 1997; Pittet, Hall-Stoodley et al. 2010).

The study hypothesis was that the patients with PCD are predisposed to biofilm-associated infections due to their impaired ciliary function.

A ciliated primary respiratory air-liquid interface (ALI) epithelial cell co-culture model using NTHi was therefore developed to investigate the relationship between ciliary function and NTHi bacterial biofilms. To allow assessment of the role of ciliary function, primary respiratory epithelial cells were obtained by nasal brushing from patients with PCD and those without the condition (termed 'non-PCD'). Once differentiated at an ALI the epithelial cell layers produced from these two patient groups had abnormal or functioning cilia respectively (see Chapter 3), allowing the study hypothesis to be investigated.

In the previous Chapters techniques to measure biofilm development and the experimental design for this co-culture model were optimised (Chapter 4). In addition, detailed comparison was made between the ALI cultured epithelial cell layers derived from PCD and non-PCD patients, both to characterise the cell layers and to look for potential confounding variables in using cells from these patients groups to consider

the study hypothesis (Chapter 3). No significant differences apart from absent or reduced ciliary beat frequency (CBF) were found.

This Chapter describes experiments using the optimised protocol for the ALI co-culture model to investigate the effect of ciliary function on NTHi biofilm development. In addition to assessing biofilm development and epithelial cell health, a number of epithelial cellular responses to NTHi infection were compared pre- and post- co-culture between the two groups, including cytokine and cationic antimicrobial peptide (CAMP) release. Biosynthesis of nitric oxide (NO) by epithelial cells was also assessed and the data presented in Chapter 6.

5.2 Experimental design

In brief, at baseline (prior to co-culture) a number of measurement were taken from the ALI cultured cell layers, including CBF and percentage of their surface area that was ciliated; trans-epithelial electrical resistance (TER) across the cell layers; and the total NO levels in apical supernatants over them. Informed by previous optimisation experiments (Chapter 4) co-culture experiments used ALI media, an MOI of 100 and a three-day time course. Daily following co-culture TER, CBF and percentage ciliation were measured. In a separate experiment, CBF and TER measurements were also taken daily on uninfected non-PCD controls (n=5). In addition, baseline levels and daily post-infection the baso-lateral and apical supernatants were collected, aliquoted, frozen at -80°C and replaced with fresh media (as described in 2.3.2) Epithelial cell layers were then washed twice to remove non-adherent bacteria, total NO levels in apical supernatant were measured and the ALI transwell membranes, with attached co-cultured cell layer, were removed from the transwell inserts. Membranes with co-cultured cell layers were divided into sections with half used to measure bacterial biofilm CFUs and remainder fixed for fluorescence *in situ* hybridisation (FISH) and scanning electron microscopy (SEM) (as described in 2.3.2 and 2.3.3).

In addition to the uninfected non-PCD controls epithelial cell layers, outlined above, the co-culture experiments were performed on 16HBE ALI cultured epithelial cell layers. 16HBE cell layers were used to further confirm the significantly greater biofilm development seen in previous optimisation experiments (4.4.2.1) and to investigate whether there were identifiable differences in the cellular responses that might contribute towards this.

The experiment was performed three times on, in total, ALI cultured epithelial cell layers from five PCD, nine non-PCD patients and seven 16HBE cell layers. However cellular responses (LL-37 and cytokine concentrations) were only measured in six 16HBE cell layers.

5.3 Experimental results

5.3.1 Biofilm development

5.3.1.1 Biofilm colony forming unit counts

Non-typeable *Haemophilus influenzae* biofilm CFUs were twice as great on PCD epithelial cell layers compared with non-PCD cell layers using the same strain inoculated at the same dose, and this was statistically significant, $P<0.05$ (mean (±SEM), $3.8x10^6$ CFUs ($±7.5x10^5$) vs. $2.0x10^6$ CFUs ($±4.8x10^5$)) (Figure 53), however there were limited numbers in these experiments and previous experiments during optimisation at a similar MOI showed a 1-log higher CFU measurement on a non-PCD cell layer.

Consistent with previous optimisation experiments (4.4.2.1), which had used an MOI of 1, 16HBE cell layers had higher biofilm CFU counts (mean (±SEM), $5x10^7$ CFUs ($±1.1x10^7$) compared with either PCD or non-PCD primary ciliated epithelial cell layers (Figure 53). However, using an MOI of 100, a 1-Log fold difference was seen, as opposed to the 3-Log fold difference seen in the previous experiments, using an MOI of 1 (4.4.2.1). Interestingly increasing the MOI from 1 to 100 resulted in an equivalent increase in NTHi biofilm CFUs for both PCD (mean, $8.4x10^4$ CFUs vs. $3.8x10^6$ CFUs) and non-PCD cell layers ($1.0x10^5$ CFUs vs. $2.0x10^6$ CFUs) but not for 16HBEs ($4.5x10^7$ vs. $5x10^7$ CFUs).

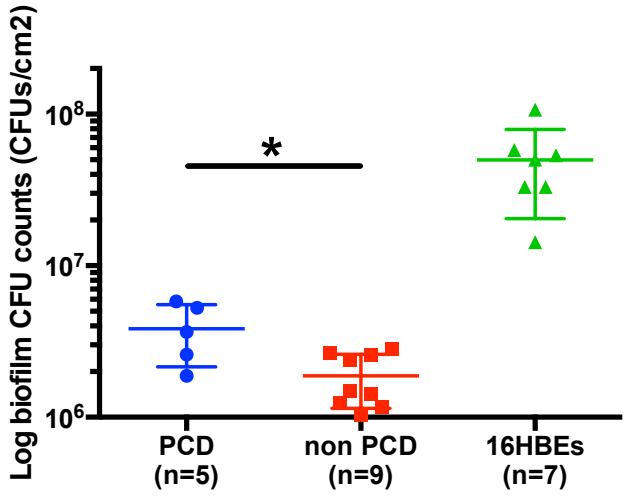

Figure 53 *– Log biofilm CFU counts of NTHi isolate four co-cultured at an MOI of 100 on ALI cultured epithelial cell layers of PCD (n=5), non-PCD (n=9) and 16HBE cells (n=7) for three days (mean, SD) (* P < 0.05)*

One of the PCD epithelial cell layers was derived from a patient with slow beating cilia (≈7 Hz) while all other PCD patients had static cilia (3.3.1, Figure 15). This rarer variant of PCD was compared with the four static PCD cell layers, acknowledging that n=1, to determine whether any clear difference would be observed (Figure 54). No difference was seen between the biofilm CFU count for this patient and the four static PCD patients (mean (±SEM), for static PCD), $3.9x10^6$ CFUs vs. $3.9x10^6$ CFUs ($±9.7x10^5$) respectively)

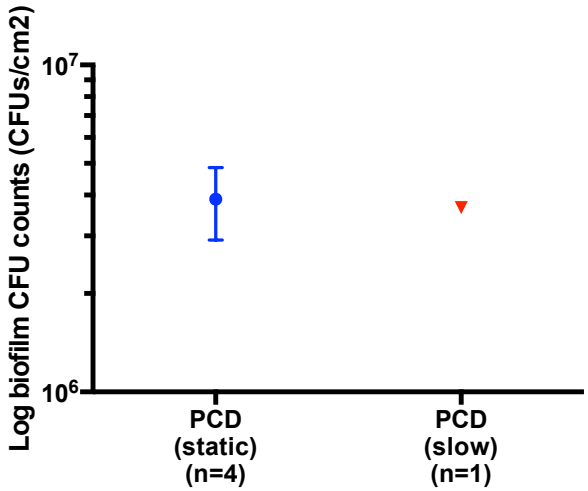

Figure 54 *- Log biofilm CFU counts of NTHi isolate 4 co-cultured at an MOI of 100 on ALI cultured epithelial cell layers of static PCD (n=4) and slow PCD (n=1) for three days (mean, SEM). No difference was seen between the two.*

5.3.1.2 Scanning electron microscopy

Each co-cultured cell layer was washed and the transwell membranes removed from the transwell inserts. These were then divided into sections, one of which fixed and processed for SEM from each cell layer (2.3.3.5).

Early biofilm development, with extracellular polymeric substance (EPS) matrix, was observed on the PCD epithelial cell layers (Figure 55 A). However NTHi biofilm development was much more extensive on the 16HBE cell layers, which was only visible between the cracks of the biofilm, see inlaid image (Figure 55 C). In contrast, the non-PCD cell layers showed little evidence of NTHi biofilm development with only occasional small micro-colonies seen (Figure 55 B).

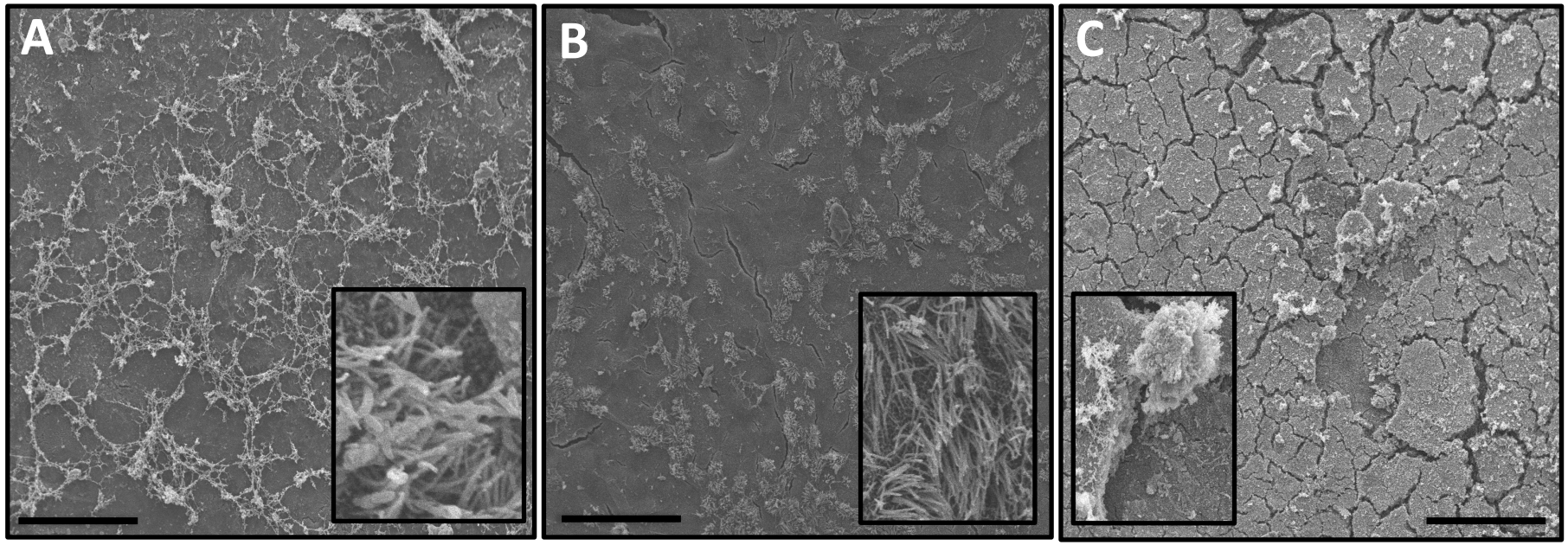

Figure 55 *- Representative SEM images of three-day NTHi isolate four biofilms co-cultured at an MOI of 100 on A) PCD, B) non-PCD and C) 16HBEs airway epithelial cell layers. Early biofilm formation with EPS matrix was seen on the PCD cell layer and a thick layer of bacterial biofilm is seen on 16HBE cell layer, the cell layer itself only visible in the cracks of the biofilm, see inlaid image, whereas on the non-PCD cell layer there was little evidence of biofilm formation with occasional small micro-colonies seen. Magnification x1200 with x3000 image inlaid, for each scale bar represents 50 µm. Images taken on a FEI Quanta 200 SEM*

5.3.1.3 Fluorescence in situ hybridization

Each co-cultured cell layer was washed and the transwell membranes removed from the transwell inserts. These were then divided into sections, one of which fixed and processed for FISH using a specific *H. Influenzae* 16S rRNA probe (green) then labelled with β-tubulin to demonstrate cilia (red) before addition of a nuclear counterstain (blue) (2.3.3.4). Individual confocal laser scanning microscopy (CLSM) image stacks were obtained for adjacent fields of view across the section. This provided a complete representation of NTHi biofilm development on the cell layer for that strip, from the edge to the centre of the transwell membrane. Each strip had between 33 and 50 fields of view dependent on the size of the section. The volume of NTHi bacterial biofilm was then calculated using Volocity software (version 6.0.1, PerkinElmer, Coventry UK) to provide quantitative image analysis data to reflect the extent of NTHi biofilms on the epithelial cell layers (2.3.3.4) (Figure 56).

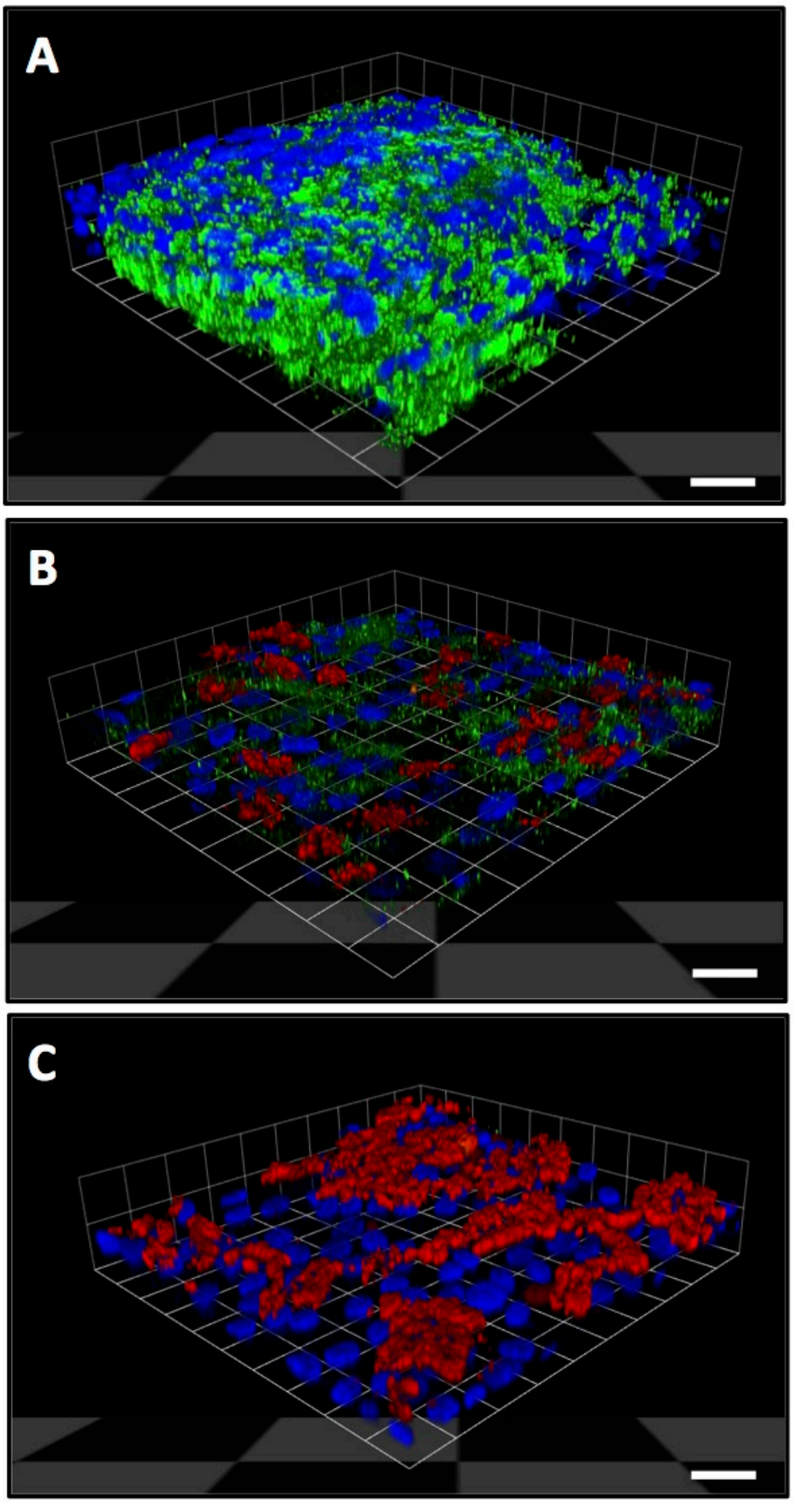
A
B
C

Figure 56 *- CLSM images (3D opacity views) of NTHi biofilms co-cultured with A) 16 HBEs and B) PCD and C) non-PCD primary ALI cultured airway epithelial cell layers at an MOI of 100 for three days. FISH using CY3 labelled H. influenzae FISH probe (green), B-tubulin antibody with a 594 Alexa fluor secondary (red) and a SYTOX® blue nuclear counter-stain (blue). The images demonstrate a thick bacterial biofilm with the 16HBE cell layer (A) and early biofilm formation with the PCD cell layer (B) whereas there is little evidence of bacteria on the non-PCD cell layer (C). Images taken on Leica SP5 CLSM at a magnification x600 and analysed using Volocity software (version 6.01). Scale bars represent 20 µm.*

Confocal laser scanning microscopy images (Figure 56) demonstrate extensive NTHi biofilm development on 16HBE cell layers (A), early biofilm development on the PCD cell layers (B) and little evidence of NTHi biofilms on non-PCD cell layers (C). The mean volume of NTHi biofilm per field of view on PCD cultured epithelial cell layers (n=5, 187 fields of view analysed) was over four times greater than on non-PCD cell layers (n=5, 206 fields of view analysed) and when considering all fields of view this was significant P<0.001 (mean±SEM, 710 (±150) μm^3 vs. 160 (±20) μm^3) (Figure 57). Co-culture on16HBE cell layers again resulted in 1-2 log fold greater mean volume of NTHi biofilm per field of view compared with both PCD and non-PCD cell layers (n=4, 134 fields of view analysed) (mean±SEM, 11250 (±1300) μm^3, P < 0.0001 for both) (Figure 57).

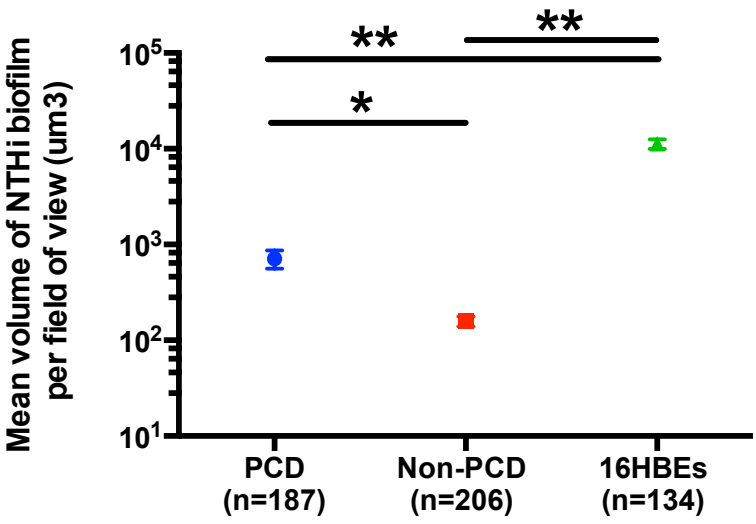

Figure 57 *- Log mean volume of NTHi isolate four per field of view following co-cultured at an MOI of 100 on ALI cultured epithelial cell layers of PCD (n=187), non-PCD (n=206) and 16HBEs (n=134) for three days (mean, SEM). Images were obtained*

*using a Leica SP5 CLSM at a magnification x400 and analysed using Volocity software (version 6.01) (*p<0.001, **p<0.0001)*

5.3.2 Epithelial cell viability and function

5.3.2.1 Ciliary beat frequency

Ciliary beat frequency was a key variable for investigating the hypothesis that in PCD ciliary dysfunction plays a role in greater susceptibility to NTHi biofilm development. However it was possible that NTHi infection might reduce CBF by means of a secondary ciliary dyskinesia during the co-culture experiments, thereby confounding the outcome of the experiments. Therefore CBF was measured daily during the co-culture experiments on the non-PCD cell layers and compared with uninfected non-PCD controls as an indication of cell function and health.

The mean CBF for non-PCD ALI co-cultured cell layer (n=9) remained in the normal range throughout the three-day time course of the co-culture experiment, with no statistical differences compared with uninfected non-PCD controls (Figure 58). The co-cultured non-PCD cell layers had a slightly higher baseline CBF compared with the uninfected non-PCD controls (mean (±SEM), 14.1Hz (±0.4) vs. 12.1Hz (±0.6) respectively, p=0.038) however both remained in the normal range (11-20 Hz).

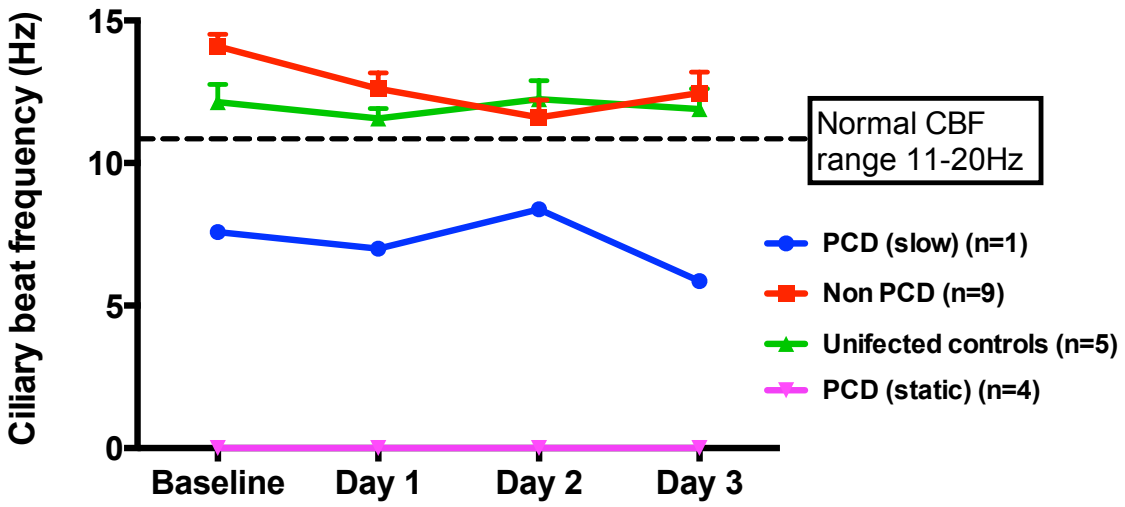

Figure 58 *- Mean ciliary beat frequency at baseline and then daily of non-PCD (n=9) slow PCD (n=1) static PCD (n=4) and uninfected controls (n=5) ALI cultured epithelial cell layers co-cultured with NTHi isolate 4 at an MOI of 100 for 3 days (mean, SEM where applicable). (Colour and lines between data points added for clarity)*

The percentage change in CBF from baseline was assessed to further investigate the effect that NTHi had on ciliary function in this co-culture model (Figure 59). Although a significant transient drop in CBF was observed by day two of co-culture compared with uninfected non-PCD controls (mean (±SEM), -15.9% (±3.4) vs. 1.1% (±4.9) respectively, P=0.001), no statistical difference was seen between the two groups by day three.

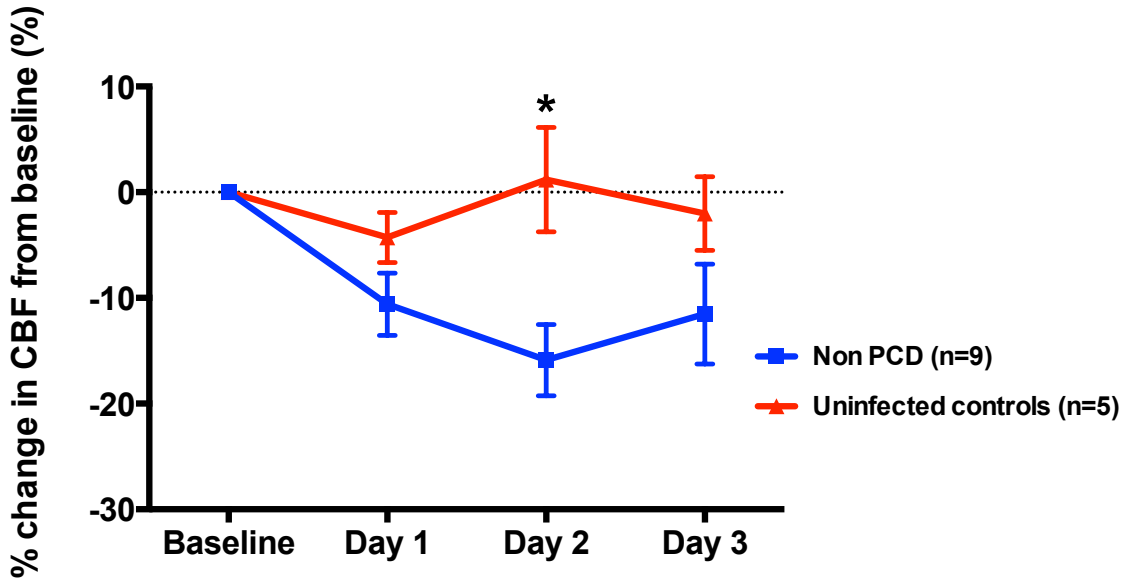

Figure 59 *- Mean ciliary beat frequency as a percentage of baseline daily across ALI cultured epithelial cell layers from non-PCD (n=9) and uninfected non-PCD controls (n=5) co-cultured with NTHi isolate 4 at MOIs of 100 for 3 days (mean, SEM) (Lines between data points added for clarity) (* P=0.001)*

5.3.2.2 Percentage ciliation

In addition to CBF, the percentage of the cell layer surface area that was ciliated was another important variable for the study hypothesis since NTHi co-culture might cause de-ciliation. This was therefore measured daily during the co-culture experiments on the infected non-PCD cell layers.

Fast Fourier transform was used to estimate the degree of ciliation from high-speed video microscopy videos at baseline and daily following co-culture (as described in 2.3.3.8.2) (Figure 60). No statistical difference was seen in the mean percentage ciliation over the three-day time course of the experiment (P values ranged from 0.52 to 0.99).

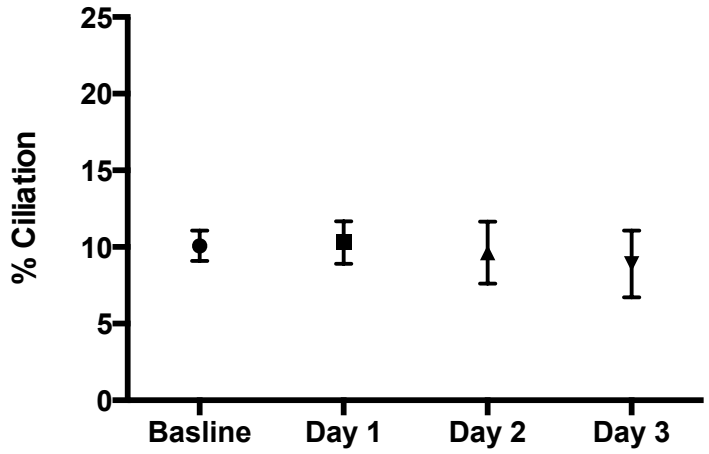

Figure 60 *- Mean percentage ciliation at baseline and then daily of non-PCD (n=8) ALI cultured epithelial cell layers co-cultured with NTHi isolate four at an MOI of 100 for 3 days (mean, SEM)*

5.3.2.3 Trans-epithelial electrical resistance

Trans-epithelial electrical resistance is a well-recognized surrogate measure of tight junction formation and hence cellular integrity and health (Xiao, Puddicombe et al. 2011) and was used as a further measure of cell layer viability and function during the three-day co-culture experiment (as described in 2.3.3.1). Additionally it could be measured for both PCD and non-PCD cell layers. Trans-epithelial electrical resistance was measured at baseline, before co-culture with NTHi, and daily following co-culture during the three-day experiment.

No statistical differences were observed between mean corrected TER levels across PCD (n=5) and non-PCD (n=9) cell layers at baseline or daily following co-culture with NTHi compared with uninfected non-PCD controls (n=5) at any time point (Figure 61) (P values ranged from 0.35 to 0.93)

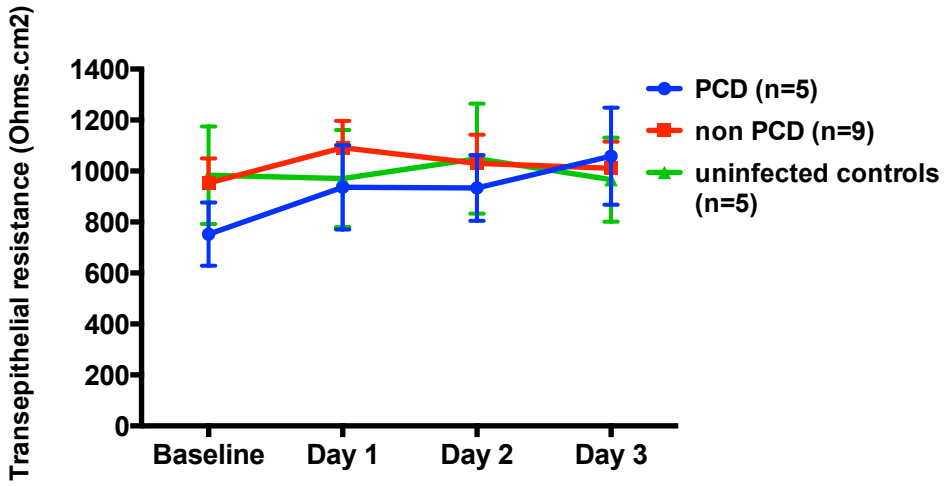

Figure 61 *- Mean corrected trans-epithelial resistance at baseline and then daily across ALI cultured epithelial cell layers from PCD (n=5), non-PCD (n=9) and uninfected non-PCD controls (n=5) co-cultured with NTHi isolate four at MOIs of 100 for three days (mean, SEM) (Lines between data points and colour added for clarity)*

However when considering the mean change in TER from baseline on the days following co-culture a significant increase at day one was observed, both by PCD and non-PCD epithelial cell layers, compared with uninfected non-PCD controls (Figure 62) (mean (±SEM), 25% (±9.6) and 16% (±5.1) vs. -1% ((±9.6), $P<0.001$ and $P<0.05$ respectively). In addition, while the TER of non-PCD cell layers decreased back towards baseline after day one, the mean TER of the PCD cell layers continued to rise and was significantly higher than both uninfected controls and non-PCD cell layers by day three (mean (±SEM), PCD 41% (±9.7) vs. non-PCD 7% (±6.6) vs. uninfected controls 1% ((±6.9), $P<0.001$ for both).

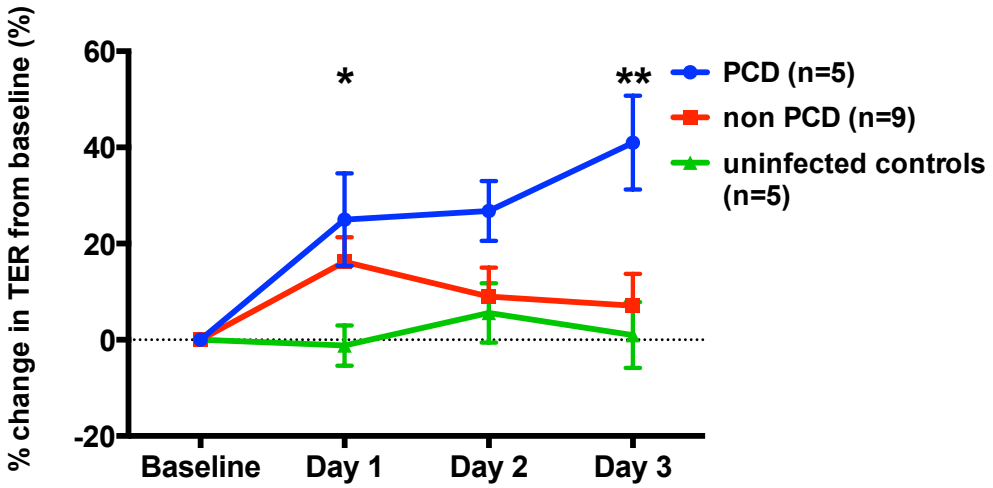

Figure 62 *- Mean corrected trans-epithelial resistance as a percentage baseline daily across ALI cultured epithelial cell layers from PCD (n=5), non-PCD (n=9) and uninfected controls (n=5) co-cultured with NTHi isolate four at MOIs of 100 for three days (mean, SEM) (Lines between data points and colour added for clarity) (*both PCD and non-PCD significantly higher TER change compared to uninfected controls, P<0.001 and P<0.05 respectively; ** PCD significant higher TER change on day three compared to non-PCD and uninfected controls, P<0.001 for both)*

5.3.3 Assessment of epithelial cell responses due to NTHi infection by biochemical assessment of the apical and basolateral supernatant

5.3.3.1 Nitric oxide

Total nitric oxide concentrations in the apical supernatant over epithelial cell layers were measured at baseline and on day three at the end of the co-culture experiment. These data are presented in chapter 6.3.

5.3.3.2 Cationic antimicrobial peptide - cathelicidin (LL-37)

Cationic antimicrobial peptides (CAMP) are produced by airway epithelial cells and not only have microbial killing effects but also recruit cells of the innate immune system (Zasloff 2002) (1.2.2.2.3). Concentrations of the CAMP cathelicidin (LL-37) were measured in the apical supernatant over PCD (n=5), non-PCD (n=9) and 16HBE (n=6) ALI cultured epithelial cell layers at baseline and daily following co-culture with NTHi (as described in 2.3.3.10). Levels of LL-37 at baseline were lower in PCD apical supernatants compared with non-PCD, however this was not significant (3.3.5) (mean±SEM) 0.62ng/ml (±0.20) vs. 1.15ng/ml (±0.20) respectively, P=0.11).

On day one of co-culture LL-37 concentrations increased significantly in PCD, non-PCD and 16HBE apical supernatants compared with baseline ((mean±SEM) 4.16ng/ml (±1.6), 3.51ng/ml (±0.4) and 3.09ng/ml (±0.5) respectively, P= 0.0004, 0.001 and 0.02 respectively) (Figure 63). LL-37 concentration stabilised for PCD and non-PCD on days two and three however continued to increase for 16HBE cell layers.

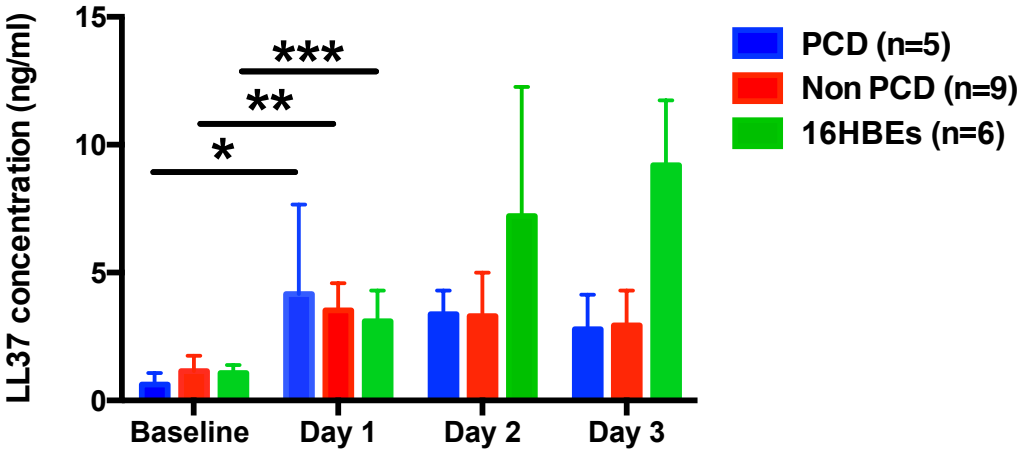

Figure 63 *– Concentrations of LL-37 (ng/ml) in the apical supernatant at baseline and then daily of PCD (n=5), non-PCD (n=9) and 16HBEs (n=6) ALI cultured epithelial cell layers co-cultured with NTHi at an MOI of 100 for three days (mean, SEM). All three increased significantly on day one compared to baseline (*P= 0.0004, **P=0.001 and ***P=0.02 respectively)*

The fold change in LL-37 from baseline was also analysed for each group. There was a greater increase in LL-37 concentration in the PCD compared with non-PCD cell layers between baseline and day one of co-culture (mean±SEM) 8.8 fold (±2.9) vs. 3.8 fold (±0.6) respectively (Figure 64).

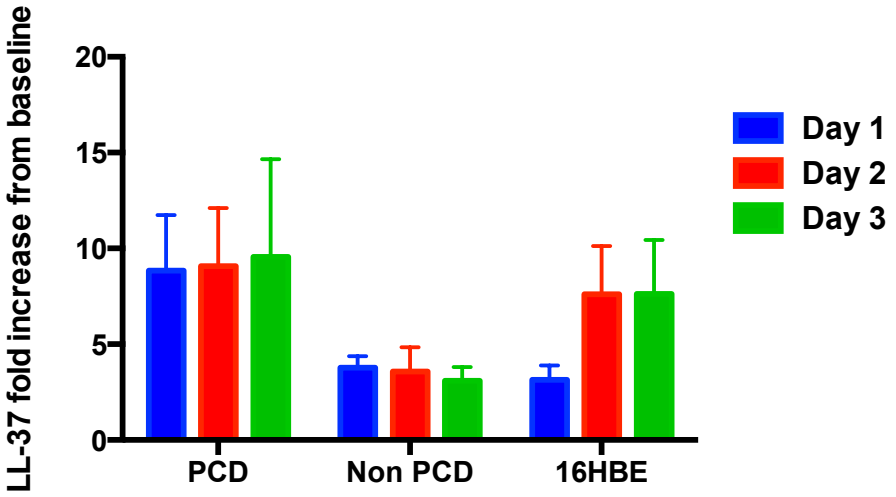

Figure 64 *- Fold increase from baseline in LL-37 concentrations in the apical supernatant daily of PCD (n=5), PCD (n=9) and 16HBEs (n=6) ALI cultured epithelial cell layers after co-culture with NTHi at an MOI of 100 for three days (mean, SEM)*

5.3.3.3 Cytokines

We hypothesised that the cytokine responses of cell layers from PCD and non-PCD cells to NTHi infection would differ, possibly to compensate for the ciliary dysfunction of the PCD cell layer.

Basolaterally applied supplemented ALI media, incubated below ALI cultured epithelial cell layers of PCD (n=5) and non-PCD (n=9) and 16HBE cells (n=6), were harvested after 24 hours, at baseline and daily following co-culture with NTHi and were frozen at -80°C. Concentrations of 19 cytokines were measured from these supernatants, 18 using a multi-analyte technique and one (IL-8) measured by a separate enzyme-linked immunosorbent assay (ELISA), as the supernatant needed to be diluted (as described in 2.3.3.11).

Ten of these cytokines had levels above the minimal detectable limit of the assay including fibroblast growth factor basic (FGFb), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)-6, IL-8 and IL-1 receptor antagonist (IL-1Ra), monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP) -1α, tumour necrosis factor (TNF) -α and vascular endothelial growth factor VEGF (Figure 65).

5.3.3.3.1 PCD versus non-PCD epithelial cell layers

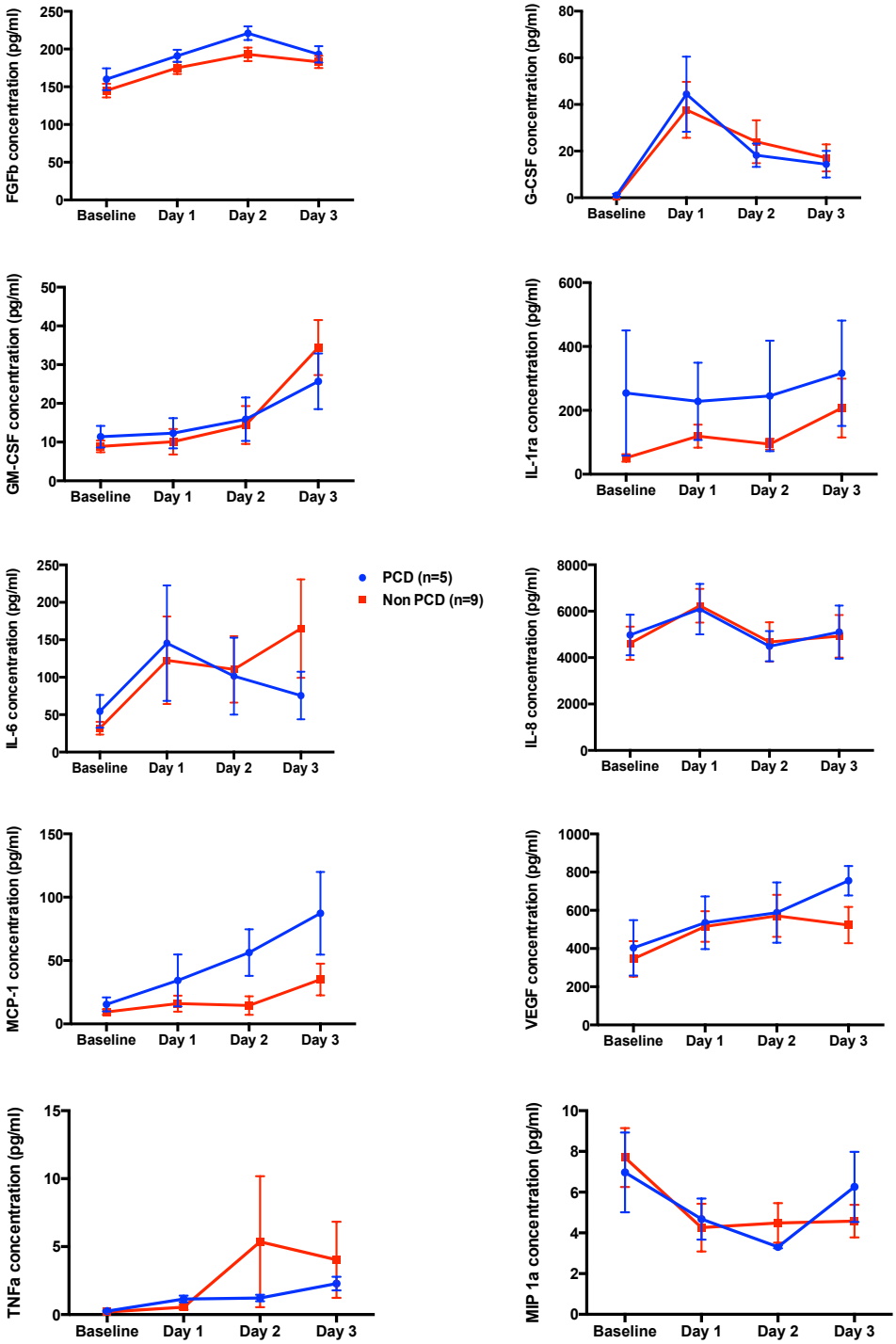

Figure 65 *– Concentrations of cytokines (pg/ml) in the basolateral supernatant at baseline and then daily of PCD (n=5) and non-PCD (n=9) ALI cultured epithelial cell layers co-cultured with NTHi at an MOI of 100 for three days (mean, SEM).*

When accounting for the multiple comparisons undertaken using the Bonferroni method, no significant differences were seen in the cytokine response between PCD and non-PCD cell layers to NTHi infection (Figure 65).

5.3.3.3.2 Primary (PCD and non-PCD) versus immortalised (16HBE) cell layers

There were, however, significant differences in the cytokine responses of 16HBE cell layer supernatants compared with the primary cell layers to NTHi infection (Figure 66 highlights examples). In particular, IL-8 concentrations were 10-20 times lower in 16HBE compared with primary cell layer supernatants.

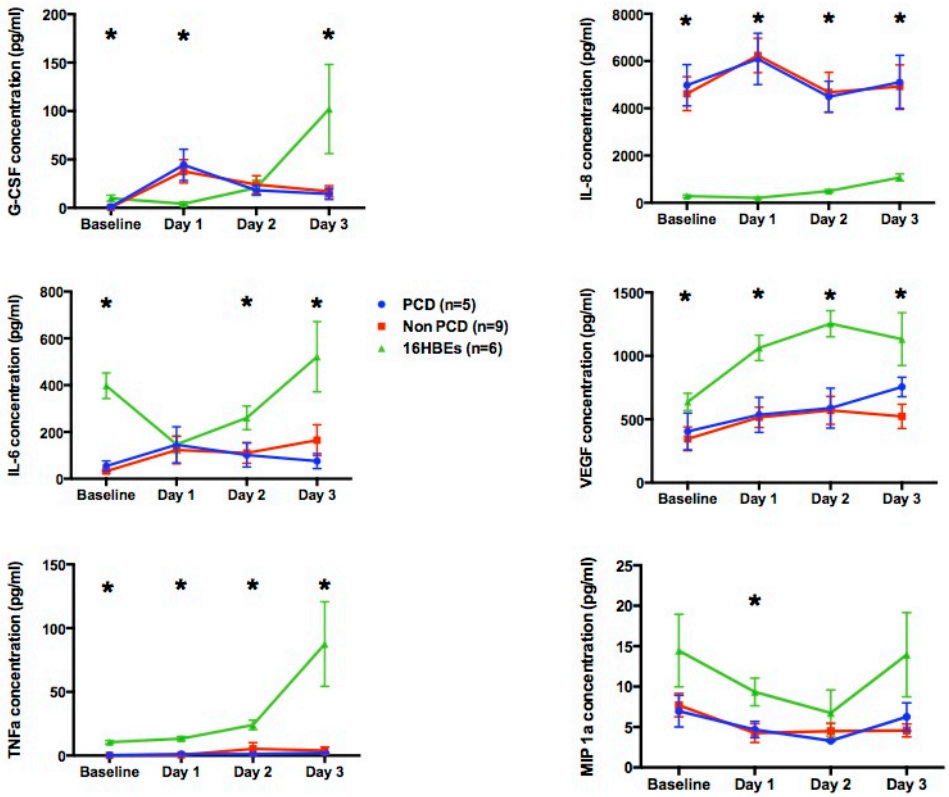

Figure 66 *- Concentrations of cytokines (pg/ml) in the basolateral supernatant at baseline and then daily of PCD (n=5), non-PCD (n=9) and 16HBE (n=6) ALI cultured epithelial cell layers co-cultured with NTHi at an MOI of 100 for three days (mean, SEM). Significant differences were seen between 16HBE and the primary cell layer supernatants for each of the cytokines presented (*P<0.05) but not between PCD and non-PCD supernatants*

Interleukins (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-10 and IL-17, interferon (IFN)-γ and macrophage inflammatory protein (MIP)-1β were not detectable by the assays used here.

5.4 Discussion

5.4.1 NTHi biofilm formation in the ALI co-culture model

These data support the study hypothesis that PCD patients are at increased risk of developing biofilm-associated infections due to impaired ciliary function.

Non-typeable *Haemophilus influenzae* biofilm development was significantly greater on PCD epithelial cells layers compared with non-PCD cell layers, evidenced by twice the NTHi biofilm CFU counts and over four times the volume of NTHi biofilm observed by FISH with CLSM and corroborated by SEM images. However it is important to note that the number of repeats in these experiments was limited and the difference between the two groups in CFU counts small.

The effect of ciliary dysfunction on biofilm development has not been investigated previously. Starner *et al* co-cultured NTHi on ALI cultured epithelial cells, using a Calu-3 cell line (Starner, Zhang et al. 2006). Notably, while forming a reasonably morphologically representative model of respiratory epithelium, these immortalised cells do not ciliate (Grainger, Greenwell et al. 2006). Consistent with this work, Starner *et al.* demonstrated increasing biofilm development by *H. influenzae* on the apical surface of the cell layers over the four-day time course assessed (Starner, Zhang et al. 2006). Galli *et al.* looked at biopsy samples from patients undergoing surgery for chronic rhinosinusitis and found an absence of cilia on samples where bacterial biofilm formation was most evident, however were unable to demonstrate whether the bacteria biofilm had caused the loss of cilia or whether absent cilia had allowed bacteria biofilm formation (Galli, Calo et al. 2008).

Consistent with data from Chapter 4 NTHi biofilm development was observe to be 10 - 20 fold greater on the unciliated immortalised 16HBE cell layers as compared with primary cells. There are a number of potential factors for this, the most important of which is that 16HBEs do not ciliate. However clear differences were observed in the cellular response of the immortalised as compared to primary cells and this may also have been a factor.

The CFU counts seen on the primary cell layers in the experiments presented in this chapter were lower than those seen in the optimisation experiments presented in chapter 4 (as seen in figure 50 compared to figure 53.) The experimental design for both was similar and hence, to a degree, this reflects the variability between different cell layers. It is therefore noteworthy that whilst 8 of the 9 ALI cell layers used in the

experiments in this chapter were derived from healthy control patients the cell layer used in the optimisation experiment was derived from a patient seen in the PCD diagnostic clinic who had recurrent chest infections but was demonstrated on testing not to have PCD. This highlights the importance of using consistent controls in future work.

While little difference was seen in the NTHi biofilm CFU counts between a time course of three and seven days, demonstrated in 4.4.2.4, the short, three-day course may not reflect *in vivo* circumstance where recurrent or persistent infection, likely mediated by bacterial biofilms, continues over longer periods. In the case of *P. aeruginosa* in cystic fibrosis, once the lower respiratory tract of patients become colonised by mucoid *P. aeruginosa* it is unlikely to be cleared and typically remains there for the rest of the patient's life (Li, Kosorok et al. 2005). It is possible that a longer time course may have demonstrated a more significant difference in biofilm formation between PCD and non-PCD cell layers. This was not evident in the optimisation work (4.4.2.3) that went to seven days however a longer time course should be considered for future work.

5.4.2 Cell viability and function following NTHi co-culture

Epithelial cell health was maintained throughout the three-days of ALI co-culture with NTHi (5.3.2), evidenced by no drop in TER and, for the non-PCD cell layers, maintenance of both CBF in the normal range and no drop in the percentage ciliation of the surface of the cell layers.

There was an increase in TER across both PCD and non-PCD cell layers on day one of co-culture compared with uninfected control cell layers. This is consistent with the findings of several other groups using both NTHi endotoxin and RSV co-culture, where an initial increase in TER following infection over the first 24 hours was also observed (Khair, Devalia et al. 1994; Masaki, Kojima et al. 2011). While the mechanism for this initial rise in TER following infection has not been elucidated, it likely to be a protective mechanism that resists breeches and maintains epithelial integrity against noxious substances. It was interesting that the TER across the PCD cell layers continued to increase on subsequent days of this co-culture experiment and was significantly higher than that across the non-PCD layer by day three. This may reflect a protective mechanism, specific to PCD epithelial cells, to maintain epithelial integrity in order to compensate for their lack of ciliary function. Alternatively, it might reflect the on-going higher bacterial load that the PCD, compared with non-PCD, cell layers were exposed to. Although there has been limited work studying co-cultures over a longer time

course, one group found that TER was maintained for up to ten days post infection with NTHi (Ren, Nelson et al. 2012).

Ciliary beat frequency of the non-PCD cell layer was maintained in the normal range throughout the three-day experiment although a significant, yet transient, 15% drop was seen in CBF on day two. Several groups have similarly found reductions in CBF following co-culture with NTHi (Janson, Carl n et al. 1999; Bailey, LeVan et al. 2012). One group co-cultured NTHi on explanted nasopharyngeal airway tissue and observed a 40% drop in CBF after two days and another group co-cultured NTHi supernatant on bovine lung tissue demonstrating a 50% reduction after one day. However, both groups studied explanted tissue as opposed to the ALI cell cultures used here and it was unclear what MOI were assessed (Janson, Carl n et al. 1999; Bailey, LeVan et al. 2012). The mechanism by which NTHi reduces CBF had been hypothesised to be toxin-mediated, possibly by lipopolysaccharide (LPS), as NTHi had been shown to cause ciliary damage without direct contact with epithelial cells (Johnson, Clark et al. 1983; Johnson and Inzana 1986; Read, Wilson et al. 1991). However Harada *et al.* found that highly purified LPS had no effect on CBF (Harada, Saida et al. 1987). More recently, Bailey *et al.* have highlighted the importance of protein kinase C epsilon activation in the reduction in CBF seen following exposure to NTHi however the bacterial product that leads to its activation remains unclear (Bailey, LeVan et al. 2012).

The percentage ciliation of the surface of the cell layers was only measured by the FFT method on the non-PCD cell layers. This technique measures ciliary movement on the surface of live cell layers and was therefore not possible on the PCD cell layers where the cilia were static. As previously described, measurement of the degree of ciliation was possible on PCD cell layers by using immunocytochemistry techniques with the cilia antibody B-tubulin, 3.3.2.1. The method requires the cell layers to be fixed. It would have been possible to fix a number of PCD cell layers at baseline then take a separate number of cell layers after the experiment undertaken in this Chapter and fixed these for such analysis. However given the variability of ciliation seen between each cell layers, see 3.3.2, the numbers required to make this statistical valid would have been inhibitive, particularly given the limitations in the availability of ALI cultured cell layers, see 4.5.6. It is therefore possible that the higher volumes of NTHi present on the PCD cell cultures might have been, in part, due to a decrease in ciliation on these cell layers that was not evident on the non-PCD cell layers. This will need to be considered further in future work.

5.4.3 Cationic antimicrobial peptide – cathelicidin (LL-37)

There was a significant increase in concentrations of LL-37 on day one for all cell layers in response to NTHi infection compared with baseline levels. While LL-37 expression by epithelial cells following *Mycobacterium tuberculosis* infection has previously been investigated (Mendez-Samperio, Miranda et al. 2008) these are the first data on its expression following NHTi infection.

Although ciliary dysfunction predisposes patients with PCD to respiratory infections, they do not die early in life from overwhelming lower respiratory tract infections. It is therefore likely that other defence mechanism(s) compensate for the absence of effective mucociliary clearance. The data here, where a significantly greater increase in LL-37 concentration from PCD as compared to non-PCD cell layers was observed on day one post infection, may point towards one such innate immune mechanism. However, the difference may have been due to the higher NTHI CFUs present on the PCD epithelial cell layers as, once corrected for, there was no significant difference seen between the two. Epithelial cells from PCD patients may compensate for the lack of ciliary function by synthesising increased amounts of CAMPs in response to infection.

5.4.4 Cytokines

We had hypothesised that the cytokine responses of PCD and non-PCD cell layers to NTHi infection may differ, possibly due to the impaired innate defence caused by ciliary dysfunction of the PCD cell layer. We therefore measured a wide spectrum of cytokines as the literature on whether many of these were produced by respiratory epithelial cells was limited and their role in infection in PCD was unknown.

Contrary to our hypothesis, we found that the cytokine responses to NTHi infection were comparable between the PCD and non-PCD cell layers. Consistent with most of the literature, concentrations of pro-inflammatory cytokines in the basolateral supernatant in both PCD and non-PCD ALI cultured epithelial cell layers increased following co-culture with NHTi (Khair, Devalia et al. 1994; Clemans, Bauer et al. 2000). However, one group using similar methods but co-culturing NTHi with an EpiAirway™ (MatTek, Ashland, USA) tissue model, found no significant rise in cytokine levels following co-culture (Ren, Nelson et al. 2012). It is possible that the passages and processing required to produce the tissue model used by this group, instead of the recently collected ALI cultured primary epithelial cell layers used in this work, may have affected the response the epithelial cells to infective stimuli. In addition, this group measured cytokines levels on day two, four and six, potentially missing the

initial rises seen in this, and other groups, work (Khair, Devalia et al. 1994; Clemans, Bauer et al. 2000).

By comparison, significant differences were seen in concentrations of cytokines produced by 16HBE cell layers compared with the primary epithelial cell layers. This was particularly evident for IL-8, a key pro-inflammatory cytokine and neutrophil chemotractant (Zeilhofer and Schorr 2000), which was 10-20 times lower in 16HBE cell layers than those derived from primary cells. The different responses to NTHi further highlights the limitations of using immortalised cell lines as an airway model, although these data may suggest clues regarding the contribution of the cytokine response to the significantly greater biofilm formation seen on the 16HBE cell layers.

Of the 19 cytokines considered, nine were not detectable by the methods used, which is consistent with work by Ren *et al.*, who were also unable to detect cytokines IL-2, 4, 10 and 17 and interferon-γ (Ren, Nelson et al. 2012). These cytokines are either not produced by epithelial cells or are produced in amounts below the detectable thresholds of the assays. Notably, Ren *et al.* found cytokine concentrations were three times higher in the apical, as opposed to basolateral, supernatants (Ren, Nelson et al. 2012) and this should be considered in future work assessing cytokine response to stimuli using ALI culture systems.

5.5 Summary

5.5.1 Biofilm development

Whilst the numbers in these experiments were relatively small, non-typeable *Haemophilus influenzae* biofilm development was found to be significantly greater on PCD epithelial cells layers compared with non-PCD cell layers, evidenced by twice the NTHi biofilm CFU counts and over four times the volume of NTHi biofilm observed by FISH with CLSM and SEM images. Ten to twenty fold greater NTHi biofilm development was observed on the unciliated immortalised 16HBE cell layers compared with the ciliated primary cell layers of both PCD and non-PCD patients.

5.5.2 Cellular response to NTHi infection

A significant increase in TER was observed across both PCD and non-PCD cell layers on day one following co-culture with NTHi compared with uninfected control cell layers. Ciliary beat frequency of the non-PCD cell layer was maintained in the normal range throughout the three-day experiment although a significant, yet transient, 15% drop

was seen in CBF on day two. There was a significant increase in concentrations of LL-37 on day one following co-culture with NTHi compared with baseline levels for all cell layers. Cytokine responses to NTHi infection were comparable between the PCD and non-PCD cell layers whereas, by comparison, significant differences were seen in concentrations of cytokines produced by 16HBE cell layers compared with the primary epithelial cell layers, which was particularly evident for IL-8.

5.6 Conclusion

The work presented in this chapter supports the study hypothesis that PCD patients are at increased risk of developing biofilm-associated infections due to impaired ciliary function. This adds to the importance of developing anti-biofilm interventions that could impact on the recurrent respiratory tract infections that occur in PCD patients.

While the use of ALI cultured primary epithelial cell co-culture models will have an important role in the development of novel anti-biofilm therapies, the use of immortalized cell lines such as 16HBE should be treated with more caution as experiments may not reflect the *in vivo* pathophysiology.

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Chapter 6

Investigating low nitric oxide in primary ciliary dyskinesia and the interaction between nitric oxide and non-typeable *Haemophilus influenzae* biofilms

6.1 Introduction

Nitric oxide (NO) is a key cellular signalling molecule with diverse physiological roles including anti-bacterial activity and modulation of biofilm development and dispersal in bacteria such as *P. aeruginosa* and *E. coli* (Barraud, Hassett et al. 2006; Major, Panmanee et al. 2010; Regev-Shoshani, Ko et al. 2010). NO is synthesized in the respiratory epithelium from L-arginine by three NO synthase (NOS) isoforms (neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS)) (Walker, Jackson et al. 2012). Infection and inflammation lead to iNOS up-regulation and hence NO biosynthesis (Ricciardolo, Sterk et al. 2004). Yet despite chronic upper and lower respiratory tract infection, nasal NO (nNO) levels are markedly reduced in the vast majority of PCD patients compared to those without the condition, so much so that nNO is used as a screening test for PCD (Lundberg, Rinder et al. 1994; Karadag, James et al. 1999; Wodehouse, Kharitonov et al. 2003; Barbato, Frischer et al. 2009). A number of potential mechanisms for the extremely low nNO in PCD have been proposed however the actual mechanism(s) remain elusive (Walker, Jackson et al. 2012).

Increasing literature shows that NO affects bacterial growth and, at varying concentrations, exhibits bacteriostatic or bactericidal activity (Barraud, Hassett et al. 2006; Major, Panmanee et al. 2010; Regev-Shoshani, Ko et al. 2010). In addition NO can modulate *P. aeruginosa* biofilm formation and cause dispersal acting via cyclic di-GMP (Barraud, Hassett et al. 2006; Barraud, Schleheck et al. 2009). The low nNO levels seen in PCD may therefore increase these patients' susceptibility to bacterial infections, and biofilm infections in particular. Consequently NO was a potentially important confounding variable in the ALI co-culture model developed in this work, where comparisons were made between epithelial cell layers from PCD and non-PCD patients to assess the role of ciliary function on biofilm formation (Chapter 5).

While exhaled nitric oxide fraction (Fe_{NO}) from the lower airway is found to be slightly lower in PCD, levels are unable to differentiate between PCD patients and healthy controls (Lundberg, Rinder et al. 1994; Karadag, James et al. 1999). It therefore remains unclear whether NO biosynthesis is reduced throughout the respiratory tract or whether the hypoplastic, blocked or sometimes absent nasal sinuses seen in patients with PCD reduce the measurable NO concentrations in the upper airway specifically (Pifferi, Bush et al. 2010). In order to assess the specific contribution of NO biosynthesis from the lower airway in more detail in the first section of this Chapter (6.2) a two-compartment model of pulmonary NO exchange dynamics was used (Tsoukias and George 1998; George, Hogman et al. 2004). This allowed separate estimates of the NO biosynthesis from the bronchial and alveolar compartments of the lung and these levels were compared between patients with PCD and healthy controls. If NO biosynthesis was found to be generally reduced throughout the respiratory tract, it would support the hypothesis that the low NO levels in PCD were due to an inability of epithelial cells to biosynthesis NO, thereby directing subsequent work. In addition, given the putative role of NO on bacterial biofilm dispersal, it would further highlight the importance of investigating the effect of NO on NTHi biofilms (Barraud, Hassett et al. 2006; Barraud, Schleheck et al. 2009). I supervised Annabelle Liew's BMedSci project and her data contributed to this section.

It is widely hypothesized that the low levels of NO seen in PCD are due to abnormal NO synthesis from PCD respiratory epithelial cells, possibly due to the absence or reduction in NOS isoforms (Narang, Ersu et al. 2002; Pifferi, Bush et al. 2011; Smith, Fadaee-Shohada et al. 2013). In the second section of this chapter (6.3) work was therefore performed firstly to compare NO biosynthesis from primary ciliated ALI cultured epithelial cell layers from PCD and non-PCD patients, both constitutively and following stimulation with both pro-inflammatory cytokines and NTHi; and secondly to examine whether all three NOS isoforms were present in the epithelial cell layers of both groups.

As discussed in section 1.4.3, a key characteristic of biofilm infections is an increased tolerance to antibiotic therapy (Brooun, Liu et al. 2000; Parsek and Singh 2003; Hall-Stoodley and Stoodley 2009). Consistent with this, it was demonstrated here that two of the clinical PCD NTHi isolates developed biofilms that were 10-fold more recalcitrant to cefotaxime than when in planktonic culture (4.3.4). NO has been demonstrated to disperse *P. aeruginosa* biofilms inducing motility, a return planktonic growth and increased susceptibility to antibiotic killing (Barraud, Hassett et al. 2006) via a specific mechanism involving energy signalling (Barraud 2009). Given the prevalence of NTHi (Edwards, Asher et al. 2003; Eastham, Fall et al. 2004; Li, Sonnappa et al. 2005; Banjar

2007) and the likely importance of bacterial biofilms in patients with PCD experiments, in the third and final section of this Chapter (6.4) were designed to establish whether NTHi biofilms were dispersed by NO, as seen with *P. aeruginosa*. Dr Aneurin Young (Academic Foundation Year 2 Doctor) undertook this work under my supervision.

6.2 Upper and lower airway nitric oxide biosynthesis in primary ciliary dyskinesia

6.2.1 Participants

Children with PCD (n=14) were recruited from the specialist PCD clinic at University Hospital Southampton (UHS, Southampton, UK). The diagnosis of PCD had been confirmed by assessing the ciliary beat frequency and pattern of airway epithelial cells by high-speed video microscopy in patients with a clinical history suggestive of PCD, further supported by assessment of ciliary ultrastructure by transmission electron microscopy interface (Lucas, Walker et al. 2011). Healthy children (n=18) were recruited from non-respiratory clinics and completed a short questionnaire to exclude diseases that might affect nitric oxide levels and were age and gender matched to the PCD participants. Inclusion criteria were that the children had to be over 6 years of age and be well on the day of testing with no evidence of respiratory tract infection in the previous four weeks. They were excluded if they had smoked in the last year or had undertaken spirometry in the previous hour. This study was approved by Southampton and South West Hampshire Research Ethics Committee (A) (REC numbers: 06/Q1702/109 and 08/H0502/126) and all subjects gave written informed consent.

6.2.2 Measurement of airway nitric oxide

Measurement of nasal and lower airway nitric oxide levels using a chemiluminescent NO analyser, NIOX® Flex (Aerocrine, Sweden) followed American Thoracic Society/European Respiratory Society (ATS/ERS) recommendations (2.4.1) (American Thoracic and European Respiratory 2005). A two-compartment model of pulmonary NO exchange dynamics, using Fe_{NO} measurements at different flow rates, was used to estimate alveolar and bronchial NO levels (as described in 2.4.1) (Tsoukias and George 1998; George, Hogman et al. 2004).

6.2.3 Results

Demographics of children with PCD and healthy controls were similar (Table 6).

Table 6 *- Demographics in subjects with primary ciliary dyskinesia compared with healthy controls (Data presented as mean (SD)*

Characteristics	**Group**	
	PCD	**Healthy controls**
Number of participants	14	18
Males (n) (%)	6 (46)	10 (56)
Age (years)	12.8 (3.9)	14.1 (2.3)
Height (cm)	155 (23)	161 (14)
Weight (kg)	48 (21)	63 (24)

All NO measurements were completed by the participants apart from one child with PCD who was unable to perform the multiple-flow Fe_{NO} protocol.

As expected nasal NO (nNO) was significantly lower in children with PCD compared with healthy control children (median (Interquartile range (IQR)) 21 (10-56) ppb *vs.* 772 (690-886) ppb, P<0.0001) (Table 7 and Figure 67a).

Table 7 *– Nitric oxide parameters in subjects with primary ciliary dyskinesia compared with healthy controls (Data presented as mean (±SD) unless stated)*

Nitric oxide parameter	**Group**		**P value**
	PCD	**Healthy controls**	
nNO (ppb)	21 (10-56)*	771 (690-886)*	<0.0001
Fe_{NO50} (ppb)	8.8 (7.3) ^	16.7 (10.8)	0.03
$J'aw_{NO}$ (pl/s)	264 (209) ^	720 (514)	0.005
$Calv_{NO}$ (ppb)	1.7 (0.8) ^	3.5 (1.3)	0.0001
^ n=13 as 1 patient was unable to perform Fe_{NO} tests * Median (IQR)			

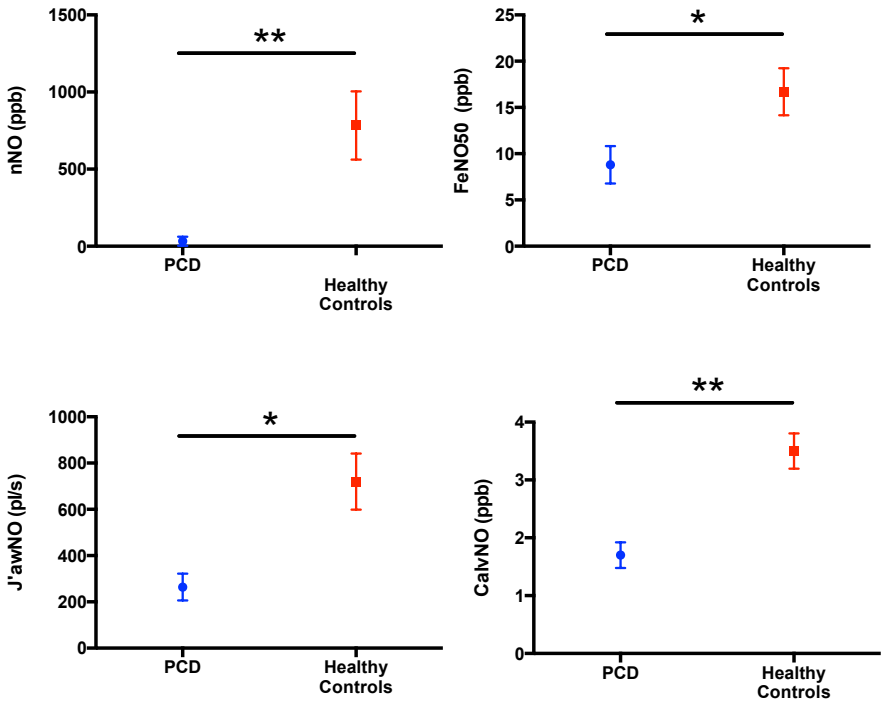

Figure 67 *- Nitric oxide concentrations in children with primary ciliary dyskinesia (PCD) (n=14) and healthy controls (n=18)* ***(a)*** *nasal nitric oxide concentration (nNO);* ***(b)*** *fractional exhaled nitric oxide concentration at 50 ml/s (Fe_{NO50});* ***(c)*** *bronchial nitric oxide flux ($J'aw_{NO}$) and* ***(d)*** *alveolar nitric oxide concentration ($Calv_{NO}$). (all mean, SEM except nNO, which shows median, IQR). (ppb, parts per billion; pl/s, picolitres per second.) (* and ** indicate P<0.05 and P<0.001 respectively)*

Exhaled (Fe_{NO50}), bronchial and alveolar NO were each significantly lower in children with PCD compared with healthy controls (mean (±SD) Fe_{NO50} - 8.8 (±7.3) ppb *vs.* 16.7 (±10.8) ppb, p=0.03, bronchial - 264 (±209) pl/s *vs.* 720 (±514) pl/s, p=0.005, and alveolar - 1.7 (±0.8) ppb *vs.* 3.5 (±1.3) ppb, P=0.0001, respectively) (Table 7 and Figure 67 b-d).

6.3 Assessment of nitric oxide biosynthesis and nitric oxide synthase localisation from PCD and non-PCD ALI cultured epithelial cell layers

6.3.1 Nitric oxide biosynthesis

6.3.1.1 PCD and non-PCD ciliated respiratory epithelial cells biosynthesise similar constitutive levels of nitric oxide

Due to the transient and volatile half-life of NO, which is rapidly broken down into its more stable products nitrate and nitrite, total NO biosynthesis was assessed by reducing nitrate to nitrite and measuring total nitrite levels. Total nitrite levels were measured at the apical surface of ALI cultured ciliated respiratory epithelial cell layers from PCD (n=11) and non-PCD (n=25) (as described in 2.4.2.1). No significant difference was seen between the two groups (mean (±SEM) 12.8 μmol/l (±1.4) vs. 16.2 μmol/l (±1.2) respectively, P=0.11) (Figure 68).

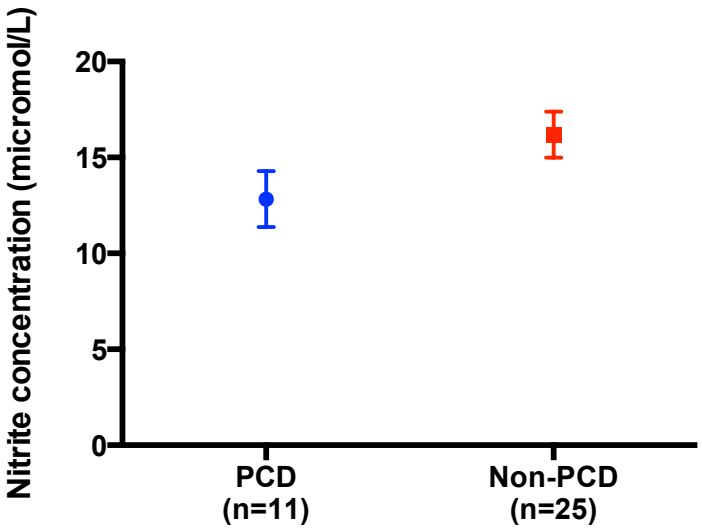

Figure 68 *– Baseline NO levels measured in apical PBS washes, following a 30-minute incubation at 37°C, of ALI cultured ciliated primary epithelial cell layers from PCD (n=11) and non-PCD (n=25) (mean, SEM). No significant difference was seen between the two groups.*

In addition, NO biosynthesis was examined using a fluorometric marker, DAF-fm diacetate, in PCD (n=3) and non-PCD (n=3) ALI cultured cell layers (as described in 2.5.2.1). DAF-fm is essentially non-fluorescent until it reacts with NO to form a fluorescent benzotriazole, which could be visualised using a fluorescent microscope.

Air-liquid interface cultured PCD and non-PCD epithelial cells were observed to fluoresce similarly, hence corroborated the total nitrite assay data indicating that there was no difference in NO biosynthesis (Figure 69).

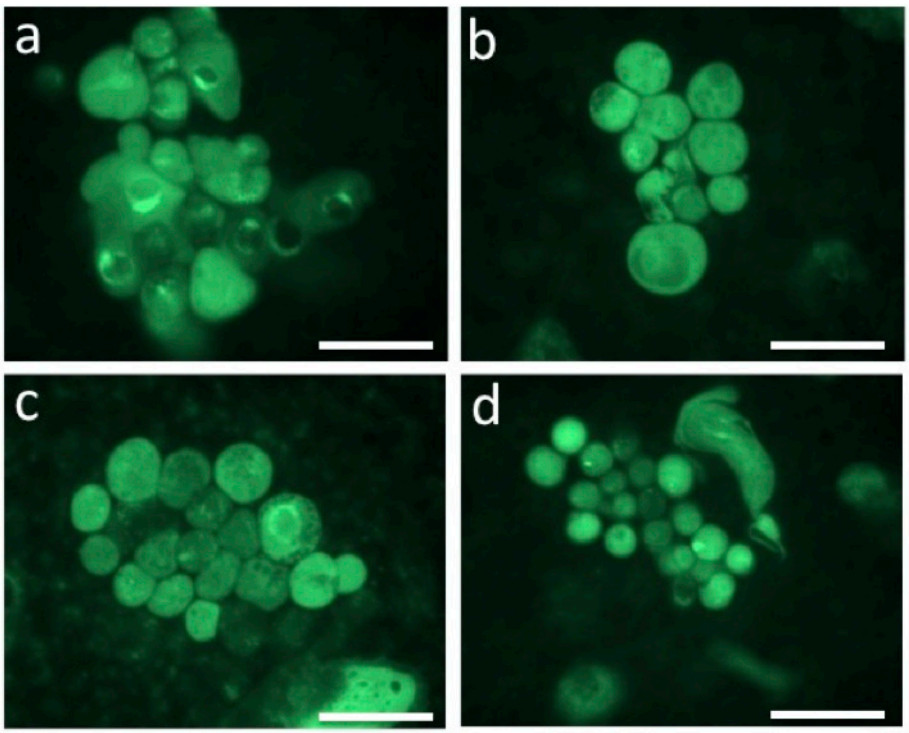

Figure 69 *– Representative fluorescent images of PCD (a and b) and non-PCD (c and d) ALI cultured ciliated primary epithelial cell following incubation with 10μM DAF-fm diacetate for 40 minutes at 37°C with 5% CO_2. Imaged using Leica DMRBE fluorescent microscope at x400 magnification. Similar levels of fluorescence were seen from the cells in the two groups suggesting that PCD epithelial cells biosynthesis similar amounts of NO to non-PCD epithelial cells. Scale bar represent 20μm.*

6.3.1.2 NO biosynthesis is up-regulated by pro-inflammatory cytokines in both PCD and non-PCD respiratory epithelial cell layers

In order to assess whether PCD epithelial cells up-regulate NO biosynthesis in response to cytokine stimulation total nitrite levels were compared in PCD (n=5) and non-PCD (n=9) epithelial cell layers following exposure to pro-inflammatory cytokines (10 ng/ml each of IL-1β, IFN-γ and TNF-α) for 18 hours, both at baseline and post stimulation (2.4.2.3).

No difference was seen in total nitrite concentration from PCD and non-PCD cell layers at baseline (mean (±SEM) 16.1 μmol/L (±1.1) vs. 19.5 μmol/L (±2.0) respectively, P=0.13)) (Figure 71). Furthermore, NO biosynthesis increased significantly by 2-3 fold following stimulation in both groups (P=0.046 and 0.002 respectively) with no

significant difference seen between PCD and non-PCD responses (mean (±SEM) 52.9 μmol/L (±15.6) vs. 57.1 μmol/L (±10.2) respectively, P=0.41)). This suggests that PCD epithelial cells are able to biosynthesise normal levels of NO in response to inflammatory mediators (Figure 70).

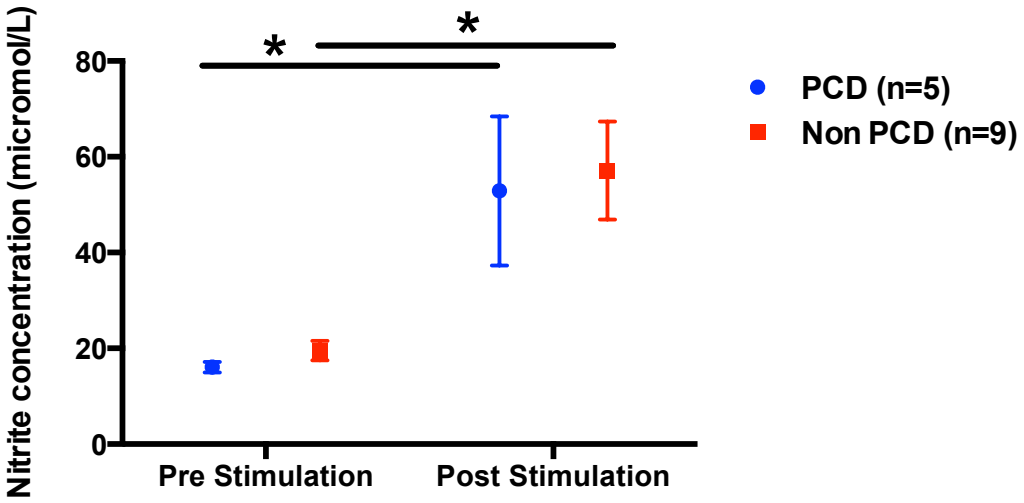

Figure 70 *– NO levels measured in apical PBS washes, following a 30 minute incubation at 37°C, of ALI cultured ciliated primary epithelial cell layers from PCD (n=5) and non-PCD (n=9) measured at baseline and 18 hours after incubation with 10 ng/ml IL-1β/IFN-γ/TNF-α in the baso-lateral media (mean, SEM). Following stimulation NO biosynthesis increased significantly by 2-3 fold in both groups (*P<0.05) but no difference was seen between PCD and non-PCD responses.*

6.3.1.3 NTHi infection stimulates NO biosynthesis in PCD and non-PCD ciliated respiratory epithelial cells

To determine whether PCD epithelial cells were capable of up-regulating NO biosynthesis in response to NTHi biofilm infection, NTHi was co-cultured on the apical surface of ciliated respiratory epithelial cell layers derived from PCD (n=5) and non-PCD (n=9) patients at an MOI of 100 for three days to allow the formation of biofilms. The apical surface was rinsed daily and TER and CBF were monitor daily to assess cell viability and function (2.4.2.4). NTHi biofilm development was examined using by CFU counts, FISH and SEM (2.3.3 and demonstrated in Chapter 5). Both TER and CBF remained stable during these experiments (5.3.2). Total nitrite levels in the apical supernatant were compared between the two groups at baseline, prior to co-culture with NTHi, and at the end of the experiment, after three days of co-culture (2.4.2.3).

Similarly to previous experiments (6.3.1.1 and 6.3.1.2), no difference was seen in total nitrite concentration between PCD (n=5) and non-PCD (n=9) (mean (±SEM) 8.4 μmol/L (±1.4) vs. 11.6 μmol/L (±1.4) respectively, P=0.16) at baseline (Figure 71). In addition, NO biosynthesis increased significantly following co-culture in both groups (p=0.026 and 0.002 respectively), but no significant differences were observed between the PCD and non-PCD cell layer biosynthesis of NO (mean (±SEM) 18.6 μmol/L (±3.8) vs. 22.7 μmol/L (±3.6) respectively, P=0.44) (Figure 71).

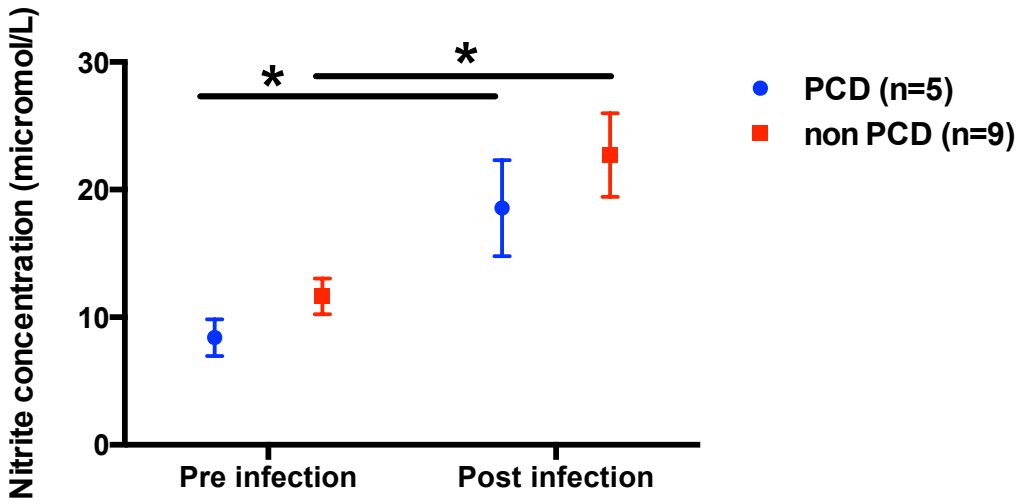

Figure 71 *– NO levels measured in apical PBS washes, following a 30 minute incubation at 37°C, of ALI cultured ciliated primary epithelial cell layers from PCD (n=5) and non-PCD (n=9) measured at baseline and 72 hours post co-culture with NTHi (Mean±SEM). Following NTHi biofilm infection NO biosynthesis increased significantly in both groups (*P<0.05) but no difference was seen between PCD and non-PCD responses.*

6.3.2 Nitric oxide synthase localisation

6.3.2.1 Ciliated respiratory epithelial cell layers cultured from both PCD and non-PCD subjects contain iNOS and eNOS

In order to compare iNOS and eNOS in PCD and non-PCD epithelial cells, ALI cultured respiratory epithelial cell layers from PCD (n=4) and non-PCD (n=4) patients were examined using immunocytochemistry techniques (as described in 2.4.2.5.2). Both PCD and non-PCD cells exhibited positive staining for both iNOS and eNOS compared with appropriate isotype controls (Figure 72).

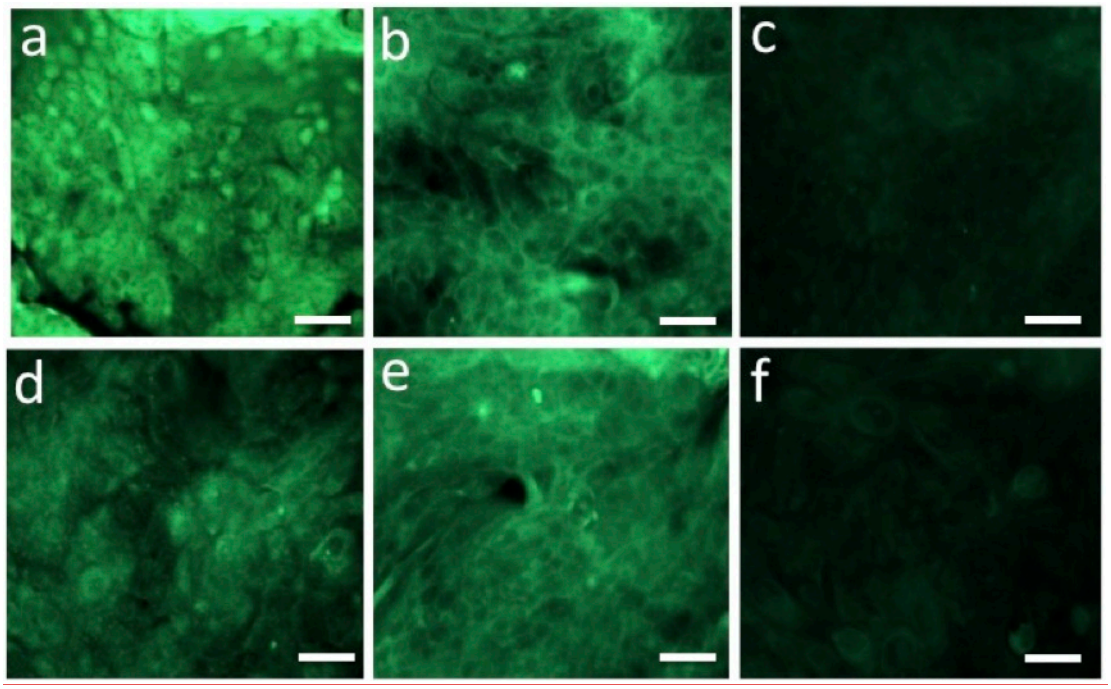

Figure 72 *– Representative fluorescent images of PCD (a, b and c) and non-PCD (d, e and f) ALI cultured ciliated primary epithelial cell layers following a 90 minute incubation with iNOS (a and d) and eNOS (b and e) rabbit polyclonal IgG primary antibodies (both 1:200) and rabbit IgG isotype control (c and f) (1:4000) followed by a 90 minute incubation with a Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (dilution 1:100). Imaged using a Leica DMRBE fluorescent microscope at x400 magnification. Positive staining was seen for both iNOS and eNOS in PCD and non-PCD cells as compared to appropriate isotype controls. Scale bar represent 20µm.*

Since heterogeneous staining was seen throughout the epithelial cells, ten random images were taken of each sample and a mean fluorescent intensity was calculated using image J (Rasband 1997-2012) and normalised against the isotype control (Figure 73). While no difference was seen in the relative mean fluorescence of PCD and non-PCD cell layers for eNOS, iNOS mean fluorescence was significantly higher in the PCD cell layers compared with non-PCD cells (mean (±SEM) 409 (±30) fluorescent units (FUs) vs. 247 (±25) FUs, $P<0.001$) (Figure 73).

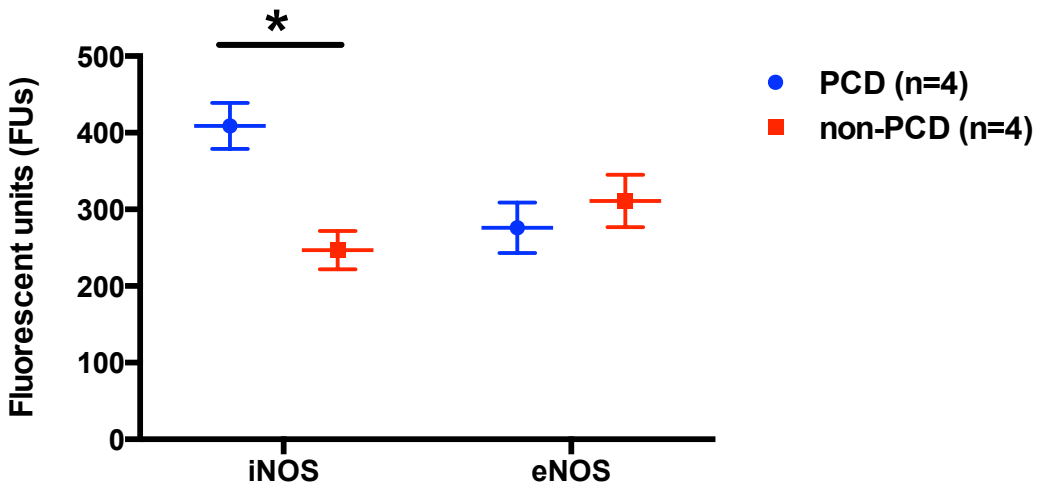

Figure 73 *– Mean fluorescence from ten random images of PCD (n=4) and non-PCD (n=4) ALI cultured epithelial cell layers labelled with iNOS and eNOS antibodies and a fluorescent secondary antibody corrected for isotype control. Imaged using a Leica DMRBE fluorescent microscope at x400 magnification, and analysed using image J (version 1.47a) (Rasband 1997-2012). No difference seen in the relative mean fluorescence of PCD and non-PCD cell layers for eNOS however iNOS mean fluorescence was higher in the PCD cell layers as compared to non-PCD (*P<0.001).*

6.3.2.2 Neuronal NOS localises to the proximal cilia in both PCD and non-PCD ciliated respiratory epithelial cell layers

In earlier experiments, bronchial biopsy tissue embedded in glycol methacrylate acrylic (GMA) resin from healthy controls (kindly gifted by Professor P. Howarth, University of Southampton, Southampton, UK) (n=4) was labelled for nNOS using immunohistochemistry techniques (as described in 2.4.2.5.1). These were research participants with no known underlying respiratory conditions. Neuronal NOS was observed to be specifically localised to the cilia (Figures 74).

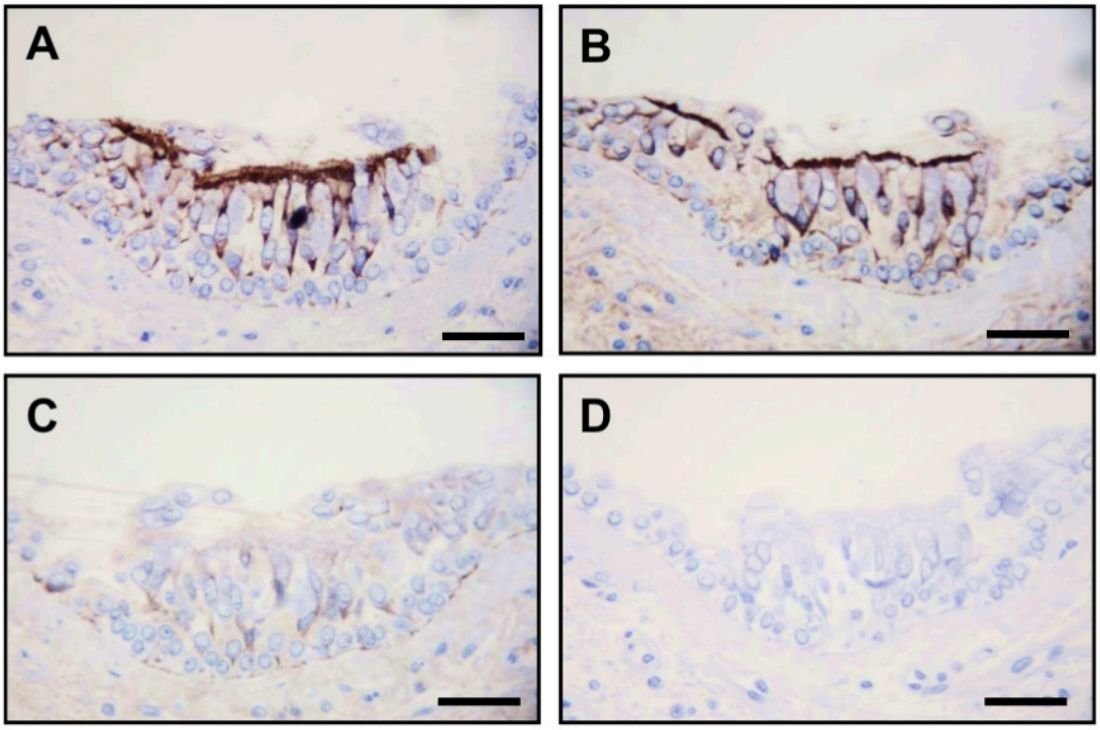

Figure 74 *– Neuronal NOS was observed to be specifically localised to the cilia: representative light microscopy images showing ciliated epithelium of bronchial biopsy tissue from a healthy control embedded in GMA; A – incubated with β-tubulin primary antibody to highlight cilia B – incubated with nNOS primary antibody (1:100 dilution) C – incubated with specific nNOS blocking peptide (1:4 dilution) added to nNOS primary antibody and D – incubated without a primary antibody as a negative control (Leica DMLB microscope at x400 magnification). Scale bar represent 20 µm.*

Given the low levels of NO seen in PCD patients where there is impaired ciliary function, the finding that the nNOS isoform specifically localised to the cilia was further investigated. Neuronal NOS localisation was compared in PCD (n=4) and non-PCD (n=4) ALI cultured respiratory epithelial cell layers using immunocytochemistry techniques (2.4.2.5.2).

Neuronal NOS was observed in both PCD and non-PCD epithelial cell layers, localised to the cilia in both groups (Figure 75 a and b). To further confirm these data, non-PCD ALI cultured cell layers were dual-labelled with antibodies to nNOS and β-tubulin, a fundamental cilial protein, to assess for co-localization. While co-localization was seen occasionally, as represented by the yellow colour (red and green merging) (Figure 75 c), in most areas nNOS was found to be proximal to b-tubulin in the base of the cilia (Figure 75 d).

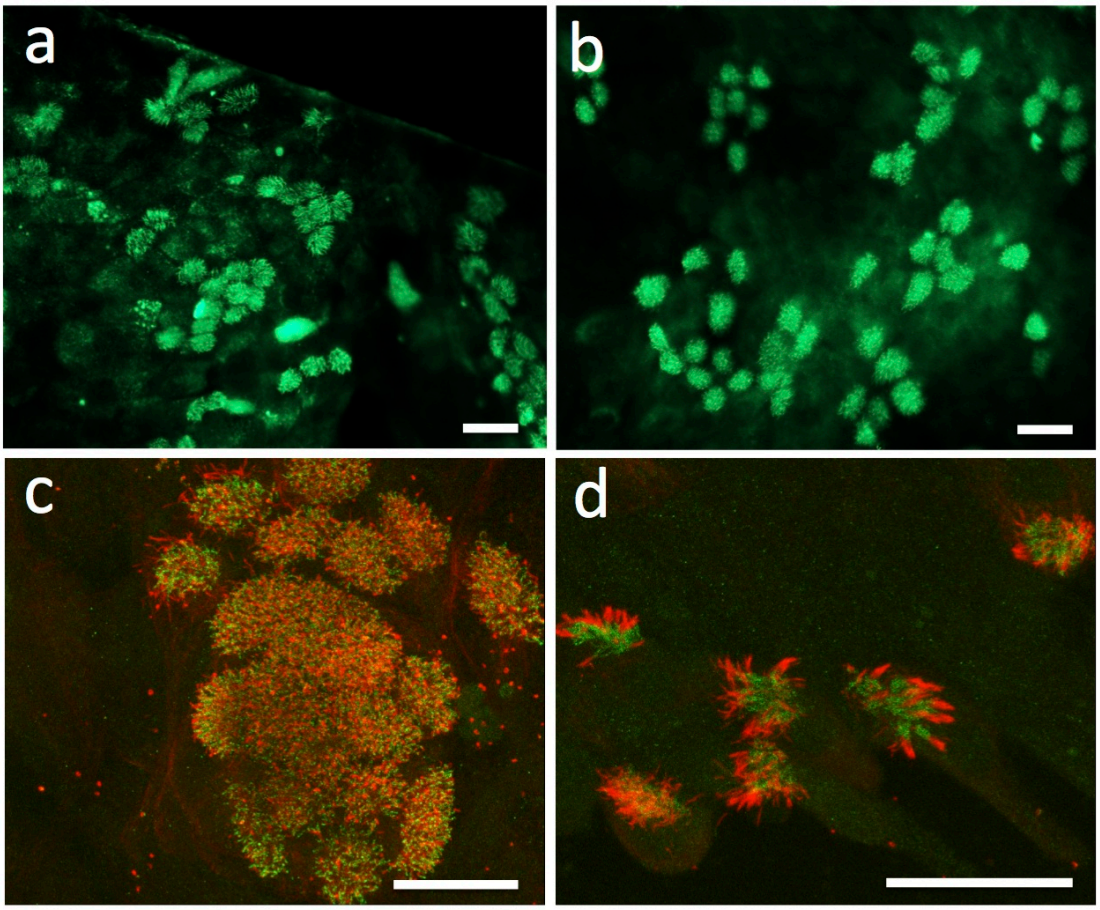

Figure 75 *– a and b) - Representative fluorescent images of (a) PCD and (b) non-PCD ALI cultured ciliated primary epithelial cell layers following a 90 minute incubation with nNOS rabbit polyclonal IgG primary antibody (1:200) followed by a 90 minute incubation with a Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (dilution 1:100). Imaged by Leica DMRBE fluorescent microscope at x400 magnification; c and d) - Representative fluorescent images of non-PCD ALI cultured ciliated primary epithelial cell following incubation with nNOS primary antibody, as above with same secondary. Then incubated for 90 minutes with a β-tubulin primary mouse monoclonal antibody (1:2000) followed by a further 90 minute with a Alexa Fluor® 594 chicken anti-mouse IgG antibody (dilution 1:500). Imaged by Leica SP5 CLSM. (nNOS – green, a β-tubulin – red, co-localisation – yellow, magnification (c) - x630, (d) - x1000). Neuronal NOS was seen localised to the cilia in both PCD and non-PCD epithelial cell layers (a and b), although co-localization was seen in some areas, as represented by the yellow colour in (c), in most areas nNOS was found to localised proximally to b-tubulin staining (d). Scale bars represent 20 µm.*

6.4 Effect of nitric oxide on non-typeable *H. influenzae* biofilm development

In this final section of this Chapter experiments were performed, using the NO donor sodium nitroprusside (SNP), to test the hypothesis that NO renders NTHi in biofilms more susceptible to antibiotic killing by causing dispersal from the biofilm. SNP has been demonstrated to produce approximately 1000th its concentration as NO (for example 100 μM SNP produces 100 nM NO) (Barraud, Schleheck et al. 2009).

6.4.1 Effects of nitric oxide on NTHI in planktonic culture

Prior to considering the affect of NO on NTHi biofilms its affects were studied in planktonic culture in order to identify a concentration of SNP that had little affect on NTHi in this phase of growth. Increasing concentrations of the NO donor SNP (10 nM – 10 mM) were therefore tested against NTHi isolate four in planktonic culture (as described in 2.4.3.1) (Figure 76).

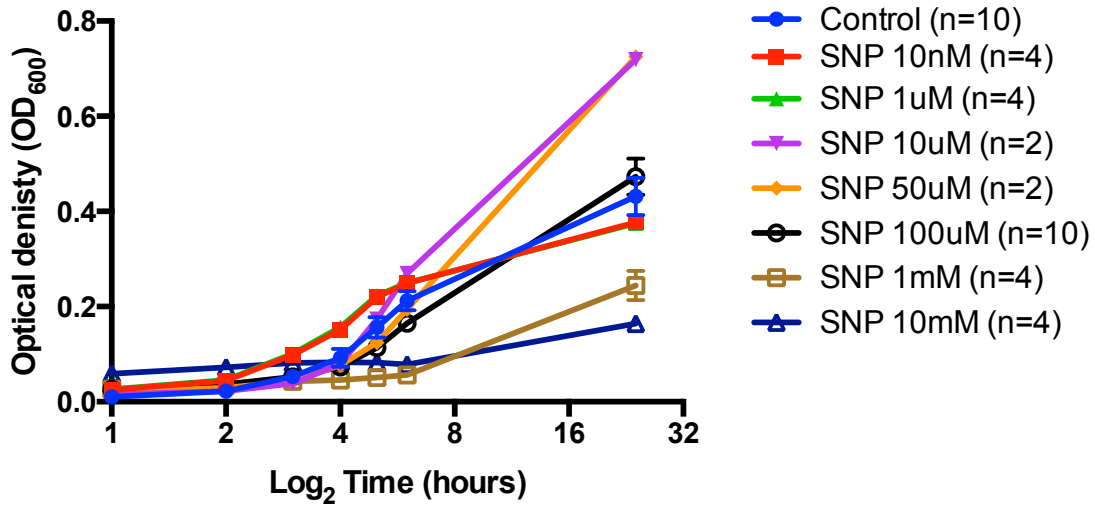

Figure 76 *– Optical density (OD_{600}) against Log_2 Time (hours) for NTHi isolate four grown in planktonic culture for 24 hours with varying concentrations of sodium nitroprusside (10 nM to 10 mM) compared to untreated controls (n = 2-10, Log_2 x-axis for time used to allow data points to be seen more clearly) colour and lines added for clarity). Low concentrations of SNP (10 nM and 1 μM, green line directly under red) had no effect on the planktonic growth, 10 μM and 50 μM SNP appeared to stimulate planktonic growth, which returned to untreated control levels with 100 μM SNP, then at higher concentrations (1 mM and 10 mM) planktonic growth was inhibited.*

No difference was seen on the planktonic growth of NTHi over the 24-hour time course when using low concentrations of SNP (10 nM and 1 μM) or 100 μM SNP compared with untreated control (P = 0.37, 0.36 and 0.55 respectively). Interestingly, 10 μM and 50 μM SNP appeared to stimulate planktonic growth (P = 0.02 and 0.01 respectively) and at higher concentrations (1 mM and 10 mM) planktonic growth was inhibited (P = 0.01 and 0.001 respectively) (as demonstrated by dose response curve, Figure 77).

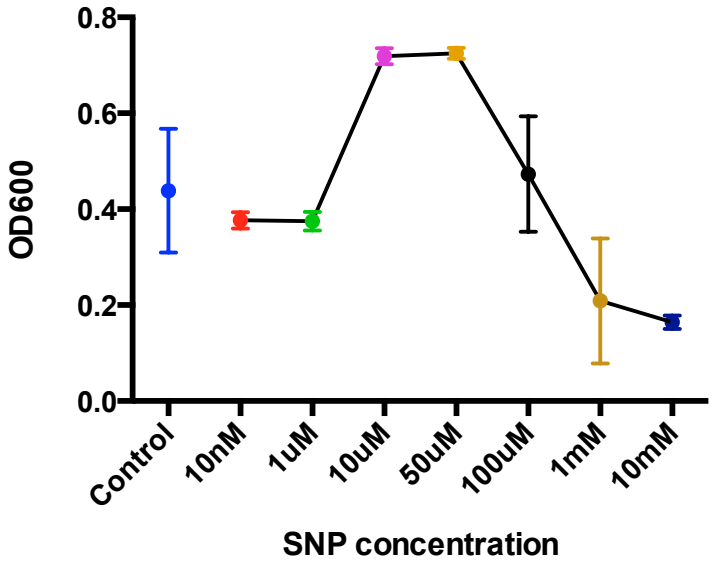

Figure 77 *– Dose response curve of SNP (10 nM to 10 mM) on NTHi isolate four grown in planktonic culture at 24 hours, as measured by optical density (OD_{600}) compared to untreated controls (n = 2-10). Low concentrations of SNP (10 nM and 1 μM) had no effect on the planktonic growth, 10 μM and 50 μM SNP appeared to stimulate planktonic growth, which returned to untreated control levels with 100μM SNP, then at higher concentrations (1 mM and 10 mM) planktonic growth was inhibited.*

In order to test whether low dose NO had a synergistic effect with antibiotic treatment 100μM SNP was chosen for the subsequent biofilm experiments, as it was the highest concentration assessed that did not inhibit planktonic growth. Hence, at this concentration, any effects seen on biofilms would not be due to the bactericidal properties of NO.

6.4.2 Effects of nitric oxide on NTHI biofilms

It had previously been demonstrated that NTHi biofilms were more tolerant to cefotaxime (4.3.4). Therefore the effect of NO on NTHi biofilms, alone and in combination with cefotaxime, were tested. In these experiments CFU counts of both the biofilm and the overlying supernatant were calculated in order to determine

whether NO had a biofilm dispersal effect, similar to *P. aeruginosa* (Barraud, Hassett et al. 2006) (Figure 78 and 79). Additionally, since the release of NO by SNP results in the production of sodium ferrocyanide, which itself has potential bactericidal/biofilm dispersal effect, experiments included potassium ferrocyanide (KFeCN) as an additional control (2.4.3.2).

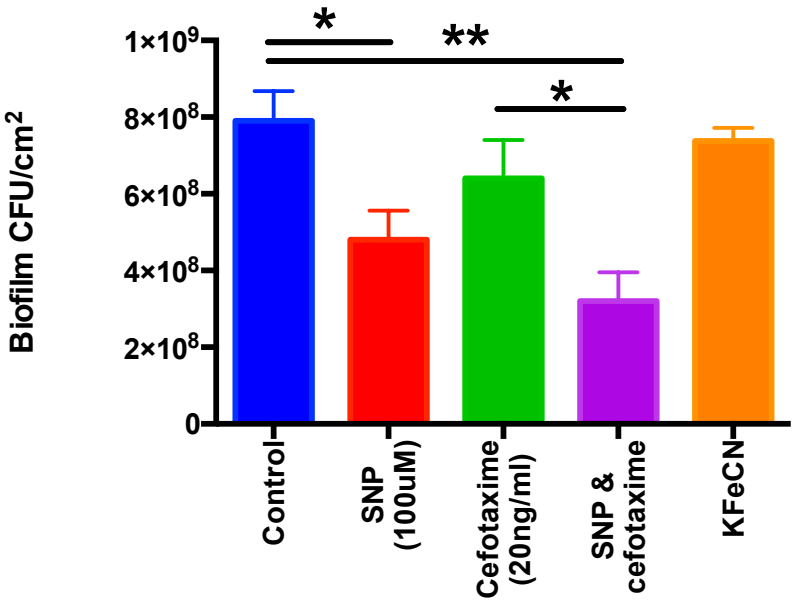

Figure 78 *– Biofilm NTHi CFU counts (CFUs/cm²) from NTHi isolate four biofilms incubated in 24-well plates for three days following six hours incubation with sodium nitroprusside (SNP) (100 μM), cefotaxime (20 ng/ml), both SNP and cefotaxime or potassium ferrocyanide (KFeCN) compared to untreated controls (n=6-9) (mean, SEM). SNP treatment caused a significant decrease in biofilm CFU counts compared to controls and the combination of SNP and cefotaxime significantly reduced biofilm CFU counts compared to both controls and cefotaxime alone (*P<0.05, **P<0.01).*

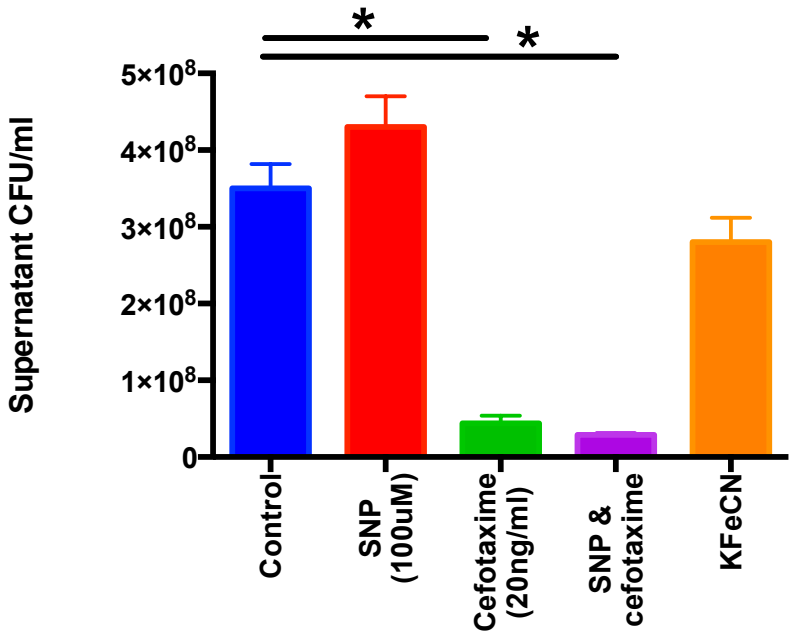

Figure 79 *- Supernatant NTHi CFU counts (CFUs/ml) from supernatant overlying NTHi isolate four biofilms incubated in 24-well plates for three days following six hours incubation with sodium nitroprusside (SNP) (100 μM), cefotaxime (20 ng/ml), both SNP and cefotaxime or potassium ferrocyanide (KFeCN) compared to untreated controls (n= 6-9) (mean, SEM). An increase was seen in the supernatant CFU counts following treatment with SNP however this was not statistically significant. Cefotaxime led to a significant, log fold, reduction in supernatant CFU counts compared to controls, its combination with SNP did not significantly reduce this further (*P<0.0001).*

Sodium nitroprusside treatment caused a significant decrease in NTHi biofilm CFU counts compared with untreated controls ((mean±SEM) $4.8x10^8$ ($±7.6x10^7$) vs. $7.9x10^8$ ($±7.8x10^7$), P=0.015) (Figure 78 and 79). Although there was a concomitant increase in the supernatant CFU counts following treatment with SNP as compared with untreated controls this was not statistically significant ((mean±SEM) $3.5x10^8$ ($±3.2x10^7$) vs. $4.3x10^8$ ($±4.0x10^7$), P=0.14).

Notably, the combination of SNP and cefotaxime significantly reduced biofilm CFU counts compared with untreated controls and cefotaxime alone (P=0.002 and P=0.028 respectively). However no synergy was observed with SNP compared with cefotaxime alone on supernatant CFU counts (P=0.18), suggesting that NO affected biofilm but not planktonic NTHi. Consistent with previous data (4.3.4), NTHi biofilm CFU counts were not significantly reduced by cefotaxime alone at a concentration of 20 ng/ml compared with untreated controls (p=0.30), however cefotaxime resulted in a significant, log fold, reduction in NTHi CFU counts in supernatant ((mean±SEM) $4.4x10^7$ ($±1.0x10^7$) vs. $3.5x10^8$ ($±3.2x10^7$), P<0.0001). There was no difference observed in

either biofilm or supernatant CFU counts between untreated controls and the potassium ferrocyanide groups (P= 0.69 and P=0.22 respectively). This suggests that any sodium ferrocyanide, released during SNP metabolism to NO, would not be a confounding variable in these experiments.

6.4.3 Summary

The NO donor SNP, at 100 μM, demonstrated little affect on planktonic growth, indicating that NO alone was not bactericidal to NTHi at this concentration. However, the addition of NO alone resulted in significantly reduced NTHi biofilm CFU counts and exhibited a synergistic effect in combination with cefotaxime. The associated increase in the supernatant CFU counts in the SNP treated group, whilst not significant, suggests that SNP may have a similar effect on NTHi biofilms to that on *P. aeruginosa* biofilms (Barraud, Hassett et al. 2006), potentially by causing dispersal of bacteria from the biofilm into the supernatant.

6.5 Discussion

6.5.1 Upper and lower airway nitric oxide biosynthesis in primary ciliary dyskinesia

Both bronchial and alveolar NO levels were significantly lower in children with PCD compared with age and gender matched healthy controls. As previously reported, nasal NO levels in this cohort were found to be significantly lower in PCD patients (Walker, Jackson et al. 2012). These findings therefore point towards a generalized mechanism for the low levels of NO that is not localized to a specific lung compartment.

Three studies have utilized the two-compartment model to assess bronchial and alveolar NO concentrations in PCD (Mahut, Escudier et al. 2006; Paraskakis, Zihlif et al. 2007; Shoemark and Wilson 2009). Consistent with these findings, all three studies demonstrated low bronchial NO in PCD patients compared to controls although only Mahut *et al.* also found low alveolar NO (Mahut, Escudier et al. 2006; Paraskakis, Zihlif et al. 2007; Shoemark and Wilson 2009). Of the other two studies that found equivalent alveolar NO levels between PCD and controls, the work by Shoemark *et al.* examined only adults (Shoemark and Wilson 2009). Interestingly data from an adult healthy control group, assessed to optimize the method, was compared to the PCD

group and similar alveolar NO levels were observed (data not shown). Paraskakis *et al.* also found comparable levels of alveolar NO between PCD and controls (Paraskakis, Zihlif et al. 2007). The difference may be explained because 63% of their PCD group were taking inhaled corticosteroids compared with only 23% of this PCD population. It is therefore possible that a higher rate of asthma co-morbidity may account for the differences in alveolar NO between studies. There may also be other differences between the study populations such as concurrent respiratory infection that may account for the differences seen.

The two-compartment model has been validated in over 20 studies across different respiratory disease groups and carries the advantage over standard Fe_{NO} measurements in allowing assessment of NO biosynthesis specifically from the bronchial and alveolar compartments of the lung (George, Hogman et al. 2004; Mahut, Escudier et al. 2006; Paraskakis, Zihlif et al. 2007; Suri, Paraskakis et al. 2007; Kerckx, Michils et al. 2008; Puckett, Taylor et al. 2010). However, despite its advantages, the two-compartment model is an idealised representation of pulmonary anatomy and function and has intrinsic limitations. In particular the model fails to account for axial molecular diffusion of bronchial NO back into the alveolar region, which may lead to a spurious rise in alveolar NO and decreased bronchial NO estimates (Shin, Condorelli et al. 2004; Shin, Condorelli et al. 2006). Nevertheless, the understanding of pulmonary NO exchange is evolving and the importance of heterogeneous ventilation and inflammation have yet to be confirmed (Suresh, Shelley et al. 2008; Barnes, Dweik et al. 2010). The cohort size assessed here is similar to those previously reported (Mahut, Escudier et al. 2006; Paraskakis, Zihlif et al. 2007; Shoemark and Wilson 2009) and included all eligible children with PCD in the UHS PCD clinic. Due to the rarity of PCD, full validation with larger numbers of subjects would require multi-centre collaboration.

Attempts to reduce contamination of Fe_{NO} with nNO were made by exhalation against a minimum mouthpiece pressure of 5cm H_20 (Silkoff, McClean et al. 1997; American Thoracic and European Respiratory 2005). This process theoretically caused velum closure and has been validated by nasal CO_2 measurement and nasal argon insufflation studies (Kharitonov and Barnes 1997; Silkoff, McClean et al. 1997). However given that nNO levels were almost 40 times greater than Fe_{NO50} in healthy children, even small amounts of contamination from the nasal cavity would lead to significant changes in Fe_{NO}. Hence, while these data suggest a generalised reduction in NO biosynthesis throughout the respiratory tract, it remains possible that contamination from the upper airway led to much of the difference seen in the lower airway NO levels.

6.5.2 Assessment of nitric oxide biosynthesis and nitric oxide synthase localisation from PCD and non-PCD ALI cultured epithelial cell layers

Contrary to the hypothesis tested, NO biosynthesis was similar in both PCD and non-PCD epithelial cell layers both constitutively, following stimulation with pro-inflammatory cytokines and NTHi infection for three days. Following stimulation and infection both groups demonstrated similar 2-3 fold increases in NO levels. Additionally, all three NOS isoforms were expressed in PCD and non-PCD ciliated epithelial cell layers and nNOS was observed to be specifically localised to the proximal cilia.

Smith *et al*. recently published findings on NO biosynthesis by PCD and healthy control ALI cultured epithelial cell layers at baseline and two hours after infection with *S. pneumoniae* (Smith, Fadaee-Shohada et al. 2013). Consistent with the constitutive NO data present here, their group found no difference in NO biosynthesis of PCD compared to non-PCD epithelial cells prior to infection. Both data therefore suggest that PCD respiratory epithelial cells are intrinsically able to biosynthesis NO. The explanation for the low NO levels seen in clinical well PCD patients therefore remains unclear.

However the groups' post infection data differed from those found here. They observed an increase in NO levels in healthy control ALI epithelial cell cultures at two hours after infection with *S. pneumoniae* but saw no such increase in PCD epithelial cell cultures (Smith, Fadaee-Shohada et al. 2013). The authors concluded that NO biosynthesis was abnormal in PCD patients following early bacterial infection. Their experimental designed differed the work here in three important ways; firstly, they used a different method of NO detection; secondly, a laboratory strain of *S. pneumoniae* was used which may account for the lack of response in their model (Smith, Fadaee-Shohada et al. 2013) as compared to the clinical PCD isolate of the gram-negative NTHi used here; and finally, the group investigated NO biosynthesis two hours post-infection while in this work NO production was examined at three days. This raises the possibility that PCD epithelial cells may exhibit a delayed NO response to bacterial stimulation.

Inducible NOS is the NOS isoform with the greatest ability to affect NO levels (Lane, Knight et al. 2004). It is therefore of interest that iNOS expression was found to be higher in PCD epithelial cells compared with non-PCD controls. However it is important to note that only limited numbers of cell layers were assessed and an unvalidated

measurement of fluorescence units was used, so the results should be interpreted with caution. Two papers have assessed *NOS2* gene expression, which codes for iNOS, in PCD patients (Pifferi, Bush et al. 2011; Smith, Fadaee-Shohada et al. 2013). One group found *NOS2* expression to be comparable in epithelial cells from PCD patients compared with healthy controls (Pifferi, Bush et al. 2011). The other, outlined above, assessed *NOS2* expression in epithelial cell cultures from PCD and healthy controls at baseline and two hours after infection with *S. pneumoniae* (Smith, Fadaee-Shohada et al. 2013). They found that although *NOS2* expression increased in the healthy control cells following infection this was not seen in the PCD cells (Smith, Fadaee-Shohada et al. 2013). However this paper did not report the absolute constitutive expression of *NOS2* and so comparison with the work here was not possible.

6.5.3 Why are nitric oxide levels low in primary ciliary dyskinesia?

In addition to the hypothesis, refuted by the data in section 6.3, suggesting that PCD respiratory epithelial cells were unable to biosynthesise NO, a number of further potential mechanisms for low levels of NO in PCD have been put forward (see section 1.6.5) (Walker, Jackson et al. 2012). One such hypothesis suggests that NO biosynthesis may be dependent on ciliary beating via a mechanochemical coupling to dynein ATPase and, given that the majority of PCD have static cilia, that this may account for the low levels seen (Narang, Ersu et al. 2002; Chilvers, Rutman et al. 2003). The novel finding that a NOS isoform (nNOS) was localised to the proximal part of the cilia was therefore of interest (6.3). However nNOS was observed to be similarly present in the cilia of both PCD and non-PCD epithelial cells and therefore does not provide evidence for a mechanism that might support this hypothesis. In addition, if this mechanical coupling hypothesis were correct, one would have predicted that bronchial but not alveolar NO levels would be reduced since bronchial epithelium is ciliated whereas the alveoli are not, and, as demonstrated in this chapter (6.2) this was not the case. Furthermore, PCD patients with different functional ciliary impairments, varying from static to hyper-frequent ciliary beat frequency, also exhibit similarly low levels of NO, suggesting that ciliary beating alone is unlikely to explain the reduced NO levels observed. It is possible, given its localisation, that nNOS has a role in basal body or transition zone function, an area important for the trafficking of proteins in and out of the cilia (Szymanska and Johnson 2012).

There are a number of other possible mechanisms for the low levels of nNO seen in PCD patients in which the nasal sinuses are thought to have a central role (1.6.5) (Walker, Jackson et al. 2012). The main source of exhaled NO is the upper airway (Lundberg, Rinder et al. 1994) and a number of conditions associated with lower nasal

NO levels involve disease of the paranasal sinuses, such as CF (Balfour-Lynn, Laverty et al. 1996), chronic sinusitis (Lindberg, Cervin et al. 1997) and PCD. In addition, PCD is associated with obstruction of the osteomeatal complex and/or a reduced storage capacity in the paranasal sinuses due to aplasia or hypoplasia (Pifferi, Bush et al. 2010). The paranasal sinuses are, therefore, likely to be important in nasal NO levels and their role in the low levels of nNO seen in PCD warrants further investigation.

6.5.4 Effect of nitric oxide on non-typeable *H. influenzae* biofilm development

The work undertaken in the final section of this Chapter (6.4) was the first work to demonstrate an effect of NO on NTHi biofilms. It suggested that NO may have a dispersal effect, as has previously been described on *P. aeruginosa* (Barraud, Hassett et al. 2006). Furthermore, NO appears to synergise with cefotaxime allowing concentrations of antibiotic that alone have little anti-biofilm effect to result in significant reductions in NTHi biofilm CFU counts. While further work is required to extend these findings, using different NTHi strains and to elucidate the mechanism for this NO-induced effect on NTHi, the role of NO as a novel anti-biofilm adjunctive therapy is of considerable interest.

6.5.5 Therapeutic role of nitric oxide in primary ciliary dyskinesia

We hypothesise, given the functional roles NO plays both in the lung and on bacterial biofilms, that exogenous NO may have a therapeutic role in patients with PCD.

A number of studies have examined if NO had any benefit to nNO, FE_{NO} and FEV_1. Initial studies used the NOS substrate L-arginine, which was observed to increase FE_{NO} levels in healthy subjects (Kharitonov, Lubec et al. 1995; Grasemann, Gartig et al. 1999; Ratjen, Gartig et al. 1999; Grasemann, Kurtz et al. 2006). In a small pilot study of PCD patients and healthy controls (n= 7 and 11 respectively) a small increase in nasal NO was observed immediately and in FE_{NO}, three hours after L-arginine infusion in PCD patients (Grasemann, Gartig et al. 1999). Another study used nebulized L-arginine in ten putative PCD patients and ten healthy controls. They reported that L-arginine not only increased nasal NO levels but also increased CBF and decreased mucociliary clearance time (Loukides, Kharitonov et al. 1998). However they did not define the functional or ciliary ultrastructural defects in their PCD group and, notably, seven of the ten patients reported to have PCD had normal CBF prior to L-arginine treatment. The two PCD patients with static/extremely slow cilia showed no change in CBF

following treatment with L-arginine (Loukides, Kharitonov et al. 1998), calling into question the diagnosis in the other study participants.
In addition to affects on ciliary function, NO has antibacterial properties (MacMicking, Nathan et al. 1995; Wei, Charles et al. 1995; Major, Panmanee et al. 2010). It is hypothesised that the lower respiratory tract is infected by micro-aspiration of respiratory pathogens from the nasopharynx (Levine, Liu et al. 2000; Bogaert, De Groot et al. 2004). One group demonstrated that reduced sinus NO concentrations were associated with sinus infection and a further case study reported that nasal application of NOS inhibitors both reduced nasal NO and led to sinus infection (Deja, Busch et al. 2003; Lundberg 2005). Increasing nasal NO levels therefore offers the potential to reduce nasopharyngeal carriage of pathogens and thereby reduce the risk of lower respiratory tract infection.

A further potential clinical benefit pertains to the effect of NO on the dispersal of bacterial biofilms. In keeping with previously reported data on the affects of NO and *P. aeruginosa* (Barraud, Hassett et al. 2006; Barraud, Schleheck et al. 2009), the data here in section 6.4 would support NO having similar dispersal affects on NTHi biofilms.

Hence whilst the evidence that augmenting NO can improve ciliary function in PCD is not compelling the findings here, supporting its role as an antibacterial and novel anti-biofilm adjunctive therapy, are of considerable interest. There is however an important balance to be struck as there is a potential risk that increasing NO levels in the upper or lower respiratory tract may drive “nitrosative stress”, in what is already a pro-inflammatory environment.

6.6 Conclusion

The data here demonstrate that epithelial cells from patients with PCD can biosynthesise normal concentrations of NO, making it unlikely that NO was a confounding variable in the ALI co-culture model developed here to examine the role of ciliary function on biofilm development. The explanation for the extremely low levels of nasal NO seen in these patients therefore remains elusive however the role of the paranasal sinuses warrants further investigation.

Bacterial biofilms are becoming increasingly recognized as important in the clinical management of patients with chronic respiratory conditions. The data here suggest that, similar to its effects on *P. aeruginosa* biofilms, NO acts to disperse NTHi biofilms. While further work will be required to extend these findings, given the lack of available

anti-biofilm therapeutic options, the role of NO augmentation, particularly in the management of PCD, merits further work in the future.

Chapter 7

Final discussion

7.1 Importance of NTHi biofilms in PCD

This work developed an ALI co-culture model to test the hypothesis that PCD, in which abnormal ciliary function leads to impaired mucociliary clearance, is a biofilm-associated disease.

Data support the study hypothesis with impaired ciliary function correlating with increased biofilm development by a PCD clinical isolate of NTHi, the most frequent pathogen cultured from the PCD patient cohort at University Hospital Southampton. Significantly more NTHi biofilm development was seen on PCD compared with non-PCD ALI cultured respiratory epithelial cell layers, evidenced by significantly higher NTHi biofilm CFUs and over four times the volume of NTHi using FISH with confocal laser scanning microscopy and image analysis and corroborated by SEM.

In addition, of the four PCD NTHi isolates assessed in this work, the two that formed bacterial biofilms were both isolated from children who had recurrent/chronic NTHi infection over three to four years as opposed to a few months in the case of the two NTHi isolates that did not.

These findings together suggest that bacterial biofilm development is likely to play an important role in the recurrence and persistence of NTHi infections in PCD patients. In addition, we hypothesise that the impaired mucociliary clearance that occurs in PCD patients will, similarly, predispose them to biofilm infections with other respiratory bacterial pathogens known to develop biofilms, such as *S. aureus* and *P. aeruginosa* (Hall-Stoodley, Costerton et al. 2004).

The clinical importance of these findings on the management of patients with PCD depends on the extent of detrimental effect recurrent/chronic *H. influenzae* infection has for PCD patients. In CF *P. aeruginosa* infection is clearly associated with decreased survival and reduced lung function (Wilmott, Tyson et al. 1985; Emerson, Rosenfeld et al. 2002; Courtney, Bradley et al. 2007; Konstan, Morgan et al. 2007) however no such longitudinal data is available assessing the effect of *H. influenzae* infection in PCD. The recently funded National PCD Management Service for children, where all children with

PCD in England will be managed by one of four national PCD centres, will allow these longitudinal microbiological and lung function data to be collated. Due to the significant consequences of *P. aeruginosa* infection in CF aggressive attempts are made to eradicate it with intensive and prolonged antimicrobial therapy, although once established this is rarely achieved. It is standard practice, in both PCD and CF, to treat positive cultures of *H. influenzae* with antibiotics however if *H. influenzae* were demonstrated to have significantly detrimental effect on lung function or mortality, particularly in those chronically infected with it, then a more aggressive approach might be warranted. In addition, it would further support the call for the development of novel adjunctive anti-biofilm therapies to be used to aid the clearance of bacterial biofilms in both conditions.

Patients with PCD have low levels of nNO. Nitric oxide has well documented antimicrobial effects, has previously been demonstrated to cause dispersal of *P. aeruginosa* biofilms and has been shown here to potentially have similar dispersal effects on NTHi biofilms. Taken together these findings would suggest a therapeutic rationale for developing NO-based adjunctive anti-biofilm therapies that might be particularly beneficial to PCD patients. In addition such treatments might also be useful in other chronic suppurative respiratory conditions that may be associated with biofilm infections (considered further in future work).

7.2 The role of mucociliary clearance in bacterial biofilm development in other respiratory conditions

While experiments focused on the importance of impaired mucociliary clearance in patients with PCD, the findings may be more widely applicable. It is well established that viral respiratory tract infections lead to ciliary dysfunction and reduced mucociliary clearance (Camner, Jarstrand et al. 1973; Tristram, Hicks et al. 1998; Pittet, Hall-Stoodley et al. 2010). There is growing interest in the condition protracted bacterial bronchitis (PBB) as a cause of chronic wet cough in children, with one paper reporting almost half of their cohort of such children had the condition (Marchant, Masters et al. 2006; Craven and Everard 2013). One hypothesis maintains that the pathogenesis of PBB begins with an acute infection, possibly viral, which leads to temporary impairment of mucociliary clearance and facilitates establishment of bacterial biofilms in the airway leading to a chronic wet cough (Everard 2012). It has been further suggested that if left unchecked, such a mechanism may be a precursor to the development of bronchiectasis (Cole 1986; Craven and Everard 2013). Our work supports this hypothesis for the development of PBB and thereby supports the

aggressive treatment of PBB in children (Marchant, Masters et al. 2012). In addition, a mechanism where acute infection impairs mucociliary clearance facilitating biofilm development may also be important in the pathogenesis of other chronic respiratory conditions, such as chronic obstructive airways disease (Shaheen, Barker et al. 1994; Sethi 2000; Murphy, Brauer et al. 2004).

7.3 Future work

7.3.1 Epithelial cellular responses to NTHi infection

7.3.1.1 Cationic antimicrobial peptides

A significant increase in the concentrations of the cationic antimicrobial peptide (CAMP) LL-37 in response to NTHi infection was observed for both PCD and non-PCD epithelial cell layers, however this was significantly greater in the PCD group. This may point towards a potential adaptive mechanism of the innate immune system of PCD patients to compensate for the lack of ciliary function by synthesising increased amounts of CAMPs in response to infection. This would be of particular relevance as, in addition to its antimicrobial properties, LL-37 has been demonstrated to potently inhibit *P. aeruginosa* biofilm formation (Overhage, Campisano et al. 2008). Future work, considering LL-37 and other important CAMPs further, in particular β-defensin (Singh, Jia et al. 1998; Harder, Meyer-Hoffert et al. 2000), will be of interest.

7.3.1.2 Cytokines

The cytokine responses to NTHi infection were comparable between the PCD and non-PCD cell layers. However the time points considered were baseline, 24, 48 and 72 hours. Future work comparing the epithelial responses both early following co-culture, over the first 12 hours, and later, in prolonged co-culture experiment, would also be of interest.

7.3.2 Novel anti-biofilm therapies

The Centers for Disease Control and Prevention (CDC, Atlanta, U.S.) estimated that 65% of human infections are associated with bacterial biofilms (Potera 1999), which have been demonstrated in many chronic infections of the upper and lower respiratory tract including chronic sinusitis, otitis media, tonsillitis and bronchiectasis as well as in cystic fibrosis (Chole and Faddis 2003; Starner, Zhang et al. 2006; Psaltis, Ha et al.

2007). This work now suggests a role for NTHi biofilms in PCD. However, despite the importance of bacterial biofilms in chronic infections, there are few, if any, effective anti-biofilm therapies apart from the use of high dose, prolonged courses of standard anti-microbials. There is therefore an urgent and unmet need for effective adjunctive anti-bacterial biofilm treatments.

Further understanding the adaptations that bacteria undergo, at a proteomic and transcriptional level, when moving from planktonic to a biofilm phase of growth and during dispersal from the biofilm, will be important in allowing development of novel therapeutic interventions that disrupt these processes. In chapter 6 NO was demonstrated to significantly reduce biofilm CFU counts, perhaps by causing biofilm dispersal. Further work, to clarify whether this is mediated by cyclic di-GMP, as seen in *P. aeruginosa* (Barraud, Hassett et al. 2006), or via an alternative mechanism will be important in developing NO as a potential anti-biofilm adjunct for the treatment of NTHi and other bacterial biofilm infections. Work beyond the scope of this thesis is therefore planned to examine the effect of NO on NTHi and *Staphylococcus aureus* biofilms using proteomic analysis alongside a 3view System (Gatan, Abingdon, UK). The 3view System is an SEM with an automated sectioning system to allow high resolution imaging of the 3D architecture of the bacterial biofilm before and after NO treatment.

Nitric oxide, used as an anti-biofilm adjunctive intervention, has now been studied in patients. Cathie *et al.* have recently reported on inhaled NO, used alongside standard antimicrobial treatment, in CF patients in a small pilot study (Cathie, Howlin et al. 2014). Twelve CF patients were randomised to receive either inhaled NO, administered for eight hours overnight for seven days whilst in hospital, in addition to standard antibiotic therapy or antibiotics alone. The amount of *P. aeruginosa* biofilm decreased significantly more in the NO treatment group as compared with those having antibiotics alone over the seven days of treatment. In addition, they observed a greater improvement in FEV_1 in the NO treated group (Cathie, Howlin et al. 2014).

However several novel, and potentially more practical, methods of delivering NO to bacterial biofilms in the respiratory tract have also recently been described. Whilst in the early stages of development, Yepuri *et al.* have reported on several cephalosporin-3'-diazeniumdiolate compounds that act as biofilm-targeted NO-donor pro-drugs (Yepuri, Barraud et al. 2013). These compounds combine cephalosporin antibiotics with an NO-donor pro-drug that selectively release NO following interaction with β-lactamase, produced by some bacteria. They have demonstrated that the NO released leads to *P. aeruginosa* biofilm dispersal *in vitro* (Yepuri, Barraud et al. 2013). This

targeted approach, while in its early phase, offers an exciting and practical way of delivering this proven anti- *P. aeruginosa* biofilm adjunct directly to bacterial biofilm.

NO has a short half-life and in the pilot study by Cathie *et al.*, outlined above, inhaled NO was therefore administered for an 8-hour period necessitating an overnight in hospital and, as such, has significant practical and cost implications (Cathie, Howlin et al. 2014). A method of exposing the bacterial biofilm to NO for prolonged periods without the need for its continuous inhalation is therefore desirable. Duong *et al.* have recently reported on a new class of core cross-linked star polymers designed to store and release NO in a slow, controlled way (Duong, Jung et al. 2014). The polymer was demonstrated to deliver NO in a controlled fashion in *P. aeruginosa* bacterial cultures, preventing both cell attachment and biofilm formation (Duong, Jung et al. 2014). If such polymers were developed into a compounds that could be safely nebulised into patients this again would offer a further exciting advance in anti-biofilm interventions. However significant work is required before either of these compounds will be available for clinical practice. The ALI co-culture model developed here could be adapted to be utilised for the pre-clinical work up of these exciting novel compounds.

Due to the persistent rhinitis and nasal congestion that patients with PCD suffer from they often regularly perform nasal rinsing treatments. This lends itself to the addition of an NO-donating compound, such as those outlined above, to the rinse solution used that would then be directly applied to the nasal passages. Many consider that the upper airways act as a reservoir for pathogens, which can then be micro-aspirated into the lower respiratory tract. This proposed treatment, delivered in this way, has the potential to improve nasal hygiene and, in doing so, have a positive impact on the rate of lower respiratory tract exacerbations.

Work in Chapter 4 suggested an association between of the production of EPS matrix and the extent of biofilm development in NTHi isolates. Several papers have demonstrated that DNA is present in the *H. influenzae* biofilm matrix (Hong, Pang et al. 2007; Jurcisek and Bakaletz 2007) with one study showing that DNase inhibited NTHi biofilm formation (Izano, Shah et al. 2009). Pulmozyme® (Genentech, California, US), a recombinant human DNase, is already used routinely in the management of patients with cystic fibrosis in order to decrease the viscosity of airway mucus. Clinical studies investigating its affects on chronic recurrent infection in PCD may now be warranted. In addition, a better understanding of the other constituents of the EPS matrix may reveal further targets for future therapeutic strategies to inhibit biofilm development or degrade existing biofilms.

7.3.3 Future roles for the primary ALI cultured epithelial co-culture model

The role of ALI co-culture model, developed in this project, could be broadened in a number of ways. It could be adapted to use both different bacterial species and primary epithelial cells from patients with different clinical phenotypes, for example co-culturing epithelial cells from patients with cystic fibrosis and *P. aeruginosa* alone or in conjunction with additional pathogens.

To date, several projects adapting the ALI co-culture model developed here are planned or underway. One will assess the responses of ALI cultured epithelial cell layers derived from PCD, CF and healthy controls to co-culture with *S. aureus* biofilms using a proteomic approach in addition to the techniques developed and used in this work. *S. aureus* is a common respiratory pathogen in both PCD and CF, where it tends to be cultured in younger children. The aim of the project is both to better understand of the role of this important respiratory pathogen and to compare the responses of PCD and CF respiratory epithelial cells in order to better understand the similarities and differences in pathophysiology between these two conditions. A further project will use the ALI co-culture model to investigate the role of different serotypes, of varying pathogenicity, of Neisseria meningitides in the colonisation of the upper respiratory tract. Ultimately these projects have the potential to identify novel therapeutic interventions for these conditions.

It would also be interesting to use the co-culture model to investigate the interaction between epithelial cells, bacteria biofilms and respiratory viruses. It is intriguing that culture-independent molecular microbiological study, longitudinally assessing the lung microbiome in CF patients, demonstrated remarkable stability in the bacterial communities found (Stressmann, Rogers et al. 2012), despite clear clinical deteriorations seen during exacerbations and type and number of antibiotics used to treat them. During episodes, bacterial communities were observed to alter temporarily before returning to pre-treatment configurations within one month of treatment (Stressmann, Rogers et al. 2012). This suggests that extrinsic factors, affecting the microbiome present in the lungs, might lead to clinical respiratory exacerbations, as opposed to novel acute bacterial infection as previously thought. There is an increasing literature on the role viruses play in respiratory exacerbations in CF, as they can be cultured using newer techniques in up to 60% of such events. (Armstrong, Grimwood et al. 1998; Wat, Gelder et al. 2008; de Almeida, Zerbinati et al. 2010). Viruses are also associated with deteriorations in lung function (van Ewijk, van der Zalm et al. 2008). One hypothesis is that respiratory viruses interact with the multi-

species bacterial biofilm in the lungs leading to the respiratory exacerbation. There are a number of potential mechanisms for this; firstly, viruses might trigger bacterial dispersal from the biofilm (Chattoraj, Ganesan et al. 2011); secondly they might affect the host inflammatory response (Sajjan, Jia et al. 2006) which either in itself or via disruption to the bacterial biofilm causes the clinical exacerbation; or finally they might facilitate bacterial adherence to the epithelial cells leading to exacerbation (Avadhanula, Rodriguez et al. 2006). A better understanding of the interaction between viruses, bacteria, potentially in biofilms, and the host respiratory epithelial cells is key to investigating these hypotheses and future novel interventions.

In addition, the co-culture model could be used to investigate the possibility of intracellular invasion by NTHi. Whilst traditionally considered to be an extracellular pathogen (Moxon, Sweetman et al. 2008) there are studies indicating that NTHi might be able to survive within airway epithelial cells (Ketterer, Shao et al. 1999; Morey, Cano et al. 2011; Nistico, Kreft et al. 2011; Lopez-Gomez, Cano et al. 2012; Ren, Nelson et al. 2012; Clementi, Hakansson et al. 2014). After internalisation bacteria have been demonstrated to remain in a quiescent state inside vacuoles (Morey, Cano et al. 2011). This might be an additional mechanism by which NTHi is able to persist in the airway, resisting both antibiotic therapy and the host immune response (Clementi, Hakansson et al. 2014). TEM images of sections through the ALI cultured epithelial cell layers following co-culture with NTHi did not reveal such vacuoles however the number of slices assessed were limited (data not shown). CSLM images using FISH 16s rRNA probes specific for *H. influenzae* could not differentiate between intra- and extra-cellular bacteria without using further cellular markers, however extracellular bacteria were seen on the cells layers by SEM. The possibility of intracellular invasion of NTHi warrants further consideration in future experiments.

7.3.4 Novel cell culture systems

Typically when using an ALI culture system only epithelial cells are included. While this is valid for looking at the respiratory epithelial cell layer it does not fully reflect the *in vivo* airway wall, as any potential crosstalk between the different cell types present would not occur. There has therefore been increasing interest in developing complex, multi-cellular culture models that include an epithelial layer alongside other cell types, potentially with a basement membrane. In addition, culture systems are being developed incorporating microfluidics and movement, to mimic the cell stresses due to breathing, referred to as “lung-on-a-chip” systems. They enable supernatant to continuously circulate and thereby allow real-time measurement of cellular responses (Nalayanda, Puleo et al. 2009; Huh, Hamilton et al. 2011). While in their infancy,

modelling the airway wall in this way will lead to advances in developing realistic *in vitro* systems that more truly replicate the *in vivo* situation and in doing so will reduce reliance on animal models. They will facilitate study of the pathophysiology of airway diseases and will increase the likelihood of novel therapies, found to be effective in pre-clinical studies, to have real benefit *in vivo*.

7.3.5 Modelling multi-species biofilms

Whilst allowing the study of this prevalent respiratory pathogen and its interaction with the respiratory epithelium, the mono-species NTHi biofilm co-culture model developed here is simplistic as it may not reflect the reality of the complex polymicrobial bacterial biofilms that are likely to dwell in the lungs of patients with chronic suppurative respiratory diseases. The importance of this was highlighted in recent work by Weimer *et al*, which demonstrated that NTHi biofilms enhanced the formation of *S. pneumoniae* biofilms (Weimer, Armbruster et al. 2010), an observation further supported by Krishnamurthy *et al* (Krishnamurthy and Kyd 2014). Therefore, alongside the development of more complex multi-cellular "lung-on-a-chip" airway wall models, there is a growing interest in developing polymicrobial, biofilm infection models, more in keeping with the reality in the lung (Dalton, Dowd et al. 2011; Ramsey, Rumbaugh et al. 2011; Xiao, Klein et al. 2012). Successful co-culture of these complex multi-cellular culture models with multispecies biofilms will give us the best understanding of the *in vivo* situation facilitating the development of novel anti-biofilm therapies that thereby benefiting patients.

7.4 Final conclusion

This work suggests that the impaired ciliary function seen in patients with PCD facilitates development of NTHi biofilms.

Despite the extremely low levels of NO observed in patents with PCD, their airway epithelial cells appear to synthesise NO normally. The mechanism for this phenomenon therefore remains elusive.

There is an unmet need for novel anti-biofilm therapies. The findings presented here suggest a possible therapeutic rationale for developing NO-based adjunctive anti-biofilm therapies for PCD and other chronic suppurative respiratory conditions that may be associated with bacterial biofilm infections.

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Appendix 1

1.1 First author papers from PhD

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Upper and lower airway nitric oxide levels in primary ciliary dyskinesia, cystic fibrosis and asthma

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Summary
Background: Patients with primary ciliary dyskinesia (PCD) have abnormal ciliary function and low nitric oxide levels. Nitric oxide (NO) biosynthesis is dependent on nitric oxide synthases (NOS). Cilia line the bronchial but not the alveolar epithelium. It has been hypothesised that NOS function relies on normal ciliary function and that in PCD bronchial but not alveolar NO might therefore be reduced. The aim of this study was to assess bronchial and alveolar NO levels primarily comparing healthy children to PCD and secondarily to cystic fibrosis (CF) and asthmatic children.
Methods: Multiple flow-rate fractional exhaled and nasal NO measurements were performed using a NIOX® Flex NO analyser (Aerocrine, Sweden) in children with PCD ($n = 14$), asthma ($n = 18$), CF ($n = 12$) and healthy controls ($n = 18$). Alveolar and bronchial NO levels were derived using a model of pulmonary NO exchange-dynamics.
Results: Both the bronchial and alveolar NO were significantly lower in PCD than healthy controls (mean (SD) 264 (209) picolitres/second (pl/s) *vs.* 720 (514) pl/s, $p = 0.024$ and 1.7 (0.8) parts per billion (ppb) *vs.* 3.5 (1.3) ppb, $p = 0.001$ respectively.) In asthmatics bronchial NO was found to be significantly higher than in healthy controls and in children with CF alveolar NO was significantly lower (2100 (1935) pl/s, $p = 0.045$ and 2.5 (1.2) ppb, $p = 0.034$ respectively.)

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Conclusion: Our findings do not support the hypothesis that NOS and ciliary function are coupled instead suggesting a more generalised mechanism for the low levels of NO seen in PCD. Our findings in CF and asthma corroborate evidence that these are diseases of the lung peripheries and bronchi respectively.

Introduction

Nitric oxide is a key cellular signalling molecule, involved in diverse physiological and pathophysiological processes such as vascular homeostasis, immune cell activity and broncho-motor tone.[1] It is synthesized in the respiratory epithelium from L-arginine by three NO synthase isoforms (NOS).[2] Infection and inflammation lead to inducible NOS stimulation and hence NO biosynthesis.[2] Airway NO, can be measured using simple non-invasive techniques via the reaction of NO with ozone and subsequent detection by chemiluminescence through commercially available equipment.[3] This allows measurement of nasal NO (nNO) and fractional exhaled NO, typically measured at a flow rate of 50 ml/s (Fe_{NO50}).

Primary ciliary dyskinesia (PCD) is an autosomal recessive condition in which abnormal ciliary structure and/or function lead to impaired mucociliary clearance, recurrent sino-pulmonary infection and, ultimately, bronchiectasis.[4] Nasal NO and, to a lesser extent, Fe_{NO} are characteristically low in PCD, so much so that nNO is used as a screening test for PCD in many specialist centres.[5] Abnormal NO concentrations have been identified in a number of other respiratory diseases including cystic fibrosis (CF), where NO levels are reduced compared to healthy controls but not to the degree seen in PCD and asthma where airway levels are elevated.[6,7]

Localising the relative contributions of different areas of the lung to total Fe_{NO} levels in these conditions would increase our understanding of the underlying pathophysiology, potentially providing a useful non-invasive marker for disease progression, and possibly help to tailor therapies. A two-compartment model of pulmonary NO exchange dynamics, using Fe_{NO} measurements at several different flow-rates, allows estimates of the relative contribution of the bronchial ($J'aw_{NO}$) and alveolar ($Calv_{NO}$) areas of the lung to total Fe_{NO}.[8,9] Bronchial NO flux is calculated as the product of the bronchial wall NO concentration and the NO diffusing capacity of the airway.[9] The alveolar NO concentration is calculated as a steady NO source, a balance between locally biosynthesised and inhaled NO *vs* NO diffusion away.[8,9]

The raised NO levels seen in asthma are attributed to eosinophilic inflammation however, it is unclear why NO is low in PCD and CF despite persistent airway infection and inflammation.[10] A number of potential mechanisms for the extremely low NO in PCD have been proposed (reviewed in Ref. [11]) however, to date, the actual mechanism(s) remains elusive. Several papers have hypothesised that it may result from a reliance of normal NOS activity, and hence NO biosynthesis, on normal ciliary ultrastructure and/or function due to a mechano-chemical coupling to dynein ATPases in the ciliary axoneme.[12,13] If this hypothesis were correct one would expect bronchial NO to be reduced in PCD, as cilia are present in the bronchial compartment and ciliary function is impaired in PCD. However cilia are not present in the alveolar compartment, so alveolar NO should not be affected by impaired ciliary function and might be comparable with healthy controls.

The three studies that have used this two-compartment model in PCD patients to date have conflicting results, but varied in age and demographics of participants and NO analysers used.[13–15] We are the first single study to assess the relative contributions of the bronchial and alveolar compartments of the lung to total Fe_{NO}, comparing healthy children to those with PCD and secondarily to children with CF and asthma.

Methods

This study was approved by Southampton and South West Hampshire Research Ethics Committee (A). REC numbers: 06/Q1702/109 and 08/H0502/126. All subjects gave written informed consent.

Participants

Children with PCD ($n = 14$), asthma ($n = 18$) and CF ($n = 12$) patients were recruited from specialist PCD and paediatric respiratory clinics. Healthy children ($n = 18$) recruited from non-respiratory clinics completed a short questionnaire to exclude disease that might affect nitric oxide levels. PCD was diagnosed by assessing the ciliary beat frequency and pattern of airway epithelial cells by high-speed video microscopy in patients with a suggestive history. Diagnosis was further supported by assessment of ciliary ultrastructure by transmission electron microscopy and, in some cases, analysis of re-differentiated cilia following culture of the airway epithelial cells at an air liquid interface.[4] CF diagnosis was based on compatible history, an abnormal sweat test and/or CF genotyping. Asthma diagnosis was based on clinical history and characteristic spirometry with reversibility.[16] Children with asthma were on different British Thoracic Society asthma management steps: step 1 ($n = 1$), step 2 ($n = 2$), step 3 ($n = 7$), step 4 ($n = 5$), step 5 ($n = 3$).[16] CF, asthma and healthy children were age and sex matched to the participants with PCD.

For inclusion the children had to be over 6 years of age and be well on the day of testing with no evidence of respiratory tract infection in the previous four weeks. They were excluded if they had multiple respiratory diagnoses, had smoked in the last year or had undertaken spirometry in the previous hour. We also assessed 14 healthy adults in order to optimise the methods (data shown in Supplementary Table 1).

Measurement of airway nitric oxide

Measurement of nasal and lower airway nitric oxide levels using a chemiluminescent NO analyser, NIOX® Flex (Aerocrine, Sweden) followed American Thoracic Society/European Respiratory Society (ATS/ERS) recommendations.[17]

Nasal NO was measured during a breath holding manoeuvre to close the velum whilst a nasal probe sampled gas aspirated from the nostril at a rate of 5 ml/s. Patients were encouraged to hold each breath for approximately 20 s until the analyser recorded a plateau in nitric oxide concentrated from the aspirated gas. Three measurements were obtained from each child using the same nostril and the mean nNO reading was recorded.

Fe_{NO} was measured at multiple flow-rates (50, 100, 200 and 250 ml/s) whilst maintaining a constant exhalation pressure >5 cm H_20 through visual feedback. Two consistent readings (within 10%) were obtained at each flow-rates. Calculation of $Calv_{NO}$ (ppb) and $J'aw_{NO}$ (nl/min) were based on the mathematical model of pulmonary NO exchange dynamics proposed by Tsoukias & George[9]:

$$V'_{NO} = V_E \times Calv_{NO} + J'aw_{NO}$$

Where NO elimination (V'_{NO}) (nl/s) is the exhaled NO concentration (ppb) × flow rate (V'_E) (ml/s), $J'aw_{NO}$ (pl/s) is bronchial NO flux, and $Calv_{NO}$ (ppb) is the steady-state NO concentration in alveolar air. Therefore the gradient and intercept of a regression line on a graph of NO elimination (V'_{NO}) against flow-rate (V'_E) represent $Calv_{NO}$ and $J'aw_{NO}$ respectively (Fig. 1).[8,9]

Spirometry was measured, following NO measurement, in respiratory participants using a Master Screen™ Body (Jaeger) in accordance with the ATS guidelines.[18]

Statistical analyses

The natural log of $J'aw_{NO}$, $Calv_{NO}$ and Fe_{NO50} were found to be normally distributed hence were used for statistical analysis. Non-parametric analysis was used for nNO. Least squares regression models were used to evaluate univariate relationships between the NO parameters and independent variables: age, sex, height, weight, FEV_1, ambient NO, use of inhaled corticosteroids and antibiotics. If log transformation was necessary to achieve normal distribution, log transformed data were used in the model. Linear regression analysis and a standard backwards model selection process were used in order to assess relationships between these variables and NO parameters and to identify confounding variables. Analysis of Variance (ANOVA) was used to compare the geometric mean of $J'aw_{NO}$, $Calv_{NO}$ and Fe_{NO50} between respiratory disease groups. Fe_{NO50} and subject demographics were also compared between groups using the ANOVA. Nasal NO was compared between groups using the Kruskal–Wallis test. Confounding variables were adjusted for during the analyses. A statistical significance level of 0.05 was used throughout. The data were evaluated using statistical analysis software SPSS version 19.0.0 (IBM, USA).

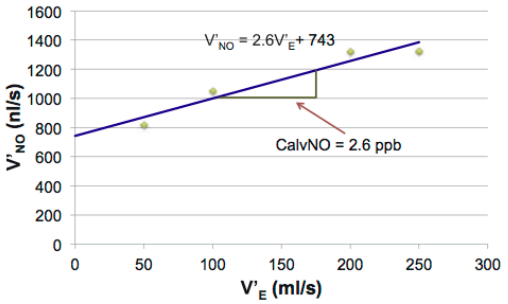

Figure 1 Example of the two-compartment mathematical technique used to estimate bronchial and alveolar contributions to Fe_{NO}. Fe_{NO} was measured at multiple expiratory flow-rates (50, 100, 200 and 250 ml/s). The gradient and intercept of a regression line between NO elimination (V'_{NO}) and flow-rate (V'_E) are recorded as $Calv_{NO}$ (ppb) and $J'aw_{NO}$ (pl/s), respectively.

Results

There were no differences seen in the demographics of children in the different respiratory groups apart from lower weight in those with CF compared to healthy controls (Table 1).

All NO measurements were completed by the participants apart from one child with PCD who was unable to perform the multiple-flow Fe_{NO} protocol and three with asthma who were unable to perform nNO measurements due to nasal obstruction.

Primary ciliary dyskinesia

As expected nNO was significantly lower in children with PCD compared to healthy control children (median (interquartile range) 27 (16–76) ppb *vs.* 772 (690–886) ppb, $p < 0.001$) (Table 2 & Fig. 2a). Both bronchial and alveolar NO were significantly lower in children with PCD compared to healthy controls (mean (SD) 264 (209) pl/s *vs.* 720 (514) pl/s, $p = 0.024$, and 1.7 (0.8) ppb *vs.* 3.5 (1.3) ppb, $p = 0.001$, respectively) (Table 2 & Fig. 2c and d). Fe_{NO50} was also lower in children with PCD, but not with significance (mean (SD) 8.8 (7.3) ppb *vs.* 16.7 (10.8) ppb, $p = 0.062$) (Table 2 & Fig. 2b).

Cystic fibrosis

There was no significant difference in nNO, Fe_{NO50} or bronchial NO between children with CF and healthy controls (Table 2, Fig. 2a–c). However, alveolar NO was significantly lower in children with CF (2.5 (1.2) ppb *vs.* 3.5 (1.3) ppb, $p = 0.001$) (Table 2 & Fig. 2d).

Asthma

There was no significant difference in nNO, Fe_{NO50} or alveolar NO between asthma and healthy controls (Table 2, Fig. 2a, b and d). However, bronchial NO was significantly higher in asthmatics (2100 (1935) pl/s v 720 (514) pl/s, $p = 0.045$) (Table 2 & Fig. 2c).

Relationships between NO parameters and independent variables

There was no significant relationship between parameters NO and FEV_1, FVC or FEF_{25-75}. Ambient NO was 6.0 (7.9) ppb (mean (SD)) and no relationship was seen between this and any other NO parameters.

Table 1 Demographics in subjects with primary ciliary dyskinesia, cystic fibrosis and asthma compared with healthy controls (* indicates $p < 0.05$) (data presented as mean (SD) unless stated).

Characteristic	Group			
	Healthy controls	PCD	CF	Asthma
Number of participants	18	14	12	18
Males (n) (%)	10 (56)	6 (46)	6 (50)	6 (33)
Age (years)	14.1 (2.3)	12.8 (3.9)	11.7 (3.1)	13.5(3.5)
Height (cm)	161 (14)	155 (23)	145 (17)	154 (17)
Weight (kg)	63 (24)	48 (21)	39 (12)*	52 (19)
FEV1 (z-score)	–	−0.89 (1.00)	−1.15 (1.2)	0.35 (1.39)
FVC (z-score)	–	−0.76 (1.64)	−0.60 (0.94)	0.28 (1.10)
FEF_{25-75} (z-score)	–	−1.39 (1.13)	−1.98 (1.05)	−1.39 (1.13)
Inhaled corticosteroids (*n*) (%)	0	3 (21)	4 (33)	17 (94)
Antibiotics (*n*) (%)	0	9 (69)	11 (92)	0 (0)
P. aeruginosa (*n*) (%)	–	0 (0)	1 (8)	–
Other microorganisms (*n*) (%)	–	5 (38)	3(25)	–

Data unavailable.

Discussion

The two-compartment model has been validated in an over 20 studies across different respiratory disease groups and carries the advantage over standard Fe_{NO} measurements in allowing assessment of NO biosynthesis specifically from the bronchial and alveolar compartments of the lung.[8,14,15,19–22] To our knowledge, this is the first study directly comparing these parameters in PCD, CF, asthma and healthy subjects within the same study thereby negating issues of methodological variation between studies when comparing these different respiratory disease groups.

In our population of children we have demonstrated that both alveolar and bronchial NO levels are reduced in PCD while only alveolar levels are reduced in CF and in asthma bronchial NO levels are raised.

Upper airway nitric oxide levels

As previously reported, we found nasal NO levels to be extremely low in PCD and reduced in CF compared to healthy and asthmatic children.[11,23]

Lower airway nitric oxide levels in primary ciliary dyskinesia

Our data do not support the study hypothesis that low levels of airway NO in PCD are due to NOS activity requiring normal ciliary function.[12,13] Also opposing the hypothesis, different PCD phenotypes including static and hyperfrequent, had similarly low levels of NO. Instead our findings point to a more generalized mechanism that is not localized to a specific lung compartment. There are a number of proposed mechanisms for this including: 1) reduced biosynthesis of NO due to reduced NOS activity[11]; or 2) increased NO breakdown to its metabolites.[11]

Three studies have utilized this two-compartment model to assess bronchial and alveolar NO concentrations in PCD.[13–15] Consistent with our findings, all three studies demonstrated low bronchial NO in PCD patients compared to controls however only Mahut et al. also found low alveolar NO.[13–15] Of the other two studies that found equivalent alveolar NO levels between PCD and controls, the work by Shoemark et al. was undertaken in adults.[13] Interestingly when we compared our adult healthy control group (assessed to optimize the methods) to our PCD group

Table 2 Nitric oxide parameters in subjects with primary ciliary dyskinesia, cystic fibrosis and asthma compared with healthy controls (* & ** indicate $p < 0.05$ & $p < 0.001$ respectively) (data presented as mean (SD) unless stated).

Nitric oxide parameter	Group			
	Healthy controls	PCD	Cystic fibrosis	Asthma
nNO (ppb)[a]	772 (690–886)	27 (16–76)**	501 (450–608)	769 (560–1124)[c]
Fe_{NO50} (ppb)	16.7 (10.8)	8.8 (7.3)[b,d]	14.4 (11.2)[b]	43.4 (41.2)[b]
$J'aw_{NO}$ (pl/s)	720 (514)	264 (209)*[b,d]	519(495)[b]	2100 (1935)*[b]
$Calv_{NO}$ (ppb)	3.5 (1.3)	1.7 (0.8)**[d]	2.5 (1.2)*	5.4 (3.5)

[a] Median (IQR).
[b] Adjusted for weight.
[c] $n = 15$ as 3 patients unable to perform nNO test.
[d] $n = 13$ as 1 patient unable to perform Fe_{NO} tests.

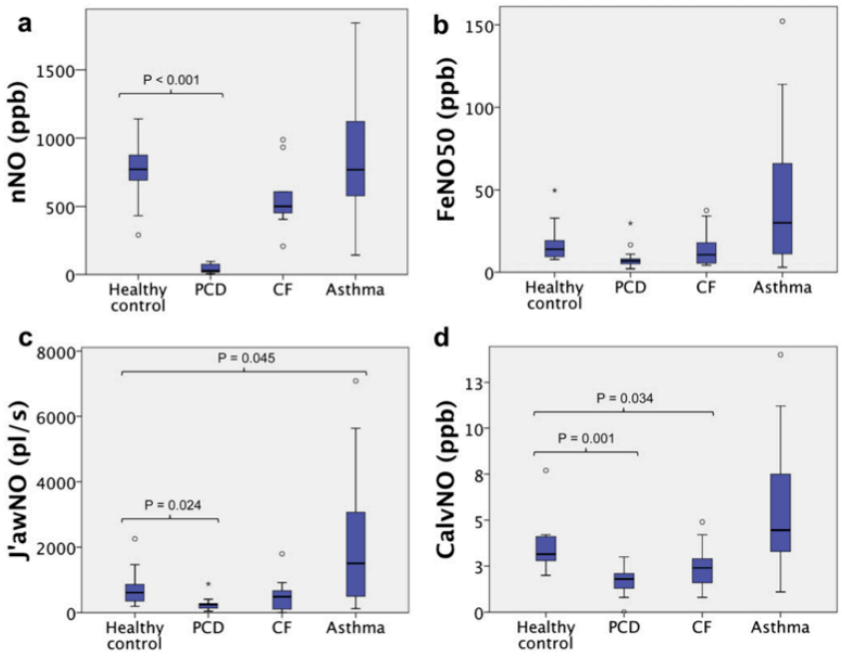

Figure 2 (a–d) - Box and whisker plot comparisons of (a) nasal nitric oxide concentration (nNO); (b) fractional exhaled nitric oxide concentration at 50 ml/s ($Fe_{NO_{50}}$); (c) bronchial nitric oxide flux ($J'aw_{NO}$) and (d) alveolar nitric oxide concentration ($Calv_{NO}$) in 18 healthy controls, 14 primary ciliary dyskinesia (PCD), 12 cystic fibrosis (CF) and 15 asthma patients. The box represents the interquartile range intersected by the median. The whiskers represent the range of data. Nasal NO is significantly lower in PCD compared to healthy subjects. $J'aw_{NO}$ is significantly lower in PCD patients and higher in asthma patients compared to healthy controls. $Calv_{NO}$ is significantly lower in PCD and CF patients compared to healthy controls. (ppb, parts per billion; pl/s, picolitres per second).

(Supplementary Table 1) we similarly found comparable alveolar NO levels. Paraskakis et al. also found comparable levels of alveolar NO between PCD and controls.[14] However 63% of their PCD group were taking inhaled corticosteroids (ICS) as compared to only 23% of our PCD population and we speculate that a higher rate of asthma co-morbidity might account for the differences in alveolar NO between studies. There may be other differences between the study populations such as concurrent respiratory infection.

Lower airway nitric oxide levels in asthma

There is a large literature on the potential role of Fe_{NO} in the pathophysiology and management of asthma.[7,20,22,24–26] It has generally been found that high Fe_{NO} levels are seen in asthma, particularly in under-treated asthmatics, and that this is reduced with ICS.[26] This has been demonstrated to originate from the bronchial region whilst alveolar NO remains relatively normal and this is consistent with our data.[27,28] A recent Cochrane review concluded that Fe_{NO}-guided steroid therapy in asthma should not be recommended.[25] However it has been suggested that bronchial NO might be more sensitive than Fe_{NO} to monitor response to ICS therapy and that these parameters may also be of benefit for disease phenotyping.[19,24]

Lower airway nitric oxide levels in cystic fibrosis

Consistent with two previous studies we found that bronchial NO in CF was similar to healthy children.[21,29] We found that alveolar NO was significantly lower in our CF group, consistent with the original work in the area by Shin et al. however contrary to data from Suri et al. who found it to be elevated.[21,29] We note that there were a number of differences in the clinical characteristics of their CF group to ours, with 50% taking ICS and 23% growing *Pseudomonas aeruginosa* (33% and 8% respectively in our CF group).[21] Nevertheless, as with the PCD group, the differences between studies may reflect other more complex mechanisms not captured in the available clinical data.

Our findings in the CF group, in contrast to the asthma group and consistent with general opinion, suggest that CF is mainly a disease of the peripheral airway. Given the antimicrobial properties of NO, the low alveolar NO levels might contribute to the chronic recurrent bacterial infections seen in CF.[30] However, alternatively, chronic peripheral lung infection with denitrifying bacteria might lead to increase NO breakdown and hence the low alveolar NO levels and further work in this area is required.

Despite its advantages, the two-compartment model is an idealised representation of pulmonary anatomy and

function and has intrinsic limitations. Notably, it fails to account for axial molecular diffusion of bronchial NO back into the alveolar region, which may lead to a spurious rise in alveolar NO and decreased bronchial NO estimates.[31–33] Nevertheless, our understanding of pulmonary NO exchange is in evolution and the importance of heterogeneous ventilation and inflammation have yet to be confirmed.[34,35] Larger numbers of subjects would have been of benefit but due to the rarity of PCD, full validation will require multi-centre collaboration The numbers we have investigated are in keeping with previously reported studies and we included all eligible children with PCD in our clinic.

Fe_{NO} was measured according to ATS/ERS guidelines to ensure accuracy and repeatability of readings.[17] Fe_{NO} is non-invasive, acceptable to patients and highly reproducible.[17] Contamination of Fe_{NO} with nNO was avoided by exhalation against a minimum mouthpiece pressure of 5 cm H_20.[17,36] This process causes velum closure has been validated by nasal CO_2 measurement and nasal argon insufflation studies.[36,37] However given that nNO levels are almost 40 times greater than Fe_{NO50} in our healthy children, even small amounts of contamination from the nasal cavity would lead to significant changes in Fe_{NO}. The NIOX® Flex analyser enables inhalation of NO-free air to control for the effect of ambient NO concentration. Furthermore a study in 1005 children found that ambient NO did not affect Fe_{NO} when measured according to ATS/ERS guidelines.[38] Similarly we found no relationship between ambient NO and NO parameters. Measures were taken to control for non-disease-related factors that may influence Fe_{NO} with groups being age and sexed matched.[17]

Due to the significant variance of bronchial and alveolar NO levels within disease groups these parameters will have a limited role in aiding the diagnosis of these respiratory conditions. To date there is little published data on their use in monitoring disease progression and the benefit of therapeutic interventions, however the limited work available in asthma has promise.[19,24] In CF the standard practice of monitoring spirometry in what is a disease of the peripheral airways is widely considered to be inadequate. While there is increasing literature on the benefit of lung clearance index in monitoring CF disease progression its availability in the clinical setting in presently limited.[39–42] The potential benefit of using alveolar NO, a non-invasive risk-free investigation, in this role might warrant further investigation.

Conclusion

Our findings do not support the hypothesis that NOS and ciliary function are coupled, instead suggesting a more generalised mechanism for the low levels of NO seen in PCD. In our population alveolar NO levels are abnormal in CF and bronchial NO levels in asthma corroborating evidence that these are diseases of the lung peripheries and bronchi respectively. While the use of these NO parameters are unlikely to aid the diagnosis of respiratory disease longitudinal studies may find benefit in their use in monitoring therapeutic interventions and disease progression.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.rmed.2012.11.021.

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REVIEW

Nitric oxide in primary ciliary dyskinesia

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ABSTRACT: Nitric oxide is continually synthesised in the respiratory epithelium and is upregulated in response to infection or inflammation. Primary ciliary dyskinesia (PCD) is characterised by recurrent sinopulmonary infections due to impaired mucociliary clearance. Despite chronic infections, nasal nitric oxide in such patients is markedly reduced and is used as a screening test for this condition. These low levels were first described >15 yrs ago but the underlying mechanisms have yet to be fully elucidated. We review epithelial nitric oxide synthesis, release and measurement in the upper airways with particular reference to PCD. The key hypotheses that have been proposed to explain the low nitric oxide levels in this condition are explored and the potential benefits of augmenting airway nitric oxide levels are considered. Further work in these patients clarifying both whether the respiratory epithelium is able to biosynthesise normal levels of nitric oxide and the role played by abnormalities in the anatomy of the paranasal sinuses is essential. While nitric oxide augmentation is unlikely to be beneficial in common PCD phenotypes, it has potential in the treatment of secondary dyskinesias and may also improve treatment of bacterial infections, particularly where biofilms are implicated.

KEYWORDS: Nasal nitric oxide, nitric oxide augmentation, nitric oxide synthase

Primary ciliary dyskinesia (PCD) is a rare autosomal recessive disorder, with considerable heterogenicity, characterised by a spectrum of corresponding defects in ciliary ultrastructure and/or ciliary function [1]. The impaired ciliary function impedes mucociliary clearance, which predisposes the patient to recurrent sinopulmonary infection [2]. Nitric oxide is a highly reactive gaseous molecule with numerous signalling roles within the airways. It is produced throughout the airways but is particularly abundant in the nasal sinuses [3]. Nitric oxide biosynthesis is typically upregulated during infection, *via* increased inducible nitric oxide synthase (iNOS) transcription and activity [4]. Despite recurrent respiratory infection, nasal concentrations of nitric oxide are markedly reduced in the vast majority of PCD patients, compared with those without the disorder. Indeed, nasal NO is now widely used as a screening test for PCD [5, 6]. Exhaled nitric oxide fraction (F_{ENO}) from the lower airway is also low in PCD but is less specific at differentiating between PCD and healthy controls [3, 7, 8]. While the association between low nasal nitric oxide and PCD has been recognised for >15 yrs, the underlying mechanisms causing this phenomenon remain unclear.

Here, we review studies of nitric oxide in the airway, focusing on epithelial-derived nitric oxide and the low levels found in PCD patients. We explore key hypotheses proposed to explain the lower nitric oxide and consider whether augmentation of nitric oxide would benefit these patients.

THE BIOSYNTHESIS AND ROLE OF NITRIC OXIDE IN THE AIRWAY

Nitric oxide is an intra- and intercellular signalling molecule involved in diverse physiological and pathophysiological processes [9], such as vascular homeostasis, immune cell activity and tumour progression [10]. Nitric oxide has a short half-life and diffuses rapidly from its point of synthesis, interacting intracellularly as well as crossing the plasma membrane and leaving the cell, where it can act extracellularly [11, 12]. Nitric oxide is synthesised *via* the oxidation of the amino acid L-arginine to L-citrulline, catalysed by three stereospecific isoenzymes in the presence of nicotinamide adenine dinucleotide phosphate, oxygen and other cofactors (fig. 1). The isoenzymes neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) are expressed constitutively and require calmodulin binding and calcium activity to produce femtomole or picomole concentrations of nitric oxide [13]. In contrast, iNOS is permanently

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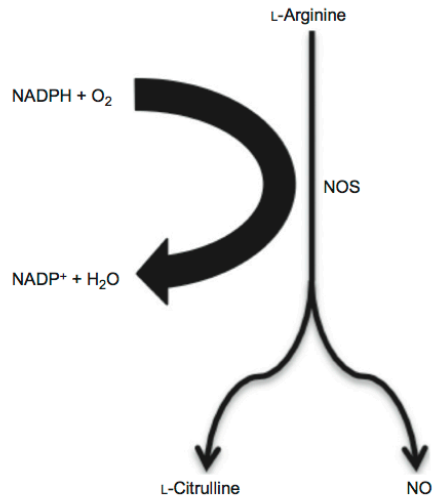

FIGURE 1. Schematic diagram to demonstrate nitric oxide biosynthesis by the conversion of L-arginine (nitric oxide synthase (NOS) substrate) to L-citrulline *via* NOS isoenzymes. During this process, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), is oxidised in the presence of oxygen to form nicotinamide adenine dinucleotide phosphate ($NADP^+$) and water. Other cofactors are also required for nitric oxide biosynthesis (not shown).

bound to calmodulin and is therefore independent of calcium activity. It may be transcriptionally induced by a number of cytokines, including interleukin-1β, tumour necrosis factor-α and interferon-γ [14], to produce nanomole concentrations of nitric oxide [4, 13]. The expression of iNOS may also be constitutive in airway epithelium [14]. NOS isoenzyme gene expression and protein localisation have been demonstrated in the airway (table 1).

Within the airway, nitric oxide is produced by numerous cell types, including epithelial cells, endothelial cells, fibroblasts, activated macrophages, nerve cells, and airway and vascular smooth muscle cells. It has diverse roles as a modulator of ciliary function, neurotransmission, bronchodilatation, vasodilatation, platelet aggregation and immune function [13]. With regard to ciliary function, *in vitro* studies of animal and human airway ciliated epithelium suggest that the induction of NOS and nitric oxide production increase ciliary beat frequency [20–26]. During oxidative stress conditions, the production of nitric oxide and reactive nitrogen species amplify inflammatory responses and, hence, may modulate chronic airway inflammatory disease [13]. Additionally, the production of nitric oxide within the airway is protective against infection, and has bacteriostatic and bactericidal activity; sodium nitrite can kill *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Burkholderia cepacia* [27]. It can also modulate biofilm formation [28], and cause biofilm dispersal, making the bacteria more susceptible to antimicrobial treatment [29].

PRIMARY CILIARY DYSKINESIA

PCD is a heterogeneous disorder, usually inherited as an autosomal recessive trait [6], causing a range of ciliary ultrastructural and functional defects, and resultant ciliary dyskinesia [30]. The prevalence of PCD is estimated to be 1 in 15,000–30,000 live births [6, 31, 32]. PCD is characterised by chronic upper and lower airway infection, and is associated with situs inversus and male infertility due to homology between respiratory cilia, embryonic nodal cilia and sperm.

The motile ciliary axoneme is formed in a "9+2" arrangement where nine peripheral microtubule doublets surround a central pair of single microtubules [33]. Over 250 other proteins maintain this structure, including radial spokes and nexin links. The inner and outer arms of the axoneme generate the force required for ciliary beating and are formed by dynein complexes, acting as mechanochemical ATPases [34]. Radial spokes structurally coordinate the ciliary beat pattern (CBP); hence, mutations affecting dynein arms or radial spokes render ciliary movement ineffective and dyskinetic [35]. Development of a genetic diagnosis for PCD has remained problematic due to the heterogenicity of the disease and, in some cases, candidate genes having large exomes. To date, there are 11 published PCD-causing gene mutations, which account for approximately one-third of PCD cases [36, 37], and genetic diagnostic techniques are being developed [6, 36].

DIAGNOSIS

Diagnosis of PCD can be confirmed by analysis of ciliated bronchial or nasal epithelia. There is no "gold standard" test that will diagnose all PCD phenotypes, and hence a diagnostic workup requires the rigorous assessment of CBF and ciliary beat pattern (CBP) by high-resolution, high-speed video microscopy and transmission electron microscopy of ciliary ultrastructure, as recommended by the European Respiratory Society Task Force consensus statement [6]. Assessment of CBP requires a high degree of experience and skill, but considering CBF in isolation risks misdiagnosing PCD. There is also an increasing literature on a population of atypical patients with PCD and normal ciliary ultrastructure, associated with mutations in

TABLE 1 Expression and protein localisation in the human airway of the nitric oxide synthase (NOS) isoenzymes

NOS isoenzyme	Protein	Gene	Chromosome	Localisation	Reference
Neuronal	nNOS	*NOS1*	12	Airway neuronal cells	[15]
Inducible	iNOS	*NOS2*	17	Airway epithelial cells	[16]
				Alveolar epithelial cells	[17]
				Paranasal epithelial cells	[18]
Endothelial	eNOS	*NOS3*	7	Airway epithelial and endothelial cells	[19]

dynein axonemal heavy chain [11], that would be missed in centres where diagnosis depends on electron microscopy without access to high-speed video microscopy [38–40]. For difficult diagnostic cases, the re-differentiation of basal epithelial cells at an air–liquid interface in cell culture allows for reassessment of ciliary function and ultrastructure that may differentiate primary from secondary dyskinesia [6, 41, 42].

SCREENING: THE ROLE OF NASAL NITRIC OXIDE

Only a small percentage of patients presenting with chronic upper and lower respiratory tract infections have PCD. Diagnostic investigation of PCD, outlined above, requires specialist skills, and is time consuming, costly and only available in a small number of specialist centres. A reliable screening test is therefore desirable [41]. For many years, the saccharin test was used, but it is difficult to perform and is unreliable in children [6, 43].

Nitric oxide detection in human exhaled breath was first described in 1991 [44]. Studies suggest that the majority of nitric oxide originates from the paranasal sinuses, with lower concentrations found in exhaled breath [18, 45]. Paranasal sinus biopsies taken from healthy controls demonstrated that iNOS isoenzyme expression was most abundant in *in situ* hybridisation and immunohistochemistry experiments, and notably more so than seen in matched nasal biopsies, suggesting iNOS as the predominant isoenzyme involved in nitric oxide biosynthesis within the paranasal sinuses [18, 46].

PCD patients have low nasal nitric oxide and $F_{E}NO$ compared with healthy controls (table 2) [3, 7, 8]. Nasal nitric oxide is sufficiently low in PCD to be used as a screening test for the condition [3, 7, 47, 48, 52, 54]. One group reported a specificity of 88%, a sensitivity of 100% and a positive predictive value of 89% for correctly diagnosing PCD when using a nasal nitric oxide cut-off level of <105 ppb [48]. While initially undertaken as a research tool, nasal nitric oxide was quickly introduced as part of the clinical diagnostic pathway in many larger European PCD centres. It has been included in the British guidelines since 2007 [5] and European consensus guidelines since 2009 [6]. Nasal nitric oxide measurement is not yet approved for clinical use in the USA where only centres with major research programmes in PCD are routinely using it. The measurement is extremely helpful in guiding the diagnostic pathway, but major drawbacks are the cost of equipment and consumables. It is also important to note that there is no widely agreed cut-off level used for the screening of PCD as levels vary significantly with age, due to the development of the paranasal sinuses over the first decade of life, and, to a lesser extent, with the device used to perform the measurement [18, 55]. Nasal nitric oxide has also been found to be useful in atypical phenotypes where normal ciliary ultrastructure makes diagnosis difficult [38, 54]. While the best validated technique for the measurement of nasal nitric oxide is performed by nasal aspiration during breath holding [56], there is recent literature on levels recorded in PCD patients during both humming and tidal breathing. Tidal measurements allow levels to be measured in children as young as 6 months of age, although there is limited experience in this age younger age group [55, 57].

Contrary to the majority of published data [3, 7, 47, 48, 52, 54], two groups [53, 58] have recently reported normal and raised levels of nasal nitric oxide in patients with PCD. A study of PCD-positive patients, confirmed by live ciliary function analysis and scrutiny of ciliary axonemal ultrastructure [6], reported five patients with nasal nitric oxide within or above the normal range [53]. A further study demonstrated that 24% of patients at an Italian PCD clinic (n=41) had nasal nitric oxide of >250 ppb, although the diagnostic evidence for PCD in these patients is unclear [58]. These studies highlight that patients with a history strongly suggestive of PCD should not be excluded from further diagnostic evaluation on the basis of nasal nitric oxide.

The value of using nasal nitric oxide as a screening tool for PCD is clear, but there are a number of other conditions in which reduced nasal nitric oxide levels occur, although usually

TABLE 2 Main studies assessing exhaled nitric oxide fraction and nasal nitric oxide in patients with primary ciliary dyskinesia (PCD) compared to healthy controls

	PCD	Healthy controls	p-value	Ref.
$F_{ENO_{50}}$ ppb	2.1 (1.3–3.5) (n=14)	6.7 (2.6–11.9) (n=37)	0.001	[47]
	2.9 (1.9–4.6)# (n=17)	6.4 (4.8–8.6)# (n=24)	0.11+	[48]
	8.1 (1.3)# (n=24)	12.5 (1.6)# (n=20)	0.033	[49]
	3.2 (0.2)# (n=15)	8.5 (0.9)# (n=14)	<0.0001	[50]
	7.1 (5.7–8.8)# (n=20)	13.9 (11.7–16.4)# (n=20)	<0.01	[51]
Nasal NO ppb	54.5 (5.0–269) (n=14)	663 (322–1343) (n=37)	<0.001	[47]
	64±36.6 (n=42)	759±145.8 (n=16)	<0.0001	[52]
	13.7 (6.8–27.8)# (n=17)	223.7 (175.5–285.2)# (n=24)	<0.05	[48]
	59.6±12.2 (n=15)	505.5±66.8 (n=14)	<0.001	[50]
	49 (34–64)# (n=20)	639 (422–890)# (n=20)	<0.01	[53]
	142±42¶ (n=45)	908±33¶ (n=49)	<0.0001	[53]
	55 (3.3–959) (n=21)	553 (116±1437) (n=20)	<0.001	[7]
	4 (1)# (n=4)	221 (14)# (n=20)	<0.001	[3]

Data are presented as median (range) or mean±SD, unless otherwise stated. $F_{ENO_{50}}$: exhaled nitric oxide measured at a set exhalation flow rate of 50 mL·s^{-1}. #: mean (95% CI); ¶: mean±SE; +: nonsignificant.

not as low as in PCD, including cystic fibrosis [59], diffuse panbronchiolitis [60], nasal polyps [61] and chronic sinusitis [62]. It is also reduced in smokers [63]. The lack of specificity requires robust diagnostic evaluation to confirm PCD in patients with low nasal nitric oxide.

MEASUREMENT OF ALVEOLAR AND BRONCHIAL NITRIC OXIDE IN HEALTH AND IN PCD

Exhaled lower airway nitric oxide as measured by F_{ENO} is typically sampled at a set exhalation flow rate of 50 $mL \cdot s^{-1}$, denoted as $F_{ENO_{50}}$ [56]. Several two-compartment mathematical models have been described, allowing estimates of alveolar nitric oxide ($C_{alv,NO}$) and conducting airway nitric oxide ($J'_{aw,NO}$) [64, 65]. The two-compartment model has been used to investigate whether low nitric oxide is confined to the upper airway in PCD, or whether it is low throughout upper and lower airways. Three published studies that have used the multiple-flow technique to estimate $J'_{aw,NO}$ and $C_{alv,NO}$ in PCD patients have conflicting findings [49, 51, 66]. All found $J'_{aw,NO}$ was reduced in PCD compared with controls but only one [66] found low $C_{alv,NO}$. While further work is needed to consider this discrepancy, the model might suggest that the lower $F_{ENO_{50}}$ seen in PCD compared to controls is principally due to a decreased bronchial biosynthesis of nitric oxide.

WHY ARE NITRIC OXIDE LEVELS REDUCED IN PCD?

Several hypotheses have been put forward at both a cellular and anatomical level for the low airway nitric oxide in PCD; some extrapolated from the finding of reduced nitric oxide levels seen in cystic fibrosis [67–70]. At the epithelial level, it has been suggested that there is reduced biosynthesis of NO [8, 50, 69] or increased breakdown, either within the cell, in a viscous mucus layer [67, 68] or by denitrifying bacteria [50]. At the anatomical level, it has been suggested that nitric oxide is sequestered in the upper respiratory tract within blocked paranasal sinuses or, alternatively, nasal nitric oxide biosynthesis or nitric oxide storage capacity is limited due to agenesis of the sinuses (fig. 2) [58, 71].

We review below four broad hypotheses that have been proposed to explain the low nitric oxide levels found in patients with PCD.

HYPOTHESIS 1: INCREASED BREAKDOWN OF NITRIC OXIDE TO METABOLITES

As nitric oxide is highly reactive, it is rapidly broken down from the breath exhalate by its reaction with, among others, oxygen, superoxides and cysteine thiols, producing reactive nitrogen species, including the potent oxidants peroxynitrite and nitrogen dioxide, and *S*-nitrosothiols [72–75].

The low nitric oxide levels seen in PCD may be associated with increased consumption of nitric oxide by superoxide anions to form reactive nitrogen species, which has been suggested as the cause of low nitric oxide in adult respiratory distress syndrome [76]. A study of 23 PCD patients and 11 healthy volunteers seemed to support this, demonstrating increased concentrations of the oxidative stress marker 8-isoprostane (8-IP) in the PCD group. However, there were a number of PCD patients with apparently normal 8-IP levels, which is inconsistent with oxidative stress associated rapid breakdown of nitric oxide in these patients [77]. CSOMA *et al.* [50] demonstrated no difference in the mean concentrations of three nitric oxide metabolites (nitrite, nitrite/nitrate or *S*-nitrosothiol) in the exhaled breath condensates of 15 PCD patients with markedly decreased nasal nitric oxide levels (table 2), compared with 14 healthy controls, suggesting that nitric oxide biosynthesis does occur in the PCD airway. Given the variability in levels of oxidative stress seen in PCD patients, it seems unlikely that this alone is responsible for the consistently low nitric oxide detection in PCD; however, further experimental work is required.

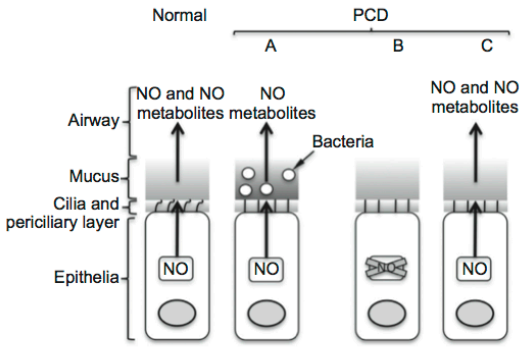

FIGURE 2. Schematic diagram to represent normal nitric oxide metabolism and release from the epithelium of healthy subjects, and hypotheses for the potential causes of low nasal nitric oxide concentrations in primary ciliary dyskinesia (PCD), based on events at the epithelium. A: increased breakdown of highly reactive nitric oxide to nitric oxide metabolites (nitrite and nitrates), either within the epithelial cell, within extra viscous sputum or by denitrifying bacteria within the mucus; hence, nitric oxide metabolites are predominantly released into the airway (hypothesis 1). B: absence of, or reduced, nitric oxide biosynthesis within the epithelial cell (hypothesis 2). C: normal nitric oxide biosynthesis within the epithelial cell; however, obstruction to the osteomeatal complex inhibits nitric oxide release from the paranasal sinuses into the nasal passage (hypothesis 3). Alternatively, hypoplasia or agenesis of the paranasal sinuses reduces nitric oxide production and storage capacity; hence, low measurable nasal nitric oxide levels are seen (hypothesis 4).

Nitric oxide is trapped and broken down in viscous sputum

Impaired mucociliary clearance in PCD causes mucus accumulation in the airways, potentially trapping nitric oxide at the periciliary level or in the mucus itself, where it is then broken down into nitric oxide metabolites. In cystic fibrosis patients, it has been suggested that the viscous mucus may trap the highly reactive nitric oxide, preventing it from being exhaled freely [67, 68]. Viscous mucus is a particular issue for cystic fibrosis patients but similar biophysical properties have been identified in sputum of patients with PCD [78]. The significance of this potential mechanism requires further evaluation.

Consumption of nitric oxide by denitrifying bacteria

Increased breakdown of airway nitric oxide by bacterial nitric oxide reductase may contribute to low F_{ENO} [79]. There is evidence in cystic fibrosis that chronic colonisation of the airway with denitrifying organisms lowers F_{ENO} and that antibiotic treatment for exacerbations increases nitric oxide concentrations [70]. PCD patients similarly suffer from recurrent infection and, to some degree, colonisation, but to our knowledge, the effect of antibiotic treatment on F_{ENO} has not been assessed. However,

denitrifying bacteria seem unlikely to be the cause of the low nitric oxide concentrations seen as cystic fibrosis patients are more chronically colonised with bacteria than patients with PCD but have significantly higher nasal nitric oxide concentrations.

HYPOTHESIS 2: REDUCED BIOSYNTHESIS OF NITRIC OXIDE

Nitric oxide synthesis occurs in patients with PCD [50]; however, a number of reasons why synthesis may be reduced in PCD epithelia have been postulated. These include an absence or decreased expression of NOS isoenzymes, a reduction in NOS isoenzyme output directly related to loss of ciliary function [8] or limitations in the availability of the NOS substrate L-arginine, either due to its reduced availability or increased metabolism [69, 80–82].

Decreased expression of NOS isoenzymes

It has been suggested that iNOS is the main contributor to $FeNO$, when looking at healthy, asthmatic and atopic children [83]. It has also been reported that patients with cystic fibrosis have reduced or absent iNOS expression in the airway epithelia [79, 84, 85]. A recent study comparing *NOS2* and *NOS3* mRNA expression (the genes for iNOS and eNOS, respectively) in the nasal mucosa of patients with PCD and secondary ciliary dyskinesia (SCD) found that there was no difference in *NOS3* expression but lower levels of *NOS2* expression in the PCD group. There was, however, significant overlap between the two groups, making these results difficult to interpret. [82]. Since PCD is a polygenic disorder, its genetic linkage to NOS gene polymorphisms is highly unlikely. Furthermore, ''next-generation sequencing'' of exomes, targeted to identify PCD causing genetic mutations, to date, has not identified the *NOS* genes (*NOS1, NOS2* and *NOS3*) as candidate genes for PCD [36].

Loss of NOS activity via mechanochemical uncoupling in PCD

NARANG *et al.* [8] proposed that normal NOS activity requires ciliary function *via* a mechanochemical coupling to dynein ATPases, which would be uncoupled in patients with PCD. This hypothesis was based on observations from patients with Duchenne muscular dystrophy (DMD) where mutations in the dystrophin gene result in an uncoupling of nNOS from the contractile apparatus, leading to the loss of contractile function seen in DMD, causing low serum nitric oxide [86–88]. While further work is required to elucidate the mechanism regulating NOS activity, an uncoupling of NOS from dynein ATPase seems unlikely as PCD phenotypes with hyper-frequent or motile dyskinetic cilia have low nitric oxide similar to patients with static cilia.

Limitations to availability of the NOS substrate L-arginine

Arginase competes with NOS isoenzymes for their common substrate L-arginine. It has been suggested in cystic fibrosis that high levels of arginase may be present and compete with NOS isoenzymes for L-arginine, hence leading to reduced nitric oxide biosynthesis [69]. In cystic fibrosis, high levels of arginase activity are seen in sputum even after 14 days of intravenous antibiotics, which significantly lowers arginase activity when compared with induced sputum from healthy control subjects ($p=0.0001$) [69]. While this has not been directly assessed in PCD patients, there have been pilot studies that demonstrate increased nasal and exhaled nitric oxide levels in PCD patients following administration of both intravenous and nebulised L-arginine, as discussed further below [82, 89]. Further work is needed to clarify the availability of this NOS substrate in PCD epithelial cells.

HYPOTHESIS 3: NITRIC OXIDE IS TRAPPED IN THE OBSTRUCTED PARANASAL SINUSES

In health, the predominant source of exhaled nitric oxide is the upper airway [45]. Evidence from studies using manoeuvres such as humming suggest nitric oxide is sequestered within the nasal cavities [71, 90, 91]. The oscillation generated by the humming is thought to increase gas exchange across the osteomeatal complex [90, 92]. During tidal breathing, it can take up to 30 min to completely clear sinus gases into the nasal cavity, but with humming, this occurs in one exhalation [93, 94]. In healthy volunteers, humming leads to a 15-fold peak in nasal nitric oxide, compared with quiet exhalation [90]. This humming peak is absent in patients with chronic sinusitis and those with cystic fibrosis [91, 95]. There is evidence, in both these conditions, to suggest this is secondary to decreased expression of iNOS, leading to decreased nitric oxide biosynthesis [79, 84, 85, 96]. Patients with PCD suffer from chronic sinusitis and, in a small study, the absence of a nasal nitric oxide humming peak has been demonstrated in PCD compared with healthy controls [71]. In this study, 13 of the 14 PCD patients underwent a computed tomography scan of the paranasal sinuses and no differences were seen in nasal nitric oxide humming peaks between those who had complete opacification of the paranasal sinuses and those with only partial opacification with patent osteomeatal complexes; however, the numbers were small [71].

A recent observational study reported five patients with PCD with normal nasal nitric oxide levels [53]. Notably, none of these five patients suffered from chronic sinusitis [53], supporting the hypothesis that a patent osteomeatal complex permits normal nasal nitric oxide levels in PCD. However, in the authors' patient population, some had PCD without sinusitis but very low nasal nitric oxide and there are very few reported cases of PCD patients with normal nasal nitric oxide levels, raising the possibility that these five patients may have an unusual phenotype or have been incorrectly diagnosed with PCD.

So, while it has been demonstrated that humming does not increase nasal nitric oxide in PCD patients, it remains unclear whether this is a consequence of the underlying inability of the airway epithelia to produce nitric oxide or that the manoeuvres did not overcome the obstruction to the osteomeatal complex.

HYPOTHESIS 4: REDUCED PRODUCTION AND STORAGE CAPACITY OF NITRIC OXIDE IN THE PARANASAL SINUSES

Aplastic or hypoplastic nasal sinuses can occur in PCD and would lead to both absent or reduced epithelial production and storage capacity of NO in the paranasal sinus, respectively. This might explain, particularly given the importance of the paranasal sinuses in nitric oxide production [45], why nasal nitric oxide concentrations are low and do not vary significantly with manoeuvres that improve sinus ventilation [58, 71, 97]. In a recent study, computed tomography scans of the paranasal sinuses at the time of diagnostic investigation for PCD demonstrated that frontal and/or sphenoidal sinuses were either aplastic or hypoplastic in 30 (73%) out of 41 PCD

TABLE 3 Clinical implications and future directions

The majority of studies indicate that more than 95% of PCD patients have very low nasal NO, confirming its suitability as a screening test
The small number of PCD patients with reportedly normal nasal NO require further evaluation, first to confirm the diagnosis of PCD and then to explain the atypical NO level in this group
Outstanding questions regarding NO in PCD
Is NO low throughout the airway, or only upper respiratory tract?
What mechanisms underlie low NO levels?
Is there a mechanistic link between ciliary beating and NO biosynthesis?
What are the implications of low NO in PCD? (*e.g.* are there effects on innate immunity?)
Does augmenting airway NO benefit patients?

PCD: primary ciliary dyskinesia.

patients, compared to only 38% of those with SCD [58]. There was a significant inverse correlation between the score for aplasia/hypoplasia of each paranasal sinus and nasal nitric oxide values in PCD ($r= -0.432$, $p=0.008$) but not in SCD ($r= -0.271$, $p=0.07$) [58], supporting the hypothesis that smaller sinuses are associated with lower nasal nitric oxide levels. However, unusually, 24% of this PCD study population had normal nasal nitric oxide; if these patients were excluded, no correlation between aplasia/hypoplasia score and nasal nitric oxide level would have been seen. There is again the possibility that these "outliers" with normal nitric oxide may have an unusual PCD phenotype or have been incorrectly diagnosed.

Further evidence in support of this hypothesis is that the paranasal sinuses develop over the first 10 yrs of life and nasal nitric oxide levels are seen to increase with age over this time period [18]. It is also noteworthy that most conditions associated with lower nasal nitric oxide levels involve disease of the paranasal sinuses, cystic fibrosis [59, 98], nasal polyps [61], chronic sinusitis [62] and PCD, which may lead to either obstruction of the osteomeatal complex or reduced storage capacity of the paranasal sinuses, or a combination of both.

THE VALUE OF AUGMENTATION OF NITRIC OXIDE LEVELS IN PCD

As the evidence that nitric oxide plays a functional role in the lung increases, research has focused on augmenting levels to observe effects. Most of the work in this field has been undertaken using the NOS substrate L-arginine, which increases $F_{E}NO$ levels in healthy subjects [81, 82, 99, 100].

Nasal nitric oxide and $F_{E}NO$ levels have been compared before and after infusion of intravenous L-arginine in PCD ($n=7$) and healthy controls ($n=11$). A 1.4-fold increase in nasal nitric oxide occurred immediately after infusion and a 1.8-fold increase in $F_{E}NO$ 3 h after infusion in PCD patients. However concentrations remained reduced compared with the baseline values of the control group [82]. Another group compared the use of nebulised L-arginine in 10 PCD patients with 10 healthy controls in a double-blinded, placebo-controlled trial. They found that L-arginine not only increased nasal nitric oxide levels but also increased CBF and decreased mucociliary clearance time [89]. However, they did not define the functional or ultrastructural defects in their PCD group and, atypically, seven of the 10 patients reported to have PCD had normal CBF, calling into question their diagnosis. The two PCD patients with the common phenotype of static or extremely slow cilia showed no change in CBF following treatment with L-arginine [89].

Another reason for augmenting nitric oxide is its antibacterial properties [27, 101, 102]. Increasing nasal nitric oxide levels potentially reduces nasopharyngeal carriage of pathogens, thereby reducing the risk of lower respiratory tract infection. There is case in the literature of a healthy volunteer showing that nasal application of NOS inhibitors both reduces nasal NO and leads to sinus infection [96, 103].

Nitric oxide has a role in the dispersal of bacterial biofilms. *P. aeruginosa* is the best characterised bacteria to undergo the genetic adaptations from motile planktonic bacteria to a noncytotoxic, nonmotile, mucoid phenotype caused by overproduction of a surface polysaccharide known as alginate. Biofilm formation also occurs with *Haemophilus influenzae* and *S. aureus*, common pathogens in the PCD population [104–106]. Bacteria embedded within biofilms can be 1,000-fold more resistant to antibiotic treatments [107]. They undergo coordinated dispersal events, in which the bacteria convert back to motile planktonic bacteria. Exposing *in vitro P. aeruginosa* biofilms to sodium nitroprusside, an nitric oxide donor, has been shown to induce biofilm dispersal, making it more susceptible to antimicrobial treatment [29]. Furthermore, recent studies have demonstrated that the slow release of nitric oxide from charged catheters inhibit *Escherichia coli* biofilm formation and also that sodium nitrite can kill *P. aeruginosa*, *S. aureus* and *B. cepacia* [27, 28].

While the evidence that augmenting nitric oxide can improve ciliary function in PCD is not compelling, the potential to promote host defence, by direct bactericidal properties and biofilm dispersal, merits further investigation. However, there is a potential risk that increasing nitric oxide levels may lead to increased nitrosative stress in what is already a pro-inflammatory environment.

SUMMARY

While the association of low nitric oxide concentrations with PCD has been recognised for >15 yrs, the underlying mechanisms remain unclear. We have reviewed the data that demonstrate low levels of nasal nitric oxide in PCD to a degree that renders this a useful screening test. The limited studies to date suggest that biosynthesis of the molecule occurs, and there is little to suggest that increased breakdown or metabolism is responsible for the low levels. There is some direct and circumstantial data to suggest the reduced size of the paranasal sinuses caused by aplasia or opacification and the lack of patency of the osteomeatal complex might contribute. However, this is supported mainly by data from patients diagnosed with PCD who had normal levels of nasal nitric oxide and, given that the vast majority of PCD patients have extremely low nitric oxide levels, it does raise questions over the reliability of their diagnosis or the possibility of them having an usual phenotype of PCD. In order to clarify the underlying cause of the low levels of nitric oxide seen, it is essential to establish both whether the airway epithelium in PCD is able to biosynthesis normal levels of nitric oxide and the role played by abnormalities in the anatomy of the paranasal sinuses.

There has been speculation that nitric oxide augmentation may be beneficial in PCD and other diseases associated with low nitric oxide. It is unlikely that ciliary function will be normalised in common PCD phenotypes, although it has potential for treatment of patients with secondary dyskinesias. More plausible is the suggestion that nitric oxide augmentation will improve treatment of bacterial infections, particularly where biofilms are implicated. A number of small observational and *in vitro* studies have been conducted, but larger clinical trials are required before this can be considered a therapeutic option. The clinical implications and future directions of nitric oxide in PCD are presented in table 3.

STATEMENT OF INTEREST

None declared.

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Ciliated Cultures From Patients With Primary Ciliary Dyskinesia Produce Nitric Oxide in Response to *Haemophilus influenzae* Infection and Proinflammatory Cytokines

To the Editor:

We read with interest the article by Smith et al[1] in an issue of *CHEST* (November 2013) examining nitric oxide (NO) biosynthesis in primary ciliated epithelial cell cultures from patients with primary ciliary dyskinesia (PCD) at baseline and 2 h after coculture with *Pneumococcus*, reporting no increase in NO. We have investigated the modulation of NO in primary cultured ciliated epithelial cells from patients with PCD following 72-h coculture with nontypeable *Haemophilus influenzae* (NTHi) isolated from infected patients with PCD and following stimulation with proinflammatory cytokines. Our study was designed to investigate NTHi biofilm development on cells from patients with PCD as opposed to the investigation of acute pneumococcal infection in Smith et al's[1] article. The study was approved by the Southampton and South West Hampshire Research Ethics Committee (06/Q1702/109).

Airway epithelium from patients with PCD and control patients without PCD was obtained by nasal brushing and was cultured at the air-liquid interface until differentiated and ciliated. We quantified the presence of NO using a total NO detection assay (Enzo Life Sciences, Inc) within phosphate-buffered saline washes applied to the apical surface of air-liquid interface cultures. NO levels were measured before and after epithelial cells were apically cocultured for 72 h with NTHi at a multiplicity of infection of 100 to evaluate biofilm infection. Cell viability was demonstrated by daily stable transepithelial electrical resistance (PCD and non-PCD) and ciliary beat frequency measurements (control subjects without PCD). We also measured levels following an 18-h incubation with a cocktail of proinflammatory cytokines (10 ng/mL each of IL-1β/interferon-γ/tumor necrosis factor-α) applied basolaterally to the cells (n = 14 for each experiment).

Our baseline data were consistent with that of Smith et al,[1] demonstrating no difference between the NO levels from PCD and non-PCD epithelia (Fig 1, Table 1). However, we found a significant twofold to threefold increase in NO levels from both PCD and non-PCD epithelia in response to NTHi and proinflammatory cytokines, with no significant difference between the two patient groups (Fig 1, Table 1, e-Fig 1). We corroborated basal NO biosynthesis in PCD and non-PCD epithelial cell cultures using 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate fluorescence (data not shown).

We speculate that the longer period of infection (72 h compared with 2 h) might account for the differences between our data and that of Smith et al.[1] The difference in organism (NTHi from infected patients with PCD rather than a laboratory strain of *Pneumococcus*) might also contribute to the difference. Taking these data alongside the findings of Smith et al,[1] we suggest that NO biosynthesis in nasal epithelia from patients with PCD may be delayed in response to infection. Results of these two studies highlight the need to investigate NO response of PCD epithelial cells to different pathogens over a range of time points. The etiology for the significantly reduced nasal NO levels in patients with PCD remains unanswered.[2]

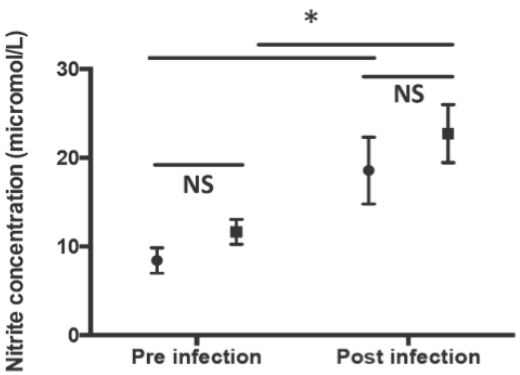

FIGURE 1. Nitric oxide levels measured in apical phosphate-buffered saline washes following a 30-min incubation at 37°C of ciliated primary epithelial cell layers from primary ciliary dyskinesia (PCD) (n = 5) and non-PCD (n = 9) cultured at an air-liquid interface, measured at baseline and 72-h post coculture with nontypeable *Haemophilus influenzae* (mean ± SEM). *$P < .05$. ● = PCD; ■ = non-PCD. NS = nonsignificant.

Table 1—*NO Levels Biosynthesized From PCD vs Non-PCD Ciliated Epithelia*

Experiment Group	Baseline, μmol/L	Poststimulation, μmol/L	*P* Values, Pre vs Post
18-h cytokine stimulation			
PCD (n = 5)	16.1 ± 1.1	52.9 ± 15.6	.046
Non-PCD (n = 9)	19.5 ± 2.0	57.1 ± 10.2	.002
P value, PCD vs non-PCD	.13	.41	...
72-h NTHi coculture			
PCD (n = 5)	8.4 ± 1.4	18.6 ± 0.3.8	.026
Non PCD (n = 9)	11.6 ± 1.4	22.7 ± 3.6	.002
P value, PCD vs non-PCD	.16	.44	...

NO levels biosynthesized from PCD vs non-PCD ciliated epithelia cultured at an air-liquid interface at baseline and following stimulation with either 10 mg/mL IL-1β/IFN-γ/TNF-α for 18 h or coculture with NTHi for 72 h (mean ± SEM). IFN = interferon; NO = nitric oxide; NTHi = nontypeable *Haemophilus influenzae*; PCD = primary ciliary dyskinesia; TNF = tumor necrosis factor.

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Additional information: The e-Figure can be found in the "Supplemental Materials" area of the online article.

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LETTER
IN PRESS | CORRECTED PROOF

Pulmonary radioaerosol mucociliary clearance in primary ciliary dyskinesia

To the Editor:

Primary ciliary dyskinesia (PCD) is a genetic condition affecting one in 10 000–40 000 people from birth [1]; cilia fail to beat, and the airway clearance of mucus and debris is severely impaired. If untreated, this results in progressive lung infection leading to bronchiectasis and ultimately respiratory failure. Additionally, delayed diagnosis has implications for genetic counselling, appropriate management of glue ear and fertility advice. Early diagnosis and appropriate treatment are believed to improve outcome. The diagnosis of PCD is highly specialised and results can remain inconclusive, despite state of the art equipment and diagnostic techniques. A European consensus statement [2, 3] highlighted that there is no "gold-standard" diagnostic test; diagnosis requires expert review of clinical history and screening tests (nasal nitric oxide measurement) alongside analysis of ciliary function and ultrastructure [2]. It is recommended that ciliary activity of respiratory epithelial cells obtained by nasal or bronchial brushing is recorded using a high-speed video camera mounted on a microscope. The images are played back in slow motion to analyse ciliary beat pattern (CBP) and frequency (CBF). Transmission electron microscopy (TEM) is used to assess ciliary ultrastructure [4]. Diagnostic uncertainty can be caused by secondary damage of the epithelium during sampling or due to infection or inflammation of epithelia; this damage can lead to abnormalities of ultrastructure, CBF and CBP. Furthermore, diagnosis is hindered by normal ciliary ultrastructure in 3–30% of cases of PCD [5, 6]. To improve diagnostic certainty, a variety of further investigations can be employed [2], including reanalysis of CBF, CBP and TEM following culture of the cells at an air–liquid interface [7, 8], or using immunofluorescence microscopy [9] to identify ciliary proteins. A single-centre study has previously reported the use of pulmonary radioaerosol mucociliary clearance (MCC) in the diagnosis of PCD [10]. The method is based on clearance patterns after the inhalation of a radioaerosol tracer. It provides a whole-lung functional test for pulmonary radioaerosol MCC. The investigation is noninvasive and has been used in thousands of patients with other lung diseases, as young as ~5 years. The authors reported that MCC was an effective noninvasive functional test for PCD [10] but the study was preliminary, and the feasibility of this complex technique and interpretation of data have not been assessed in other centres. We therefore conducted a study to replicate the results using a standardised protocol. This study was approved by the National Research Ethics Service (South Central committee 11/SC/0192) and all subjects gave written informed consent.

We assessed pulmonary radioaerosol MCC in six adult patients previously diagnosed with "classical" PCD (table 1) according to European Respiratory Society consensus guidelines [2] compared with four healthy controls. PCD and control groups were similar in age and sex distribution but PCD patients had impaired lung function (mean FEV_1 74% (range 36–105%) predicted in PCD patients and 97% (range 93–100%) predicted in controls) (table 1). Participants inhaled nebulised technetium-99m labelled nanocoll (GE Healthcare, Little Chalfont, UK) *via* a DeVilbiss 646 nebuliser (DeVilbiss Healthcare, Somerset, PA, USA) while breathing to ensure the best deposition in the central airways, as described previously [11]. Planar

TABLE 1 Primary ciliary dyskinesia subject characteristics

Subject	Age years	HSV findings	TEM findings	nNO ppb	FEV_1 L	FEV_1 % predicted
1	19.0	Static and twitching	Normal	100	3.74	84
2	16.8	Static	ODA defect	86	2.95	67
3	21.6	Static	ODA defect	43	3.26	105
4	61.1	Static and twitching	ODA and IDA defect	57	0.6	36
5	43.4	Static	ODA and IDA defect	30	1.82	71
6	18.9	Dyskinetic and uncoordinated	Axonemal disorganisation and absent IDAs	40	3.12	80

HSV: high-speed video microscopy; TEM: transmission electron microscopy; nNO: nasal nitric oxide; FEV_1: forced expiratory volume in 1 s; ODA: outer dynein arm; IDA: inner dynein arm.

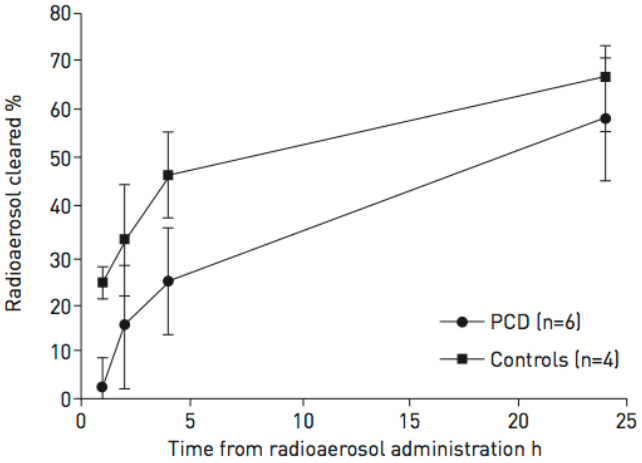

FIGURE 1 Mean percentage of radioaerosol cleared at 1, 2, 4 and 24 h in the primary ciliary dyskinesia (PCD) and control groups. Error bars represent 95% confidence intervals.

gamma camera images were taken at time 0, 1, 2, 4 and 24 h on a dual-head GE Infinia gamma camera (GE Systems, Milwaukee, WI, USA). A single-photon emission computed tomography/computed tomography scan and krypton-81 ventilation images were also obtained to characterise the initial distribution, allow attenuation correction and define the lung outline to assist interpretation of clearance data [12]. Geometric mean image data were interpreted after applying attenuation correction. Image analysis was carried out blind to patient group.

The mean percentage radioaerosol cleared was significantly greater in the control than in the PCD group at both 1 h (mean 24.0% (95% CI 3.4%) *versus* 2.3% (95% CI 6.1%)) and 4 h (46.3% (95% CI 8.7%) *versus* 24.2% (95% CI 11.1%)) (fig. 1). By 24 h, clearance was similar in the two groups (66.2% (95% CI 7.1%) *versus* 57.9% (95% CI 12.8%)). The technique was well tolerated by the subjects and there were no adverse events. Cooperation is needed from participants and it is unlikely that the test would be possible in preschool children.

Our study adds to the limited data that pulmonary radioaerosol MCC might be a useful diagnostic adjunct for diagnosing PCD. The expertise required to conduct the studies and interpret the data are likely to limit it to specialist nuclear medicine centres. The normal clearance of the aerosol by 24 h demonstrates alternative methods of airway clearance in PCD than MCC, including chest clearance physiotherapy (the subjects were advised to undertake their normal physiotherapy regimen between the 4- and 24-h images), cough and macrophage activity. It provides reassurance of the safety of this radioaerosol technique. Further data are required to confirm whether the technique will be useful for differentiating patients with considerable secondary ciliary damage or for diagnosing patients with "atypical PCD" (*e.g.* normal ciliary ultrastructure).

@ERSpublications
Pulmonary radioaerosol mucociliary clearance is reduced in PCD patients and might prove useful in diagnosis http://ow.ly/uP5Vg

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1.2 Relevant co-authored papers and chapters from PhD

Chapter 12

Primary ciliary dyskinesia

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Summary

Primary ciliary dyskinesia (PCD) is an autosomal recessive disease with an incidence estimated between 1:2,000 and 1:40,000. Ciliated epithelia line the airways, nasal and sinus cavities, Eustachian tube and fallopian tubes. Congenital abnormalities of ciliary structure and function impair mucociliary clearance. As a consequence, patients present with chronic sinopulmonary infections, recurrent glue ear and female subfertility. Similarities in the ultrastructure of respiratory cilia, nodal cilia and sperm result in patients with PCD also presenting with male infertility, abnormalities of left-right asymmetry (most commonly *situs inversus totalis)* and congenital heart disease. Early diagnosis is essential to ensure specialist management of the respiratory and otological complications of PCD. Diagnostic tests focus on analysis of ciliary function and electron microscopy structure. Analysis is technically difficult and labour intensive. It requires expertise for interpretation, restricting diagnosis to specialist centres. Management is currently based on the consensus of experts, and there is a pressing need for randomised clinical trials to inform treatment.

Keywords: Diagnosis, Kartagener's syndrome, primary ciliary dyskinesia, treatment

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Primary ciliary dyskinesia (PCD) is a relatively rare hereditary disorder characterised by chronic infection of the upper and lower airways [1, 2]. In addition, approximately half the patients have *situs inversus* and male infertility is common. The airway symptoms are caused by impaired mucociliary clearance (MCC) that results in accumulation of airway secretions often containing bacteria and allergens, leading to inflammation and chronic infection. The impaired MCC is a consequence of abnormal ciliary beat function that is usually, but not always, associated with abnormal ciliary axoneme structure as seen with electron microscopy (EM) [1, 2].

Although the association of chronic sinopulmonary disease with *situs inversus* has been known for the last 100 yrs, it is only in recent decades that an understanding of the underlying pathophysiology has been appreciated. A case of bronchiectasis associated with *situs inversus* was first reported by SIEWART in 1904 [3]. Kartagener reported 11 cases of *situs inversus* and bronchiectasis that were all associated with sinusitis in 1933 (reviewed in [4]). It was not until 1975 that ciliary disease was first

described in two males who had immotile sperm with abnormal ultrastructure on EM. One of the males had significant lung disease and within a year the hypothesis was proposed that the symptoms of Kartagener's syndrome were due to a congenital problem of MCC associated with ultrastructural anomalies of the respiratory cilia, sperm and embryonic nodal cilia (reviewed in [4]). Within the next few years, defects in ciliary activity including static cilia, reduced beat frequency, hyperfrequency and asynchronous beating pattern were described. As the understanding of the underlying pathophysiology developed, the name changed from Kartagener's syndrome to immotile cilia syndrome and later to PCD. Kartagener's syndrome is part of the wider condition named PCD, which is its basic pathophysiological mechanism, and designates PCD with *situs inversus*. The term Kartagener's syndrome is generally no longer used.

Recent technological advances in PCD diagnostics have improved our ability to recognise and appropriately treat patients with this disorder. However, prompt diagnosis requires a high index of suspicion and access to highly specialised diagnostic services [5]. A European Respiratory Society (ERS) Task Force on PCD in children has recently published a consensus statement in an attempt to improve the diagnosis and treatment of PCD in children [1]. A European survey by this Task Force indicated that PCD in children is under-diagnosed and diagnosed late, particularly in countries with low health expenditures [6]. Early diagnosis of PCD is important, because appropriate, early management is thought to improve long-term prognosis [7, 8]. Access to specialist facilities in most of Europe depends on the specialist interests of local physicians. The ERS PCD Task Force is committed to ensuring a uniform approach to diagnosis and treatment of PCD throughout Europe, facilitating diagnosis in countries where this is not yet optimal. It is currently developing a European Registry with the overall aim of facilitating research and improving outcome for people with this rare disorder.

Pathophysiology

Ciliated epithelia line the airways, nasal and sinus cavities, Eustachian tube and fallopian tubes. In healthy individuals, cilia are bathed in a layer of periciliary fluid and beat in a coordinated pattern at a frequency of 11–18 Hz. During the forward beat they extend into the overlying mucus, propelling it towards the oropharynx where it is swallowed or expectorated. This process is known as MCC. MCC forms a key role in the innate immunity of the respiratory tract by clearing mucus (fig. 1). Impaired MCC predisposes patients with PCD to recurrent respiratory infections, rhinosinusitis and ear infections [9].

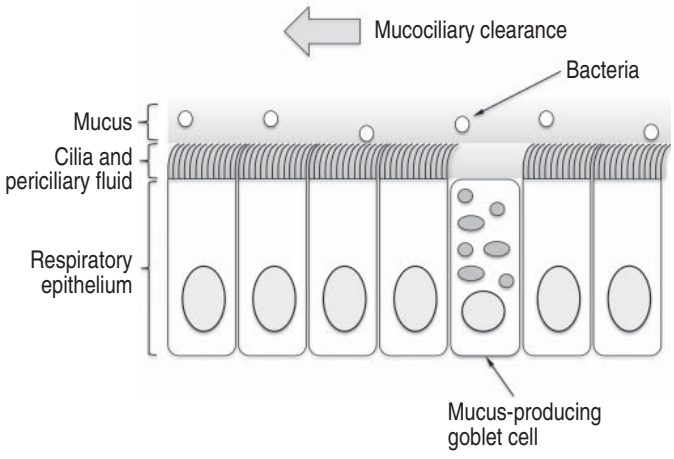

Figure 1. Schematic image of mucociliary clearance in the lung. The cilia, which are bathed in periciliary fluid, make coordinated sweeps into the overlaying mucus, directing the mucus and associated particles towards the oropharynx.

Normal ciliary ultrastructure

Motile cilia (as found in the upper and lower airway and fallopian tubes) and sperm have a highly organised "9+2" arrangement with nine peripheral microtubule doublets (A and B) surrounding a central pair of single microtubules running the length of the ciliary axoneme (figs 2 and 3a). The structure of the axoneme is maintained by protein cross-links; each doublet is connected to the adjacent one by nexin links and to the central pair by radial spokes. The central singlets are connected together by a bridge.

Motile cilia have inner and outer dynein arms (IDA and ODA,

respectively) attached to their peripheral microtubule doublets. Dynein is a mechanochemical ATPase and generates the force for ciliary beating. The ATPase activity of the dynein arms slides the A microtubules relative to the B microtubules, generating the force required for ciliary beating and bending [10]. Hence, abnormalities of the dynein arms affect ciliary beating.

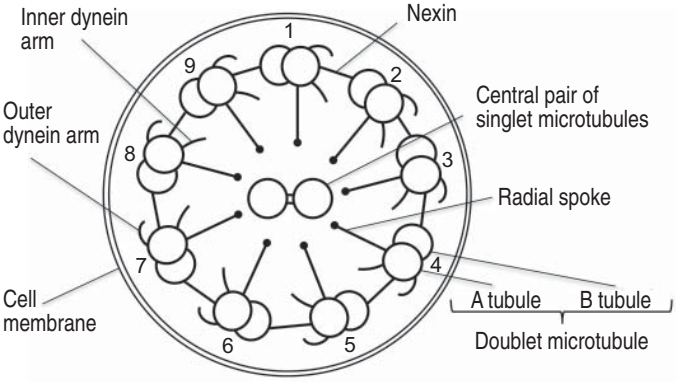

Figure 2. Diagram of the transverse section of a respiratory cilium axoneme.

Ciliary ultrastructural abnormalities in PCD

70–80% of patients with PCD have ultrastructural defects involving absent IDA and/or ODA (fig. 3b) [11–13]. Less common ultrastructural abnormalities include radial spoke defects (fig. 3c), with associated loss of microtubular arrangement, and transposition defects, where the missing central pair is replaced by a peripheral doublet of microtubules [11, 12]. Complete ciliary aplasia has also been reported [14]. The ultrastructural defects are associated with predictable abnormalities in the ciliary function. For example, ODA defects are associated with predominantly static and extremely slow ciliary beating, while transposition defects result in a circular beat pattern [15]. In normal individuals, defects are seen in 3–5% of cilia [16] and secondary defects, due to infection, pollutants and inflammation, have been shown to lead to abnormalities in up to 10% of cilia [11].

The structure of the sperm flagella is similar to the ultrastructure of cilia, accounting for the male infertility that is commonly associated with PCD [17]. Nodal cilia, important for left-right asymmetry during embryonic development [18], are also structurally similar to respiratory cilia, but have a "9+0" arrangement, lacking the central pair of microtubules. Nodal cilia dysfunction accounts for the *situs inversus* and complex cardiac disorders that are associated with PCD [19]. Rarely, PCD is also associated with diseases in which primary sensory cilia (*e.g.* polycystic kidney disease, retinitis pigmentosa) or ependymal cilia defects (hydrocephalus) are implicated (reviewed in [20]).

Many cells of the body contain non-motile (primary or sensory) cilia that are structurally very similar to motile cilia, but with a "9+0" structure [20]. PCD, caused by defects in motile cilia, can

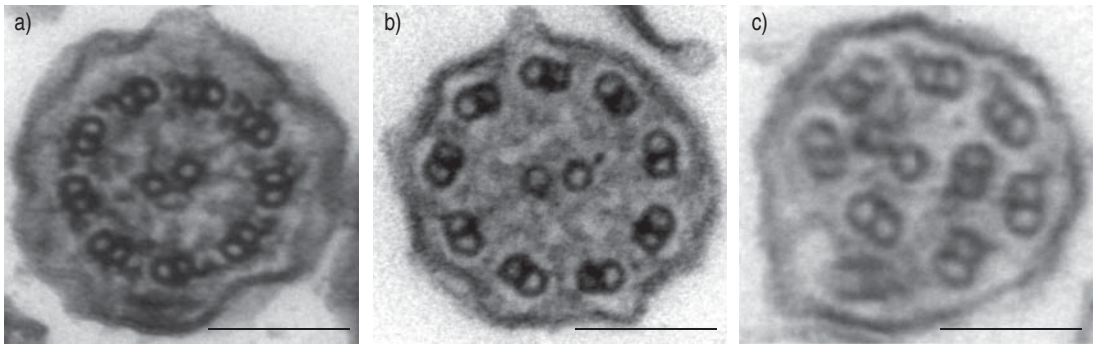

Figure 3. Transmission electron micrographs of ciliary ultrastructure showing a) normal ultrastructure, b) inner and outer dynein arm defects in primary ciliary dyskinesia, and c) radial spoke defect in primary ciliary dyskinesia (images obtained using FEI Tecnai 12 transmission electron microscope (FEI UK Limited, Cambridge, UK) at 80 kV). Scale bars=80 nm. Images provided courtesy of P. Goggin (Primary Ciliary Dyskinesia Centre, Biomedical Imaging Unit, Southampton University Hospitals NHS Trust, Southampton, UK; personal communication).

rarely be associated with disorders of non-motile cilia, for example Bardet–Biedl syndrome, polycystic kidney disease and retinitis pigmentosa. The inherited disorders related to dysfunction of motile and non-motile cilia have been collectively termed "ciliopathies" [21, 22] (www.ciliopathyalliance.org.uk).

There is an increasing case literature on a group of patients with a PCD phenotype and ciliary hyperfrequency associated with normal ciliary ultrastructure [23, 24]. This atypical form of PCD can be caused by mutations in dynein axonemal heavy chain (*DNAH*)*11*. These patients are likely to remain undiagnosed, particularly in centres where diagnosis depends on EM, with no access to high-speed video analysis. A recent large series estimated that $\leqslant$10% of patients with PCD and no laterality defects were being missed due to normal ciliary ultrastructure [11].

Genetics

PCD is inherited as an autosomal recessive disease. Over 250 proteins are involved in the formation of cilia and the disease-causing genes are commonly very large, making genetic diagnosis of PCD a challenge [25]. To date, mutations have been identified in 10 genes, mainly leading to dynein arm or radial spoke defects (reviewed in [1] and summarised in table 1). The commonest of these mutations, *DNAH5* mutation and dynein axonemal intermediate chain (*DNAI*)*1*, have a combined prevalence of 17–35% in patients with PCD [26, 34–37]. Other known mutations are rare. Therefore, the sensitivity of genetic testing is currently low but should increase with the identification of new genes. With concurrent simplification of genetic analyses, genetic testing is likely to become more widespread.

Epidemiology

Prevalence

Estimates of incidence and prevalence of PCD vary widely (table 2). This is explained by the broad range of clinical severity in PCD and the lack of a diagnostic gold standard, resulting in considerable under-diagnosis. In 1947, TORGERSEN [38] examined chest radiographs from one-third of Norway's population. He found *situs inversus* in one person per 8,000 inhabitants and signs of bronchiectasis in 10% of these. Assuming that 50% of PCD patients have *situs inversus*, he estimated a prevalence of PCD of 1:40,000. This is probably an underestimate, as not all PCD patients have bronchiectasis and chest radiographs are insensitive for its detection. AFZELIUS and STENRAM [40] compared the number of paediatric PCD patients known to them in Sweden with population figures for 1976–1990, and calculated a prevalence of 1:22,000. Considering that they

Table 1. The genes implicated in primary ciliary dyskinesia (PCD)

Gene	Ultrastructure defect	Locus	Phenotype	[Ref.]
DNAH5	ODA	5p15	PCD/KS	[26]
DNAH11	Normal	7p15.3–21	PCD/KS	[23]
DNAI1	ODA	9p21–p13	PCD/KS	[27]
DNAI2	ODA	17q25.1	PCD/KS	[28]
TXNDC3	ODA	7p14.1	KS	[29]
KTU	ODA and IDA	14q21.3	PCD/KS	[30]
RPGR	Variable	Xp21.1	PCD with retinitis pigmentosa	[31]
OFD1	Unknown	Xp22	PCD with mental retardation	[32]
RSPH9	RS	6p21	PCD	[33]
RSPH4A	RS	6q22	PCD	[33]

DNAH: dynein axonemal heavy chain; DNAI: dynein axonemal intermediate chain; TXNDC3: thioredoxin domain containing 3 (spermatozoa) gene; KTU: kintoun; RPGR: retinitis pigmentosa guanosine triphosphate regulator gene; OFD1: oral-facial-digital type 1 syndrome; RSPH: radial spoke head protein; ODA: outer dynein arms; IDA: inner dynein arms; RS: radial spoke; KS: Kartagener's syndrome. Adapted from [1].

Table 2. Estimates of prevalence of primary ciliary dyskinesia (PCD) in the general population, using different methodological approaches

First author [ref.]	Country	Population	Methods	Prevalence of PCD
Torgersen [38]	Norway	General population of Norway	Chest radiographs, examining *situs inversus* bronchiectasis	1:40000
Katsuhara [39]	Japan	4 cases among 16566 survivors of atomic bomb	Chest radiographs and physical examination	1:4100
Afzelius [40]	Sweden	General population of Sweden	Comparison of known paediatric PCD patients (nominator) with population figures for this age group (denominator)	Measured 1:22000, estimated 1:10000
O'Callaghan [41]	UK	South Asian population of Bradford, UK, high level of consanguinity	Comparison of known paediatric PCD patients (nominator) with population figures for this age group (denominator)	1:2265
Kuehni [6]	26 European countries	Clinician-reported PCD cases among 5–14-yr-olds	Comparison of diagnosed paediatric PCD patients per country (nominator) with population figures for this age group in the same country (denominator)	Measured 1:10000 in Cyprus, 1:20000 in Denmark and Switzerland

Provided courtesy of M-P.F. Strippoli (Division of International and Environmental Health, University of Bern, Bern, Switzerland; personal communication).

might not know all patients, they estimated the true prevalence to be nearer to 1:10,000. A long-term study on delayed atom bomb effects in Japan based on chest radiographs and physical examinations detected four cases of PCD in 16,566 persons (1:4,100) [39]. In a South Asian population living in the UK with relatively high levels of consanguinity, prevalence has been reported as 1:2,265 [41]. Recently (2007–2008), prevalence of diagnosed PCD in children was assessed in a European-wide survey of all paediatric respiratory centres in Europe [6]. Prevalence of diagnosed PCD in 5–14-yr-olds varied widely between countries. The highest frequencies were reported from Cyprus (111 cases per million inhabitants (1:10,000)), Switzerland and Denmark (both about 47 cases per million inhabitants (1:20,000)). Considering that a proportion of cases must have been missed in these countries, the true prevalence in Europe is likely to be at least 1:10,000.

Age at diagnosis

In the aforementioned survey by the ERS Task Force on PCD in children, nearly 1,200 patients currently in follow-up were reported among the participating paediatric respiratory centres, 1,003 in the age group of 0–19 yrs. Of these, 57% were males and 48% had *situs inversus* [6]. The median age at diagnosis was 5.3 yrs, significantly lower in children with *situs inversus* compared with those without (3.5 *versus* 5.8 yrs). The distribution of age at diagnosis was skewed, with a first narrow peak at age 0–2 yrs, mainly accounted for by children with *situs inversus*, and a broader peak at age 4–8 yrs followed by a long tail (fig. 4). Only 9% of children had been diagnosed in the neonatal period, 16% of those with *situs inversus* and 4% of those without. The mean age at diagnosis was lower in patients cared for by larger centres and varied considerably between countries, from 4.8 yrs in the British Isles to 6.8 yrs in Eastern Europe, with a significant negative correlation between mean age at diagnosis and general government expenditure on health in these countries. A relatively high age at diagnosis had also been found in independent publications from the UK and Denmark, which reported a mean age at diagnosis of 4.4 and 8.1 yrs, respectively, significantly higher than in patients with cystic fibrosis (CF) treated in the same centres [42, 43].

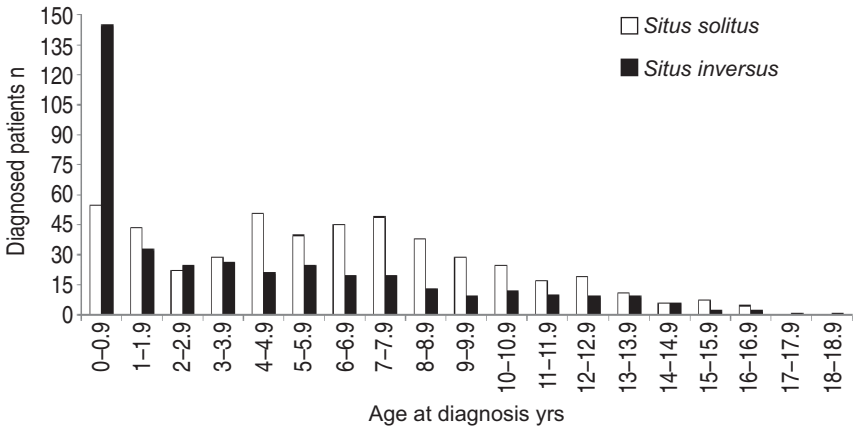

Figure 4. Distribution of age at diagnosis of patients with primary ciliary dyskinesia aged 0–19 yrs at the time of the survey, separately for patients with *situs solitus* and *situs inversus* (n=897). Adapted from [6], provided courtesy of M-P.F. Strippoli (Division of International and Environmental Health, University of Bern, Bern, Switzerland; personal communication).

Natural history

Little is known about the range of clinical severity and functional limitations at different ages, or about the natural history of PCD [1, 7, 8, 44]. While early case reports describing mainly paediatric patients suggested a relatively benign course of the disease, more recent data including adult populations are less positive. A cross-sectional survey by NOONE *et al.* [44] conducted in 1994–2002 in the USA describes 78 persons with PCD, including 31 children. In this sample, 61% of children and 98% of adults had bronchiectasis, 25% of adults were colonised with mucoid *Pseudomonas aeruginosa* and 25% of the cohort (38% of adults) had very severe disease with respiratory failure. All of these very severe patients had a forced expiratory volume in 1 s (FEV_1) $\leqslant$40% predicted, and had had a lung transplantation, were listed for transplantation or were dependent on oxygen. FEV_1 (% pred) declined with age (0.8% per year). Although this was less than the average lung function decline in CF patients in the USA (3.6% per year), the drop was statistically and clinically significant.

Recently, MARTHIN *et al.* [43] described long-term results for 74 paediatric and adult patients followed for a median of 9.5 yrs in the national Danish PCD centre. At diagnosis, 36% of the population had an FEV_1 of $<$80% pred, and lung function was significantly worse for patients diagnosed at a later age. Per year delay in diagnosis, FEV_1 (% pred) was 0.8% lower, almost identical to that described in the USA. After diagnosis, all patients were followed in the national PCD centre with standardised procedures, including twice-daily physiotherapy, monthly sputum examinations, 3-monthly clinical examinations and rapid antibiotic treatment of exacerbations. Time-courses of lung function following diagnosis varied greatly between patients, with 34% of patients showing a decline of $>$10% over 10 yrs, 57% being stable and 10% of patients showing an improvement of $\geqslant$10%. The change in lung function over time was not correlated to age at diagnosis or initial lung function. Although the median slope after diagnosis suggested by the longitudinal analysis was less steep than the slope suggested by the cross-sectional analysis, it still declined, suggesting that even state-of-the-art treatment only slowed down but did not reverse the decline in lung function. This is in contrast with earlier reports that suggested that lung function could be stabilised after diagnosis and initiation of treatment [7, 8]. In addition to the lack of evidence on long-term effectiveness of different treatments, it is also unknown how far environmental exposures, such as passive or active smoking, air pollution or viral infections, affect clinical severity and long-term prognosis [1]. This underlines the necessity of setting up large and representative cohort studies to know more about disease severity, evolution over time and the impact of concomitant factors and different treatments.

Clinical presentation and consequences of PCD

Most PCD patients have persistent upper and lower respiratory tract symptoms. Delayed diagnosis may occur because of the nonspecific nature of symptoms, such as rhinitis, cough and otitis media. As diagnosis becomes more readily accessible, physicians should maintain a high index of suspicion to ensure early diagnosis and appropriate management.

Respiratory disease

In up to 75% of patients, PCD manifests itself early in life as neonatal respiratory distress or pneumonia [8, 13, 44], although it is generally not diagnosed at this time unless *situs inversus* is discovered incidentally. From childhood to adult life, the respiratory clinical picture is characterised by chronic productive cough and recurrent infections of the upper and lower airways. Digital clubbing is present in 19–50% [45]. *Pectus excavatum* has been reported in 9% of PCD patients compared with 0.3% in the general population [46].

Reduced mean FEV_1 and forced vital capacity have been demonstrated in large series of PCD patients [43, 44, 47]. However, only a few small studies provide more detailed analyses of lung function [8, 48, 49]. Obstructive ventilatory defects have been reported in the vast majority of patients [8]. Significant response to β_2-agonists has been found in 25–55% [8, 47]. Most patients have increased residual volume and normal total lung capacity [8]. Restrictive ventilatory defects may occur after extensive lung resection [49].

Bronchiectasis is found in 50–70% of children with PCD when investigated by chest computed tomography (fig. 5) and in virtually all adults [46, 50]. The anatomical distribution of bronchiectasis differs significantly from CF, which is characterised by frequent upper lobe involvement [50]. In PCD, the middle lobe and lingula are most frequently and severely affected, followed by the lower lobes, while the upper lobes appear relatively spared [50]. This probably results from a better drainage of the upper lobes due to gravity. Severity of bronchiectasis is correlated with older age, worse lung function and colonisation by mucoid *P. aeruginosa* [46, 50]. Mucus plugging of central and peripheral airways is found in 75% [50, 51]. Other features include lobar atelectasis, peribronchial consolidation and air trapping. Emphysema is uncommon [46].

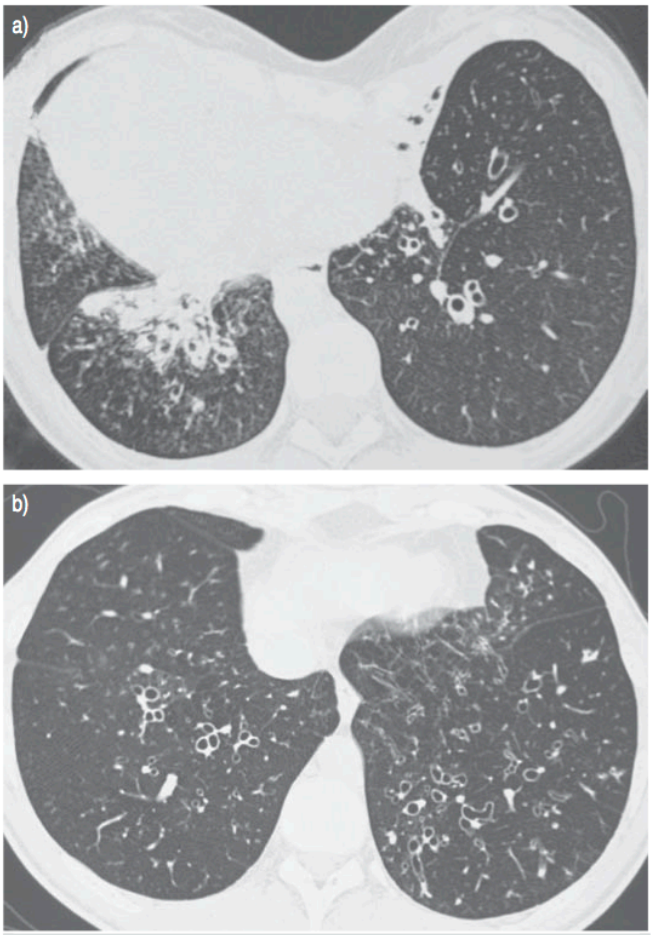

Figure 5. High-resolution computed tomography scans typical of those seen in adults with primary ciliary dyskinesia. a) *Situs inversus*, middle lobe atelectasis on the left side, bilateral bronchiectasis with peribronchial infiltrates on the right and *pectus excavatum* are shown. b) Moderate bilateral bronchiectasis is visible.

The most frequent pathogens isolated from sputum in PCD are *Haemophilus influenzae* (37–72%), *Streptococcus pneumoniae* (16–24%), *Staphylococcus aureus* (20–22%), *P. aeruginosa* (0–25%) and nontuberculous mycobacteria (0–7%) [7, 44, 52]. The prevalence of *P. aeruginosa* increases with age; in one series it was found in 38% of children and 62% of adults [46].

Ear, nose and throat

The epithelium lining the middle ear, paranasal sinuses and nasal pharynx is ciliated, and defective MCC caused by PCD results in chronic rhinitis and sinusitis and recurrent otitis media [1]. A study of 70 consecutive patients diagnosed with PCD [13] reported 88% to have nasal symptoms, 46% glue ear, 30% had required grommets and 36% reported hearing difficulties. Similarly, a study that reviewed the clinical history notes of 55 children at the time of their diagnosis [42] reported that 76% had rhinitis that was typically present from the neonatal period, 51% had serous otitis media and 25% reported hearing loss at the time of diagnosis. Sinusitis is commonly associated with PCD [53]. More recently, an association with aplastic or hypoplastic sinuses has been reported [54].

Cardiac and abdominal *situs*

Abnormalities of right-left asymmetry in the thorax and abdomen occur in ~50% of patients [44]. The organs are usually a mirror image of normal (*situs inversus totalis*). KENNEDY *et al.* [19] investigated the prevalence of right-left asymmetry defects in 337 patients with PCD by retrospective review of radiograph and ultrasound records. Normal *situs* was identified in 46% of patients and 48% had *situs inversus totalis*. The remaining 6% had heterotaxy (*situs ambiguous*). A range of heterotaxy abnormalities was identified, predominantly left or right isomerism, with or without congenital heart disease. A range of abnormalities within the abdominal cavity was also identified, including dextrogastria, polysplenia, asplenia and abnormally placed liver. One patient had pancreatic hypoplasia and another had intestinal malrotation. Pulmonary *situs* abnormalities also occur.

The random *situs* is a consequence of loss of nodal ciliary function during embryonic development [18]. Nodal cilia have a "9+0" structure, explaining why PCD caused by central pair microtubule defects do not affect laterality [11].

Fertility

PCD may present with male infertility secondary to absent or impaired motility of the sperm tail, which usually, but not always, bears the same ultrastructural and functional defect as the respiratory cilia [55]. However, some males are fertile [56]. In females, fertility may be reduced as a result of altered oviduct transport due to impaired ciliary function [55]. Either absent or dyskinetic motion with reduced beat frequency has been observed in ciliated cells of fallopian tubes of PCD patients [57]. Ectopic pregnancy has been reported in PCD and attributed to reduced oviduct transport [58].

Psychosocial aspects of PCD

There is little data available on the psychological and mental health impact of PCD. Unlike most lung diseases, PCD is commonly complicated by significant rhinosinusitis, hearing disorders and fertility issues. A study of 78 Italian patients investigated the impact of PCD on health-related quality of life using the St George's Respiratory Questionnaire to assess respiratory symptoms and the medical outcomes study short form-36 (SF-36) to investigate generic quality of life in adults and children [59]. The researchers found that a delay in diagnosis was associated with poorer quality of life, perhaps reflecting poor adaptation to the diagnosis at a later age. Interestingly, for most, the quality of life improved following confirmation of the diagnosis. Symptoms, activity and respiratory impact scores deteriorated with age and this was associated with a decline in the physical and mental health components of the quality-of-life scale. The decline in quality of life started in childhood and adolescence, with patients with the highest treatment burden having poorer quality of life. A study

from the UK similarly used the St George's Respiratory Questionnaire and SF-36 [60]. Researchers investigated the pattern of symptoms, describing their variability and development through life. In contrast to the Italian study, these researchers reported little abnormality in generic quality of life during childhood and adolescence. The authors of the Italian study [59] suggested that the reasons for the discrepancies could be earlier diagnosis in the UK, more aggressive early treatment and better continuity between paediatric and adult care.

Once diagnosed, results from both studies suggest that care by a specialised team can improve quality of life. A PCD nurse specialist can help to integrate the management between specialties by providing a point of contact for support services and additionally accessing financial assistance if this is available [61]. The nurse can provide counselling for adolescents and adults on issues including smoking and fertility, and nurses are often seen as an advocate for adolescents, empowering them to participate actively in their healthcare management.

Family support and peer groups also provide practical help and reassurance to patients with PCD and their families. In the UK, the PCD Family Support Group has an informative website (www.pcdsupport.org.uk). The site includes a forum where patients can present questions and advice to share with others undergoing similar experiences.

Diagnostic testing for PCD

Clinical history and examination are central to the diagnosis, and conventional diagnostic clues, as previously discussed, include *situs inversus*, significant neonatal respiratory distress, rhinorrhoea from the newborn period, recurrent "wet" cough and recurrent otitis media with effusion. Patients with a suggestive history should be referred to a service with the specialist facilities and expertise to diagnose PCD [5]. Specialist centres should be able to perform all necessary investigations and have a high enough throughput to maintain the expertise to interpret them. Notably, there is an increasing case literature on PCD associated with abnormal ciliary beat pattern (CBP) but normal ciliary ultrastructure; hence, centres only able to perform transmission EM will miss the diagnosis in 10–20% of cases [11, 23, 24]. The ERS PCD Task Force has published consensus recommendations for the diagnosis of PCD.

Screening

The detailed diagnostic investigations are time consuming and costly; therefore, screening tests are widely utilised. For many years, the saccharin test was conducted to give an assessment of MCC. It involved placing a micro-tablet of saccharin on the inferior turbinate and recording the time taken for the subject to taste it, normally <60 min. However, it is difficult to perform and can be unreliable in children and, therefore, is not recommended in this age group [1].

In the mid-1990s, it was demonstrated that nasal nitric oxide (NO) concentrations were significantly lower in patients with PCD compared with healthy controls, and it has subsequently been demonstrated, in a number of studies, that nasal NO can be reliably used as a screening test for PCD [62–66]. One group found a specificity of 88%, a sensitivity of 100% and a positive predictive value of 89% for correctly diagnosing PCD when using a nasal NO cut-off level of <105 [62]. It has also been demonstrated that nasal NO is low in both atypical PCD cases [67] and in children as young as 6 months of age [68]. However, recent studies have reported the rare occurrence of normal and raised levels of nasal NO in some patients confirmed to have PCD [54, 65]. It is important that the screening threshold of NO levels for a positive result is specific to the analyser and the technique used at that centre, and patients with a strongly suggestive history despite normal nasal NO levels should not be excluded from further diagnostic evaluation, particularly if they do not suffer from sinus disease.

Radio-aerosol MCC studies use a radioactive tracer to assess pulmonary and nasal MCC. Despite being performed for >25 yrs [69], there is limited experience on their use in PCD; therefore, they tend to be reserved as a supplementary screening tool [1, 70].

Diagnostic testing

If there is clinical suspicion of PCD, particularly if screening tests are suggestive, diagnosis can be confirmed by analysis of ciliated bronchial or nasal epithelia obtained by brushings using a cytology brush. Patients should not be brushed within 4 weeks of an upper respiratory tract infection, to minimise secondary dyskinesia and poor quality samples that may require repeat brushing. There is no ''gold standard'' test that will diagnose all PCD phenotypes; hence, a diagnostic work-up requires the rigorous assessment of ciliary beat frequency (CBF) and CBP with transmission EM of ciliary ultrastructure [13]. For difficult diagnostic cases, the re-differentiation of basal epithelial cells at an air–liquid interface in cell culture may permit a second diagnostic work-up with cultured ciliated epithelium [5, 71–73].

CBF and CBP analysis

The measurement of CBF has been used in the diagnosis of PCD for the last 20 yrs [74]. More recently, progress in high-speed video technology has allowed assessment of CBP by high-resolution, high-speed video microscopy with slow-motion replay [75]. This advance is of particular benefit, as an estimated 10–15% of patients with PCD have normal CBF but abnormal CBP [1]. It also allows a permanent record to be made, which permits reassessment and audit of the analysis.

Normal CBF is between 11–18 Hz (at 37°C), but in patients with PCD, this is typically, but not always, markedly reduced to ~3 Hz or static [15]. In patients with ODA or combined IDA and ODA defects, the majority of cilia are static (55% and 80%, respectively), with a mean CBF of 2.3 Hz and 0.8 Hz, respectively [15]. In patients with isolated IDA or radial spoke defects, the CBP appeared stiff, with reduced amplitude [15]. Ciliary transposition defects led to circular beat patterns and a mean CBF of 10.7 Hz [15]. There is also a group of PCD patients with the *DNAH11* mutation [24] who have hyperkinetic beat frequency, associated with an altered CBP.

It is generally accepted that the diagnosis of PCD can be excluded if CBF and CBP are normal, without going on to perform transmission EM analysis, which is time consuming, costly and requires a high level of specialist knowledge [1, 15].

Transmission EM

Assessment of the ultrastructure of the cilia by transmission EM (fig. 3) is essential in patients with abnormal CBF or CBP [1]. It requires specialist knowledge to interpret the ultrastructural changes. Centres and studies vary in the number of cilial transverse sections examined, from 20–300 cilia [11, 76]; most centres would assess $\geqslant$100 healthy cilia. The method of quantifying a primary ciliary defect also varies between centres. Normal patients can have defects in $\leqslant$10% of cilia but are distinguishable from PCD by lack of universality of the defect [11].

While ultrastructural defects are important in the diagnosis, there are well documented groups of patients with PCD who have normal cilial ultrastructure [11, 24]. This stresses the importance of a full diagnostic work-up in cases where clinical suspicion is high.

Air–liquid interface cell culture

Cell culture techniques have been used to aid the diagnosis of PCD for over a decade. Re-differentiation of basal epithelial cells to ciliated cells is achieved using culture at an air–liquid interface and has also been described in suspension [73, 77]. It is of particular benefit when the original sample has possible secondary ciliary defects, for example due to chronic infection, or has few cilia. It allows a second diagnostic work-up with reduced environmental factors that may have compromised ciliary function in the original sample [71]. It can also help to confirm rarer or atypical PCD phenotypes [71]. While air–liquid interface culture is a technically demanding,

expensive and time-consuming process, it does reduce the need for repeat brushing and reduces false-positive diagnoses.

Genetic analysis

Although genetic analyses are now available in a number of research centres for some of the mutations seen in PCD, genetic testing is not routinely used in Europe, as the genes responsible for most cases have not yet been identified [1]. Genetic testing can be reserved until after the clinical diagnosis has been proven and directed for the specific PCD ultrastructural defect found [1]. However, as further PCD-causing mutations in the numerous candidate PCD genes are identified, clinical genetic testing for PCD will become a more practical option. A technique using immunofluorescence microscopy to visualise and localise the proteins along the ciliary axoneme can facilitate genetic analysis by directing the search [78].

Treatment

Although gene transfer has successfully restored normal ciliary beating in human PCD airway epithelial cells *in vitro* [79], no specific treatment exists yet to correct ciliary dysfunction in the clinical setting. Therefore, therapy aims at improving mucus clearance, treating infections, improving or stabilising lung function and preventing chronic lung damage. As only a few randomised trials are available [80–82], most recommendations are based on expert opinion [1, 2] or extrapolated from available evidence in CF, although this is unsatisfactory because PCD and CF have different pathogenesis. Guidelines for the management of PCD in children have been recently provided by the ERS PCD Task Force [1]. No specific guidelines are currently available for PCD in adults. The recommendations outlined here are, therefore, based on the expert opinion of the ERS PCD Task Force [1], unless referenced otherwise.

PCD patients should be regularly followed by a multidisciplinary team at an expert centre with a caseload of $\geqslant$10–15 patients [1]. A protocol-based care system is recommended [1]. Visits should take place every 2–3 months in children and 6–12 months in adults [9]. Relevant disciplines include paediatric or adult respiratory medicine, chest physiotherapy, ear-nose-throat, genetic counselling and fertility clinics, in liaison with the local paediatrician or general practitioner [9]. Cardiology with expertise in congenital heart disorders may be needed in some patients. A specialised nurse may be a key link between the patient and the various specialties [61]. Social services may be needed in some cases.

Respiratory

Observations that lung function deteriorates before diagnosis but stabilises when the disease is recognised and treated [7, 8, 83] has suggested that lung damage results from under-treatment, and that it can be prevented by early diagnosis and therapy [7]. A recent longitudinal study has partly challenged this view by showing that lung function deteriorates in one-third of cases despite therapy [43] and that early diagnosis does not necessarily protect from deterioration. However, it also showed that improvement occurs with treatment in 10% of cases [43], confirming previous observations [52], and that FEV_1 decline seems reduced by therapy [43].

Improvement of airway mucus clearance

Physiotherapy is considered an important component of PCD management, although a recent survey showed that only 75% of patients actually perform it daily [59]. It consists of postural drainage, chest percussion, breathing manoeuvres, cough and use of oscillating devices, ideally performed twice daily at home (self-administered sessions or with parental assistance) and with regular support of a physiotherapist [2]. Physiotherapy should be increased during infections.

Physical exercise before physiotherapy is encouraged as it induces bronchodilation superior to β_2-agonists [47], and because deep breathing improves mucus clearance. *N*-acetylcysteine has not been shown to be beneficial and should not be used [84]. Nebulised recombinant human DNase is anecdotally beneficial in isolated cases [85–87]. Nebulised normal or hypertonic saline may be considered [1], but there are no clinical trials to support general use in PCD. β_2-agonists do not appear to be clearly beneficial in the whole PCD population [80], but the FEV_1 reversibility observed in some series suggests that they might be useful in a subgroup of patients [8]. Antitussives should be avoided.

Treatment and prevention of acute airway infections

Acute infection of the upper or lower airways should be promptly treated with high-dose oral antibiotics, guided by sputum cultures [1]. Fibreoptic bronchoscopy may be necessary occasionally to obtain samples when sputum cannot be obtained. Intravenous antibiotics are indicated when response to oral treatment is not satisfactory.

When *P. aeruginosa* is isolated, most physicians would attempt an eradication regimen similar to CF [1] and consider long-term nebulised anti-*Pseudomonas* antibiotics for established chronic colonisation [2], although the impact of *P. aeruginosa* infection on lung function in PCD remains unclear. Continuous prophylactic oral antibiotics given in cycles are usually not recommended except in particular cases with very frequent recurrent infections [1, 2]. A regular 3-monthly intravenous therapy may be considered in a minority of cases. Vaccinations against influenza and *S. pneumoniae* should be administered, in addition to the regular immunisation plan [1]. General health measures include avoidance of smoking and other environmental exposures [1].

Reduction of airway inflammation

No data are currently available to recommend the routine use of inhaled corticosteroids to reduce airway inflammation in PCD [1]. Asthma may, however, coexist with PCD in some patients, and should be recognised and treated. Anti-inflammatory macrolides have proven beneficial in CF, and might represent a therapeutic option in other causes of bronchiectasis. A beneficial effect of macrolides has been reported in PCD [88], although conflicting data exist [89].

Surgery and lung transplantation

Lobectomy has been reported in 10–41% of PCD patients in retrospective series [44, 46, 52], although many of these interventions were performed in an older era of medical and surgical practice, before doubt arose about validity of surgery for bronchiectasis. One small retrospective series from 15 yrs ago reports subjective benefit in operated patients [90]. Lobectomy in diffuse advanced bronchiectasis is currently not recommended [1], although it might be considered in rare cases where morbidity can be clearly attributed to a localised area of severe bronchiectasis. Lung transplantation has been reported in 1–3% of PCD patients in large series [44] but little is known about outcome. Only few data are available on the technical features of lung transplantation in the context of *situs inversus* [91, 92].

Monitoring

Spirometry and pulse oxymetry should be performed at regular intervals. Sputum analyses are recommended at the time of exacerbation and routinely at regular intervals in stable condition [1]. Chest high-resolution computed tomography might be of interest to document the presence of bronchiectasis, as this information may lead to management changes including more aggressive use of antibiotics and more intensive physiotherapy. However, frequent high-resolution computed tomography scans should be avoided to reduce irradiation.

Ear, nose and throat

Episodes of acute middle ear infections must be promptly treated with antibiotics. Chronic otitis media with mucus accumulation in the Eustachian tubes is common in children with PCD and leads to variable hearing loss. Therefore, children should undergo regular audiometric assessment and receive hearing aids and speech therapy when needed. These can usually be stopped at adolescence, as chronic otitis media spontaneously becomes milder.

Tympanostomy tubes are not recommended routinely, since they can lead to chronic otorrhoea and evidence for their benefit is conflicting [93, 94]. The management of serous otitis media in PCD relating to grommet insertion has recently been reviewed [93]. The literature search identified eight relevant publications, most with small numbers of patients and only two papers published in the preceding 10 yrs. The authors highlighted the poor evidence base for our management of otitis media effusions in PCD. They challenged the commonly held belief that grommet insertion is disproportionately associated with complications in PCD, suggesting that complications are no more common than in the non-PCD population. Since the review, a retrospective observational study reviewed the otological features of PCD in 58 children and young adults [94]. The frequency of acute otitis media, conductive hearing loss and otorrhoea was reduced with increasing age, but was still present in the young adults. In all age groups, >70% of patients had serous otitis media but, again, this was less common in young adults than in children. Half of the cohort had a history of tympanostomy tube insertion. All of the children with grommets had at least one episode of otorrhoea and half developed tympanic perforation. The researchers concluded that their data support conservative management of serous otitis media in PCD, whereas surgical intervention is rarely associated with significant improvement in hearing [94]. This controversy demonstrates the lack of well-defined clinical trials in PCD. Powered, randomised trials are urgently needed.

Chronic rhinorrhoea is present lifelong in most patients with PCD and varies from watery to mucoid. Regular clearance of nasal mucus is recommended. Saline nasal douches may be helpful [9]. Long-term nasal corticosteroids may improve rhinosinusitis but the benefit for rhinorrhoea is unclear. Occasional use of anti-cholinergic drugs has been reported. Mucolytic agents are not recommended.

Episodes of acute sinusitis require antibiotic therapy. Frequently relapsing sinus infections should prompt ear-nose-throat advice to consider surgery to improve sinus drainage and aeration [9].

Fertility

Patients should be informed early about possible subfertility or infertility, and referred to appropriate services. Treatment should be individualised. Successful ovular intracytoplasmic sperm injection [95, 96] and *in vitro* fertilisation [97] have been reported in isolated cases. As PCD is an autosomal recessive disorder, genetic counselling should be offered to patients consulting for infertility, to provide information about the risk of disease transmission to the offspring.

Conclusions

The impaired MCC associated with PCD leads to bronchiectasis, chronic rhinosinusitis and ear disease. Our understanding of this disease remains poor, with limited data on prevalence, and much less about natural history. The technology to aid the diagnosis of PCD has improved in recent years but diagnosis of PCD can be challenging, particularly in atypical phenotypes. Once diagnosed, there is little evidence to inform management practice. Best practice is generally based on anecdotal experience of experts and observational cohorts. Conflicts in recommendation are emerging in the literature [93, 94], reflecting the urgent need for well-conducted randomised studies. International collaboration will be necessary to achieve sufficient power for trials of treatments to improve long-term outcome and management of intercurrent exacerbations.

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Statement of interest

None declared.

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Nasal nitric oxide screening for primary ciliary dyskinesia: systematic review and meta-analysis

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ABSTRACT Nasal nitric oxide (nNO) concentrations are low in patients with primary ciliary dyskinesia (PCD) providing a noninvasive screening test.

We conducted a systematic review of the literature to examine the utility of nNO in screening for PCD, in particular 1) different respiratory manoeuvres during sampling (velum closure, tidal breathing, *etc.*), 2) accuracy in screening young/uncooperative children, 3) stationary *versus* portable analysers, and 4) nNO in "atypical" PCD.

96 papers were assessed according to modified PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) criteria and 22 were included in this review.

Meta-analysis of 11 studies comparing nNO during a velum closure breath hold gave a mean±SD nNO of 19.4±18.6 nL·min^{-1} in PCD (n=478) and 265.0±118.9 nL·min^{-1} in healthy controls (n=338). Weighted mean difference for PCD *versus* healthy controls was 231.1 nL·min^{-1} (95% CI 193.3–268.9; n=338) and 114.1 nL·min^{-1} (95% CI 101.5–126.8; n=415) for PCD *versus* cystic fibrosis. Five studies of nNO measurement during tidal breathing demonstrated that this is an acceptable manoeuvre in young children where velum closure is not possible, but the discriminatory value was reduced. Four small studies of portable NO analysers suggest these are reliable tools for screening for PCD. However, nNO must be interpreted alongside clinical suspicion. Future studies should focus on standardising sampling techniques and reporting.

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Introduction

Primary ciliary dyskinesia (PCD) is a rare, autosomal recessive disease characterised by abnormal ciliary function. The resulting impaired mucus clearance from the lungs, upper airways and middle ear typically leads to respiratory symptoms from soon after birth, with ongoing chronic sinopulmonary infection and progressive loss of lung function. Approximately half of those with PCD have *situs inversus* and male

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infertility is common [1]. A European Respiratory Society (ERS) consensus statement on diagnostic techniques includes high-speed video microscopy to assess function and assessment of ciliary ultrastructure by transmission electron microscopy. Both are time-consuming, technically demanding investigations that are only available in specialist centres [2]. Difficulty accessing specialist diagnostic services frequently contributes to a delay in diagnosis [3]; therefore, an ERS PCD Task Force recommended measurement of nasal nitric oxide (nNO) as a screening test [2] to improve preselection of patients to travel to centralised diagnostic centres. A survey prior to the ERS consensus statement suggested only 46% of European PCD centres were measuring nNO [4].

LUNDBERG *et al.* [5] first reported very low levels of nNO in PCD patients in 1994, but development of nNO as a screening test was slow to establish with only a handful of studies over the next decade [6–10]. American Thoracic Society (ATS)/ERS guidelines for the measurement of nNO were published in 1999 and updated in 2005 [11]. These recommended aspiration of gas from one nostril with gas entrained *via* the other naris during a velum closure manoeuvre in order to avoid contamination by lower airway gases. Subsequently, a number of studies have suggested that nNO is not only sensitive and specific in differentiating PCD patients from healthy controls but that manoeuvres other than velum closure, which is not suitable for young children, may be discriminatory.

The mechanism by which nNO is reduced in PCD has yet to be elucidated, several hypotheses have been proposed and have been more fully reviewed elsewhere [12]. Cultured respiratory epithelial cells have been studied for their ability to synthesise NO, as well as their levels of expression of the three NO producing enzymes (nitric oxide synthases). The results have been conflicting [13–15], but cultured epithelial cells from PCD patients seem to produce equivalent amounts of NO, at baseline, as cells from non-PCD patients. Further work is needed to assess the role of the paranasal sinuses in reduced production of NO [5, 16].

The ATS/ERS guidelines recommend measurement using a stationary chemiluminescence analyser [11]; however, the recent availability of hand-held analysers with an electrochemical sensor provides a cheaper and more portable option, making screening more accessible [17]. The reliability of these portable devices has only been assessed in a handful of small studies and data from exhaled NO studies suggest readings differ between handheld and stationary devices [18]. We undertook a systematic literature review to assess the diagnostic accuracy of nNO measurement, highlighting the different analysers and respiratory manoeuvres currently used. We also considered the evidence for nNO as a screen for PCD in young, uncooperative children.

Our aim was to consider the effectiveness of nNO measurement as a screening tool for PCD by reviewing: 1) nNO measured in PCD patients compared with healthy and disease control groups using stationary analysers during a velum closure technique (as recommended in ATS/ERS guidelines [2]), including a meta-analysis; 2) nNO in patients with atypical PCD (normal electron microscopy); 3) different respiratory manoeuvres to measure nNO including breath holding, velum closure by forced expiration against resistance, tidal breathing and humming; 4) measurement of nNO in young children (<5 years) unable to cooperate with respiratory manoeuvres; and 5) reliability of handheld NO analysers for screening compared with stationary chemiluminescence analysers.

Methods

A protocol for the systematic review was developed prospectively (online supplementary material) and is briefly described in the following sections.

Search strategy

The following databases were searched from inception until April 16, 2014: MEDLINE, EMBASE, PreMEDLINE In-Process & Other Non-Indexed Citations, Web of Knowledge Science Citation Index (SCI), Web of Knowledge ISI Proceedings and Cochrane Systematic Reviews Database. Additional references were sought through citations listed by the identified studies.

Study selection

Manuscripts were included if nNO was measured in PCD patients along with details of nNO sampling technique, analyser, sampling rate, patient age and method of PCD diagnosis.

Data analysis and synthesis

The sampling rate of NO analysers is a determinant of nNO concentration. In an attempt to standardise between studies using different sampling rates we converted reported concentrations (ppb) to $nL \cdot min^{-1}$ using the formula ppb × sampling rate ($L \cdot min^{-1}$). Two studies have reported good agreement between different analysers when using this conversion estimate [17, 19].

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Studies that reported mean nNO values with either standard deviation or standard error (SE/SEM) were included in the meta-analysis. Data were analysed using STATA 11.0 (StataCorp LP, College Station, TX, USA) to perform a generalised inverse variance analysis of mean difference between nNO in PCD patients and healthy or cystic fibrosis (CF) controls. We also assessed studies for heterogeneity.

Results

Study selection and risk of bias

We identified 96 publications, excluding duplicates, reporting nNO as an outcome in PCD patients (fig. S1). 35 were original research studies of nNO and were assessed for eligibility (table S1), with 22 included in the narrative synthesis and 11 in the meta-analysis (table 1).

Using QUADAS principles [27], apart from lack of blinding, the risk of bias was assessed as low for all included studies. Further details of the QUADAS criteria are given in the online supplementary material.

Ability of nNO to discriminate between PCD and healthy or disease controls

We identified 19 manuscripts that reported nNO measured by velum closure manoeuvres with a stationary analyser (n=634 for PCD); of these 19 papers, 18 compared PCD with healthy controls (n=582) and 11 compared PCD with CF patients (n=510). In addition to these 18 papers, one paper used a portable analyser to compare PCD with bronchiectasis [28]. All studies were included in the narrative synthesis and reported significantly lower nNO levels in PCD than healthy controls and CF patients (tables 1 and 2).

The meta-analysis included 478 PCD patients, 338 healthy controls and 415 patients with CF (table 1). The combined mean$\pm$SD nNO were: PCD 19.4$\pm$18.6 nL·min^{-1}, healthy controls 265.0$\pm$118.9 nL·min^{-1}, and CF 123.2$\pm$62.7 nL·min^{-1}. These measurements appear consistent with data from studies where nonparametric data was reported; lower median values than means are consistent with negatively skewed data (table 2).

All 11 studies in the PCD *versus* healthy control meta-analysis reported considerably lower levels of nNO in PCD, and this is reflected in the weighted mean difference of 231.1 nL·min^{-1} (95% CI 193.3–268.9) (fig. 1). The difference between PCD and CF was also significant across studies, but with a smaller weighted mean difference of 114.1 nL·min^{-1} (95% CI 101.5–126.8) (fig. 2). There was a high degree of heterogeneity, with an I^2 (variation attributable to heterogeneity) of 93.9% for PCD *versus* healthy conrols, and a moderate degree for PCD *versus* CF (I^2 of 40.8%). This reflects differences in the study populations, such as age, and methodological issues such as breath hold with CO_2 monitoring *versus* sampling during exhalation against resistance.

Nine studies calculated cut-off values with sensitivity and specificity for diagnostic accuracy within their populations (table 3). LEIGH *et al.* [19] developed a cut-off of 77 nL·min^{-1} using data from their centre and subsequently correctly identified 70 of 71 PCD patients (>98%) across six other sites. This suggests that, when using standardised protocols, cut-off values are valid in different settings, analysers and populations.

Four studies reported nNO levels in consecutive referrals to a PCD diagnostic service; two were excluded (lack of sampling details and unclear PCD diagnosis) [19, 33]. CORBELLI *et al.* [10] reported 34 referrals, half of whom proved PCD positive; mean nNO for PCD positive patients was 16.4 nL·min^{-1} *versus* 159.2 nL·min^{-1} for PCD negative referrals ($p<0.05$). MARTHIN and NIELSEN [25] found a highly significant difference ($p<0.0001$) in nNO levels between 12 referrals whose diagnostic tests were positive and 46 whose tests were negative; medians (95% CI) were 15.9 (3–162) nL·min^{-1} for positive *versus* 204.3 (34.5–386.1) nL·min^{-1} for negative results. Their cut-off of 52.5 nL·min^{-1} identified 55 out of 59 PCD cases from consecutive referrals (sensitivity of 92%, specificity of 96%) [25].

Seven studies compared nNO levels in PCD patients with those in non-CF bronchiectasis (table 4). Five reported similar nNO levels in bronchiectasis to healthy controls [7–9, 23, 26] and one used only a portable analyser in a small number of patients [28]. MORENO GALDÓ *et al.* [24] reported lower nNO in bronchiectasis than in healthy participants (mean 90.3 nL·min^{-1} *versus* 224.5 nL·min^{-1}), but levels were significantly higher than in PCD patients. It is unclear why this study found bronchiectasis patients to have lower levels than those reported in other studies, but participant numbers were low [24] and the bronchiectasis group may have contained a number of undiagnosed PCD patients. PCD patients nNO was also significantly lower than patients with asthma [19, 24, 30, 31], chronic obstructive pulmonary disease (COPD) [19, 28] and humoral immunodeficiency [31].

Nasal nitric oxide in patients with atypical PCD

LEIGH *et al.* [19] describe 10 PCD patients with *DNAH11* mutations and normal ciliary ultrastructure that all had nNO levels below 77 nL·min^{-1}. PIFFERI *et al.* [34] found four patients with normal ultrastructure, but

TABLE 1 Summary of studies measuring nNO in PCD, healthy control and cystic fibrosis groups using a stationary analyser with a velum closure manoeuvre

	PCD		Healthy controls		Cystic fibrosis	
	Subjects n	Mean±SD nL·min^{-1}	Subjects n	Mean±SD nL·min^{-1}	Subjects n	Mean±SD nL·min^{-1}
Study						
Wodehouse *et al.* [9], 2003	42	16.4±9.2	16	189.8±36.5	15	139.5±40.7
Csoma *et al.* [20], 2003	15	14.9±11.8	14	126.4±62.5		
Noone *et al.* [21], 2004	64	19±17	27	376±124	11	184±109
Corbelli *et al.* [10], 2004	17	16.4±4.35	24	268.4±138.5		
Piacentini *et al.* [22], 2008	10	8.9±5.7	26[¶]	195±92.8		
Shoemark *et al.* [23], 2009	20	12.3±8.6	20	159.8±133.5		
Moreno Galdó *et al.* [24], 2010	9	22±29.9	37	224.5±37.1	210	109.5±27
Marthin and Nielsen [25], 2011	45	19.0±13.6	57	272.4±76.0	49	124.8±103.7
Mateos-Corral *et al.* [26], 2011	20	17.9±13.7	19	366.5±131.7	32	138.8±84.1
Leigh *et al.* [19], 2013	149	20.7±24.1	78	304.6±118.8	77	134.0±73.5
Leigh *et al.* [19], 2013[#]	71	23.3±18.0				
Marthin and Nielsen [17], 2013	16	23.7±22.8	20	267±74.4	21	150.3±58.8
Combined	478	19.4±18.6	338	265.0±118.9	415	123.2±62.7
ppb equivalents[¶]						
NIOX (Flex/MINO)[+]		64.7		883.3		410.7
Eco Medics CLD 88[§]		58.8		803.0		373.3
Sievers[ƒ]		38.8		530.0		246.4
LR2000[##]		77.6		1060.0		492.8

nNO: nasal nitric oxide; PCD: primary ciliary dyskinesia. [#]: replication; [¶]: results from co-operative healthy controls only; [+]: Aerocrine AB, Solna, Sweden; [§]: Eco Medics AG, Duernten, Switzerland; [ƒ]: GE Analytical Instruments, Boulder, CO, USA; [##]: Logan System, Rochester, UK.

abnormal ciliary beat frequency, had nNO levels consistently below 22 nL·min^{-1} with no overlap with secondary ciliary dyskinesia or healthy subjects. Knowles *et al.* [35] recently identified patients with *RSPH1*

TABLE 2 Studies reporting nonparametric summary statistics for nasal nitric oxide in PCD, healthy control and cystic fibrosis groups measured *via* stationary analyser with velum closure manoeuvre

Study	PCD		Healthy controls		Cystic fibrosis	
	Subjects n	Outcome nL·min^{-1}	Subjects n	Outcome nL·min^{-1}	Subjects n	Outcome nL·min^{-1}
Karadag *et al.* [6], 1999	21	Median 13.75 (range 0.83–239.8)	60	Median 138.3 (range 29–359.3)		
Narang *et al.* [7], 2002	31	Median 15.1 (range 0.8–230)	53	Median 179 (range 99.5–359.3)	17	Median 122.8 (range 7.8–285)
Horvath *et al.* [8], 2003	14	Median 13.6 (range 1.3–67.3)	37	Median 165.8 (range 80.5–335.8)	20	Median 85.8 (range 7.5–249.3)
Santamaria *et al.* [29], 2008	14	Median 3.3 (IQR 3.3)	14	Median 90.1 (IQR 67.2)		
Marthin and Nielsen [25], 2011[#]	12	Median 15.9 (95% CI 3–162)				
Walker *et al.* [30], 2013	14	Median 8.1 (IQR 4.8–22.8)	18	Median 231.6 (IQR 207–265.8)	12	Median 150.3 (IQR 135–182.4)
Boon *et al.* [31], 2014	38	Median 16.8 (IQR 8.1–35.7)	49	Median 236.4 (IQR 198.3–295.8)	46	Median 109.5 (IQR 75.6–169.5)
Harris *et al.* [32], 2014	11	Median 12.3 (IQR 7.5–15.9)	15	208.2 (IQR 181.2–240)	6	Median 139.5 (IQR 99–227.7)

PCD: primary ciliary dyskinesia; IQR: interquartile range. *: calculated by using mean in nl/min/machine sampling rate; [#]: consecutive referrals.

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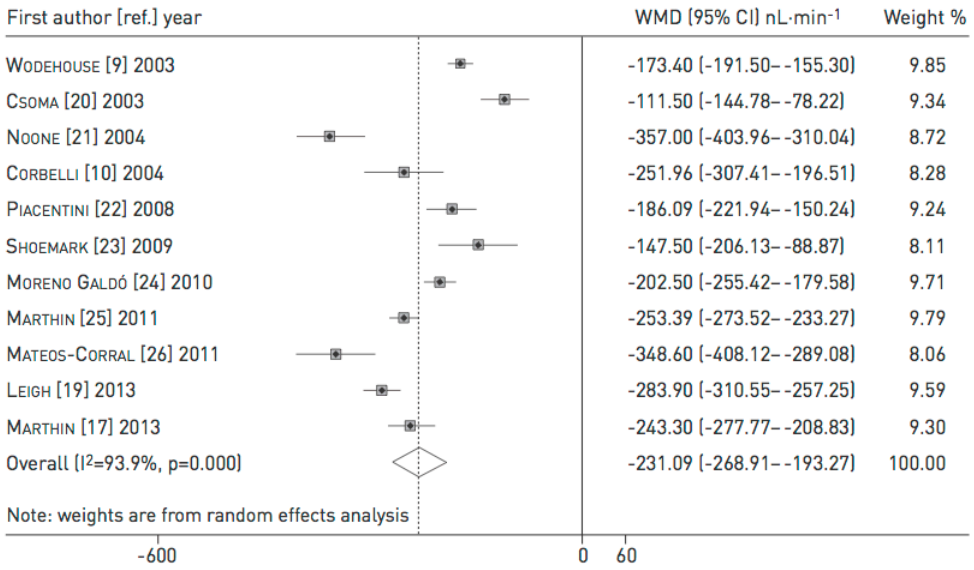

FIGURE 1 Forest plot showing the weighted mean difference (WMD) in mean nasal nitric oxide between healthy controls and primary ciliary dyskinesia patients using a stationary analyser and velum closure technique.

mutations causing PCD that have a milder phenotype and higher nNO levels (mean 98.3 nL·min^{-1}). 12 of the 18 patients identified with this mutation have nNO above a diagnostic cut-off of 77 nL·min^{-1} [35, 36]. The reason for higher nNO levels in this genotype is unclear, but may provide insights into the mechanisms of low nNO in PCD.

Comparison of manoeuvres used during measurement of nasal nitric oxide

Four studies have compared tidal breathing with a velum closure technique (table 5). Tidal breathing produces consistently lower nNO levels in both PCD and control patients, but preserves the significant difference between PCD and other groups [17, 22, 25, 26].

Meta-analysis of three studies [17, 25, 26] gave statistically significant differences in mean nNO between tidal breathing and velum closure for healthy controls (182.2 nL·min^{-1} *versus* 278.8 nL·min^{-1}; p<0.0001) and CF patients (91.5 nL·min^{-1} *versus* 132.7 nL·min^{-1}; p<0.0001), but not PCD (21.6 nL·min^{-1} *versus*

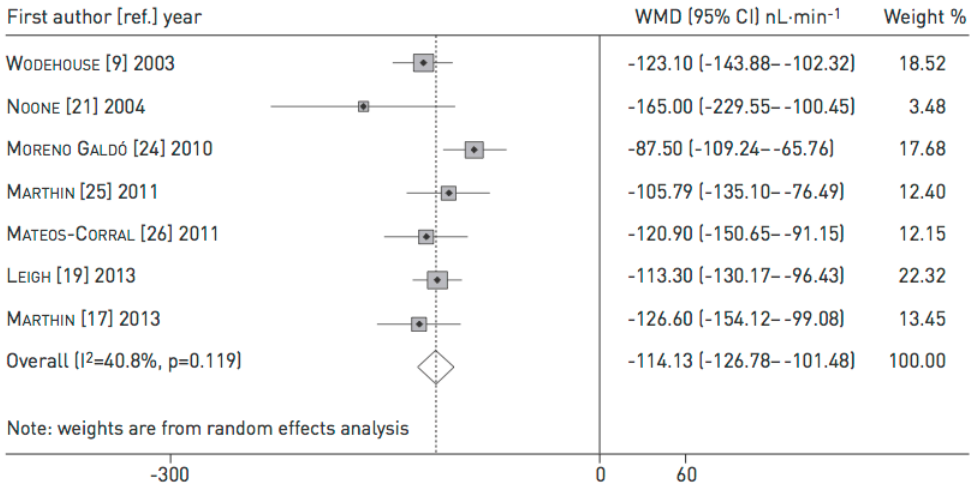

FIGURE 2 Forest plot showing weighted mean difference (WMD) in mean nasal nitric oxide between cystic fibrosis and primary ciliary dyskinesia patients using a stationary analyser and velum closure technique.

TABLE 3 Summary of studies presenting sensitivity and specificity of their cut-off values for nNO for PCD *versus* healthy patients

Study	Subjects n	nNO cut-off nL·min^{-1}	Sensitivity %	Specificity %
Mateos-Corral *et al.* [26], 2011[#]	44	60.8	100	100
Narang *et al.* [7], 2002	157	25	75	96
		62.5	97	90
Horvath *et al.* [8], 2003	102	46.8	93	95
Corbelli *et al.* [10], 2004	34	126	94	88
Marthin and Nielsen [25], 2011	94	Breath hold 52.5	91.1	100
		Oral exhalation 72.6	94.3	100
		Tidal breathing 47.4	94.4	100
Leigh *et al.* [19], 2013	227	77	98	>99.9
Marthin and Nielsen [17], 2013	57	78.6	100	100
Harris *et al.* [32], 2014	47	38	100	95
Boon *et al.* [31], 2014	226	90	89.5	87.3

nNO: nasal nitric oxide; PCD: primary ciliary dyskinesia. [#]: bronchiectasis patients included in healthy control group.

19.8 nL·min^{-1}). The standard deviations for PCD and controls are also larger in tidal breathing (48.6 and 127.4 nL·min^{-1}) than in velum closure (18.2 and 99.4 nL·min^{-1}), diminishing discriminatory value.

Three studies investigated nNO levels during a humming exhalation [26, 29, 38]. Humming rapidly empties the paranasal sinuses if the osteomeatal complex is patent and causes a peak in nNO in healthy controls but not in PCD patients [39]. Sampling rates and methods were variable; therefore, it was not possible to reliably convert ppb readings to nL·min^{-1} and differences between studies should be interpreted with caution. Montella *et al.* [38] found significant differences in mean levels between PCD (n=14), healthy controls (n=13) and CF (n=11) at 5.1 ppb, 165.5 ppb and 45.6 ppb. Mateos-Corral *et al.* [26] showed a significant ($p<0.0001$) difference in mean nNO during humming between PCD and healthy controls or CF children over 5 years of age (PCD 34.4 ppb, healthy controls 3034.9 ppb and CF 402.3 ppb; n=20, 19 and 32, respectively), with a 123 ppb cut-off being 100% sensitive. Santamaria *et al.* [29] also found a significant difference between PCD and healthy controls using humming exhalation with means of 2.8 ppb in PCD (n=14) and 212.4 ppb in healthy controls (n=14). They report a sensitivity and specificity of 100% using a nasal humming NO cut-off of 48.7 ppb.

Use of nNO as a screen for PCD in young children

Very young children are unable to cooperate with specific breathing manoeuvres. Measurement during tidal breathing is, therefore, used in this age group.

TABLE 4 nNO values in PCD, bronchiectasis patients and healthy controls

Study	Bronchiectasis		Healthy controls		PCD	
	Subjects n	nNO nL·min^{-1}	Subjects n	nNO nL·min^{-1}	Subjects n	nNO nL·min^{-1}
Narang *et al.* [7], 2002	21	133.4 (20–513.3)	53	179 (99.5–359.3)	31	15.1 (0.8–230)
Horvath *et al.* [8], 2003	31	170 (77.5–250)	37	165.8 (80.5–335.8)	14	13.6 (1.3–67.3)
Wodehouse *et al.* [9], 2003	20	220.1±49.1	16	227.7±43.7	42	19.7±11.0
Wodehouse *et al.* [9], 2003[#]	12	193.2±39.0				
Shoemark *et al.* [23], 2009	20	223±127.8	20	159.8±133.5	20	12.3±8.6
Moreno Galdó *et al.* [24], 2010	8	90.3±(63–117.5)	37	224.5±37.1	9	22.0±29.9
Mateos-Corral *et al.* [26], 2011	6	376.2±346.4	10	366.5±131.7	150	17.9±13.7
Harrison *et al.* [28], 2012[¶]	4	75.3±81.9	5	125.7±22.8	12	15.6±11.6

Data are presented as median (range) or mean±SD, unless otherwise stated. PCD: primary ciliary dyskinesia; nNO: nasal nitric oxide. [#]: Young's syndrome; [¶]: measured by portable NIOX MINO analyser.

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TABLE 5 Studies reporting measurements using the tidal breathing technique of nasal nitric oxide sampling for PCD, healthy and cystic fibrosis patients *via* a stationary analyser, velum closure measurements are shown if they were performed in the same set of patients

Study	PCD			Healthy controls			Cystic fibrosis		
	Subjects n	Tidal breathing nL·min^{-1}	Velum closure nL·min^{-1}	Subjects n	Tidal breathing nL·min^{-1}	Velum closure nL·min^{-1}	Sunjects n	Tidal breathing nL·min^{-1}	Velum closure nL·min^{-1}
PIACENTINI *et al.* [22], 2008				15	100.8±17.4	206.3±29.1			
PIACENTINI *et al.* [37], 2010				43	86.3±39.1				
DEGANO *et al.* [13], 2011	5	6.5 (4.5–8)		10	105 (83.5–127)				
MARTHIN and NIELSEN [25], 2011	54	25.8±61.7	19.0±13.6	52	160.2±64.9	272.4±76.0	17	72.9±48.2	124.8±103.7
MATEOS-CORRAL *et al.* [26], 2011	20	Mouth open: 13.3±9.5 Mouth closed: 10.0±6.5	17.9±13.7	19	Mouth open: 281.3±231.9 Mouth closed: 234.7±191.8	366.5±131.7	32	Mouth open: 107.6±58.6 Mouth closed: 85.7±54.6	138.8±84.1
MARTHIN and NIELSEN [17], 2013	16	17.7±16.8	23.7±22.8	21	145.8±46.7	267±74.4	21	81.9±44.0	150.3±58.8
BOON *et al.* [31], 2014	38	8.4 (6–22.2)	16.8 (8.1–35.7)	49	150 (106.5–180)	236.4 (198.3–295.8)	46	54 (36–87)	109.5 (75.6–169.6)

Data are presented as mean±SD or median (interquartile range), unless otherwise stated. PCD: primary ciliary dyskinesia.

BARALDI *et al.* [40] reported two infants that were evaluated for PCD (at 4 and 6 months of age) and compared them with five healthy infants with a mean age of almost 4 months (range 1.3–7 months). nNO levels in the two PCD patients were 9.4 and 12.7 nL·min^{-1}, while the mean for the healthy controls was 32.5 nL·min^{-1} (range 24.8 to 41.7 nL·min^{-1}). The lower levels in healthy controls make false positive results more likely [40]. STEHLING *et al.* [41] report a PCD neonate who had a nNO of less than 5 ppb on day 4 of life (sampling method not stated), while six healthy neonates (2–24 days) had a mean nNO of 171.2 ppb. MARTHIN and NIELSEN [25] reported a 16-day-old with nNO of 2.7 nL·min^{-1} and a 16-week-old with nNO of 3.3 nL·min^{-1}, both with PCD. The overall false positive rate was 39% in children under 6 years of age performing nNO during tidal breathing, nevertheless the negative predictive value was 99% [25].

PIACENTINI *et al.* [22] measured nNO in two uncooperative PCD patients and 50 healthy children less than 1 year of age during tidal breathing. The two infants with PCD had nNO levels of 2.2 and 12.5 nL·min^{-1}. They reported mean±SD nNO levels of 38.4±4.9 nL·min^{-1} in healthy children under 6 months of age (n=26) and 92.7±13.8 nL·min^{-1} for those aged 6–12 months (n=24). Their analysis showed that a cut-off of 16.2 nL·min^{-1} for less than 6 months of age had a sensitivity of 90% and specificity of 81% for diagnosis of PCD [22].

The role of portable nitric oxide meters in screening for PCD

Four studies used a portable nNO analyser; three compared this portable analyser with a stationary chemiluminescent analyser (table 6).

MONTELLA *et al.* [38] assessed stationary and portable analysers in PCD, CF and healthy control patients using nasal exhalation. HARRIS *et al.* [32] reported that many healthy controls were unable to hold their breath for sufficient time to measure nNO using the portable device (45 s was required for sampling as opposed to 20 s for the stationary device), so only tidal breathing measurements were used. MARTHIN and NIELSEN [17] reported successful breath hold for 45 s by 70% of participants in their study (*versus* 86% for the stationary device and 100% for tidal breathing).

Portable sampling at 2 mL·s^{-1} showed no advantage over 5 mL·s^{-1} and breath hold could not be performed with the former [17, 32].

MARTHIN and NIELSEN [17], similar to the other three studies [28, 32, 38], confirmed significant differentiation between PCD, CF and healthy controls was obtained using the portable analyser ($p<0.0001$) using both tidal breathing and velum closure. Three studies performed receiver operating characteristic (ROC) analysis. MONTELLA *et al.* [38] reported a cut-off of 6.9 nL·min^{-1} for silent nasal exhalation gave a sensitivity of 100% and a specificity of 85% to detect patients with PCD. HARRIS *et al.* [32] reported a cut-off of 30 nL·min^{-1} had 100% sensitivity and 95% specificity. MARTHIN and NIELSEN [17] had cut-offs of

TABLE 6 Summary of studies reporting nasal nitric oxide measurements with a portable nitric oxide analyser in PCD, healthy controls and cystic fibrosis patients, readings from a stationary analyser are shown for comparison if they were measured

Study	PCD		Healthy controls		Cystic fibrosis	
	Stationary	Portable	Stationary	Portable	Stationary	Portable
MONTELLA *et al.* [38], 2011[#]	Median 1.5 (range 1.0–2.3), n=14	Median 2.6 (range 1.9–3.5), n=14	Median 13.7 (range 9.6–19.5), n=13	Median 12.4 (range 8.9–17.6), n=13	Median 9.5 (range 7.0–13.0), n=11	Median 13.4 (range 10.4–17.3), n=11
HARRISON *et al.* [28], 2012[¶]		Mean±SD 7.2±4.1, n=4		Mean±SD 125.7±22.8, n=5		Mean±SD 12±11.7, n=6
MARTHIN and NIELSEN [17], 2013[+]	Mean±SD 23.7±22.8, n=16	Mean±SD 19.2±18.7, n=12	Mean±SD 267±74.4, n=20	Mean±SD 180.9±57.7, n=21	Mean±SD 150.3±58.8, n=21	Mean±SD 97.2±41.6, n=8
HARRIS *et al.* [32], 2014[§]	Median 12.3 (IQR 8.4), n=11	Median 5.4 (IQR 3.3), n=12	Median 208.2 (IQR 58.8), n=15	Median 112.6 (IQR 88.0), n=15		

All nasal nitric oxide measurements have units of nL·min^{-1}. PCD: primary ciliary dyskinesia; IQR: interquartile range. [#]: nasal exhalation; [¶]: tidal breathing; [+]: breath hold; [§]: tidal breathing (MINO Breathhold – Flex).

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64 nL·min^{-1} for breath hold (sensitivity 100%, specificity 95.2%) and 43 nL·min^{-1} for tidal breathing (sensitivity 100%, specificity 100%).

Discussion

This systematic review and meta-analysis confirms that nNO is significantly lower in PCD than in healthy individuals or patients with respiratory diseases including CF. Screening for PCD by measuring nNO during a velum closure manoeuvre is accurate with good sensitivity and specificity. Measurement of nNO during tidal breathing is relatively easy to perform in non-cooperative children and provides a reasonable screening test, but is less sensitive than velum closure. Healthy young children have much lower nNO than adults or older children and false positives during screening are, therefore, more common. Although most studies have used stationary chemiluminescence analysers, a handful of small studies suggest that portable analysers are reliable for measuring nNO as a screening test for PCD. It is important for those using portable analysers to understand the limitations of these devices. As well as differing in the means of analysis, portable analysers do not display the sampling in real-time so there is no way of checking the validity of the measurement (for example ensuring a steady plateau reading is taken). Plateau visualisation can ensure the reading is not affected by nares leak or lower airway contamination. NIOX portable *versus* stationary analysers are compared more fully by HARRIS *et al.* [32]. There is a need for standardised protocols and training at sites distant to the PCD diagnostic centre whether using portable or stationary analysers.

In assessing diagnostic accuracy of nNO, choosing a cut-off nNO level that is three standard deviations from the mean would include all but 0.15% of PCD patients; using data from the meta-analysis (table 1) this cut-off is 75.2 nL·min^{-1}. Individual studies suggested cut-offs ranging from 38 to 126 nL·min^{-1} (table 3), with the largest study (n=227) calculating that a cut-off of 77 nL·min^{-1} has sensitivity of 98% [42]; while MARTHIN and NIELSEN [17] found a cut-off of 78.6 nL·min^{-1} provided them with a sensitivity of 100%. 77 nL·min^{-1} is equivalent to 257 ppb on NIOX Flex/MINO, 233 ppb on Eco Medics CLD 88sp, 308 ppb on LR2000 and 144 ppb on Sievers. High sensitivity is important to select all PCD patients for further diagnostic testing, but it is important to note that the cut-off level of 77 nL·min^{-1} is for children >5 years of age and adults using velum closure manoeuvres. Further work is needed to establish cut-offs for younger children, for tidal breathing and for portable devices.

Respiratory manoeuvres during measurement are one of the topics requiring standardisation. For example, a variety of methods are used to achieve velum closure that is required for a "true" plateau measurement. Velum closure can be achieved during voluntary manoeuvres (making a "k" sound) or during breath hold, but is easier to achieve while blowing against a resistor. If a plateau is not reached the measures will be lower and the cut-off less discriminatory. This is demonstrated by results from MARTHIN and NIELSEN [25] where the cut-off for measurement during velum closure during exhalation against a resistor is higher than during breath hold (table 3).

Rarely patients with PCD have nNO levels in the normal range [19, 25]. MARTHIN and NIELSEN [25] examined the characteristics of the five subjects in their study with PCD and normal nNO, and found all had classical symptoms such as chronic wet cough and persistent nasal secretions from birth; all were subsequently discovered to have both beat frequency and ultrastructure abnormalities. This highlights need for PCD diagnostic testing in those with strong clinical suspicion even if nNO is normal.

A number of studies suggest that patients with CF have nNO concentrations that are lower than in healthy controls (tables 1 and 2). Although we have demonstrated that levels are generally greater than in PCD, occasional CF patients have nNO in a similar range to PCD patients [19, 25, 26]. This may be due to nasal polyposis [31], reduced levels of inducible nitric oxide synthase [43] or changes in arginine metabolism [44].

In the small numbers of patients studied, there remains a highly significant difference in nNO levels between PCD, CF and healthy groups measured during tidal breathing despite the influence of lower airway gas mixing (table 5), but the discrimination is poorer than measurement during velum closure [17, 19, 25, 32]. Tidal breathing is the only option for screening younger children who are unable to cooperate with breathing manoeuvres; as nNO levels are lower in healthy children than healthy adults [37] false positive rates are as high as 39% in children under 6 years of age [25]. However, PIACENTINI *et al.* [22] found a cut-off of 16.2 nL·min^{-1} was 90% sensitive for a PCD diagnosis in children less than 6 months of age. nNO measured during tidal breathing is, therefore, a useful adjunct to clinical suspicion in the screening of young children for PCD with good negative predictive value, but must be interpreted with caution and alongside clinical history, particularly in those under 12 months-old. It should be noted that children as young as 3 years of age were able to cooperate with velum closure in some studies [22], but in the experience of the authors, success is low in preschool children.

The cost, maintenance and training required for stationary NO analysers has led to the development of handheld sensors. The studies comparing portable and stationary analysers are small, but suggest they are a reliable means of measuring nNO [17, 32]. ROC analysis by HARRIS *et al.* [32] and MARTHIN and NIELSEN [17] produced cut-offs of 30 and 64 $nL \cdot min^{-1}$, respectively (100 and 213 ppb on NIOX, 91 and 194 ppb on Eco Medics CLD88sp), for tidal breathing nNO *via* portable analyser; both achieving sensitivity of 100%.

There were a number of limitations to the data available for the meta-analysis. The meta-analysis found a high degree of heterogeneity within and between studies; therefore, combining means and standard deviations must be interpreted with caution. The higher degree of heterogeneity in the healthy control comparison may reflect that most studies included children. This may skew results in controls, as STRUBEN *et al.* [xx] reported that nNO is age related under 12 years of age and found the mean nNO in 6–17 year-olds to be 135 $nL \cdot min^{-1}$ (meta-analysis healthy control mean: 271.6 $nL \cdot min^{-1}$). Also, many studies report parametric summary statistics for an item that cannot be less than zero but with a relatively large standard deviation, and we have not been able to interrogate the source data to validate this assumption of normality. The studies that reported nonparametric summary statistics could not be incorporated into the meta-analysis. In addition, we converted a number of different sampling techniques into $nL \cdot min^{-1}$ values and, although there is some evidence that this is a valid conversion [17, 19], differing sampling rates may have different effects depending on the size of the nasal cavity and whether there is a patent sinus meatus with normally developed sinuses beyond.

In conclusion, there is strong evidence that nNO is reduced in PCD compared with healthy controls and other patient groups, supporting the international expansion of nNO as a screening test for PCD. Our review has demonstrated that measurement of nNO as a screening test is accurate using a variety of manoeuvres and analysers. Efforts are now needed to standardise the reporting of measurements. The review confirms the following. 1) nNO measurement during velum closure *via* breath hold or oral exhalation has the best discriminatory ability, but measurement during tidal breathing is simpler to perform. 2) A nNO level below 77 $nL \cdot min^{-1}$ (NIOX 257 ppb; Eco Medics 233 ppb; Sievers 144 ppb; and LR2000 308 ppb) should be considered highly suggestive of PCD and warrants further assessment in a specialist diagnostic centre. However normal nNO levels are occasionally reported in PCD. Further studies are required to develop cut-offs for children <5 years of age, for tidal breathing and for portable devices. 3) nNO levels are low in those with "atypical" PCD, including those with normal ultrastructure on electron microscopy. 4) nNO levels are lower in all preschool children, therefore, they must be interpreted with the clinical picture. There are a high number of false positives, particularly in infants. 5) Readings using a hand-held analyser appear to provide an accurate screening test for PCD patients, but the numbers of patients in these studies have been small, and experience from the clinical setting is now needed. The hand-held analyser that is currently available requires a long breath hold, with resultant poor success rates for obtaining a measurement using this manoeuvre. 6) Tidal breathing nNO measurement can be a useful adjunct in the screening of young children or adults who cannot cooperate with velum closure. It should be noted that the rate of false positive results appears high in infants, although data are sparse.

This review has demonstrated a lack of uniformity for methods and reporting. All methods to measure nNO provided good discrimination between PCD and non-PCD groups, but the accuracy of some methods was better than others. Moving forward, we advocate that evidence-based or consensus guidelines are urgently required for methods at different ages and in different clinical settings (*e.g.* specialist PCD centre *versus* satellite referral centre). This will be one of the areas for consideration by a newly commissioned ERS Task Force: Diagnosis of PCD in a molecular age: a practice guideline for diagnosing patients with PCD).

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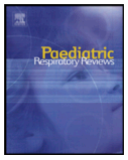

Short Communication

Overcoming challenges in the management of primary ciliary dyskinesia: The UK model

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ARTICLE INFO

SUMMARY

Primary Ciliary Dyskinesia (PCD) is an autosomal recessive disease associated with bronchiectasis, chronic rhinosinusitis, infertility and *situs inversus*. Estimates of prevalence vary widely, but is probably between 1:10,000- 1:40,000 in most populations. A number of observational studies indicate that access to services to diagnose and manage patients with PCD vary both between and within countries. Diagnosis is often delayed and frequently missed completely. The prognosis of patients with PCD is variable, but evidence suggests that it is improved by early diagnosis and specialist care. This article briefly reviews the literature concerning PCD and the evidence that specialist care will improve healthcare outcomes. The article specifically refers to a new national service in the UK.

Primary Ciliary Dyskinesia (PCD) is an autosomal recessive disease associated with bronchiectasis, chronic rhinosinusitis, infertility and *situs inversus*. Estimates of prevalence[1] vary widely, but is probably between 1:10,000- 1:40,000, with much higher prevalence in certain ethic groups, for example the Asian population of Bradfordwhere it has been shown to be 1 in 2,200.[2] PCD is often diagnosed late after a prolonged period of recurrent upper and lower respiratory infections which can lead to irreversible lung damage. The lack of recognition of signs and symptoms is common, frequently resulting in misdiagnosis and treatment as 'difficult asthma'. Age at diagnosis of patients in UK and Denmark has been reported as 4.4 and 8.1 years respectively,[3,4] significantly older than patients diagnosed with cystic fibrosis. Early diagnosis and specialist management is necessary, amongst other things, to prevent the progression of suppurative lung disease, ensure appropriate treatment of hearing impairment and counsel the family regarding genetic inheritance. For older patients the issues of fertility need careful consideration.

The diagnosis of PCD requires specialist assessment of cilia function and ultrastructure, which until 2006 was primarily undertaken within three research establishments in the UK. Respiratory paediatricians from these three centres recognised the vulnerability caused by lack of clinical infrastructure. Over several years they worked with the PCD Family Support Group, establishing multidisciplinary networks to develop standards of care, conduct surveys and identifying the clinical need. The data formed the basis of a business plan to lobby for national funding. In 2006 the National Specialist Commissioning Team (NSCT) funded a specialist service for the diagnosis of PCD in England.[5] The diagnostic service comprises three centres, with state of the art diagnostic facilities and a multidisciplinary team. Testing includes[6] measurement of nasal nitric oxide, assessment of ciliary function using high-speed video analysis of ciliary beat pattern in addition to ciliary beat frequency and ultrastructural analysis of cilia by electron microscopy. The UK diagnostic service has successfully diagnosed over 200 children with the condition, but subsequent follow-up has remained a post-code lottery. Although some patients receive excellent management in line with national and international consensus guidelines[6,7] many receive inappropriate

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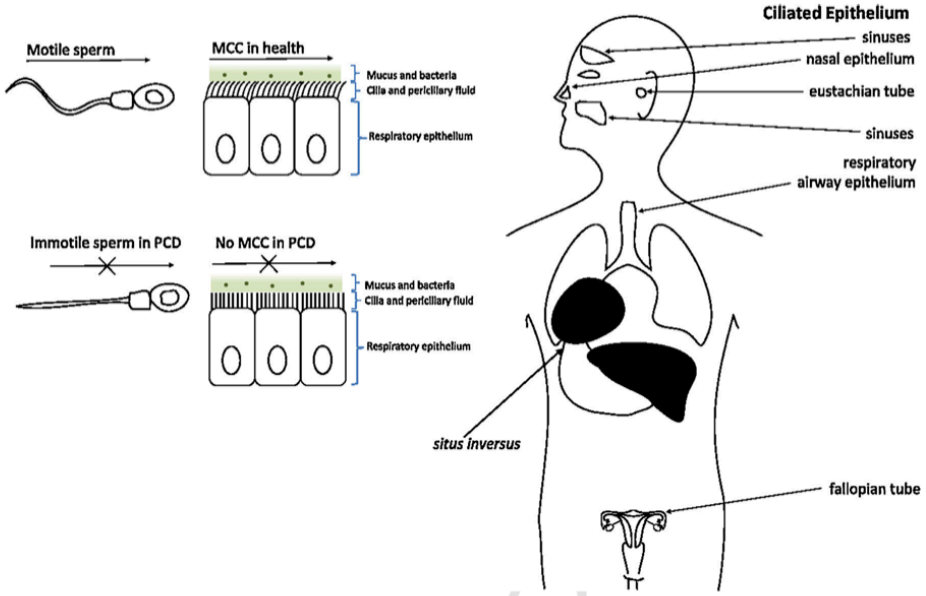

Figure 1. In healthy individuals respiratory cilia lining the upper and lower respiratory tracts beat in a coordinated sweeping pattern, moving mucus and debris, including bacteria towards the oropharynx for swallowing or expectorating. Cilia lining the Eustachian tubes are responsible for clearing fluid from the middle ear and those lining the fallopian tubes facilitate transport of the ova to the uterus. PCD cilia do not beat effectively, and mucus, pathogens and allergens persist in the airways leading to chronic lung disease and rhinosinusitis. Glue ear is common, sperm are often immotile, and 50% of patients have situs inversus caused by impairment of motile cilia in the embryonic node. (Figure provided by Robert Scott).

or no respiratory or ENT care following diagnosis (PCD Support Group Survey, 2011). The multidisciplinary PCD specialists worked with the PCD Support Group to gather supporting evidence for a national service to manage children following diagnosis. Case studies demonstrating the outcome of poor management were recorded. Support was obtained from a number of organisations including Royal College of Paediatrics and Child Health, British Thoracic Society and British Paediatric Respiratory Society. A business case was made based on health, social and financial benefits of a national service. This led NSCT to commission a service to provide specialist multidisciplinary team (MDT) care annual review and advice service for PCD, which will become fully operational from 2013. The four centres, based in Leeds/ Bradford, Leicester, London and Southampton, will provide annual review by a MDT of ENT and respiratory PCD specialists for children from England and Scotland. Specialist advice will be available to professionals involved in the care of these patients. Children from other nations will be welcome following funding agreements.

WHAT IS PRIMARY CILIARY DYSKINESIA?

Motile cilia are microscopic organelles on epithelial cells lining the respiratory airways, nasal and sinus cavities, Eustachian tubes, brain ventricular system and fallopian tubes. The coordinated sweeping movement of the cilia clear mucus and debris [Figure 1]. Mutations in genes encoding ciliary proteins lead to ultrastructural defects that impair ciliary function, and thus mucociliary clearance.[1] The resulting syndrome of PCD was formally known as Immotile Cilia Syndrome, or if *situs inversus* is present, Kartagener's syndrome.

Newborns with PCD commonly fail to clear airway secretions efficiently and may present with neonatal respiratory distress, tachypnoea and nasal congestion. They continue to have a persistent wet cough and nasal symptoms. Failure to clear secretions, particles and bacteria leads to chronic inflammation and infection which eventually cause bronchiectasis and chronic rhinosinusitis. Inadequate clearance of middle ear fluid along the Eustachian tubes causes serous otitis media and can lead to hearing deficit and delayed speech. Due to defects in the embryonic nodal cilia, approximately 46% of patients have *situs inversus*, and 6% have ambiguous *situs* with a high incidence of congenital heart defects. Sperm flagella have similar ultrastructure to cilia, and male infertility is usual. Female subfertility and a higher risk of ectopic pregnancy may be caused by impaired ciliary activity in the fallopian tubes. Abnormal function of motile ependymal and sensory cilia explain the rare associations of PCD with hydrocephalus, polycystic kidney disease and retinitis pigmentosa.

HOW SHOULD PCD BE MANAGED?

Patients with a suggestive history [Table 1] should be referred to a PCD diagnostic centre where investigation will include screening by nasal nitric oxide levels and assessment of ciliary function and ultrastructure using specimens obtained by nasal brushing with a cytology brush.[1,6] Culture of the epithelial cells with re-differentiation of cilia at an air-liquid interface allows reassessment of the cilia to exclude environmental reasons for dyskinesia in the original sample (eg. infection). PCD is a polygenic disorder; to date mutations have been identified in 20 genes accounting for approximately 50% of families with PCD. Routine genetic testing is therefore not part of the current diagnostic portfolio in UK, although the rapid increase in reported genes is soon likely to lead to genetics testing becoming part of the work-up.

In common with other rare diseases, the evidence base for managing PCD is primarily limited to consensus of experts,[7–9]

Table 1
Who should be referred for PCD Diagnostic testing?

Neonate
Situs inversus plus respiratory or nasal symptoms
Neonatal respiratory distress of unknown cause
Persistent rhinorrhoea and cough
Sibling with PCD, particularly if symptomatic
Childhood
Persistent life-long wet cough (also consider CF, immunity defects etc.) commonly associated with rhinitis since infancy and sinusitis in older children
Unexplained bronchiectasis (also consider CF, immunity defects etc.)
Serous otitis media in association with lower and upper airway symptoms; in these patients prolonged discharge if grommets are inserted is common
Adults
Symptoms as above since early childhood, often with infertility or subfertility.

NB. a lower threshold for referral should be considered if the patient is from a consanguineous background, or is of an ethnic origin known to have increased prevalence of PCD.

using data extrapolated from more common conditions. The respiratory management is commonly based on evidence from cystic fibrosis. However the underlying pathophysiology, disease pattern and prognostic indicators are all different and disease-specific evidence is urgently required. Similarly, ENT problems associated with PCD do not respond as predicted to 'conventional' treatments, and for example grommets may increase morbidity without improving hearing. For these reasons, management led by specialists with experience specific to PCD is required.

Paediatricians managing PCD have historically considered it a 'mild' disease. Children typically have an unremitting wet cough despite physiotherapy and antibiotics, but rarely complain. This is unlike other respiratory problems such as cystic fibrosis where the occurrence of a wet cough prompts review and treatment to ensure it is cleared. They also have continual ENT symptoms, but remain systemically well. Data from a cohort of children and adults with PCD highlight the need for aggressive respiratory management to prevent the insidious decline in respiratory function which leads to 38% of adults experiencing respiratory failure of whom half are severe enough to consider for lung transplant.[10] Indeed, bronchiectasis can occur in early infancy and is present in up to 80% of children on chest CT, demonstrating the need for early instigation of appropriate treatment. A number of longitudinal studies have demonstrated that lung function deteriorates before the diagnosis of PCD, but stabilises following diagnosis and appropriate treatment.[11–13] This suggests that lung damage results from under-treatment and can be prevented by early intervention and intensive therapy. Although randomised controlled trials are urgently required to direct optimal treatment, the consensus opinion of specialists indicates that regular effective airway clearance, elimination of *Pseudomonas aeruginosa* and aggressive management of respiratory exacerbations are essential.

HOW WILL A SPECIALIST MDT IMPROVE HEALTHCARE OUTCOMES IN PCD?

The PCD service will ensure that all patients in England and Scotland have access annually to a specialist multidisciplinary team (MDT). The annual review will culminate in an individualised management plan, which will be implemented through self-care, regular review and management at the patient's local hospital or management by a specialist respiratory MDT. It will thus provide specialist review, in conjunction with local delivery of care. The PCD MDT will be available to provide advice to local providers and respiratory teams. If necessary they will assess patients between reviews and will, for example, provide bronchoscopy services. Physiotherapy equipment, nebulisers, CT scans and ultrasound scans relating to PCD are also funded. The service is not funded for the inpatient care of children with PCD and routine clinic follow up appointments.

Annual review clinics will be held at the four PCD centres. The PCD MDT will provide a small number of out-reach clinics at geographically strategic centres to ensure accessibility, for example in Birmingham. Each team includes a PCD respiratory paediatrician and specialist ENT surgeon supported by a PCD clinical nurse specialist, physiotherapist, audiologist and respiratory physiologist.

The health benefits of the service will be monitored by the NSCT using reports of lung function, audiometry and health related quality of life. Geographical monitoring and accessibility for minority groups will also be reviewed. The service will allow longitudinal monitoring of patients through a national PCD database, improving our prognostic understanding for the disease. It will also provide a registry for recruitment of patients for urgently needed clinical trials.

HOW CAN PATIENTS BE REFERRED?

As new patients are diagnosed with PCD they will be provided with follow-up options, including shared care between a PCD centre and their local services. Patients who have previously been diagnosed are invited to access the specialist PCD service, by contacting any of the PCD centres. As the service is nationally commissioned, financial agreements will not be needed for patients from England and Scotland. Children from other nations will be welcome in accordance with local and national commissioning policies. The PCD Support Group can provide advice to parents or patients regarding management options and other aspects of care (http://www.pcdsupport.org.uk).

In summary, the proposed service establishes a clinical care pathway, led by specialists in PCD management, to ensure optimal treatment of this multisystem disease. The PCD MDT will work in collaboration with local care providers to prevent or reduce severe lung disease and hearing problems. This in turn will reduce the long-term burden on health care resources.

CONTRIBUTION STATEMENT

Jane Lucas drafted the manuscript. All authors provided comment and approved the final version.

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Review

Genetic Testing in the Diagnosis of Primary Ciliary Dyskinesia: State-of-the-Art and Future Perspectives

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Abstract: Primary ciliary dyskinesia (PCD) is a heterogeneous autosomal recessive condition affecting around 1:15,000. In people with PCD, microscopic motile cilia do not move normally resulting in impaired clearance of mucus and debris leading to repeated sinopulmonary infection. If diagnosis is delayed, permanent bronchiectasis and deterioration of lung function occurs. Other complications associated with PCD include congenital heart disease, hearing impairment and infertility. A small number of longitudinal studies suggest that lung function deteriorates before diagnosis of PCD but may stabilise following diagnosis with subsequent specialist management. Early diagnosis is therefore essential, but for a number of reasons referral for diagnostic testing is often delayed until older childhood or even adulthood. Functional diagnostic tests for PCD are expensive, time consuming and require specialist equipment and scientists. In the last few years, there have been considerable developments to identify genes associated with PCD, currently enabling 65% of patients to be identified by bi-allelic mutations. The rapid identification of new genes continues. This review will consider the evidence that early diagnosis of PCD is beneficial. It will review the recent advances in identification of PCD-associated genes and

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will discuss the role of genetic testing in PCD. It will then consider whether screening for PCD antenatally or in the new born is likely to become a feasible and acceptable for this rare disease.

Keywords: primary ciliary dyskinesia; cystic fibrosis; genetic testing; screening; mutation; cilia; diagnosis

1. Introduction

Primary ciliary dyskinesia (PCD) is an inherited disorder of the function of motile cilia and sperm flagella, usually associated with abnormalities of the cilial ultrastructure as observed by electron microscopy (EM). It has an incidence of around 1 in 15,000 live births [1,2], however it is considerably more prevalent in certain populations, for example a consanguineous British Asian population has a prevalence of 1:2265 [3]. The majority of patients are symptomatic from birth and go on to have persistent or recurrent sinopulmonary infection. Around half of patients have associated situs inversus (Kartagener's syndrome) or other disorders of left-right asymmetry [4]. Diagnosis is often delayed with only half of cases identified before 5 years of age [2]. Evidence that later diagnosis is associated with poorer lung function and quality of life [5,6] highlights the need for early diagnosis. Cystic fibrosis (CF) shares a number of features with PCD including progressive bronchiectatic disease and decline in lung function. The advent of neonatal screening for CF has allowed earlier diagnosis with potential improvements in long-term lung function and morbidity [7].

Siewert first described the triad of situs inversus, bronchiectasis and sinusitis in 1904, with the term Kartagener's syndrome coined following his 1935 description. In 1976, Afzelius was the first to link this disease to cilia and termed the disease "immotile cilia syndrome" [8]. *DNAH5* was identified as a candidate gene for PCD in 2000 [9] and shortly after was characterized and confirmed to be associated with PCD and randomization of left-right symmetry [10]. Rapid advances in understanding the molecular genetic basis of PCD have been made in recent years.

Motile cilia are found in respiratory epithelium, brain ependymal cells, spinal cord and fallopian tubes whilst sharing a common axonemal structure with spermatozoa flagella. Cilial axonemes of healthy individuals have a "9 + 2" arrangement with nine pairs of microtubule doublets surrounding a central pair running the length of the ciliary axoneme (Figures 1 and 2). Attached to the peripheral microtubules are inner and outer dynein arms in which dynein, a mechanochemical ATPase, generates the force for ciliary beating. The organized structure is maintained by nexin and radial spokes. Defects in the ultrastructure cause the cilia to beat abnormally, impairing mucociliary clearance leading to sinopulmonary disease, glue ear and female sub-fertility. Abnormalities of the ultrastructure of sperm flagella can lead to dysmotility and infertility. PCD in humans rarely causes hydrocephalus. The "9 + 0" motile cilia (no central pair) are present on the embryonic ventral node and have a role in determining left-right symmetry. Abnormal ciliary function in these embryonal cilia can lead to the classic situs inversus described by Kartagener where the organs are a mirror image or other disorders of situs termed heterotaxy (including left isomerism, right isomerism, isolated dextrocardia and abdominal situs inversus) [4,11].

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Figure 1. Diagram showing the main structural elements of the human motile cilium with the "9 + 2" arrangement.

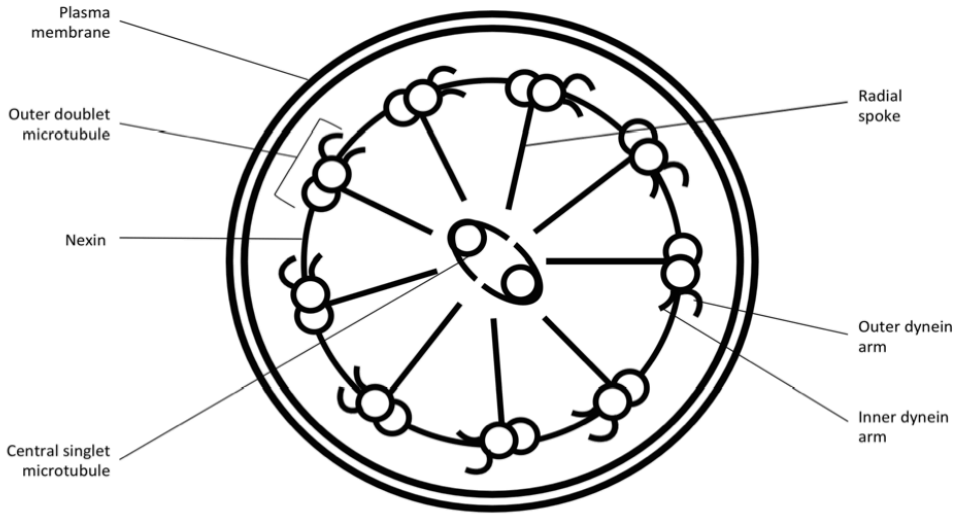
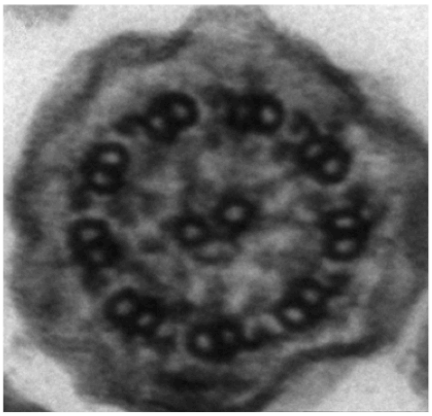


Figure 2. Scanning electron microscope image of a human cilium with normal ultrastructure.



Consistent with the many proteins involved in ciliary structure, protein assembly and intraflagellar transport PCD is a markedly heterogeneous disorder. To date, almost 30 genes have been found to be associated with PCD [12], mostly linked to specific ultrastructural defects, but it is anticipated that several hundred genes may code for proteins responsible for normal ciliary function.

Expanding knowledge of PCD genetics has raised the possibility of genetic screening for the condition, similarly to neonatal CF screening programs. Approximately 50%–60% of PCD patients have bi-allelic mutations in genes that are currently known to be associated with PCD making testing to support diagnosis a reality [12]. Current hurdles include the large number of PCD genes that are as yet unidentified, lack of a good supporting screening test for neonates, and the relatively low incidence of the disease in most populations. This manuscript reviews the need for, and possibility of, early screening for PCD in high-risk populations.

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2. Diagnosis of PCD

Diagnostic investigations for PCD are highly specialised, requiring expensive equipment and an experienced team of clinicians and scientists [13]. European consensus guidelines [14] reflect the need for access to a number of methods to ensure robust diagnosis, as there is no single "gold standard" investigation.

Ciliary defects in PCD were first identified using electron microscopy [8] and this remained the "gold standard" diagnostic tool for many years. Samples of ciliated respiratory epithelium can be collected from nasal or bronchial brushing biopsy and approximately 100 cilia examined in transverse section to identify abnormalities of the outer or inner dynein arms, central pairs or microtubular arrangement [15]. A 3%–30% of patients with PCD are reported to have normal ultrastructure [16,17] and assessment of ciliary function is therefore necessary to exclude PCD. Ciliated respiratory cells obtained by brush biopsy can be imaged by high resolution, high-speed video (HSV) microscopy [18]. The images are played back at slower speed to allow analysis of beat pattern. Ciliary beat frequency is normally 11–18 Hz (measured at 37 °C) but in patients with PCD the beat frequency is typically static, slow or hyperfrequent. Less often the beat frequency is normal but the sweep or beat pattern abnormal [15]. These dysmotile patterns are often associated with specific transmission electron microscope (TEM) defects [19], for example in outer dynein arm (ODA) or combined inner dynein arm (IDA) and ODA defects, the majority of cilia are static, whilst with central pair defects cilia make a rotating motion rather than sweeping. Recently, subtle abnormalities of beat pattern [20] have been shown to be associated with the full PCD phenotype demonstrating the need for analysis of ciliary beat pattern by experienced technicians. HSV analysis is frequently complicated by secondary ciliary dyskinesia, which is common in patients with viral infections or simply due to damage to the epithelium during sampling [21].

In recent years, additional diagnostic tests have been added to support the diagnostic portfolio, particularly where HSV analysis and electron microscope (EM) examination are inconclusive. Cell culture of biopsy samples prior to re-analysis by HSV and EM minimises secondary ciliary defects [22] and immunofluorescent analysis of proteins can define ciliary defects [23]. Radioaerosol mucociliary clearance provides an *in vivo* assessment of ciliary function [24]. However, these additional investigations are only available at a handful of centres, often as a research rather than clinical tool. Genetic testing is not yet routine in most countries but the recent rapid increase in identified mutations that is reviewed below makes it likely that PCD genetics testing will soon become part of the diagnostic pathway.

Nasal nitric oxide (nNO) is extremely low in most, but not all, patients with PCD and therefore provides a good screening test [25–28]. Nasal NO can be measured using commercially available analysers, which sample gas from the upper airway (transnasal flow) during breath-holding or tidal breathing [29]. Infants with PCD have low nNO [30,31], and measurement during tidal breathing is possible in most young children [32]. The main drawback of nNO as a screening test in infants is poor specificity since approximately 40% of healthy young children have low levels [33]. There is therefore no reliable screening test in the group who would benefit most from screening.

In summary, ciliary EM and HSV are technically challenging, labour intensive processes that should be undertaken only in specialist referral centres. Expanded genetic testing might help in

identifying patients that need functional assessment and improving the diagnostic process, thus impacting on the need to diagnose patients at an early age.

3. The Need for Early Diagnostics

The majority of PCD patients have neonatal symptoms and around half have situs inversus but, despite these signs, diagnosis is often delayed. This prevents early onset of regular airway clearance therapy, aggressive management of infections, monitoring and treatment of hearing impairment and genetic counselling for the family. In a series of 55 cases, Coren *et al.* found the median age at diagnosis to be 4 years, even though 67% had neonatal respiratory distress and 69% had abnormal situs [5] (with a prevalence of 50% in PCD, this suggests under-recognition in those with normal situs). Although 45% of patients had both neonatal symptoms and situs inversus, only half of these children were diagnosed before the age of 1 year [5]. Recent survey data from across Europe [2] showed the average age at diagnosis to be 5.8 years in those without situs inversus and 3.5 years in those with it. It was also noted that diagnosis was earlier in centres caring for more than 20 PCD patients (3 years *vs.* 4 years) emphasizing the importance of clinical suspicion and access to diagnostic tests [2]. By comparison, the average age of diagnosis for CF is 1.3 years even though it is unusual for these patients to have any neonatal symptoms unless they present with meconium ileus [5]. This emphasises the importance of clinical suspicion and identification of those at risk as any advances in genetic testing will only be useful once applied to the appropriate group of patients.

Early diagnosis of PCD has the potential to improve patient outcomes; 12 of the patients in the Coren series already had bronchiectasis at diagnosis [5]. Age at diagnosis has been shown to affect long-term lung function [6] whilst an observational study from North America [34] highlighted that PCD can lead to severe respiratory disease in adulthood with a high percentage developing respiratory failure and requiring lung transplant. Ellerman and Bisgaard showed that, unlike CF, lung function was relatively stable once therapy with antibiotics and airway clearance was initiated [6]. It is anticipated that whilst early diagnosis will delay disease progression and improve morbidity, knowledge of the diagnosis can also direct appropriate therapy choice; for example, treatment of hearing impairment and rhinosinusitis should be treated by specialists with an understanding of PCD as treatment options may be different to the general population.

PCD is therefore a disease that presents with early symptoms and can progress to significant, irreversible lung disease but which is amenable to early intervention meaning there are significant potential benefits from early diagnosis.

4. Genetics of PCD

PCD is primarily an autosomal recessive disease. Unlike CF, PCD is a markedly genetically heterogeneous condition with mutations in the 27 known genes (Table 1) accounting for 50%–60% of PCD cases [12]. Whilst the *CFTR* gene associated with CF was identified in 1989 [35], it was not until 2000 that the first gene associated with PCD was reported [9]. The gene was *DNAH5* which is a cause of defects of the dynein arms. *DNAI1* identification followed soon after [36]. *DNAI1* or *DNAH5* mutations account for the majority of genetic mutations in North America [10,37]. Approximately 50%–60% of PCD patients have bi-allelic mutations in a known PCD gene. Most of these mutations

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correspond to a specific ultrastructural defect. For example, *ZMYND10* [38] and *DYX1C1* [39] are associated with inner and outer dynein arm defects whilst mutations in *CCDC39* and *CCDC40* lead to axonemal disorganisation and absent inner dynein arms [40]. *DNAH11* was identified in 2002 in a patient with normal ciliary ultrastructure on EM [41] and accounts for 22% of those with normal ultrastructure [42]. Recently, mutations of the *HYDIN* gene were noted to be associated with apparently normal ultrastructure using conventional EM but by using a tomography approach an abnormality of the central pair apparatus was seen [20]. Many of the early identifications of genes used a candidate gene approach but recent discoveries such as *HEATR2* and *ARMC4* have been made through ciliome, exome or whole genome sequencing [43,44].

Table 1. Genes with mutations linked to primary ciliary dyskinesia. ODA—outer dynein arms, IDA—inner dynein arms.

Gene	Structural Defect
Abnormalities in dynein proteins	
DNAI1	ODA defect (+/− IDA)
DNAH5	ODA defect (+/− IDA)
DNAH11	Beat abnormalities (normal structure)
DNAI2	ODA defect
DNALI1	ODA defect
TXNDC3	ODA defect
ARMC4	ODA defect
Genes coding for proteins responsible for assembly or transport of axonemal proteins	
KTU	ODA and IDA defects
LRRC50	ODA and IDA defects
DNAAF3	ODA and IDA defects
CCDC39	ODA and IDA defects
CCDC40	Axone disorganisation and IDA defect
CCDC103	ODA and IDA defects
CCDC114	ODA defect
HEATR2	Absent ODA
CCDC65	Cilial vibration, normal structure
ZMYND10	Absent ODA + IDA
SPAG1	Absent ODA + IDA
C21orf59	Absent ODA + IDA
Central pair abnormalities	
RSPH9	Central pair defects
RSPH4A	Central pair defects
RSPH1	Central pair defects
HYDIN	Central pair defects
Nexin-dynein complex defects	
DRC CCDC164	Nexin link missing
CCDC65	Beat abnormalities
Genes causing PCD with associated syndromes	
OFD1	Unknown
RPGR	Variable

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The majority of PCD associated genes are rare and sometimes linked to only one or two affected families. Genetic diagnostics might therefore focus on a few of the more common mutations, much as CF screening programs concentrate on only a few of the almost 2000 known *CFTR* mutations [45].

Patients with identified mutations generally have biallelic mutations at a single locus, for example two mutated copies of *DNAH5*. Given the number of different possible mutations at each locus, the disease phenotype will often be the result of compound heterozygosity with the potential of mutations at different loci combining to cause a clinical phenotype. Indeed, single allele *DNAH5* mutations are found in around 7% of PCD patients with no other mutation found [37]. Similarly, a patient with a single allele *DNAH11* mutation has been reported with a classical PCD phenotype [46].

A challenge facing researchers seeking new mutations is the huge volume of genetic data generated by advances in sequencing and the need for sophisticated bioinformatics. Techniques such as whole exome sequencing produce a huge number of variations and require sophisticated algorithms to analyse and process these variations. International collaborations advancing our knowledge of disease associated variation ensure data can be appropriately analysed and verified [47].

5. Comparison of Primary Ciliary Dyskinesia and Cystic Fibrosis

Although PCD and CF are both recessively inherited causes of chronic suppurative lung disease, there are a number of differences in both pathogenesis and aetiology. CF is associated with defects of a single gene, the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which encodes the chloride channel of the same name. To date, almost 2000 *CFTR* mutations have been identified [45]. Targeted mutation analysis with the American College of Medical Genetics 25 mutation panel detects at least one mutation in 88% of CF cases [48] whilst sequence analysis detects up to 98.7% of known *CFTR* mutations [49]. Neonatal CF screening is now in place in a number of countries worldwide including the U.S., UK and Australia, and many parts of Europe, Russia and Canada; though not all of these include DNA analysis within their newborn screening program. All screening protocols rely on the collection of blood spots in the newborn period for analysis of immunoreactive trypsin (IRT) levels. In the UK, those with IRT above the 99.5th centile are sent for analysis of the four commonest mutations with two mutations in a patient resulting in a label of "probably CF". The presence of a single mutation leads to further 29–31 gene analysis whilst the presence of no mutations leads to further IRT analysis [50,51]. The U.S. approach varies across states whilst European practice varies widely, for example, Poland use an initial 640 mutation panel with complete sequencing of the *CFTR* gene if only one mutation is found [52]. Although very sensitive, this last approach can yield a high number of mutations of variable penetrance and expression. The potential benefits of NBS in PCD were demonstrated by a study of CF in London that NBS showed a reduction in the median age at diagnosis (excluding those with meconium ileus) from 2.4 years to 3 weeks [53].

PCD is currently associated with 27 different genes but with several hundred different proteins potentially affected, the task of identifying disease causing mutations is made all the more difficult. This difficulty is reflected in the fact that only 60% of North American PCD cases have an identified genetic mutation [54], the same proportion that are accounted for by a single mutation in CF (δF508 homozygotes) [50]. Targeted mutation analysis has not been effective in PCD as each gene may have many different mutations associated with clinical disease, each of which is very rare; however next

generation sequencing panels have been developed that can screen known genes by comparing them to reference genomes. Next generation sequencing techniques, such as whole exome sequencing, may also expand the number of known disease associated loci [43].

Another major difference in CF is that the NBS has IRT as a reliable investigation to screen patients prior to genetic testing [55]. Effective PCD screening should also rely on a simple screening test with nasal nitric oxide (NO) a seemingly good candidate as it forms part of the European consensus guidelines [14]. However, specificity is unacceptably low in young children, precisely the group that screening needs to target. At the moment, we are therefore dependent on increased awareness of PCD by neonatologists and family doctors who should refer to specialist diagnostic centres when concerned [13]. As more genes are recognized and the cost of genetic testing comes down, it is likely that patients with even mild neonatal symptoms will be able to have DNA samples sent for screening with less need to travel to a specialist centre for brushing biopsy.

Screening and genetic testing does not replace formal diagnostic studies; sweat testing still forms part of the CF diagnostic pathway and it is likely that functional ciliary assessment will continue to be required for a diagnosis of PCD to be made. However, CF patients have benefited from increasing genotype-phenotype correlation and, in the most striking example, genotype specific treatment with ivacaftor for *G551D* mutations [56]. Greater genotype-phenotype classification is an area where PCD patients may derive most benefit from genetic testing.

6. Conclusions

The combination of low incidence and relatively low sensitivity of genetic testing in PCD means that general population screening is not likely to be viable in the near future, however it may be appropriate in populations where PCD is common, for example the Asian population of Bradford [3]. The incidence of situs inversus in the general population is thought to be around 1 in 10,000 with many of these cases associated with PCD [11], therefore all cases with any respiratory symptoms should be referred for assessment and the availability of rapid genetic testing may aid diagnosis. It may also be possible to screen neonates with persistent tachypnoea and other features suggestive of PCD with rapid genetic sequencing; though it would be important to consider the sensitivity of genotyping so the correct children could also be assessed using HSV and EM assessments.

PCD and CF are both autosomal recessive disorders causing chronic suppurative lung disease, raising the possibility of applying some of the CF genetic screening successes to PCD. The difficulties in achieving this reflect that CF is related to just one gene (*CFTR*) with mutation detection possible in up to 98.7% of cases whilst PCD involves 27 known and several hundred potential genes, a variety of mutations within each of these genes and, at present, the ability to detect a mutation in only 60% of cases. Additionally, in CF IRT provides a good newborn screening test, but in PCD low nNO, which is a good screening test in older children and adults, is not sufficiently specific in infancy. Genetic testing does not currently form part of the European diagnostic pathway in PCD [14], however, next generation sequencing techniques will both expand the known disease loci in PCD and improve the feasibility of rapid gene sequencing; thus increasing the role of genetic testing in PCD. Additionally, as genotype-phenotype correlation is improved, patients may benefit from more specific information on disease characteristics and, potentially, mutation specific treatments. The need for cilial structure and

beat analysis is only likely to be reduced once NO screening, genetic testing sensitivity and genotype-phenotype correlation are suitably robust.

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Author Contributions

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Conflicts of Interest

The authors declare no conflict of interest.

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Complexity, Temporal Stability, and Clinical Correlates of Airway Bacterial Community Composition in Primary Ciliary Dyskinesia

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Primary ciliary dyskinesia (PCD) is a genetic disease characterized by abnormalities in ciliary function, leading to compromised airway clearance and chronic bacterial infection of the upper and lower airways. The compositions of these infections and the relationships between their characteristics and disease presentation are poorly defined. We describe here the first systematic culture-independent evaluation of lower airway bacteriology in PCD. Thirty-three airway samples (26 from sputum, 7 from bronchoalveolar lavage [BAL] fluid) were collected from 24 PCD patients aged 4 to 73 years. 16S rRNA quantitative PCR and pyrosequencing were used to determine the bacterial loads and community compositions of the samples. Bacterial loads, which ranged from 1.3×10^4 to 5.2×10^9 CFU/ml, were positively correlated with age ($P = 0.002$) but not lung function. An analysis of ~7,000 16S rRNA sequences per sample identified bacterial species belonging to 128 genera. The concurrently collected paired samples showed high bacterial community similarity. The mean relative abundance of the dominant genera was 64.5% (standard deviation [SD], 24.5), including taxa reported through standard diagnostic microbiology (members of the genera *Pseudomonas*, *Haemophilus*, and *Streptococcus*) and those requiring specific *ex vivo* growth conditions (members of the genera *Prevotella* and *Porphyromonas*). The significant correlations observed included a positive relationship between *Pseudomonas aeruginosa* relative abundance and age and a negative relationship between *P. aeruginosa* relative abundance and lung function. Members of the genus *Ralstonia* were also found to contribute substantially to the bacterial communities in a number of patients. Follow-up samples from a subset of patients revealed high levels of bacterial community temporal stability. The detailed microbiological characterization presented here provides a basis for the reassessment of the clinical management of PCD airway infections.

Primary ciliary dyskinesia (PCD) is a genetic disease characterized by abnormal ciliary structure and function. While variations in diagnostic approaches make PCD prevalence difficult to determine accurately, the reported incidence rates range from 1 in 2,000 to 1 in 40,000 (1).

Abnormal ciliary function leads to impaired mucociliary clearance, chronic airway infection and inflammation, bronchiectasis, and chronic otitis media (2). The consequence is airway stasis, which is a unique characteristic of PCD compared with those of other chronic lung diseases. In addition, the vast majority of patients with PCD have low nasal nitric oxide (nNO) levels, which have a potential impact on innate immunity (3). Chronic productive cough and nasal congestion are almost universal, with chronic bronchitis, recurrent pneumonia, and bronchiectasis also being common in PCD patients. Lung disease in PCD begins early in childhood, with considerable variation in the progression and severity between individuals (1, 4). Situs inversus is present in approximately half of patients (4).

While chronic infections of the PCD airways are strongly associated with morbidity and mortality (5), relatively little is known about their bacterial composition. An observational culture-based study from the United States reported nontypeable *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and oropharyngeal species to be most frequently isolated from children and adolescents, with *Pseudomonas aeruginosa* and nontuberculous mycobacteria being more common in adults and patients with advanced lung disease (6). However, the extent to which these findings are representative of PCD patients more widely, and the prevalence of species that are refractory to growth under conditions that are used in standard diagnostic microbiology, are not known.

Culture-independent analytical techniques, including next-generation sequencing, have allowed for the detailed characterization of lower airway bacterial communities associated with a range of obstructive airway diseases. Such analyses have typically revealed much greater bacterial diversity than is reported through standard diagnostic microbiology (7–12), with a substantial contribution often made by species requiring anaerobic conditions for growth, including members of the genera *Prevotella*, *Veillonella*, *Propionibacterium*, and *Actinomyces* (8, 13).

In most cases, evidence of a direct pathogenic role in lower

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TABLE 1 Summarized characteristics of study population

Subject characteristics[a]	Data
Male/female ratio	8/16
Age range (median) (yr)	4–73 (15)
FEV_1 % pred (% [no.])	
>70	60 (15)
40–69	32 (8)
<40	4 (1)
Stable at time of sampling (% [no.])	84 (21)
Antibiotic therapy at time of sampling (% [no.])	72 (18)
ICS inhaler (% [no.])	48 (12)
Wet cough (% [no.])	92 (23)
Rhinitis (% [no.])	84 (21)
Sinusitis (% [no.])	40 (10)
Situs inversus (% [no.])	48 (12)

[a] One patient was too young for spirometry. Information regarding antibiotic treatment and inhaled corticosteroid inhaler use was not available for one patient. FEV_1 pred (%), forced expiratory volume in 1 s, expressed as a percentage of the predicted volume; ICS, inhaled corticosteroid.

airway infections for any single species remains to be demonstrated. However, in cystic fibrosis (CF) (10, 14–16) and non-CF bronchiectasis (17), the characteristics of the bacterial community as a whole, such as the number of bacterial species present (richness) or their relative abundance (evenness), have been correlated with clinical measures of disease severity and progression.

Whether similar bacterial communities develop in PCD airways as a result of impaired clearance has not been reported previously. Obtaining a comprehensive and detailed characterization of PCD lower airway bacteriology is therefore important, both to identify the species that might contribute directly to airway disease and to determine the potential markers of disease progression. Our aim was to perform the first systematic evaluation of lower airway bacteriology in PCD. In keeping with other conditions where airway clearance is compromised, we hypothesized that a culture-independent analysis of PCD respiratory samples would reveal a greater bacterial diversity than that reported through diagnostic microbiology, and further, that the composition of this airway microbiota would correlate with clinical measures of disease.

MATERIALS AND METHODS

Twenty-four patients were recruited from the adult and pediatric PCD clinics at University Hospital Southampton. All had been diagnosed with PCD by the national PCD service according to international diagnostic guidelines (4, 18). Written informed consent was provided by patients or parents (local and national research and development [R&D] and ethical approvals CHI395 and 07/Q1702/109). The study population data are summarized in Table 1 and the details, including those of the follow-up samples, shown in Table S1 in the supplemental material. Ciliary abnormalities and cases of situs inversus are detailed in Table S2 in the supplemental material.

Twenty-six spontaneously expectorated sputum samples were collected between July 2012 and February 2013 during routine appointments (if stable) or during exacerbations. Exacerbations were defined as a change in respiratory symptoms that the PCD specialist considered to be caused by a lower respiratory tract infection requiring antibiotic therapy. In addition, 7 bronchoalveolar lavage (BAL) fluid samples had been collected from 5 PCD patients between 2002 and 2010. The sputum and BAL fluid samples for molecular analysis were transferred to the laboratory on ice and stored at −80°C.

Standard diagnostic microbiology was performed on sample aliquots at the Health Protection Agency South East Laboratory, in accordance with standard protocols. Bacteria were pelleted from the BAL fluid samples by centrifugation, and the sputa and BAL fluid pellets were transported to King's College London for molecular analysis.

Molecular analysis of bacterial community composition. DNA extractions were performed as described previously (17). An estimation of bacterial cell numbers per unit volume of respiratory samples was achieved by quantitative PCR (qPCR) as described previously (10). Amplicon pyrosequencing (bacterial tag-encoded FLX amplicon pyrosequencing [bTEFAP]) was also performed as described previously (19). The detailed protocols for all analytical steps are provided in the supplemental material. Statistical analysis was performed using a variety of computer packages, including XLStat, NCSS 2007, R, and NCSS 2010. Alpha and beta diversity analyses were conducted as described previously (19–22). Unless otherwise confirmed by species-specific PCR, the detected bacteria are reported at the genus level.

A genus-specific PCR assay for *Ralstonia* was used as described previously (23). Specific PCR assays for *Ralstonia respiraculi*, *Ralstonia insidiosa*, *Ralstonia pickettii*, and *Ralstonia mannitolilytica* were performed as described previously (23, 24). *P. aeruginosa*-specific PCR was performed as described previously (25) and used to confirm the identifications achieved through pyrosequencing. The primer details are given in Table S3 in the supplemental material, and the reaction conditions are provided in the supplemental material.

RESULTS AND DISCUSSION

By performing the first systematic assessment of PCD airway microbiota, our aim was to examine the extent to which relationships exist between the features of airway bacterial community composition and clinical measures of disease. Further, we aimed to provide a microbiological basis for the management of chronic infections in this patient group.

A cross-sectional analysis was performed on samples from 24 patients (20 sputum, 4 BAL fluid). The study population was broadly reflective of the PCD population, with a range of ciliary phenotypes, ages, and severity of disease. Approximately half of the patients were receiving prophylactic antibiotics, and a similar proportion was receiving inhaled corticosteroids.

Comparison of culture-independent and culture-based analyses. The culture-based diagnostic microbiology data are shown in Table S4 in the supplemental material. In a number of instances where the recognized respiratory pathogens were reported by culture-based analysis, they were also found to have a high relative abundance in sequencing profiles. For example, *P. aeruginosa* was detected by culture in patients 5 and 15, representing 96.7 and 98.7 of the sequences, respectively. However, discrepancies were also observed. For example, in patient 16, *P. aeruginosa* represented 96.2% of sequences but was not reported by culture. Further, *P. aeruginosa* was detected in a further 8 patients at a low relative abundance but was not reported by diagnostic microbiology. Similar discrepancies were observed for *H. influenzae* and *S. pneumoniae*.

Determination of total bacterial load. The initial stage of culture-independent analysis involved an assessment of the bacterial density in the airway samples. Here, the total numbers of bacterial cells per unit volume of respiratory secretion were determined by qPCR. Bacterial loads ranged from 1.3×10^4 to 5.2×10^9 CFU/ml (mean ± standard deviation [SD], $1.0 \times 10^9 \pm 1.7 \times 10^9$ CFU/ml) and were significantly lower in BAL fluid samples than in sputum

TABLE 2 Genera detected in ≥10 patients

Genus	No. detected
Streptococcus	21
Neisseria	20
Haemophilus	20
Prevotella	20
Veillonella	20
Porphyromonas	19
Ralstonia	17
Actinomyces	16
Campylobacter	14
Corynebacterium	13
Blautia	13
Pseudomonas	12
Staphylococcus	11
Oribacterium	11
Candidate division tm7	11
Capnocytophaga	11
Selenomonas	11
Derxia	10
Tannerella	10

samples (mean ± SD, $2.2 \times 10^6 \pm 4.8 \times 10^6$ CFU/ml and $1.2 \times 10^9 \pm 1.8 \times 10^9$ CFU/ml, respectively; $P = 0.005$). These bacterial cell densities are broadly consistent with those reported in other chronic respiratory bacterial infections, such as CF and non-CF bronchiectasis, as assessed using the same analytical approach (9, 17).

The observed bacterial loads were positively correlated with patient age ($P = 0.002$, Spearman's correlation). However, a significant correlation between age and lung function decline has been reported previously (6) but was not detected here. Intuitively, increasing bacterial load might be linked with increasing disease severity, and therefore the absence of a significant relationship between bacterial load and lung function in our study is perhaps surprising. However, longitudinal studies of PCD patients suggest that the courses of lung function vary greatly between patients (26), suggesting that larger studies are required to fully define this relationship.

Airway bacterial community composition. The types of bacteria present in the PCD airway samples were assessed by 16S rRNA gene pyrosequencing. A total of 311,996 sequences were derived from the sample collection. After stringent sequence curation, a total of 187,071 bacterial sequences were carried forward, representing an average of 7,482 sequences per sample (range, 902 to 17,586).

Across the sample collection, 128 separate genera were detected, with an average of 24 per sample (SD, 11.2; range, 5 to 57). Of these, 39 genera were detected only in one patient. The detected genera were predominantly from the phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*, representing 30.4%, 28.9%, 17.2%, and 9.4% of the sequences, respectively. The occupancy of the genera ranged from 1 to 21 patients, with a mean frequency of 4.9 patients (SD, 5.1). Those genera detected in ≥10 patients are listed in Table 2. Bacterial diversity was assessed using three separate measures (richness, Shannon index, and Chao1) (see Fig. S1 in the supplemental material). However, no significant correlations were found between community diversity and clinical measures.

Given that complex microbial communities exist in the oropharynx and upper respiratory tract, and that there is the potential for bacteria to be introduced to the lower airways from the wider environment, the detection of a broad phylogenetic range of bacterial species was expected. However, the relative abundances of the detected genera varied greatly. The mean relative abundance of the "dominant genus" (the genus representing the greatest proportion of total sequence identities in each sample) was 64.5% of all detected sequences (SD, 24.5; range, 19.9 to 99.5%). However, many of the detected genera were present at a low relative abundance; when rank ordered according to relative abundance, typically, only the seven most prevalent genera in each patient contributed >1% of the total bacterial signal.

TABLE 3 Predominant genera detected

Genus	No. of patients in whom the listed genus was dominant
Pseudomonas	5
Neisseria	5
Ralstonia	4
Haemophilus	4
Streptococcus	2
Prevotella	2

While the clinical significance of a particular bacterial taxon can be disproportionate to its relative abundance, in the lower airways, a context thought to be free from substantial bacterial colonization in healthy individuals, an argument can be made for focusing first on those bacteria that are greatest in number (Table 3). These dominant genera included both those likely to be reported through standard diagnostic microbiology (e.g., *Pseudomonas*, *Haemophilus*, and *Streptococcus*) and those unlikely to be detected without specific growth conditions being present (e.g., *Prevotella*). Further, the majority of genera detected in a high proportion of patients, or at a high relative abundance, were those commonly associated with the airways or oropharynx (*Pseudomonas*, *Haemophilus*, *Streptococcus*, *Neisseria*, *Prevotella*, *Veillonella*, *Porphyromonas*, and *Actinomyces*) or gastrointestinal tract or oral cavity (*Blautia* and *Campylobacter*). In a number of cases, however, genera that did not fall into either of these groups were also detected with a high relative abundance. For example, *Ralstonia* species were detected in 17 of the 24 patients (68%) by 16S rRNA gene sequencing analysis (see Table S5 in the supplemental material), with a mean relative abundance of 20.4% (SD, 31.9; range, 0.1% to 89.6%), and represented the dominant genus in four patients.

The high prevalence of *Ralstonia* in PCD airway secretions observed here warrants further consideration. While other nonfermenting Gram-negative bacilli, including *P. aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, and *Burkholderia cepacia* complex, have been recognized as common causes of respiratory infection, the role of *Ralstonia* species is less well understood. Some *Ralstonia* species have, however, previously been recovered from a range of infection contexts (27), including the CF airway, most commonly from the species *R. mannitolilytica*, *R. pickettii*, and *R. respiraculi* (23). Here, the relative abundance of *Ralstonia* species was significantly higher in BAL fluid samples than in sputum samples ($P = 0.01$, one-way analysis of variance [ANOVA]). Due to the unanticipated high incidence of the genus in the PCD samples analyzed here, genus-specific PCR was used to confirm

TABLE 4 Significant correlations between relative genus abundance levels

Data by correlate:		Spearman's coefficient	Significance (*P*)
1	2		
Pseudomonas	*Porphyromonas*	−0.574[b]	0.003
Stenotrophomonas	*Prevotella*	−0.574[b]	0.003
Stenotrophomonas	*Haemophilus*	−0.420[a]	0.036
Stenotrophomonas	*Massilia*	0.444[a]	0.026
Stenotrophomonas	*Nocardioides*	0.541[b]	0.005
Stenotrophomonas	*Ralstonia*	0.613[b]	0.001
Porphyromonas	*Streptococcus*	0.538[b]	0.006
Porphyromonas	*Moryella*	0.461[a]	0.020
Porphyromonas	*Neisseria*	0.602[b]	0.001
Porphyromonas	*Prevotella*	0.683[b]	0.000
Prevotella	*Neisseria*	0.597[b]	0.002
Prevotella	*Streptococcus*	0.453[a]	0.023
Prevotella	*Eikenella*	0.494[a]	0.012
Eikenella	*Neisseria*	0.413[a]	0.040
Haemophilus	*Streptococcus*	0.470[a]	0.018

[a] *P* value of <0.05.
[b] *P* value of <0.01.

TABLE 5 Significant correlations between genus relative abundance and patient age, FEV_1 percent predicted, and the relative abundance of dominant genus

Data by correlate:		Spearman's coefficient	Significance (*P*)
1	2		
Pseudomonas	Age	0.422[a]	0.036
Blautia		−0.399[a]	0.048
Corynebacterium		−0.594[b]	0.002
Ralstonia		−0.614[b]	0.001
Staphylococcus		−0.491[a]	0.013
Blautia	FEV_1 % pred[c]	0.459[a]	0.024
Haemophilus		0.405[a]	0.050
Tannerella		0.445[a]	0.029
Pseudomonas		−0.432[a]	0.035
Pseudomonas	Dominant genus abundance	0.413[a]	0.040
Actinomyces		−0.447[a]	0.025
Blautia		−0.521[b]	0.008
Campylobacter		−0.445[a]	0.026
Capnocytophaga		−0.603[b]	0.001
Haemophilus		−0.425[a]	0.034
Oribacterium		−0.434[a]	0.030
Porphyromonas		−0.548[b]	0.005
Prevotella		−0.409[a]	0.042
Streptococcus		−0.756[b]	0.000
Tannerella		−0.559[b]	0.004
Veillonella		−0.547[b]	0.005

[a] *P* value of <0.05.
[b] *P* value of <0.01.
[c] FEV_1 % pred, forced expiratory volume in 1 s, expressed as a percentage of the predicted volume.

the detection of *Ralstonia* species. A positive PCR result was obtained for 17 of the 18 respiratory sample extracts in which *Ralstonia* was detected by pyrosequencing and in a further three samples in which it was not (see Table S5 in the supplemental material). Species-specific PCR detected *R. pickettii* in 18 samples and *R. mannitolilytica* in two samples. *R. respiraculi* and *R. insidiosa* were not detected.

While not normally considered to be associated with virulence (27) (and not observed to be negatively correlated with lung function in this study), the clinical significance of the detection of *Ralstonia* species in PCD airway secretions remains to be determined. The previous occurrence of "pseudo-outbreaks" due to contaminated diagnostic reagents (27) means that the detection of *Ralstonia* species must be interpreted with some caution. However, the detection of more than one *Ralstonia* species within the patient group perhaps suggests this was not the case here.

The high abundance of the genus *Blastococcus* in one patient is also noteworthy. Members of this genus have been isolated from a range of environmental contexts (5), with the species detected here, *Blastococcus aggregatus*, associated with brackish water in particular (28). The potential clinical significance of this species in the PCD airway is not yet known.

Correlations of species relative abundance and hierarchical cluster analysis. We investigated whether significant relationships existed between the relative abundance of different bacterial community members. A number of significant correlations were identified and are listed in Table 4. Of particular note are the significant negative correlation between the relative abundances of *Pseudomonas* and *Porphyromonas* and the significant positive correlations between a number of anaerobic genera, including *Prevotella*, *Neisseria*, *Porphyromonas*, and *Streptococcus*. There are several potential explanations for the existence of these relationships. *P. aeruginosa* was present at a high relative abundance in a number of samples, and the predominance of a single member of the bacterial community in this way can result in a reduction of the relative abundances of other bacteria. However, in addition, these negative correlations may also reflect differences in airway conditions (particularly oxygen tension in the cases of anaerobic genera), the ecological interactions between different bacterial species, and the extent of the oropharyngeal contribution to sputum. The predominance of either *Pseudomonas* or a mixed group of facultative and obligate anaerobes has been reported in airway samples from other contexts, including in CF (16).

Correlation of relative species abundance and clinical measures. Hierarchical cluster analysis was used to assess the extent to which bacterial community structure correlated with clinical measures. No clear relationships between patient data and community clustering were identified, although samples obtained by BAL tended to cluster within a broad subgroup, suggesting a more discrete composition compared to that for sputum samples (see Fig. S2 in the supplemental material). A comparison of the composition of BAL fluid samples and sputum samples revealed that the former contained significantly lower numbers of bacterial cells ($P = 0.001$, Wilcoxon rank sum test) and contained significantly higher relative levels of the genera *Ralstonia*, *Corynebacterium*, *Staphylococcus*, *Anaerococcus*, *Propionibacterium*, and *Acinetobacter* ($P < 0.05$). This divergence between sample types might reflect bacterial community differences across the airway regions, the contribution of oropharyngeal bacteria to expectorated sputum, and the younger age of patients sampled using this approach.

Both positive and negative significant correlations were identified between the relative abundances of particular genera and patient age, FEV_1 (percent predicted), and dominant genus relative abundance (used here as a marker of bacterial overgrowth) (Table 5). In particular, there were significant positive correlations between *P. aeruginosa* relative abundance and both patient

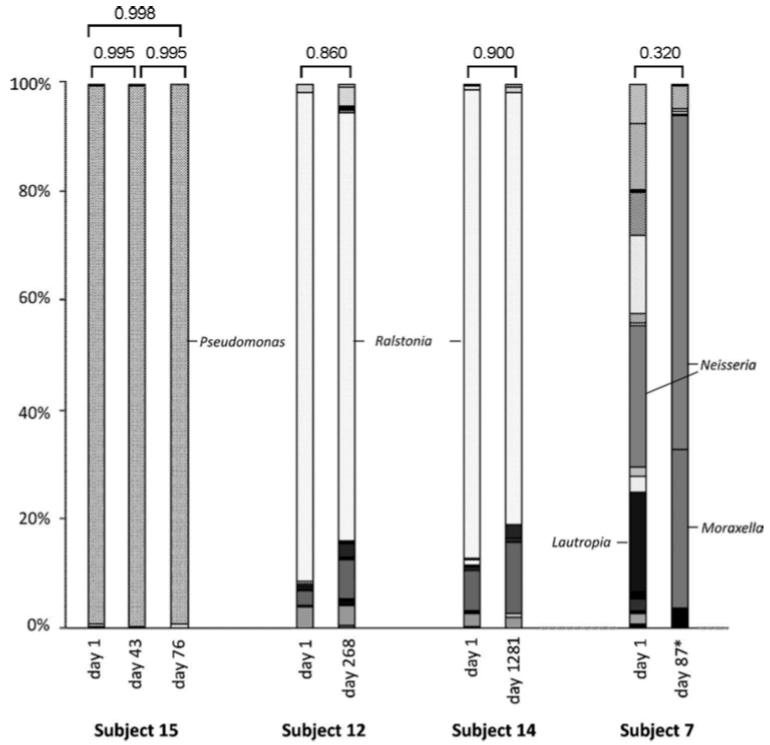

FIG 1 Comparison of paired sputum samples obtained on the same day, with dominant genera indicated. The Bray-Curtis similarity coefficient is given for each pair.

age and dominant genus abundance and significant negative correlations with FEV_1 (percent predicted). These findings are in line with the similar strong associations reported for *Pseudomonas* with disease progression across a range of obstructive airway diseases (6, 29, 30). Further, *P. aeruginosa* infection has been shown to be associated with bronchiectasis and air trapping in PCD (31).

Significant positive associations were observed between the genera *Fusobacterium*, *Prevotella*, *Mycoplasma*, *Abiotrophia*, and *Selenomonas* and the occurrence of sinusitis. Of these, only the association with *Fusobacterium* remained significant following continuity correction ($P = 0.014$, Pearson's chi-square test). While members of this genus would be unlikely to be isolated by standard microbiological analysis of respiratory samples, they are commonly associated with sinus infections (32).

Bacterial community temporal stability. Chronic infections of CF lower airways can vary relatively little in terms of bacterial community composition over periods of a year or more when patients are clinically stable (10, 33). At the same time, the occurrence of exacerbations and the associated antibiotic therapies result in large-scale changes in community composition (34). We therefore investigated whether a similar pattern also exists in PCD infections through the analysis of a limited number of follow-up samples.

First, analysis was performed on paired sputum samples, which were collected at the same hospital visit and obtained from three of the patients and used to assess intersample variation and technical reproducibility (Fig. 1). Bray-Curtis similarity scores were used to determine similarities between the paired samples. Scores of 0.93, 0.85, and 0.53 were obtained from the three sample pairs, respectively, compared to 0.19 between the samples from all patients.

Bacterial community temporal stability was then assessed through the analysis of follow-up samples obtained from four patients in the study (Fig. 2). In three cases, both sample points were obtained during times of stability, and in one case, the follow-up sample was obtained during a period of exacerbation. Stable follow-up samples showed a high level of community stability, even where the interval period between samples was >3.5 years (Bray-Curtis similarity score, 0.9). However, when the second sample was obtained during exacerbation, community similarity was greatly reduced (Bray-Curtis similarity score, 0.32) despite an intervening period of only 87 days. While based on only a small group of patients, these longitudinal observations suggest that bacterial community composition typically varies substantially between individual patients but can remain remarkably stable for given patients over long periods.

The relatively small size of the patient groups from whom sam-

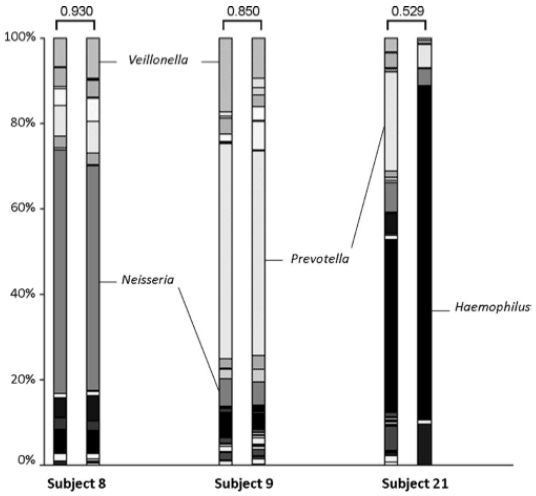

FIG 2 Comparison of serially obtained samples from four patients. Days are numbered from the initial sample obtained. *, sample collected during exacerbation (subject 7, day 87). All other samples were collected during clinical stability. The Bray-Curtis similarity coefficient is given for each pairwise comparison.

ples were obtained represents a limitation of our study; this in part reflects the rarity of PCD. The application of the analysis described here to a larger patient population would present an opportunity to compare the airway microbiology of PCD patients with that of patients with other chronic lower respiratory infections, such as CF and non-CF bronchiectasis. Such comparative analyses might aid in the delineation of relationships between bacterial community composition and airway characteristics, such as airway stasis in the case of PCD. As such, this represents an important area for future investigation. In addition, the study described here was primarily cross-sectional; more detailed longitudinal analyses are also likely to provide important additional insights into the relationships between bacterial community dynamics and disease progression.

In conclusion, in providing an in-depth culture-independent analysis of PCD lower airway microbiology, we highlight the potential contributions of species that are unlikely to be isolated through conventional diagnostic microbiology to disease progression. The data presented here provide insight into the processes that take place in a respiratory system when bacterial clearance is compromised, with relationships between bacterial community composition and clinical measures of disease identified. These findings and their implications for treatment now warrant further investigation.

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Validation of a portable nitric oxide analyzer for screening in primary ciliary dyskinesias

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Abstract

Background: Nasal nitric oxide (nNO) levels are very low in primary ciliary dyskinesia (PCD) and it is used as a screening test.

Methods: We assessed the reliability and usability of a hand-held analyser in comparison to a stationary nitric oxide (NO) analyser in 50 participants (15 healthy, 13 PCD, 22 other respiratory diseases; age 6–79 years). Nasal NO was measured using a stationary NO analyser during a breath-holding maneuver, and using a hand-held analyser during tidal breathing, sampling at 2 ml/sec or 5 ml/sec. The three methods were compared for their specificity and sensitivity as a screen for PCD, their success rate in different age groups, within subject repeatability and acceptability. Correlation between methods was assessed.

Results: Valid nNO measurements were obtained in 94% of participants using the stationary analyser, 96% using the hand-held analyser at 5 ml/sec and 76% at 2 ml/sec. The hand-held device at 5 ml/sec had excellent sensitivity and specificity as a screening test for PCD during tidal breathing (cut-off of 30 nL/min,100% sensitivity, >95% specificity). The cut-off using the stationary analyser during breath-hold was 38 nL/min (100% sensitivity, 95% specificity). The stationary and hand-held analyser (5 ml/sec) showed reasonable within-subject repeatability(% coefficient of variation = 15).

Conclusion: The hand-held NO analyser provides a promising screening tool for PCD.

Keywords: Nasal nitric oxide, Primary ciliary dyskinesia, Nitric oxide analyser

Background

Primary ciliary dyskinesia (PCD) is an autosomal recessive condition in which abnormal ciliary function leads to impaired mucociliary clearance and consequent recurrent upper and lower respiratory tract infection [1]. Approximately half the patients have *situs inversus* and male infertility is common [1]. Early diagnosis is important to ensure appropriate management and counselling; several studies suggest that early diagnosis and treatment improves long-term prognosis. To comply with European consensus guidelines diagnosis of PCD includes investigations requiring access to highly specialised equipment and diagnostic scientists [2-4]. Such facilities are not widely available, contributing to the inequality of diagnosed cases throughout Europe [5]. Nasal NO (nNO) is characteristically low in PCD [6,7], so much so that nNO is recommended as a pre-diagnostic screening test for the condition [2,3,8-10]. Until recently, the only commercially available analyzers for measuring nNO were non-portable desktop analyzers which are extremely expensive. A reasonably priced, practical method of measuring nNO might improve the disparity in diagnosis and reduce diagnostic costs by reliably identifying patients for referral to specialist centres. A number of hand-held devices have proved beneficial for measuring fractional exhaled nitric oxide in patients with asthma [11-14] in clinical and research settings. Commercially available hand-held analyzers have recently been adapted to measure nNO in breath-hold and tidal breathing modes [15,16]. A study of PCD and CF patients reported a hand-held device to be as effective as

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the stationary analyser for assessing nNO during silent and humming exhalation [17]. Furthermore, a recent Danish study has showed that tidal breathing nNO measured by a hand-held NO device discriminates between PCD, cystic fibrosis (CF) and healthy controls (HC) [18]. PCD is rare and no center has access to large numbers of patients. The two published studies using hand-held devices have respectively assessed nNO in only 14 PCD patients during silent and humming single breath exhalation [17] and 16 PCD patients during tidal breathing and during a velum closure maneuver [18]. Our data study therefore adds to the limited data, using a similar tidal breathing maneuver to that used in the Danish study. The aim of our single center cross-sectional study was to evaluate the ability of a commercially available analyzer (NIOX MINO®) to discriminate between PCD, other respiratory diseases and healthy controls in comparison to a 'gold standard' chemiluminescence analyser (NIOX® Flex).

Methods

This study was approved by Southampton and South West Hampshire Research Ethics Committee A (06/Q1702/109 and 08/H0502/126). All subjects gave written informed consent.

Participants

Fifty people consented to participate in this study, including 15 healthy volunteers, 13 with PCD and 22 with other respiratory disorders. Children and adults with PCD, cystic fibrosis (CF) (n = 6), and non-CF non-PCD chronic suppurative lung disease (CSLD) (n = 7) were recruited from specialist PCD and respiratory clinics. Those with asthma +/− hay fever (n = 9) were recruited from amongst staff/students and from respiratory clinics.

Healthy participants completed a short questionnaire to exclude disease that might affect nitric oxide levels. PCD was diagnosed at Southampton's national PCD centre by analysing respiratory epithelia ciliary beat frequency and pattern using high-speed video microscopy in patients with a suggestive history [2,4,19]. Diagnosis was supported by assessment of ciliary ultrastructure by transmission electron microscopy (TEM). In some cases diagnosis was further clarified by analysis of re-differentiated cilia following culture of the airway epithelial cells at an air liquid interface. CF diagnosis was based on compatible history, an abnormal sweat test and/or CF genotyping. CSLD participants were recruited from patients with a chronic history of purulent sputum referred to the PCD diagnostic service who had the diagnosis of PCD and CF excluded. They had not necessarily had a HRCT; bronchiectasis had therefore not been radiologically excluded nor confirmed. Asthma diagnosis was self-reported amongst staff and students.

Measurement of airway nitric oxide

Two different devices were used: NIOX MINO® and NIOX® Flex analysers. Measurement of nNO levels using NIOX® Flex (Aerocrine, Sweden) uses chemiluminescence with sampling at 5 ml/s [20], whilst the NIOX MINO® uses an electrochemical sensor and provides a result after sampling for 90 seconds at 2 ml/s or for 45 seconds at 5 ml/s. The analyser only provides a result if uninterrupted sampling has been maintained throughout this sampling time. NIOX MINO® can also sample during tidal breathing.

The NIOX® Flex analyser was maintained, calibrated and tested according to manufacturer's guidelines. Measurements were made by health care professionals who had been trained following a standard operating procedure derived from the manufacturer's guidelines.

In brief, ambient NO was noted before testing patients. All patients were symptomatically free of viral infection or respiratory exacerbation. Participants were requested to blow their nose before testing. A nasal probe sampled gas aspirated from the nostril at a rate of 5 ml/sec during a breath-holding maneuver. Patients held their breath for approximately 20 seconds until the real-time analyser recorded a plateau in nitric oxide concentrated from the aspirated gas. The measurement was recorded from a steady nNO concentration plateau of at least 4 seconds. Three measurements within 10% were obtained for each participant using the same nostril and the maximum nNO reading was recorded. nNO concentration in parts per billion (ppb) were converted to NO production (nL/min) using the equation NO production (nL/min) = nNO concentration (ppb) x sampling flow rate (litres/min).

The NIOX MINO® was used in nasal mode. A nasal probe sampled gas aspirated from the nostril at 5mls/sec and was then repeated at 2 ml/sec. Following ATS [20] and manufacturer's guidelines we initially attempted these measurements during breath-holding manoeuvres To obtain a test result the participant needed to achieve uninterrupted sampling for 45 seconds whilst sampling at 5 ml/sec or 90 seconds at 2 ml/sec. Breath holding for this duration was not achievable even by healthy adult volunteers and it was decided to abandon this maneuver. Instead we followed the alternative method suggested by the manufacturers of sampling nasal gas at 2 ml/sec and 5 ml/sec during open mouth breathing. This non-velum closure technique has previously been confirmed as reproducible and valid using a stationary chemiluminescence analyser [21]. Three recordings at each rate were attempted, and the highest was taken.

Usability and reliability of analysers

The multidisciplinary clinical PCD team who undertook these measurements (paediatrician, PCD nurse specialist, PCD respiratory physiotherapist and respiratory

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technicians) discussed the pros and cons of the analysers. Their consensus opinions were recorded.. The success rate for obtaining a measurement with each analyser was recorded.

Statistical analyses

The highest of three measurements for each method was recorded. Nasal NO values were $\log_{10}$ transformed where appropriate because data was not normally distributed. Comparisons between disease groups were made using unpaired t-tests. Comparisons between analyzers were made using paired t-tests. Receiver Operating Characteristic (ROC) curves were used to determine cut of values, sensitivity and specificity. Pearson correlation coefficients were calculated to investigate correlation between nNO values obtained with the NIOX® Flex and NIOX MINO®. Brand Altman plots were constructed with the difference of measurements from the two analyzers plotted against the mean of the two methods. The reproducibility of analyzers was reported as the intra-subject% Coefficient of Variability (%CV); this was calculated by calculating the standard deviation for the three measurements taken using each protocol, and dividing that by the triplicate mean, and multiplying by 100. Data were analyzed using statistical analysis software SPSS version 19.0.0 (IBM, USA).

Results

Valid readings were obtained in 47 participants using the NIOX® Flex breath-hold protocol. Three participants (aged 5, 8 and 8 years) were unable to breath-hold for 20 seconds to obtain a technically acceptable measurement. Using the NIOX MINO® healthy adults were unable to manage the breath-hold protocol of 45 seconds for 5 ml/sec or 90 seconds for 2 ml/sec and we abandoned these protocols with no resulting data. However measurement during tidal mouth breathing using the NIOX MINO® successfully provided data for 48 participants at a sampling rate of 5 ml/sec and 38 participants at 2 ml/sec. The mean (range) ages of participants in each group were: healthy controls 31 years (8–65), PCD 23 years (5–71), CF 15 years (6–29), asthma 35 years (12–59) and CSLD 36 years (8–79).

Nasal NO as a screening tool for PCD

Using the NIOX® Flex, patients with PCD had significantly lower levels of nNO than healthy controls ($p < 0.001$), CF ($p = 0.003$), asthma ($p < 0.001$) and CSLD ($p = 0.005$). Statistically lower nNO values were similarly obtained in patients with PCD when using the NIOX MINO® at 5 ml/sec (HC $p < 0.001$; CF $p = 0.028$; asthma $p < 0.001$; CSLD $p = 0.002$). Using the NIOX MINO® sampling at 2 ml/ sec, patients with PCD had significantly lower nNO measurements than HC ($p < 0.001$), CF ($p = 0.001$) and asthma ($p < 0.001$). However, two participants with CSLD had very low levels (<6 nL/min) and there was no statistical difference in nNO values between this group and those with PCD ($p = 0.11$). One participant with CF and normal ciliary function and ciliary ultrastructure, had very low levels of nNO (<30 nL/min) measured using the NIOX® Flex and NIOX MINO®.

Receiver operating characteristic (ROC) curves were generated for the NIOX® Flex and NIOX MINO® at 2 ml/ sec and 5 ml/sec in cases with PCD versus participants without PCD (curves not shown) and cut-off values were determined for optimal sensitivity and specificity (Table 1). Using NIOX® Flex, a nNO cut off levels of 38 nL/min had 100% sensitivity and 95% specificity for distinguishing PCD patients from non-PCD patients. A cut off value of 30 nL/min when using the NIOX MINO® sampling at 5 ml/sec, provided 100% sensitivity and 95% specificity; using the same analyser, sampling at 2 ml/sec, a cut of value of 43 nL/min provided 100% sensitivity and 93% specificity for differentiating PCD patients from the other groups.

Comparison of nNO values between analysers

Comparing analysers, within patients, the NIOX MINO® sampling at 5 ml/sec or 2 ml/sec consistently measured lower ($p < 0.001$, $p < 0.001$) than the NIOX® Flex (Figures 1, 2, 3). Pearson correlation analysis showed excellent linear association between nNO readings obtained by NIOX® Flex and NIOX MINO® at 5 ml/sec or 2 ml/sec ($r = 0.93$ $p < 0.001$ and $r = 0.93$ $p < 0.001$ respectively). The relationship between the nNO measured by NIOX® Flex and NIOX MINO® at 5 ml/sec were further investigated by Bland-Altman plot (Figure 4). The Bland-Altman plots show a high degree of difference between readings from the two analysers, particularly in participants with very high levels of nNO. However the patients with PCD all had low nNO levels, and this group all demonstrated low variability of readings between analysers.

Reproducibility within-analyser

The intra-subject%CV showed good repeatability between three measurements taken during the same visit using NIOX® Flex (%CV = 9) and reasonable repeatability

Table 1 Nasal nitric oxide cut-off values to discriminate between PCD and non-PCD groups using different analysers

	NIOX® Flex Breath-hold	Niox Mino$_{5ml/sec}$ Tidal breathing	Niox Mino$_{2ml/sec}$ Tidal breathing
nNO cut-off value (nL/min)	38	30	43
Sensitivity%	100	100	100
Specificity%	95	95	93

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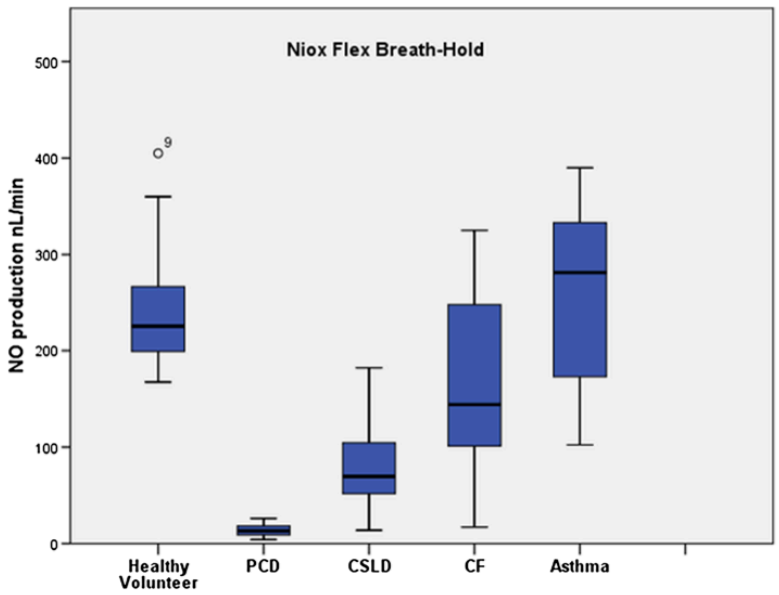

Figure 1 Box plots demonstrating nasal NO production (nL/min) measured by NIOX® Flex using a breath-hold manoeuver in healthy volunteers (n = 15), PCD (n = 11), CSLD (n = 6), CF (n = 6) and asthma (n = 9) (line: median, box: quartiles, whiskers: minimum and maximum.

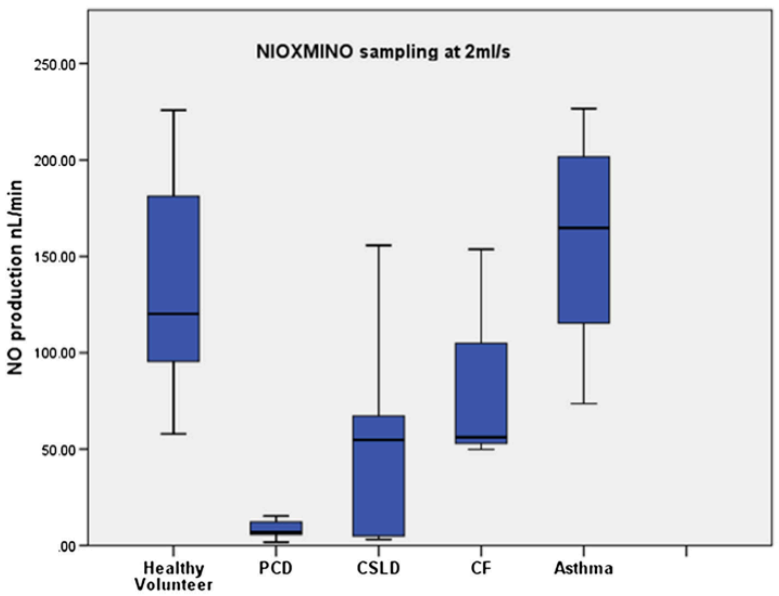

Figure 2 Box plots demonstrating nasal NO production (nL/min) measured by NIOX MINO® during mouth breathing with nasal sampling at 2 ml/ sec in healthy volunteers (n = 13), PCD (n = 9), CSLD (n = 5), CF (n = 3) and asthma (n = 8).

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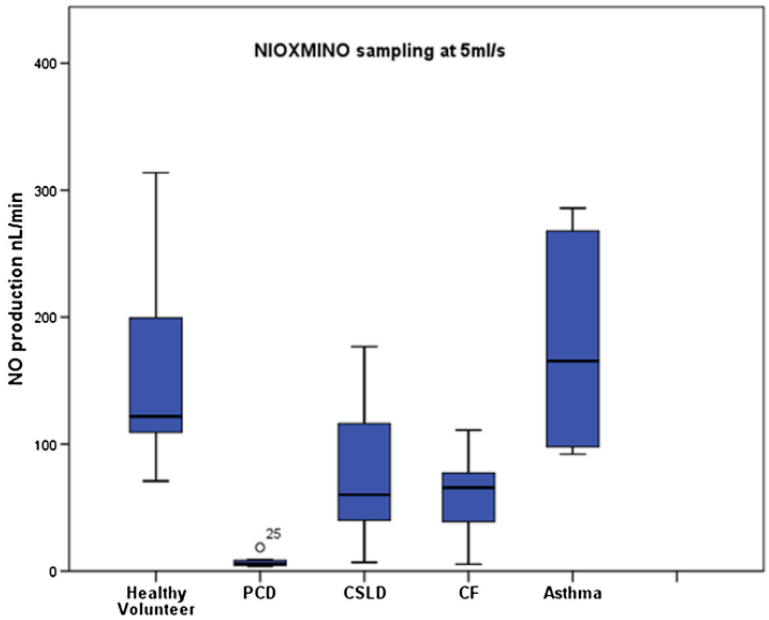

Figure 3 Box plots demonstrating nasal NO production (nL/min) measured by NIOX MINO® during mouth breathing with nasal sampling at 5 ml/ sec in healthy volunteers (n = 15), PCD (n = 12), CSLD (n = 7), CF (n = 5) and asthma (n = 9).

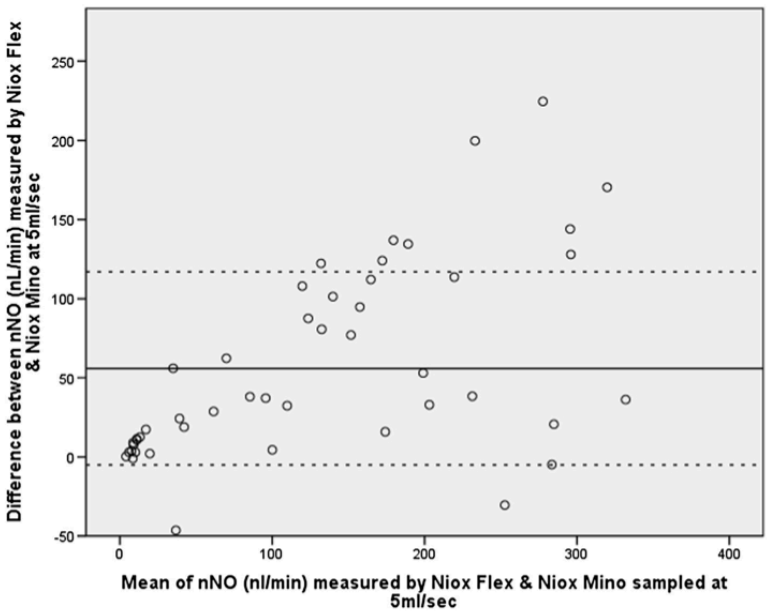

Figure 4 Bland Altman plots comparing the NIOX® Flex with NIOX MINO® at 5 ml/sec. Each point represents the difference between the nNO readings (nL/min) obtained from a patient using the two analysers versus the mean of the two measurements. The reference lines represent the mean (and 1.96*SD) inter-analyser differences.

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using the NIOX MINO® at 5 ml/sec (%CV = 15) and NIOX MINO® at 2 ml/sec (%CV = 14).

Usability of the analysers

Prior to the study the nurse specialist and physiotherapist were highly experienced obtaining nNO readings from adults and children using the NIOX® Flex breath-hold protocol. They were unable to obtain readings with the NIOX MINO® using the breath-hold technique because even healthy adults were unable to breath-hold for the requisite time. Their assessment (Table 2) was that younger children were sometimes able to achieve nNO readings using the NIOX MINO® by using the mouth breathing technique with sampling at 5 ml/sec, even when acceptable reading were not obtained using the NIOX® Flex. However, the NIOX MINO® sampling at 2 ml/sec was a very lengthy procedure and fewer participants achieved it.

The portability of the NIOX MINO® was considered a benefit by health care professionals involved in the study, although the need to be placed on a firm, flat surface and the short sampling tube were poorer ergonomic features.

Discussion and conclusions

This study confirmed that the hand-held NIOX MINO® provides a reliable screening tool for PCD compared to the NIOX® Flex in this study population. This is consistent with two previous studies using a hand held device in PCD patients, one of which collected nasal gas via a tight fitting nasal mask during single expiration silent breathing or humming [15]. The other study compared a tidal breathing and breath-hold maneuvers (+/− velum closure) [18]. We were unable to obtain readings using the NIOX MINO® during breath-holding but found NIOX MINO® nNO readings taken during mouth breathing correlated well with measurements from the NIOX® Flex during breath-holding manoeuvres. This was particularly true for patients with PCD. Within our study population, we were more successful (96% versus 76% success) and quicker (15–30 minutes versus 20–40 minutes) measuring nNO using the NIOX MINO® at 5 ml/sec than at 2 ml/sec; we found no advantage for measuring at 2 ml/sec and would use 5 ml/sec in the future. A recent Danish study [18] using the NIOX MINO® similarly reported that the method recommended by ATS/ERS guidelines for measuring nNO [20] (aspiration of nasal gas during velum closure and breath-hold) was impossible at a sampling rate of 2 ml/sec, but they had a success rate of 70% during breath-hold at a rate of 5 ml/sec. This compared to 100% success when sampling at 5 ml/sec during tidal breathing [18].

We noted that all three methods gave reproducible results. Although the methods were well correlated, there were differences in the mean nNO levels between analysers. nNO production recorded by the NIOX MINO® was lower than that recorded by the NIOX® Flex. This is likely to reflect contamination of the nasal sample by lower

Table 2 Comparison of analysers and methods reflecting the opinions of the multidisciplinary specialist PCD team having conducted the research

	NIOX® Flex 20 seconds	NIOX MINO®
Ease of use	Easily used by children over 8 years and by some younger using breath hold (BH)	Breath hold difficult for 45 sec/ 90 sec by all ages. Tidal breathing (TB) easily achieved in 5 ml/sec sampling mode
Time to complete 3 readings	5-10 min (BH)	20-40 min (TB)
Practical issues	Needs stable environment and dedicated work space.	Needs to be used on a flat surface
	Poor portability	Excellent portability
	Breath-holding technique difficult for young children and advanced lung disease	Most people could manage the technique but it resulted in a dry mouth. Short sampling catheter
Cost of machine	Very Expensive. Approx £30,000	Expensive. Approx £2,100
Need for calibration gases	Yes. Approx £1,500 per annum	No- autocalibrates
Need for maintenance service	Yes. Approx £3,800 per annum	No
Consumables	Nasal sampling olives: £40 per 100	Nasal sampling olives: £40 per 100 patients
Longevity	Good	Sensor needs replacing after 100 or 300 readings (using our protocol 30 or 100 patients). Many readings failed but were still 'counted'. Sensor expires after 1 year Approx. £1,215 for sensor 300.
Range of nNO (ppb)	25-2000 ppb	5-1700 ppb
Effected by other electronic devices in room e.g. mobile phones	No	Yes

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airway gas during mouth breathing and short mucosal contact time. This demonstrates the need for different diagnostic cut-offs for different breathing methods. Using a stationary chemiluminescence NO analyser, a study [21] of 85 children including 20 with PCD similarly demonstrated that non-velum closure methods yielded lower values that during the velum closure technique recommended in ATS/ ERS guidelines [20]. As confirmed by our study, the authors reported open mouth tidal breathing provides reproducible and discriminatory nNO levels. Since measurement during tidal breathing is easier to perform, allowing its use in young children, standardisation of this approach and reference data is called for.

In our population, a cut-off value of 38 nL/min provided 100% sensitivity and 95% specificity using the NIOX® Flex during breath-hold. A cut-off of 30 nL/min using the NIOX MINO® (tidal breathing with sampling at 5 ml/sec) provided 100% sensitivity and 95% specificity. These cut off values are somewhat lower that those reported in a large study investigating the use of nNO as a screening test for PCD [9]. Leigh *et al.* determined a cut off value of 77 nL/min was 98% sensitive and >99.9% specific when screening patients at their PCD centre. The authors then used the same protocol to measure nNO in 155 patients referred for PCD diagnostic testing at six other hospitals. They found that this level correctly identified 70 of the 71 participants who were confirmed to have PCD. A study using the NIOX® Flex and NIOX MINO® to measure nNO during silent and humming exhalations [2,17] calculated lower cut-off values than in our study. This is likely to reflect greater contamination by lower airway gases during these exhalation manoeuvres. Importantly, the key findings of both manuscripts were in line with our paper, i.e. PCD patients have lower nNO than healthy controls or CF patients [9,17], and the hand-held device is effective for screening to differentiate PCD from other groups [17].

Whilst there is now substantial data to demonstrate that nNO measurement is helpful in guiding the diagnostic pathway, we need to recognize limitations of this measurement [10]. Standardized methods to measure nNO are not appropriate for younger children, precisely the age group that need targeting for diagnostic measurement. Sampling during tidal breathing provides a potential method in this group, but data is limited [22]. Low nNO has previously been described in patients with CF and other respiratory conditions. One of our patients with CF and negative PCD diagnostics had very low levels of nNO (NIOX® Flex 17 nL/min, NIOX MINO® 5 ml/Sec 5 nL/min). Two patients with CSLD also had low levels of nNO. Both had PCD excluded using protocols that follow ERS guidelines [2]. They had normal ciliary beat frequency and pattern before and following culture at air liquid interface. They also had normal ultrastructure by TEM. These data demonstrate that although nNO is useful as a screening test, it is not a substitute for formal assessment of ciliary function and assessment of ciliary ultrastructure. It is noteworthy that although all PCD patients in this study had very low levels of nNO, occasionally PCD patients with normal levels have been described [8,22,23]. This highlight that patients with a history strongly suggestive of PCD should not be excluded from further diagnostic evaluation on the basis of nNO.

An important observation by the authors has arisen from referrals to our diagnostic service where nNO has been measured by the referring team using NIOX MINO®. A number of patients have been noted to have extremely low levels of nNO at the referring hospital, but normal/ high levels when measured using the NIOX MINO® or NIOX® Flex at our centre. We suspect this might reflect leaks at the nares or excessive lower airway contamination, and highlights the need for training and standardised protocols if the NIOX MINO® is to be used as a screening test at satellite hospitals.

In summary, nNO measurements using the NIOX MINO® successfully discriminates PCD patients from healthy individuals and those with other lung conditions. Nasal NO values were reproducible and correlated well with the NIOX® Flex. However, we consistently recorded lower readings using the tidal breathing maneuver. The NIOX MINO® is easily portable and relatively cost-effective as compared to the desktop NIOX® Flex. Health care professionals and patients found it acceptable. Hand-held devices therefore provide a promising screening tool for PCD although further studies will be required to establish reference data for each breathing maneuver and analyser. One study has successfully attempted to standardise methodology across a number of collaborating sites with different analysers [9]. With the array of sampling techniques and analysers available, standardised protocols and cut off data should be developed. Due to the rarity of PCD it is likely that a coordinated international approach will be required to achieve this.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AH had the idea for the research and designed the study; EB, KG, RJ & JP undertook clinical testing and data recording; AH, HE and JSL undertook data analysis and interpretation; HE, WW & JSL recruited patients and are clinical leads for the PCD diagnostic service; all authors contributed to and approved the manuscript; JSL takes responsibility for the integrity of the study data and analysis. All authors read and approved the final manuscript.

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Appendix 2

Table 1 – PCD diagnostic data for patients and samples used in this work

Subject	nNO (ppb)	CBF (Hz)	CBP	Ciliary ultrastructure defect	Co-culture experiment (n=5)*	B-tubulin ciliation (n=5)	PAS staining (n=5)	Variable NO study (n=14)	NO assay at baseline (n=11)	NO assay after stimulation (n=5)	DAF-fm (n=3)	NOS isoenzymes (n=4)
NB1057	n/a	Static	Static	ODA					■	■		
NB1077	n/a	Static	Static/twitching	ODA					■	■		
NB1082	n/a	Static	Static	ODA					■			
NB1144	87	8.3	Slow/static	ODA					■	■		
NB1165	n/a	26	Hyper-frequent	Normal					■	■		
NB1174	n/a	Static	Static	ODA & IDA					■	■		
NB1219	258	7.8	Slow/dyskinetic	Normal	■	■	■		■			
NB1254	57	10	Static/dyskinetic	ODA & IDA			■					
NB1257	26.6	Static	Static	Normal	■				■			
NB1350	41	Static	Static	ODA		■						■
NB1355	41	Static	Static	Normal		■	■				■	
NB1365	6	Static	Static/dyskinetic	ODA		■	■	■			■	
NB1377	n/a	Static	Static/dyskinetic	ODA		■	■				■	

*Main co-culture experiment in chapter 5 includes biofilm CFU counts, scanning electron microscopy, Fluorescence in situ hybridisation, trans-epithelial electrical resistance (TER), cytokines, antimicrobial peptides (AMP) and NO assay. Also baseline AMP, cytokines and TER in chapter 3. ODA – outer dynein arm defect, IDA – inner dynein arm defect.

Table 1 (continued)– PCD diagnostic data for patients and samples used in this work

Subject	nNO (ppb)	CBF (Hz)	CBP	Ciliary ultrastructure defect	Co-culture experiment (n=5)*	B-tubulin ciliation (n=5)	PAS staining (n=5)	Variable NO study (n=14)	NO assay at baseline (n=11)	NO assay after stimulation (n=5)	DAF-fm (n=3)	NOS isoenzymes (n=4)
PCD410	65	Static	Static	ODA								
PCD411	86	Static	Static	ODA								
PCD414	43	Static	Static	ODA								
PCD415	n/a	Static	Static	ODA & IDA								
PCD416	29	Static	Static	ODA								
PCD417	5	Static	Static	ODA								
PCD418	21	Static	Static	ODA								
PCD420	36	Static	Static	ODA								
PCD421	65	Static	Static	ODA								
PCD422	96	Static	Static	Normal								
PCD424	66	Static	Static	ODA								
PCD425	75	Static	Static	ODA								
PCD427	16	Static	Static	ODA								
PCD429	20	Static	Static/twitching	ODA & IDA								

*Main co-culture experiment in chapter 5 includes biofilm CFU counts, scanning electron microscopy, Fluorescence in situ hybridisation, trans-epithelial electrical resistance (TER), cytokines, antimicrobial peptides (AMP) and NO assay. Also baseline AMP, cytokines and TER in chapter 3. ODA – outer dynein arm defect, IDA – inner dynein arm defect.

Table 2 – Non-PCD patients clinical phenotype data and samples used in this work

Subject	Clinical phenotype	nNO (ppb)	CBF (Hz)	CBP	TEM	Co-culture experiment (n=9)*	B-tubulin ciliation (n=5)	PAS staining (n=9)	NO assay at baseline (n=25)	NO assay after stimulation (n=9)	NO assay pre/post infection (n=9)	DAF-fm (n=4)	NOS isoenzymes (n=4)	nNOS immunohisto-chemistry (n=5)
HV79	Healthy Control	UK	UK	UK	UK				■		■			
HV88	Healthy Control	UK	UK	UK	UK				■		■			
HV101	Healthy Control	UK	UK	UK	UK	■			■	■				
HV103	Healthy Control	UK	UK	UK	UK	■			■	■				
HV104	Healthy Control	UK	UK	UK	UK	■			■	■				
HV105	Healthy Control	UK	UK	UK	UK				■					
HV108	Healthy Control	UK	UK	UK	UK	■			■	■				
HV111	Healthy Control	UK	UK	UK	UK	■			■	■				
HV114	Healthy Control	UK	UK	UK	UK	■			■	■				
HV115	Healthy Control	UK	UK	UK	UK	■			■	■				
HV117	Healthy Control	UK	UK	UK	UK	■			■	■				
HV118	Healthy Control	UK	UK	UK	UK				■					
HV156	Healthy Control	UK	UK	UK	UK							■		
HV165	Healthy Control	UK	UK	UK	UK			■				■		
AHNP37	Healthy Control	UK	UK	UK	UK									■
AMNP8	Healthy Control	UK	UK	UK	UK									■

Subject	Clinical phenotype	nNO (ppb)	CBF (Hz)	CBP	TEM	Co-culture experiment (n=9)*	B-tubulin ciliation (n=5)	PAS staining (n=9)	NO assay at baseline (n=25)	NO assay after stimulation (n=9)	NO assay pre/post infection (n=9)	DAF-fm (n=4)	NOS isoenzymes (n=4)	nNOS immunohisto-chemistry (n=5)
DS58i	Healthy Control	UK	UK	UK	UK									
HL133AW	Healthy Control	UK	UK	UK	UK									
NB1037	Cough	NP	13.0	Normal	NP									
NB1092	Nasal polyps	463	14.7	Normal	NP									
NB1130	Cough & grommets	NP	12.9	Normal	NP									
NB1154	Cough	NP	14.5	Normal	NP									
NB1168	Cough	NP	15.5	Normal	Normal									
NB1186	Cough & sinusitis	136	17.4	Normal	Normal									
NB1189	Cough	NP	13.3	Normal	NP									
NB1192	Sinusitis	NP	14.6	Normal	Normal									
NB1193	Bronchiectasis	240	13.5	Normal	Normal									
NB1195	Bronchiectasis	245	15.7	Normal	Normal									
NB1196	TGA	NP	12.9	Normal	Normal									
NB1221	Recurrent LRTI	NP	15.5	Normal	Normal									
NB1223	Recurrent LRTI	925	26.8	Normal	NP									
NB1241	Recurrent LRTI	NP	12.2	Normal	Normal									

Subject	Clinical phenotype	nNO (ppb)	CBF (Hz)	CBP	TEM	Co-culture experiment (n=9)*	B-tubulin ciliation (n=5)	PAS staining (n=9)	NO assay at baseline (n=25)	NO assay after stimulation (n=9)	NO assay pre/post infection (n=9)	DAF-fm (n=4)	NOS isoenzymes (n=4)	nNOS immunohisto-chemistry (n=5)
NB1366	Situs inversus	NP	15	Normal	NP									
NB1367	Recurrent cough	570	18.8	Normal	Normal									
NB1354	Recurrent cough	890	14.6	Normal	NP									
NB1356	Cough	260	15.8	Normal	NP									
NB1357	Recurrent cough	NP	15.6	Normal	NP									
NB1371	Cough and sinusitis	176	15.5	Normal	NP									
NB1379	Neonatal pneumonia	NP	14.56	Normal	Normal									
NB1382	Rhinitis	NP	15.7	Normal	Normal									
NB1398	Recurrent LRTI	NP	18.7	Normal	Normal									
NB1397	Recurrent LRTI	NP	14.3	Normal	Normal									

*Main co-culture experiment in chapter 5 includes biofilm CFU counts, scanning electron microscopy, Fluorescence in situ hybridisation, trans-epithelial electrical resistance (TER), cytokines, antimicrobial peptides (AMP) and NO assay. Also baseline AMP, cytokines and TER in chapter 3. ODA – outer dynein arm defect, IDA – inner dynein arm defect. UK – unknown, NP - not performed

Appendix 3

Table showing a working example of the calculation using the fast fourier transform method to estimate the average percentage ciliation of an ALI cultured cell layer (as per 2.3.3.8.2)					
FoV (as per figure 11)	Percentage ciliation of FoV	Average ciliation of the two FoV of that ring (ie 1 & 16, 2 & 15 etc, figure 11)	Multiplier at that diameter (as per table 3)	Total ciliation at that diameter	Average ciliation across cell layer (sum of previous column divided by total number of FoV (453)
1	24.10	17.33	101	1750.7	**8.84%**
2	1.15	8.6	88	756.8	
3	0.06	3.70	75	277.6	
4	0.38	4.78	63	301.0	
5	0.44	5.84	50	292.1	
6	3.74	7.43	38	282.3	
7	1.88	7.61	25	190.2	
8	9.32	12.16	13	158.1	
9	14.99				
10	13.33				
11	11.12				
12	11.25				
13	9.173				
14	7.34				
15	16.05				
16	10.57				
SUM				**4008.8**	
FoV – field of view					